Untersuchungen zur Regulation des Lipid- und Energiestoffwechsels durch den Peroxisomenproliferator-aktivierten Rezeptor α

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Für meine Kinder,
das beste Experiment meines Lebens

Inhaltsverzeichnis

Abkürzungsverzeichnis

BBD	γ-Butyrobetain-Dioxygenase
CACT	Carnitin-Acylcarnitin-Translokase
CLA	konjugierte Linolsäuren
CPT	Carnitin-Palmitoyltransferase
FAS	Fettsäuresynthase
FGF	*fibroblast growth factor*
HMG	3-Hydroxy-3-Methylglutaryl
13-HPODE	13-Hydroperoxy-9,11-Octadecadiensäure
Insig	*insulin-induced gene*
LDL	*low density lipoprotein*
LH	Luteinisierendes Hormon
LXR	*liver X receptor*
m	mitochondrial
MCT	Monocarboxylat-Transporter
OCTN	*novel organic cation transporter*
PPAR	Peroxisomenproliferator-aktivierter Rezeptor
PPRE	PPAR *response element*
PUFA	mehrfach ungesättigte Fettsäuren
RXR	Retinsäurerezeptor
SREBP	*sterol regulatory element–binding protein*
TML	Trimethyllysin
TSH	Thyreoidea-stimulierendes Hormon
VLDL	*very low density lipoprotein*

1 Einführung und Fragestellung

Eine effiziente Bereitstellung von Energie ist Voraussetzung für das Überleben aller Organismen. Hierbei spielt die Fähigkeit, Energieressourcen entsprechend Angebot und Nachfrage zu speichern oder zu mobilisieren, eine fundamentale Rolle. Glukose und langkettige Fettsäuren aus der Nahrung stellen die Hauptenergiequellen für Säuger dar. So haben sich im Laufe der Zeit komplex regulierte Systeme für die Speicherung und Mobilisierung dieser natürlichen Energiequellen entwickelt [1]. Dabei liefern Fettsäuren und andere Nahrungskomponenten nicht nur Energie, sondern lösen auch eine Vielzahl zellulärer Signale aus, unter anderem durch die Beeinflussung der Transkription von Genen [2-5]. Die Peroxisomenproliferator-aktivierten Rezeptoren (PPAR) stellen dabei einen bedeutenden Mediator der Regulation der Genexpression durch Fettsäuren dar [6, 7]. Sie wurden ursprünglich 1990 [8] als nukleare Rezeptoren für Peroxisomenproliferatoren, zu denen die lipidsenkenden Fibrate und auch Weichmacher zählen [9], beschrieben. Kurze Zeit später konnte gezeigt werden, dass verschiedene Fettsäuren, Fettsäurederivate und Eicosanoide ebenfalls als Liganden und Aktivatoren der PPAR wirken und somit ihre physiologischen Agonisten darstellen [6, 10, 11]. Bisher sind die drei PPAR-Subtypen α, β/δ und γ bekannt, die von separaten Genen kodiert werden und sich hinsichtlich Gewebeverteilung und Ligandenbindung unterscheiden [12]. Die transkriptionelle Aktivierung von Genen durch PPAR erfolgt im Heterodimerkomplex mit dem Retinsäurerezeptor (RXR) durch Bindung an spezifische DNA-Sequenzen, die PPAR *response elements* (PPRE) [13, 14], und wird durch verschiedene Co-Repressoren und Co-Aktivatoren reguliert [15, 16]. Umfangreiche Untersuchungen zu Struktur, Funktion und Wirkungsweise der PPAR zeigten ihre zentrale Beteiligung an der Regulation von Lipid- und Lipoproteinstoffwechsel, Glukosehomöostase, Energiehaushalt, zellulärer Differenzierung und inflammatorischen Prozessen und verdeutlichen somit ihr therapeutisches Potential und ihre Schlüsselstellung in der Therapie von z. B. Fettstoffwechselstörungen oder Diabetes mellitus [17-24].

Fibrate, die bereits seit etwa 40 Jahren zur Therapie von Fettstoffwechselstörungen eingesetzt werden, bewirken über eine Aktivierung des PPARα eine Verringerung der Konzentration an zirkulierenden Triglyzeriden und eine Erhöhung der Konzentration an HDL-Cholesterol. Die triglyzeridsenkende Wirkung wird dabei durch die transkriptionelle Modulation der Lipolyse von triglyzeridreichen Lipoproteinen, der zellulären Aufnahme und β-Oxidation von Fettsäuren sowie eine verringerte Synthese von Fettsäuren und Triglyzeriden erreicht [25]. Dabei sind die Mechanismen, die zur verringerten Synthese von Fettsäuren und Triglyzeriden führen, teilweise noch unklar. Neben der triglyzeridsenkenden Wirkung wurde sowohl für Fibrate als auch natürliche PPARα-Agonisten eine Senkung der Cholesterol-

Konzentrationen in Leber und Plasma von Menschen und Säugern beobachtet, deren molekulare Ursachen bisher ebenfalls weitgehend unbekannt sind [26-29].

Die zentrale Bedeutung des PPARα im Stoffwechsel besteht in seiner Funktion als Sensor für die Konzentration an freien Fettsäuren in der Zelle. So führen freie Fettsäuren, die infolge von Energiemangelzuständen wie z. B. Hunger aus dem Fettgewebe freigesetzt werden, zur Aktivierung des PPARα in der Leber, welcher durch die transkriptionelle Regulation einer Vielzahl von Genen den Säugerorganismus an diese Mangelsituation anpasst [23]. Die kritische Rolle des PPARα im Hungerzustand konnte mit Hilfe von PPARα-defizienten (*knockout*) Mäusen gezeigt werden, die im Fastenzustand u. a. eine schwere Hypoglykämie und eine Fettleber entwickeln [30-32]. Gesteuert durch PPARα werden im Hungerzustand langkettige Fettsäuren aus dem Fettgewebe in den Mitochondrien zur Energiebereitstellung oxidiert. Dies erfordert den Transport der Fettsäuren über Plasmamembranen, ihre Bindung im Zytosol und den Transport in die Mitochondrienmatrix in Form von Carnitin-Derivaten. Für eine Vielzahl von Enzymen und Proteinen, die maßgeblich an diesen komplexen Mechanismen und der nachfolgenden β-Oxidation beteiligt sind, konnte eine transkriptionelle Regulation der kodierenden Gene durch PPARα bereits gezeigt werden [23]. So weisen sowohl die Acyl-CoA-Synthase als auch die Carnitin-Palmitoyltransferase (CPT)-I, welche die Umwandlung der Fettsäuren in die entsprechenden Carnitin-Derivate katalysieren, funktionelle PPRE in ihren Promotorregionen auf [33-35]. Ob auch der nachfolgende Transport des Acylcarnitins in die mitochondriale Matrix, der durch die Carnitin-Acylcarnitin-Translokase (CACT) vermittelt wird, einer Regulation durch PPARα unterliegt, ist nahe liegend, wurde bisher jedoch nicht untersucht.

Aufgrund seiner Rolle beim Transport der langkettigen Fettsäuren in die mitochondriale Matrix stellt Carnitin einen essenziellen Metabolit für die β-Oxidation von Fettsäuren dar [36-38]. Alle Gewebe, die Fettsäuren als Energiequelle nutzen, benötigen Carnitin für ihre normale Funktion. Carnitin steht dem Körper zum einen aus der Nahrung, zum anderen aus der körpereigenen Biosynthese, die hauptsächlich in der Leber stattfindet, zur Verfügung [39]. Gewebe, die nicht zur Biosynthese befähigt sind, müssen Carnitin aus dem Blut aufnehmen. Die Verteilung des Carnitins im Körper und die intrazelluläre Carnitin-Homöostase werden durch spezifische Membrantransporter, die *novel organic cation transporters* (OCTN) kontrolliert, die in der apikalen Zellmembran lokalisiert sind [40, 41]. Interessanterweise wurden in den Lebern von Ratten, die gefastet oder mit dem synthetischen PPARα-Agonisten Clofibrat behandelt wurden, erhöhte Carnitin-konzentrationen gefunden [42-45], was eine Rolle des PPARα in der Regulation der Carnitinhomöostase vermuten lässt. Inwiefern diese erhöhten Carnitinkonzentrationen in der Leber von Ratten bei Nahrungsentzug bzw. nach Clofibratbehandlung tatsächlich durch eine

Beeinflussung der Carnitinaufnahme und/oder der Carnitin-Biosynthese durch PPARα zustande kommen, ist bislang unbekannt.

Das aus der β-Oxidation der langkettigen Fettsäuren stammende Acetyl-CoA wird unter anderem zur Synthese von Ketonkörpern verwendet, die im Hungerzustand eine bedeutende Energiequelle darstellen. Der damit normalerweise einhergehende Anstieg der Konzentration an zirkulierenden Ketonkörpern im Plasma konnte bei PPARα-defizienten Mäusen nicht oder kaum beobachtet werden [30-32]. Ursächlich hierfür ist die fehlende Induktion der mitochondrialen 3-Hydroxy-3-Methylglutaryl-(mHMG)-CoA-Synthase [32], dem Schlüsselenzym der Ketonkörpersynthese, welches ebenfalls über ein PPRE im Promotor durch PPARα reguliert wird [46, 47]. Nach ihrer Synthese in der Leber werden die Ketonkörper in die peripheren Gewebe transportiert und dort oxidiert. Der Transport der Ketonkörper über Membranen wird durch die Familie der Monocarboxylat-Transporter (MCT) bewerkstelligt, die zur Genfamilie der *solute carrier*-16 gehören [48-51]. Der bisher am umfangreichsten charakterisierte Vertreter MCT1 wird ubiquitär exprimiert und transportiert neben Ketonkörpern auch Laktat, Pyruvat und Butyrat [48]. Die funktionelle Assoziation des MCT1 mit der Ketogenese und seine Rolle in der Energieversorgung der Zellen im Hungerzustand lassen vermuten, dass MCT1 ebenso wie die mHMG-CoA-Synthase einer Regulation durch PPARα unterliegt. Studien hierzu existieren bislang nicht.

PPARα wird vor allem in Geweben mit hohen Raten der Fettsäureoxidation wie Leber, Niere und Herz, aber z. B. auch im Darm und im Gehirn exprimiert [23, 52]. So konnte in zahlreichen Studien gezeigt werden, dass PPARα-Agonisten sowohl vorbeugende als auch akute neuroprotektive Effekte aufweisen, die auf den anti-inflammatorischen und anti-oxidativen Effekten bei PPARα-Aktivierung basieren [53]. Desweiteren wird eine Beteiligung des PPARα an der Regulation der Expression des Prolaktins in Hypophysenzellen diskutiert [54]. Der im Gehirn lokalisierte PPARα spielt darüber hinaus offenbar eine entscheidende Rolle bei der Regulation der Glukosehomöostase des gesamten Körpers im Hungerzustand [55]. Bisher ist allerdings nicht bekannt, inwiefern der PPARα im Hungerzustand nicht nur in der Leber, sondern auch im Gehirn aktiviert wird.

Die für PPAR namensgebenden Peroxisomenproliferatoren, zu denen auch die Fibrate gehören, lösen eine Hepatomegalie, begleitet von einem Anstieg der Zahl und Größe der Peroxisomen, und bei längerer Gabe Lebertumoren aus [56-58]. Dieses Phänomen ist allerdings auf so genannte proliferierende Spezies, wie Ratte und Maus, beschränkt [59, 60]. Bei nicht-proliferierenden Spezies, zu denen z. B. der Mensch, das Schwein, das Meerschweinchen und der Affe gehören, führt die Gabe von Fibraten zwar zu den beschriebenen lipidsenkenden Effekten, eine Peroxisomenproliferation bleibt dagegen aus, was den therapeutischen Einsatz der Fibrate ermöglicht. Mitglieder der nicht-proliferierenden

Spezies weisen eine im Verhältnis geringere Expression des PPARα in der Leber auf und die Induktion PPARα-regulierter Gene ist teilweise schwächer als in proliferierenden Spezies [61-66]. Aus diesem Grund können PPARα-vermittelte Effekte und Zusammenhänge, die meist in Ratten oder Mäusen beobachtet wurden, nur bedingt auf den Menschen übertragen werden. Das Schwein, das wie der Mensch zu den nicht-proliferierenden Spezies gehört, weist eine dem Menschen vergleichbare PPARα-Expression in der Leber [67] sowie ähnliche Stoffwechselmerkmale und anatomische Gegebenheiten auf [68]. Somit stellt das Schwein ein geeignetes Modelltier dar, um die biochemischen Effekte von PPARα-Agonisten auf den Stoffwechsel zu untersuchen und ihre Relevanz für den Menschen einzuschätzen.

Neben der Bedeutung des Schweins als Modelltier für den Menschen hinsichtlich der Analyse von Stoffwechselvorgängen sind solche Forschungsergebnisse aber auch relevant, um Informationen zur Ernährungsphysiologie des Schweins als Nutztier zu erhalten. Es existieren bereits einige Studien, die sich mit der Rolle des PPARα und seiner Stimulierbarkeit durch Futterzusätze bei Nutztieren, wie z. B. dem Rind, beschäftigen [69-71]. Dagegen existieren bisher keine Studien zum Einfluss einer PPARα-Aktivierung durch Futterzusätze auf den Lipidstoffwechsel bei Legehennen, die sich durch eine besonders hohe Rate der Synthese von Triglyzeriden, Phospholipiden und Cholesterol im Vergleich zu Säugern auszeichnen [72].

Die Stärke der Bindung und Aktivierung der PPAR durch Fettsäuren variieren mit ihrer Kettenlänge und dem Sättigungsgrad [73-75]. Als besonders potente PPARα-Agonisten wurden neben mehrfach ungesättigten Fettsäuren (PUFA) auch chemisch modifizierte Fettsäuren wie oxidierte Fettsäuren und zyklische Fettsäuremonomere sowie konjugierte Linolsäuren (CLA) identifiziert [76-80]. Oxidierte Fettsäuren und zyklische Fettsäuremonomere entstehen in fetthaltigen Lebensmitteln zum Beispiel durch Erhitzen, Weiterverarbeitung und unsachgemäße Lagerung [81-83]. Die Aufnahme von oxidierten Fettsäuren und zyklischen Fettsäuremonomeren über den steigenden Konsum an frittierten Lebensmitteln hat dabei eine besondere Relevanz für die Humanernährung [81]. CLA sind positionelle und geometrische Isomere der Linolsäure mit konjugierten Doppelbindungen, die überwiegend über den Verzehr von Wiederkäuerprodukten wie Fleisch und Milch, aber auch in synthetischer Form als Nahrungsergänzungsmittel aufgenommen werden [84-88]. Es konnte bereits gezeigt werden, dass die Aufnahme eines oxidierten Fettes zu einer Aktivierung des PPARα verbunden mit einer Absenkung der Plasmatriglyzeride durch eine gesteigerte β-Oxidation in der Ratte führt [89, 90]. Ebenso existiert eine große Anzahl von Studien, die sich mit den biologischen Wirkungen der CLA auf den tierischen Metabolismus beschäftigen [91-93]. Eine Einbeziehung dieser in der Humanernährung relevanten

natürlichen PPARα-Agonisten in die Untersuchung PPARα-abhängiger Effekte erscheint daher sinnvoll.

Die vorliegende Arbeit beschäftigt sich schwerpunktmäßig mit der Aufklärung neuer Zusammenhänge hinsichtlich der Regulation des Lipid- und Energiestoffwechsels durch PPARα. Aufgrund der erwähnten speziesspezifischen Unterschiede bezüglich einer PPARα-Aktivierung wurden verschiedene Spezies in die Untersuchungen einbezogen. Schließlich wurde auch die Wirkung von natürlichen nutritiven PPARα-Agonisten auf die untersuchten Stoffwechselwege betrachtet.

Insgesamt liegen dieser Arbeit 14 Originalarbeiten zugrunde. Das Ziel dieser Originalarbeiten war die Beantwortung der folgenden Fragen:

- Welche Mechanismen unterliegen den verringerten Cholesterol- und Triglyzeridkonzentrationen in der Leber von Ratten bei Clofibratgabe?
- Bewirken oxidierte Fette als natürliche PPARα-Agonisten vergleichbare Änderungen des Lipidstoffwechsels wie Clofibrat und sind die Wirkungen von PPARα-Agonisten abhängig von der verwendeten Spezies?
- Wird die CACT ebenso wie die Acyl-CoA-Synthase und CPT-I durch PPARα reguliert?
- Welche Wirkmechanismen liegen den erhöhten Carnitin-Konzentrationen in der Leber nach Clofibratgabe bzw. Fasten zugrunde? Können vergleichbare Effekte auch mit natürlichen PPARα-Agonisten beobachtet werden?
- Wird MCT1 ebenfalls durch PPARα reguliert und gibt es dabei speziesspezifische Unterschiede? Welche Effekte sind bei Verwendung von nutritiven PPARα-Agonisten zu beobachten?
- Kommt es während des Fastens zu einer Aktivierung des PPARα im Gehirn, und ist PPARα in die Regulation der Expression von Hypophysenhormonen involviert?

2 Eigene Originalarbeiten (A1 – A14)

A1 **König B**, Koch A, Spielmann J, Hilgenfeld C, Stangl GI, Eder K. Activation of PPARα lowers synthesis and concentration of cholesterol by reduction of nuclear SREBP-2. *Biochemical Pharmacology* 2007;73:574-585.

A2 **König B**, Koch A, Spielmann J, Hilgenfeld C, Hirche F, Stangl GI, Eder K. Activation of PPARα and PPARγ reduces triacylglycerol synthesis in rat hepatoma cells by reduction of nuclear SREBP-1. *European Journal of Pharmacology* 2009;605:23-30.

A3 Koch A, **König B**, Spielmann J, Leitner A, Stangl GI, Eder K. Thermally oxidized oil increases the expression of insulin-induced genes and inhibits activation of sterol regulatory element-binding protein-2 in rat liver. *Journal of Nutrition* 2007;137:2018-2023.

A4 Luci S, **König B**, Giemsa B, Huber S, Hause G, Kluge H, Stangl GI, Eder K. Feeding of a deep-fried fat causes PPARα activation in the liver of pigs as a non-proliferating species. *British Journal of Nutrition* 2007;97:872–882.

A5 **König B**, Eder K. Differential action of 13-HPODE on PPARα downstream genes in rat Fao and human HepG2 hepatoma cell lines. *Journal of Nutritional Biochemistry* 2006;17:410-418.

A6 **König B**, Kluge H, Haase K, Brandsch C, Stangl GI, Eder K. Effects of clofibrate treatment in laying hens. *Poultry Science* 2007;86:1187-1195.

A7 **König B**, Spielmann J, Haase K, Brandsch C, Kluge H, Stangl GI, Eder K. Effects of fish oil and conjugated linoleic acids on expression of target genes of PPARα and sterol regulatory element-binding proteins in the liver of laying hens. *British Journal of Nutrition* 2008;100:355-363.

A8 Gutgesell A, Wen G, **König B**, Koch A, Spielmann J, Stangl GI, Eder K, Ringseis R. Mouse carnitine-acylcarnitine translocase (CACT) is transcriptionally regulated by PPARα and PPARδ in liver cells. *Biochimica et Biophysica Acta – General subjects* 2009;1790:1206-1216.

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A10 Koch A, **König B**, Stangl GI, Eder K. PPARα mediates transcriptional upregulation of novel organic cation transporters-2 and -3 and enzymes involved in hepatic carnitine synthesis. *Experimental Biology and Medicine* 2008;233; 356-365.

A11 Koch A, **König B**, Luci S, Stangl GI, Eder K. Dietary oxidized fat up regulates the expression of organic cation transporters in liver and small intestine and alters carnitine concentrations in liver, muscle and plasma of rats. *British Journal of Nutrition* 2007;98:882-889.

A12 **König B**, Koch A, Giggel K, Dordschbal B, Eder K, Stangl GI. Monocarboxylate transporter (MCT)-1 is up-regulated by PPARα. *Biochimica et Biophysica Acta – General subjects* 2008;1780:899-904.

A13 **König B**, Fischer S, Schlotte S, Wen G, Eder K, Stangl GI. Monocarboxylate transporter 1 and CD147 are up-regulated by natural and synthetic peroxisome proliferator-activated receptor α agonists in livers of rodents and pigs. *Molecular Nutrition & Food Research* 2010;54:1248-1256.

A14 **König B**, Rauer C, Rosenbaum S, Brandsch C, Eder K, Stangl GI. Fasting upregulates PPARα target genes in brain and influences pituitary hormone expression in a PPARα dependent manner. *PPAR Research* 2009;2009:801609.

A1 **König B**, Koch A, Spielmann J, Hilgenfeld C, Stangl GI, Eder K:

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Activation of PPARα lowers synthesis and concentration of cholesterol by reduction of nuclear SREBP-2

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ABSTRACT

To elucidate the mechanisms underlying the cholesterol lowering effects of PPARα agonists we investigated key regulators of cholesterol synthesis and uptake in rats and in the rat hepatoma cell line Fao after treatment with the PPARα agonists clofibrate and WY 14,643, respectively. In rat liver as well as in Fao cells, PPARα activation led to a decrease of transcriptionally active nuclear SREBP-2. mRNA concentrations of the key regulators of SREBP processing, Insig-1 in rat liver and Insig-1 and Insig-2a in Fao cells, were increased upon PPARα activation. Thus we suggest, that the observed reduction of the amount of nuclear SREBP-2 was due to an inhibition of the processing of the precursor protein. Both, in rat liver and in Fao cells, mRNA concentrations of the SREBP-2 target genes HMG-CoA reductase (EC1.1.1.34) and LDL receptor were reduced after treatment with the PPARα agonists. Furthermore, treatment of Fao cells with WY 14,643 reduced cholesterol synthesis. As a result, the amount of total cholesterol in liver, plasma and lipoproteins of clofibrate treated rats and in WY 14,643 treated Fao cells was decreased compared to control animals and cells, respectively. In conclusion, we could show a novel link between PPARα and cholesterol metabolism by demonstrating that PPARα activation lowers cholesterol concentration by reducing the abundance of nuclear SREBP-2.

1. Introduction

In animal cells, lipid homeostasis is maintained by a feedback mechanism that regulates the transcription of genes involved in lipid synthesis and uptake. Transcription factors that regulate the coordinated expression of these genes are the family of sterol regulatory element binding proteins (SREBPs) [1]. Three isoforms of SREBP are known in mammals, SREBP-1a, SREBP-1c and SREBP-2. While SREBP-1c, the predominant isoform in adult liver, preferentially activates genes required for fatty acid synthesis and their incorporation into triacylglycerols and phospholipids, SREBP-2 preferentially activates the low density lipoprotein (LDL) receptor gene and various genes required for cholesterol synthesis such as 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase (EC1.1.1.34) [2]. SREBP-1a is an activator of both, the cholesterol and fatty acid biosynthetic pathway, but it is present in much lower amounts in liver than the other two forms [3]. SREBPs are

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Abbreviations: ACO, acyl-CoA oxidase; Cyp, cytochrome P450; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HDL, high density lipoprotein; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; Insig, insulin-induced gene; LDL, low density lipoprotein; PPAR, peroxisome proliferator-activated receptor; SCAP, SREBP cleavage activating protein; SREBP, sterol regulatory element binding protein; VLDL, very low density lipoprotein

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synthesized as 120-kDa integral membrane proteins of the endoplasmic reticulum and form a complex with SREBP cleavage activating protein (SCAP). When sterol concentrations in cells are high, the SCAP/SREBP complex is retained in the ER. When cells are depleted of sterols, SCAP escorts SREBP to the Golgi for proteolytic processing. In the Golgi, sequential cleavages occur, releasing the mature N-terminal domain of SREBP that then translocates to the nucleus and activates transcription of sterol regulatory element-containing genes [2,4,5].

Retention of the SCAP/SREBP complex in the ER is mediated by sterol-dependent binding of the complex to one of two ER retention proteins designated insulin-induced gene (Insig)-1 and -2 [6,7]. Insig-1 and Insig-2 are integral membrane proteins that are expressed in most tissues with especially high expression in the liver [6,7]. Insig-1 differs from Insig-2 in its requirement of nuclear SREBPs for its expression providing a feedback mechanism for lipid homeostasis [7]. Furthermore, Insig-1 but not Insig-2 is able to cause ER retention of the SCAP/SREBP complex in the absence of sterols at high expression levels [7]. Unlike Insig-1, Insig-2a, the liver-specific isoform of Insig-2, is suppressed by insulin and induced by fasting [8]. Recently it has been shown that activation of the peroxisome proliferator-activated receptor (PPAR)-γ by rosiglitazone induced the expression of Insig-1 in white adipose tissue via a PPAR response element in the promoter region of Insig-1 [9].

PPAR are transcription factors belonging to the superfamily of nuclear receptors that can be activated by fatty acids and their metabolic derivatives. They are implicated in the regulation of lipid and lipoprotein metabolism, glucose homeostasis, cellular differentiation, cancer development as well as in the control of the inflammatory response [reviewed in 10–12]. There are three PPAR isotypes, PPARα, PPARβ/δ and PPARγ, all of which regulate the expression of target genes by binding to DNA sequence elements as heterodimers with the 9-cis retinoic acid receptor after activation [13]. PPARα is highly expressed in tissues with high fatty acid oxidation, in which it controls a comprehensive set of genes that regulate most aspects of lipid catabolism [14]. Furthermore, the action of fibrates, a class of hypolipidemic drugs, on lipid metabolism is mediated by the activation of PPARα [15].

Both, natural and synthetic ligands of PPARα are known to lower the plasma and liver cholesterol concentrations in man and animals [16–19]. Mice in which the PPARα gene has been disrupted (PPARα-null (KO) mice) are hypercholesterolemic [20] and show a dysregulation in the hepatic expression of HMG-CoA reductase during the diurnal variation of cholesterogenesis [21]. Also other studies suggested an involvement of PPARα in the regulation of cholesterol synthesis; however, both, stimulatory and inhibitory effects of fibrates on hepatic HMG-CoA reductase and cholesterol synthesis in rats have been reported depending on fibrates and model used [22–24].

The objective of the present study was to further evaluate the mechanisms underlying the cholesterol lowering effects of PPARα activation in rat liver. Therefore we treated rats with the PPARα agonist clofibrate. Based on their central role in the regulation of the cholesterol metabolism, we focused on effects of PPARα activation on the gene expression and nuclear concentration of SREBP-2 and on the gene expression of Insig-1 and Insig-2a as key regulators of SREBP-2 activity. Furthermore, we analyzed the expression of SREBP-2 target genes HMG-CoA reductase and LDL receptor. To verify the obtained results, we used the Fao cell model which is commonly used to study the effects of PPARα agonists on hepatic lipid metabolism *in vitro* [25–27]. We examined the influence of WY 14,643, another PPARα agonist with high specificity [28], on Insigs, SREBP-2 and its target genes and on cholesterol synthesis in Fao cells.

2. Material and methods

2.1. Materials

WY 14,643, DMSO, MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide; Thiazol blue), TRIZOL™ reagent, SYBR® Green I, protease inhibitor mix and the anti-rabbit-IgG peroxidase conjugate antibody were purchased from Sigma-Aldrich (Steinheim, Germany). Ethyl 2-(4-chlorophenoxy)-2-methylpropionate (clofibrate) was obtained from Fluka Chemie GmbH (Buchs, Switzerland). Rat hepatoma Fao cell line was purchased from ECACC (Salisbury, UK). F-12 Nutrient Mixture (Ham), gentamycin and fetal calf serum (FCS) were obtained from Invitrogen (Karlsruhe, Germany). Reverse transcriptase was supplied by MBI Fermentas (St. Leon-Rot, Germany), and Taq polymerase by Promega (Mannheim, Germany). Bicinchoninic acid assay reagent was a product of Interchim (Montfucon, France). The nitrocellulose blotting membrane was from Pall (Pensacola, FL, USA), and the ECL-reagent kit from GE Healthcare (München, Germany). The anti-SREBP-2 antibody (rabbit polyclonal IgG) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and the anti-β-Actin antibody (rabbit polyclonal IgG) was purchased from Abcam Ltd. (Cambridge, UK). Autoradiography film for Western blot analysis (Agfa Cronex) was from Roentgen Bender (Baden-Baden, Germany). Male Sprague–Dawley rats were supplied by Charles River (Sulzfeld, Germany). Radioactive [1,2-^{14}C] acetate (specific activity 110 mCi/mmol) was from Hartmann Analytic (Braunschweig, Germany), and TLC sheets (Si 60 aluminium sheets) were from VWR International (Darmstadt, Germany).

2.2. Cell culture

Fao rat hepatoma cells were cultured in Ham-F12 medium supplemented with 10% FCS and 0.05 mg/ml gentamycin. Cells were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. For experiments, Fao cells were seeded in 24- or 6-well culture plates at a density of 2.1×10^5 and 1.05×10^6 cells, respectively, per well and used prior reaching confluence (usually 3 days after seeding). Experiments were carried out in low-serum medium (0.5% FCS) as commonly used for PPARα activation studies with several agonists since PPARα activation is more pronounced under these conditions [29–31]. Furthermore, expression of SREBP-2 target genes is upregulated compared to full-serum medium [32]. The cells were preincubated with low-serum medium for 16 h and then stimulated for 6 and 24 h with WY 14,643. WY 14,643 was

added to the low-serum medium from a stock solution in DMSO. Final DMSO concentration did not exceed 0.1% (v/v). Cells treated with the appropriate vehicle concentration were used as a control. Cell viability of Fao cells was not reduced by 24 h incubation with WY 14,643 up to a concentration of 100 μM as demonstrated by the MTT assay ([33]; data not shown).

2.3. Animals, diets and sample collection

Male Sprague–Dawley rats, with an average initial body weight of 366 g (±28; S.D.), were randomly assigned to two groups ($n = 8$) and kept individually in Macrolon cages in a room controlled for temperature (22 ± 2 °C), relative humidity (50–60%) and light (12 h light/dark cycle). All experimental procedures described followed established guidelines for the care and handling of laboratory animals and were approved by the council of Saxony-Anhalt. The animals were treated with 250 mg/kg of clofibrate in 1 ml sunflower oil or with an equal volume of the vehicle sunflower oil by gavage once a day 2 h after beginning of the light cycle. All rats were fed a commercial standard basal diet (''altromin 1324'', Altromin GmbH, Lage, Germany). To standardize food intake, the diets were fed daily in restricted amounts of 18 g per day. Water was available ad libitum from nipple drinkers during the whole experiment. At day 4 of treatment, animals received the last dose of clofibrate or vehicle alone and 9 g of the diet and were killed 4 h later by decapitation under light anaesthesia with diethyl ether. Rats were non-fasted before killing because food deprivation before killing leads to a significant down regulation of the genes involved in cholesterol metabolism to be considered in this study [34]. Blood was collected into heparinized polyethylene tubes. The liver was excised. Plasma was obtained by centrifugation of the blood (1100 × g, 10 min, 4 °C) and stored at −20 °C. Liver samples for RNA isolation and lipid extraction were snap-frozen in liquid nitrogen and stored at −80 °C.

2.4. RT-PCR analysis

Total RNA was isolated from Fao cells after the incubation in 24-well plates and rat livers, respectively, by TRIZOL™ reagent according to the manufacturer's protocol. cDNA synthesis was carried out as described [31]. The mRNA expression of genes was measured by real-time detection PCR using SYBR® Green I. Real-time detection PCR was performed with 1.25 U Taq DNA polymerase, 500 μM dNTPs and 26.7 pmol of the specific primers (Operon Biotechnologies, Cologne, Germany; Table 1). For determination of mRNA concentration a threshold cycle (C_t) was obtained from each amplification curve using the software RotorGene 4.6 (Corbett Research, Australia). Calculation of the relative mRNA concentration was made using the $\Delta\Delta C_t$ method as previously described [35]. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH; EC1.2.1.12) was used for normalization.

2.5. Immunoblot analysis

Whole cell extracts of Fao cells were prepared by lysis in 20 mM Tris, 150 mM NaCl, 0.1% Triton X-100, 1% protease inhibitor mix, pH 7.5, after the incubation in six-well-plates. The protein content was determined by the bicinchoninic acid assay. Nuclear extracts of rat livers were prepared from fresh tissue samples (150 mg) according to Woo et al. [36] and equal amounts of proteins were pooled from four rats per treatment group. Thirty to fifty micrograms of Fao cell proteins and 100 μg of pooled rat liver nuclear extracts, respectively, were separated on 10% sodium dodecylsulfate acrylamide gel electrophoresis according to the method of Laemmli et al. [37] and electrotransferred to a nitrocellulose membrane. After blocking in 50 mM Tris, 150 mM NaCl, 0.2% Tween-20, pH 7.5, containing 3% nonfat dry milk, bands corresponding to nuclear SREBP-2 (for Fao cell and rat liver samples) and β-Actin (for Fao cell samples, as a loading control) were visualized with

Table 1 – Characteristics of the specific primers used for RT-PCR analysis

Gene	Forward and reverse primers	bp	Annealing temperature	NCBI GenBank
ACO	5′ CTTTCTTGCTTGCCTTCCTTCTCC 3′ 5′ GCCGTTTCACCGCCTCGTA 3′	415	60 °C	NM017340
Cyp4A1	5′ CAGAATGGAGAATGGGGACAGC 3′ 5′ TGAGAAGGGCAGGAATGAGTGG 3′	460	65 °C	NM175837
GAPDH	5′ GCATGGCCTTCCGTGTTCC 3′ 5′ GGGTGGTCCAGGGTTTCTTACTC 3′	337	60 °C	BC059110
HMG-CoA reductase	5′ AAGGGGCGTGCAAAGACAATC 3′ 5′ ATACGGCACGGAAAGAACCATAGT 3′	406	57 °C	BC064654
Insig-1	5′ ATTTGGCGTGGTCCTGGCTCTGG 3′ 5′ GCGTGGCTAGGAAGGCGATGGTG 3′	389	62 °C	NM022392
Insig-2a	5′ GACGGATGTGTTGAAGGATTTCT 3′ 5′ TGGACTGAAGCAGACCAATGTC 3′	83	59 °C	AY156086
LDL receptor	5′ AGAACTGCGGGGCCGAAGACAC 3′ 5′ AAACCGCTGGGACATAGGCACTCA 3′	490	65 °C	NM175762
SREBP-2	5′ ATCCGCCCACACTCACGCTCCTC 3′ 5′ GGCCGCATCCCTCGCACTG 3′	312	65 °C	BC101902

enhanced chemiluminescence reagents and exposed to autoradiography film. Films were analyzed with the Gel-Pro Analyzer software (Intas, Upland, CA, USA).

2.6. Determination of triacylglycerol and cholesterol concentrations in Fao cells, liver, plasma and lipoproteins

Lipid extraction from Fao cells after 24 h of incubation with 100 μM of WY 14,643 or vehicle alone and measurement of cellular and secreted triacylglycerols and cellular cholesterol was carried out as described [31]. Rat liver lipids were extracted using a mixture of *n*-hexane and isopropanol (3:2, v/v) [38]. Aliquots of the lipid extracts were dried and dissolved in a small volume of Triton X-100 [39]. Plasma lipoproteins were separated by stepwise ultracentrifugation (900,000 × *g*, 1.5 h, 4 °C; very low density lipoproteins (VLDL) + chylomicrons: $\delta < 1.006$ g/ml; LDL: $1.006 < \delta < 1.063$ g/ml; high density lipoproteins (HDL): $\delta > 1.063$ g/ml) using a Micro-Ultracentrifuge (Sorvall Products, Bad Homburg, Germany).

Concentrations of total cholesterol and triacylglycerols were determined using an enzymatic reagent kit (Ecoline S+, Merck, Darmstadt, Germany).

2.7. Determination of cholesterol synthesis

After a pre-incubation of 22 h at 37 °C, 5% CO_2 with the different concentrations of WY 14,643, 0.2 μCi [1,2-^{14}C]acetate (specific activity 110 mCi/mmol) were added in order to measure the newly synthesized cholesterol [40,41]. Cells were further incubated for 2 h at 37 °C, 5% CO_2. After incubation the cells were washed twice with cold PBS. The lipids were extracted twice with a mixture of hexane and isopropanol (3:2, v/v) [38]. After removing the solvents in a vacuum centrifugal evaporator the lipids were dissolved in 80 μl chloroform, 4 μL of which were applied to 10 × 20 cm^2 TLC using a TLC spotter PS01 (Desaga, Heidelberg, Germany). Plates were developed with a mixture of hexane, diethyl ether and acetic acid (80:20:3, v/v/v) [42]. Lipid-bound radioactivity was detected and quantified by autoradiography (Fuji imager system, Tina 2 software, Raytest, Straubenhart, Germany).

2.8. Statistical analysis

Treatment effects were analyzed by one-way ANOVA using the Minitab Statistical Software (Minitab, State College, PA, USA). In the cell culture experiments, means of the four treatments were compared by Fisher's multiple range test if the *F* value was significant ($P < 0.05$). Differences with $P < 0.05$ were considered to be significant.

3. Results

3.1. Activation of PPARα in rat liver and Fao cells

We treated male Sprague–Dawley rats with the PPARα agonist clofibrate for 4 days. Animals were killed 4 h after the last dose of clofibrate and typical characteristics of PPARα activation were analyzed. Treatment with clofibrate led to an increase in the relative liver masses of the rats of about 22% compared to control animals ($P < 0.001$; Table 2). Rats treated with the PPARα agonist had higher mRNA concentrations of the PPARα downstream genes acyl-CoA oxidase (ACO; EC1.3.3.6) and cytochrome P450 (Cyp) 4A1 (EC1.14.15.3) of about 9- and 18-fold, respectively, in their livers compared to control animals ($P < 0.001$; Fig. 1). Furthermore, the triacylglycerol concentrations of the livers of rats treated with clofibrate were about 40% lower than those of control rats ($P < 0.05$; Table 2). The concentrations of triacylglycerols in plasma and VLDL + chylomicrons were also reduced about 59 and 81%, respectively, in clofibrate treated rats compared to control animals ($P < 0.001$).

Next, we incubated rat hepatoma Fao cells with increasing amounts of the PPARα agonist WY 14,643 for 6 and 24 h. Incubation of Fao cells with WY 14,643 for 6 and 24 h, respectively, led to a significant increase of the mRNA concentration of ACO and Cyp4A1 ($P < 0.05$; Fig. 2). Increase of ACO mRNA concentration by incubation of Fao cells with WY 14,643 was more pronounced after 6 h compared to 24 h treatment, whereas Cyp4A1 mRNA increased more after 24 h than after 6 h treatment, compared to control cells. Furthermore, we analyzed the influence of the PPARα agonist on the concentration of cellular and secreted triacylglycerols in Fao

Table 2 – Relative liver mass and triacylglycerol and cholesterol concentrations in liver, plasma and lipoproteins of rats treated with clofibrate compared to control rats

	Control	Clofibrate
Relative liver mass (g/kg body mass)	35.7 ± 2.7	45.7 ± 3.1**
Triacylglycerols		
Liver (μmol/g)	32.43 ± 7.26	19.75 ± 4.85*
Plasma (mM)	0.92 ± 0.19	0.38 ± 0.13**
VLDL + chylomicrons (mM)	0.74 ± 0.17	0.14 ± 0.03**
Total cholesterol		
Liver (μmol/g)	14.92 ± 2.50	12.85 ± 3.54*
Plasma (mM)	1.60 ± 0.34	0.49 ± 0.11**
VLDL + chylomicrons (mM)	0.09 ± 0.03	0.01 ± 0.00**
LDL (mM)	0.41 ± 0.12	0.12 ± 0.02**
HDL (mM)	0.92 ± 0.20	0.20 ± 0.05**

Values are means ± S.D. ($n = 8$). The asterisks indicate significant differences from control animals (*$P < 0.05$; **$P < 0.001$).

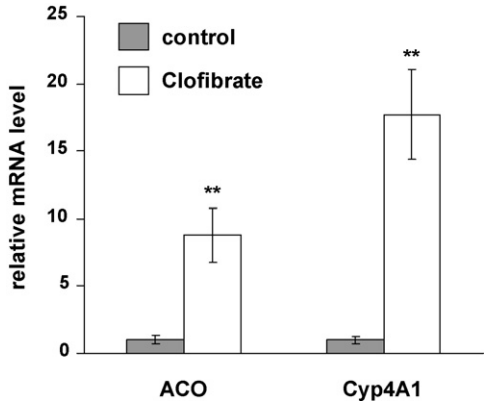

Fig. 1 – Effect of the PPARα agonist clofibrate on the mRNA concentration of PPARα downstream genes ACO and Cyp4A1 in rat liver. Rats were treated orally with 250 mg/kg of clofibrate for 4 days. Control animals obtained the appropriate volume of the vehicle sun flower oil. Total RNA was extracted from rat livers and relative mRNA concentrations were determined by realtime detection RT-PCR analysis using GAPDH mRNA concentration for normalization. Values are means ± S.D. ($n = 8$). **Significantly different from control animals, $P < 0.001$.

cells. The concentrations of cellular triacylglycerols of Fao cells incubated with WY 14,643 for 24 h was about 13% lower compared to control cells treated with vehicle alone [control: 41.3 ± 0.9 nmol mg cell protein^{-1}, 100 μM WY 14,643: 35.8 ± 4.4 nmol·mg cell protein^{-1}; values are means ± S.D. ($n = 3$); $P < 0.05$]. The amount of secreted triacylglycerols in VLDL was measured in the culture medium after incubation. Cells incubated with WY 14,643 for 24 h secreted about 24% less triacylglycerols than control cells [control: 411 ± 30 nmol·mg cell protein^{-1} 24 h^{-1}, 100 μM WY 14,643: 313 ± 41 nmol·mg cell protein^{-1} 24 h^{-1}; values are means ± S.D. ($n = 3$); $P < 0.05$].

3.2. *Influence of PPARα agonists on Insig-1, Insig-2a and SREBP-2 in rat liver and Fao cells*

To study the influence of PPARα activation on key regulators of cholesterol synthesis and uptake, we analyzed the mRNA concentrations of Insig-1, Insig-2a and SREBP-2. Furthermore, we determined the amount of the nuclear form of SREBP-2 by Western-blotting. In rats treated with clofibrate, the relative mRNA concentration of Insig-1 in the liver was about 80% higher than in control animals ($P < 0.05$; Fig. 3A), whereas the mRNA concentration of Insig-2a, the liver specific transcript of the Insig-2 gene, was about 80% lower in the liver of clofibrate treated rats than in control rats ($P < 0.001$; Fig. 3A). The concentration of SREBP-2 mRNA in the liver of rats treated with the PPARα agonist was about 40% lower than that of control rats ($P < 0.05$; Fig. 3A). To analyze the amount of nuclear SREBP-2, we isolated the nuclear fractions of livers of the rats. The relative protein level of the mature SREBP-2 in the livers of rats treated with clofibrate was about 70% lower than in control animals ($P < 0.05$; Fig. 3B and C).

Treatment of Fao cells with WY 14,643 for 6 h led to a significant and concentration dependent increase of Insig-1 mRNA concentration compared to control cells, and this effect was abolished after 24 h of treatment (Fig. 4A). At the highest concentration of WY 14,643 used, Insig-1 mRNA concentration was about 80% higher than that of control cells after 6 h of incubation ($P < 0.05$). Likewise, incubation of Fao cells with 100 μM WY 14,643 for 6 h increased the mRNA concentration of Insig-2a about 50% compared to control cells ($P < 0.05$). After 24 h of incubation, Insig-2a mRNA concentration in WY 14,643 treated cells still tended to be higher (about 20%, $P < 0.10$) than in control cells (Fig. 4A). SREBP-2 mRNA concentration was unchanged in cells incubated with WY 14,643 for 6 h, whereas it was about 20% lower after 24 h of treatment with all WY 14,643 concentrations used compared to control cells ($P < 0.05$; Fig. 4A). Western blot analysis of whole Fao cell lysates revealed that the relative protein concentration of the mature SREBP-2 after treatment of Fao cells with 100 μM of WY 14,643

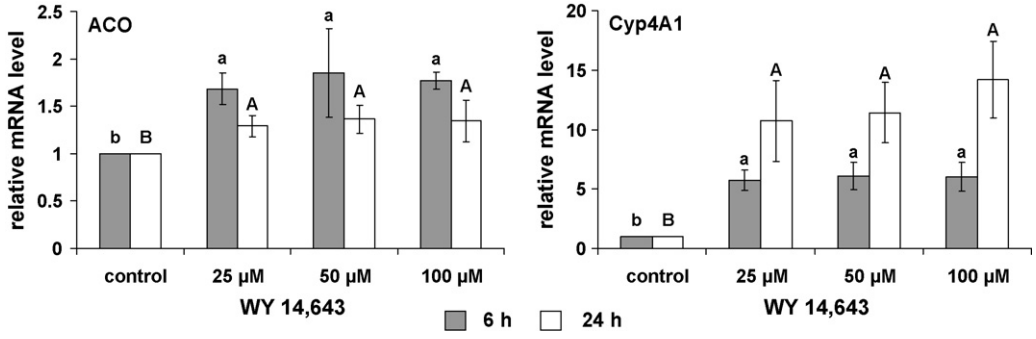

Fig. 2 – Effect of the PPARα agonist WY 14,643 on the mRNA concentration of PPARα downstream genes ACO and Cyp4A1 in Fao cells. Fao cells were grown in culture medium until subconfluent state. Medium was then changed to low-serum medium (0.5% fetal calf serum). After 16 h preincubation in this medium, cells were incubated with increasing concentrations of the PPARα agonist WY 14,643 from 25 to 100 μM for 6 and 24 h, respectively. Control cells were incubated with low-serum medium containing vehicle alone. Total RNA was extracted from cells and relative mRNA concentrations were determined by real time detection RT-PCR analysis using GAPDH mRNA concentration for normalization. Values are means ± S.D. ($n = 3$). Bars without the same superscript letter are significantly different, $P < 0.05$. Small letters (a and b) denote differences in 6 h incubation, capital letters (A and B) denote differences in 24 h incubation.

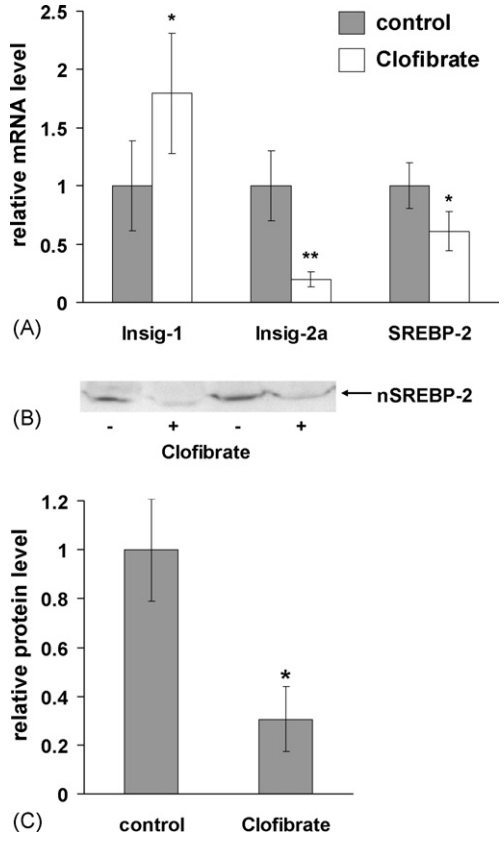

Fig. 3 – Effect of clofibrate treatment on Insig-1, Insig-2a and SREBP-2 in rat liver. Rats were treated orally with 250 mg/kg of clofibrate for 4 days. Control animals obtained the appropriate volume of the vehicle sun flower oil. (A) Total RNA was extracted from rat livers and Insig-1, Insig-2a and SREBP-2 mRNA concentrations were determined by real time detection RT-PCR analysis using GAPDH mRNA concentration for normalization. Values are means ± S.D. ($n = 8$). (B) Liver nuclear extracts of four animals per group were pooled and 100 μg of the samples were separated by 10% SDS-PAGE and immunoblotted with anti-SREBP-2 antibodies. (C) Relative intensity of the bands in (B) was quantified by densitometry. The asterisks indicate significant differences from control animals ($^{*}P < 0.05$; $^{**}P < 0.001$).

for 6 h was not different from that of the control cells (Fig. 4B and C). After 24 h of treatment, mature SREBP-2 concentration was about 35% lower compared to untreated cells ($P < 0.05$).

3.3. *Influence of PPARα agonists on the mRNA concentration of HMG-CoA reductase and LDL receptor in rat liver and Fao cells*

We analyzed the effect of PPARα agonists on the expression of SREBP-2 target genes involved in cholesterol synthesis and uptake. In rats treated with clofibrate, the relative mRNA concentration of HMG-CoA reductase in the liver was about 40% lower than in control animals ($P < 0.05$; Fig. 5). Furthermore, treatment of rats with the PPARα agonist led to a reduction of LDL receptor mRNA concentration about 27% compared to control rats ($P < 0.05$; Fig. 5).

The level of HMG-CoA reductase mRNA in Fao cells after treatment with different concentrations of WY 14,643 for 6 h was not changed compared to control cells. Incubation of Fao cells with WY 14,643 for 24 h led to a reduction of HMG-CoA reductase mRNA concentration about 38, 35 and 24% at 25, 50 and 100 μM WY 14,643, respectively, compared to untreated cells ($P < 0.05$; Fig. 6). LDL receptor mRNA concentration was unchanged in cells incubated with WY 14,643 for 6 h compared to control cells. After 24 h treatment, LDL receptor mRNA concentration was about 30, 35 and 33% lower in Fao cells incubated with 25, 50 and 100 μM WY 14,643, respectively, than in untreated cells ($P < 0.05$; Fig. 6).

3.4. *Effect of PPARα agonists on cholesterol concentration in liver, plasma and lipoproteins of rats and on cholesterol concentration and synthesis in Fao cells*

PPARα activation in rats by clofibrate reduced the amount of total cholesterol in rat liver about 14% compared to control rats ($P < 0.05$; Table 2). Also, the cholesterol concentration of the plasma of clofibrate treated rats was about 69% lower than that of untreated animals ($P < 0.001$). The amount of total cholesterol in VLDL + chylomicrons, LDL and HDL was decreased about 89, 70 and 78%, respectively, by treatment of rats with the PPARα agonist ($P < 0.001$; Table 2).

In Fao cells treated with 100 μM WY 14,643 for 24 h, the concentration of cellular cholesterol was about 16% lower compared to control cells incubated with vehicle alone [control: 21.9 ± 1.5 nmol·mg cell protein^{-1}, 100 μM WY 14,643: 18.4 ± 3.1 nmol·mg cell protein^{-1}; values are means ± S.D. ($n = 3$); $P < 0.05$]. Furthermore, cholesterol synthesis was significantly lower about 27, 25 and 44% in Fao cells incubated with 25, 50 and 100 μM WY 14,643, respectively, for 24 h compared to control cells ($P < 0.05$; Fig. 7).

4. Discussion

The cholesterol concentration in mammalian cells is tightly controlled by a feedback mechanism involving Insigs, SCAP and SREBPs [43]. The aim of this study was to elucidate the mechanism by which PPARα ligands influence cholesterol synthesis and uptake in rat liver. For that, we first examined the effect of clofibrate treatment on the cholesterol metabolism of rats. Clofibrate is known to be a hypolipidemic drug and its plasma triacylglycerol and cholesterol lowering effects are well reported [18]. Treatment of rats with clofibrate for 4 days led to a strong PPARα activation as indicated by an upregulation of the PPARα target genes ACO and Cyp4A1 and increased relative liver weights which are due to the induced peroxisome proliferation [44,45]. Both, ACO and Cyp4A1 are typical PPARα downstream genes and are considered as marker genes for PPARα activation [46,47]. The increased expression of these genes involved in fatty acid β-oxidation is one of the mechanisms underlying the hypotriglyceridemic effect upon PPARα activation. Others are increased hydrolysis of plasma triglycerides, stimulation of cellular fatty acid uptake, decreased synthesis of fatty acids and triglycerides and

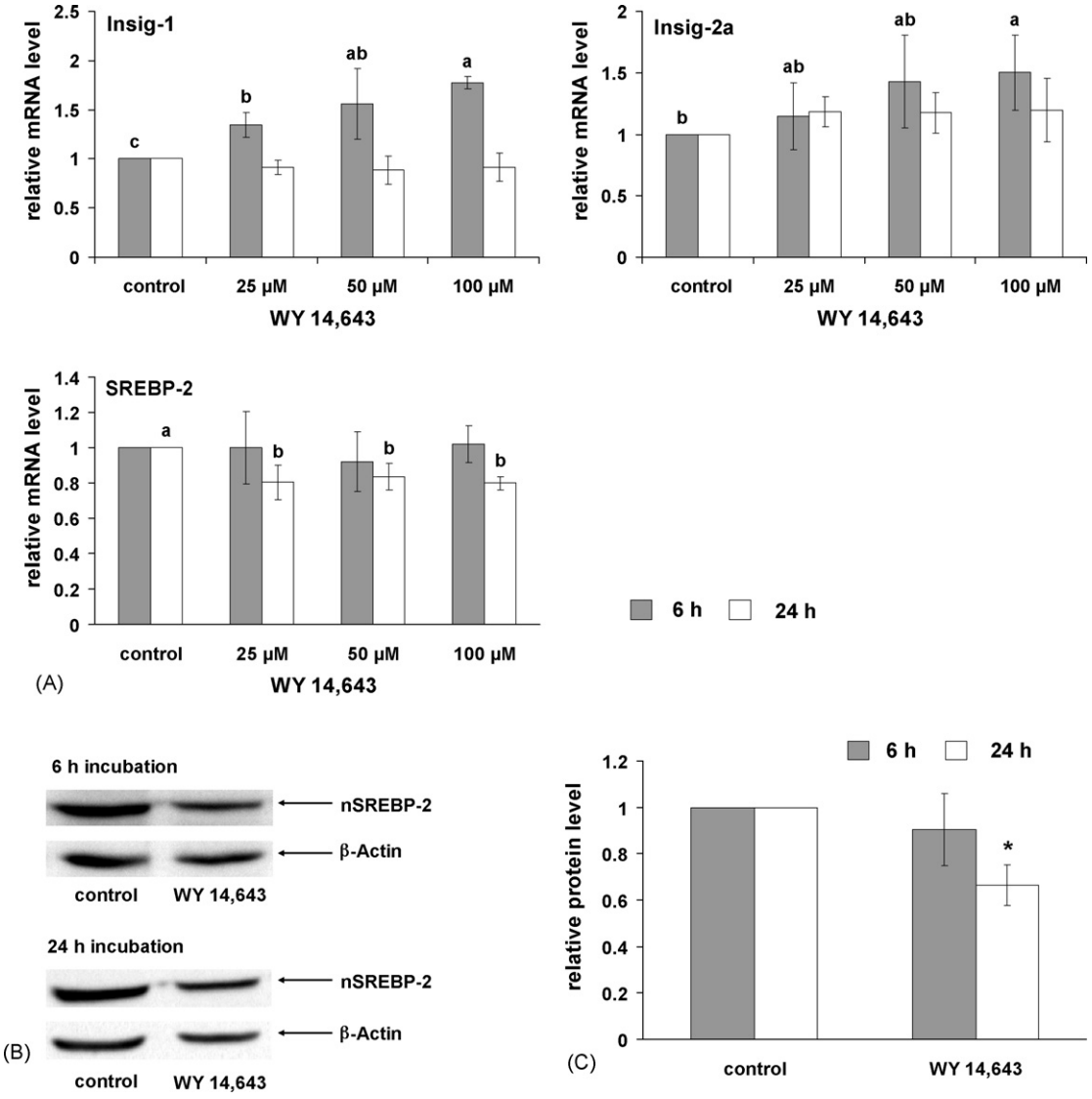

Fig. 4 – Effect of WY 14,643 on Insig-1, Insig-2a and SREBP-2 in Fao cells. Fao cells were grown in culture medium until subconfluent state. Medium was then changed to low-serum medium (0.5% fetal calf serum). After 16 h preincubation in this medium, cells were incubated with increasing concentrations of the PPARα agonist WY 14,643 from 25 to 100 μM for 6 and 24 h, respectively. Control cells were incubated with low-serum medium containing vehicle alone. (A) Total RNA was extracted from cells and Insig-1, Insig-2a and SREBP-2 mRNA concentrations were determined by realtime detection RT-PCR analysis using GAPDH mRNA concentration for normalization. (B) After cell lysis, equal amounts of proteins were separated by 10% SDS-PAGE and immunoblotted with anti-SREBP-2 and anti-β-Actin antibodies. Representative immunoblots after 6 and 24 h incubation of Fao cells with or without 100 μM WY 14,643 are shown. (C) Relative intensity of the bands in (B) was quantified by densitometry using β-Actin-specific band as loading control. Values are means ± S.D. ($n = 3$). Bars without the same superscript letter (a–c) are significantly different, $P < 0.05$. *Significantly different from control cells (*$P < 0.05$).

decreased production of VLDL [15]. Indeed, rats treated with the PPARα agonist had markedly reduced triacylglycerol levels in liver, plasma and VLDL + chylomicrons. Second, to verify the results obtained in the rat study we used an *in vitro* model and the PPARα agonist WY 14,643. Compared to clofibrate, WY 14,643 is a more potent PPARα agonist and exhibits a more strict PPAR subtype specificity [28,48] allowing us to assign the observed effects actually to an activation of PPARα. Incubation of the Fao cells with WY 14,643 for 6 and 24 h led to a strong activation of PPARα as indicated by several fold increased mRNA concentrations of ACO and Cyp4A1. Furthermore, treatment of cells with WY 14,643 largely reduced the concentration of intracellular and secreted triacylglycerols.

Next, we analyzed the effect of PPARα activation on key regulators of cholesterol synthesis and uptake. Both, in the liver of rats treated with clofibrate and in Fao cells treated with WY 14,643 for 24 h, the amount of the transcriptionally active form of SREBP-2 in the nucleus was reduced compared to

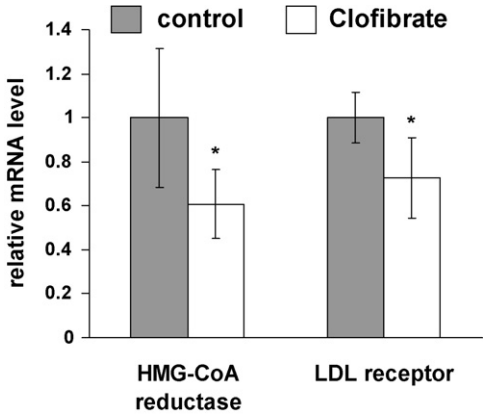

Fig. 5 – Effect of clofibrate treatment on the mRNA concentrations of HMG-CoA reductase and LDL receptor in rat liver. Rats were treated orally with 250 mg/kg of clofibrate for 4 days. Control animals obtained the appropriate volume of the vehicle sun flower oil. Total RNA was extracted from rat livers and relative mRNA concentrations were determined by realtime detection RT-PCR analysis using GAPDH mRNA concentration for normalization. Values are means ± S.D. (n = 8). *Significantly different from control animals, $P < 0.05$.

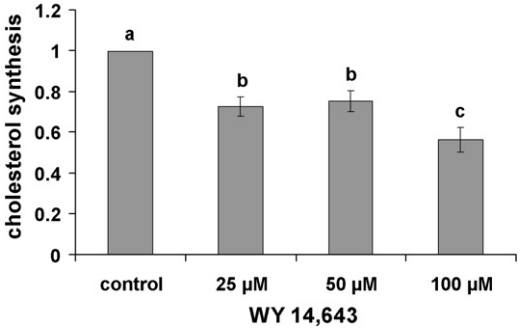

Fig. 7 – Effect of WY 14,643 on the relative cholesterol synthesis rate in Fao cells. Cells were pre-incubated for 22 h with different concentrations of WY 14,643 or with vehicle alone (control). Thereafter, cells were incubated for further 2 h with or without the indicated concentrations of WY 14,643 with addition of [1,2-^{14}C]acetate in order to measure the newly synthesized cholesterol. Cellular lipids were extracted with a mixture of hexane and isopropanol. Lipids were separated by thin-layer chromatography and lipid-bound radioactivity was detected and quantified by autoradiography. Values are means ± S.D. (n = 3). Bars without the same superscript letter (a–c) are significantly different, $P < 0.05$.

control animals and cells, respectively. This can be due on the one hand to a reduced transcription of the gene or reduced stability of the transcript resulting in lowered mRNA concentrations and reduced availability of the SREBP-2 precursor protein. However, also a reduced amount of nuclear SREBP-2 can lead to decreased mRNA concentrations of the SREBP-2 gene since SREBP-2 contains a sterol regulatory element in its enhancer/promoter region and the nuclear form can activate its own gene in an autoregulatory loop [49]. In the liver of clofibrate treated rats, the mRNA concentration of SREBP-2 was reduced compared to control rats. In Fao cells, after 6 h of incubation with WY 14,643 the mRNA concentration of SREBP-2 was unchanged, whereas it was significantly reduced after 24 h of incubation compared to control cells. This observation in Fao cells indicates, that a reduction of SREBP-2 mRNA did not precede the decrease of its nuclear form and thus there may be another reason for the observed reduction of nuclear SREBP-2 upon PPARα activation.

The integral membrane proteins Insig-1 and -2 have been previously identified as modulators of SREBP activity [6,7]. They anchor the SCAP/SREBP complex in the endoplasmic reticulum in the presence of sterols. Overexpression of Insig-1 in the liver of transgenic mice inhibited processing of SREBPs [50]. Inversely, reduction of both Insig mRNAs by RNA interference or by mutational inactivation led to an increase in nuclear SREBPs [51–53]. We could show that in the liver of

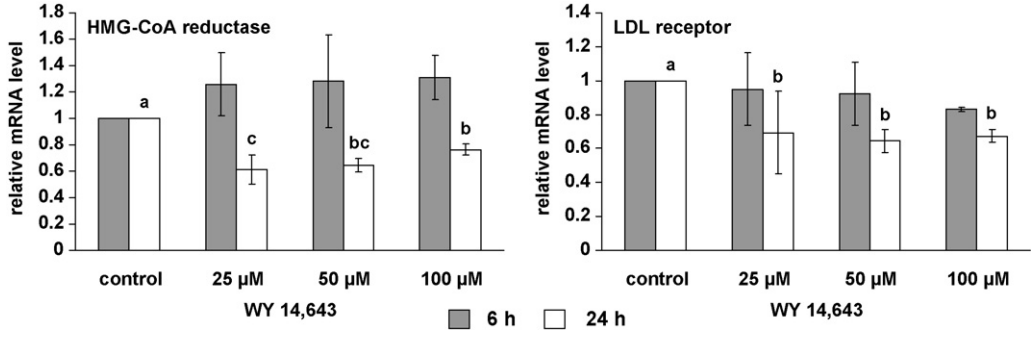

Fig. 6 – Effect of WY 14,643 on the mRNA concentration of HMG-CoA reductase and LDL receptor in Fao cells. Fao cells were grown in culture medium until subconfluent state. Medium was then changed to low-serum medium (0.5% fetal calf serum). After 16 h preincubation in this medium, cells were incubated with increasing concentrations of the PPARα agonist WY 14,643 from 25 to 100 μM for 6 and 24 h, respectively. Control cells were incubated with low-serum medium containing vehicle alone. Total RNA was extracted from cells and relative mRNA concentrations were determined by real time detection RT-PCR analysis using GAPDH mRNA concentration for normalization. Values are means ± S.D. (n = 3). Bars without the same superscript letter (a–c) are significantly different, $P < 0.05$.

rats treated with clofibrate the mRNA concentration of Insig-1 was increased compared to control animals. Furthermore, incubation of Fao cells with WY 14,643 for 6 h led to a significant and dose-dependent increase of the mRNA concentrations of Insig-1 and, to a lesser extent, of Insig-2a, the liver-specific transcript of the Insig-2 gene. The induction of Insig-1 mRNA concentration in WY 14,643 treated Fao cells observed after 6 h of incubation was completely abolished after 24 h. We suggest that this is due to a decline in Insig-1 transcription caused by reduced nuclear SREBP-2. The transcription of Insig-1 requires nuclear SREBPs [7]. In contrast to Insig-1, the expression of Insig-2 is not dependent on nuclear SREBPs permitting feedback regulation of cholesterol synthesis over a wide range of sterol concentrations by the concerted action of both Insig-1 and Insig-2 [7]. The level of Insig-2a mRNA after 24 h of incubation of the Fao cells with WY 14,643 was not as high as after 6 h of incubation but tended to be still elevated over control.

Taken together these data suggest that the reduced amount of nuclear SREBP-2 upon PPARα activation in rat liver and Fao cells, respectively, may be rather due to increased expression of Insigs which retard SREBP-2 processing than to decreased transcription of the SREBP-2 gene. Recently it was demonstrated that Insig-1 is regulated by PPARγ in white adipose tissue of diabetic mice via a PPAR response element in its promoter region [9]. In Fao cells, the increase of Insig-1 and -2a mRNA concentrations was observed simultaneously with ACO and Cyp4A1 induction after short term incubation of the Fao cells with WY 14,643. Considering the existence of a PPAR response element in the Insig-1 promotor one could speculate, that the upregulation of Insig-1 and Insig-2a may be directly mediated by PPARα. Further experiments are required to prove this hypothesis.

In contrast to our results obtained with the Fao cell model, PPARα activation in rats markedly reduced the mRNA concentration of Insig-2a in the liver. The expression of Insig-2a, the liver-specific transcript of Insig-2, is specifically down-regulated by insulin [8]. It has been reported that activation of PPARα improved insulin sensitivity in different models of insulin resistance, probably by reducing lipid accumulation in tissues due to increased fatty acid oxidation and by down-regulation of a gene involved in insulin receptor signalling in hepatocytes [54–57]. Thus we suggest that improvement of insulin sensitivity in the liver of clofibrate treated rats may account for the down-regulation of Insig-2a. Nevertheless, the reduced expression of Insig-2a did not interfere with the inhibition of SREBP-2 processing in clofibrate treated rats, indicating that Insig-1 is more important than Insig-2a in the regulation of SREBP-2 activity. Yabe et al. [8] discussed a special role of Insig-2a in processing of SREBP-1c in the liver of mice allowing the SREBP-1c to exit the ER to stimulate fatty acid synthesis, even at elevated hepatic cholesterol concentrations.

The reduced abundance of transcriptionally active SREBP-2 in the nucleus upon PPARα activation in the livers of clofibrate treated rats and in Fao cells after 24 h of incubation with WY 14,643 was mirrored by lowered mRNA concentrations of two SREBP-2 target genes encoding proteins for cholesterol synthesis and uptake. In clofibrate treated rats as well as in Fao cells stimulated with WY 14,643 for 24 h, the mRNA concentrations of both, HMG-CoA reductase and LDL receptor were decreased compared to control animals and cells, respectively. Furthermore, cholesterol synthesis rate in Fao cells incubated with WY 14,643 for 24 h was decreased compared to control cells which is in agreement with the reduced mRNA concentration of HMG-CoA reductase, the rate-limiting enzyme of cholesterol synthesis, upon PPARα activation.

In clofibrate treated rats, the reduced expression of genes involved in cholesterol synthesis and uptake was reflected by decreased concentrations of total cholesterol in the liver, plasma and lipoproteins. A similar decline in serum cholesterol levels associated with decreased HMG-CoA reductase activity in liver microsomes was observed in rats fed 0.3% clofibrate in the diet for 3–7 days [58]. Also in Fao cells, reduced cholesterol synthesis and uptake resulted in a decrease of the total cholesterol concentration after 24 h of incubation with WY 14,643.

Thus, our data show for the first time that PPARα activation lowers the cholesterol concentration in rat liver, plasma and lipoproteins and in Fao cells by reducing the amount of nuclear SREBP-2 thereby decreasing cholesterol synthesis and uptake. Further, our data indicate that this reduction of nuclear SREBP-2 is mediated by increased expression of Insigs (Fig. 8).

Several reports indicated an involvement of PPARα in the regulation of cholesterol synthesis in the liver. In wild-type mice, an antiparallel relationship exists between the expression of the PPARα gene and that of HMG-CoA reductase and LDL receptor genes; in PPARα-null (KO) mice, the diurnal variation of cholesterogenic gene expression was abolished [21]. These observations are in agreement with our study that shows that PPARα activation inhibits the expression of HMG-CoA reductase and LDL receptor by reducing the amount of nuclear SREBP-2.

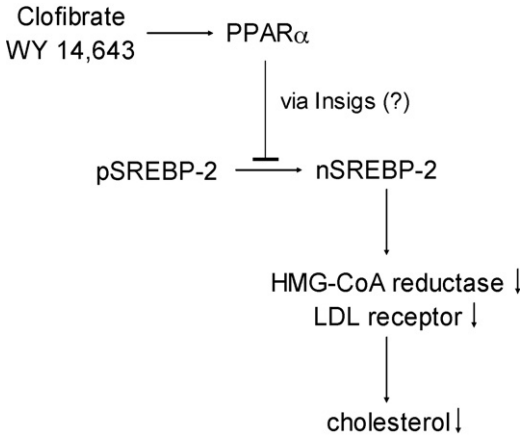

Fig. 8 – Schematic diagram of the proposed pathways leading to decreased cholesterol synthesis and concentration upon PPARα activation. PPARα activation by clofibrate or WY 14,643 reduces the amount of nuclear SREBP-2, probably via an upregulation of Insigs. In turn, this leads to a decreased expression of SREBP-2 target genes HMG-CoA reductase and LDL receptor implicated in cholesterol synthesis and uptake and finally to reduced cholesterol concentrations.

Also other studies supported our results on the mechanism underlying the cholesterol lowering effect of PPARα agonists by indicating that clofibrate inhibited HMG-CoA reductase activity and decreased cholesterol synthesis in rats and in cultured hepatocytes [22,24,58–60]. However, there are also few studies which are in contrast to our results. The PPARα agonists gemfibrozil and ciprofibrate upregulated cholesterol synthesis and HMG-CoA reductase activity or mRNA concentration in rats and in cultured hepatocytes [23,24,61]. WY 14,643 treatment of wild-type mice resulted in a decreased rate of cholesterol synthesis, whereas in PPARα-null (KO) mice cholesterol synthesis was unaffected by WY 14,643 treatment [62]. While this reduction of cholesterol synthesis by PPARα is consistent with our data, the authors found increased HMG-CoA reductase mRNA levels in wild-type mice but not PPARα-null (KO) mice treated with WY 14,643 [62]. The reasons for the conflicting results concerning the effects of PPARα agonists on cholesterol synthesis and HMG-CoA reductase are difficult to explain but may depend on experimental conditions, species and type of fibrate used. Furthermore, HMG-CoA reductase is regulated by a complex feedback mechanism including transcriptional, translational and posttranslational levels and the sterol-dependent ubiquitination and proteolytic degradation of the protein mediated by Insigs [63,64]. Thus, measurements of HMG-CoA reductase mRNA may not always reflect the actual activity of the enzyme.

In conclusion, data from the rat experiment and the *in vitro* study strongly suggest that PPARα activation lowers the cholesterol concentration by reducing the abundance of nuclear SREBP-2, probably via an upregulation of Insigs. This leads in turn to diminished expression of the SREBP-2 target genes HMG-CoA reductase and LDL receptor and reduced cholesterol synthesis and uptake. Thus, these date give important insights in the complex regulation of lipid homeostasis in liver cells by providing a novel link between PPARα and cholesterol metabolism. Moreover, these results may help to explain the cholesterol lowering effects of natural ligands of PPARα such as polyunsaturated fatty acids, conjugated linoleic acids and oxidized fatty acids in man and animals [16,17,65].

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Activation of PPARα and PPARγ reduces triacylglycerol synthesis in rat hepatoma cells by reduction of nuclear SREBP-1

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ABSTRACT

Fibrates and thiazolidinediones, agonists of PPARα and PPARγ, respectively, reduce triglyceride concentrations in rat liver and plasma. Fatty acid and triacylglycerol synthesis in mammals is regulated by sterol regulatory element-binding protein (SREBP)-1c. Recently, it was shown that insulin-induced gene (Insig)-1, the key regulator of SREBP activity, is up-regulated by both activation of PPARα and PPARγ. In order to elucidate whether inhibition of SREBP-1 activation may contribute to the triacylglycerol lowering effect of PPARα and PPARγ agonists, we incubated rat hepatoma Fao cells with WY 14,643 and troglitazone, strong and selective agonists of PPARα and PPARγ, respectively. Activation of both, PPARα and PPARγ led to increased concentrations of Insig-1 and Insig-2a, with the most prominent effect on Insig-2a after troglitazone incubation. As a result, the amount of nuclear SREBP-1 was reduced in Fao cells by both WY 14,643 and troglitazone treatment. The reduction of nuclear SREBP-1 was associated with decreased mRNA concentrations of its target genes fatty acid synthase and glycerol-3-phosphate acyltransferase, implicated in fatty acid and triacylglycerol synthesis. This was finally reflected in reduced rates of newly synthesized triacylglycerols from de novo-derived fatty acids and decreased intracellular and secreted triacylglycerol concentrations in Fao cells treated with WY 14,643 and troglitazone, respectively. Thus, these data suggest that the triacylglycerol reducing effect of fibrates and thiazolidinediones is partially caused by inhibition of SREBP-1 activation via up-regulation of Insig.

1. Introduction

Fibrates are a class of hypolipidemic drugs that are effective in lowering elevated plasma triacylglycerol and cholesterol levels (Frick et al., 1987; Zimetbaum et al., 1991). Thiazolidinediones are used in the treatment of insulin-resistant states (reviewed in Quinn et al., 2008). Their mode of action is based on their structural similarity to free fatty acids which enables them to bind to peroxisome proliferator-activated receptors (PPAR). PPAR are transcription factors belonging to the superfamily of nuclear receptors and regulate nutrient metabolism and energy homeostasis (reviewed in Desvergne and Wahli, 1999; Chinetti et al., 2000; Duval et al., 2002). There are three PPAR isotypes, PPARα, PPARβ/δ and PPARγ (Schoonjans et al., 1997). PPARα is highly expressed in tissues with high fatty acid oxidation (Mandard et al., 2004); PPARγ is predominantly expressed in adipose tissue but also to a lesser extent in other tissues like skeletal muscle and liver (Fajas et al., 1997). Both, fibrates and thiazolidinediones, decrease plasma triacylglycerol concentrations in rats (Naderali et al., 2004; Lee et al., 2004; Festuccia et al., 2006; Laplante et al., 2007; König et al., 2007). Upon binding to PPARα, fibrates decrease circulating triacylglycerol levels by transcriptional modulation of genes involved in lipolysis, cellular uptake and β-oxidation of fatty acids and decreased synthesis of fatty acids and triacylglycerols (reviewed in Schoonjans et al., 1996; Staels et al., 1998). Mechanisms underlying the reduced fatty acid and triacylglycerol synthesis are, however, not completely understood. Induction of the β-oxidation pathway results in lower availability of fatty acids for triacylglycerol synthesis. Furthermore, PPARα activation leads to reduced expression of fatty acid synthase (FAS) in the liver of rats (Eder et al., 2003). The triacylglycerol lowering effect of thiazolidinediones results at least in part from an increase in adipose lipoprotein lipase activity and triacylglycerol-derived fatty acid uptake and retention in adipose tissue (Laplante et al., 2007).

In mammals, lipogenesis is regulated by the transcription factor family designated sterol regulatory element-binding proteins (SREBP; Brown and Goldstein, 1999). Three isoforms of SREBP are known in mammals, SREBP-1a, SREBP-1c and SREBP-2. SREBP-1c, the predominant isoform in adult liver, preferentially activates genes required for fatty acid synthesis and their incorporation into triacylglycerols and phospholipids (Horton et al., 2002). Retention of the SCAP/SREBP complex in the endoplasmic reticulum is mediated by sterol-dependent

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binding of the complex to one of two endoplasmic reticulum retention proteins designated insulin-induced gene (Insig)-1 and -2 (Yang et al., 2002; Yabe et al., 2002).

Recently we could show that activation of PPARα up-regulates mRNA concentration of Insig-1 in the liver of rats and in rat hepatoma cells which leads to reduced concentration of the nuclear form of SREBP-2. As a result, expression of SREBP-2 target genes implicated in cholesterol synthesis and uptake is decreased leading to reduced cholesterol concentrations in the liver, plasma and lipoproteins and hepatoma cells, respectively (König et al., 2007). Consistent with our observation of up-regulation of Insig-1 by PPARα it has been shown that activation of PPARγ by rosiglitazone induced the expression of Insig-1 in white adipose tissue via a PPRE in the promoter region of Insig-1 (Kast-Woelbern et al., 2004).

As the maturation of SREBP-1c is regulated in the same way as that of SREBP-2, namely by Insig, we hypothesized that not only the maturation of SREBP-2 but also that of SREBP-1c is reduced by PPARα agonists. This would lead to inhibition of fatty acid and triacylglycerol synthesis and may contribute to the reduced triacylglycerol concentrations observed upon administration of PPARα agonists. Interestingly, also the PPARγ agonist troglitazone lowered cholesterol synthesis in two human cell lines via reduced concentration of nuclear SREBP-2 (Klopotek et al., 2006). In light of the observed up-regulation of Insig-1 by PPARγ (Kast-Woelbern et al., 2004) we suggested that also PPARγ activation may influence triacylglycerol level by inhibition of SREBP-1c maturation.

Thus, in this study, we incubated Fao cells with the PPARα agonist WY 14,643 and the PPARγ agonist troglitazone, respectively, and analysed the expression of Insig, the expression and nuclear concentration of SREBP-1c and the expression of its target genes FAS and glycerol-3-phosphate acyltransferase (GPAT) coding for enzymes that catalyse important steps in fatty acid and triacylglycerol synthesis, respectively. Furthermore, we measured the rate of newly synthesized triacylglycerols from de novo-derived fatty acids and concentration of triacylglycerols in Fao cells after incubation with WY 14,643.

2. Materials and methods

2.1. Materials

WY 14,643 (4-Chloro-6-(2,3-xylidino)-2-pyrimidinylthioacetic acid), troglitazone, dimethylsulfoxide (DMSO), MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide; Thiazol blue), TRIZOL™ reagent, SYBR® Green I, protease inhibitor mix and the anti-rabbit-IgG peroxidase conjugate antibody were purchased from Sigma-Aldrich (Steinheim, Germany). Rat hepatoma Fao cell line was purchased from ECACC (Salisbury, UK). F-12 Nutrient Mixture (Ham), gentamycin and fetal calf serum (FCS) were obtained from Invitrogen (Karlsruhe, Germany). Taq polymerase was from Promega (Mannheim, Germany). Bicinchoninic acid assay reagent was a product of Interchim (Montfucon, France). The nitrocellulose blotting membrane was from Pall (Pensacola, FL, U.S.A), and the ECL-reagent kit from Amersham-Pharmacia (Freiburg, Germany). The anti-SREBP-1 antibody (K-10; rabbit polyclonal IgG) was from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.) and the anti-β-actin antibody (rabbit polyclonal IgG) was purchased from Abcam Ltd. (Cambridge, UK). Autoradiography film for Western blot analysis (Agfa Cronex) was from Roentgen Bender (Baden-Baden, Germany).

2.2. Cell culture

Fao rat hepatoma cells were cultured in Ham-F12 medium supplemented with 10% FCS and 0.05 mg/ml gentamycin. Cells were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. For experiments, Fao cells were seeded in 24- or 6-well culture plates at a density of $2.1 \cdot 10^5$ and $1.05 \cdot 10^6$ cells, respectively, per well and used prior reaching confluence (usually 3 days after seeding). The cells were preincubated with low-serum medium (0.5% FCS) for 16 h and then stimulated for the times indicated with WY 14,643 and troglitazone, respectively. Agonists were added to the low-serum medium from a stock solution in DMSO. Final DMSO concentration did not exceed 0.1% (v/v). Cells treated with the appropriate vehicle concentration were used as a control. Cell viability after treatment with WY 14,643 or troglitazone was assessed by the MTT assay (Mossman, 1983). Cell viability of Fao cells was not reduced by 24 h incubation with WY 14,643 up to a concentration of 100 μM and with troglitazone up to a concentration of 20 μM (data not shown).

2.3. RT-PCR analysis

Total RNA was isolated from Fao cells after the incubation in 24-well plates by TRIZOL™ reagent according to the manufacturer's protocol. cDNA synthesis was carried out as described (König and Eder, 2006). The mRNA expression of genes was measured by real-time detection PCR using the Rotor Gene 2000 system (Corbett Research, Mortlake, Australia). Real-time detection PCR was performed with 1.25 U Taq DNA polymerase, 500 μM dNTPs and 26.7 pmol of the specific primers (Operon Biotechnologies, Cologne, Germany; Table 1). Calculation of the relative mRNA concentration was made using the $\Delta\Delta C_t$ method as previously described (Pfaffl, 2001). The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for normalisation. The primers used in the experiments were purchased from Operon (Köln, Germany) and are shown in Table 1.

2.4. Immunoblot analysis

Whole cell extracts of Fao cells were prepared by lysis in 20 mM Tris, 150 mM NaCl, 0.1% Triton X-100, 1% protease inhibitor mix, pH 7.5, after the incubation in 6-well-plates. The protein content was determined by the bicinchoninic acid assay. 30–50 μg of Fao cell proteins were separated on 10% sodium dodecylsulfate acrylamide gel electrophoresis and electrotransferred to a nitrocellulose membrane. After blocking in 50 mM Tris, 150 mM NaCl, 0.2% Tween-20, pH 7.5, containing 3% nonfat dry milk, bands corresponding to nuclear SREBP-1 and β-actin (as a loading control) were visualized with enhanced

Table 1
Characteristics of the specific primers used for RT-PCR analysis

Gene	Forward and reverse primers	bp	Annealing temperature	NCBI GenBank
ACO	5′ CTTTCTTGCTTGCCTTCCTTCTCC 3′ 5′ GCCGTTTCACCGCCTCGTA 3′	415	60 °C	NM017340
Cyp4A1	5′ CAGAATGGAGAATGGGGACAGC 3′ 5′ TGAGAAGGGCAGGAATGAGTGG 3′	460	65 °C	NM175837
FAS	5′ CCTCCCCTGGTGGCTGCTACAA 3′ 5′ CCTGGGGTGGGCGGTCTTT 3′	224	60 °C	X62888
GAPDH	5′ GCATGGCCTTCCGTGTTCC 3′ 5′ GGGTGGTCCAGGGTTTCTTACTC 3′	337	60 °C	BC059110
GPAT	5′ CAGCGTGATTGCTACCTGAA 3′ 5′ CTCTCCGTCCTGGTGAGAAG 3′	194	60 °C	AF021348
Insig-1	5′ ATTTGGCGTGGTCCTGGCTCTGG 3′ 5′ GCGTGGCTAGGAAGGCGATGGTG 3′	389	62 °C	NM022392
Insig-2a	5′ GACGGATGTGTTGAAGGATTTCT 3′ 5′ TGGACTGAAGCAGACCAATGTC 3′	83	59 °C	AY156086
PPARγ	5′ CCCTGGCAAAGCATTTGTAT 3′ 5′ CCCTGGCAAAGCATTTGTAT 3′	222	60 °C	AF156665
SREBP-1a	5′ ACACAGCGGTTTTGAACGACATC 3′ 5′ ACGGACGGGTACATCTTTACAG 3′	268	60 °C	L16995[a]
SREBP-1c	5′ GGAGCCATGGATTGCACATT 3′ 5′ AGGAAGGCTTCCAGAGAGGA 3′	191	60 °C	XM_213329

[a] Primer sequence from Kakuma et al. (2000).

chemiluminescence reagents and exposed to autoradiography film. Films were analysed with the Gel-Pro Analyzer software (Intas, Upland, CA, U.S.A.). The polyclonal antibody used for detection of SREBP-1 (K-10) recognizes an internal fragment of SREBP-1 and is commonly used for detection of its transcriptionally active nuclear form (Yu-Poth et al., 2005; Knight et al., 2005; Hsu et al., 2007).

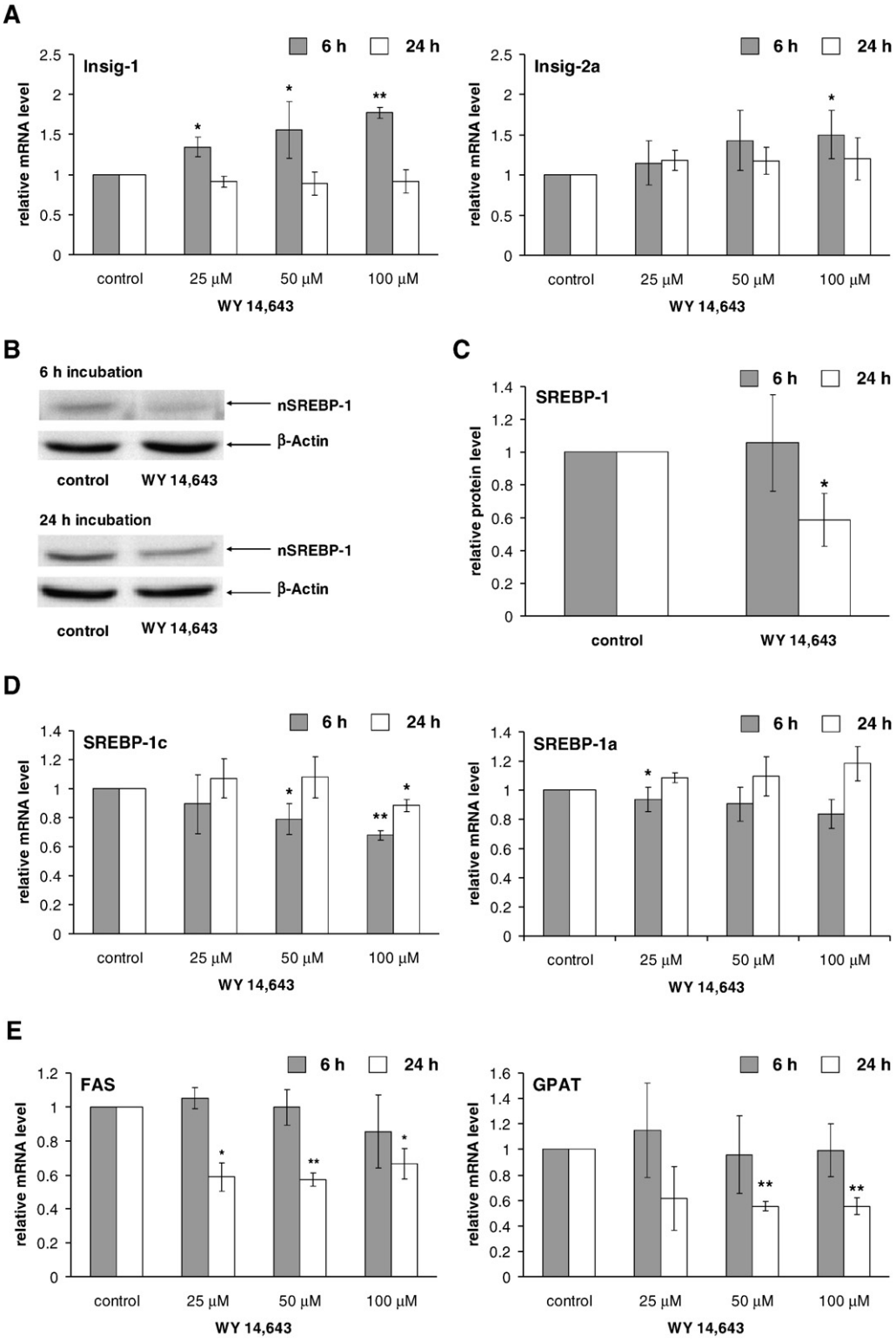

Fig. 1. PPARα agonist WY 14,643 down-regulates lipogenic enzymes in Fao cells. Fao cells were incubated with increasing concentrations of WY 14,643 from 25 to 100 µM for 6 and 24 h, respectively. Control cells were incubated with low-serum medium containing vehicle alone. Total RNA was extracted from cells and Insig-1, Insig-2a (A), SREBP-1c, SREBP-1a (D) and FAS and GPAT (E) mRNA concentrations were determined by realtime detection RT-PCR analysis using GAPDH mRNA concentration for normalisation. (B) Fao cells were incubated as described and after cell lysis, equal amounts of proteins were separated by 10% SDS-PAGE and immunoblotted with anti-SREBP-1 and anti-β-actin antibodies. Figure shows representative immunoblots after 6 and 24 h incubation of Fao cells with or without 100 µM WY 14,643. (C) Relative intensity of the bands in (B) was quantified by densitometry using β-actin-specific band as loading control. Values are means ± SD ($n=3$). Symbols indicate significant differences from control cells (*$P<0.05$; **$P<0.001$).

Table 2
Concentration of triacylglycerols in Fao cells

	Cellular triacylglycerols (nmol/mg cell protein)	Secreted triacylglycerols (VLDL; nmol/mg cell protein)
WY 14,643 (24 h)		
Control	41.3±0.9	411±30
100 μM	35.8±4.4[a]	313±41[a]
Troglitazone (6 h)		
Control	41.3±2.0	401±8
20 μM	37.3±3.5[a]	287±30[a]

[a] Significantly different from control cells, $P<0.05$.

2.5. Determination of triacylglycerol concentrations

Lipid extraction from Fao cells after incubation with the PPAR agonists or vehicle alone and measurement of cellular and secreted triacylglycerols were carried out as described (König and Eder, 2006).

2.6. Determination of the rate of newly synthesized triacylglycerols from de novo-derived fatty acids

After a pre-incubation of 22 h (WY 14,643) or 4 h (troglitazone) at 37 °C, 5% CO_2 with the different concentrations of the PPAR agonists, 0.2 μCi [1,2-^{14}C] acetate (specific activity 108 mCi/mmol) was added in order to measure the rate of synthesis of triacylglycerols from de novo-derived fatty acids (17, 18). Cells were incubated for 2 h at 37 °C, 5% CO_2. After incubation the cells were washed twice with cold PBS. The lipids were extracted twice with a mixture of hexane and isopropanol (3:2, v/v) (19). After removing the solvents in a vacuum centrifugal evaporator the lipids were dissolved in 80 μl chloroform, 4 μL of which were applied to 10×20 cm^2 TLC using a TLC spotter PS01 (Desaga, Heidelberg, Germany). Plates were developed with a mixture of hexane, diethyl ether and acetic acid (80:20:3, v/v/v) (20). Incorporation of [1,2-^{14}C] acetate into triacylglycerols was detected and quantified by autoradiography by analysis of the triacylglycerol spot (Fuji imager system, Tina 2 software, Raytest, Straubenhart, Germany).

2.7. Statistical analysis

Means of treatments and control were compared by Student's *t* test using the Minitab Statistical Software (Minitab, State College, PA, U.S.A.). Differences with $P<0.05$ were considered to be significant.

3. Results

3.1. WY 14,643 up-regulated Insig-1 and Insig-2a and reduced SREBP-1 and its target genes in Fao cells

We incubated rat hepatoma Fao cells with increasing amounts of the PPARα agonist WY 14,643 for 6 and 24 h. Incubation of Fao cells with WY 14,643 led to a strong increase of mRNA concentrations of the PPARα target genes acyl-CoA oxidase (ACO; mean value±SD; control cells=1.0; 6 h incubation: 25 μM WY 14,643, 1.7±0.2*; 50 μM, 1.9±0.5*; 100 μM, 1.8±0.1*; 24 h incubation: 25 μM, 1.2±0.1*; 50 μM, 1.4±0.1*; 100 μM, 1.3±0.2; *significantly different from control, $P<0.05$) and cytochrome P450 4A1 (Cyp4A1; mean value±SD; control cells=1.0; 6 h incubation: 25 μM WY 14,643, 5.7±0.8*; 50 μM, 6.1±1.1*; 100 μM, 6.0±1.2*; 24 h incubation: 25 μM, 10.7±3.4*; 50 μM, 11.4±2.6*; 100 μM, 14.1±3.2*; *significantly different from control, $P<0.05$) compared to control cells. Treatment of Fao cells with WY 14,643 for 6 h led to a significant and concentration-dependent increase of Insig-1 mRNA concentration compared to control cells, and this effect was abolished after 24 h of treatment (Fig. 1A). At the highest concentration of WY 14,643 used, Insig-1 mRNA concentration was about 80% higher than that of control cells after 6 h of incubation ($P<0.05$). Likewise, incubation of Fao cells with 100 μM WY 14,643 for 6 h increased the mRNA concentration of Insig-2a about 50% compared to control cells ($P<0.05$). After 24 h of incubation, Insig-2a mRNA concentration in WY 14,643 treated cells still tended to be higher (about 20%, $P<0.10$) than in control cells (Fig. 1A). Western blot analysis of Fao cell lysates revealed that the relative protein concentration of the mature SREBP-1 after treatment of Fao cells with 100 μM of WY 14,643 for 6 h was not different from that of the control cells (Fig. 1B,C). After 24 h of treatment, mature SREBP-1 concentration was 41% lower compared to untreated cells ($P<0.05$; Fig. 1B,C). mRNA concentration of SREBP-1c was 20% ($P<0.05$) and 32% ($P<0.001$) lower in cells incubated with 50 and 100 μM WY 14,643, respectively, for 6 h than in control cells (Fig. 1D). After 24 h of incubation, mRNA concentration of SREBP-1c was 12% lower in cells treated with 100 μM WY 14,643 than in control cells (Fig. 1D; $P<0.05$). mRNA concentration of SREBP-1a was only marginally decreased after 6 h of incubation (about 7% at 25 μM WY 14,643; $P<0.05$) and it was unchanged after 24 h of incubation compared to control cells (Fig. 1D). However, comparison of baseline C_t values of both SREBP isoforms (SREBP-1c, 16.5; SREBP-1a, 22.2) in realtime PCR analysis showed that abundance of SREBP-1c mRNA in Fao cells was about 50-fold higher than that of SREBP-1a mRNA. mRNA concentration of the SREBP-1c target gene FAS was unchanged after 6 h of incubation of Fao cells with WY 14,643 but was 41% ($P<0.05$), 43% ($P<0.001$) and 34% ($P<0.05$) lower after 24 h of incubation with 25, 50 and 100 μM WY 14,643, respectively, than in control cells (Fig. 1E). GPAT mRNA concentration was unchanged in cells incubated with WY 14,643 for 6 h compared to control cells. After 24 h treatment, GPAT mRNA concentration was 45% lower in Fao cells incubated with 50 and 100 μM WY 14,643, respectively, than in untreated cells ($P<0.001$; Fig. 1E).

3.2. WY 14,643 reduced triacylglycerol synthesis in Fao cells

The concentrations of cellular triacylglycerols of Fao cells incubated with 100 μM WY 14,643 for 24 h was 13% lower compared to control cells treated with vehicle alone ($P<0.05$; Table 2). The amount of secreted triacylglycerols in VLDL was measured in the culture

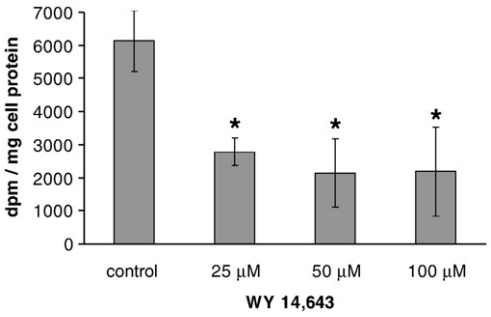

Fig. 2. PPARα agonist WY 14,643 reduces triglyceride synthesis rate in Fao cells. Cells were pre-incubated for 22 h with different concentrations of WY 14,643 or with vehicle alone (control). Thereafter, cells were incubated for further 2 h with or without the indicated concentrations of WY 14,643 with addition of [1,2-^{14}C]acetate in order to measure the newly synthesized triacylglycerols. Cellular lipids were extracted with a mixture of hexane and isopropanol. Lipids were separated by thin-layer chromatography and lipid-bound radioactivity was detected and quantified by autoradiography. Values are means±SD ($n=3$). *Significantly different from control cells (*$P<0.05$).

medium after incubation. Cells incubated with WY 14,643 for 24 h secreted about 24% less triacylglycerols than control cells ($P<0.05$; Table 2). The rate of newly synthesized triacylglycerols from de novo-derived fatty acids was significantly lower about 55 to 65% in Fao cells incubated with 25, 50 or 100 μM WY 14,643 for 24 h compared to control cells ($P<0.05$; Fig. 2).

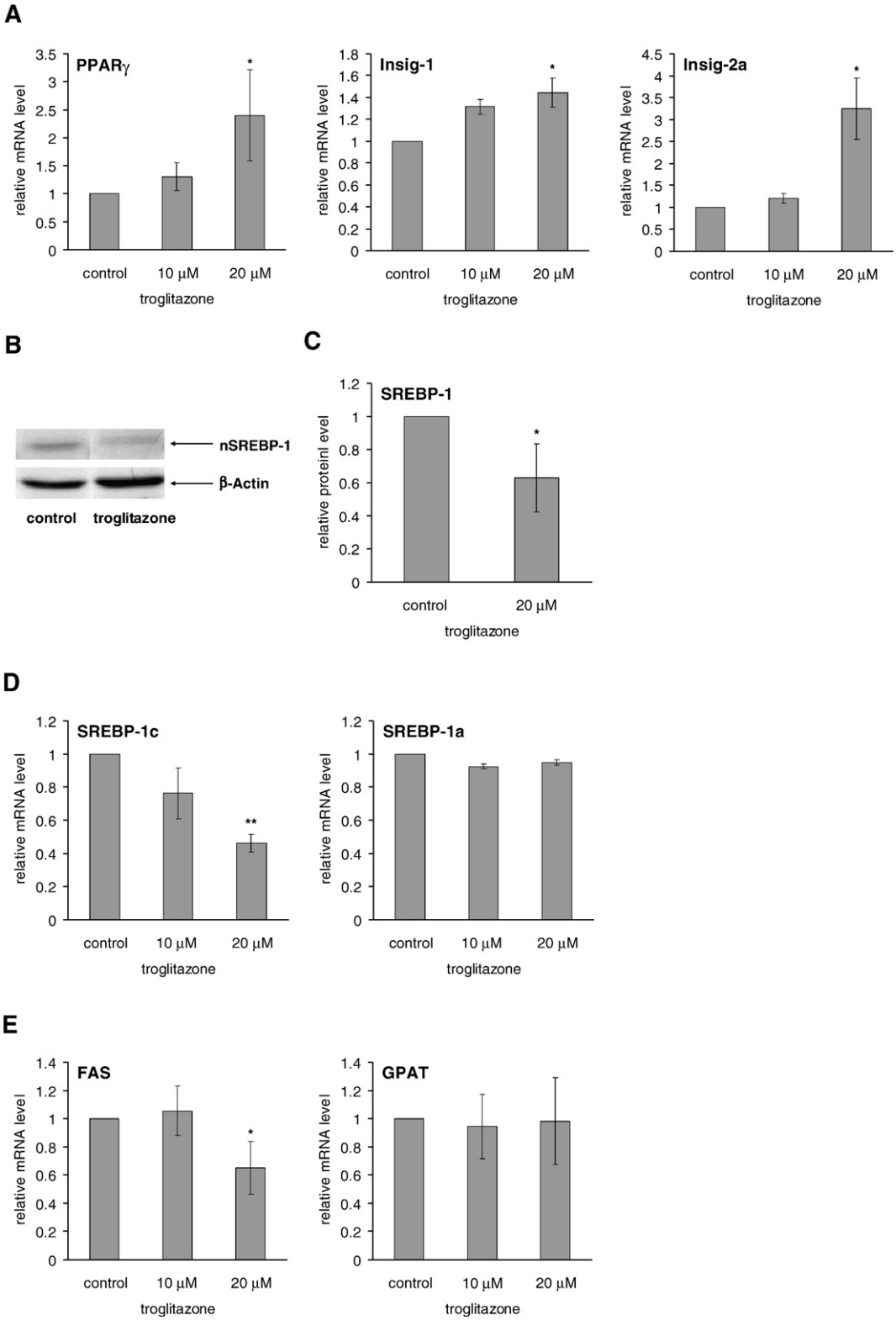

Fig. 3. PPARγ agonist troglitazone down-regulates lipogenic enzymes in Fao cells. Fao cells were incubated with 10 or 20 μM of troglitazone for 6 h. Control cells were incubated with low-serum medium containing vehicle alone. Total RNA was extracted from cells and PPARγ, Insig-1, Insig-2a (A), SREBP-1c, SREBP-1a (D) and FAS and GPAT (E) mRNA concentrations were determined by realtime detection RT-PCR analysis using GAPDH mRNA concentration for normalisation. (B) Fao cells were incubated as described and after cell lysis, equal amounts of proteins were separated by 10% SDS-PAGE and immunoblotted with anti-SREBP-1 and anti-β-actin antibodies. Figure shows representative immunoblots after 6 h incubation of Fao cells with or without 20 μM troglitazone. (C) Relative intensity of the bands in (B) was quantified by densitometry using β-actin-specific band as loading control. Values are means ± SD ($n=3$). Symbols indicate significant differences from control cells (*$P<0.05$; **$P<0.001$).

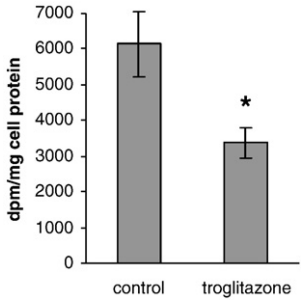

Fig. 4. PPARγ agonist troglitazone reduces triglyceride synthesis rate in Fao cells. Cells were pre-incubated for 4 h with 20 μM troglitazone or with vehicle alone (control). Thereafter, cells were incubated for further 2 h with or without troglitazone with addition of [1,2-^{14}C]acetate in order to measure the newly synthesized triacylglycerols. Cellular lipids were extracted with a mixture of hexane and isopropanol. Lipids were separated by thin-layer chromatography and lipid-bound radioactivity was detected and quantified by autoradiography. Values are means±SD (n=3). *Significantly different from control cells (*P<0.05).

3.3. Troglitazone up-regulated Insig-1 and Insig-2a and reduced SREBP-1 and its target genes in Fao cells

Next, we incubated Fao cells with 10 and 20 μM of the PPARγ agonist troglitazone for 6 h. mRNA concentration of PPARγ was 2.4-fold higher in cells incubated with 20 μM troglitazone than in control cells (P<0.05; Fig. 3A). Insig-1 mRNA concentration was 44% higher in cells treated with 20 μM troglitazone compared to control cells (P<0.05; Fig. 3A). mRNA concentration of Insig-2a was 3.3-fold higher in Fao cells treated with 20 μM troglitazone for 6 h than in control cells treated with vehicle alone (P<0.05; Fig. 3A). Western blot analysis of whole Fao cell lysates showed that incubation of the cells with 20 μM troglitazone for 6 h reduced the amount of mature nuclear SREBP-1 about 37% compared to control cells incubated with vehicle alone (P<0.05; Fig. 3B,C). In Fao cells incubated with 20 μM of troglitazone mRNA concentration of SREBP-1c was 55% lower than in control cells (P<0.001; Fig. 3D). mRNA concentration of SREBP-1a was not influenced by troglitazone treatment (Fig. 3D). FAS mRNA concentration was 35% lower in Fao cells treated with 20 μM troglitazone than in control cells (P<0.05) whereas mRNA concentration of GPAT was unchanged (Fig. 3E).

3.4. Troglitazone reduced triacylglycerol synthesis in Fao cells

The concentrations of cellular triacylglycerols of Fao cells incubated with 20 μM troglitazone for 6 h was 10% lower compared to control cells treated with vehicle alone (P<0.05; Table 2). The amount of secreted triacylglycerols in VLDL in the culture medium of Fao cells after incubation with 20 μM troglitazone for 6 h was 28% lower than in control cells (P<0.05; Table 2). The rate of newly synthesized triacylglycerols from de novo-derived fatty acids was about 45% lower in Fao cells incubated with 20 μM troglitazone for 6 h compared to control cells (P<0.05; Fig. 4).

4. Discussion

Agonists of PPARα and PPARγ decrease the concentration of plasma and liver triacylglycerols in rats (Naderali et al., 2004; Lee et al., 2004; Festuccia et al., 2006; Laplante et al., 2007; König et al., 2007). This study was designed to find out whether this is in part caused by decreased synthesis of fatty acids and triacylglycerols due to inhibition of SREBP-1 pathway. First we incubated rat hepatoma Fao cells with the PPARα agonist WY 14,643 which resulted in a strong up-regulation of the typical PPARα target genes ACO and Cyp 4A1 indicative of PPARα activation. Recently we observed that the mRNA concentration of Insig-1 and Insig-2a was up-regulated by activation of PPARα leading to inhibition of SREBP-2 dependent cholesterol synthesis (König et al., 2007). Here we could show for the first time that also the amount of the transcriptionally active form SREBP-1 in the nucleus was reduced upon PPARα activation after 24 h in Fao cells. Thus we suggest that this is at least in part due to the up-regulation of Insig-1 and Insig-2a, which have been previously identified as modulators of SREBP activity. Insig anchor the SCAP/SREBP complex in the endoplasmic reticulum in the presence of sterols (Yang et al., 2002; Yabe et al., 2002). Overexpression of Insig-1 in the liver of transgenic mice inhibited processing of SREBP (Engelking et al., 2004). Inversely, reduction of both Insig mRNAs by RNA interference or by mutational inactivation led to an increase in nuclear SREBP (Sever et al., 2003; Adams et al., 2004; Lee et al., 2005). Nevertheless, we cannot exclude that the observed reduction of nuclear SREBP-1 is additionally caused by decreased expression of its precursor. Indeed, mRNA concentration of SREBP-1c is also down-regulated by PPARα activation even after 6 h of incubation with WY 14,643. A reduced amount of nuclear SREBP-1 can lead to decreased mRNA concentrations of the SREBP-1c gene since SREBP-1c contains a sterol regulatory element in its enhancer/promoter region and the nuclear form can activate its own gene in an autoregulatory loop (Amemiya-Kudo et al., 2002). Recently it was shown that PPARα activation can suppress SREBP-1c promoter through reduction of liver X receptor/RXR heterodimer formation, the activator of SREBP-1c promoter (Yoshikawa et al., 2003). However, this suppression was only observed upon concomitant LXR agonist treatment in mice and primary rat hepatocytes, but not upon WY 14,643 treatment alone (Yoshikawa et al., 2003) suggesting that also in Fao cells incubated with WY 14,643 this mechanism may not be relevant for the observed down-regulation of nuclear SREBP-1. In liver, the predominant SREBP-1 isoform is SREBP-1c whereas SREBP-1a seems to predominate in several cultured cells (Shimomura et al., 1997). However, SREBP-1a isoform, which was only slightly influenced by WY 14,643, is considerably less expressed than SREBP-1c in Fao cells indicating that it is does not significantly influence fatty acid and triglyceride synthesis.

The reduced abundance of transcriptionally active SREBP-1 in the nucleus upon PPARα activation in Fao cells after 24 h of incubation with WY 14,643 was reflected by lowered mRNA concentrations of two SREBP-1c target genes encoding enzymes for fatty acid and triacylglycerol synthesis. Both, FAS, catalysing a committed step in fatty acid synthesis, and GPAT, one of the key enzymes of triacylglycerol synthesis, were down-regulated after 24 h of incubation of Fao cells with WY 14,643. Furthermore, rate of newly synthesized triacylglycerols from de novo-derived fatty acids in Fao cells incubated with WY 14,643 for 24 h was decreased compared to control cells which is in agreement with the reduced mRNA concentration of GPAT. Finally, these data agree well with the reduced intracellular and secreted triacylglycerol concentrations in Fao cells after 24 h of incubation with WY 14,643.

Thus, these data strongly indicate that the triacylglycerol-reducing effect of fibrates is partially caused by a decrease in fatty acid and triacylglycerol synthesis due to inhibition of SREBP-1 maturation by up-regulation of Insig.

Based on the fact that a functional PPRE has been described in human Insig-1 gene that is regulated by PPARγ (Kast-Woelbern et al., 2004) we also analysed Fao cells incubated with the PPARγ agonist troglitazone. Expression of PPARγ in liver cells is relatively low (Fajas et al., 1997). In agreement with Davies et al. (2002) we observed a strong up-regulation of PPARγ mRNA after 6 h of incubation with 20 μM troglitazone indicative of activation of PPARγ. In agreement with the study of Kast-Woelbern et al. (2004) we could observe an up-regulation of Insig-1 mRNA in Fao cells at 20 μM troglitazone in rat Fao cells. Interestingly, Insig-2a mRNA was also up-regulated by PPARγ and the effect was much stronger than that observed for Insig-1 or in Fao cells incubated with WY 14,643. The strong increase of Insig mRNA concentration was associated with a reduction of the concentration of the transcriptionally active form of SREBP-1 in the

nucleus already after 6 h of incubation of Fao cells with 20 μM troglitazone. Also mRNA concentration of FAS and that of SREBP-1c itself were reduced after 6 h of incubation with 20 μM troglitazone which is consistent with the decreased concentration of nuclear SREBP-1. However, GPAT mRNA concentration was not changed after 6 h of incubation of Fao cells with 20 μM troglitazone. Nevertheless, the rate of newly synthesized triacylglycerols from de novo-derived fatty acids in Fao cells incubated with 20 μM troglitazone for 6 h was strongly reduced which was also reflected in decreased concentrations of intracellular and secreted triacylglycerols. The reason for unchanged GPAT mRNA concentration after 6 h of incubation of Fao cells with troglitazone is unclear. Interestingly, GPAT mRNA was significantly reduced after 24 h of incubation of Fao cells with 20 μM troglitazone compared to control cells (data not shown).

In contrast to WY 14,643, the down-regulation of SREBP-1 and its target genes was much faster in Fao cells incubated with troglitazone. Whereas effects of WY 14,643 were obvious after 24 h of incubation, troglitazone-induced down-regulation of SREBP-1 pathway was already observed after 6 h of incubation. We suggest that this is due to the strong up-regulation of Insig-2a by troglitazone. Yabe et al. (2003) discussed a special role for Insig-2a in SREBP-1c activation which allows cells to stimulate fatty acid synthesis by processing of SREBP-1c even when hepatic cholesterol levels are relatively high. Thus, our data indicate that also in case of PPARγ the inhibition of SREBP-1c activation by up-regulation of Insig, particularly Insig-2a, may contribute to the observed triacylglycerol-reducing effect of PPARγ agonists in rats. In parallel, we observed in human HepG2 and Caco-2 cells that troglitazone reduced cholesterol synthesis by reduction of the nuclear form of SREBP-2 (Klopotek et al., 2006).

These data match well with the findings in rats that PPARα activation down-regulates FAS expression and activity (Eder et al., 2003). However, in mice, the effects of PPARα agonists on SREBP-1 related pathways are inconsistent and do not match the results of this study. For example, there was either no effect of WY 14,643 or fenofibrate treatment on hepatic FAS expression and/or nuclear SREBP-1 (Yahagi et al., 1999; Sekiya et al., 2003; Li et al., 2005a) or both nuclear SREBP-1 and FAS mRNA concentration were increased in liver upon WY 14,643 treatment in wild-type but not in PPARα knockout mice (Knight et al., 2005). Furthermore, PPARα activation in pigs did also not influence hepatic mRNA concentration of SREBP-1 and its target genes (Luci et al., 2007). Thus, species-specific differences may exist regarding the influence of PPARα activation on SREBP-1 and its target genes. Also, effects of PPARγ agonists on SREBP-1 and FAS seem to be species-specific. In Zucker diabetic rats, troglitazone treatment decreased concentration of nuclear SREBP-1 and that of FAS mRNA in the liver (Li et al., 2005b). In contrast, no effect on hepatic nuclear SREBP-1 was observed upon troglitazone treatment of mice (Yahagi et al., 1999). In a mouse-derived hepatocyte cell line, expression of PPARγ2 increased expression of both SREBP-1c and FAS and increased triacylglycerol synthesis (Schadinger et al., 2005).

In conclusion, the data of this study demonstrate for the first time, that activation of both, PPARα and PPARγ, in rat Fao hepatoma cells lead to inhibition of SREBP-1 activation by up-regulation of Insig-1 and Insig-2a thereby decreasing synthesis of fatty acids and triacylglycerols. This may represent an additional mechanism by which fibrates and thizolidinediones decrease triacylglycerol concentration in rat liver and plasma.

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Thermally Oxidized Oil Increases the Expression of Insulin-Induced Genes and Inhibits Activation of Sterol Regulatory Element-Binding Protein-2 in Rat Liver[1,2]

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Abstract

Administration of oxidized oils to rats or pigs causes a reduction of their cholesterol concentrations in liver and plasma. The reason for this effect is unknown. We tested the hypothesis that oxidized oils lower cholesterol concentrations by inhibiting the proteolytic activation of sterol regulatory element-binding protein (SREBP)-2 in the liver and transcription of its target genes involved in cholesterol synthesis and uptake through an upregulation of gene expression of insulin-induced genes (Insig). For 6 d, 18 rats were orally administered either sunflower oil (control group) or an oxidized oil prepared by heating sunflower oil. Rats administered the oxidized oil had higher messenger RNA (mRNA) concentrations of acyl-CoA oxidase and cytochrome P450 4A1 in the liver than control rats ($P < 0.05$), indicative of activation of PPARα. Furthermore, rats administered the oxidized oil had higher mRNA concentrations of Insig-1 and Insig-2a, a lower concentration of the mature SREBP-2 in the nucleus, lower mRNA concentrations of the SREBP-2 target genes 3-hydroxy-3-methylglutaryl CoA reductase and LDL receptor in their livers, and a lower concentration of cholesterol in liver, plasma, VLDL, and HDL than control rats ($P < 0.05$). In conclusion, this study shows that reduced cholesterol concentrations in liver and plasma of rats administered an oxidized oil were due to an inhibition of the activation of SREBP-2 by an upregulation of Insig, which in turn inhibited transcription of proteins involved in hepatic cholesterol synthesis and uptake. J. Nutr. 137: 2018–2023, 2007.

Introduction

Oxidized lipids as components of heated or fried foods play an important role in nutrition in industrialized countries (1). Lipid peroxidation products present in oxidized oils influence animal metabolism in several ways, including the metabolism of lipids. Recently, we and others have shown that feeding oxidized oils to rats causes a reduction of concentrations of triacylglycerols and cholesterol in liver and plasma (2–4). The reduction of triacylglycerols in liver and plasma may be due to a stimulation of hepatic β-oxidation triggered by an activation of PPARα and a reduced hepatic de novo fatty acid synthesis (3–5). The molecular mechanisms underlying the reduction of cholesterol concentrations in liver and plasma, however, have not yet been elucidated.

Cholesterol homeostasis in mammalian cells is regulated by sterol regulatory element-binding protein (SREBP)[3]. SREBP belong to a large class of transcription factors containing basic helix-loop-helix-Zip domains, of which 3 isoforms have been characterized: SREBP-1a, -1c, and -2 (reviewed in 6,7). Whereas SREBP-1c, the predominant isoform in adult liver, preferentially activates genes required for fatty acid synthesis, SREBP-2 preferentially activates the LDL receptor gene and various genes required for cholesterol synthesis, such as 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase (6,7). SREBP-1a is an activator of both the cholesterol and fatty acid biosynthetic pathways, but it is present in much lower amounts in liver than the other 2 forms (8). After synthesis in membranes of the endoplasmic reticulum, SREBP form a complex with SREBP-cleavage activating protein (SCAP). When cells are depleted of sterols, SCAP escorts SREBP from the endoplasmic reticulum to the Golgi. Within the Golgi, 2 resident proteases, site-1 protease and site-2 protease, sequentially cleave the SREBP, release the amino-terminal basic helix-loop-helix-Zip-containing domain from the membrane, and allow it to translocate to the nucleus and activate transcription of their target genes. Recently, insulin-induced genes (Insig)-1 and -2 were identified as membrane proteins that reside in the endoplasmic reticulum and play a central role in the regulation of SREBP cleavage (9,10). When intracellular sterol concentrations are increased, SCAP binds to Insig, an action that prevents the translocation of the SREBP-SCAP complex from the endoplasmic reticulum to Golgi and the

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[3] Abbreviations used: ACO, acyl-CoA oxidase; Cyp4A1, cytochrome P450 4A1; HMG-CoA, 3-hydroxy-3-methylglutaryl CoA; Insig, insulin-induced gene; mRNA, messenger RNA; SCAP, SREBP-cleavage activating protein; SREBP, sterol regulatory element-binding protein.
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proteolytic activation of SREBP. As a result, the synthesis of cholesterol and fatty acids declines.

We recently observed in rats that activation of PPARα caused an upregulation of the expression of Insig-1 in the liver, which in turn inhibited proteolytic activation of SREBP-2 and lowered hepatic cholesterol synthesis and liver and plasma cholesterol concentrations (5). We and others have found that feeding an oxidized fat causes an activation of PPARα in the liver of rats or pigs and in rat fetuses (4,11–13). Therefore, we assume that oxidized fats affect cholesterol metabolism in a similar way as clofibrate did in our recent study. Our hypothesis is that the reduced concentrations of cholesterol in liver and plasma observed in rats fed an oxidized oil are mediated by an increased gene expression of Insig in the liver. An upregulation of Insig is expected to lower the concentration of the transcriptionally active SREBP-2 in the nucleus, which in turn leads to a reduced expression of its target genes involved in hepatic cholesterol synthesis (e.g. HMG-CoA reductase) and cholesterol uptake (LDL receptor) and explains reduced plasma and liver cholesterol concentrations. To proof this hypothesis, we performed an experiment with rats that were orally administered either a fresh or an oxidized oil. For an oxidized oil, we used an oil treated at a relatively low temperature over a long period, because such oils have high concentrations of primary lipid peroxidation products such as hydroxy- and hydroperoxy fatty acids, which are regarded as very potent PPARα agonists (14–16).

Materials and Methods

Animals and diets. Male Sprague-Dawley rats supplied by Charles River with an initial body weight of 115 ± 14 g (mean ± SD) were randomly assigned to 2 groups of 9 rats each. They were kept individually in Macrolon cages in a room controlled for temperature (22 ± 2°C), relative humidity (50–60%), and light (12-h-light/-dark cycle). All experimental procedures described followed established guidelines for the care and handling of laboratory animals and were approved by the council of Saxony-Anhalt. All rats were orally administered 2 mL fresh or oxidized sunflower oil by gavage once per day 2 h after the beginning of the light cycle. All rats were fed a commercial standard basal diet (altromin 1324). According to the declaration of the manufacturer, this diet contained (per kilogram) 11.9 MJ metabolizable energy, 190 g crude protein, 60 g crude fiber, 40 g crude fat, and 70 g crude ash. The vitamin E concentration of this diet was 75 mg/kg. To standardize food intake, the diets were fed daily in restricted amounts of 12 g/d, equivalent to an intake of 143 kJ metabolizable energy per day. Rats consumed water ad libitum from nipple drinkers during the entire experiment.

Preparation of the oxidized oil. The thermoxidized oil was prepared by heating sunflower oil (from a local supermarket) in an electric fryer (Saro Gastro-Products) for 25 d by 60°C. Throughout the heating process, air was continuously bubbled through the oil. The extent of lipid peroxidation was determined by assaying the peroxide value (17), concentration of TBARS (18), concentration of conjugated dienes (19), acid values (17), the percentage of total polar compounds (20), and the concentration of total carbonyls (21). The fatty acid composition of the dietary fats was determined by GC. Fats were methylated with trimethylsulfonium hydroxide (22). Fatty acid methyl esters were separated by GC using a system (HP 5890, Hewlett Packard) equipped with an automatic on-column injector, a polar capillary column (30-m FFAP, 0.53-mm i.d., Macherey and Nagel) and a flame ionization detector (23).

Sample collection. At d 6, rats received the last dose of fresh or oxidized oil and 9 g of the diet again 2 h after the beginning of the light cycle and were killed 4 h later by decapitation under light anesthesia with diethyl ether. Blood was collected into heparinized polyethylene tubes. The liver was excised. Plasma was obtained by centrifugation of the blood (1100 × *g*; 10 min, 4°C) and stored at −20°C. Liver samples for RNA isolation and lipid extraction were snap-frozen in liquid nitrogen and stored at −80°C.

Real-time RT-PCR analysis. Total RNA was isolated from rat liver by TRIZOL reagent (Life Technologies) according to the manufacturer's protocol. cDNA synthesis was carried out as described (16). The messenger RNA (mRNA) expression of genes was measured by real-time detection PCR using SYBR Green I and the Rotor Gene 2000 system (Corbett Research). Real-time detection PCR was performed with 1.25 units Taq DNA polymerase, 500 μmol desoxy ribonucleotide triphosphates, and 26.7 pmol of the specific primers (Operon Biotechnologies). For determination of mRNA concentration, a threshold cycle was obtained from each amplification curve using the software RotorGene 4.6 (Corbett Research). Calculation of the relative mRNA concentration was made using the ΔΔ threshold cycle method as previously described (24). We used the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (EC1.2.1.12) for normalization. The primer sequences used for real-time detection PCR were described previously (5).

Immunoblot analysis. Nuclear extracts of rat livers were prepared from fresh tissue samples (150 mg) according to Woo et al. (25). The protein content of the samples was determined by the bicinchoninic acid assay. We purchased bicinchoninic acid reagent from Interchim. Equal amounts of proteins were pooled from 5 and 4 rats, respectively, per group and 80 μg protein per lane was separated on 10% SDS-polyacrylamide gels according to the method of Laemmli et al. (26) and electrotransferred to a nitrocellulose membrane (Pall). Polyclonal anti-SREBP-2 antibody (Abcam) was used to detect nuclear SREBP-2 using enhanced chemiluminescence reagent (GE Healthcare) and a chemiluminescence imager camera (Biostep). Signals were analyzed with the Phoretix TotalLab TL100 software. The anti-rabbit-IgG peroxidase conjugate antibody was purchased from Sigma-Aldrich.

Liver, plasma, and lipoprotein cholesterol. Rat liver lipids were extracted with a mixture of n-hexane and isopropanol (3:2, v:v) (27). Aliquots of the lipid extracts were dried and dissolved in a small volume of Triton X-100 (28). Plasma lipoproteins were separated by stepwise ultracentrifugation as described (5). Total cholesterol concentrations of liver, plasma, and lipoproteins were determined using the enzymatic reagent kit (Ecoline S^+, DiaSys).

Statistical analysis. Means of treatments and control were compared by Student's *t* test using the Minitab Statistical software (Minitab). Values in the text are means ± SD. Means were considered significantly different at $P < 0.05$.

Results

Characterization of the experimental oil. Palmitic (16:0), stearic (18:0), oleic (18:1), and linoleic acid [18:2 (n-6)] were the major fatty acids in both oils, accounting for >98 g/100 g total fatty acids. Due to loss of PUFA by oxidation during heat treatment of the oil, the oxidized oil had a lower proportion of linoleic acid and slightly higher proportions of SFA and oleic acid (**Table 1**). The oxidized oil had much higher concentrations of peroxides (125-fold), conjugated dienes (>2740-fold), TBARS (11-fold), total carbonyls (32-fold), polar compounds (4-fold), and a higher acid value (14-fold) than the fresh oil (Table 1).

Body weight and relative liver weight of rats. Final body weight did not differ between groups of rats (fresh oil, 144 ± 14 g; oxidized oil, 133 ± 14 g; $n = 9$). However, body weight gain over the feeding period was lower in rats administered the oxidized oil (17.5 ± 6.4 g; $n = 9$) than in rats administered the fresh oil (29.7 ± 4.5 g; $n = 9$) ($P < 0.05$). The relative liver weight, expressed per kilogram body mass, was higher in rats administered the oxidized oil (49.7 ± 3.4 g/kg; $n = 9$) than in those administered the fresh oil (39.4 ± 2.0 g/kg) ($P < 0.001$).

Relative mRNA concentrations of PPARα and PPARα downstream genes in the liver. Relative mRNA concentration

TABLE 1 Characteristics of the experimental oils

	Fresh oil	Oxidized oil
Major fatty acids, *g/100 g* fatty acids		
16:0	6.1	8.5
18:0	3.3	4.9
18:1	32.2	35.7
18:2 (n-6)	55.9	41.7
18:3 (n-3)	0.1	<0.1
Peroxidation products		
Peroxide value, *mEq O_2/kg*	3.0	378.6
Conjugated dienes, *mmol/kg*	<0.1	273.6
TBARS, *mmol/kg*	1.1	13.1
Total carbonyls, *mmol/kg*	2.9	96.9
Total polar compounds, *%*	5.1	27.8
Acid value, *g KOH/kg*	0.4	5.8

of PPARα in the liver did not differ between groups (**Fig. 1**). However, rats administered the oxidized oil had higher relative mRNA concentrations of the PPARα downstream genes acyl-CoA oxidase (ACO) and cytochrome P450 4A1 (Cyp4A1) than rats administered fresh oil ($P < 0.05$; Fig. 1).

Relative mRNA concentrations of Insig in the liver. Relative mRNA concentrations of Insig-1 and Insig-2a, the liver-specific isoform of Insig-2, in the liver were higher in rats administered the oxidized oil than in those administered the fresh oil ($P < 0.05$; **Fig. 2**).

Relative concentration of nuclear SREBP-2 and relative mRNA concentrations of SREBP-2, HMG-CoA reductase, and LDL receptor in the liver. Rats administered the oxidized oil had lower protein concentrations of nuclear SREBP-2 in the liver than rats administered the fresh oil (relative values are the mean of 2 pools for each group: fresh oil, 1.00 ± 0.45; oxidized oil, 0.26 ± 0.02; $P < 0.05$; **Fig. 3***A*). Relative mRNA concentrations of SREBP-2 and its target genes HMG-CoA reductase and LDL receptor were also lower in rats administered the oxidized oil than in those administered the fresh oil ($P < 0.05$; Fig. 3*B*).

Cholesterol concentrations in liver, plasma, VLDL, LDL, and HDL. Rats administered the oxidized oil had lower concentrations of cholesterol in liver, plasma, VLDL, and LDL than rats administered the fresh oil ($P < 0.05$); the concentration of cholesterol in LDL did not differ between groups (**Table 2**).

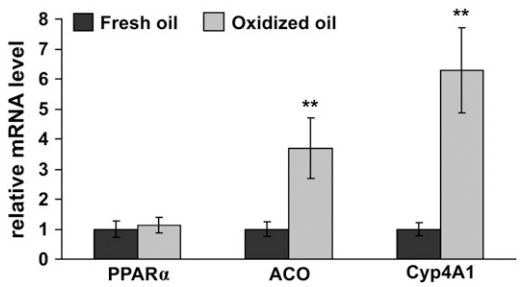

FIGURE 1 Relative mRNA concentrations of PPARα, ACO, and Cyp4A1 in rat livers treated with fresh or oxidized oil. Values are means ± SD, $n = 9$. **Significantly different from rats treated with fresh oil, $P < 0.001$.

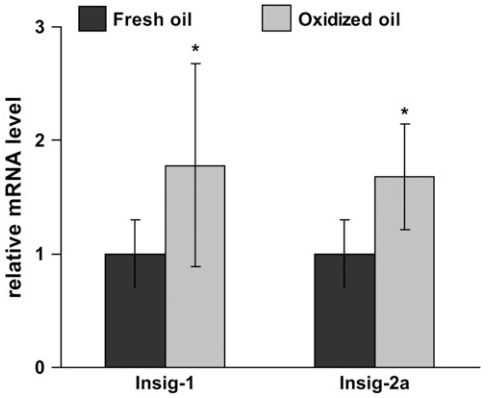

FIGURE 2 Relative mRNA concentrations of Insig-1 and Insig-2a in rat livers treated with fresh or oxidized oil. Values are means ± SD, $n = 9$. *Significantly different from rats treated with fresh oil, $P < 0.05$.

Discussion

We recently found that treatment with clofibrate inhibits the proteolytic activation of SREBP-2 by an upregulation of the expression of Insig-1, which in turn lowered transcription of SREBP-2 target genes involved in hepatic cholesterol synthesis and uptake and reduced liver and plasma cholesterol concentrations in rats (5). This effect was probably caused by PPARα activation. In this study, we investigated the hypothesis that oxidized fats are able to exert similar effects due to their ability to activate PPARα. Hydroxy- and hydroperoxy fatty acids, such as hydroxyoctadecadienoic and hydroperoxyoctadecadienoic acid, occurring in oxidized fats are very potent PPARα agonists (14–16). These fatty acids are produced during the early stage of lipid peroxidation. Because they are unstable and decompose at high temperatures (29), fats treated at low temperature have much higher concentrations of these primary lipid peroxidation products than fats treated at high temperatures (4). The high peroxide value and the high concentration of conjugated dienes

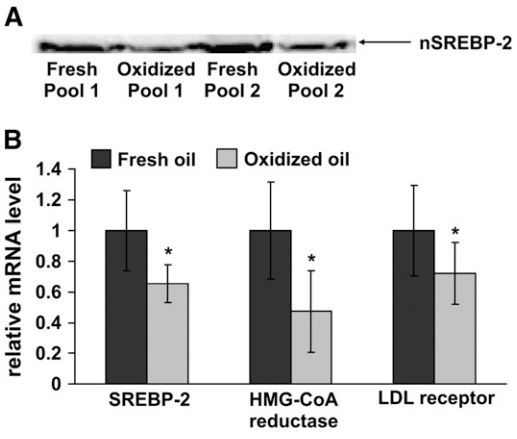

FIGURE 3 Effect of an oxidized oil on SREBP-2 and its target genes in the liver of rats. Concentration of nuclear SREBP-2 (~68 kDa) in the liver of rats treated with fresh or oxidized oil was determined by western blot (*A*). Liver nuclear extracts of 5 and 4 rats, respectively, from each group were pooled. Relative mRNA concentrations of SREBP-2, HMG-CoA reductase, and LDL receptor in the liver of rats treated with fresh or oxidized oil (*B*). Values are means ± SD, $n = 9$. *Significantly different from rats treated with fresh oil, $P < 0.05$.

TABLE 2 Concentration of cholesterol in liver, plasma, and plasma lipoproteins of rats treated with fresh or oxidized oil[1]

	Fresh oil	Oxidized oil
Liver, *μmol/g*	7.56 ± 0.42	6.15 ± 0.68**
Plasma, *mmol/L*	2.07 ± 0.23	1.68 ± 0.18*
VLDL, *mmol/L*	0.16 ± 0.08	0.02 ± 0.01**
LDL, *mmol/L*	0.56 ± 0.11	0.54 ± 0.10
HDL, *mmol/L*	1.27 ± 0.17	1.10 ± 0.17*

[1] Results are means ± SD, $n = 9$. Asterisks indicate significant differences from group fed fresh oil, *$P < 0.05$, **$P < 0.001$.

indicate that the oxidized oil used in this study indeed had high concentrations of hydroxy- and hydroperoxy fatty acids. Due to the loss of PUFA during the heating process, the concentrations of PUFA (i.e. linoleic acid) in the oxidized oil were slightly lower than in the fresh. Although intake of PUFA can influence cholesterol metabolism, i.e. plasma cholesterol concentration (30), we assume that the small difference in the intake of PUFA cannot be the main reason for the differences in cholesterol metabolism observed in this study. This assumption is confirmed by the observation that liver and plasma cholesterol concentrations were also reduced by oxidized oils in a similar extent in recent studies in which fresh and oxidized oils were equalized for their fatty acid composition (4,31–33). So that all rats obtained the same dose of oxidized oil, we administered it orally by gavage. The oxidized and fresh oil, respectively, accounted for about 25% of total energy of the total daily feed. Because it was observed in a previous rat study that even short term application of a PPARα agonist led to the typical changes known for PPARα activation [such as upregulation of classical target genes involved in β-oxidation and reduction of triacylglycerol concentration (16)], we decided to give the oxidized oil during a relatively short period of 6 d. The intake of oxidized fats could cause a reduction of the food intake in rats, which could in turn cause secondary effects that interact with the effect of treatment (34,35). To ensure an identical food intake in both groups of rats, we used a controlled feeding system in which each rat consumed 12 g diet/d. This amount of diet is slightly below that rats would consume ad libitum but is about 50% in excess of that necessary to meet the maintenance energy requirement (36) and therefore ensures adequate growth. The finding that rats administered the oxidized oil gained less weight during the experimental period than those administered the fresh oil even though both groups received an identical amount of diet indicates that the oxidized oil impaired the feed conversion ratio. This finding agrees with other reports that also showed that feeding of oxidized fats impairs growth of rats (37–40). We did not investigate the reason for this. Previous studies, however, have shown that oxidized fats lower the digestibility of nutrients and this may be the reason for the reduced body weight gains of the rats administered the oxidized oil observed in this study (37,41). However, daily body weight gains of the rats administered the oxidized oil were also within the normal physiological range and as these rats appeared quite normal, we assume that the oxidized oil did not cause general toxicity.

Studies in rats and pigs have shown that feeding oxidized oils lowers plasma and tissue tocopherol concentrations and causes oxidative stress (4,32,34,35,42). In this study, we did not determine the vitamin E status of the animals. According to these recent studies, administration of the oxidized fat probably also lowered plasma and tissue vitamin E concentrations compared with control animals. Nevertheless, because the diet used in this study had a relatively high vitamin E concentration and because the experimental period was relatively short, we assume that the rats administered the oxidized oil had an adequate vitamin E status in spite of the vitamin E consuming effect of the oxidized oil. Therefore, it is unlikely that the results in this study were confounded by vitamin E deficiency in the rats administered the oxidized fat.

The finding of increased mRNA concentrations of the typical PPARα downstream genes ACO and Cyp4A1 (43) in the liver and increased liver masses indeed indicates that the oxidized oil caused an activation of PPARα in the liver of the rats. This indication agrees with recent studies in rats and pigs and in rat fetes, which also showed that intake of oxidized fats leads to an activation of PPARα in the liver (3,4,12,44,45). Activation of PPARα by the oxidized oil may be due to the presence of hydroxy- and hydroperoxy fatty and cyclic fatty acids, all of which have been shown to be potent PPARα activators (14–16,46). We recently showed that the effect of oxidized fats on activation of PPARα is independent of the dietary vitamin E concentration (4). The finding that oxidized fats also exert a PPARα-activating effect at very high dietary vitamin E concentrations (which suppress the induction of oxidative stress) indicates that activation of PPARα is not caused by oxidative stress but by lipid peroxidation products present in the oxidized oil.

This study shows for the first time, to our knowledge, that administration of an oxidized oil upregulates the gene expression of Insig-1 and Insig-2a in the liver. Because Insig are able to retain the SCAP-SREBP-complex within the endoplasmic reticulum, thus inhibiting the proteolytic activation of SREBP in the Golgi (9,10), this event is probably the reason for the lower concentration of the mature SREBP-2 in the nucleus, which in turn leads to a reduced transcription of HMG-CoA reductase, the rate-limiting enzyme of de novo synthesis of cholesterol, and LDL receptor. Reduced cholesterol concentrations in liver and plasma, therefore, are likely the result of a reduction of hepatic cholesterol synthesis. Reduced hepatic cholesterol concentrations, moreover, may be in part due to a reduced uptake of LDL into liver cells. Besides nuclear concentrations of SREBP-2, mRNA concentration of SREBP-2 was also reduced in the liver of rats administered the oxidized oil. Because SREBP-2 contains a sterol-regulatory element in its enhancer/promoter region and thus the nuclear form can activate its own gene in an autoregulatory loop (47), this reduction is probably the effect of the reduced nuclear SREBP-2 concentration. In previous experiments with Fao cells treated with the PPARα agonist WY 14643, we demonstrated that the decreased SREBP-2 mRNA concentration did not precede the decrease of its nuclear form, indicating that it is due, rather, to increased expression of Insig (5).

Considering that similar effects were observed in the liver of rats treated with the synthetic PPARα agonist clofibrate and in rat hepatoma cells treated with the more potent and selective PPARα agonist WY 14,643 (5), we propose that the oxidized oil upregulated Insig in the liver of rats by PPARα activation. A functional PPAR response element that is regulated by PPARγ has already been identified in the human Insig-1 gene (48). Analysis of the 5′ flanking region of rat Insig-1 using the PPAR response element consensus sequence from literature revealed 2 putative PPAR response elements at positions −592 and −1181 upstream of the transcription start site of the reported cDNA. The functionality of these PPAR response elements should be examined in future experiments.

We have recently observed that troglitazone, a synthetic PPARγ agonist, also lowers the mature SREBP-2 concentration

and inhibits cholesterol synthesis in HepG2, a human hepatoma cell line (49). Because oxidized fatty acids are also able to bind to and activate PPARγ (50,51), we cannot exclude the possibility that the oxidized oil induced the effects observed in this study by activating PPARγ, whose expression in the liver is, however, much lower than that of PPARα (52). The expression of Insig is also regulated by insulin. Insig-1 is upregulated by insulin, an effect caused by the insulin-induced stimulation of SREBP-1c gene transcription (53,54), which in turn leads to increased transcription of Insig-1 that is an obligatory SREBP target gene (9). In contrast, the Insig-2a transcript in the liver is strongly repressed by insulin. Thus, during fasting and feeding, Insig-1 and Insig-2a are regulated reciprocally (55). It has been shown that dietary oxidized frying oil lowers postprandial plasma concentration of insulin and induces glucose intolerance in rats and mice (56). As reduced plasma insulin concentrations would be expected to lower gene expression of Insig-1, it is unlikely that the upregulation of Insig-1 in the liver of rats administered the oxidized oil was mediated by insulin. Whether or not the observed upregulation of Insig-2a in the liver of rats treated with oxidized oil is mediated by reduced insulin concentrations or by PPARα activation remains unclear. In Fao cells treated with the PPARα agonist WY 14,643, mRNA concentration of Insig-2a was also increased, indicating that PPARα activation may also play a role in upregulation of Insig-2a.

The results of this study disagree with a recent study that investigated the effect of a moderately oxidized fat on triacylglycerol and cholesterol metabolism in pigs (45). In that study, the oxidized fat caused a moderate activation of PPARα but did not alter expression of genes involved in cholesterol metabolism, including SREBP-2, Insig, HMG-CoA reductase, and LDL receptor. That study and our study may disagree because of at least 2 reasons. First, the animal model used, pigs, belong to the group of nonproliferating species and have a lower expression of PPARα in the liver and a much weaker response of many genes to PPARα activation than rats, which belong to the group of proliferating species (57). Second, the fat used in the recent study performed with pigs was, according to concentrations of lipid peroxidation products, less oxidized than the fat used in this study. In the pig study, we used a mildly oxidized fat in which concentrations of peroxides (4-fold), conjugated dienes (4-fold), carbonyls (10-fold), and thiobarbituric acid reactive substances (30-fold) were only moderately increased compared with the fresh control fat. The oxidized fat used in this study had much higher concentrations of lipid peroxidation products, particularly of primary lipid peroxidation products, than that used in the pig study.

Although this study in rats shows that oxidized fats influence cholesterol metabolism via an upregulation of Insig, an effect probably mediated by activation of PPARα, it remains to be investigated whether such an effect also occurs in humans. With respect to expression and activation of PPARα, humans behave similarly to pigs. Humans and pigs have a similar expression of PPARα in the liver that is, however, 90% lower than in rats (58). Accordingly, upregulation of PPARα target genes in the liver by PPARα agonists is much weaker in pigs and humans than in rats (59,60). Therefore, it is expected that effects of oxidized fats on cholesterol metabolism, mediated by PPARα activation, in humans are weaker than those in rats observed in this study.

The fat used in this study prepared by heating at a relatively low temperature over a long period does not directly reflect the oxidized fats in human nutrition that originate predominantly from deep frying of foods. However, we have recently shown that fats produced under deep frying conditions lower liver and plasma cholesterol concentrations in rats to a similar extent as fats heated at a low temperature over a long period such as that used in this study (61). Moreover, it has been shown that fats prepared by deep frying are able to activate PPARα in the liver of rats (3,44). Therefore, it is likely that deep-fried fats influence the cholesterol metabolism in a similar way as fats prepared at a lower temperature for a longer period.

In conclusion, this study shows that oxidized oils are able to affect the activation of SREBP-2 by an upregulation of Insig-1 and Insig-2a in the liver of rats, which in turn lowers transcription of genes involved in cholesterol synthesis and uptake. This provides an explanation for reduced concentrations of cholesterol in liver and plasma observed in rats in this and recent studies. Although we assume that these effects are triggered by activation of PPARα, this must be proven in future studies.

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Feeding of a deep-fried fat causes PPARα activation in the liver of pigs as a non-proliferating species

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Recent studies have shown that dietary oxidised fats influence the lipid metabolism in rats by activation of PPARα. In this study, we investigated whether a mildly oxidised fat causes activation of PPARα in pigs which are non-proliferators like man. Eighteen pigs were assigned to two groups and received either a diet containing 90 g/kg of a fresh fat or the same diet with 90 g/kg of an oxidised fat prepared by heating for 24 h at 180°C in a deep fryer. Pigs fed the oxidised fat had a higher peroxisome count, a higher activity of catalase and a higher mRNA concentration of mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase in the liver and a higher concentration of 3-hydroxybutyrate in plasma than pigs fed the fresh fat ($P<0{\cdot}05$). Hepatic mRNA concentrations of acyl-CoA oxidase and carnitine palmitoyltransferase-1 tended to be increased in pigs fed the oxidised fat compared to pigs fed the fresh fat ($P<0{\cdot}10$). Pigs fed the oxidised fat, moreover, had higher mRNA concentrations of sterol regulatory element-binding protein (SREBP)-1 and its target genes acetyl-CoA carboxylase and stearoyl-CoA desaturase in the liver and higher mRNA concentrations of SREBP-2 and its target genes 3-hydroxy-3-methylglutary-CoA reductase and LDL receptor in liver and small intestine. In conclusion, this study shows that even a mildly oxidised fat causes activation of PPARα in the liver of pigs. Up-regulation of SREBP and its target genes in liver and small intestine suggests that the oxidised fat could stimulate synthesis of cholesterol and TAG in these tissues.

Oxidised fat: Pig: PPARα: Cholesterol: Triacylglycerols

The typical western diet contains large quantities of PUFA that are heated or processed to varying degrees. In fast-food restaurants fat is heated in fryers for up to 18 h daily, at temperatures close to 180°C (Frankel *et al.* 1984). Several studies with animals have been performed to investigate the effects of oxidised fats on the metabolism (reviewed in Cohn, 2002). Recently, it has been shown in rats that oxidised fats are able to influence the lipid metabolism by activation of PPARα (Chao *et al.* 2001, 2004, 2005; Sülzle *et al.* 2004), a transcription factor belonging to the nuclear hormone receptor superfamily (Schoonjans *et al.* 1996). This is probably due to the occurrence of hydroxy- and hydroperoxy fatty acids such as hydroxy octadecadienoic acid and hydroperoxy octadecadienoic acid which are potent activators of PPARα (Delerive *et al.* 2000; Mishra *et al.* 2004; König & Eder, 2006). Activation of PPARα leads to an increase in the transcription of genes related to fatty acid transport across the cell membrane, intracellular lipid trafficking, mitochondrial and peroxisomal fatty acid uptake, and both mitochondrial and peroxisomal fatty acid β-oxidation, gluconeogenesis and ketogenesis (Mandard *et al.* 2004). Recently, it has been shown that PPARα activation influences also the expression or the proteolytic activation of sterol regulatory element-binding proteins (SREBP), transcription factors which control fatty acid synthesis and cholesterol homeostasis (Patel *et al.* 2001; Guo *et al.* 2001; Knight *et al.* 2005; König *et al.* 2006). Therefore, PPARα activation stimulates not only the degradation of fatty acids by enhancing β-oxidation but affects also the synthesis of cholesterol and TAG. Reduced liver and plasma concentrations of TAG and cholesterol are typical effects observed in animals treated with PPARα agonists, and such effects have been also observed in rats administered oxidised fats (Huang *et al.* 1988; Eder & Kirchgessner, 1998; Eder, 1999; Chao *et al.* 2001, 2004, 2005; Sülzle *et al.* 2004).

Regarding the expression of PPARα in tissues and the effects of PPARα activation on transcription of its target genes, there are great differences between various species. In rodents, PPARα is highly expressed, and activation of PPARα not only induces many genes involved in various metabolic pathways such as β-oxidation, ketogenesis and

Abbreviations: ACC, acetyl-CoA carboxylase; ACO, acyl-CoA oxidase; CPT-1, carnitine palmitoyltransferase-1; CYP7, cholesterol 7α-hydroxylase; HMG-CoA-R, 3-hydroxy-3-methylglutaryl-CoA reductase; L-FABP, liver fatty acid binding protein; mHMG-CoA-S, mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase; MTP, microsomal TAG transfer protein; SCD, stearoyl-CoA desaturase; SOD, superoxide dismutase; SREBP, sterol regulatory element-binding protein; TBARS, thiobarbituric acid-reactive substances.

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gluconeogenesis but also causes severe peroxisome proliferation in the liver (Peters *et al.* 2005). In contrast to rodents, PPARα agonists do not induce peroxisome proliferation in the liver of many other species, such as guinea pigs, swine, monkeys and man (Holden & Tugwood, 1999). These non-proliferating species have a lower expression of PPARα in the liver and the response of many genes to PPARα activation is much weaker than in proliferating species. For that reason, effects related to PPARα activation observed in rodents cannot be directly applied for non-proliferating species such as man. Therefore, it remains unknown whether oxidised fats are able to cause PPARα activation also in non-proliferating species.

The aim of the present study was to investigate whether a dietary oxidised fat, prepared by heating sunflower oil under usual deep-frying conditions (180°C) for 24 h in a deep fryer, is able to activate PPARα and to cause peroxisome proliferation in pigs. Pigs have been chosen as a model since they belong – like man – to the non-proliferating species (Yu *et al.* 2001; Peffer *et al.* 2005) and since pig liver cells show a similarity to human liver cells in the gene response to PPARα agonists (Cheon *et al.* 2005). We focused our analyses on liver and small intestine as both tissues exhibit a high expression of PPARα (Braissant *et al.* 1996; Lemberger *et al.* 1996). Moreover, both tissues play an important role in whole body lipid homeostasis, i.e. synthesis and secretion of lipoproteins rich in TAG and cholesterol (Lindsay & Wilson, 1965; Dietschy *et al.* 1993). We examined the expression of various genes involved in lipid metabolism which have been already shown to be influenced by PPARα activation. Furthermore, in both tissues we determined gene expression of SREBP and important SREBP target genes involved in fatty acid synthesis and cholesterol uptake and synthesis.

Materials and methods

Animals

For the experiment, eighteen male 8-week-old crossbred pigs ((German Landrace × Large White) × Pietrain) were kept in a room under controlled temperature at 23 ± 2°C and 55 ± 5 % relative humidity with light from 06.00 to 18.00 hours. One day before the beginning of the experimental feeding period, the pigs were weighed and randomly allocated to two groups with body weights of 12·0 (SD 1·1) kg in the control group and 12·2 (SD 0·9) kg in the treatment group. All experimental procedures described followed established guidelines for the care and use of laboratory animals and were approved by the local veterinary office.

Diets and feeding

Both groups of pigs received a nutritionally adequate diet for growing pigs containing (in g/kg) wheat (400), soyabean meal (230), wheat bran (150), barley (100), sunflower oil or test oil (90), and mineral premix including L-lysine, DL-methionine and L-threonine (30). This diet contained 14·4 MJ metabolisable energy and 185 g crude protein/kg. Diet intake was controlled, and each animal in the experiment was offered an identical amount of diet per day. During the feeding period, the amount of diet offered each day was increased continuously from 400 to 1200 g. The pigs had free access to water via nipple drinking systems. The experimental diets were administered for 28 d.

Preparation of the test fats

To prepare the oxidised fat, sunflower oil obtained from a local supermarket was heated at a temperature of 180°C for 24 h in a deep fryer. This treatment caused a loss of PUFA and tocopherols. The major fatty acids in the fresh and the oxidised fat, respectively, were (g/100 g total fatty acids): palmitic acid (16 : 0), 6·30 *v.* 6·70; stearic acid (18 : 0), 4·0 *v.* 4·2; oleic acid (18 : 1*n*-9), 22·8 *v.* 23·8; linoleic acid (18 : 2*n*-6), 63·6 *v.* 59·9. Other fatty acids were present only in small amounts (< 0·5 g/100 g fatty acids). To equalise the fatty acid composition of the fresh and the oxidised fat, the fresh fat was composed of a mixture of sunflower oil and palm oil (93 : 7, w/w). To adjust dietary vitamin E concentrations, we analysed the native concentrations of tocopherols in the fresh fat and in the oxidised fats after the thermal treatment. With consideration of the native tocopherol concentrations of the dietary fats, the diets were supplemented individually with all-*rac*-α-tocopheryl acetate (the biopotency of all-*rac*-α-tocopheryl acetate is considered to be 67 % of that of α-tocopherol). The final vitamin E concentration was 620 mg α-tocopherol equivalents/kg in both fats. Concentrations of lipid peroxidation products were determined after the fats have been already included into the diets. Therefore, lipids of the diets were extracted by *n*-hexane and isopropanol (3 : 2, v/v; Hara & Radin, 1978). Concentration of thiobarbituric acid-reactive substances (TBARS; Sidwell *et al.* 1954), conjugated dienes (Recknagel & Glende, 1984), peroxide value (Deutsche Gesellschaft für Fettwissenschaft, 1994), acid value (Deutsche Gesellschaft für Fettwissenschaft, 1994) and concentration of total carbonyls (Endo *et al.* 2001) were determined in the extracted fat.

Sample collection

After completion of the feeding period the animals were killed under light anaesthesia. Each pig was fed its respective diet 4 h before being killed. After killing, blood was collected into heparinised polyethylene tubes. Plasma was obtained by centrifugation of the blood (1100 ***g***, 10 min, 4°C). Plasma lipoproteins were separated by step-wise ultracentrifugation (Mikro-Ultrazentrifuge; Sorvall Products, Bad Homburg, Germany) at 900 000 ***g*** at 4°C for 1·5 h. Plasma densities were adjusted by sodium chloride and potassium bromide and the lipoprotein fractions $\delta < 1{\cdot}006$ kg/l VLDL plus chylomicrons, $1{\cdot}006 < \delta < 1{\cdot}063$ kg/l LDL and $\delta > 1{\cdot}063$ kg/l HDL were removed by suction. The liver was dissected and weighted and samples were stored at −80°C until analysis. For preparation of liver homogenate, 1 g liver tissue was homogenised in PBS by TissueLyser (Qiagen, Haan, Germany), centrifuged at 600 ***g*** for 10 min at 4°C and the supernatant was stored at −20°C until analysis. For isolation of intestinal epithelial cells, the abdomen was immediately opened after killing, and a 35 cm intestinal segment was dissected starting at 30 cm distal to the pyloric sphincter, and flushed twice with ice-cold wash buffer (PBS containing 0·2 mM-phenylmethylsulphonyl fluoride and 0·5 mM-dithiothreitol, pH 7·4).

The isolation of porcine intestinal epithelial cells was performed by the modified distended intestinal sac technique according to Fan *et al.* (2004). In brief, the intestinal segments were filled with 100 ml preincubation buffer (PBS containing 27 mM-sodium citrate, 0·2 mM-phenylmethylsulphonyl fluoride and 0·5 mM-dithiothreitol, pH 7·4), sealed with strings and filled intestinal segments were incubated in a saline bath (154 mM-NaCl) for 15 min at 37°C. Afterwards, the pre-incubation buffer was discarded, and the intestinal segments were filled with isolation buffer (PBS containing 1·5 mM-Na_2EDTA, 0·2 mM-phenylmethylsulphonyl fluoride, 0·5 mM-dithiothreitol and 2 mM-D-glucose, pH 7·4). Two major cell fractions, consisting of the upper and the crypt cell fraction, were sequentially isolated from intestinal segments through two consecutive incubations with isolation buffer at 37°C for 40 (upper cell fraction) and 60 min (crypt cell fraction), respectively. Each cell fraction was collected separately, and washed twice with ice-cold PBS. Afterwards, cells were retained by centrifugation (400 ***g***, 4 min, 4°C) and immediately frozen at −80°C. For further analysis, we used the crypt cell fraction as it has been shown that these cells have a 6–8-fold higher capacity of lipid synthesis than villus cells (Shakir *et al.* 1978).

Lipid analysis

Lipids from liver were extracted with a mixture of *n*-hexane and isopropanol (3 : 2, v/v; Hara & Radin, 1978). For determination of the concentrations of lipids in liver, aliquots of the lipid extracts were dried and the lipids were dissolved using Triton X-100 (De Hoff *et al.* 1978). Concentrations of TAG and cholesterol in plasma and lipoproteins and those of liver were determined using enzymatic reagent kits (cat. no. 113009990314 for cholesterol and cat. no. 157609990314 for TAG; Ecoline S^+, DiaSys, Holzheim, Germany).

Preparation of liver microsomal and cytosolic fractions

Liver (1 g) was homogenised in 10 ml 0·1 M-phosphate buffer, pH 7·4, containing 0·25 M-sucrose using a Potter-Elvehjem homogeniser. Homogenates were centrifuged at 1000 ***g*** for 10 min at 4°C, and the supernatant was centrifuged at 15 000 ***g*** for a further 15 min. The microsomal pellet was obtained by centrifugation of the 15 000 ***g*** supernatant at 105 000 ***g*** for 60 min. The resulting cytosolic fraction in the supernatant was separated, microsomal pellets were resuspended in the homogenisation buffer and all samples were stored at −20°C for further analysis. The protein concentrations of cytosolic and microsomal fractions were determined with the BCA reagent according to the protocol of the supplier (Interchim, Montelucon, France) using bovine serum albumin as standard.

RT–PCR analysis

Total RNA from liver tissue and enterocytes, respectively, was isolated by the TissueLyser (Qiagen) using Trizol reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocol. RNA concentration and purity were estimated from the optical density at 260 and 280 nm (SpectraFluor Plus; Tecan, Crailsheim, Germany). The quality of all RNA samples was furthermore assessed by agarose gel electrophoresis. Total RNA (1·2 μg) was used for cDNA synthesis as described previously (König & Eder, 2006). The mRNA concentration of genes was measured by real-time detection PCR using SYBR® Green I and the Rotor Gene 2000 system (Corbett Research, Mortlake, Australia). Real-time detection PCR was performed with 1·25 U Taq DNA polymerase, 500 μM-dNTP and 26·7 pmol of the specific primers. For determination of mRNA concentration a threshold cycle (C_t) and amplification efficiency was obtained from each amplification curve using the software RotorGene 4·6 (Corbett Research). Calculation of the relative mRNA concentration was made using the $\Delta\Delta C_t$ method as previously described (Pfaffl, 2001). The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase was used for normalisation. The PCR primers used for real-time RT–PCR were obtained from Operon (Köln, Germany) and Roth (Karlsruhe, Germany), respectively, and are listed in Table 1.

Enzyme assays

Superoxide dismutase (SOD) activity in liver cytosol was determined according to the method of Marklund & Marklund (1974) with pyrogallol as the substrate. One unit of SOD activity is defined as the amount of enzyme required to inhibit the autoxidation of pyrogallol by 50 %. The activity of glutathione peroxidase in liver cytosol was determined with *t*-butyl hydroperoxide as substrate according to the method of Paglia & Valentine (1967). One unit of glutathione peroxidase activity is defined as 1 μmol reduced β-nicotinamide adenine dinucleotide phosphate oxidised/min. The activity of glutathione *S*-transferase was determined using 1-chloro-2,4-dinitrobenzene as substrate as described by Habig *et al.* (1974). One unit of glutathione *S*-transferase is defined as one nmol substrate consumed/min. Catalase activity in liver homogenate was determined using H_2O_2 as substrate according to the method of Aebi (1986). One unit of catalase activity is defined as the amount consuming 1 mmol H_2O_2/min.

Determination of conjugated dienes, thiobarbituric acid-reactive substances and α-tocopherol

Lipids from liver were extracted using a mixture of *n*-hexane and isopropanol (3 : 2, v/v; Hara & Radin, 1978). After drying the lipid extracts, 1 mg extract was dissolved in 1 ml *n*-hexane. The concentrations of conjugated dienes were calculated by using the molar extinction coefficient for conjugated dienes at 234 nm ($\epsilon = 29\,500$ mol/cm). The concentrations of TBARS were measured in liver homogenates as described (Brandsch *et al.* 2002). The concentration of α-tocopherol in liver tissue was determined by HPLC (Brandsch *et al.* 2002).

Determination of H_2O_2

To determine the H_2O_2 content in liver homogenates, the method for cell culture systems described by Royall & Ischiropoulos (1993) was modified, using dihydrorhodamine 123 as substrate. Homogenates were incubated with 27·5 μM-dihydrorhodamine 123 for 1 h at 37°C in a final volume of 400 μl. After incubation, the fluorescence of rhodamine 123, the oxidation product of dihydrorhodamine 123, was measured (excitation wavelength 485 nm, emission wavelength 538 nm).

Table 1. Characteristics of the specific primers used for RT–PCR analysis

Gene	Forward primer (from 5′ to 3′)	Reverse primer (from 5′ to 3′)	bp	Annealing temp. (°C)	NCBI GenBank
ACC	CTCCAGGACAGCACAGATCA	GCCGAAACATCTCTGGGATA	170	60	AF175308
ACO	CTCGCAGACCCAGATGAAAT	TCCAAGCCTCGAAGATGAGT	218	60	AF185048
apo CIII	GACACCTCCCTTCTGGACAA	TCCCAGAAGTCGGTGAACTT	185	60	NM_001002801
CPT-1	GCATTTGTCCCATCTTTCGT	GCACTGGTCCTTCTGGGATA	198	60	AF288789
CYP7	TATAGGGCACGATGCACAGA	ACCTGACCAGTTCCGAGATG	200	60	NM_001005352
FAS	GAACACGGCCTAGAAGTGGA	ATCTGGATCCTGCAGATGG	199	62	NM_213839
FATP	GGTTCCAGCCTGTTGAATGT	AACAAAACCTTGGTGCTTGG	275	60	DQ192231
FDPS	GAAAGGCAGGATTTCATCCA	AGAAGGCTTGGAGCAGTTCA	259	60	AY609787
GAPDH	AGGGGCTCTCCAGAACATCATCC	TCGCGTGCTCTTGCTGGGGTTGG	446	60	AF017079
Glutathione peroxidase	CAAGAATGGGGAGATCCTGA	GATAAACTTGGGGTCGGTCA	190	60	NM_214201
Glutathione *S*-transferase	TTTTTGCCAACCCAGAAGAC	GGGGTGTCAAATACGCAATC	246	60	NM_214300
HMG-CoA-R	GGTCAGGATGCGGCACAGAACG	GCCCCACGGTCCCGATCTCTATG	127	65	S79678
I-FABP	TACAGCCTCGCAGACGGAACTG	TGCTTGATGAGGAGAGGAAAACAG	276	59	AY960624
Insig-1	AGAGGGAGTGGGCCAGTGTGATGC	ACGGGAGCCAGGAGCGGATGTAG	276	65	AY336601
Insig-2	AAATCACGCCAGCGCTAAAGTG	TCCTACTCCAAGGCCAAAACCAC	127	60	AY585269
LDL receptor	AGAACTGGCGGCTGAAGAGCATC	GAGGGGTAGGTGTAGCCGTCCTG	115	60	AF118147
L-FABP	TTCGGTGCATGTCTAAGCTG	TGAGAGGGAGAGGATGAGGA	200	60	DQ182323
mAAT	TATGTCACCGTGCAGACCAT	CTCCTTCCACTGCTCAGGAC	309	60	M11732
mHMG-CoA-S	GGACCAAACAGACCTGGAGA	ATGGTCTCAGTGCCCACTTC	198	62	U90884
MTP	CAGGACGGCAAAGAAAGAAGG	ATGGGAAGCAAAACCACAAGG	199	60	AY217034
NPC1	ACGCGGTATCTTTGGTCAAC	AGTGGCTCCCAGCAAGACTA	266	60	AF169635
NPC2	GGAGGGGAGGAGAAATCAAG	ATTCGGGTCTTGTCTGGTTG	267	60	NM_214206
PPARα	CAGCCTCCAGCCCCTCGTC	GCGGTCTCGGCATCTTCTAGG	382	58	DQ437887
SCD	ACGTTGTGCCAGTGAGTCAG	GTCTTGGCCTCTTGTGCTTC	206	62	NM_213781
SOD	TCCATGTCCATCAGTTTGGA	CTGCCCAAGTCATCTGGTTT	250	60	AF396674
SREBP-1	CCTCTGTCTCTCCTGCACC	ACAAAGAGAAGCGCCAAGAA	213	62	NM_214157
SREBP-2	CGCTCGCGAATCCTGCTGTG	GGTGCGGGTCCGTGTCGTG	103	65	DQ020476

ACC, acetyl-CoA carboxylase; ACO, acyl-CoA oxidase; CPT-1, carnitine palmitoyltransferase-1; CYP7, cholesterol 7α-hydroxylase; FAS, fatty acid synthase; FATP, fatty acid transport protein; FDPS, farnesyl diphosphate synthase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HMG-CoA-R, 3-hydroxy-3-methylglutaryl-CoA reductase; I-FABP, intestinal fatty acid binding protein; Insig, insulin-induced gene; L-FABP, liver fatty acid binding protein; mAAT, mitochondrial aspartate aminotransferase; mHMG-CoA-S, mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase; MTP, microsomal TAG transfer protein; NPC, Niemann-Pick type C; SCD, stearoyl-CoA desaturase; SOD, superoxide dismutase; SREBP, sterol regulatory element-binding protein.

As previously shown by Walrand *et al.* (2003), dihydrorhodamine 123 is specifically oxidised by H_2O_2.

Determination of 3-hydroxybutyrate

Concentration of 3-hydroxybutyrate in plasma was determined using an enzymatic assay (cat. no. 10907979035; R-Biopharm AG, Darmstadt, Germany).

Transmission electron microscopy

Liver tissues were fixed in 3 % sodium cacodylate-buffered glutaraldehyde (pH 7·2) and post-fixed with 1 % osmium tetroxide. After washing three times, probes were dehydrated in an ethanol series and embedded in Spurr's epoxy resin. For observations with an EM 900 transmission electron microscope (Carl Zeiss SMT, Oberkochen, Germany), ultrathin sections (80 nm) were mounted on copper grids. Catalase is known to be located in peroxisomes specifically and was marked for a better visualisation of peroxisomes. For immunohistochemistry, ultrathin sections were blocked for 30 min with 1 % bovine serum albumin and 0·1 % Tween in PBS and incubated overnight with sheep polyclonal anti-catalase serum (1 : 50; Biotrend, Köln, Germany). For detection of primary antibody, sections were incubated for 1 h with a gold-marked donkey–anti-sheep antibody (1 : 25; Biotrend) and finally stained with uranyl acetate/lead citrate. Peroxisomes were counted in 1000 different prints per liver sample for each animal with a magnification of 12 000×.

Statistics

The results were analysed using Minitab (State College, PA, USA) statistical software (release 13). Statistical significance of differences of the mean values of the two groups of pigs was evaluated using Student's *t* test. Mean values were considered significantly different for $P<0·05$.

Results

Fatty acid composition and concentration of lipid peroxidation products in the dietary fats

Palmitic, stearic, oleic and linoleic acid were the major fatty acids in the dietary fats. The sum of these fatty acids accounted for about 95 g/100 g total fatty acids in the fats (Table 2). Amounts of stearic, oleic and linoleic acid were nearly identical in both fats; the amount of stearic acid was slightly higher in the fresh fat than in the oxidised fat. Peroxide value, acid value and concentration of conjugated dienes were 4–5-fold higher in the oxidised than in the fresh fat included in the diet (Table 2). The concentration of total carbonyls was 10-fold higher and that of TBARS was 30-fold higher in the oxidised than in the fresh fat (Table 2).

Body weights, antioxidant status and concentrations of lipid peroxidation products in the liver

Body weights of the pigs at the end of the experiment on day 28 did not differ between the two groups (25·6 (SD 1·4) *v.* 26·0 (SD 1·5) kg in pigs fed the oxidised fat *v.* pigs fed the fresh fat; nine pigs per group). Pigs fed the oxidised fat had a higher mRNA concentration and a higher activity of SOD and a lower activity of microsomal glutathione *S*-transferase in the liver than pigs fed the fresh fat ($P<0·05$; Table 3). Activities of glutathione peroxidase and cytosolic glutathione *S*-transferase as well as mRNA concentrations of these enzymes in the liver did not differ between both groups of pigs (Table 3). Concentrations of total, reduced and oxidised glutathione in the liver also did not differ between the two groups of pigs whereas the concentration of α-tocopherol was lower in pigs fed the oxidised fat than in pigs fed the fresh fat ($P<0·05$; Table 3). Concentration of TBARS in the liver did not differ between the two groups of pigs whereas the concentration of conjugated dienes was slightly but significantly higher in pigs fed the oxidised fat than in pigs fed the fresh fat ($P<0·05$; Table 3).

Table 2. Major fatty acids and concentrations of some lipid peroxidation products in the fresh and the oxidised fat after inclusion into the diet

	Fresh fat	Oxidised fat
Major fatty acids (g/100 g fatty acids)		
16 : 0	9·0	6·7
18 : 0	4·1	4·2
18 : 1*n*-9	23·7	23·8
18 : 2*n*-6	59·8	59·9
Peroxidation products		
Conjugated dienes (mmol/kg)	22·7	89·1
TBARS (μmol/kg)	9	271
Peroxide value (mEq O_2/kg)	2·5	10·0
Acid value (g KOH/kg)	1·6	8·0
Total carbonyls (mmol/kg)	2·5	24·5

TBARS, thiobarbituric acid-reactive substances.

Indices of peroxisome proliferation

Liver weights of the pigs were not different between the two groups but pigs fed the oxidised fat had a higher peroxisome count and a higher activity of catalase in the liver than pigs fed the fresh fat ($P<0·05$; Table 4). Relative mRNA concentration of acyl-CoA oxidase (ACO), a peroxisomal enzyme, in the liver, was 34 % higher in pigs fed the oxidised fat than in control animals ($P=0·062$; Table 4). The concentration of H_2O_2 which is mainly released from peroxisomal oxidases was not different between the two groups of pigs (Table 4).

mRNA concentrations of genes in liver and intestine

In liver, mRNA concentrations of PPARα and genes involved in fatty acid transport and oxidation [liver fatty acid binding protein (L-FABP), carnitine palmitoyltransferase-1 (CPT-1)], fatty acid and cholesterol synthesis [SREBP-1 and -2, insulin-induced gene-1 and -2, fatty acid synthase, acetyl-CoA carboxylase (ACC), stearoyl-CoA desaturase (SCD), 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA-R)], cholesterol uptake (LDL receptor), bile acid synthesis [cholesterol 7α-hydroxylase (CYP7)], lipoprotein assembly and secretion [microsomal TAG transfer protein (MTP)], inhibition of lipoprotein lipase (apo CIII) and ketogenesis [mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase (mHMG-CoA-S)]

Table 3. mRNA concentrations and activities of antioxidant enzymes and concentrations of antioxidants and lipid peroxidation products in livers of pigs fed a diet with a fresh fat or an oxidised fat

(Mean values and standard deviations)

	Fresh fat (*n* 9)		Oxidised fat (*n* 9)	
	Mean	SD	Mean	SD
Superoxide dismutase				
mRNA concentration (relative)	1·00	0·22	1·24*	0·14
Activity (U/mg protein)	42·7	8·4	58·8*	6·0
Glutathione *S*-transferase	202	42	144*	13
mRNA concentration (relative)	1·00	0·31	1·27	0·23
Activity in microsomes (U/mg protein)	202	42	144*	13
Activity in cytosol (U/mg protein)	760	262	761	186
Glutathione peroxidase				
mRNA concentration (relative)	1·00	0·13	1·14	0·22
Activity (U/mg protein)	4·72	0·77	5·14	0·75
Glutathione, total (nmol/mg)	2·13	0·51	2·17	0·37
Glutathione, reduced (nmol/mg)	1·70	0·50	1·84	0·55
Glutathione, oxidised (nmol/mg)	0·21	0·12	0·17	0·10
α-Tocopherol (nmol/g)	14·5	2·5	11·9*	1·8
Conjugated dienes (μmol/mg protein)	16	1	18*	3
TBARS (mmol/g)	7·2	1·6	7·3	2·8

TBARS, thiobarbituric acid-reactive substances.
Mean values were significantly different from those of the fresh fat group: *$P<0·05$.

were determined (Fig. 1). Pigs fed the oxidised fat had significantly higher mRNA concentrations of mHMG-CoA-S, a classical PPARα target gene, SREBP-1 and its target genes ACC and SCD, and SREBP-2 and its target genes HMG-CoA-R and LDL receptor than control pigs fed the fresh fat ($P<0·05$). mRNA concentration of CYP7 was lower in pigs fed the oxidised fat than in pigs fed the fresh fat ($P<0·05$). mRNA concentrations of CPT-1 and MTP, two other PPARα target genes, tended to be increased in pigs fed the oxidised fat ($P=0·074$ and $P=0·065$, respectively) compared to pigs fed the fresh fat whereas mRNA concentrations of PPARα, L-FABP, apo CIII, insulin-induced gene-1 and -2, and fatty acid synthase were not different between pigs fed the fresh fat and those fed the oxidised fat (Fig. 1).

In enterocytes, relative mRNA concentrations of PPARα and of proteins involved in fatty acid uptake (L-FABP, intestinal fatty acid binding protein, fatty acid transport protein, mitochondrial aspartate aminotransferase), fatty acid oxidation (ACO, CPT-1), intracellular trafficking of cholesterol (Niemann-Pick type C1 and 2) and fatty acid synthesis (SREBP-1, fatty acid synthase) were not different between pigs fed the oxidised fat and those fed the fresh fat (Fig. 2). However, mRNA concentration of SREBP-2 and its target genes HMG-CoA-R and LDL receptor, involved in cholesterol synthesis and uptake, were higher in pigs fed the oxidised fat than in pigs fed the fresh fat ($P<0·05$; Fig. 2). mRNA concentration of farnesyl diphosphate synthase did not differ between the two groups of pigs (Fig. 2).

Concentrations of TAG and cholesterol in liver, plasma and lipoproteins

Concentrations of TAG in liver, plasma and TAG-rich lipoproteins did not differ between pigs fed the fresh fat and those fed the oxidised fat. Concentrations in pigs fed the oxidised fat *v*. pigs fed the fresh were (nine pigs per group): liver, 88 (SD 20) *v*. 91 (SD 19) μmol/g; plasma, 0·96 (SD 0·26) *v*. 1·09 (SD 0·17) mmol/l; chylomicrons + VLDL, 0·80 (SD 0·25) *v*. 0·93 (SD 0·16) mmol/l. Concentrations of cholesterol in liver, plasma, LDL and HDL were also not different between the two groups of pigs. Concentrations in pigs fed the oxidised fat *v*. pigs fed the fresh were: liver, 73 (SD 14) *v*. 69 (SD 10) μmol/g; plasma, 2·63 (SD 0·32)

Table 4. Indices of peroxisome proliferation in livers of pigs fed a diet with a fresh fat or an oxidised fat

(Mean values and standard deviations)

	Fresh fat (*n* 9)		Oxidised fat (*n* 9)	
	Mean	SD	Mean	SD
Liver weight (g)	673	63	700	64
Peroxisome count (number/print)	366	67	515*	91
Acyl-CoA oxidase mRNA concentration (relative)	1·00	0·33	1·34†	0·37
Catalase (U/mg protein)	0·75	0·14	0·89*	0·13
H_2O_2 (fluorescence/g liver)	29 372	12 343	29 437	8361

Mean values were significantly different from those of the fresh fat group: †$P<0·1$; *$P<0·05$.

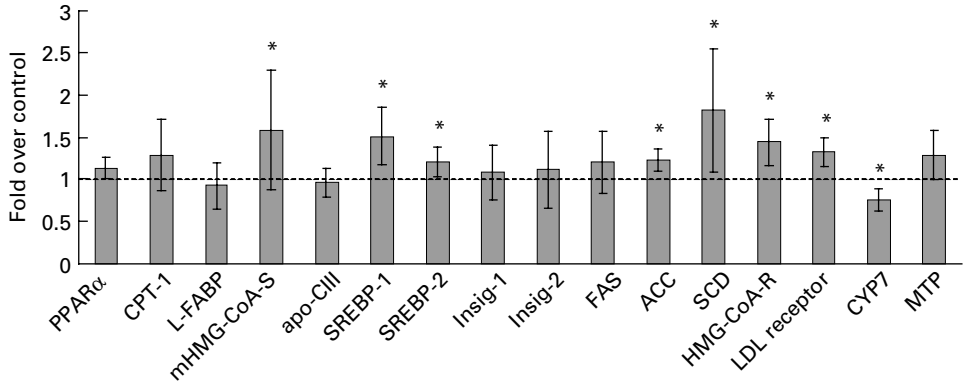

Fig. 1. Relative mRNA concentrations (--- represents 1·00) of various genes involved in hepatic lipid metabolism in enterocytes of pigs fed a diet with a fresh fat or an oxidised fat. Values were determined by real-time detection RT–PCR using the mRNA concentration of glyceraldehyde-3-phosphate dehydrogenase for normalisation Values are means with their standard deviations depicted by vertical bars (n 9) obtained for the pigs fed the oxidised fat relative to the values of the control group fed fresh fat. ACC, acetyl-CoA carboxylase; CPT-1, carnitine palmitoyltransferase-1; CYP7, cholesterol 7α-hydroxylase; FAS, fatty acid synthase; HMG-CoA-R, 3-hydroxy-3-methylglutaryl-CoA reductase; Insig, insulin-induced gene; L-FABP, liver fatty acid binding protein; mHMG-CoA-S, mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase; MTP, microsomal TAG transfer protein; SCD, stearoyl-CoA desaturase; SREBP, sterol regulatory element-binding protein. Mean values were significantly different from those of the fresh fat group: $^*P < 0{\cdot}05$.

v. 2·83 (SD 0·22) mmol/l; LDL, 0·96 (SD 0·16) *v*. 0·97 (SD 0·15) mmol/l; HDL, 1·02 (SD 0·18) *v*. 1·13 (SD 0·11) mmol/l.

Concentration of 3-hydroxybutyrate in plasma

Pigs fed the oxidised fat had a higher concentration of 3-hydroxybutyrate in plasma than pigs fed the fresh fat (1·23 (SD 0·58) *v*. 0·52 (SD 0·27) mmol/l; $P < 0{\cdot}05$).

Discussion

In the present study, pigs were fed a diet containing an oxidised fat prepared under usual deep-frying conditions. The relatively low concentrations of lipid peroxidation products (conjugated dienes, TBARS, peroxides and carbonyls) in the oxidised fat indicate that this fat was mildly oxidised. Concentrations of peroxidation products in this fat were indeed even lower than in soyabean oil or hydrogenated animal–vegetable oil blends used for frying of potatoes at 190°C over a period of 24 h (Frankel, 1998). The reason for the relatively low degree of oxidation is that we did not add foodstuffs to be fried during the preparation of the oil as we wanted to avoid contamination of the oil with food ingredients. It is well known that ingredients of foodstuffs, i.e. metal ions, enhance the lipid peroxidation process during frying of fats (Kubow, 1992). The concentration of conjugated dienes which include the potent PPARα activators hydroxy- and hydroperoxy fatty acids (Delerive *et al.* 2000; König & Eder, 2006) was approximately four times higher in the oxidised fat than in the fresh fat. The finding of an increased activity of SOD and a slightly elevated concentration of conjugated dienes, together with the observation of a slightly reduced concentration of α-tocopherol, indicates that the oxidised fat produced oxidative stress in the liver of the pigs. It has been demonstrated that under

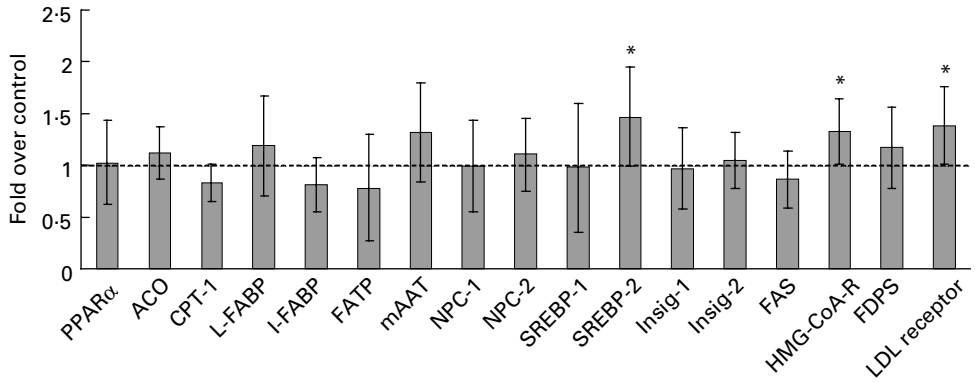

Fig. 2. Relative mRNA concentrations (--- represents 1·00) of various genes involved in intestinal lipid metabolism in livers of pigs fed a diet with a fresh fat or an oxidised fat. Values were determined by real-time detection RT–PCR using the mRNA concentration of glyceraldehyde-3-phosphate dehydrogenase for normalisation. Values are means with their standard deviations depicted by vertical bars (n 9) obtained for the pigs fed the oxidised fat relative to the values of the control group fed fresh fat. ACO, acyl-CoA oxidase; CPT-1, carnitine palmitoyltransferase-1; FAS, fatty acid synthase; FATP, fatty acid transport protein; FDPS, farnesyl diphosphate synthase; HMG-CoA-R, 3-hydroxy-3-methylglutaryl-CoA reductase; I-FABP, intestinal fatty acid binding protein; Insig, insulin-induced gene; L-FABP, liver fatty acid binding protein; mAAT, mitochondrial aspartate aminotransferase; NPC, Niemann-Pick type C; SREBP, sterol regulatory element-binding protein. Mean values were significantly different from those of the fresh fat group: $^*P < 0{\cdot}05$.

oxidative stress, expression of SOD is stimulated and concentration of α-tocopherol is reduced due to an enhanced consumption (Liu & Huang, 1995; Ruiz-Gutierrez *et al.* 1999; Atalay *et al.* 2000). However, the oxidative stress produced by the oxidised fat was very moderate as concentrations of TBARS and glutathione remained completely unchanged. In rodents treated with PPARα agonists such as fibrates or WY-14,643, production of H_2O_2 is largely increased due to a strong up-regulation of peroxisomal oxidases, and this causes oxidative stress and contributes to hepatocarcinogenesis in these species (Peters *et al.* 2005). In the present study, feeding the oxidised fat did not increase the concentration of H_2O_2 in the liver. This was probably due to two reasons: first, there was only a slight increase in the mRNA concentration of ACO, one of the enzymes producing H_2O_2; second, activity of catalase, the key enzyme of decomposition of H_2O_2 in peroxisomes was increased. Therefore, generation of H_2O_2 did not contribute to oxidative stress in animals treated with oxidised fat. The reason for the moderate oxidative stress may be that a part of the dietary lipid peroxidation products is absorbed in the intestine and reaches the liver via lipoproteins (Staprans *et al.* 2005). Production of oxidative stress by intake of strongly oxidised fats has been shown several times in rodents (Yoshida & Kajimoto, 1989; Liu & Huang, 1996; Liu & Lee, 1998; Ammouche *et al.* 2002; Eder *et al.* 2004; Keller *et al.* 2004*a*, *b*). The present study shows for the first time that even a mildly oxidised fat, as used in human nutrition, can induce moderate oxidative stress in pigs as a non-proliferating species.

To find out whether the mildly oxidised fat caused activation of PPARα in the liver of pigs, we determined mRNA concentrations of the classical PPARα target genes ACO, CPT-1 and mHMG-CoA-S as well as peroxisome count, activity of catalase and plasma concentration of 3-hydroxybutyrate. Recent studies in pigs have shown that activation of PPARα in pigs, by either treatment with clofibrate or by fasting, leads to an increased expression of these PPARα target genes, and in turn stimulates mitochondrial and peroxisomal β-oxidation and ketogenesis (Yu *et al.* 2001; Peffer *et al.* 2005; Cheon *et al.* 2005). The finding of an increased peroxisome count together with increased activity of catalase, a peroxisomal enzyme, a significantly increased mRNA concentration of mHMG-CoA-S and an increased plasma concentration of 3-hydroxybutyrate strongly indicate that the oxidised fat caused PPARα activation in the liver of the pigs. The finding that mRNA concentrations of ACO and CPT-1, two other classical PPARα target genes were also increased by 34 and 29 %, although not significantly different to control, supports the assumption that the oxidised fat induced hepatic PPARα activation in the pigs. It has been shown that these two enzymes are only moderately up-regulated in pig liver by PPARα agonists. For instance, in pigs treated with clofibrate, a strong PPARα agonist, hepatic gene expression of CPT-1 and ACO was only 1·89- and 1·42-fold, respectively, increased over control while gene expression of mHMG-CoA-S was increased 3·32-fold (Cheon *et al.* 2005). This presents an explanation for the observations that mHMG-CoA-S was significantly increased in pigs treated with oxidised fat and that ACO and CPT-1 were only slightly increased. The finding that mRNA concentration of MTP, a gene recently shown to be up-regulated by PPARα activation (Ameen *et al.* 2005), tended to be increased in the liver of pigs fed the oxidised fat also indicates that the oxidised fat caused PPARα activation in the liver. Recently, studies in rats have already shown that oxidised fats are able to activate PPARα in the liver (Huang *et al.* 1988; Chao *et al.* 2001; Sülzle *et al.* 2004). In these rat studies, up-regulation of PPARα target genes in the liver was much stronger than in pigs of the present study. This may have two different reasons: first, most PPARα target genes respond stronger to PPARα activation in rats than in non-proliferating species such as pigs or man; second, fats used in the rat studies were more strongly oxidised than the mildly oxidised fat used in the present study. The present study shows for the first time that even a mildly oxidised fat causes activation of PPARα in pigs which are, as man, less sensitive to PPARα agonists than rodents.

To study whether the oxidised fat caused PPARα activation in small intestine, we considered in addition to the classical PPARα target genes ACO and CPT-1, several genes involved in fatty acid transport (L-FABP, intestinal fatty acid binding protein, fatty acid transport protein and mitochondrial aspartate aminotransferase) and cholesterol trafficking (Niemann-Pick type C1 and 2) in intestinal tissue. All these genes have been shown to be up-regulated by PPARα activation (Darimont *et al.* 1998; Motojima *et al.* 1998; Mochizuki *et al.* 2001; Chinetti-Gbaguidi *et al.* 2005). The finding that none of these genes was up-regulated in cells of small intestine indicates that oxidised fat caused no or even weak PPARα activation and does not influence intestinal fatty acid transport and cholesterol trafficking.

Synthesis of lipids in mammalian cells is controlled by a network involving the action of insulin-induced genes and SREBP, and it has been recently shown in several studies that this network is influenced by PPARα activation (Guo *et al.* 2001; Patel *et al.* 2001; Knight *et al.* 2005; König *et al.* 2006). The present study shows that feeding a mildly oxidised fat increased the mRNA concentration of SREBP-1 and its target genes ACC and SCD, two key enzymes of *de novo* fatty acid synthesis, in the liver. These alterations may be caused by activation of PPARα in the liver. Knight *et al.* (2005) found that treatment with WY 14,643, a synthetic PPARα agonist, causes a strong up-regulation of enzymes involved in hepatic fatty acid synthesis and stimulates fatty acid synthesis in wild-type mice but not in PPARα null mice. Knight *et al.* (2005) suggest that up-regulation of hepatic fatty acid synthesis is a compensatory response on the increased fatty acid oxidation to maintain a constant cellular TAG level. The finding that TAG levels in liver and plasma were not reduced in pigs fed the oxidised fat compared to control pigs indeed suggests that an increased β-oxidation of fatty acids was compensated by an increased fatty acid synthesis. As there is no evidence for a direct action of PPARα on the promoter regions of SREBP-1 and ACC genes, it is likely that the increased mRNA concentrations of these genes are an indirect result of PPARα activation. In contrast, SCD is not only dependent on SREBP-1 but has also a PPAR response element in its promoter (Miller & Ntambi, 1996). Therefore, its transcription may have been in part directly stimulated by PPARα activation. An up-regulation of SCD which catalyses the formation of MUFA from SFA has also been observed in pigs treated with clofibrate (Cheon *et al.* 2005). These findings of the effects of the oxidised fat on gene expression of lipogenic enzymes observed in pigs are opposite to those observed in rats in which a dietary oxidised fat causes a down-regulation of lipogenic enzymes and a strong reduction of liver and plasma TAG (Eder & Kirchgessner, 1998; Eder *et al.* 2003).

It is moreover shown that feeding the mildly oxidised fat led to a moderate but significant up-regulation of SREBP-2, and its target genes HMG-CoA-R and LDL receptor, in both liver and small intestine. The present findings suggest that the oxidised fat could have stimulated synthesis and uptake of cholesterol in these tissues. As this effect occurs not only in the liver but also in the small intestine where no PPARα activation was found in pigs fed the oxidised fat, it is questionable whether these effects are linked to PPARα activation. The finding that hepatic genes involved in cholesterol synthesis were not altered in pigs treated with clofibrate indeed suggests that PPARα activation does not influence SREBP-2 controlled transcription of genes involved in cholesterol homeostasis (Cheon *et al.* 2005). On the other hand, treatment with the PPARα agonist WY 14,643 caused an up-regulation of genes involved in hepatic cholesterol synthesis in wild-type mice but not in PPARα null mice, indicating that PPARα activation indeed could directly stimulate cholesterol synthesis (Knight *et al.* 2005). It should be noted, however, that there is also another study that found a suppression of gene expression and proteolytic activation of SREBP-2, and a strong down-regulation of its target genes accompanied by reduced cholesterol synthesis in rats (König *et al.* 2006). The effect of PPARα activation on SREBP-2-dependent cholesterol synthesis is not yet clear and may also be different between various species. Besides an up-regulation of genes involved in synthesis and uptake of cholesterol, we found a down-regulation of CYP7, the key enzyme of bile acid formation, in the liver. It has been shown in human and rat liver cells that PPARα agonists lower CYP7 expression probably by reducing the availability of hepatic nuclear factor 4α which is required for binding to a DR-1 in CYP7 promoter (Marrapodi & Chiang, 2000; Patel *et al.* 2000). Therefore, we assume that down-regulation of CYP7 in the liver of pigs fed the oxidised fat was caused by PPARα activation induced by the oxidised fat. Increased hepatic cholesterol synthesis and uptake of cholesterol into the liver, together with a decreased bile acid synthesis, is expected to increase hepatic cholesterol concentration. In contradiction to this, liver and plasma cholesterol concentrations were unchanged in pigs fed the oxidised fat compared to pigs fed the fresh fat. We assume that the changes in gene expression were too small to induce phenotypical alterations of cholesterol concentrations.

In conclusion, the present study shows that a mildly oxidised fat causes PPARα activation in the liver of pigs as indicated by an increased peroxisome count, a moderate up-regulation of PPARα target genes and a stimulation of ketogenesis. Moreover, the oxidised fat led to an up-regulation of the expression of SREBP-1 and SREBP-2 and their target genes involved in TAG and cholesterol synthesis, suggesting a stimulation of lipid synthesis. As the fat used in the present study was even less oxidised than fats used for deep-frying of foods, and as there exists a similarity in the gene response to PPARα agonists between pig and human liver cells, deep-fried fats could exert similar effects in man.

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Differential action of 13-HPODE on PPARα downstream genes in rat Fao and human HepG2 hepatoma cell lines

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Abstract

In rats, oxidized fats activate the peroxisome proliferator-activated receptor α (PPARα), leading to reduced triglyceride concentrations in liver, plasma and very low density lipoproteins. Oxidation products of linoleic acid constitute an important portion of oxidized dietary fats. This study was conducted to check whether the primary lipid peroxidation product of linoleic acid, 13-hydroperoxy-9,11-octadecadienoic acid (13-HPODE), might be involved in the PPARα-activating effect of oxidized fats. Therefore, we examined the effect of 13-HPODE on the expression of PPARα target genes in the rat Fao and the human HepG2 hepatoma cell lines. In Fao cells, 13-HPODE increased the mRNA concentration of the PPARα target genes acyl-CoA oxidase (ACO), cytochrome P450 4A1 and carnitine-palmitoyltransferase 1A (CPT1A). Furthermore, the concentration of cellular and secreted triglycerides was reduced in Fao cells treated with 13-HPODE. Because PPARα mRNA was not influenced, we conclude that these effects are due to an activation of PPARα by 13-HPODE. In contrast, HepG2 cells seemed to be resistant to PPARα activation by 13-HPODE because no remarkable induction of the PPARα target genes ACO, CPT1A, mitochondrial HMG-CoA synthase and Δ9-desaturase was observed. Consequently, cellular and secreted triglyceride levels were not changed after incubation of HepG2 cells with 13-HPODE. In conclusion, this study shows that 13-HPODE activates PPARα in rat Fao but not in human HepG2 hepatoma cells.

Keywords: 13-HPODE; PPARα; Fao; HepG2

1. Introduction

Oxidized fats are generated during processing and storage of foods, and constitute an important portion of Western diets. Increased intake of oxidized fats has been linked to an enhanced incidence of coronary heart disease, endothelial dysfunction and cancer in humans [1,2]. Several studies examined the physiological effects of oxidized dietary fats in animal models [3–6]. In rats, reduced concentrations of triglycerides in liver, plasma and very low density lipoproteins (VLDL) were observed after feeding a diet rich in oxidized fats [6–9]. This is in part due to the reduced expression of lipogenic enzymes in the rat liver [6]. Furthermore, the reduced triglyceride concentrations resulted from an enhanced fatty acid oxidation in the liver. It was shown recently that dietary oxidized fats as well as cyclic fatty acid monomers, components of heated fats, lead to activation of the peroxisome proliferator-activated receptor α (PPARα) and cause an increased expression of its target genes [10–12]. This resembles the mechanism of action of fibrates, a class of hypolipidemic drugs [13].

Peroxisome proliferator-activated receptors are transcription factors belonging to the superfamily of nuclear receptors and are implicated in the regulation of lipid and lipoprotein metabolism, glucose homeostasis, cellular differentiation, cancer development as well as in the control of the inflammatory response [14–16]. They can be activated by peroxisome proliferators, including fibrates, fatty acids and eicosanoids, and regulate the expression of target genes by binding to DNA sequence elements as heterodimers with the 9-*cis*-retinoic acid receptor [17]. PPARα downstream genes participate in aspects of lipid catabolism such as fatty acid uptake and binding, peroxisomal, mitochondrial and

Abbreviations: 13-HPODE, 13-hydroperoxy-9,11-octadecadienoic acid; ACO, acyl-CoA oxidase; CPT1A, carnitine-palmitoyltransferase 1A; Cyp4A1, cytochrome P450 4A1; FAS, fatty acid synthase; FCS, fetal calf serum; HMGCoAS2, mitochondrial HMG-CoA synthase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PPAR, peroxisome proliferator-activated receptor; PUFAs, polyunsaturated fatty acids; SREBP, sterol regulatory element-binding protein; VLDL, very low density lipoprotein.

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microsomal fatty acid oxidation and lipoprotein assembly and transport [14].

Oxidized fats include a mixture of primary and secondary lipid peroxidation products depending on their thermal treatment. Heating fats at relatively low temperatures over a long period results in high concentrations of primary lipid peroxidation products. These fats have a more pronounced effect on the lipid metabolism of rats than fats heated at a high temperature for a shorter period [6]. Linoleic acid is the most predominant fatty acid among dietary polyunsaturated fatty acids (PUFAs). Its primary autooxidation product is 13-hydroperoxy-9, 11-octadecadienoic acid (13-HPODE). In plants and mammals, 13(*S*)-HPODE is the product of linoleic acid oxidation by lipoxygenases.

This study was designed to test whether 13-HPODE might be involved in the observed activation of PPARα by oxidized fats. So far, only cyclic fatty acid monomers are suggested to act as peroxisome proliferator analogs [10]. As a model system, the rat hepatoma cell line Fao was chosen, which is commonly used for examination of interactions of different substances with PPARα [18–20]. Considering the known species-specific differences of the action of fibrates and other peroxisome proliferators [21], we used the human hepatoma cell line HepG2 to evaluate the relevance of the obtained results for humans. HepG2 and Fao cells have been already used for evaluation of species-specific differences in peroxisome proliferator action [20,22,23].

Thus, this study was focused on the ability of 13-HPODE to activate PPARα in the rat and human hepatoma cell lines, Fao and HepG2, respectively. Therefore, we analyzed the mRNA concentrations of PPARα and selected PPARα downstream genes like acyl-CoA oxidase (ACO), cytochrome P450 4A1 (Cyp4A1), carnitine-palmitoyltransferase 1A (CPT1A), mitochondrial HMG-CoA synthase (HMGCoAS2) and Δ9-desaturase using a semiquantitative PCR. Considering the observed suppression of lipogenic enzymes by oxidized fats [6], we further examined the genes coding for sterol regulatory element-binding proteins (SREBPs) and fatty acid synthase (FAS). In addition, we examined the effect of 13-HPODE on the concentrations of cellular cholesterol and cellular and secreted triglycerides in Fao and HepG2 cells.

2. Materials and methods

2.1. Materials

Linoleic acid, soybean lipoxygenase (type V) and 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (Munich, Germany). F-12 Nutrient Mixture (Ham), RPMI 1640, trypsin-EDTA (0.05% trypsin, 0.53 mM EDTA), gentamycin and fetal calf serum (FCS) were obtained from Invitrogen (Karlsruhe, Germany). Rat hepatoma Fao cell line was obtained from ECACC (Salisbury, UK) and human HepG2 hepatoma cell line was from DSMZ (Braunschweig, Germany).

2.2. Cell culture

HepG2 human hepatoma cells were grown in RPMI 1640 medium supplemented with 10% FCS and 0.05 mg ml^{-1} gentamycin. Fao rat hepatoma cells were cultured in Ham-F12 medium supplemented with 10% FCS and 0.05 mg ml^{-1} gentamycin. Cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2. For experiments, cells were seeded in 24-well cell culture plates at a density of 2.1×10^5 (Fao) and 1.7×10^5 (HepG2) cells per well and used prior reaching confluence (usually 3 days after seeding). The cells were preincubated with low-serum medium (0.5% FCS) for 16 h and then stimulated for the times indicated. Fatty acids were added to the low-serum medium from a stock solution in ethanol. Cells treated with the appropriate vehicle concentration were used as a control.

Cell viability after treatment with fatty acids was assessed by the MTT assay [24].

2.3. Preparation of 13-HPODE

13-HPODE was prepared by oxidation of linoleic acid with soybean lipoxygenase according to Funk et al. [25]. Stock solution of linoleic acid was prepared in absolute ethanol and then diluted in PBS. The linoleic acid was incubated with soybean lipoxygenase (100 U/100 nmol, 2 h at 37°C). The formation of 13-HPODE was monitored spectrophotometrically between 200 and 300 nm, using PBS as reference. The conversion of linoleic acid into 13-HPODE is observed as an increase in absorbance at 234 nm. Subsequently, the 13-HPODE was extracted twice with *n*-hexane. The *n*-hexane was evaporated and 13-HPODE was dissolved in ethanol. The concentration of the stock solution was calculated using the extinction coefficient of 23 mM^{-1} cm^{-1} for conjugated diens.

2.4. RT-PCR analysis

Measurement of mRNA concentration was done as a semiquantitative analysis using β-actin for normalization. Total RNA was isolated from cells by Trizol reagent (Life Technologies, Karlsruhe, Germany) according to the manufacturer's protocol. Total RNA (1.2 μg) was used for cDNA synthesis with 60 U Revert Aid M-MuLV Reverse Transcriptase (Fermentas, St. Leon-Rot, Germany) and 0.5 μg oligo dT18 primer (Qiagen, Hilden, Germany) for 1 h at 42°C followed by an inactivation step at 70°C for 10 min. The PCR reaction was carried out in 20 μl PCR buffer containing 2 μl RT reaction, 1 U BioTherm DNA Polymerase (Genecraft, Lüdinghausen, Germany), 200 μM dNTPs and 20 pmol of the specific primers. Sequences and characteristics of the specific primers used for cDNA amplification are listed in Table 1. After initial denaturation at 95°C for 3 min, cycling was performed with

Table 1
Characteristics of the specific primers used for RT-PCR analysis

Gene	Forward primer	Reverse primer	bp	Annealing temperature (°C)	No. of cycles	Genbank accession no.
ACO, rat	5′ CTTTCTTGCTTGCCTTCCTTCTCC 3′	5′ GCCGTTTCACCGCCTCGTA 3′	415	60	27	NM017340
Cyp4A1, rat	5′ CAGAATGGAGAATGGGGACAGC 3′	5′ TGAGAAGGGCAGGAATGAGTGG 3′	460	65	29	NM175837
CPT1A, rat	5′ GGAGACAGACACCATCCAACATA 3′	5′ AGGTGATGGACTTGTCAAACC 3′	416	60	28	NM031559
PPARα, rat	5′ CCCTCTCTCCAGCTTCCAGCCC 3′	5′ CCACAAGCGTCTTCTCAGCCATG 3′	555	65	29	M88592
SREBP-1c, rat	5′ GGAGCCATGGATTGCACATT 3′	5′ AGGAAGGCTTCCAGAGAGGA 3′	191	60	33	XM213329
SREBP-2, rat	5′ CCGGTAATGATGGGCCAAGAGAAAG 3′	5′ AGGCCGGGGGAGACATCAGAAG 3′	400	60	29	XM216989
FAS, rat	5′ CCTCCCCTGGTGGCTGCTACAA 3′	5′ CCTGGGGTGGGCGGTCTTT 3′	224	60	27	X62888
β-Actin, rat	5′ ATCGTGCGTGACATTAAAGAGAAG 3′	5′ GGACAGTGAGGCCAGGATAGAG 3′	429	60	22	NM031144
ACO, human	5′ GTGGGCGCATACATGAAGGAGACC 3′	5′ GTGGCTGGATGCGCTGACTGG 3′	367	65	26	X71440
CPT1A, human	5′ AATCATCAAGAAATGTCGCACGA 3′	5′ AGGCAGAAGAGGTGACGATCG 3′	309	65	32	NM001876
HMGCoAS2, human	5′ AACGCCTAGCCTCCCGAAAGTG 3′	5′ CCATAAGCCCAGGACAGTGATTGC 3′	401	60	36	NM005518
Δ9-Desaturase, human	5′ TTCCTGGCTCTACCCTGTCTGTCC 3′	5′ GGGCACCCTCACCAAGTAAGC 3′	480	60	27	NM005063
FAS, human	5′ CATCGGCGACGTGGGCATTTTG 3′	5′ CCGGGTTCACCAGCAGGGAGCG 3′	494	65	26	BC007909
PPARα, human	5′ TGTGGCTGCTATCATTTGCTGTGG 3′	5′ CTCCCCCGTCTCCTTTGTAGTGC 3′	344	60	31	L02932
SREBP-1, human	5′ GTGGCGGCTGCATTGAGAGTGAAG 3′	5′ AGGTACCCGAGGGCATCCGAGAAT 3′	362	60	30	U00968
SREBP-2, human	5′ CGCCACCTGCCCCTCTCCTTCC 3′	5′ TGCCCTGCCACCTATCCTCTCACG 3′	390	65	28	NM004599
β-Actin, human	5′ GAGCGGGAAATCGTGCGTGAC 3′	5′ GCCTAGAAGCATTTGCGGTGGAC 3′	518	60	19	NM001101

denaturation for 30 s at 95°C, annealing for 30 s at a primer-specific temperature (Table 1) and elongation for 1 min at 72°C, followed by a final extension step for 7 min at 72°C. Initially, the linearity of PCR for each specific primer pair was tested to ensure that amplification of target cDNA remained in the exponential range. The final PCR products were electrophoresed on a 1% agarose gel and visualized by ethidium bromide staining. The relative intensities of the bands were quantified by densitometric analysis and corrected for the corresponding β-actin band densities. Data are expressed relatively to mRNA levels of control cells.

2.5. Lipid extraction and concentration determination

Total cellular lipids were extracted with a mixture of *n*-hexane and isopropanol (3:2). Lipid extracts were dried and dissolved in a small volume of Triton X-100 [26]. For determination of triglyceride concentration in VLDL, cell supernatants were collected after 24 h incubation and centrifuged for 5 min at 350×*g* to remove residual cells. Concentrations of cholesterol and triglycerides were determined using an enzymatic reagent kit (Ecoline S+, Merck, Darmstadt, Germany).

2.6. Statistical analysis

Effects of various concentrations of 13-HPODE or linoleic acid were analyzed using one-way ANOVA using the Minitab Statistical Software (Minitab, State College, PA). For significant *F* values, means were compared by Fisher's multiple range test. Furthermore, the direct comparison of the effects of 13-HPODE and linoleic acid at equal concentration was done by pairwise Student's *t* test. Differences with $P<.05$ were considered to be significant.

3. Results

3.1. Effect of 13-HPODE and linoleic acid on mRNA concentrations of selected genes involved in lipid metabolism of Fao cells

Cell viability of Fao cells was not reduced by 24 h incubation with 13-HPODE up to a concentration of 50 μM as demonstrated by the MTT assay. Incubation of Fao cells with increasing amounts of 13-HPODE led to a significant and concentration-dependent increase of mRNA levels of the PPARα downstream genes ACO, Cyp4A1 and CPT1A (Fig. 1). At the highest 13-HPODE concentration used, mRNA levels of these genes were about 50–70% higher than those of the control cells. mRNA concentration of PPARα itself did not change. At 50 μM 13-HPODE, a slight but significant increase of mRNA concentration was further found for SREBP-1c and SREBP-2, whereas FAS mRNA was not influenced at all (Fig. 1). For comparison, Fao cells were stimulated with linoleic acid under identical conditions. After 6 h incubation, a significant increase of 10–27% of ACO and Cyp4A1 mRNA levels was observed. Pairwise comparison of the relative mRNA levels of cells treated with equal concentrations of 13-HPODE and linoleic acid, respectively, revealed that the effects of 13-HPODE are significantly stronger than those of linoleic acid. mRNA concentrations of all other genes tested were not affected by incubation with linoleic acid (Fig. 1). After 12 h incubation of Fao cells with 13-HPODE, Cyp4A1 and CPT1A mRNA levels remained elevated over control, but to a lesser extent, compared to 6 h incubation, whereas mRNA concentrations of ACO, PPARα, SREBP-1c, SREBP-2 and FAS remained constant (data not shown). No significant changes in mRNA levels of all examined

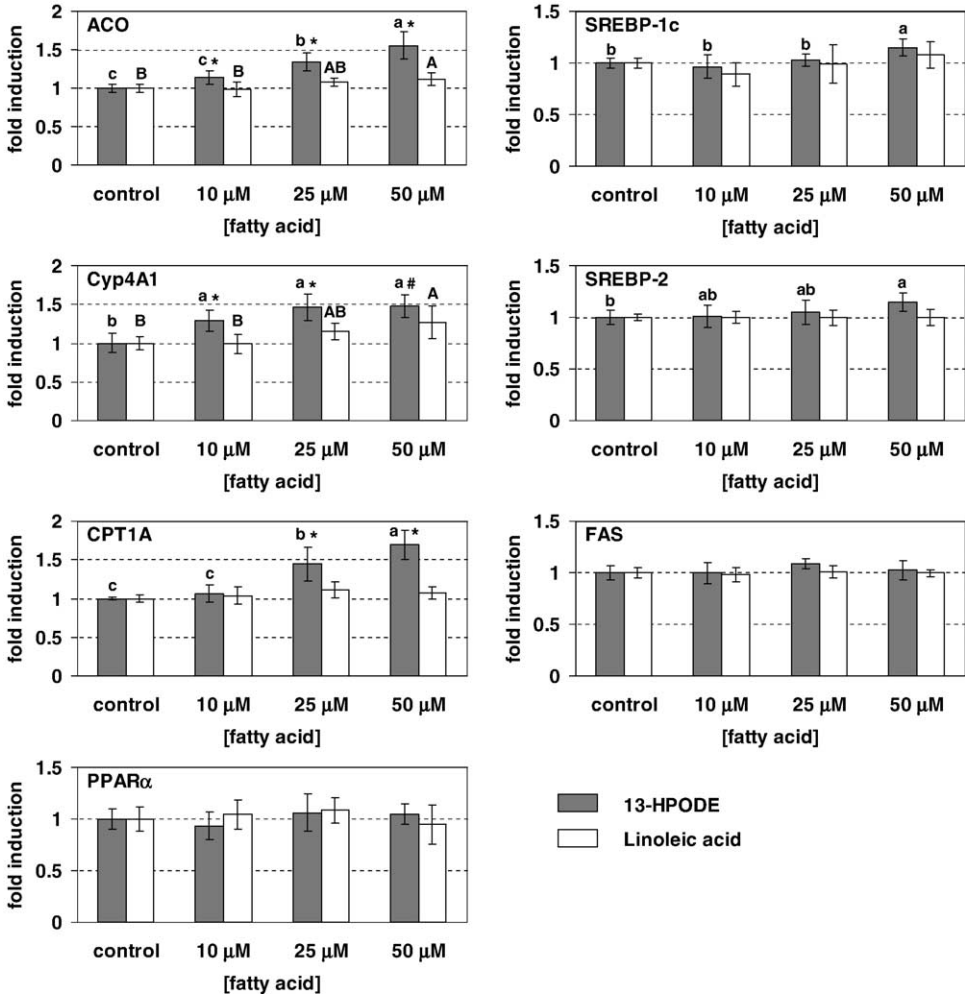

Fig. 1. Effect of 13-HPODE and linoleic acid on mRNA concentrations of selected genes involved in lipid metabolism of Fao cells. Fao cells were grown in culture medium until subconfluent state. Medium was then changed to low-serum medium (0.5% FCS). After 16 h preincubation in this medium, 13-HPODE or linoleic acid were added from ethanolic stock solution to give final concentrations of 10, 25 and 50 µM, respectively, and cells were incubated for 6 h. Control cells were incubated with low-serum medium containing vehicle alone. Acyl-CoA oxidase, Cyp4A1, CPT1A, PPARα, SREBP-1c, SREBP-2 and FAS mRNA levels were determined by semiquantitative RT-PCR analysis using β-actin mRNA concentration for normalization as described in the experimental procedures section. Values are means±S.D. ($n=6$). Data are expressed relative to mRNA levels of control cells. Small letters (a, b, c) denote differences in 13-HPODE; capital letters (A, B) denote differences in linoleic acid ($P<.05$). The symbols * and # indicate significant differences at $P<.05$ and $P<.1$, respectively, from linoleic acid at equal concentration.

genes were observed after 24 h incubation. Stimulation of Fao cells with linoleic acid for 12 and 24 h did not influence mRNA concentrations of ACO, Cyp4A1, CPT1A, PPARα, SREBP-1c, SREBP-2 and FAS (data not shown). β-Actin mRNA that was used as a control for normalization was not influenced at all by treatment of the cells with 13-HPODE and linoleic acid, respectively.

3.2. Effect of 13-HPODE on mRNA concentrations of selected genes involved in lipid metabolism of HepG2 cells

Incubation of HepG2 cells with 13-HPODE concentrations above 25 µM strongly reduced cell viability as tested by the MTT assay (data not shown). Consequently, HepG2 cells were incubated with 13-HPODE concentrations up to 25 µM for mRNA analysis. As with Fao cells, the PPARα downstream genes ACO and CPT1A were analyzed. Cyp4A11, the gene corresponding to rat Cyp4A1, was not examined because no Cyp4A11 mRNA was found in HepG2 cells [27,28]. Furthermore, the PPARα target genes Δ9-desaturase and HMGCoAS2 were included. The mRNA concentrations of ACO, CPT1A and Δ9-desaturase were not affected after stimulation of HepG2 cells with 13-HPODE for 6 h. For HMGCoAS2, a slight but significant concentration-dependent induction of mRNA level after 6 h incubation was observed (Fig. 2). No changes in mRNA levels of all examined PPARα downstream genes could be seen after 24 h incubation of HepG2 cells with 13-HPODE (Fig. 2). Peroxisome proliferator-activated receptor α mRNA was readily detectable by RT-PCR in HepG2 cells used in this study, indicating that the absence of effects on PPARα downstream genes was not due to lacking expression of PPARα.

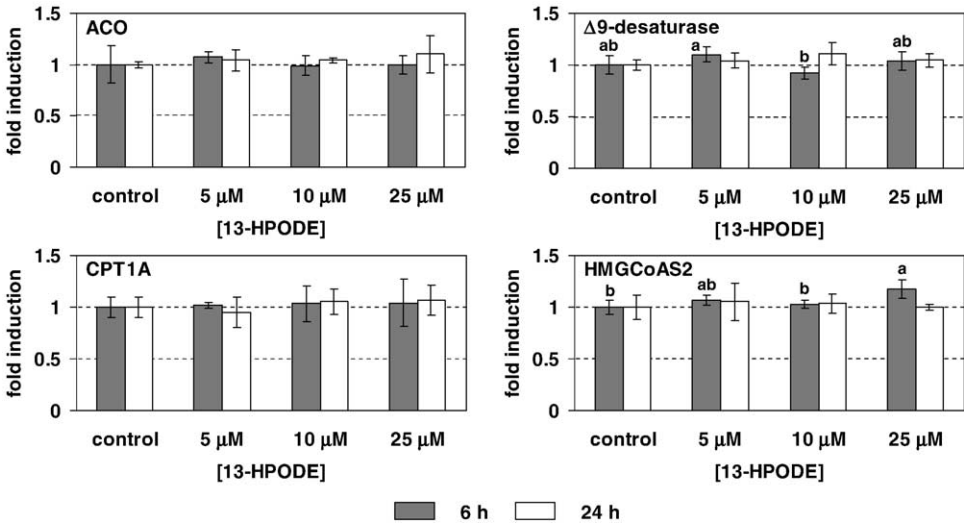

Fig. 2. Effect of 13-HPODE on mRNA concentrations of selected genes involved in lipid metabolism of HepG2 cells. HepG2 cells were grown in culture medium until subconfluent state. Medium was then changed to low-serum medium (0.5% FCS). After 16 h preincubation in this medium, 13-HPODE was added from ethanolic stock solution to give final concentrations of 5, 10 and 25 μM, respectively, and cells were incubated for 6 and 24 h. Control cells were incubated with low-serum medium containing vehicle alone. Acyl-CoA oxidase, CPT1A, Δ9-desaturase and mitochondrial hydroxymethylglutaryl-CoA synthase (HMGCoAS2) mRNA levels were determined by semiquantitative RT-PCR analysis using β-actin mRNA concentration for normalization as described in the Materials and Methods. Values are means±S.D. ($n=4$). Data are expressed relative to mRNA levels of control cells. Means with unlike letters differ, $P<.05$.

Furthermore, no alterations of SREBP-1, SREBP-2 and FAS mRNA levels occurred after stimulation of HepG2 cells with 13-HPODE for 6 and 24 h (data not shown). Finally, no effect on mRNA concentrations of PPARα downstream genes was observed after incubation of HepG2 cells with linoleic acid at concentrations up to 25 μM (data not shown).

3.3. Influence of 13-HPODE on the concentrations of cellular and secreted lipids of Fao and HepG2 cells

To test whether the observed induction of PPARα downstream genes resulted in alterations of cellular and secreted lipids, we examined the concentrations of cellular cholesterol and cellular and secreted triglycerides after

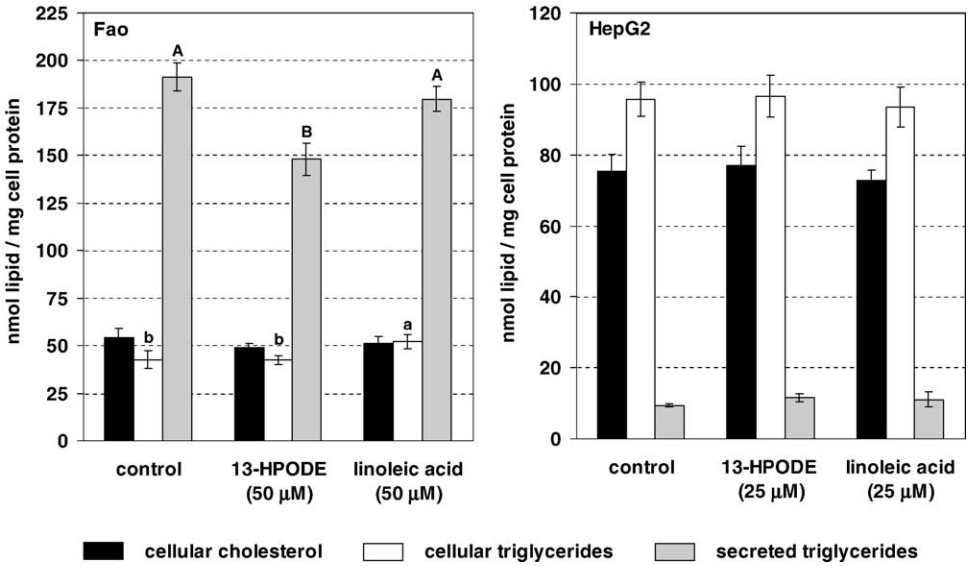

Fig. 3. Influence of 13-HPODE and linoleic acid on concentrations of cellular cholesterol and cellular and secreted triglycerides in Fao and HepG2 cells. Cells were incubated with 13-HPODE and linoleic acid, respectively, for 24 h in low-serum medium. Final fatty acid concentrations were 50 μM (Fao) and 25 μM (HepG2). Control cells were treated with low-serum medium containing vehicle alone. Cell supernatant after incubation was used for determination of triglyceride concentration in VLDLs. Total cellular lipids were extracted with a mixture of *n*-hexane and isopropanol (3:2), extracts were dried and lipids were dissolved in a small volume of Triton X-100. Cholesterol and triglyceride concentrations were determined using an enzymatic reagent kit. Values are means±S.D. ($n=4$). Small letters (a, b) denote differences in cellular triglycerides, capital letters (A, B) denote differences in secreted triglycerides ($P<.05$).

stimulating the cells with 13-HPODE. For comparison, cells were incubated with linoleic acid at equal concentration. Control cells were treated with vehicle alone. Final concentrations of fatty acids during incubation were 50 μM for Fao and 25 μM for HepG2 cells, respectively.

Cholesterol concentration in Fao cells was not influenced by incubation with 13-HPODE and linoleic acid, respectively (Fig. 3). The cellular triglyceride concentration was about 20% higher in cells treated with linoleic acid compared to 13-HPODE-treated and control cells. 13-HPODE-treated cells secreted about 20% less triglycerides compared to cells incubated with linoleic acid and control cells (Fig. 3).

Remarkably, the ratio of cellular to secreted triglyceride concentration was much higher in HepG2 compared to Fao cells (Fig. 3). We could not find any difference in cellular cholesterol and cellular and secreted triglyceride concentrations of HepG2 cells treated with 13-HPODE and linoleic acid, respectively, for 24 h.

4. Discussion

Lipid hydroperoxides are the fundamental primary products of autooxidation of PUFA. They can further react with oxygen and decompose to form a great variety of secondary oxidation products. Linoleic acid oxidation products represent an important portion of oxidized dietary fats. The aim of this study was to evaluate a possible involvement of the primary lipid peroxidation product of linoleic acid, 13-HPODE, in the observed activation of PPARα by oxidized fats in rat liver. Our data demonstrate that incubation of rat hepatoma Fao cells with 13-HPODE for 6 h led to a significant and concentration-dependent increase of mRNA concentrations of the PPARα downstream genes ACO, Cyp4A1 and CPT1A. Linoleic acid, the precursor of 13-HPODE, did also slightly enhance ACO and Cyp4A1 mRNA levels, but clearly less effective than its oxidized form. Acyl-CoA oxidase catalyses the rate-limiting step of peroxisomal β-oxidation and represents a direct target of PPARα. Induction of ACO is generally considered as marker of peroxisome proliferation [29]. Cyp4A1 catalyses the microsomal ω-hydroxylation of fatty acids [30]. CPT1A is involved in the first limiting step of mitochondrial β-oxidation, the entry flux of fatty acids into the mitochondria by catalyzing the formation of fatty acyl carnitine for translocation across the inner mitochondrial membrane. Both Cyp4A1 and CPT1A are regulated by PPARα and strongly induced by fibrates and other peroxisome proliferators [30,31]. Because PPARα mRNA was not affected by 13-HPODE and linoleic acid, respectively, the observed increased expression of PPARα downstream genes was not due to a higher PPARα expression. Hence, the up-regulation of ACO, Cyp4A1 and CPT1A genes should be the result of an activation of PPARα by 13-HPODE and linoleic acid, respectively. The observed changes in mRNA levels of PPARα downstream genes are very small. This is consistent with the described PPAR-mediated effects of other fatty acids [32,33]. Compared to lipid- and glucose-lowering drugs, PPAR binding and activation by fatty acids are weaker, which is also in accordance with the high plasma concentrations of fatty acids [34]. The finding that linoleic acid, which was used as a control, had a negligible effect on expression of PPARα downstream genes further supports that the observed effect was specific for 13-HPODE.

Up-regulation of ACO, Cyp4A1 and CPT1A genes by PPARα activation results in an enhanced fatty acid oxidation in the cells followed by reduced triglyceride levels. This phenomenon is also part of the mechanism of hypolipidemic action of fibrates [13,35]. Reduced triglyceride levels in liver, plasma and VLDL were also observed in rats fed oxidized fats and could be attributed to activation of PPARα [6,12]. The cellular triglyceride level was elevated in Fao cells incubated 24 h with linoleic acid compared to vehicle-treated cells. This may be attributed to enhanced triglyceride synthesis by addition of a fatty acid to the cells incubated in low-serum medium. In 13-HPODE-treated cells, triglyceride level was reduced compared to cells treated with linoleic acid. We suggest that this is due to the strong activation of PPARα downstream genes by 13-HPODE leading to enhanced fatty acid oxidation. Furthermore, the level of secreted triglycerides was lower when cells were incubated with 13-HPODE instead of linoleic acid. This result corresponds also well with the stronger induction of genes implicated in β-oxidation of fatty acids by 13-HPODE compared to its nonoxidized form. These data further support our finding that 13-HPODE enhances PPARα downstream genes by activation of PPARα in Fao cells. The weak induction of PPARα downstream genes by linoleic acid was not sufficient to cause measurable changes in triglyceride concentrations.

Several endogenous oxidized lipids were recently identified as PPAR ligands. 8*S*-hydroxy-5,9,11,14-eicosatetraenoic acid [8(*S*)-HETE], an arachidonic acid metabolite from lipoxygenation, has been shown to be a strong activator of the human PPARα [36] and plays an important role in keratinocyte differentiation [37]. Lipoxygenase products of linoleic acid are constituents of oxidized LDL and have been shown to be present in atherosclerotic lesions [38]. 9*S*-hydroxy-10,12-octadecadienoic acid and 13*S*-hydroxy-9,11-octadecadienoic acid (9- and 13-HODE) are activators of PPARγ [39,40] that is involved in the differentiation of several cell types including adipocytes, monocytes and macrophages [14]. Furthermore, 9- and 13-HODE were found to activate PPARα in human primary endothelial cells in transient transfection experiments [41]. To our knowledge, 13-HPODE has not been described as a ligand of PPARs in liver cells. The implication of 13-HPODE in the pathogenesis of atherosclerosis has been reported. It increased the expression of cell adhesion molecules and inflammatory chemokines in vascular smooth muscle cells via activation of nuclear factor-kappa

B [42,43]. Furthermore, 13-HPODE activated NAD(P)H oxidases in smooth muscle cells [44] and affected NO synthase activity in endothelial cells and macrophages [45,46]. Considering these literature data on PPAR activation by structurally related compounds, we suggest that the observed induction of PPARα downstream genes in Fao cells is the result of a direct activation of PPARα by 13-HPODE. Nevertheless, we cannot exclude the possibility that PPARα was not directly activated by 13-HPODE, but by another compound that has been intermediately formed from 13-HPODE.

In our experiments, linoleic acid at the highest concentration of 50 μM also slightly enhanced ACO and Cyp4A1 mRNAs in Fao cells, but the effect of 13-HPODE at the same concentration on PPARα downstream genes was much stronger than that of its nonoxidized form (Fig. 1). Linoleic acid is known to bind all PPAR subtypes at micromolar concentrations, but with the highest affinity to PPARα [47]. Thus, we can conclude that 13-HPODE is a more potent activator of PPARα than its precursor. Analogously, 8(*S*)-HETE has a 50-fold higher affinity for *Xenopus laevis* PPARα than its precursor, arachidonic acid [34].

As mentioned above, feeding of rats with oxidized fats did also suppress the expression of lipogenic enzymes like FAS in the liver [6]. The SREBP family of transcription factors plays a key role in the regulation of lipogenic enzymes and cholesterol synthesis [48,49]. SREBP-1 has emerged as a regulator of fatty acid and triglyceride synthesis, whereas SREBP-2 regulates cholesterol synthesis [49]. At the highest 13-HPODE concentration used, we observed a small but significant increase of SREBP-1c and SREBP-2 mRNA concentration in Fao cells. Increase of SREBP-1c and SREBP-2 after fibrate treatment was observed in rats and mice and was abolished in PPARα null mice [50–52]. Possibly, the depletion of fatty acids due to stimulation of β-oxidation by fibrates causes an increase in SREBP-1c, which is required for stimulating fatty acid synthesis [52]. Nevertheless, the level of FAS mRNA, which is regulated by SREBP-1c, was not changed in our experiments.

To assess the impact of the observed effect of PPARα activation by 13-HPODE in human cells, we further examined the influence of 13-HPODE on human hepatoma HepG2 cells. In contrast to Fao cells, no induction of PPARα downstream genes was observed after stimulating HepG2 cells for 6 h with 13-HPODE. Only HMGCoAS2 mRNA was slightly enhanced after 6 h incubation at the highest 13-HPODE concentration used. HMGCoAS2 is the main enzyme involved in ketone body formation and is directly controlled by PPARα [53]. Also, longer incubation times (24 h) did not result in changes of the mRNA levels of the examined genes. Furthermore, no change of SREBP-1 and SREBP-2 mRNA levels was observed after 13-HPODE treatment in contrast to Fao cells. It is well established that species-specific differences exist in PPARα function (reviewed in Ref. [21]). Mediated by PPARα, fibrates and other peroxisome proliferators cause hypolipidemic, peroxisome proliferation and liver carcinogenic effects [13,14,35]. Rats and mice are extremely responsive to these effects, whereas humans are resistant to the proliferative and carcinogenic effects of these drugs, but the hypolipidemic effect still manifests [21,29]. There is no conclusive evidence that humans are not responsive to peroxisome proliferation. Only few studies exist demonstrating a weak induction of the peroxisome proliferation marker enzyme ACO by fibrates and other peroxisome proliferators in HepG2 cells [22,54,55]. Most studies revealed that ACO expression is not induced in HepG2 cells and human hepatocytes after treatment with these substances [20,23,27,28,56]. On the other hand, genes encoding enzymes that catalyze branch points or rate-limiting steps in the utilization of fatty acids for ketone body formation, the long chain fatty acyl-CoA synthetase, CPT1A and HMGCoAS2, are responsive to PPARα activation in HepG2 cells and human hepatocytes [23,27,28]. Controversial data exist on PPARα-mediated activation of Δ9-desaturase, a key enzyme in fatty acid biosynthesis, in HepG2 cells by fibrates [28,55]. Δ9-desaturase gene itself is regulated by PPARα in rodents [57]. Considering the much more potent action of fibrates on PPARα compared to fatty acids [34], the lack of effects of 13-HPODE on PPARα downstream genes of HepG2 cells is consistent with previous reports and reflects the known species-specific differences of peroxisome proliferators action.

In summary, we present indirect evidence that 13-HPODE, the primary lipid peroxidation product of linoleic acid, activates PPARα in rat hepatoma Fao cells. Thus, 13-HPODE might be one of the components that are responsible for the observed PPARα-activating effect of oxidized fats in rat liver. In contrast, HepG2 cells did not respond to a stimulation with 13-HPODE indicating that also the effect of oxidized fats in humans may be different from that observed in rats.

Acknowledgement

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Effects of Clofibrate Treatment in Laying Hens

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ABSTRACT Expression of peroxisome proliferator-activated receptor-α (PPARα) has been shown in liver of chicks, but effects of its activation have not yet been investigated. In this study, laying hens were treated with clofibrate, a synthetic PPARα agonist, to investigate the effects of PPARα activation on liver lipid metabolism. Hens receiving a diet containing 5 g of clofibrate/kg had a lower food intake and higher liver mRNA concentrations of typical PPARα target genes (carnitine palmitoyltransferase 1A, acyl-coenzyme A oxidase, bifunctional enzyme, lipoprotein lipase) involved in hepatic mitochondrial and peroxisomal β-oxidation and plasma triglyceride clearance than control hens that received the same diet without clofibrate ($P < 0.05$). Hens treated with clofibrate also had lower mRNA concentrations of fatty acid synthase, 3-hydroxy-3-methylglutaryl-coenzyme A reductase, and low-density lipoprotein receptor, proteins involved in fatty acid biosynthesis and cholesterol biosynthesis and uptake, than hens fed the control diet ($P < 0.05$). These changes in clofibrate-treated hens were accompanied by reduced liver triglyceride concentrations, strongly diminished very low density triglyceride and cholesterol concentrations ($P < 0.05$), a disturbed maturation of egg follicles, a complete stop of egg production, and a markedly reduced plasma 17-β-estradiol concentration ($P < 0.05$). In conclusion, it is shown that clofibrate has complex effects on hepatic lipid metabolism in laying hens that mimic PPARα activation in mammals, affect maturation of egg follicles, and lead to a stop of egg production. Because clofibrate treatment strongly reduced food intake in the hens, some of these effects (i.e., egg production) may have been due to a low energy and nutrient intake.

Key words: peroxisome proliferator-activated receptor-α, clofibrate, laying hen, triglyceride, cholesterol

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INTRODUCTION

Peroxisome proliferator-activated receptor- (**PPAR**) α is a member of the nuclear receptor superfamily. In mammals, it is highly expressed in tissues with high fatty acid oxidation such as liver or muscle (Desvergne and Wahli, 1999). Peroxisome proliferator-activated receptor-α regulates the expression of target genes by binding to DNA sequence elements as heterodimers with the 9-*cis* retinoic acid receptor after activation. Peroxisome proliferator-activated receptor-α target genes are mainly involved in cellular fatty acid uptake and intracellular fatty acid transport, mitochondrial and peroxisomal fatty acid oxidation, ketogenesis, and gluconeogenesis (Mandard et al., 2004). Peroxisome proliferator-activated receptor-α is activated by lipid soluble compounds such as eicosanoids, fatty acids, or fibrates (Desvergne and Wahli, 1999). Recently, it has been shown that activation of PPARα does not only stimulate catabolism of fatty acids but also affects synthesis of triglycerides and cholesterol by interacting with gene expression and proteolytic activation of sterol regulatory element-binding proteins (**SREBP**), transcription factors that have been identified and recognized as key regulators of lipid synthesis and homeostasis (Patel et al., 2001; Guo et al., 2005; Knight et al., 2005; König et al., 2007). It has been found that SREBP-1c preferentially activates genes required for fatty acid synthesis, whereas SREBP-2 preferentially activates the low-density lipoprotein (**LDL**) receptor gene and various genes required for cholesterol synthesis such as 3-hydroxy-3-methylglutaryl coenzyme A (**HMG-CoA**) reductase (Horton et al., 2002). Sterol regulatory element-binding proteins are synthesized as inactive integral endoplasmic reticulum membrane proteins and are activated by proteolytic cleavages in the Golgi, releasing the mature N-terminal domain of SREBP that then translocates to the nucleus and activates transcription of sterol regulatory element-containing genes (Horton et al., 2002).

Compared with mammals, laying hens have a very high rate of hepatic synthesis of triglycerides, phospholipids, and cholesterol, which plays a crucial role in lipid deposition in egg yolk (Walzem et al., 1999). Hepatic triglyceride and phospholipid synthesis in birds is strongly stimulated in bird liver by estrogens that are formed in theca cells of small white follicles (Kudzma et al., 1975; Dashti et al., 1983). Lipids synthesized in the

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liver are incorporated into triglyceride-rich lipoproteins that are secreted into the blood. Plasma of laying hens therefore contains extremely high concentrations of triglycerides, most of which are localized in very LDL (**VLDL**; Hermier et al., 1989). Very LDL with a particle diameter of 25 to 44 nm are bound to specific oocyte receptors and are deposited in developing egg yolk follicles (Walzem et al., 1999).

It has been recently shown that chick liver also expresses PPARα which has a high homology with mouse, rat, and human PPARα (Diot and Douaire, 1999; Meng et al., 2005). However, the function of PPARα in laying hens has not yet been elucidated. If activation of PPARα has similar effects on hepatic gene expression as observed in mammals, we expect that it stimulates β-oxidation of fatty acids in the liver and lowers hepatic and plasma triglyceride concentrations, which in turn may affect lipid deposition into egg follicles. Because estrogens are produced in theca cells of small white follicles (Robinson and Etches, 1986), an inhibition of follicle maturation could also affect formation of estrogens.

The aim of this study was therefore to investigate the effect of a synthetic PPARα agonist on the lipid metabolism of laying hens. We focused our analyses mainly on lipid concentrations of liver and plasma and on hepatic expression of genes that were shown in rat studies to be upregulated by PPARα activation. These included carnitine palmitoyltransferase-1A (**CPT-1A**), acyl-coenzyme A oxidase (**ACO**), bifunctional enzyme, all genes involved in mitochondrial or peroxisomal β-oxidation, and lipoprotein lipase (**LPL**), the key enzyme of plasma triglyceride clearance (Mandard et al., 2004). Recently, it has been shown that chick liver also expresses SREBP-1 and -2 (Gondret et al., 2001; Assaf et al., 2003). To find out whether there is also a functional link between PPARα and SREBP, we also determined gene expression of insulin-induced genes (**Insig**), SREBPs, and the important SREBP target genes involved in fatty acid synthesis [fatty acid synthase (**FAS**)] and cholesterol synthesis and uptake (HMG-CoA reductase, LDL receptor). Due to the close relationship between hepatic lipid metabolism and deposition of lipids in egg yolk via VLDL, we also determined amounts of triglycerides and cholesterol in egg yolks.

MATERIALS AND METHODS

Birds and Treatment

An experiment was conducted with 18 Lohmann White layers with an age of 64 wk and an average BW of 1,750 g (±140, SD). The hens were allotted to 2 groups of 9 each, a control group and a treatment group. They received a nutritionally adequate diet consisting of (in g/kg of diet) wheat (465), extracted soybean meal (130), corn (120), peas (80), Ca carbonate (75), extracted sunflower meal (70), sunflower oil (30), dicalcium phosphate (12.5), vitamin and mineral premix (10), fiber (5), NaCl (2), and DL-Met (0.5). This diet contained 11.4 MJ of ME and 169 g of CP per kilogram [as determined by the official German Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten methods (Naumann and Bassler, 1976)]. Concentrations of nutrients of the diet, including vitamins and minerals, were in agreement with recommendations for laying hens (Gesellschaft für Ernährungsphysiologie, 1999). The diet of the treatment group was supplemented with 5 g of clofibrate [ethyl 2-(4-chlorophenoxy)-2-methylpropionate; Fluka Chemie GmbH, Buchs, Switzerland] per kilogram.

The hens were kept 1 bird per cage in an environmentally controlled room at 18°C. The room was lit for 14 h daily at an intensity of 20 to 30 lx. Feed and water (via nipple drinkers) were available ad libitum. The experiment was conducted over a 5-wk period. All procedures followed established guidelines for the care and handling of animals and were approved by the veterinary council of Saxony-Anhalt. The following data were recorded: BW at the start and end of the experiment, weekly food intake, and number of eggs daily.

Sample Collection

At the end of each week, a blood sample was drawn from the jugular vein of each hen (after fasting for 7 h). To determine egg yolk weight and concentrations of yolk lipids and fatty acid composition, 2 eggs from each hen were sampled at the end of wk 2. Eggs were cooked in water for 10 min. After the end of wk 5, overnight-fasted hens were anesthetized and then decapitated. Blood was collected in heparinized tubes; plasma was separated by centrifugation at 1,500 × g for 10 min at 4°C. Liver was excised, weighed, and immediately snap-frozen in liquid N. Aliquots of liver for RNA isolation were stored at –80°C; other samples were stored at –20°C. From few hens of each group, 1 ovary was excised and photographed. To separate VLDL from the remaining lipoproteins, plasma density was adjusted by NaCl and KBr to δ = 1.022 kg/L as a density cut proposed by Rodriguez-Vico et al. (1992) for chick VLDL. After ultracentrifugation at 900,000 × g at 4°C for 1.5 h, VLDL were removed by suction.

Determination of Lipids in Plasma, Lipoproteins, and Egg Yolk

Lipids from liver and cooked egg yolks were extracted with a mixture of n-hexane and isopropanol (3:2, vol/vol; Hara and Radin, 1978). For determination of the concentrations of lipids in liver and egg yolks, aliquots of the lipid extracts were dried, and the lipids were dissolved using Triton X-100 (De Hoff et al., 1978). Concentrations of triglycerides and cholesterol in plasma and lipoproteins and those of liver and egg yolk were determined using enzymatic reagent kits (1.14830, 1.14856, VWR International, Darmstadt, Germany). The fatty acid composition of egg yolk total lipids was determined by gas chromatography of fatty acid methyl esters that were prepared by methylation with trimethylsulfonium hydroxide (Brandsch et al., 2002).

Table 1. Characteristics of the specific primers used for reverse transcription-PCR analysis

Gene[1]	Forward primer (from 5′ to 3′)	Reverse primer (from 5′ to 3′)	Base pairs	Annealing temperature (°C)	GenBank no.
ACO	ACGCCCAAATTACTCAGGTG	GGATTTCTTTGCCCACTCAA	173	60	NM_001006205
β-actin	ATGAAGCCCAGAGCAAAAGA	GGGGTGTTGAAGGTCTCAAA	223	62	L08165
Bifunctional enzyme	ATTCTTGCAGTCTGGCACCT	CCTGTGGTCATAGCCTGGTT	255	62	BG713425
CPT-1A	GATTTGGACCTGTGGCTGAT	CTGCTTTCATTCGCTGTTCA	262	62	NM_001012898
FAS	GCTGAGAGCTCCCTAGCAGA	TCCTCTGCTGTCCCAGTCTT	164	60	NM_205155
Hepatic lipase	CACATGGGTCTCAGTGTTGG	TCATGGGCACATTTCACAGT	161	62	XM_425067
HMG-CoA reductase	TCCCTGAACCCTCATCTTTG	TCTGCAAGAATACGGCTCCT	250	60	NM_204485
Insig-1	CGACCCATCCAAGAAGATGT	GCATTTGAGGAAGGATGGAA	212	60	NM_001030966
Insig-2	GCTCGGATACGGATTTGTGT	TTGAACTCCTTCAGGGATGG	174	60	NM_001031261
LDL receptor	GCAGTCACAGCATCAGCTTC	ACTCGTTGTGTCGCACACTC	150	60	NM_204452
LPL	CAACTTCCGAAAGGCTTGAG	GGCCAGGAGAACAACAAAAA	229	62	NM_205282
PPARα	AGGCCAAGTTGAAAGCAGAA	GTCTTCTCTGCCATGCACAA	217	60	NM_001001464
SREBP-1	GGTGTCAGGGTGCAGTTTTT	TCACTAGAGGTCCCCACGTC	165	60	AY029224
SREBP-2	CCAAGGAGAGCCTGTACTGC	CCCATTGAGTCCAGGAAAGA	217	60	XM_416222

[1]ACO = Acyl-CoA oxidase; CPT = carnitine palmitoyltransferase; FAS = fatty acid synthase; HMG-CoA = 3-hydroxy-3-methylglutaryl coenzyme A; Insig = insulin-induced gene; LDL = low-density lipoprotein; LPL = lipoprotein lipase; PPAR = peroxisome proliferator-activated receptor; SREBP = sterol regulatory element-binding protein.

Determination of Plasma 17-β-Estradiol Concentration

Concentration of 17-β-estradiol in plasma was determined with an ELISA (RE 52041, IBL GmbH, Hamburg, Germany).

Reverse Transcription-PCR Analysis

Total RNA was isolated from livers by Trizol reagent (Sigma-Aldrich, Steinheim, Germany) according to the protocol of the manufacturer. Complementary DNA synthesis was carried out as described (König and Eder, 2006). The mRNA concentration of genes was measured by real-time detection PCR using SYBR Green I and the RotorGene 2000 system (Corbett Research, Mortlake, Australia). Real-time detection PCR was performed with 1.25 U of Taq DNA polymerase (Promega, Mannheim, Germany), 500 μM deoxyribonucleotide triphosphate, and 26.7 pmol of the specific primers (Operon Biotechnologies, Cologne, Germany; Table 1). For determination of mRNA concentration, a threshold cycle and amplification efficiency were obtained from each amplification curve using the software RotorGene 4.6 (Corbett Research). Calculation of the relative mRNA concentration was made using the $\Delta\Delta$ threshold cycle method as previously described (Pfaffl, 2001). The housekeeping gene β-actin was used for normalization.

Statistical Analyses

Means of the 2 groups were compared by Student's *t*-test. Means were considered significantly different for $P < 0.05$. To test correlations between food intake and other variables within the group of hens treated with clofibrate, linear correlation analysis was performed. Values in the text are given as means ± SD.

RESULTS

BW, Food Intake, and Laying Performance

Initial BW of the hens did not differ between control- and clofibrate-treated hens (Table 2). Hens fed the diet supplemented with clofibrate consumed less feed during the whole feeding period than hens of the control group ($P < 0.05$, Table 2). Moreover, food intake in the group treated with clofibrate showed a large variation among the individual hens. It ranged from 945 g during the experiment in the hen with the lowest food intake to 2,975 g in that with the highest intake. Due to the low food intake, hens treated with clofibrate lost BW markedly during the 5-wk feeding period (Table 2). Within the group of hens treated with clofibrate, there was a positive linear correlation between final BW and food intake during the experimental period ($R^2 = 0.58$, $P < 0.05$). Therefore, final BW was lower in hens treated with clofibrate

Table 2. Food intake, BW, and egg production of control hens and hens treated with clofibrate for 5 wk[1]

Item	Control	Clofibrate
Initial weight (g)	1,729 ± 138	1,771 ± 143
Final weight at wk 5 (g)	1,728 ± 141	1,495 ± 289*
Food intake (g)		
wk 1	823 ± 96	548 ± 241*
wk 2	828 ± 80	362 ± 243*
wk 3	818 ± 86	359 ± 185*
wk 4	854 ± 85	309 ± 81*
wk 5	859 ± 130	335 ± 137*
wk 1 to 5	4,133 ± 372	1,912 ± 646*
Egg production (%)		
wk 1	82 ± 9	60 ± 19*
wk 2	88 ± 15	14 ± 25*
wk 3	93 ± 8	9 ± 20*
wk 4	86 ± 15	2 ± 5*
wk 5	82 ± 18	0
wk 1 to 5	85 ± 11	17 ± 12*

[1]Values are means ± SD (n = 9/group).

*$P < 0.05$.

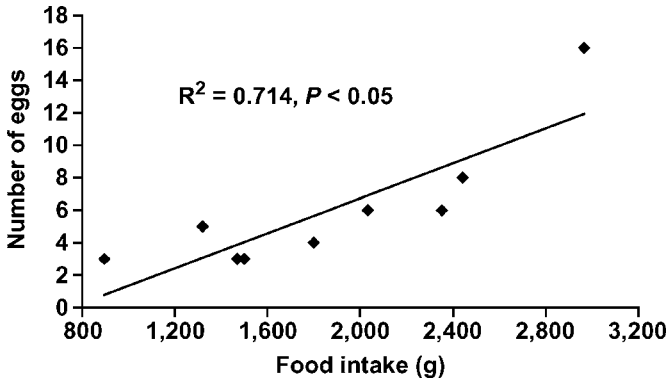

Figure 1. Relationship between food intake and egg production of hens treated with 5 g of clofibrate per kilogram of diet for 5 wk. Total food intake was compared with the number of eggs produced over the feeding period. Each point represents values from an individual hen.

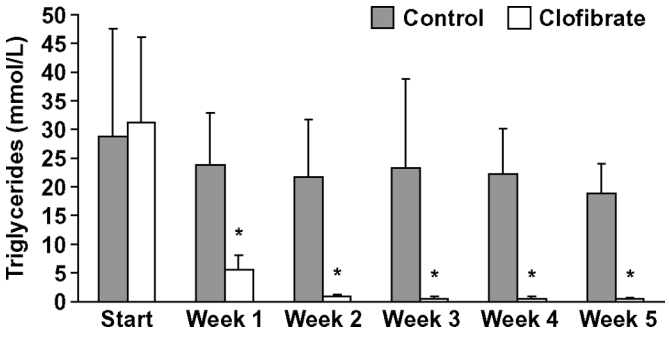

Figure 3. Effect of clofibrate treatment of laying hens on the concentration of triglycerides in plasma. Hens obtained a diet with or without (control) the addition of 5 g of clofibrate per kilogram for 5 wk. Plasma samples were drawn from the jugular vein at the beginning of the feeding period and at the end of each week. Values are means ± SD (n = 9/group). *P < 0.05.

than in control hens (P < 0.05, Table 2). Egg production in the control group was at a normal level during the whole experimental period. In contrast, egg production in the group treated with clofibrate was even reduced in the first week of treatment (Table 2). At wk 2, most hens completely stopped production of eggs. Within the group of hens treated with clofibrate, there was a positive linear correlation between egg production and food intake during the experimental period (R^2 = 0.71, P < 0.05, Figure 1).

The ovary of control hens showed a normal distribution of follicles of different size (i.e., the existence of large yellow follicles containing egg yolk and small yellow and small white follicles). In the ovary of hens treated with clofibrate, large follicles were completely absent, and there were also fewer small yellow and white follicles than in control hens (Figure 2).

Concentrations of Triglycerides and Cholesterol in Plasma, VLDL, and Liver

Initial concentrations of triglycerides in plasma did not differ between both groups of hens (Figure 3). Already after 1 wk of treatment, hens treated with clofibrate had much lower concentrations of triglycerides in plasma than hens of the control group (P < 0.05, Figure 3). During the second week of treatment, plasma triglycerides in hens treated with clofibrate declined further and stayed at values below 1 mmol/L for the remaining feeding period (Figure 3). At wk 5, hens treated with clofibrate also had a strongly reduced concentration of triglycerides in VLDL compared with control hens (0.13 ± 0.14 vs. 8.52 ± 2.45 mmol/L, P < 0.05). Liver weight and hepatic triglyceride concentrations were also significantly lower in hens treated with clofibrate than in control hens (P < 0.05), whereas relative liver weight did not differ between both groups (Table 3). Within the group of hens treated with clofibrate, there was a positive linear correlation between liver weight and food intake during the experimental period (R^2 = 0.63, P < 0.05). Liver triglyceride concentration, plasma triglyceride concentrations from wk 1 to 5, and VLDL triglyceride concentration did not show any significant correlation with food intake in the group of hens treated with clofibrate (P > 0.05).

Plasma cholesterol concentration of hens treated with clofibrate was not different from that of control hens (control: 3.62 ± 0.74 mmol/L; clofibrate: 3.50 ± 0.86 mmol/L). However, hens treated with clofibrate had a strongly reduced VLDL cholesterol concentration compared with

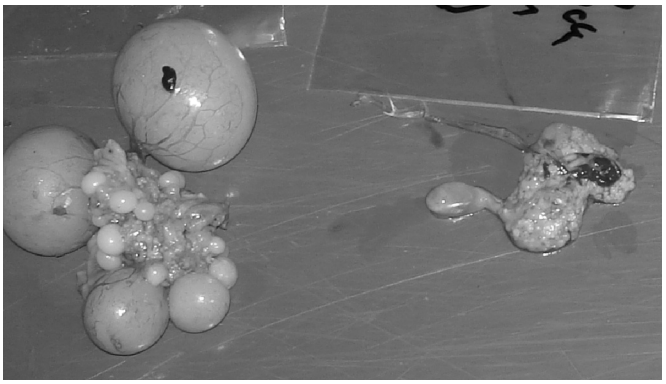

Figure 2. Typical aspect of an ovary of a control hen (left) and of a hen treated with 5 g of clofibrate per kilogram of diet for 5 wk (right).

Table 3. Weights of livers and egg yolks and concentrations of triglycerides and cholesterol in livers and egg yolks of control hens and hens treated with clofibrate[1]

Item	Control	Clofibrate
Liver[2]		
Weight (g)	44.0 ± 8.8	32.9 ± 9.1*
Relative weight (g/100 g of BW)	2.52 ± 0.34	2.62 ± 0.69
Triglycerides (μmol/g)	43.9 ± 10.9	15.6 ± 5.0*
Cholesterol (μmol/g)	7.8 ± 1.2	8.3 ± 1.4
Egg yolk[3]		
Weight (g)	18.2 ± 1.1	18.0 ± 0.3
Triglycerides (μmol/g)	169 ± 17	165 ± 20
Cholesterol (μmol/g)	36.1 ± 3.0	37.7 ± 2.2

[1]Values are means ± SD (n = 9/group).
[2]Livers were analyzed after 5 wk of treatment.
[3]Egg yolks were analyzed after 2 wk of treatment.
*P < 0.05.

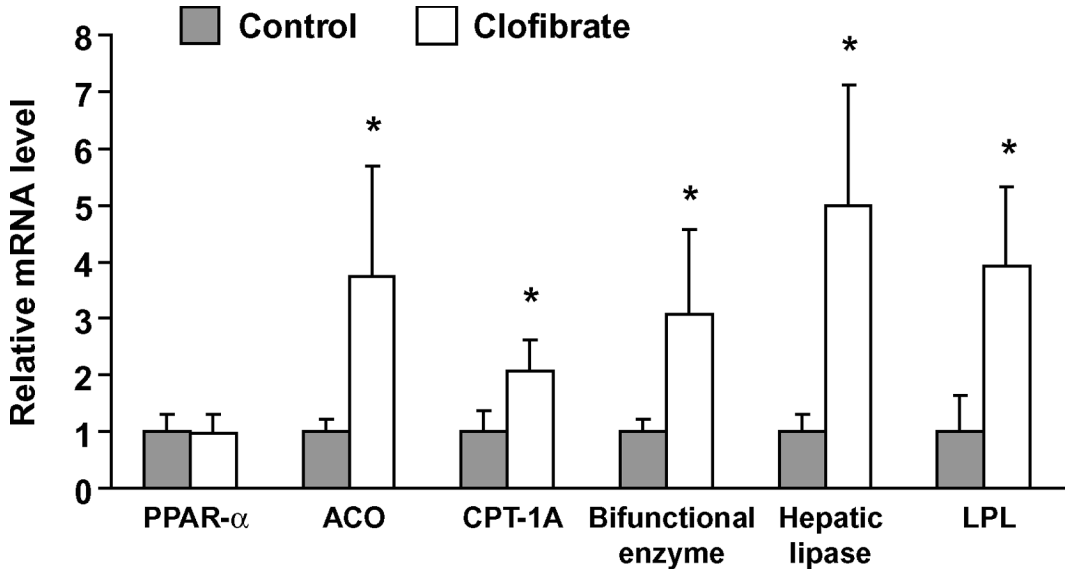

Figure 4. Effect of clofibrate treatment of laying hens on the mRNA concentration of peroxisome proliferator-activated receptor-α (PPAR-α), acyl-coenzyme A oxidase (ACO), carnitine palmitoyltransferase-1A (CPT-1A), bifunctional enzyme, lipoprotein lipase (LPL), and hepatic lipase in the liver. Hens obtained a diet with or without (control) the addition of 5 g of clofibrate per kilogram for 5 wk. Total RNA was extracted from the liver, and relative mRNA concentrations of the genes were determined by real-time detection reverse transcription-PCR analysis using β-actin mRNA concentration for normalization. Values are means ± SD (n = 9/group). *$P < 0.05$.

control hens (0.04 ± 0.02 vs. 0.93 ± 0.24 mmol/L, $P < 0.05$). Moreover, there was a positive linear correlation between VLDL cholesterol concentration and food intake during the experimental period in the group of hens treated with clofibrate ($R^2 = 0.68$, $P < 0.05$). Cholesterol concentration in the liver did not differ between both groups of hens (Table 3).

Weights of Egg Yolks, Concentrations of Triglycerides and Cholesterol in Egg Yolk, and Fatty Acid Composition of Egg Yolk Total Lipids

Eggs sampled from hens treated with clofibrate at wk 2 did not differ in yolk weights and concentrations of triglycerides and cholesterol in yolk from eggs of control hens (Table 3). Eggs of control hens and those of hens treated with clofibrate did not show significant differences in their yolk fatty acid composition. Amounts of fatty acids in yolk total lipids in average of both groups were as follows (g/100 g of total fatty acids): 14:0, 0.25 ± 0.03; 16:0, 24.0 ± 1.2; 16:1 (n-9), 0.76 ± 0.13; 16:1 (n-9), 1.88 ± 0.17; 18:0, 9.53 ± 0.61; 18:1 (n-9), 40.2 ± 0.9; 18:2 (n-6), 16.8 ± 0.9; 18:3 (n-3), 0.18 ± 0.02; 20:4 (n-6), 2.24 ± 0.16; 22:4 (n-6), 0.22 ± 0.04; 22:5 (n-6), 0.53 ± 0.05; and 22:6 (n-3), 0.45 ± 0.05.

mRNA Concentrations of Genes Involved in Hepatic Fatty Acid and Cholesterol Metabolism

Peroxisome proliferator-activated receptor-α mRNA was detected in the liver of laying hens by reverse transcription-PCR, and its concentration did not differ between both groups of hens (Figure 4). Also, all other genes to be analyzed were well expressed in the liver according to reverse transcription-PCR data, with the only exception of LPL, which was weakly expressed in the liver of control hens. Hens treated with clofibrate had higher relative mRNA concentrations of ACO, CPT-1A, bifunctional enzyme, LPL, and hepatic lipase in the liver (Figure 4) and lower mRNA concentrations of Insig-1, SREBP-2, FAS, LDL receptor, and HMG-CoA reductase (Figure 5) than hens fed control diet ($P < 0.05$). Hepatic mRNA concentrations of Insig-2 and SREBP-1 did not differ between both groups of hens (Figure 5). In the groups of hens treated with clofibrate, there was a positive correlation between mRNA concentration of bifunctional enzyme in the liver and food intake during the experimental period ($R^2 = 0.75$, $P < 0.05$). The mRNA concentrations of all the other genes determined did not show significant correlations with food intake within the group of hens treated with clofibrate.

Concentration of 17-β-Estradiol in Plasma

After 1 wk of treatment, hens treated with clofibrate had significantly lower plasma 17-β-estradiol concentrations than control hens ($P < 0.05$, Figure 6). In the plasma of hens treated with clofibrate, 17-β-estradiol concentration further declined until the end of the experiment, whereas it remained on a constant level during the whole experiment in control hens (Figure 6). At the end of the experiment (wk 5), plasma 17-β-estradiol concentration was about 70% lower in hens treated with clofibrate than in control hens ($P < 0.05$, Figure 6). In the group of hens treated with clofibrate, concentration of 17-β-estradiol at wks 1, 2, or 5, respectively, did not show any significant correlation with food intake ($P > 0.05$).

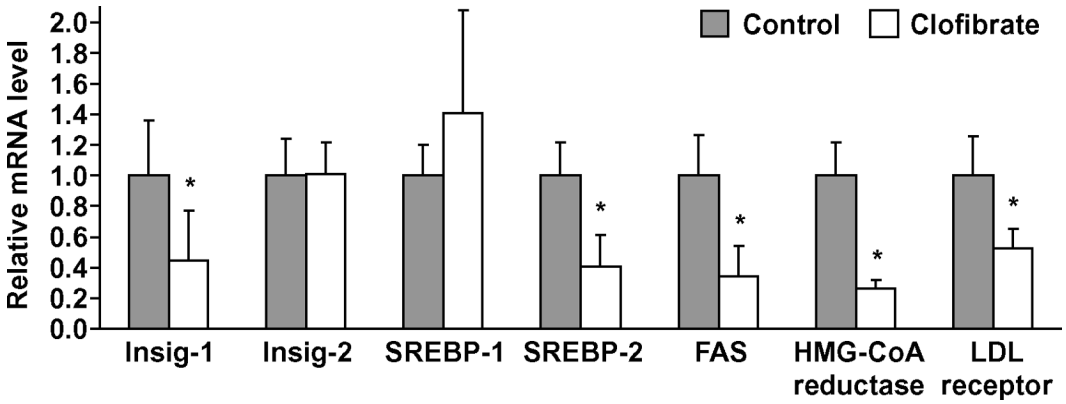

Figure 5. Effect of clofibrate treatment of laying hens on the mRNA concentration of insulin-induced gene- (Insig) 1 and 2, sterol regulatory element-binding protein- (SREBP) 1 and 2, fatty acid synthase (FAS), 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, and low-density lipoprotein (LDL) receptor in the liver. Hens obtained a diet with or without (control) the addition of 5 g of clofibrate per kilogram for 5 wk. Total RNA was extracted from the liver, and relative mRNA concentrations of the genes were determined by real-time detection reverse transcription-PCR analysis using β-actin mRNA concentration for normalization. Values are means ± SD (n = 9/group). *$P < 0.05$.

DISCUSSION

To our knowledge, this is the first study in which laying hens were treated with clofibrate, a synthetic PPARα agonist. It is shown that clofibrate treatment of hens caused a strong depression of food intake, BW loss, a decline in plasma estrogen concentration, a strong reduction of plasma triglycerides, and a complete stop of egg production. Detection of PPARα mRNA in the liver of hens confirmed recent findings that showed the expression of this transcription factor in chick liver (Diot and Douaire, 1999; Meng et al., 2005). Gene expression analyses in the liver demonstrated that clofibrate treatment upregulated ACO, CPT-1A, bifunctional enzyme, and LPL. In mammals, all of these enzymes have a PPAR response element in their promoter regions. Their transcription is stimulated by the binding of the PPARα-retinoid X receptor heterodimer, formed by activation of PPARα, to the PPAR response element (reviewed in Mandard et al., 2004). The finding that the same enzymes are upregulated by clofibrate treatment in hens suggests that the avian form of these enzymes also contains PPAR response elements in their promoter regions. In mammals, LPL is expressed predominantly in adipose tissue and skeletal muscle and in the liver of newborn animals (reviewed in Merkel et al., 2006). In adult animals, LPL expression in the liver can be induced by activation of PPARα (Schoonjans et al., 1996). This is consistent with our data showing that LPL, which was weakly expressed in the liver of control hens, was strongly upregulated upon clofibrate treatment. In our study, PPARα mRNA concentration in the liver was not increased by clofibrate treatment. A functional PPAR response element has been identified in the human PPARα promoter (Pineda Torra et al., 2002). Nevertheless, in agreement with our study, several other studies have shown that PPARα activation does not necessarily upregulate expression of PPARα (Ribas et al., 2005; Morimura et al., 2006; König et al., 2007).

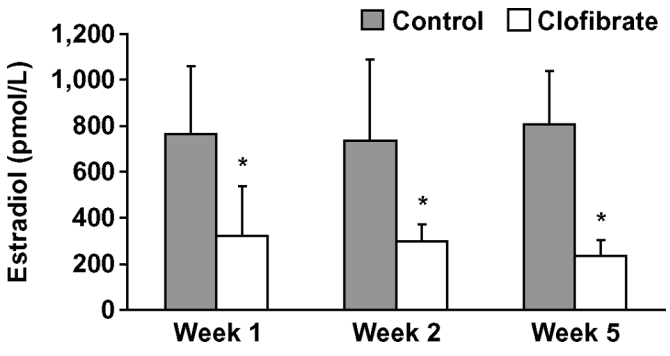

Figure 6. Effect of clofibrate treatment of laying hens on the concentration of 17-β-estradiol in the plasma. Hens obtained a diet with or without (control) the addition of 5 g of clofibrate per kilogram for 5 wk. Plasma samples were drawn from the jugular vein at the end of wk 1, 2, and 5. Values are means ± SD (n = 9/group). *$P < 0.05$.

One finding of this study was that clofibrate treatment caused a strong reduction of food intake already in the first week of treatment. This finding agrees with a recent study by Fu et al. (2003) that showed that treatment of mice with various PPARα agonists reduces food intake and lowers BW compared with control mice. The finding that this effect does not occur in PPARα-null mice strongly suggests that this effect is due to PPARα activation. The molecular mechanisms underlying the appetite-suppressing effect of PPARα agonists have not been completely elucidated (Lo Verme et al., 2005). Nevertheless, these studies in mice suggest that the reduction of food intake by clofibrate in hens, leading to a strong reduction of BW during the experimental period, may have also been induced by PPARα activation.

Another impressing effect observed in this study was that clofibrate strongly reduced triglyceride concentration in liver, plasma, and VLDL. Hepatic gene expression analysis strongly suggests that these effects are at least in part due to increased hepatic mitochondrial and peroxisomal β-oxidation (as shown by increased mRNA concentrations of CPT-1A, bifunctional enzyme, and ACO). Markedly reduced plasma triglyceride concentrations may also

be due to an increased expression of LPL, the key enzyme of clearance of plasma triglycerides, and hepatic lipase, which hydrolyzes triglycerides and phospholipids in chylomicron remnants, intermediate and high density lipoproteins (Santamarina-Fojo et al., 2004). Because enzymes involved in β-oxidation as well as LPL are target genes of PPARα, upregulation of these enzymes might be mediated by PPARα activation. It is well known that nonesterified fatty acids released from adipose tissue are also able to bind to and activate PPARα (Kersten et al., 1999). Because hens treated with clofibrate had a strongly negative energy balance, it is likely that they had increased concentrations of nonesterified fatty acids in plasma that were released from adipose tissue. Therefore, the possibility existed that plasma nonesterified fatty acids contributed to PPARα activation in hens treated with clofibrate. The finding that mRNA concentrations of PPARα target genes were not inversely correlated with food intake, however, suggests that nonesterified fatty acids released from adipose tissue due to negative energy balance did not play a significant role for PPARα activation in the liver. Interestingly, mRNA concentration of bifunctional enzyme, one of the PPARα target genes, was even positively correlated with food intake. We assume that hens with a low food intake had a lower upregulation of that enzyme than hens with a higher food intake, because they took in less clofibrate. Because there were no correlations between food intake and mRNA concentrations of all the other PPARα target genes, we assume that the amount of clofibrate consumed by the hens with the lowest food intake was already sufficient to induce maximum upregulation of these genes.

As indicated by reduced mRNA concentration of FAS, one of the key enzymes of fatty acid synthesis, reduced triglyceride concentrations, in hens treated with clofibrate is also caused by reduced rate of fatty acid synthesis. In avians, like in mammals, hepatic gene expression of FAS is controlled by SREBP-1 (Gondret et al., 2001). However, in contrast to mammals, chicken seem to express a single form of SREBP-1 (Zhang and Hillgartner, 2004). It is known that SREBP-1-dependent gene expression of FAS is downregulated by fasting or a low food intake (Horton et al., 1998). The lack of a correlation between FAS mRNA in the liver and food intake, however, suggests that the reduced food intake in hens treated with clofibrate was not the major reason for the strong downregulation of FAS expression. Because hepatic triglyceride biosynthesis in birds is strongly stimulated by estrogens (Kudzma et al., 1975; Chan et al., 1976), the strongly reduced plasma concentrations of 17-β-estradiol may be mainly responsible for the downregulation of FAS expression. Because mRNA concentration of SREBP-1 was not reduced in the liver of hens treated with clofibrate compared with control hens, we assume that concentration of mature SREBP-1 in the nucleus was reduced, which led in turn to reduced mRNA concentration of FAS.

The observation that follicles remained small and immature in hens treated with clofibrate and that these hens stopped egg production shortly after the beginning of clofibrate treatment may be the consequence of the strongly reduced plasma triglyceride concentration. Plasma VLDL bound to oocyte receptors are required for lipid filling of follicles and egg yolk formation (Walzem et al., 1999). Stop of egg production in hens treated with clofibrate, however, was probably also due to their very low food intake, because we found a positive correlation between food intake and number of eggs in these hens. Interestingly, eggs produced in the first and the second week of hens treated with clofibrate did not differ in size, lipid concentrations, and fatty acid composition from those of control hens. This confirms that egg yolk composition is highly conserved (Kuksis, 1992). Inhibition of the maturation of follicles by clofibrate could also contribute to the low plasma concentrations of estrogens, which are predominantly produced in theca cells of small white follicles (Robinson and Etches, 1986). Estrogen production in follicle theca cells is stimulated by luteinizing hormone (Robinson and Etches, 1986). It has been shown that luteinizing hormone production is reduced in hens with restricted diet intake (Bruggeman et al., 1998). However, because there was no correlation between food intake and plasma 17-β-estradiol concentration in hens treated with clofibrate, it is likely that the reduced food intake was not the major reason for reduced plasma estradiol concentration.

The present study moreover shows that clofibrate treatment caused a strong downregulation of SREBP-2 and its target genes, HMG-CoA reductase, the key enzyme of hepatic cholesterol synthesis, and LDL receptor. This indicates that clofibrate lowers hepatic cholesterol synthesis and uptake of cholesterol into the liver. We assume that hepatic SREBP-2-dependent cholesterol synthesis and uptake of LDL into the liver were downregulated, because less cholesterol was required for VLDL synthesis and VLDL assembly and secretion was strongly reduced by clofibrate. It has been shown that expression and proteolytic activation of SREBP-2 depends on cellular requirement for cholesterol in mammals. When cholesterol requirement is high and cells are depleted of cholesterol, proteolytic processing of SREBP-2 is activated, whereas it is inhibited when cellular cholesterol concentration is high (Horton et al., 2002). Similarly, downregulation of nuclear SREBP-2 has been demonstrated in chicken fed a high cholesterol diet, indicating that chicken nuclear form of SREBP-2 is controlled by cholesterol levels in a similar manner as in mammals (Matsuyama et al., 2005). Furthermore, it has been shown that levels of nuclear SREBP-2 controlled the expression of HMG-CoA reductase and LDL receptor also in chicken liver (Matsuyama et al., 2005). Thus, we assume that the reduced mRNA concentrations of HMG-CoA reductase and LDL receptor in hens fed clofibrate are due to reduced amounts of nuclear SREBP-2. Whether this reduction in nuclear SREBP-2 is made up by reduced transcription of the gene, as indicated by reduced SREBP-2 mRNA concentration, or by posttranslational processes remains unclear. In mammals, proteolytic activation of SREBP-2 is controlled by Insig-1 and Insig-2 (Yabe et al., 2002; Yang et al., 2002).

Although Insig-2 mRNA concentration was not changed upon clofibrate treatment, Insig-1 mRNA concentration was reduced in hens fed clofibrate compared with control hens. In mammals, Insig-1 but not Insig-2 is also a target gene of SREBP-2. Its upregulation by SREBP-2 provides a feedback mechanism for cholesterol homeostasis (Yabe et al., 2002). Thus, the reduced mRNA concentration of Insig-1 in chicken upon clofibrate treatment is possibly mediated by decreased nuclear SREBP-2 levels. Reduction of nuclear SREBP-2 levels by inhibition of its proteolytic activation and a subsequent decrease of the transcription of its target genes has also been found upon PPARα activation in rat liver (König et al., 2007).

In conclusion, this study shows for the first time that PPARα activation by clofibrate causes a strong reduction of food intake and complex alterations of the lipid metabolism, namely an increase of hepatic fatty acid oxidation and a reduction of fatty acid and cholesterol synthesis. These changes in turn lead to a dramatic reduction of VLDL triglyceride and cholesterol concentrations, which, in conclusion, affect lipid deposition in yolk and lead to a stop of egg production.

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A7 **König B**, Spielmann J, Haase K, Brandsch C, Kluge H, Stangl GI, Eder K:

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Effects of fish oil and conjugated linoleic acids on expression of target genes of PPARα and sterol regulatory element-binding proteins in the liver of laying hens

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In mammals, (*n*-3) PUFA and conjugated linoleic acids (CLA) act as activators of PPARα and alter nuclear concentrations of sterol regulatory element-binding proteins (SREBP) in the liver, and thereby influence hepatic lipid catabolism and synthesis. In this study, we investigated the hypothesis that (*n*-3) PUFA and CLA exert similar effects in the liver of laying hens. Thirty hens (64 weeks old) were fed diets containing 30 g/kg of sunflower oil (control), fish oil (salmon oil) or CLA in TAG form (containing predominantly *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA) for 5 weeks. Hens fed fish oil had a higher expression of some PPARα target genes and a lower nuclear concentration of SREBP-2 in the liver and lower concentrations of cholesterol and TAG in plasma than control hens. Nuclear concentration of SREBP-1 and its target genes involved in lipogenesis were not altered in hens fed fish oil. Hens fed CLA had increased concentrations of TAG and cholesterol in the liver. However, their mRNA levels of PPARα target genes and nuclear concentrations of SREBP-1 and SREBP-2 as well as mRNA levels of their target genes in the liver were largely unchanged compared to control hens. The results of this study suggest that (*n*-3) PUFA cause a moderate activation of PPARα and lower cholesterol synthesis but do not impair fatty acid synthesis in the liver of laying hens. CLA lead to an accumulation of TAG and cholesterol in the liver of hens by mechanisms to be elucidated in further studies.

Laying hens: PPARα: Sterol regulatory element-binding proteins: Fish oil: Conjugated linoleic acid

In laying hens, hepatic lipid metabolism plays an important role for the production of eggs. Compared with mammals, laying hens have a very high rate of hepatic synthesis of TAG, phospholipids and cholesterol, which plays a crucial role in lipid deposition in egg yolk[1]. Lipids synthesised in the liver are incorporated into TAG-rich lipoproteins that are secreted into the blood. Plasma of laying hens therefore contains extremely high concentrations of TAG, most of which are localised in VLDL[2]. VLDL with a particle diameter of 25 to 44 nm are bound to specific oocyte receptors and are deposited in developing egg yolk follicles[1]. An impairment of hepatic lipid synthesis leads to a stop of egg production[3].

Hepatic lipid metabolism is regulated by transcription factors such as PPARα and sterol regulatory element-binding proteins (SREBP). PPARα is a member of the nuclear receptor superfamily. In mammals, it is highly expressed in tissues with high fatty acid oxidation such as liver or muscle[4]. PPARα regulates the expression of target genes by binding to DNA sequence elements as heterodimers with the 9-*cis* retinoic acid receptor after activation. PPARα target genes in rodents and human subjects are mainly involved in cellular fatty acid uptake and intracellular fatty acid transport, mitochondrial and peroxisomal fatty acid oxidation, ketogenesis and gluconeogenesis[5]. PPARα is activated by lipid soluble compounds such as eicosanoids, fatty acids or fibrates[4]. In mammals, PPARα activation leads to a strong reduction of plasma and liver TAG concentration due to an enhanced β-oxidation of fatty acids, an inhibition of TAG synthesis and an increased activity of lipoprotein lipase[6].

SREBP are transcription factors which have been identified and recognised as key regulators of lipid synthesis and homeostasis. SREBP-1c preferentially activates genes required for fatty acid synthesis while SREBP-2 preferentially activates the LDL receptor gene and various genes required for cholesterol synthesis such as 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase[7]. SREBP are synthesised as inactive integral endoplasmic reticulum membrane proteins and are activated by proteolytic cleavages in the Golgi releasing the mature N-terminal domain of SREBP that then translocates to the nucleus and activates transcription of sterol regulatory element-containing genes[7–9].

Abbreviations: ACO, acyl-CoA oxidase; ACC, acetyl-CoA carboxylase; CLA, conjugated linoleic acids; CPT, carnitine palmitoyltransferase; FAS, fatty acid synthase; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; Insig, insulin-induced gene; SREBP, sterol regulatory element-binding protein.

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Recently, it has been shown that both PPARα and SREBP are expressed in chicken liver and that they have a high homology with mouse, rat and human PPARα and SREBP, respectively[10–13]. We have recently shown that feeding laying hens clofibrate, a synthetic PPARα agonist, causes up-regulation of several genes recognised as typical PPARα target genes in rodents and human subjects which in turn led to dramatically reduced concentrations of TAG in liver and plasma of the hens[3]. Moreover, it has also been shown that SREBP-1 is the major regulator of lipogenesis and that SREBP-2 controls the cholesterol biosynthetic pathway in chick liver as in mammals[14].

In mammals, both SREBP and PPARα are also activated by various naturally occurring lipids such as *n*-3 fatty acids or conjugated linoleic acids (CLA)[15–17]. Long-chain *n*-3 PUFA activate PPARα, which stimulates mitochondrial and peroxisomal β-oxidation of fatty acids, and reduces the transcription of SREBP-1, which leads to an inhibition of *de novo* fatty acid synthesis due to a down-regulation of lipogenic enzymes[18–20]. These events are involved in the hypotriacylglycerolaemic effects of *n*-3 PUFA in mammals. CLA have also be shown to be activators of PPARα[15,16,21]; in contrast to *n*-3 PUFA, CLA however do not reduce but rather increase transcription of SREBP-1 and SREBP-2 in mammalian liver cells which leads to an increased *de novo* synthesis of fatty acids and cholesterol in the liver[22,23]. Whether *n*-3 PUFA or CLA are able to activate PPARα also in laying hens or to influence the action of SREBP, and thereby influence the lipid metabolism of laying hens, has not yet been investigated. Therefore, we performed an experiment in which hens were fed diets with fish oil or a CLA oil in comparison to sunflower oil which was used as a reference. To study the PPARα-activating effect of these oils, we determined mRNA levels of various genes which were shown to be up-regulated by PPARα activation in rat models. These included carnitine palmitoyltransferase (CPT)-1A, acyl-CoA oxidase (ACO) and the peroxisomal bifunctional enzyme (enoyl-CoA hydratase/L-3-hydroxyacyl-CoA dehydrogenase), all genes involved in mitochondrial or peroxisomal β-oxidation[5]. To assess whether fish oil or CLA influence gene expression or proteolytic activation of SREBP, we determined mRNA levels and nuclear concentrations of SREBP-1c and SREBP-2. Moreover, we determined gene expression of insulin-induced genes (Insig), proteins that are involved in the proteolytic activation of SREBP[24,25] and the important SREBP target genes involved in fatty acid synthesis (acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS)) and cholesterol uptake (LDL receptor) and synthesis (HMG-CoA reductase). To study whether potential effects on PPARα or SREBP lead to phenotypic alterations in the lipid metabolism, we also determined concentrations of TAG and cholesterol in liver, plasma and egg yolk.

Materials and methods

Animals and treatment

An experiment was conducted with thirty Lohmann White layers (64 weeks old) with an average body weight of 1732 (SD 136) g. The hens were allotted to three groups of ten each. They received a nutritionally adequate diet which was supplemented with (30 g/kg) sunflower oil (from a local supermarket; control group), salmon oil (Caesar & Loretz GmbH, Hilden, Germany) or CLA oil (BASF, Ludwigshafen, Germany) (Table 1). The CLA oil contained 60 g CLA isomers per 100 g CLA oil in TAG form with *cis*-9, *trans*-11 and *trans*-10, *cis*-12 as the main isomers. The fatty acid composition of total lipids of the three experimental diets is given in Table 1.

The basal diet contained (g/kg diet): 465 wheat, 130 extracted soya bean meal, 120 corn, 80 peas, 75 $CaCO_3$, 70 extracted sunflower meal, 30 oil, 12·5 Ca_2PO_4, 10 vitamin and mineral premix, 5 fibre, 2 NaCl and 0·5 DL-methionine. This diet contained 11·4 MJ metabolisable energy/kg and 169 g crude protein/kg (as determined by the official German VDLUFA methods)[26]. Concentrations of essential amino acids, minerals and vitamins were in accordance with recommendations of the German Nutrition Society[27] for laying hens. The amount of vitamin E supplied by the premix was 40 mg/kg (as all-rac-α-tocopheryl acetate).

The hens were kept one bird per cage in an environmentally controlled room at 18°C. The room was lit for 14 h daily. Feed (mash) and water (via nipple drinkers) were available *ad libitum*. The experiment was conducted over a 5-week period. All procedures followed established guidelines for the care and handling of animals and were approved by the veterinary council of Saxony-Anhalt. Body weight at the start and end of the experiment, total egg production and weekly feed consumption were monitored.

Sample collection

After the end of week 5, overnight-fasted hens were anaesthetised by a strike on the head and were then decapitated. Blood was collected in heparinised tubes; plasma was separated by centrifugation at 1500 ***g*** for 10 min at 4°C. Liver was excised, weighed and immediately snap frozen in liquid N_2. Aliquots of

Table 1. Fatty acid composition of total lipids (g/100 g total fatty acids) of the experimental diets

Fatty acid	Control diet	Salmon oil diet	CLA oil diet
C14 : 0	0·11	4·26	0·14
C16 : 0	9·27	15·85	9·40
C16 : 1	0·15	4·87	0·21
C18 : 0	3·11	3·18	3·16
C18 : 1 *cis*-9 + *cis*-11	27·97	19·09	23·06
C18 : 2 (*n*-6)	55·10	22·25	22·04
C18 : 2 *cis*-9, *trans*-11	–*	–	18·47
C18 : 2 *trans*-10, *cis*-12	–	–	18·69
C18 : 2 *cis*-10, *cis*-12	–	–	1·03
C18 : 2 *trans*-9, *trans*-11	–	–	0·23
C18 : 3 (*n*-3)	1·46	1·58	1·48
C18 : 4 (*n*-3)	–	1·49	–
C20 : 0	0·29	0·45	0·38
C20 : 1 (*n*-11)	0·40	3·19	–
C20 : 4 (*n*-3)	–	0·81	–
C20 : 5 (*n*-3)	–	8·07	–
C22 : 0	0·56	0·19	0·46
C22 : 1 (*n*-9)	–	3·59	–
C22 : 5 (*n*-3)	–	1·67	–
C22 : 6 (*n*-3)	–	8·19	–

CLA, conjugated linoleic acids.
* < 0·1 g/100 g total fatty acids.

liver for RNA isolation were stored at −80°C; other samples were stored at −20°C. To determine egg yolk weight and concentrations of yolk lipids and fatty acid composition, two eggs from each hen were sampled at the end of week 5. Eggs were cooked in water for 10 min. The two eggs from each hen were pooled for determination of TAG and cholesterol concentrations in egg yolk.

Analysis of TAG, cholesterol and fatty acids

Lipids from liver and cooked egg yolks were extracted with a mixture of n-hexane and isopropanol (3:2, v/v)[28]. For determination of the concentrations of TAG and cholesterol in the liver and egg yolks, aliquots of the lipid extracts were dried and the lipids were dissolved using Triton X-100[29]. Concentrations of TAG and cholesterol in plasma, liver and egg yolks were determined using enzymatic reagent kits (VWR International, Darmstadt, Germany, catalogue no. 1.14830, 1.14856). The fatty acid composition of dietary oils was determined by gas chromatography of fatty acid methyl esters which were prepared by methylation with trimethylsulfonium hydroxide[30]. Concentrations of CLA isomers in the CLA oil were analysed by Ag^+-HPLC-DAD[31].

RT–PCR analysis

Total RNA was isolated from livers by TRIZOL reagent (Sigma-Aldrich, Steinheim, Germany) according to the manufacturer's protocol. cDNA synthesis was carried out as described[32]. The mRNA level of genes was measured by realtime detection PCR using SYBR® Green I and the Rotor Gene 2000 system (Corbett Research, Mortlake, Australia). PCR was performed with 1·25 U Taq DNA polymerase (Promega, Mannheim, Germany), 500 μM desoxyribonucleoside triphosphates and 26·7 pmol of the specific primers (Operon Biotechnologies, Cologne, Germany; Table 2). For determination of mRNA level a threshold cycle (C_t) and amplification efficiency was obtained from each amplification curve using the software RotorGene 4.6 (Corbett Research, Australia). Calculation of the relative mRNA level was made using the amplification efficiencies and the C_t values[33]. The housekeeping gene β-actin was used for normalisation.

Immunoblot analysis

Nuclear extracts of livers were prepared from fresh tissue samples[34]. The protein content of the samples was determined by the bicinchoninic acid assay. Bicinchoninic acid reagent was purchased from Interchim (Montfucon, France). Equal amounts of proteins were pooled from five hens per group and 90 μg protein per lane were separated on 10 % SDS-polyacrylamide gels and electrotransferred to a nitrocellulose membrane (Pall, Pensacola, FL, USA). Polyclonal anti-SREBP-1 antibody (Santa Cruz Biotechnology, Inc., Heidelberg, Germany) and polyclonal anti-SREBP-2 antibody (Abcam plc, Cambridge, UK) were used to detect nuclear SREBP-1 and nuclear SREBP-2, respectively, using enhanced chemiluminescence reagent (GE Healthcare, Munich, Germany) and a chemiluminescence imager (biostep GmbH, Jahnsdorf, Germany). Signals were analysed with the Phoretix TotalLab TL100 software. The anti-rabbit-IgG peroxidase conjugate antibody was purchased from Sigma-Aldrich (Steinheim, Germany).

Statistical evaluation

Treatment effects were evaluated by one-factorial ANOVA. For significant F values ($P<0·05$), means of the treatments (fish oil, CLA) were compared pairwise with the control group by Student's t test. Means were considered significantly different for $P<0·05$. Values are given as means with their standard deviation.

Results

Body and liver weights, feed intake and egg production

Initial body weights of the hens did not differ between the three groups (control, 1747 (SD 146) g; fish oil, 1723 (SD 175) g; CLA, 1719 (SD 95) g, n 10 for each group). Liver weights of hens fed diets containing either fish oil or CLA did not differ from those of control hens (control, 44·0 (SD 8·8) g; fish oil, 40·6 (SD 4·6) g; CLA, 48·6 (SD 5·9) g). However, liver weights of hens fed the diet containing CLA were higher than those of hens fed the diet containing fish oil ($P<0·05$). Feed intake of the hens during the experimental period (control, 4059 (SD 372) g; fish oil, 4121 (SD 273) g;

Table 2. Characteristics of the specific primers used for RT-PCR analysis

Gene	Forward primer (from 5′ to 3′)	Reverse primer (from 5′ to 3′)	bp	Annealing temperature (°C)	NCBI GenBank
ACO	ACGCCCAAATTACTCAGGTG	GGATTTCTTTGCCCACTCAA	173	60	NM_001006205
ACC	TGTGGCTGATGTGAGCTTTC	ACTGTCGGGTCACCTTCAAC	152	60	NM_205505
β-Actin	ATGAAGCCCAGAGCAAAAGA	GGGGTGTTGAAGGTCTCAAA	223	62	L08165
Bifunctional enzyme	ATTCTTGCAGTCTGGCACCT	CCTGTGGTCATAGCCTGGTT	255	62	BG713425
CPT-1A	GATTTGGACCTGTGGCTGAT	CTGCTTTCATTCGCTGTTCA	262	62	NM_001012898
FAS	GCTGAGAGCTCCCTAGCAGA	TCCTCTGCTGTCCCAGTCTT	164	60	NM_205155
HMG-CoA-R	TCCCTGAACCCTCATCTTTG	TCTGCAAGAATACGGCTCCT	250	60	NM_204485
Insig-1	CGACCCATCCAAGAAGATGT	GCATTTGAGGAAGGATGGAA	212	60	NM_001030966
Insig-2	GCTCGGATACGGATTTGTGT	TTGAACTCCTTCAGGGATGG	174	60	NM_001031261
LDL receptor	GCAGTCACAGCATCAGCTTC	ACTCGTTGTGTCGCACACTC	150	60	NM_204452
PPARα	AGGCCAAGTTGAAAGCAGAA	GTCTTCTCTGCCATGCACAA	217	60	NM_001001464
SREBP-1	GGTGTCAGGGTGCAGTTTTT	TCACTAGAGGTCCCCACGTC	165	60	AY029224
SREBP-2	CCAAGGAGAGCCTGTACTGC	CCCATTGAGTCCAGGAAAGA	217	60	XM_416222

ACO, acyl-CoA oxidase; ACC, acetyl-CoA carboxylase; CPT, carnitine palmitoyltransferase; FAS, fatty acid synthase; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; Insig, insulin-induced gene; SREBP, sterol regulatory element-binding protein.

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CLA, 4041 (SD 348) g) and final body weights after 5 weeks (control, 1735 (SD 158) g; fish oil, 1749 (SD 136) g; CLA, 1701 (SD 143) g) did not differ between the three groups. Egg production rate was not different between the three groups of hens (control, 86 (SD 11) %; fish oil, 86 (SD 10) %; CLA, 87 (SD 9) %). However, eggs of hens fed the diet containing CLA had heavier yolks than eggs of control hens or hens fed the diet containing fish oil ($P<0{\cdot}05$; control, 18·2 (SD 1·1) g; fish oil, 17·7 (SD 0·8) g; CLA, 20·9 (SD 1·5) g).

Expression of PPARα and its target genes in the liver

Gene expression of PPARα was detected in the liver of the hens, but PPARα mRNA level did not differ between the three groups of hens (Fig. 1). Hens fed the diet containing fish oil had significantly higher mRNA levels of ACO (+45 %) and bifunctional enzyme (+50 %) in the liver than control hens ($P<0{\cdot}05$, Fig. 1). mRNA level of CPT-1A was not different between hens fed the diet containing fish oil and control hens (Fig. 1). In hens fed the diet containing CLA none of the PPARα target genes was significantly up-regulated in the liver relative to control hens (Fig. 1). There was however a tendency towards increased mRNA levels of ACO (+29 %, $P=0{\cdot}10$) and bifunctional enzyme (+30 %, $P=0{\cdot}06$) in hens fed the diet containing CLA compared to control hens. The mRNA level of CPT-1A in the liver was not different between hens fed the diet containing CLA and control hens (Fig. 1).

mRNA levels of Insig, SREBP, concentration of nuclear SREBP and mRNA levels of SREBP target genes in the liver

Hens fed the diet containing fish oil showed a moderately increased level of SREBP-1 mRNA compared to control hens (+31 %, $P<0{\cdot}05$; Fig. 2) but concentration of nuclear SREBP-1 protein in the liver was slightly decreased (−12 %, Fig. 3 (A)). Hepatic mRNA levels of the SREBP-1 target genes ACC and FAS were unchanged in hens fed the diet containing fish oil compared to control hens (Fig. 2). Hepatic mRNA level of SREBP-2 did not differ between hens fed the diet containing fish oil and those fed the control diet (Fig. 2). The concentration of the nuclear SREBP-2 protein, however, was lower in hens fed the diet containing fish oil than in control hens (−42 %, Fig. 3 (B)). Hepatic mRNA levels of the SREBP-2 target genes Insig-1 (−36 %, $P<0{\cdot}05$) and HMG-CoA reductase (−24 %, $P<0{\cdot}05$) were moderately reduced in hens fed the diet containing fish oil compared to hens fed the control diet; mRNA level of the SREBP-2 target gene LDL receptor, however, did not differ between these two groups of hens (Fig. 2). Hepatic mRNA level of Insig-2 did also not differ between hens treated with fish oil and control hens (Fig. 2).

In hens fed the diet containing CLA, mRNA level of SREBP-1 in the liver was markedly increased in comparison to control hens (+70 %, $P<0{\cdot}05$; Fig. 2), while concentration of nuclear SREBP-1 in the liver of hens fed CLA containing diet was only slightly increased (+23 %; Fig. 3 (A)). However, mRNA levels of the SREBP-1 target genes ACC and FAS did not differ between these two groups of hens (Fig. 2). Hens fed the diet containing CLA did not differ in hepatic mRNA level of SREBP-2 (Fig. 2) and had slightly reduced concentration of the nuclear SREBP-2 protein (−17 %, Fig. 3 (B)) and a higher mRNA level of the SREBP-2 target gene LDL receptor (+30 %, $P<0{\cdot}05$; Fig. 2). mRNA levels of SREBP-2 target genes Insig-1 and HMG-CoA reductase as well as that of Insig-2 did not differ between these two groups of hens (Fig. 2).

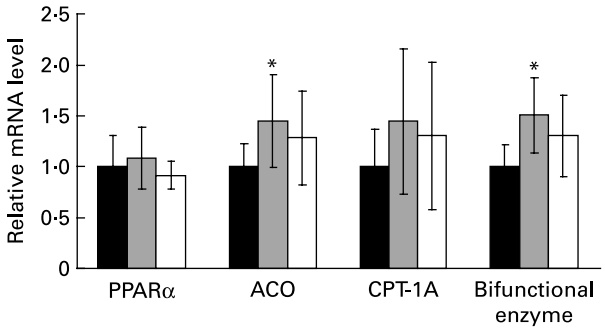

Fig. 1. Effect of feeding laying hens diets containing (30 g/kg) sunflower oil (control, ■), fish oil (▨) or conjugated linoleic acids (CLA; □) for 5 weeks on the mRNA levels of PPARα, acyl-CoA oxidase (ACO), carnitine palmitoyltransferase (CPT)-1A and bifunctional enzyme in the liver. Total RNA was extracted from the liver and relative mRNA levels of the genes were determined by RT-PCR analysis using β-actin mRNA level for normalisation. Values are means with standard deviation indicated by vertical bars for ten hens per group. Mean values were significantly different from control group: *$P<0{\cdot}05$.

Concentrations of TAG and cholesterol in liver, plasma and egg yolk

Hens fed the diet containing fish oil had lower concentrations of TAG and cholesterol in plasma than control hens ($P<0{\cdot}05$; Table 3). Concentration of TAG and cholesterol in liver did not differ in hens fed fish oil compared to control hens (Table 3). Hens fed the diet containing fish oil moreover tended to have lower concentrations and absolute amounts of TAG in egg yolk than hens fed the control diet ($P=0{\cdot}07$ and $P=0{\cdot}06$, respectively; Table 3). Concentrations and absolute amounts of cholesterol in egg yolk did not differ between these two groups of hens.

Hens fed the diet containing CLA had higher concentrations of TAG and cholesterol in liver ($P<0{\cdot}05$) and tended to have higher concentration of cholesterol in plasma than control hens ($P=0{\cdot}06$; Table 3). Concentration of TAG in plasma was not altered in hens fed the diet containing CLA compared to control hens. Moreover, both concentration and absolute amount of TAG in egg yolks tended to be lower in hens fed the diet containing fish oil compared to control hens ($P=0{\cdot}07$ and $P=0{\cdot}06$, respectively); concentration of cholesterol in egg yolk did not differ between those two groups of hens (Table 3). Hens fed the diet containing CLA tended to have higher concentrations of TAG and cholesterol in egg yolk than control hens ($P=0{\cdot}06$ and $P=0{\cdot}10$, respectively). Absolute amounts of TAG and cholesterol in egg yolk were higher in hens fed the diet containing CLA than in control hens ($P<0{\cdot}05$, Table 3).

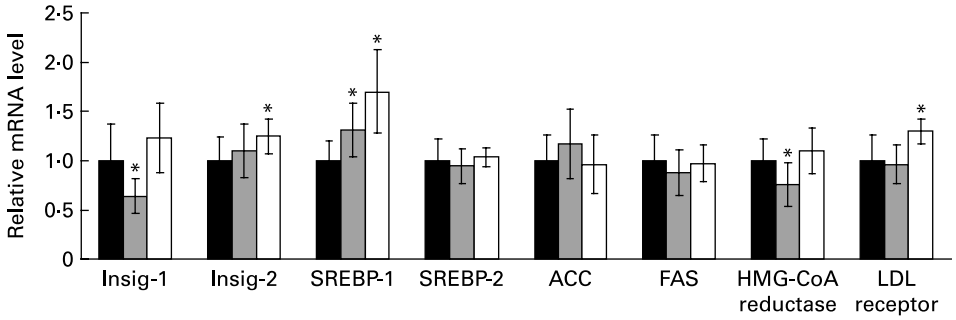

Fig. 2. Effect of feeding laying hens diets containing (30 g/kg) sunflower oil (control, ■), fish oil (▩) or conjugated linoleic acids (CLA; □) for 5 weeks on the mRNA levels of insulin-induced gene (Insig)-1 and -2, sterol regulatory element-binding protein (SREBP)-1 and -2, acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase and LDL receptor in the liver. Total RNA was extracted from the liver and relative mRNA levels of the genes were determined by RT-PCR analysis using β-actin mRNA level for normalisation. Values are means with standard deviation indicated by vertical bars for ten hens per group. Mean values were significantly different from control group: $^{*}P < 0{\cdot}05$.

Discussion

To our knowledge, this is the first study which investigated the effects of fish oil and CLA in laying hens on the expression of genes controlled by PPARα and SREBP, transcription factors that control whole-body lipid homeostasis. Hens received diets containing sunflower oil, fish oil or CLA for 5 weeks. To study whether the dietary oils activated PPARα, we determined mRNA levels of classical PPARα target genes, namely ACO, CPT-1A and the peroxisomal bifunctional enzyme. Feeding fish oil to laying hens caused a significant up-regulation of ACO and bifunctional enzyme compared to feeding sunflower oil which was used as a control fat. Although we did not perform a direct PPARα activation assay, this finding suggests that *n*-3 PUFA present in fish oil were able to bind to and activate PPARα. It should be noted that, since the up-regulation of these genes was relatively weak, being 45 and 50 % for ACO and bifunctional enzyme, respectively, *n*-3 PUFA probably caused only a slight activation of PPARα in the liver of hens. This is in contrast to rodents in which administration of fish oil causes a much stronger up-regulation of PPARα target genes in the liver. For example, in the study of Nakatani *et al.*[19], feeding a diet containing fish oil for one week increased mRNA level of ACO in the liver of mice four-fold.

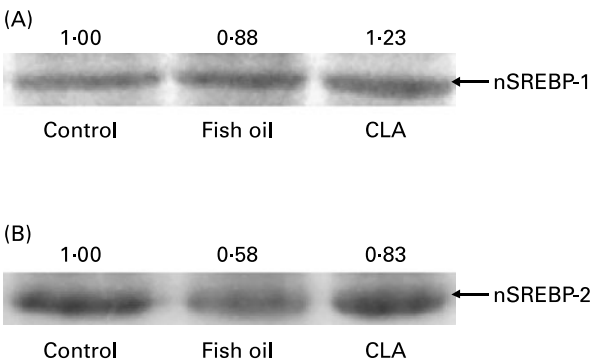

Fig. 3. Effect of feeding laying hens diets containing (30 g/kg) sunflower oil (control), fish oil or conjugated linoleic acids (CLA) for 5 weeks on the concentration of nuclear sterol regulatory element-binding protein (SREBP)-1 (A) and SREBP-2 (B) in the liver. Liver nuclear extracts of five hens per group were pooled and 90 μg of the samples were separated by 10 % SDS-PAGE and immunoblotted with anti-SREBP-1 and anti-SREBP-2 antibodies, respectively. Numbers indicate intensity of the SREBP-specific bands relative (about 68 kDa) to control (= 1·00) as obtained from densitometric analysis.

The data of the present study, moreover, show that the CLA mixture administered to the hens, with *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA as the main isomers, did not significantly up-regulate gene expression of PPARα target genes although there were some numerical increases in mRNA levels of ACO and bifunctional enzyme. It is therefore concluded that dietary CLA does not activate PPARα, or at most to a very small extent, in the liver of laying hens under *in vivo* conditions. This is in contrast to studies in rat liver cells which demonstrated that both of these isomers are able to bind to and activate PPARα[15,16]. The disagreement between the present study and the rat liver cell study may be that rat liver cells have a very high gene expression of PPARα in the liver, which is several-fold greater than in other species[35,36].

In rodents, PPARα activation leads to a reduction of liver and plasma TAG concentration[37]. The increased expression of genes involved in fatty acid β-oxidation is one of the mechanisms underlying the hypotriacylglycerolaemic effect upon PPARα activation[5]. In laying hens, treatment with clofibrate also caused a strong reduction of liver and plasma TAG concentration which suggests that PPARα activation may affect fatty acid and TAG metabolism in a similar way as in rodents[3]. Thus, it is likely that PPARα activation contributed to the reduction of plasma TAG concentrations observed in hens fed the diet containing fish oil. It should be noted, however, that fish oil has several other effects on lipid metabolism such as inhibiting assembly of VLDL or stimulating the expression of PPAR cofactors[38,39]. These effects could also contribute to alterations in plasma lipid concentrations observed in hens fed fish oil.

In mammals, *n*-3 PUFA cause a down-regulation of SREBP-1 at the transcriptional level which in turn inhibits transcription of genes involved in hepatic *de novo* fatty acid synthesis such as FAS or ACC[40,41]. The present study shows that *n*-3 PUFA do not exert such an effect in the liver of laying hens. It is shown that mRNA level of SREBP-1 is even increased by fish oil in the liver of hens. It has been shown that gene expression and activation of SREBP-1c in the liver are triggered by activation of liver X-receptor, a receptor which is activated by oxysterols[42]. Studies in rats

Table 3. Concentrations of TAG and total cholesterol in liver, plasma and egg yolks of laying hens fed diets containing (30 g/kg) sunflower oil (control), fish oil or conjugated linoleic acids (CLA) for 5 weeks

(Mean values with standard deviations for ten hens per group)

	Control		Fish oil		CLA	
	Mean	SD	Mean	SD	Mean	SD
TAG						
Liver (μmol/g)	43·9	10·9	36·5	8·7	62·0*	20·8
Plasma (mmol/l)	20·0	4·1	15·3*	2·9	21·8	9·5
Egg yolk (μmol/g)	125	26	106**	17	160**	50
Egg yolk (mmol/yolk)	2·29	0·59	1·87**	0·32	3·36*	1·17
Total cholesterol						
Liver (μmol/g)	7·75	1·16	7·87	1·54	10·38*	1·62
Plasma (mmol/l)	3·62	0·74	2·79*	0·62	4·61*	1·28
Egg yolk (μmol/g)	15·9	1·8	16·2	2·2	17·2**	1·5
Egg yolk (μmol/yolk)	288	26	286	39	360*	49

Mean values were significantly different from control group: *$P<0·05$, **$P<0·10$.

have shown that induction of oxidative stress, i.e. by administration of fish oil, enhances the formation of oxysterols in the liver[43]. Although the diet used in this study contained an adequate amount of vitamin E, it is possible that administration of fish oil enhanced the formation of oxysterols which in turn caused a liver X-receptor-induced activation of SREBP-1 in the liver of the hens. Nevertheless, an elevated mRNA level of SREBP-1 in the liver of hens fed a diet containing fish oil did not result in increased concentration of nuclear SREBP-1 which was instead marginally decreased. Consistent with that, mRNA levels of SREBP-1 target genes ACC and FAS were also unchanged. These data suggest that fish oil did not inhibit lipogenesis in the liver of hens.

In addition to reduced plasma TAG concentration we found that the TAG concentration of egg yolks also tended to be decreased by fish oil feeding. TAG from the liver are secreted via VLDL into the blood, and they are taken up by oocyte receptors. Thus we suggest that, due to enhanced fatty acid oxidation, VLDL secretion from the liver and uptake by oocyte receptors was reduced leading to reduced TAG concentration in egg yolk.

For SREBP-2, no alteration in mRNA level was observed upon fish-oil feeding but the concentration of nuclear SREBP-2 in the liver was decreased. In agreement with that, mRNA level of the SREBP-2 target gene HMG-CoA reductase and that of Insig-1, whose transcription is also regulated by SREBP, was reduced in hens treated with fish oil. In agreement with reduced expression of HMG-CoA reductase, the key enzyme of cholesterol synthesis, cholesterol concentration in the plasma of hens fed fish oil was reduced. A similar reduction of plasma cholesterol concentration was also observed in another study by feeding fish oil to young chicks[44]. In rats, it has been shown that activation of PPARα causes an inhibition of the processing of the immature SREBP-2, leading to a reduced SREBP-2 activity in the liver which in turn causes a reduction of hepatic cholesterol synthesis and plasma cholesterol concentration[37,45]. Thus, the possibility exists that processing of SREBP-2 in hens fed fish oil was also inhibited by activation of PPARα.

The present study reveals that feeding CLA up-regulates LDL receptor in the liver of laying hens. This effect that could contribute to the increased cholesterol concentration in the liver observed in hens fed the diet containing CLA. A similar up-regulation of LDL receptor expression by *trans*-10, *cis*-12 CLA was also observed in HepG2 cells and was dependent on the up-regulation of SREBP-2[23]. In hens fed CLA, level of SREBP-2 mRNA was unchanged and that of nuclear SREBP-2 was marginally decreased indicating that up-regulation of LDL receptor was probably not mediated by SREBP-2 in the liver of hens fed CLA. Consistent with almost unchanged concentration of nuclear SREBP-2, mRNA levels of SREBP-2 target genes HMG-CoA reductase and Insig-1 were also not altered. In mammals, LDL receptor expression is also regulated via specificity protein-1 and activator protein-1 binding sites and by liver X-receptor α[46–48]. Data on effects of dietary CLA on plasma and liver lipids in mammals are inconsistent revealing decreased, unchanged and increased cholesterol levels[49–52]. Taken together, the reasons for the increased liver and plasma cholesterol concentrations in hens fed CLA cannot be completely explained by the data of this study.

The present study shows that CLA, like fish oil, caused an up-regulation of SREBP-1 mRNA expression in the liver of hens. Nevertheless, the concentration of mature SREBP-1 protein in the nucleus was only marginally increased and the mRNA levels of both SREBP-1 target genes ACC and FAS remained unchanged. Thus, elevated mRNA levels of SREBP-1 did not result in increased nuclear concentration of the protein. This may be due to the increased expression of Insig-2. Dependent on cholesterol concentration, Insig retard the SREBP precursor proteins in the endoplasmic reticulum thereby preventing their proteolytic activation[24,25]. In mice, Insig-2a, the liver-specific transcript of Insig-2, is regulated by insulin and thus plays a special role in regulation of SREBP-1c maturation allowing fatty acid synthesis even at elevated cholesterol concentrations[53]. The finding that CLA increases hepatic TAG concentration agrees with recent studies in laying hens[54,55]. In these studies, even a low concentration of a mixture of CLA (5 g/kg diet) caused an increase of the number of lipid vacuoles in the liver of laying hens and an increased concentration of liver total lipids. Nevertheless, the reason for the increased hepatic TAG concentration in hens fed CLA remains unclear. Studies in mice demonstrated that *trans*-10, *cis*-12 CLA induces hyperinsulinaemia and fatty liver which may be caused by reduced leptin and adiponectin

plasma concentrations and an up-regulation of PPARγ, adipocyte lipid-binding protein, fatty acid transporter mRNA and FAS genes[56,57]. It could be that *trans*-10, *cis*-12 CLA which made up approximately 50 % of total dietary CLA isomers exerted similar effects in the liver of hens.

Interestingly, we found also an increase in egg yolk weights and in yolk TAG and cholesterol concentrations in hens fed the diet containing CLA. Effects of CLA on egg yolk weight reported in literature are variable. In some studies dietary CLA lowered egg or yolk weights[58–60] whereas in others CLA did not alter egg or yolk weights[61,62]. Results about the effects of CLA on egg yolk lipids are also variable[55,61,62]. In our study, both TAG and cholesterol concentrations in egg yolks were increased by dietary CLA. It is possible that the increased yolk weights and increased concentrations of TAG and cholesterol in egg yolk are linked to increased TAG concentrations in the liver and increased cholesterol concentrations in liver and plasma. It is possible that VLDL secretion from the liver and uptake into oocytes were increased in hens fed CLA which resulted in increased cholesterol and TAG transfer into egg yolk.

In conclusion, this study shows for the first time that fish oil causes a moderate up-regulation of PPARα target genes in the liver of laying hens but had less effect on the concentration of nuclear SREBP-1 in the liver and its target genes involved in TAG synthesis. Possibly, up-regulation of PPARα target genes contributed to reduced plasma TAG concentration observed in hens fed fish oil. It is also shown that the concentration of nuclear SREBP-2 and expression of HMG-CoA reductase is decreased by fish oil. This suggests that fish oil lowers hepatic cholesterol synthesis, an effect that might contribute to decreased plasma cholesterol concentration in these hens. It is furthermore shown that a mixture of CLA with *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA does not significantly up-regulate PPARα target genes and has less effect on nuclear concentrations of SREBP-1 and SREBP-2 and their target genes. This suggests that CLA do not influence synthesis of TAG and cholesterol in the liver. Nevertheless, hens fed CLA had higher concentrations of TAG in liver and of cholesterol in liver and plasma and a higher concentration of TAG and cholesterol in egg yolks than control hens. The mechanisms underlying these effects have to be elucidated in further studies.

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Mouse carnitine-acylcarnitine translocase (CACT) is transcriptionally regulated by PPARα and PPARδ in liver cells.

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Mouse carnitine–acylcarnitine translocase (*CACT*) is transcriptionally regulated by *PPARα* and PPARδ in liver cells

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ABSTRACT

Background: Hepatic *PPARα* acts as the primary mediator of the adaptive response to fasting by upregulation of a number of genes involved in fatty acid catabolism. Whether carnitine–acylcarnitine translocase (*CACT*), which mediates the import of acylcarnitines into the mitochondrial matrix for subsequent β-oxidation of fatty acid moieties, is also regulated by *PPARα* in the liver has not yet been investigated.
Methods and Results: Herein, we observed that hepatic mRNA abundance of *CACT* was increased by both, fasting and treatment with *PPARα* agonist WY-14,643 in wild-type mice but not *PPARα*-knockout mice ($P<0.05$). Cell culture experiments revealed that *CACT* mRNA abundance was higher in liver cells treated with either WY-14,643 or PPARδ agonist GW0742, but not with PPARγ agonist troglitazone (TGZ) than in control cells ($P<0.05$). In addition, reporter assays revealed activation of mouse *CACT* promoter by WY-14,643 and GW0742, but not TGZ. Moreover, deletion and mutation analyses of *CACT* promoter and 5′-UTR revealed one functional PPRE in the 5′-UTR of mouse *CACT*.
General significance: CACT is upregulated by *PPARα* and PPARδ, probably by binding to a functional PPRE at position +45 to +57 relative to the transcription start site. The upregulation of *CACT* by *PPARα* and PPARδ, which are both important for the regulation of fatty acid oxidation in tissues during fasting, may increase the import of acylcarnitine into the mitochondrial matrix during fasting.

1. Introduction

During prolonged fasting mitochondrial oxidation of long-chain fatty acids (LCFA) provides the major source of energy for the organism. LCFA are derived from adipose tissue, from which they are released into the plasma by the action of hormone-sensitive lipase and taken up across the plasma membrane into cells by fatty acid transporters. Inside the cell, the fatty acids are bound to cytosolic fatty acid-binding proteins (*c-FABP*) and transported from the plasma membrane to cell organelles such as the mitochondria. The transfer of LCFA into the mitochondrial matrix, where β-oxidation takes place, is accomplished by a complicated mechanism depending on the availability of carnitine [1]. In the first step, free LCFA are transformed into their CoA derivatives by acyl-CoA synthase (*ACS*), an enzyme located on the outer most part of the mitochondrial membrane. The LCFA-CoA derivatives are then converted into carnitine derivatives by carnitine-palmitoyltransferase I (CPT I), an integral protein located on the outer mitochondrial membrane. In the next step, acylcarnitines are imported into the mitochondrial matrix through carnitine–acylcarnitine translocase (*CACT*) [2], which is embedded in the inner mitochondrial membrane. This protein catalyzes a mole-to-mole exchange of carnitines and acylcarnitines so that the fatty acid moieties can be translocated into the mitochondrial matrix [3,4]. In the mitochondrial matrix, LC acylcarnitines are finally reconverted to the respective LC acyl-CoAs by CPT II, an enzyme associated with the inner face of the inner mitochondrial membrane.

Convincing evidence exists that FA themselves are able to regulate the expression of genes involved in their catabolism via nuclear transcription factors such as peroxisome proliferator-activated receptors (PPAR). The *PPARα* isotype, which is abundantly expressed in tissues with high rates of fatty acid oxidation such as the liver [5], acts as the primary mediator of the adaptive response to fasting. The crucial role of *PPARα* during fasting is evidenced by the fact that *PPARα* knockout mice cannot sustain long-term food deprivation [6]. During fasting, *PPARα* is activated by free fatty acids released from adipose tissue and taken up into tissues and, thereby, stimulates the transcription of a large number of genes encoding proteins involved in all aspects of fatty acid catabolism such as fatty acid transporters, c-FABPs, *ACS*, CPT I, and CPT II [7,8]. In addition, activation of *PPARα* also causes upregulation of the carnitine transporter novel organic cation transporter 2 (*OCTN2*) in tissues with abundant *PPARα* expression and enzymes involved in carnitine biosynthesis in liver and kidney [9–12]. Upregulation of *OCTN2* and enzymes involved in carnitine synthesis by

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PPARα activation is probably a means to supply body cells with sufficient carnitine required for carnitine-dependent transport of fatty acids into the mitochondrion.

Due to the fundamental role of the import of acylcarnitine into mitochondrial matrix through *CACT* for lipid catabolism, it should be expected that *CACT* is also regulated by *PPARα* in tissues like the liver, which mediates the metabolic response to fasting. Although data from DNA microarray analyses indicated that *CACT* might be regulated by *PPARα* [13,14], the exact regulation of *CACT* by *PPARα* in the liver has, however, not yet been investigated. Thus, the present study aimed to explore the regulation of *CACT* by *PPARα* in the liver. For this end we performed two experiments with *PPARα* knockout and wild-type mice which were either fasted for 48 h or treated with WY-14,643. Since several *PPARα*-regulated genes such as *CPT I* are also regulated by other PPAR isotypes [15,16], namely PPARγ and PPARδ, which have distinct but also partially overlapping biological functions compared to *PPARα*, we also carried out experiments with cultured liver cells which were treated with WY-14,643 and compared the effect on *CACT* expression with that of troglitazone (TGZ), a selective agonist of PPARγ, and GW0742, a selective agonist of PPARδ. In addition, we performed *CACT* promoter activation studies, and gel shift assays.

2. Materials and methods

2.1. Animal experiments

Two animal experiments with male (first experiment) and female (second experiment), respectively, *PPARα*-knockout mice (129S4/SvJae-*Ppara*^{tm1Gonz}/J) and corresponding wild-type control mice (129S1/SvImJ) purchased from Jackson Laboratory (Bar Harbor, ME, USA) were performed. All animals were fed a commercial standard rodent chow ("altromin 1324", Altromin GmbH, Lage, Germany). Water was available ad libitum from nipple drinkers during both experiments. At the start of the experiments, mice of each genotype with an average initial body weight of 24.3 ± 3.2 g, means ± SD ($n = 36$, first experiment) and 27.5 ± 1.4 g ($n = 40$, second experiment) were randomly assigned to two groups and kept individually in Macrolon cages in a room with controlled temperature (22 ± 2 °C), relative humidity (50–60%), and light (12:12-h light:dark cycle). All experimental procedures described followed established guidelines for the care and handling of laboratory animals and were approved by the council of Saxony-Anhalt.

In the first experiment, one group ("fed") of each genotype received the standard rodent chow ad libitum for the next 48 h (fed wild-type mice, $n = 10$; fed *PPARα*-knockout mice, $n = 10$), whereas from the other group ("fasted") the diet was removed and mice were fasted for the next 48 h (fasted wild-type mice, $n = 10$; fasted *PPARα*-knockout mice, $n = 10$). Mice were then killed by decapitation under light anesthesia with diethyl ether at the morning of the next day either in the fed status (fed) or after the 48 h fasting period (fasted).

In the second experiment, mice in the treatment groups ("WY") (WY-treated wild-type mice, $n = 8$; WY-treated *PPARα*-knockout mice, $n = 8$) received 40 mg/kg WY-14,643 once daily 2 h after the beginning of the light cycle on 4 consecutive days. WY-14,643 was dissolved in DMSO and sunflower oil (50:50, v/v) at a final concentration of 8 mg/ml as described [17]. The daily dose of WY-14,643 (in 0.12 ml) was given by gavage. Animals of the control groups ("control") (control wild-type mice, $n = 8$; control *PPARα*-knockout mice, $n = 8$) were given the same volume of the vehicle DMSO/sunflower oil. Additionally, all mice received the standard rodent chow in controlled amounts of 4 g per day. On day 4 of treatment, mice received the last dose of WY-14,643 or vehicle alone and 1 g of the diet and were killed 4 h later by decapitation under light anesthesia with diethyl ether.

2.2. Sample collection

Blood was collected into ethylenediaminetetraacetic acid-containing tubes, and plasma was obtained by centrifugation (1100 *g*, 10 min, 4 °C). Samples of the liver destined for RNA isolation and plasma were immediately snap-frozen in liquid nitrogen and stored at −80 °C.

2.3. Cell culture experiments

HepG2 cells purchased from German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) were grown in RPMI 1640 medium (Invitrogen, Karlsruhe, Germany) supplemented with 10% FCS (Biochrom, Berlin, Germany) and 0.5% gentamicin (Invitrogen). Fao rat hepatoma cells obtained from the European Collection of Cell Cultures (Salisbury, UK) were cultured in Ham's-F12 medium (Invitrogen) supplemented with 10% FCS and 0.05 mg/mL gentamicin. Cells were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

For experiments on mRNA expression, HepG2 cells and Fao cells were seeded in 24-well culture plates at a density of 2.1×10^5 and 2×10^5 cells per well, respectively, and used prior to reaching confluence (usually 3 days after seeding). HepG2 cells and Fao cells were then preincubated with low-serum medium (0.5% FCS) for 16 h and subsequently treated for 6 h with either WY-14,643, TGZ or GW0742 (all from Sigma-Aldrich, Steinheim, Germany). WY-14,643, TGZ and GW0742 were added to the low-serum medium from stock solutions in DMSO. Final DMSO concentration did not exceed 0.1% (v/v). Cells treated with the same vehicle concentration were used as a control. Viability of the cells was not reduced by 24 h incubation with

Table 1
Characteristics of the primers used for real-time reverse transcriptase polymerase chain reaction analysis.

Gene	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)	PCR product size (bp)	NCBI GenBank
CACT (human)	TGACATCCTGCACCGTGTAT	GCCTCCAGTGAGAACCTGAG	216	NM_000387
L-CPT I (human)	AATCATCAAGAAATGTCGCACGA	AGGCAGAAGAGGTGACGATCG	309	NM_001876
β-actin (human)	GAGCGGGAAATCGTGCGTGAC	GCCTAGAAGCATTTGCGGTGGAC	518	NM_001101
PPARα (human)	TGTGGCTGCTATCATTTGCTGTGG	CTCCCCCGTCTCCTTTGTAGTGC	344	L02932
PPARγ (human)	GCAGGAGCAGAGCAAAGAGGTG	AAATATTGCCAAGTCGCTGTCATC	352	NM_138711
PPARδ (human)	TGCAGGCTTAGGTCCTCACT	AGGATCAGTTGGGTCAGTGG	256	NM_006238
CACT (mouse)	TGGACACTGTTGCTGAGAGG	TTGGCCAAAGGTATCGAGTC	225	NM_020520
L-CPT I (mouse)	CCAGGCTACAGTGGGACATT	GAACTTGCCCATGTCCTTGT	209	NM_013495
CYP4A10 (mouse)	TGAGGGAGAGCTGGAAAAGA	CTGTTGGTGATCAGGGTGTG	208	NM_010011
β-actin (mouse)	ACGGCCAGGTCATCACTATTG	CACAGGATTCCATACCCAAGAAG	87	NM_007393
CACT (rat)	AGCCCACCTGTTATCCACTG	TGTGCAAAAAGAGCCTTCCT	178	NM_053965
L-CPT I (rat)	GGAGACAGACACCATCCAACATA	AGGTGATGGACTTGTCAAACC	416	NM_031559
β-actin (rat)	ATCGTGCGTGACATTAAAGAGAAG	GGACAGTGAGGCCAGGATAGAG	429	NM_031144
PPARα (rat)	CCCTCTCTCCAGCTTCCAGCCC	CCACAAGCGTCTTCTCAGCCATG	555	M88592
PPARγ (rat)	CCCTGGCAAAGCATTTGTAT	ACTGGCACCCTTGAAAAATG	222	NM_013124
PPARδ (rat)	CAGAATTCCTCCCCTTCCTC	TTGCGGTTCTTCTTCTGGAT	230	U75918

either PPAR agonist at the concentrations indicated as evidenced by the MTT assay [18] (data not shown).

For experiments on *CACT* promoter activity, HepG2 cells were seeded in 96-well culture plates at a density of 4–5 × 10^5 cells per well, and used for transient transfection at a confluence of 70%.

2.4. RT-PCR analysis

Total RNA was isolated from cells and tissue samples using Trizol™ reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocol. Total RNA concentration and purity were estimated from the optical density at 260 and 280 nm, respectively. Synthesis of cDNA and determination of mRNA abundance by real-time detection PCR were performed as recently described in detail [19]. For absolute quantification of mRNA abundance of PPAR subtypes, standard curves were generated with purified PCR products of *PPARα*, PPARγ and PPARδ which were obtained by extraction of cut ethidium bromide-stained bands following 2% agarose gel electrophoresis by MinElute Gel Extraction Kit (Qiagen, Hilden, Germany). Quantification of double-stranded DNA concentration of purified PCR products was performed using the PicoGreen DNA Quantitation Kit (Molecular Probes) and a spectrofluorometer (excitation: 480 nm, emission: 520 nm). Relative quantification of mRNA abundance of *CACT*, L-CPT I and CYP4A10 was performed using the $2^{-\Delta\Delta Ct}$-method using *β-actin* as reference gene [20]. Ct-values of target genes and the reference gene were obtained using Rotorgene Software 5.0. Relative mRNA abundance of the genes investigated is expressed as fold change in the treatment groups compared to the control group. Characteristics of gene-specific primers obtained from Operon (Köln, Germany) are shown in Table 1.

2.5. In silico analysis of mouse CACT promoter and 5′-UTR for putative PPRE

To identify putative PPRE in the mouse *CACT* promoter and 5′-UTR, approximately 2 kb of the 5′-flanking region of mouse *CACT* from positions −1806 to +133 relative to the transcription start site (NCBI GenBank CT571271) was analyzed using NUBIScan [21] and MatInspector (Genomatix) [22].

2.6. Promoter reporter gene constructs

Five mouse *CACT* promoter constructs, pGL4.10-m*CACT*_1128, pGL4.10-m*CACT*_803, pGL4.10-m*CACT*_645, pGL4.10-m*CACT*_421, and pGL4.10-m*CACT*_170 were generated. The parental promoter construct pGL4.10-m*CACT*_1128 containing four putative PPRE was generated by PCR-amplification of the mouse *CACT* promoter from positions −995 to +133 using mouse BAC clone RP23-334M10 (imaGene, Berlin Germany), and subcloning of the generated PCR product containing two adapters of XhoI and HindIII site into the XhoI and HindIII sites of pGL4.10[luc2] basic reporter vector (encoding the synthetic *Firefly* luciferase reporter gene; Promega). The PCR primer sequences were as follows: 5′-ATACTCGAGCTCTGTAAGAGCAGCCAGTT-3′ and 5′-ATAAAGCTTGCTCAGTCTTCTGTCTGTCT-3′.

The mouse *CACT* promoter truncation constructs pGL4.10-m*CACT*_803 containing three PPRE (sequence spanning from −670 to +133), pGL4.10-m*CACT*_645 containing two PPRE (sequence spanning from −512 to +133), pGL4.10-m*CACT*_421 containing one PPRE (sequence spanning from −288 to +133) and m*CACT*_170 containing one PPRE (sequence spanning from −37 to +133) were PCR-amplified from the parental pGL4.10-m*CACT*_1128 promoter construct using specific 5′ primers flanking the putative PPRE (m*CACT*_170: 5′-ATACTCGAGAAGTAGACTTCAGGGCGGAA-3′, m*CACT*_421: 5′-ATACTCGAGCAAAGACAGGGACCTCTATG-3′, m*CACT*_645: 5′-ATACTCGAGCTTCTGCCAGAAAGGTCCTT-3′, m*CACT*_803: 5′-ATACTCGAGGACTGATGTCCTCACAGTTC-3′) and one common 3′ primer (m*CACT*_HindIII: 5′-ATAAAGCTTGCTCAGTCTTC-TGTCTGTCT-3′). The generated PCR products containing two adapters of XhoI and HindIII site in the end of PCR fragments were subcloned into the XhoI and HindIII sites of pGL4.10[luc2]. After cloning, the integrity and fidelity of all *CACT* promoter constructs were verified by DNA sequencing.

The mouse *CACT* promoter mutant constructs pGL4.10-m*CACT*_1128mut, pGL4.10-m*CACT*_803mut, pGL4.10-m*CACT*_421mut, and pGL4.10-m*CACT*_170mut were generated by site-directed mutagenesis using the QuickChange mutagenesis kit from Stratagene Europe (Amsterdam, Netherlands) according to the manufacturer's protocol. The targeted mutation was introduced in the PPRE at position +45 to +57 (AGGTGAAAGGTCG) using the following

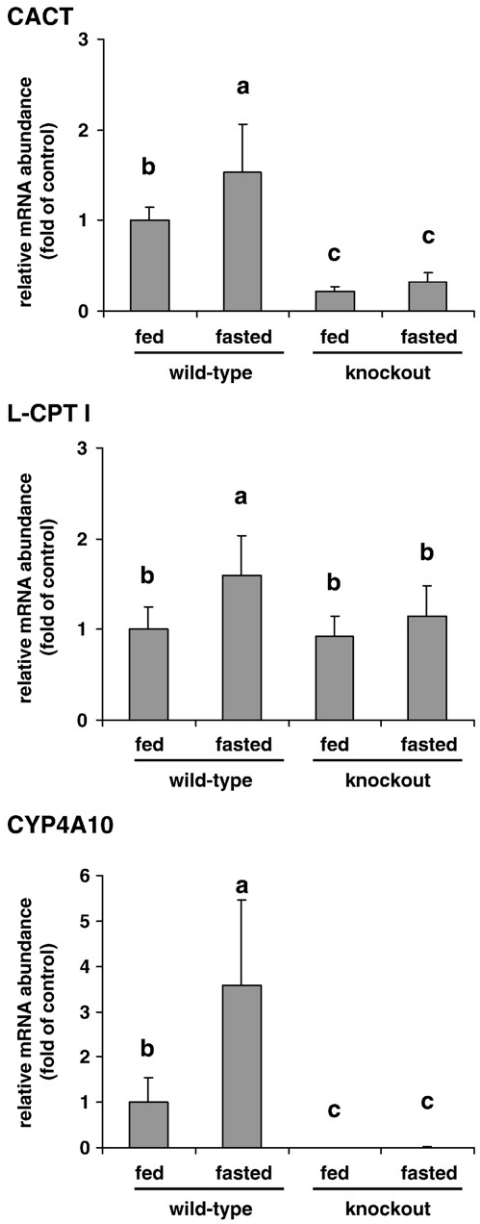


Fig. 1. Effect of fasting on mRNA abundances of carnitine–acylcarnitine translocase (CACT), liver-type carnitine-palmitoyltransferase I (L-CPT I), and cytochrome P450 4A10 (CYP4A10) in the liver of wild-type and PPARα knockout mice. Mice of both genotypes were either fasted for 48 h (fasted group) or fed a standard rodent diet ad libitum for 48 h (fed group). Total RNA was extracted from liver and mRNA abundances were determined by real-time detection RT-PCR analysis using β-actin for normalization. Bars represent means ± SD ($n = 10$). Means without a common letter differ, $P < 0.05$. Significant effects ($P < 0.05$) from two-way ANOVA: CACT: fasting, genotype, fasting × genotype; L-CPT I: fasting, genotype; CYP4A10: fasting, genotype, fasting × genotype.

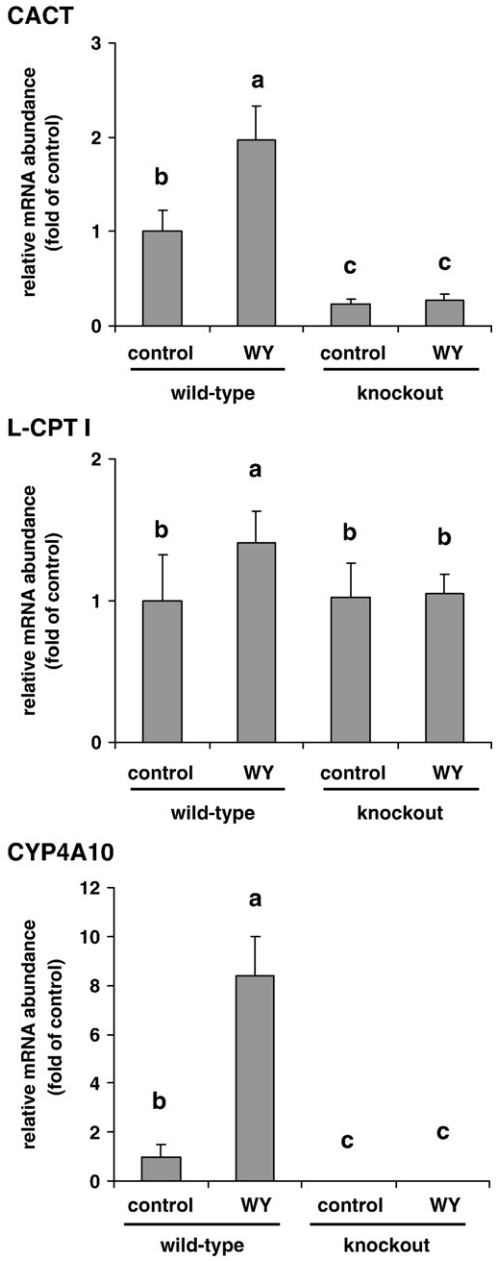


Fig. 2. Effect of WY-14,643 on mRNA abundances of carnitine–acylcarnitine translocase (CACT), liver-type carnitine-palmitoyltransferase I (L-CPT I), and cytochrome P450 4A10 (CYP4A10) in the liver of wild-type and PPARα knockout mice. Mice of both genotypes were treated orally for 4 days with either 40 mg/kg of WY-14,643 (WY-14,643 group) or the appropriate volume of vehicle only (control group). Total RNA was extracted from liver and mRNA abundances were determined by real-time detection RT-PCR analysis using β-actin for normalization. Bars represent means ± SD ($n = 8$). Means without a common letter differ, $P < 0.05$. Significant effects ($P < 0.05$) from two-way ANOVA: CACT: WY-14,643, genotype, WY-14,643 × genotype; L-CPT I: WY-14,643; CYP4A10: WY-14,643, genotype, WY-14,643 × genotype.

primers (m*CACT*-PPREmut_forward: 5′-AGCGGCTCCGCCGACCATA-CACCTACATTCAGGTG-3′, m*CACT*-PPREmut_reverse: 5′-CACCTGAATG-TAGGTGTATGGTCGGCGGAGCCGCT-3′). The mutant constructs were tested for the presence of the desired mutation and the absence of any unexpected mutations by DNS sequencing.

2.7. Transient transfection and dual luciferase assay

HepG2 cells were transiently transfected with either the generated *CACT* promoter constructs, negative control vector pGL4.10-m*CACT*_0 or positive control vector 3× ACO-PPRE (containing three copies of consensus PPRE from the ACO promoter in front of a luciferase reporter gene; this vector was a generous gift from Dr. Sander Kersten, Nutrigenomics Consortium, Top Institute (TI) Food and Nutrition, Wageningen, Netherlands), and pGL4.74[hRluc/TK] (encoding the *Renilla* luciferase reporter gene; Promega), which was used as an internal control reporter vector to normalize for differences in transfection efficiency, using FuGENE 6 transfection reagent (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol. In addition, in experiments investigating the role of *PPARα*, co-transfections with mouse *PPARα* expression plasmid pCMX-m*PPARα* and mouse RXRα expression plasmid pCMX-mRXRα (both, generous gifts from R.M. Evans, Salk Institute for Biological Studies, San Diego, CA, USA) were carried out. Subsequently, cells were treated with either 100 μM WY-14,643, 20 μM TGZ, 1 μM GW0742 or vehicle (DMSO = control) for 24 h. Afterwards the cells were washed with phosphate-buffered saline and lysed with passive lysis buffer (Promega). Luciferase activities were determined with the Dual-Luciferase Reporter Assay System from Promega according to the manufacturer's instructions using a Mithras LB940 luminometer (Berthold Technologies, Bad Wildbad, Germany) as described recently in more detail [23].

2.8. Nuclear protein extraction

Nuclear extracts were prepared from Fao cells. After cells were washed and harvested with ice-cold PBS by scraping, cells were centrifuged, and the pellets suspended in buffer 1 (25 mM HEPES, 5 mM KCl, 0.5 mM $MgCl_2$, + protease inhibitor) followed by buffer 2 (1:99 mixture of NP-40 and buffer 1, + protease inhibitor), and mixed at 4°C for 15 min. After centrifugation, pellets were suspended in buffer 3 (1:1 mixture of buffer 1 and buffer 2, + protease inhibitor), and mixed gently. After a further centrifugation step, the resulting pellets were suspended in buffer 4 (25 mM HEPES, 10% sucrose,

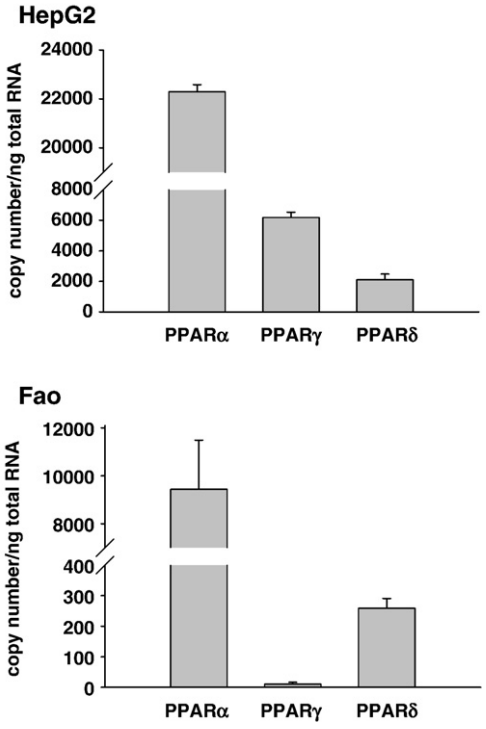


Fig. 3. Absolute mRNA abundances of PPARα, PPARγ and PPARδ in HepG2 cells, and Fao cells. Total RNA was extracted from cells and PPAR isotype-specific mRNA abundances were quantified by real-time detection RT-PCR analysis using standard curves generated with purified PCR products of human and rat, respectively, PPARα, PPARγ and PPARδ. Bars represent means ± SD for $n = 3$.

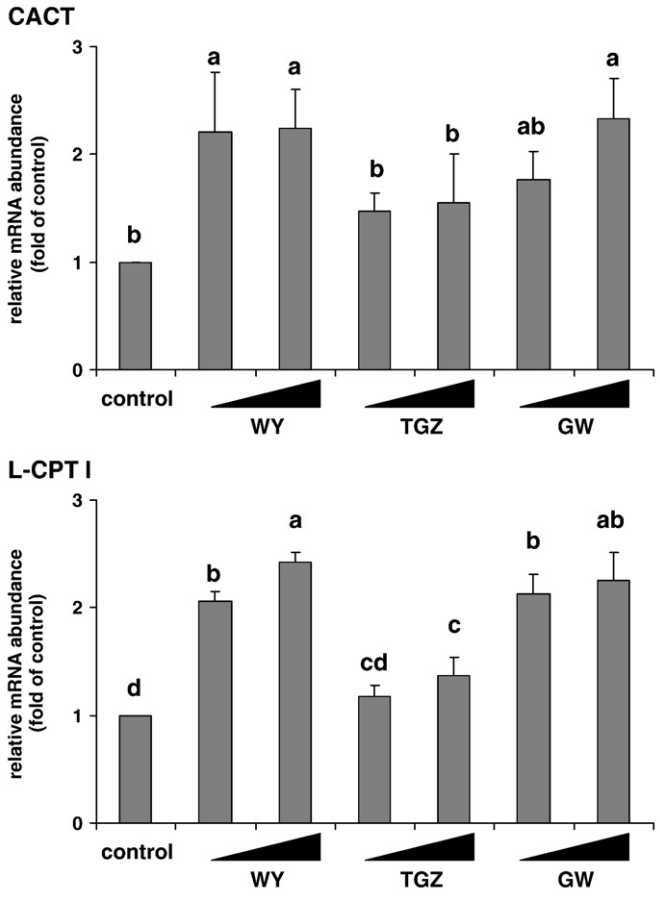


Fig. 4. Effect of PPARα agonist WY-14,643, PPARγ agonist TGZ, and PPARδ agonist GW0742 on the mRNA abundances of carnitine–acylcarnitine translocase (CACT) and liver-type carnitine-palmitoyltransferase I (L-CPT I) in HepG2 cells. HepG2 cells were grown in culture medium until subconfluent state. Medium was then changed to low-serum medium (0.5% fetal calf serum). After 16 h preincubation in this medium, cells were incubated with increasing concentrations of WY-14,643 (50 and 100 μM), TGZ (10 and 20 μM), and GW0742 (0.5 and 1 μM) for 6 h. Control cells were incubated with medium containing vehicle alone. Total RNA was extracted from cells and mRNA abundances were determined by real-time detection RT-PCR analysis using β-actin for normalization. Bars represent means ± SD of at least three independent experiments. Means without a common letter differ, $P<0.05$.

350 mM NaCl, 0.01% NP-40, + protease inhibitors), mixed by rotation at 4°C for 1 h. The nuclear protein was obtained by a final centrifugation step. The nuclear protein (supernatant) was stored at − 80 °C until analysis. The protein concentration of the nuclear extracts was determined by the BCA protein assay kit (Pierce, Rockford, IL).

2.9. Electrophoretic mobility shift assay (EMSA)

The following oligonucleotides used in EMSA were annealed: *CACT*-PPRE, 5′-CGCCCACCTGAATGTAGGTGAAAGGTCGGCGG-3′ and 5′-GGCTCCGCCGACCT TTCACCTACATTCAGGTG-3′; *CACT*-PPREmut, 5′-CGCCCACCTGAATGTAGGTG TATGGTCGGCGG-3′ and 5′-GGCTCCG CCGACCATACACCTACATTCAGGTG-3′; for specific competition rACO-PPRE, 5′-TTCCCGAACGTGACCTTTGTCCTGGTCCC CTTTGATC-3′ and 5′-AAAGGGGACCAGGACAAAGGTCACGTTCGGGAAGATC-3′; for non-specific competition PPRE-cont: 5′-GACAGTGACTCTTGTGGGGATACTCTCT GACTCTACT-CTTGAAGAATGATATATAC-3′ and 5′-ATATCATTCTTCAAGAG-TAGA GTCAGAGAGTATCCCCACAAGAGT*CACT*GTCTGTT-3′. After annealing, 100 ng of double-stranded DNA probes were labelled with 0.05 mM DIG-ddUTP in 1× labelling buffer (0.2 M potassium cacodylate, 25 mM Tris–HCl, 0.25 ng/ml bovine serum albumin, pH 6.6), 5 mM $CoCl_2$, 20 U/μl terminal transferase (Roche, Penzberg, Germany), and incubated at 37 °C for 15 min. The DIG-labelled DNA probes (4 ng) were incubated with 5 to 10 μg of nuclear protein and 5-, 50- and 100-fold molar excess of unlabelled specific DNA probes for competition in EMSA binding buffer (10 mM Tris–HCl, 120 mM KCl, 0.5 mM EDTA, 0.1% Triton X-100, 12.5% glycerol, 0.2 mM DTT) in the presence of 1 μg poly(dI–dC) for 30 min at RT. The protein–DNA complexes were resolved by 6% non-denaturing (native) polyacrylamide gel electrophoresis, and transferred to a positive charged nylon membrane. The DIG-labelled DNA was detected by chemiluminescence using anti-Digoxigenin-AP conjugate and CSPD (both from Roche) and a Bio-Imaging system (Biostep, Jahnsdorf, Germany).

2.10. Statistical analysis

Data of all experiments were analyzed using the Minitab Statistical Software (Minitab, State College, PA, USA). Treatment effects of animal experiments experiment were analyzed by two-way ANOVA with classification factors being treatment (WY-14,643 or fasting), genotype and the interaction of treatment (WY-14,643 or fasting) and genotype. Treatment effects of cell culture experiments were analyzed by one-way ANOVA. For significant *F*-values, means were compared

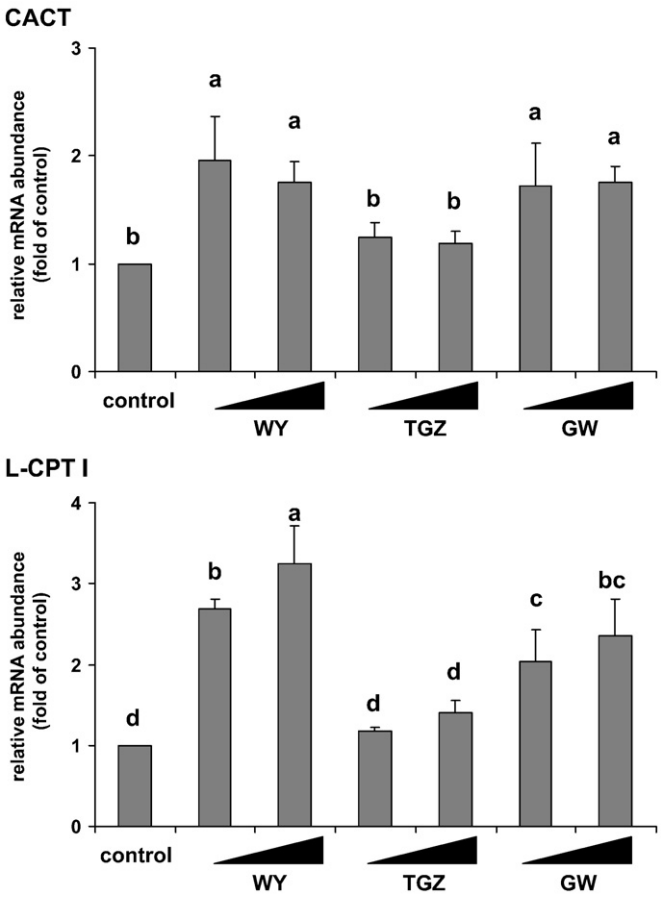


Fig. 5. Effect of PPARα agonist WY-14,643, PPARγ agonist TGZ, and PPARδ agonist GW0742 on the mRNA abundances of carnitine–acylcarnitine translocase (CACT) and liver-type carnitine-palmitoyltransferase I (L-CPT I) in Fao cells. Fao cells were grown in culture medium until subconfluent state. Medium was then changed to low-serum medium (0.5% fetal calf serum). After 16 h preincubation in this medium, cells were incubated with increasing concentrations of WY-14,643 (50 and 100 μM), TGZ (10 and 20 μM), and GW0742 (0.5 and 1 μM) for 6 h. Control cells were incubated with medium containing vehicle alone. Total RNA was extracted from cells and mRNA abundances were determined by real-time detection RT-PCR analysis using β-actin for normalization. Bars represent means ± SD of at least three independent experiments. Means without a common letter differ, $P<0.05$.

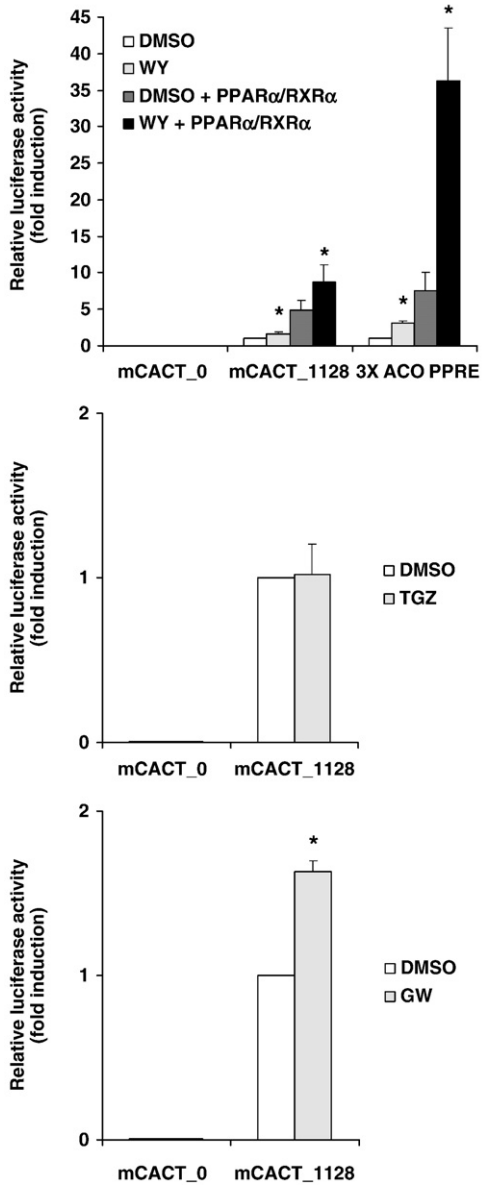


Fig. 6. Effect of PPARα agonist WY-14,643, PPARγ agonist TGZ, and PPARδ agonist GW0742 on the promoter activity of mouse carnitine–acylcarnitine translocase (CACT) in HepG2 cells. HepG2 cells were transiently transfected with either pGL4.10-mCACT_1128 construct containing four PPRE of mouse CACT promoter, negative control vector pGL4.10-mCACT_0 or positive control vector 3× ACO-PPRE, and internal control vector pGL4.74. In addition, in experiments investigating the role of PPARα, co-transfections with mouse PPARα expression plasmid pCMX-mPPARα and mouse RXRα expression plasmid pCMX-mRXRα were carried out. Subsequently, cells were treated with either 100 μM WY-14,643, 20 μM TGZ, 1 μM GW0742 or vehicle (DMSO = control) for 24 h. Afterwards cells were lysed, and luciferase activities determined. Relative luciferase activity is shown as the ratio of *Firefly*/*Renilla* luciferase and bars represent means ± SD of at least three independent experiments. The asterisks indicate significant differences from DMSO-treated control cells ($P<0.05$).

by Fisher's multiple range test. In cell culture and animal experiments, means were considered significantly different for $P<0.05$.

3. Results

3.1. Final body weights of mice in the fasting experiment

Final body weights were significantly influenced by fasting and the genotype (wild-type control, 26.4 ± 1.4 g; wild-type fasting, 23.5 ± 1.5 g; *PPARα*-knockout control, 27.8 ± 1.4 g; *PPARα*-knockout fasting, 24.4 ± 1.4 g; mean ± SD; $n = 10$). Final body weights of fasted mice were lower than those of non-fasted mice ($P<0.05$); final body weights of *PPARα*-knockout mice were higher than those of wild-type mice ($P<0.05$). The interaction of fasting and genotype had no effect on final body weights.

3.2. Final body weights of mice in the WY-14,643 experiment

Final body weights were not influenced by either WY-14,643, genotype or the interaction of both factors (wild-type control, 25.1 ± 2.7 g; wild-type WY-14,643, 25.2 ± 1.9 g; *PPARα*-knockout control, 23.9 ± 3.1 g; *PPARα*-knockout WY 14,643, 23.2 ± 4.3 g; mean ± SD; $n = 8$).

3.3. Effect of fasting on mRNA abundance of CACT, L-CPT I and CYP4A10 in the liver of PPARα-knockout mice

To elucidate the involvement of *PPARα* in the regulation of *CACT* by fasting in the liver, we determined the mRNA abundance of *CACT* and the known *PPARα* target genes *L-CPT I* and *CYP4A10* in the liver of fasted wild-type and *PPARα*-knockout mice. Fasting caused an increase in the mRNA abundance of *CACT*, L-CPT I and CYP4A10 in the liver of wild-type mice ($P<0.05$; Fig. 1), but not in the liver of *PPARα*-knockout mice (Fig. 1). The mRNA abundance of *CACT* and CYP4A10 in the liver was markedly lower in fed *PPARα*-knockout mice than in fed wild-type mice ($P<0.05$). In contrast, mRNA abundance of L-CPT I in the liver did not differ between fed *PPARα*-knockout mice and fed wild-type mice (Fig. 1).

3.4. Effect of WY-14,643 on mRNA abundance of CACT, L-CPT I and CYP4A10 in the liver of PPARα-knockout mice

Similar as fasting, treatment with the synthetic *PPARα* agonist WY-14,643 increased mRNA abundance of *CACT*, L-CPT I and CYP4A10 in the liver of wild-type mice ($P<0.05$; Fig. 2), whereas in the liver of *PPARα*-knockout mice treatment with WY-14,643 did not increase mRNA abundance of those genes (Fig. 2). Hepatic mRNA abundance of *CACT* and CYP4A10 was markedly lower in untreated *PPARα*-knockout mice than in untreated wild-type mice ($P<0.05$), whereas hepatic mRNA abundance of L-CPT I did not differ between these two groups (Fig. 2).

3.5. Abundance of PPARα, PPARγ and PPARδ mRNA in HepG2 and Fao cells

Since the expression of the different PPAR isotypes is a prerequisite for the mediation of PPAR isotype-specific gene transcription, we first analyzed the mRNA abundance of the different PPAR isotypes in HepG2 and Fao cells (Fig. 3). In both cell lines *PPARα* had the highest mRNA abundance of all PPAR isotypes. In contrast, PPARδ had the lowest mRNA abundance in HepG2 cells (Fig. 3), whereas in Fao cells PPARγ had the lowest mRNA abundance of the three PPAR subtypes (Fig. 3). In HepG2 cells, mRNA abundance of *PPARα* was about 4-fold higher than that of PPARγ and about 10-fold higher than that of PPARδ (Fig. 3). In Fao cells, mRNA abundance of *PPARα* was about 36-fold higher than that of PPARδ and about 900-fold higher than that of PPARδ (Fig. 3).

3.6. Effect of WY-14,643, TGZ and GW0742 on mRNA abundance of CACT and L-CPT I in HepG2 cells

In the next step, we investigated the effects of the PPAR isotype-specific agonists WY-14,643, TGZ, and GW0742 on mRNA abundance of *CACT* and the known PPAR target gene *L-CPT I* in HepG2 cells. HepG2 cells treated with 50 and 100 μM WY-14,643 had an about 2.2.-fold higher mRNA abundance of *CACT* than control cells ($P<0.05$; Fig. 4). The mRNA abundance of L-CPT I was about 2.1 and 2.4-fold higher in HepG2 cells treated with 50 and 100 μM WY-14,643, respectively, than in control cells ($P<0.05$; Fig. 4). Cells treated with 10

and 20 μM TGZ did not differ from control cells with respect to the mRNA abundance of *CACT* (Fig. 4). The mRNA abundance of L-CPT I was 1.4-fold higher in cells treated with 20 μM TGZ compared to control cells ($P<0.05$; Fig. 4), but did not differ between cells treated with 10 μM TGZ and control cells. In cells treated with 0.5 and 1 μM GW0742 the mRNA abundance of *CACT* was about 1.7 and 2.3-fold, respectively, higher than in control cells ($P<0.05$; Fig. 4). The mRNA abundance of L-CPT I was about 2.1 and 2.3-fold higher in HepG2 cells treated with 0.5 and 1 μM GW0742, respectively, than in control cells ($P<0.05$; Fig. 4).

3.7. Effect of WY-14,643, TGZ and GW0742 on mRNA abundance of CACT and L-CPT I in Fao cells

We further investigated the effects of the PPAR isotype-specific agonists WY-14,643, TGZ, and GW0742 on mRNA abundance of *CACT* and the known PPAR target gene L-CPT I in Fao cells. In Fao cells treated with 50 and 100 μM WY-14,643 the mRNA abundance of *CACT* was about 2.0 and 1.8-fold, respectively, higher than in control cells ($P<0.05$; Fig. 5). The mRNA abundance of L-CPT I was about 2.7 and 3.2-fold higher in Fao cells treated with 50 and 100 μM WY-14,643, respectively, than in control cells ($P<0.05$; Fig. 5). Fao cells treated with 10 and 20 μM TGZ did not differ from control cells with respect to mRNA abundance of *CACT* and L-CPT I (Fig. 5). Fao cells treated with 0.5 and 1 μM GW0742 had an about 1.7-fold higher mRNA abundance of *CACT* than control cells ($P<0.05$; Fig. 5). The mRNA abundance L-CPT I was about 2.0 and 2.4-fold higher in Fao cells treated with 0.5 and 1 μM GW0742, respectively, compared to control cells ($P<0.05$; Fig. 5).

3.8. Identification of putative PPRE in the mouse CACT promoter and 5′-UTR

Sequence analysis of the 5′-flanking region of mouse *CACT* from positions −1806 to +133 using NUBIScan and MatInspector revealed four putative PPRE at positions −808 to −796, −580 to −568, −332 to −324, and +45 to +57.

3.9. Effect of WY-14,643, TGZ and GW0742 on CACT promoter activity

To test whether mouse *CACT* promoter is activated by PPAR ligands, we cloned the 5′-regulatory region of mouse *CACT* (sequence spanning from −995 to +133) into a luciferase reporter vector, and transiently transfected HepG2 cells with this promoter reporter construct, which was named pGL4.10-m*CACT*_1128. As shown in Fig. 6, the PPAR ligands WY-14,643 (100 μM) and GW0742 (1 μM), but not TGZ (20 μM) compared to vehicle alone (DMSO) increased luciferase activity in HepG2 cells transiently transfected with the pGL4.10-m*CACT*_1128 construct indicative of activation of mouse *CACT* promoter ($P<0.05$). In addition, co-transfection of mouse *PPARα* expression plasmid pCMX-m*PPARα* and mouse RXRα expression

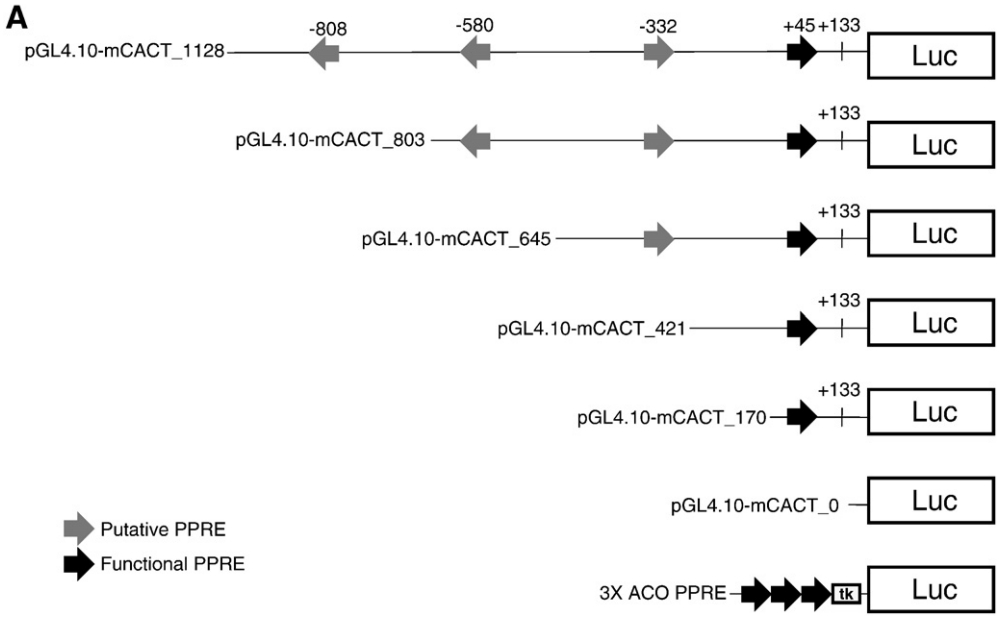


Fig. 7. Identification of functional PPRE in mouse CACT promoter: (A) Mouse CACT promoter constructs used. Numbers of the constructs (1128, 803, 645, 421, 170, 0) denote the 5′-deletion end point of the constructs. (B) Effect of PPARα agonist WY-14,643, PPARγ agonist TGZ, and PPARδ agonist GW0742 on the promoter activity of mouse carnitine–acylcarnitine translocase (CACT) in HepG2 cells. HepG2 cells were transiently transfected with either pGL4.10-mCACT truncation constructs or negative control vector pGL4.10-mCACT_0, and internal control vector pGL4.74. In addition, in experiments investigating the role of PPARα, co-transfections with mouse PPARα expression plasmid pCMX-mPPARα and mouse RXRα expression plasmid pCMX-mRXRα were carried out. Subsequently, cells were treated with either 100 μM WY-14,643, 20 μM TGZ, 1 μM GW0742 or vehicle (DMSO = control) for 24 h. Afterwards cells were lysed, and luciferase activities determined. Relative luciferase activity is shown as the ratio of *Firefly*/*Renilla* luciferase and bars represent means ± SD of at least three independent experiments. The asterisks indicate significant differences from DMSO-treated control cells ($P<0.05$). (C) Effect of PPARα agonist WY-14,643, PPARγ agonist TGZ, and PPARδ agonist GW0742 on the promoter activity of mouse carnitine–acylcarnitine translocase (CACT) in HepG2 cells. HepG2 cells were transiently transfected with either wild-type or mutated pGL4.10-mCACT truncation constructs, and internal control vector pGL4.74. In addition, in experiments investigating the role of PPARα, co-transfections with mouse PPARα expression plasmid pCMX-mPPARα and mouse RXRα expression plasmid pCMX-mRXRα were carried out. Subsequently, cells were treated with either 100 μM WY-14,643, 20 μM TGZ, 1 μM GW0742 or vehicle (DMSO = control) for 24 h. Afterwards cells were lysed, and luciferase activities determined. Relative luciferase activity is shown as the ratio of *Firefly*/*Renilla* luciferase and bars represent means ± SD of at least three independent experiments. The asterisks indicate significant differences from DMSO-treated control cells ($P<0.05$). (D) Binding of the PPARα/RXRα heterodimer to the putative CACT-PPRE (+45 to +57) as determined by gel shift assay. Aliquots of Fao nuclear extracts were incubated with DIG-labelled oligonucleotides corresponding to either the PPRE at position +45 to +57 (CACT-PPRE) or the mutated CACT-PPRE +45 to +57 (PPREmut) in the presence or absence of a 5-, 50-, and 100-fold molar excess of unlabelled competitor DNA (oligonucleotide corresponding to the ACO-PPRE). The protein–DNA complexes were resolved by 6% non-denaturing (native) polyacrylamide gel electrophoresis, and transferred to a positive charged nylon membrane. The DIG-labelled DNA was detected by chemiluminescence. These experiments were performed two times and the results of one representative experiment are shown. (E) Sequence alignment of the PPRE at position +45 to +57 of mouse, rat and human CACT. The PPRE, which is comprised of two hexanucleotides separated by a single nucleotide (AGGTGAnAGGTCG), termed direct repeat 1, is underlined. Matching nucleotides are shown by asterisks.

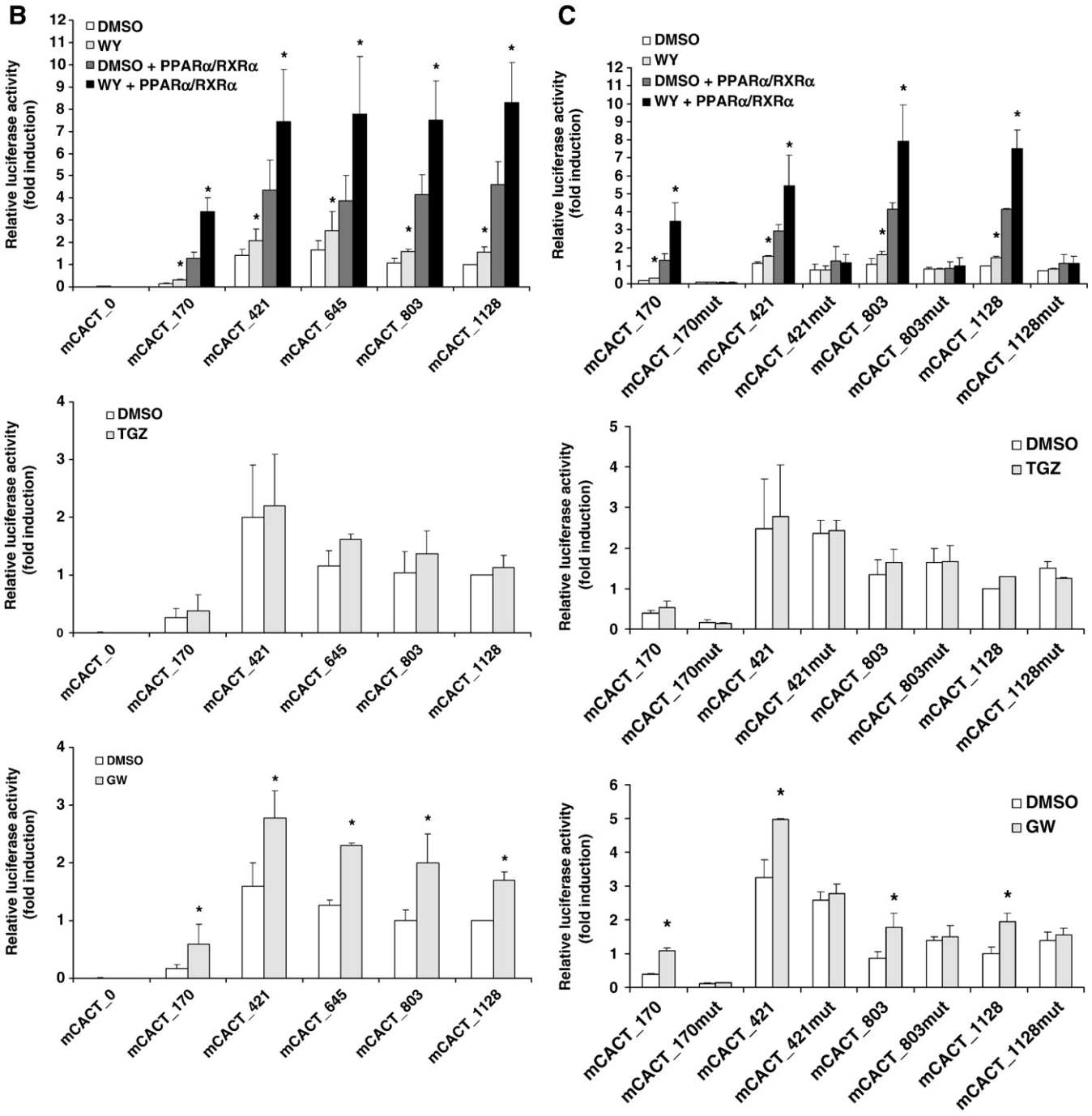


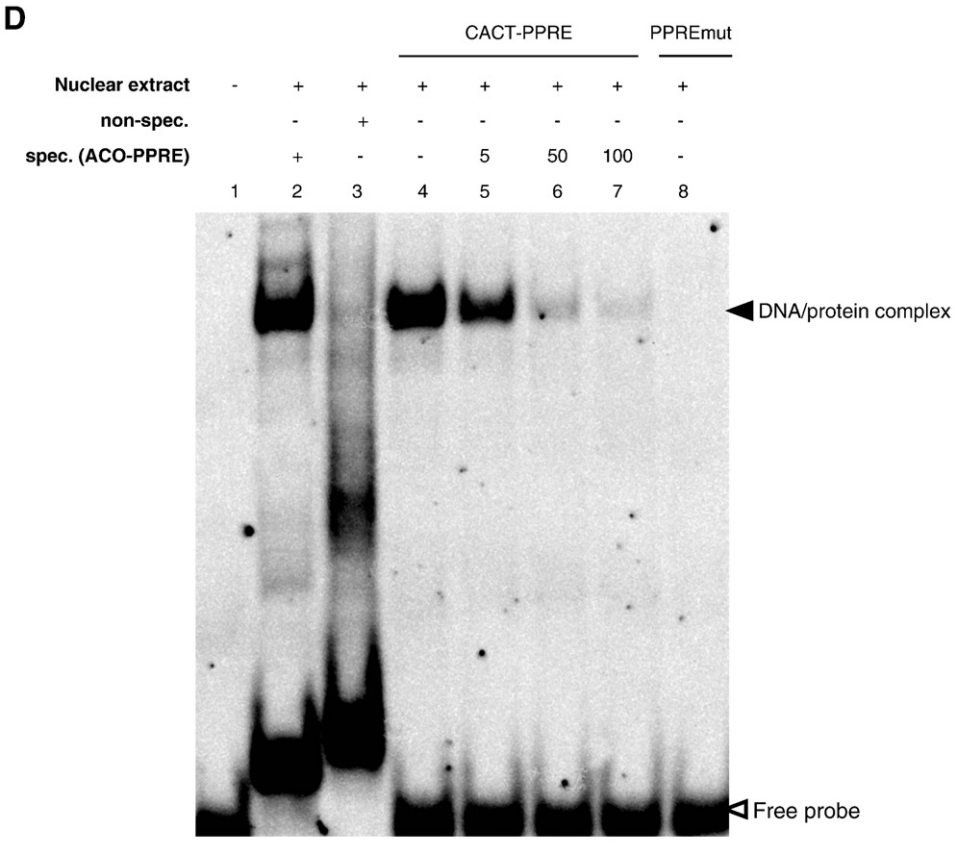
Fig. 7 (*continued*).

plasmid pCMX-mRXRα further increased transcriptional activity of the mouse *CACT* promoter both, in the presence and absence of WY-14,643 ($P<0.05$, Fig. 6).

3.10. Identification of functional PPRE in mouse CACT promoter

To demonstrate the functionality of putative PPAR, we generated serial truncation constructs of the 5′-regulatory region of mouse *CACT* containing three putative PPRE (construct pGL4.10-m*CACT*_803), two putative PPRE (construct pGL4.10-m*CACT*_645), and one putative PPRE (construct pGL4.10-m*CACT*_421 and construct pGL4.10-m*CACT*_170; Fig. 7A). As shown in Fig. 7B, the PPAR ligands WY-14,643 (100 μM) and GW0742 (1 μM), but not TGZ (20 μM) compared to vehicle alone (DMSO) increased luciferase activity in HepG2 cells transiently transfected with these mouse *CACT* promoter truncation constructs ($P<0.05$). The magnitude of induction of promoter activity by WY-14,643 and GW0742 between the different constructs was almost identical indicating that the most proximal PPRE at position +45 to +57 is functional.

To confirm that this PPRE is actually the functional PPRE, we generated *CACT* promoter mutant constructs (pGL4.10-m*CACT*_1128-mut, pGL4.10-m*CACT*_803mut, pGL4.10-m*CACT*_421mut, and pGL4.10-m*CACT*_170 mut), each of which contained a targeted mutation within PPRE at position +45 to +57. As a consequence, induction of



E

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Consensus PPRE                AACTAGGTCAAAGGTCA

Human          GCCCCGCCCACCCAAAAGTGGGTGAAAGGTCGGCGGCGCCGGCACTGCAGC

Mouse          GCCCCGCCCACCTGAATGTAGGTGAAAGGTCGGCGGAGCCGCTCGGGCAGC

Rat            GCCCCGCCCACCTTAATGTAGGTGAAAGGTCGGTGTAGCCGCTTGGACAGC

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Fig. 7 (*continued*).

promoter activity by WY-14,643 and GW0742 in HepG2 cells transfected with the *CACT* mutant promoter constructs was completely abolished (Fig. 7C). These results demonstrate that the PPRE at position +45 to +57 may be functional in mouse *CACT*.

To confirm that this PPRE at +45 to +57 binds the *PPARα*/RXRα heterodimer *in vitro*, we performed a gel shift assay (EMSA) using Fao nuclear extracts together with DIG-labelled oligonucleotide corresponding to the PPRE at position +45 to +57. As shown in Fig. 7D, the labelled oligonucleotide corresponding to PPRE +45 to +57 formed a DNA–protein complex in the presence of nuclear extract containing *PPARα*/RXRα proteins. Formation of the DNA–protein complex was efficiently blocked in a competitive manner in the presence of an excess of unlabelled specific oligonucleotide (Fig. 7D). The mutated PPRE (+45 to +57) did not cause DNA–protein complex formation (Fig. 7D). These results indicate that *PPARα*/RXRα heterodimer binds specifically to the PPRE at position +45 to +57 of mouse *CACT*.

In addition, sequence alignment of the PPRE at position +45 to +57 of mouse, rat and human *CACT* revealed that this PPRE is highly homologous between species and has a high similarity to the consensus PPRE known from the literature (AGGTCAAAGGTCA) (Fig. 7E).

4. Discussion

The present study aimed to explore whether *CACT* is regulated by *PPARα* in the liver. We therefore performed two experiments with *PPARα*-knockout and wild-type mice which were either fasted for 48 h or treated with WY-14,643. To demonstrate *PPARα* activation we determined hepatic mRNA abundance of the known *PPARα* target genes *L-CPT I* and *CYP4A10*. As expected, both genes were significantly upregulated by fasting and treatment with WY-14,643 in the liver of wild-type mice but not of *PPARα*-knockout mice. Upregulation of CYP4A10 by WY-14,643 in female mice was even higher than that by fasting in male mice indicating that *PPARα* was strongly activated by WY-14,643 in this experiment. As *PPARα* is generally less activated in females than in males [24], our findings suggest that the effects induced by WY-14,643 in female mice in the present study might be even stronger in male mice. Determination of mRNA abundance of *CACT* also revealed a significant increase by both, fasting (1.5-fold) and WY-14,643 treatment (2-fold) in the liver of wild-type mice but not of *PPARα*-knockout mice. Although upregulation of *CACT* by fasting and WY-14,643 was rather low when compared to CYP4A10, the magnitude of upregulation is in the range of other *PPARα*

regulated genes [13]. Therefore, our findings indicate that upregulation of *CACT* by fasting and WY-14,643 treatment in the liver is mediated by *PPARα*, and that *CACT* is regulated by *PPARα* at the transcriptional level. As also observed with CYP4A10, the hepatic mRNA abundance of *CACT* was markedly lower in *PPARα*-knockout mice than in wild-type mice. This however is no direct proof that expression of *CACT* is regulated by *PPARα* because it is well documented that several known *PPARα* target genes are expressed in tissues of *PPARα*-knockout mice at levels comparable to those of wild-type mice [25,26], as also evidenced in the present study for L-CPT I.

In contrast to the liver, upregulation of *CACT* by fasting in the small intestine of mice was demonstrated not to be regulated by *PPARα* as shown in a recent study [27]. Contradictory to that study [27], *CACT* was identified as a *PPARα*-regulated gene in small intestine of mice treated with the synthetic *PPARα* activator WY-14,643 [28]. Although the reason for this differential regulation of *CACT* by fasting and WY-14,643 in the small intestine is unknown, these observations suggest that *PPARα*-independent regulation of *CACT* by fasting is unique to the small intestine and that fasting-induced regulation of *CACT* in the liver, the central organ mediating the metabolic response to fasting, is different from that in the small intestine.

Upregulation of *CACT* and L-CPT I could be also observed in two liver cell lines, human HepG2 cells and rat Fao cells, treated with WY-14,643. These findings from cell culture experiments concur well with the results from our mice experiments, and, thus, provide a further indication that *CACT* is regulated by *PPARα* in liver cells. The comparable extent of upregulation of *CACT* and L-CPT I by WY-14,643 in liver cells suggests that both genes are similar responsive to *PPARα* activation.

Our cell culture experiments further revealed that *CACT* is also significantly induced by the selective PPARδ agonist GW0742 but not by the selective PPARγ agonist TGZ. These findings indicate that regulation of *CACT* expression by PPARs in liver cells is not only restricted to the *PPARα* isotype. This however is not surprising since it is well established that several PPAR-regulated genes are responsive to different PPAR isotypes; e.g. CPT I is well known to be upregulated by *PPARα* and PPARδ agonists and slightly by PPARγ agonists [15,16]. Furthermore, albeit expression of the *PPARα* isotype is predominating in HepG2 and Fao cells, as evidenced in the present study, both liver cell lines also express other PPAR isotypes in significant amounts, which is a prerequisite for the mediation of PPAR-regulated gene transcription. Upregulation of *CACT* by different PPAR isotype-specific agonists such as WY-14,643 and GW0742 is also comprehensible considering that the different PPAR isotypes have partially overlapping functions, e.g. the PPARδ isotype also plays an important role in the regulation of fatty acid oxidation during fasting, especially in skeletal muscle, where PPARδ expression is increased during fasting leading to the upregulation of genes involved in fatty acid oxidation [29]. Therefore, the marked upregulation of *CACT* in response to the PPARδ-selective agonist GW0742 may reflect the important role of PPARδ for fatty acid catabolism. Whether or not PPARδ is also involved in the regulation of *CACT in vivo*, however, deserves further studies. In contrast, the lack of response of *CACT* to TGZ probably reflects the lower importance of PPARγ, which primarily triggers the expression of genes responsible for adipogenesis [30] and triglyceride storage in adipose cells [31], for fatty acid catabolism.

Transcriptional regulation by PPAR is mediated by binding of activated PPAR/retinoid X receptor heterodimers to specific DNA sequences, called peroxisome proliferator response elements (PPRE) present in and around (i.e. 5′-UTR, first intron) the promoter of PPAR target genes [32,33]. Whereas functional PPRE have been documented for mouse CYP4A10 and mouse CPT I [34,35], these have not yet been described for *CACT*. In silico analysis of mouse *CACT* promoter, however, revealed four putative PPRE at positions −808 to −796, −580 to −568, −332 to −324, and +45 to +57 relative to the transcription start site with high similarity to the consensus PPRE (AGGTCAAAGGTCA) indicating that mouse *CACT* is directly regulated by PPAR. To determine whether these putative PPRE are functional, we tested the responsiveness of different mouse *CACT* promoter truncation constructs containing different size fragments of mouse *CACT* promoter with either 4, 3, 2 or 1 PPRE to WY-14,643, TGZ, and GW0742. These experiments revealed that the mouse *CACT* promoter is only activated by WY-14,643 and GW0742, but not TGZ, which is in good accordance with the effects of the PPAR ligands on *CACT* mRNA abundance. The finding that the magnitude of induction of *CACT* promoter activity by WY-14,643 and GW0742 between the different *CACT* promoter truncation constructs was almost identical indicates that the PPRE at +45 to +57 is functional. To confirm that this PPRE is actually the functional PPRE, we generated *CACT* promoter mutant constructs that contained a targeted mutation within the PPRE at +45 to +57. As a consequence, induction of promoter activity by WY-14,643 and GW0742 in HepG2 cells transfected with these mutant constructs was completely abolished indicating that this PPRE is functional in mouse *CACT*. In addition, alignment of this PPRE sequence revealed that it is extremely well conserved between human, mouse and rat *CACT* 5′-regulatory region, which provides a further indication that this sequence is important for transcriptional regulation of *CACT*.

To determine whether this *CACT*-PPRE binds the *PPARα*/RXRα heterodimer *in vitro*, we performed a gel shift assay. In the presence of nuclear extract containing *PPARα* and RXRα, a strong band appeared representing the DNA-*PPARα*/RXRα complex which disappeared in the presence of an excess of unlabelled specific oligonucleotide. No band for the DNA-*PPARα*/RXRα complex was observed when a mutated *CACT*-PPRE was used. These findings indicate that the *PPARα*/RXRα heterodimer binds specifically to the PPRE at +45 to +57.

In conclusion, the present study shows for the first time that *CACT* promoter activity and transcription are induced by *PPARα* and PPARδ in liver cells. Activation of *CACT* promoter by *PPARα* and PPARδ agonists is probably mediated by binding of activated PPAR to a functional PPRE located at +45 to +57 in the 5′-UTR of mouse *CACT*. Future studies are necessary to demonstrate whether upregulation of *CACT* by *PPARα* and PPARδ, which are both important for the regulation of fatty acid oxidation in tissues, may increase the import of acylcarnitine into the mitochondrial matrix during fasting.

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PPARα agonists up-regulate organic cation transporters in rat liver cells

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Abstract

It has been shown that clofibrate treatment increases the carnitine concentration in the liver of rats. However, the molecular mechanism is still unknown. In this study, we observed for the first time that treatment of rats with the peroxisome proliferator activated receptor (PPAR)-α agonist clofibrate increases hepatic mRNA concentrations of organic cation transporters (OCTNs)-1 and -2 which act as transporters of carnitine into the cell. In rat hepatoma (Fao) cells, treatment with WY-14,643 also increased the mRNA concentration of OCTN-2. mRNA concentrations of enzymes involved in carnitine biosynthesis were not altered by treatment with the PPARα agonists in livers of rats and in Fao cells. We conclude that PPARα agonists increase carnitine concentrations in livers of rats and cells by an increased uptake of carnitine into the cell but not by an increased carnitine biosynthesis.

Keywords: Carnitine; Peroxisome proliferator activated receptor α; Rat; Organic cation transporter

Carnitine (L-3-hydroxy-4-*N*-*N*-*N*-trimethylaminobutyrate) is an essential metabolite, which has a number of indispensable functions in intermediary metabolism. The most prominent function lies in its role in the transport of activated long-chain fatty acids from the cytosol to the mitochondrial matrix where β-oxidation takes place. Other functions of carnitine include the transfer of products of peroxisomal β-oxidation to the mitochondria for oxidation in the citrate cycle, the modulation of the acyl-CoA/CoA-ratio, and the storage of energy as acetylcarnitine [1–3].

All tissues that use fatty acids as a fuel source require carnitine for normal function. Carnitine is derived from dietary sources and endogenous biosynthesis [4]. Carnitine biosynthesis involves a complex series of reactions involving several tissues [5]. Lysine provides the carbon backbone of carnitine. Lysine in protein peptide linkages undergoes methylation of the ε-amino group to yield trimethyllysine (TML), which is released upon protein degradation. Muscle is the major source of TML. The released TML is further oxidized to butyrobetaine by the action of trimethyllysine dioxygenase (TMLD), 3-hydroxy-*N*-TML aldolase, and 4-*N*-trimethylaminobutyraldehyde dehydrogenase (TMABA-DH). Butyrobetaine is hydroxylated by γ-butyrobetaine dioxygenase (BBD) to form carnitine. The last reaction which is rate-limiting for carnitine synthesis occurs primarily in liver and kidney [6].

Distribution within the body and intracellular homeostasis of carnitine are controlled by membrane transporters. The organic cation transporters (OCTNs), in particular OCTN-2, physiologically the most important one, operate on the intestinal absorption and renal reabsorption of carnitine and play a major role in tissue distribution by catalyzing the uptake of carnitine into body cells. In most tissues, carnitine concentrations are much higher than in plasma, and the high tissue-to-plasma concentrations (up to about 100:1 in muscle) are maintained by carnitine transporters [7]. The fact that inborn or acquired defects of OCTNs lead to primary or secondary systemic carnitine deficiency demonstrates their essential role in carnitine homeostasis [8].

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It has been shown that treatment of rats with clofibrate increases the concentration of carnitine in the liver [9], and it was suggested that this effect is caused by increased hepatic carnitine synthesis due to an increased availability of TML from muscle [10]. Clofibrate belongs to a group of hypolipidemic compounds which exert their effects by activation of peroxisome proliferator activated receptor (PPAR)-α, a transcription factor belonging to the nuclear hormone receptor superfamily [11]. Activation of PPARα causes an up-regulation of carnitine palmitoyltransferase (CPT)-1 and CPT-2 in the liver to enhance β-oxidation of fatty acids [12,13]. Since carnitine is a cofactor of these enzymes, activation of PPARα should increase the need for carnitine which could be met either by an increased de novo carnitine synthesis in the liver or by an increased uptake of carnitine from blood into the liver by membrane carnitine transporters, OCTN-1 and OCTN-2 [14]. The possibility that the clofibrate induced increase in hepatic carnitine concentration could have been mediated by an activation of PPARα has not yet been investigated. We hypothesized that activation of PPARα causes either up-regulation of enzymes involved in hepatic carnitine biosynthesis or increases carnitine uptake into liver cells by an up-regulation of OCTNs. The hypothesis that activation of PPARα could be involved in clofibrate induced increase of hepatic carnitine concentration is supported by the finding that hepatic carnitine concentration is also increased during starvation [15,16], a state in which PPARα is activated by increased hepatic concentrations of unesterified fatty acids [17]. In order to investigate this hypothesis, we performed experiments with rats and Fao rat hepatoma cells. In the first experiment, we treated rats with the synthetic PPARα agonist clofibrate and determined hepatic mRNA concentrations of the carnitine transporters OCTN-1 and -2, and enzymes involved in carnitine biosynthesis (TMLD, TMABA-DH, BBD). In order to explore whether effects of PPARα agonists on carnitine concentration are dependent on other tissues (e.g., muscle which provides TML for hepatic carnitine biosynthesis) or not, the second experiment was performed with rat hepatoma Fao cells which were treated with WY-14,643, another synthetic PPARα agonist.

Materials and methods

Animal experiment. Male Sprague–Dawley rats, with an average initial body weight of 366 g (±28; SD), were randomly assigned to two groups ($n = 8$) and kept individually in Macrolon cages in a room controlled for temperature (22 ± 2 °C), relative humidity (50–60%), and light (12 h light/dark cycle). All experimental procedures described followed established guidelines for the care and handling of laboratory animals and were approved by the council of Saxony-Anhalt. The animals were treated with 250 mg/kg of clofibrate (Fluka Chemie GmbH, Buchs, Switzerland) in 1 mL sunflower oil or with an equal volume of the vehicle sunflower oil by gavage once a day 2 h after beginning of the light cycle. All rats were fed a commercial standard basal diet (''altromin 1324'', Altromin GmbH, Lage, Germany). To standardize food intake, the diets were fed daily in restricted amounts of 18 g per day. Water was available *ad libitum* from nipple drinkers during the whole experiment. At day 4 of treatment, animals received the last dose of clofibrate or vehicle alone and 9 g of the diet and were killed 4 h later by decapitation under light anesthesia with diethyl ether. Blood was collected into heparinized polyethylene tubes. Liver and gastrocnemius muscles were quickly removed, frozen with liquid nitrogen, and stored at −80 °C pending further analysis. Plasma was obtained by centrifugation of the blood (1100*g*, 10 min, 4 °C) and stored at −20 °C.

Cell culture experiment. Fao rat hepatoma cells (ECACC, Salisbury, UK) were cultured in Ham-F12 medium supplemented with 10% FCS and 0.05 mg/mL gentamycin (Invitrogen, Karlsruhe, Germany). Cells were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. For experiments, Fao cells were seeded in 6-well culture plates at a density of 1.06×10^6 cells per well and used prior reaching confluence (usually 3 days after seeding). The cells were then stimulated for 6 and 20 h with 50 μM WY-14,643 (Sigma–Aldrich, Steinheim, Germany). WY-14,643 was added to the medium from a stock solution in DMSO. Final DMSO concentration did not exceed 0.1% (v/v). Cells treated with the appropriate vehicle concentration were used as a control. Cell viability after treatment with WY-14,643 was assessed by (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (MTT) [18]. Cell viability of Fao cells was not reduced by treatment with WY-14,643 (data not shown).

Carnitine analysis. Concentrations of free carnitine and acetyl carnitine in rat liver, muscle, plasma, and Fao cells were analyzed using tandem mass spectrometry according to Vernez et al. [19]. Quantitative analysis was achieved by use of stable isotope-labeled internal standard carnitine-d_3 (Larodan Fine Chemicals, Malmö, Sweden). Plasma or cells (50 μL) were added with methanol containing the internal standard; freeze-dried liver and muscle tissues (50 mg) were added with a water:methanol mixture (2:1, v/v) containing the internal standard. For extraction, the specimen was first sonified in an ultrasound bath for 20 min, then shaken in a water bath for 30 min at a temperature of 50 °C and finally centrifuged at 13,000*g* for 10 min at 4 °C. The supernatant was used for further analysis. A 1100-er series HPLC (Agilent Technologies, Waldbronn, Germany) equipped with a Kromasil 100 column (5 μm particle size, 125 mm length, 2 mm internal diameter, CS-Chromatographie Service, Langerwehe, Germany) and an API 2000 LC-MS/MS-System (Applied Biosystems, Darmstadt, Germany) were used for quantification of free carnitine and acetyl carnitine. For detection, the analytes were ionized by positive ion (5500 V) electrospray. As eluents, methanol and a methanol:water:acetonitrile mixture (50:45:5) were used.

RT-PCR analysis. Total RNA was isolated from Fao cells and rat livers, respectively, by TRIZOL™ reagent (Sigma–Aldrich, Steinheim, Germany) according to the manufacturer's protocol. cDNA synthesis was carried out as described [20]. The mRNA concentration of genes was measured by realtime detection PCR using SYBR® Green I and a MJ Research Opticon system (Biozym Diagnostik GmbH, Oldendorf, Germany). Realtime detection PCR was performed with 1.25 U Taq DNA polymerase (Promega, Mannheim, Germany), 500 μM dNTPs and 26.7 pmol of the specific primers (Operon Biotechnologies, Cologne, Germany; Table 1). Annealing temperature for all primers was 60 °C. Amplification efficiencies for all primer pairs were determined by template dilution series. Calculation of the relative mRNA concentration was made using the amplification efficiencies and the C_t values [21]. The housekeeping gene β-actin was used for normalization.

Statistical analysis. Means of treatments and control were compared by Student's t test using the Minitab Statistical Software (Minitab, State College, PA, USA). Differences with $P < 0.05$ were considered to be significant.

Results

Carnitine concentrations in rat liver, plasma, and muscle and in Fao cells

Rats treated with clofibrate had a higher concentration of free carnitine in the liver than control rats ($P < 0.05$,

Table 1
Characteristics of the specific primers used for RT-PCR analysis

Gene	Forward primer (from 5′ to 3′)	Reverse primer (from 5′ to 3′)	bp	NCBI GenBank
β-Actin	ATCGTGCGTGACATTAAAGAGAAG	GGACAGTGAGGCCAGGATACAG	429	BC063166
CPT-1	GGAGACAGACACCATCCAACATA	AGGTGATGGACTTGTCAAACC	416	NM_031559
CPT-2	TCCTCGATCAAGATGGGAAC	GATCCTTCATCGGGAAGTCA	237	NM_012930
OCTN-1	AGCATTTGTCCTGGGAACAG	ACTCAGGGATGAACCACCAG	200	NM_022270
OCTN-2	CCTCTCTGGCCTGATTGAAG	CTCCGCTGTGAAGACGTACA	226	NM_012930
TMLD	GCCCTGTGGCATTCAAGTAT	GGTCCAACCCCTATCATGTG	201	AF374406
TMABA-DH	TTTGAGACTGAAGCCGAGGT	CACCGGGCTGACGTTATAGT	156	NM_022273
BBD	ATTCTGCAAAAGCTCGGAAA	CTCCTTGGAGTCCTGCTCTG	183	NM_022629

Table 2
Concentrations of total carnitine in liver, plasma, and gastrocnemius muscle of control rats and rats treated with clofibrate

	Control	Clofibrate
Liver		
Free carnitine (nmol/g)	282 ± 39	900 ± 145*
Acetyl carnitine (nmol/g)	12 ± 6	3 ± 1*
Plasma		
Free carnitine (μmol/L)	55 ± 8	28 ± 4*
Acetyl carnitine (μmol/L)	20 ± 4	8 ± 2*
Gastrocnemius		
Free carnitine (nmol/g)	631 ± 71	637 ± 63
Acetyl carnitine (nmol/g)	208 ± 52	144 ± 36*

Values are means ± SD ($n = 8$). An asterisk (*) indicates a significant difference from control rats ($P < 0.05$).

Table 2). The concentration of acetyl carnitine which made up however only a very small percentage of total carnitine was lower in rats treated with clofibrate than in control rats ($P < 0.05$, Table 2). Concentrations of free and acetyl carnitine in plasma were both lower in rats treated with clofibrate than in control rats ($P < 0.05$, Table 2). In gastrocnemius muscle, concentrations of free carnitine did not differ between both groups of rats whereas the concentration of acetyl carnitine was lower in rats treated with clofibrate than in control rats ($P < 0.05$, Table 2).

Fao cells treated with 50 μM WY-14,643 for either 6 or 20 h had higher concentrations of free carnitine than control cells treated with medium alone ($P < 0.05$, Table 3). Moreover, cells treated with WY-14,643 for 20 h had a higher concentration of acetyl carnitine than control cells ($P < 0.05$, Table 3). After 6 h incubation, the concentration of acetyl carnitine did not differ between cells treated with WY-14,643 and control cells (Table 3).

Table 3
Concentrations of free carnitine and acetyl carnitine in Fao cells treated either with vehicle alone (control) or with WY-14,643 for 6 or 20 h

	Control	WY-14,643
Six hours incubation		
Free carnitine (pmol/mg protein)	23 ± 8	33 ± 7*
Acetyl carnitine (pmol/mg protein)	87 ± 20	81 ± 17
Twenty hours incubation		
Free carnitine (pmol/mg protein)	40 ± 7	63 ± 25*
Acetyl carnitine (pmol/mg protein)	78 ± 20	97 ± 30*

Values are means ± SD ($n = 3$). An asterisk (*) indicates a significant difference from control cells ($P < 0.05$).

Relative mRNA concentrations of CPTs, OCTNs, and enzymes involved in carnitine biosynthesis in rat liver and Fao cells

Rats treated with clofibrate had higher relative mRNA concentrations of CPT-1, CPT-2, OCTN-1, and OCTN-2 in the liver than control rats ($P < 0.05$) whereas relative mRNA concentrations of genes encoding enzymes of hepatic carnitine synthesis (TMLD, TMABA-DH, BBD) did not differ between both groups of rats (Fig. 1).

Fao cells treated with 50 μM WY-14,643 for either 6 or 20 h had also higher relative mRNA concentrations of CPT-1, CPT-2, and OCTN-2 than control cells ($P < 0.05$, Fig. 2). The extent of up-regulation of expression of these genes by WY-14,643 was similar in cells treated for 6 h and those treated for 20 h, indicating that a period of 6 h was already sufficient for maximum up-regulation. Relative mRNA concentrations of OCTN-1 and of genes encoding enzymes of hepatic carnitine synthesis (TMLD, TMABA-DH, BBD) did not differ

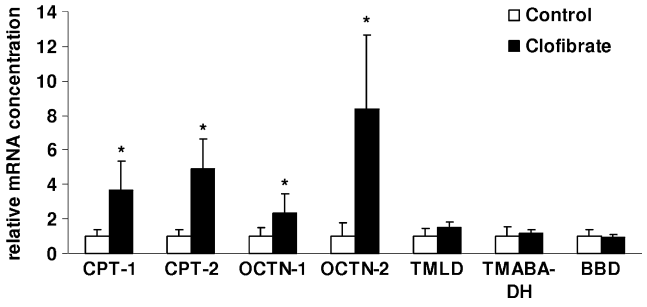

Fig. 1. Effect of clofibrate on the relative mRNA concentrations of carnitine palmitoyltransferases (CPT-1, CPT-2), organic cation transporters (OCTN-1, OCTN-2), and enzymes of carnitine biosynthesis (trimethyllysine dioxygenase, TMLD; 4-*N*-trimethylaminobutyraldehyde dehydrogenase, TMABA-DH; γ-butyrobetaine dioxygenase, BBD) in the liver of rats. Rats were treated orally with 250 mg/kg of clofibrate for 4 days. Control animals obtained the appropriate volume of the vehicle sunflower oil. Total RNA was extracted from rat livers and mRNA concentrations were determined by realtime detection RT-PCR analysis using β-actin mRNA concentration for normalization. Values are means ± SD ($n = 8$). An asterisk (*) indicates a significant difference from control rats ($P < 0.05$).

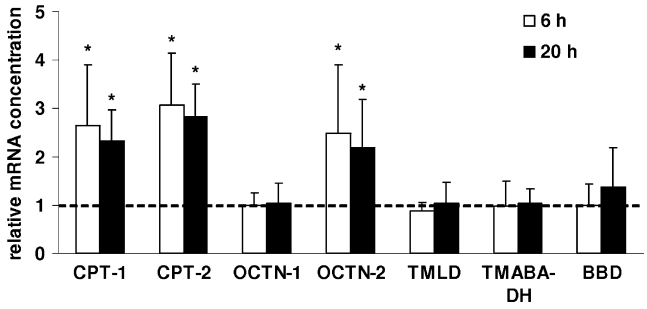

Fig. 2. Effect of WY-14,643 treatment for either 6 or 20 h on the relative mRNA concentrations of carnitine palmitoyltransferases (CPT-1, CPT-2), organic cation transporters (OCTN-1, OCTN-2), and enzymes of carnitine biosynthesis (trimethyllysine dioxygenase, TMLD; 4-*N*-trimethylaminobutyraldehyde dehydrogenase, TMABA-DH; γ-butyrobetaine dioxygenase, BBD) in Fao cells. Fao cells were grown in culture medium until subconfluent state and were then incubated with 50 μM of the PPARα agonist WY-14,643 for 6 and 20 h, respectively. Control cells were incubated with low-serum medium containing vehicle alone. Total RNA was extracted from cells and mRNA concentrations were determined by realtime detection RT-PCR analysis using β-actin mRNA concentration for normalization. mRNA concentrations of the genes in the treated cells are shown relative to control cells (=1.00; dotted line) treated for 6 and 20 h, respectively. An asterisk (*) indicates a significant difference from control cells ($P < 0.05$).

between cells treated with WY-14,643 and control cells (Fig. 2).

Discussion

In this study, rats and Fao cells were treated with the PPARα agonists clofibrate and WY-14,643, respectively. CPT-1 and CPT-2 have been identified as PPARα target genes [22–24], and the observation that expression of these genes was strongly up-regulated in the liver of rats and in Fao cells by treatment with the agonists indicated PPARα activation. The rat study confirms the previous observation that clofibrate treatment increases the concentration of carnitine in the liver of rats [9,10]. The novel finding of this study is that clofibrate treatment caused a strong up-regulation of OCTN-2 (8.4-fold) and a less strong up-regulation of OCTN-1 (2.4-fold) whereas mRNA concentrations of genes encoding enzymes of hepatic carnitine biosynthesis remained unchanged. These observations strongly indicate that the increased carnitine concentration in the liver of rats treated with clofibrate was rather due to an increased uptake of carnitine from the blood into the liver than to an increased synthesis of carnitine. This indication accords with the observation that carnitine concentrations in plasma were reduced by clofibrate treatment, probably due to an increased uptake into cells. More than 95% of the total carnitine in the body is localized in the muscle which serves as a carnitine storage. When plasma carnitine concentrations are lowered such as by treatment with pivalate, carnitine can be mobilized from the muscle in order to normalize plasma carnitine concentrations [25]. The finding that the concentration of acetyl carnitine, the storage form of carnitine, was reduced in gastrocnemius of rats treated with clofibrate indeed indicates that carnitine might have been mobilized from muscle.

The in vitro study in which Fao cells were treated with WY-14,643 confirms most of the observations of the rat study. It is shown that treatment of cells with WY-14,643 increased gene expression of OCTN-2 and increased the intracellular concentration of free carnitine whereas gene expression of enzymes of hepatic carnitine biosynthesis was not altered, too. The only disagreement between the cell and the rat study was that expression of OCTN-1 was not up-regulated by the PPARα agonist in Fao cells. This may have two reasons: first, the effect of WY-14,643 in Fao cells was generally weaker than the effect of clofibrate in rats on the respective parameters; second, OCTN-1 was generally less responsive to PPARα agonists than OCTN-2. As gene expression of OCTN-1 remained unchanged in Fao cells treated with WY-14,643, it can be concluded that the increased concentration of free carnitine was exclusively the result of an increased carnitine uptake into the cell by OCTN-2.

The cell culture study disproves the hypothesis that the increase of the carnitine concentration in livers of rats treated with clofibrate is caused by an increased hepatic carnitine synthesis due to an increased availability of TML derived from muscle [10]. Carnitine concentration in Fao cells was increased by WY-14,643 although concentrations of TML in the media of treated and control cells were identical. This means that carnitine concentrations in Fao cells were increased by WY-14,643 independent of the availability of TML from muscle or other tissues.

The observation that OCTN-2 expression was up-regulated and that carnitine concentration was increased in liver and cells treated with two different PPARα agonists indicates that these effects were caused by PPARα activation. This indication provides also an explanation for the observation of increased hepatic carnitine concentrations in fasted rats [15,16]. During fasting, non-esterified fatty acids are liberated from adipose tissue and act as activators of PPARα when they have entered the liver. Activation of PPARα up-regulates many genes involved in hepatic mitochondrial and peroxisomal β-oxidation of fatty acids to supply acetyl-CoA used for the generation of ATP via citrate cycle and for the generation of ketone bodies, an important fuel for the brain during fasting [17,26]. These metabolic adaptations during fasting triggered by PPARα aim to minimize the use of protein and carbohydrates as fuel and allow mammals to survive long periods of energy deprivation. CPTs are rate-limiting for β-oxidation of fatty acids [22,24]. The up-regulation of CPTs, which is essential for the metabolic adaptations occurring during fasting, might increase the demand of carnitine in liver cells. We postulate that up-regulation of OCTNs by PPARα activation is a means to supply liver cells with sufficient carnitine required for transport of excessive amounts of fatty acids into the mitochondrion, and therefore plays an important role in the adaptive response of liver metabolism to fasting.

In conclusion, the present study shows for the first time that treatment of rats and rat Fao hepatoma cells with PPARα agonists clofibrate and WY-14,643, respectively, causes an up-regulation of OCTNs whereas expression of genes encoding enzymes involved in hepatic carnitine biosynthesis remain unchanged. Up-regulation of OCTNs may enhance carnitine uptake from blood or medium, respectively, into liver cells and this may be the reason for the increased carnitine concentrations in livers of rats treated with clofibrate.

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PPARα mediates transcriptional upregulation of novel organic cation transporters-2 and -3 and enzymes involved in hepatic carnitine synthesis.

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PPARα Mediates Transcriptional Upregulation of Novel Organic Cation Transporters-2 and -3 and Enzymes Involved in Hepatic Carnitine Synthesis

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We tested the hypothesis that transcription of novel organic cation transporters (OCTNs) is directly regulated by peroxisome proliferator–activated receptor (PPAR)-α. Therefore, wild-type mice and mice deficient in PPARα (PPARα$^{-/-}$) were treated with the PPARα agonist WY 14,643. Wild-type mice treated with WY 14,643 had a greater abundance of OCTN2 mRNA in their liver, muscle, kidney, and small intestine and a greater abundance of OCTN3 mRNA in kidney and small intestine than did untreated wild-type mice ($P < 0.05$). Moreover, wild-type mice treated with WY 14,643 had greater mRNA abundances of enzymes involved in hepatic carnitine synthesis (4-N-trimethylaminobutyraldehyde dehydrogenase, γ-butyrobetaine dioxygenase) and increased carnitine concentrations in liver and muscle than did untreated wild-type mice ($P < 0.05$). Untreated PPARα$^{-/-}$ mice had a lower abundance of OCTN2 mRNA in liver, kidney, and small intestine and lower carnitine concentrations in plasma, liver, and kidney than did untreated wild-type mice ($P < 0.05$). In PPARα$^{-/-}$ mice, treatment with WY 14,643 did not influence mRNA abundance of OCTN2 and OCTN3 and carnitine concentrations in all tissues analyzed. The abundance of OCTN1 mRNA in all the tissues analyzed was not changed by treatment with WY 14,643 in wild-type or PPARα$^{-/-}$ mice. In conclusion, this study shows that transcriptional upregulation of OCTN2 and OCTN3 in tissues and of enzymes involved in hepatic carnitine biosynthesis are mediated by PPARα. It also shows that PPARα mediates changes of whole-body carnitine homeostasis in mice by upregulation of carnitine transporters and enzymes involved in carnitine synthesis. Exp Biol Med 233:356–365, 2008

Key words: peroxisome proliferator–activated receptor (PPAR)-α; carnitine; novel organic cation transporter (OCTN); mice

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Introduction

Carnitine (L-3-hydroxy-4-N-N-N-trimethylaminobutyrate) is an essential metabolite, which has a number of indispensable functions in intermediary metabolism (1–4). Carnitine is derived from dietary sources and endogenous biosynthesis (4, 5). Carnitine biosynthesis involves a complex series of reactions (6). Lysine provides the carbon backbone of carnitine. Lysine in protein peptide linkages undergoes methylation of the ε-amino group to yield trimethyllysine (TML), which is released upon protein degradation. The released TML is further oxidized to butyrobetaine by the action of trimethyllysine dioxygenase (TMLD), 3-hydroxy-N-trimethyllysine aldolase and 4-N-trimethylaminobutyraldehyde dehydrogenase (TMABA-DH). γ-Butyrobetaine (BB) is hydroxylated by γ-butyrobetaine dioxygenase (BBD) to form carnitine. In mice, considerable activity of that enzyme is found only in the liver (7). From tissues that lack BBD, BB is excreted and transported via the circulation to the liver, where it is converted into carnitine (6).

Distribution of carnitine within the body and intracellular homeostasis of carnitine are controlled by novel organic cation transporters (OCTNs) that belong to the solute carrier 22A family, localized on the apical membrane of cells (8, 9). Three OCTNs have been identified so far: OCTN1, OCTN2, and OCTN3 (10–12). OCTNs are polyspecific; they transport several cations and L-carnitine (13, 14). OCTN1 and OCTN2 are expressed in several tissues such as kidney, intestine, skeletal muscle, heart, liver, and brain (12, 15, 16). In contrast, OCTN3 is expressed exclusively in testes, kidney, and intestine (12, 17). Among the three OCTNs, OCTN3 has the highest specificity for carnitine, and OCTN1 has the lowest (12). OCTNs operate on the intestinal absorption and renal reabsorption of carnitine and play a major role in tissue distribution by carrying carnitine into body cells. Because of its high binding affinity for carnitine and its wide expression, OCTN2 is the most important carnitine transporter. OCTN1 contributes less to carnitine

transport than OCTN2 because of its low carnitine transport activity. OCTN3 may be important for carnitine uptake into testes and may contribute to reabsorption of carnitine in kidney (12). The fact that inborn or acquired defects of OCTNs lead to primary or secondary systemic carnitine deficiency demonstrates their essential role in carnitine homeostasis (9).

We have shown that treatment of rats or rat Fao hepatoma cells with clofibrate or WY 14,643, respectively, both synthetic agonists of peroxisome proliferator–activated receptor (PPAR)-α, causes an upregulation of OCTN2 and an increase in carnitine concentration in liver cells (18). We also have found that feeding oxidized fats to rats causes an upregulation of OCTN1 and OCTN2 and increases carnitine concentrations in the liver (19). As dietary oxidized fats are also able to activate PPARα in the liver (20–23), we hypothesize that transcriptional upregulation of OCTNs is mediated by PPARα, a transcription factor belonging to the nuclear hormone receptor superfamily (24). The present study aims to test this hypothesis. Therefore, we performed an experiment with mice deficient in PPARα (PPARα$^{-/-}$ mice) that were treated as wild-type mice with WY 14,643 and determined OCTN1 and OCTN2 mRNA abundance in the liver, skeletal muscle, kidney, small intestine, and testes. In the kidney, testes, and small intestine, we also analyzed OCTN3 mRNA abundance. To show the consequences of an alteration in OCTN gene expression on carnitine homeostasis, we also determined carnitine concentrations in these tissues. To elucidate whether the increased hepatic carnitine concentration observed in rats treated with PPARα agonists (18, 19, 25) could be due to increased carnitine biosynthesis in the liver, we also considered the mRNA abundance of enzymes involved in carnitine biosynthesis in the liver and tissue concentrations of TML and BB, which are precursors for carnitine synthesis in the liver.

Material and Methods

Materials. WY 14,643, dimethylsulfoxide (DMSO), TRIZOL reagent and SYBR Green I were purchased from Sigma-Aldrich (Steinheim, Germany). Reverse transcriptase was supplied by MBI Fermentas (St. Leon-Rot, Germany) and Taq polymerase by Promega (Mannheim, Germany). All primers were purchased from Operon Biotechnologies (Cologne, Germany).

Animals, Diets, and Sample Collection. For all experiments, we used male PPARα$^{-/-}$ mice (129S4/SvJae-*Ppara*^{tm1Gonz}/J) and corresponding wild-type control mice (129S1/SvImJ) purchased from the Jackson Laboratory (Bar Harbor, ME, USA). The mice were 11–12 weeks old with an average initial body weight (±SD) of 24.3 ± 3.2 g. Mice of each genotype were randomly assigned to two groups and kept individually in Macrolon cages in a room with controlled temperature (22°C ± 2°C), relative humidity (50%–60%), and light (12:12-hr light:dark cycle). All experimental procedures described followed established guidelines for the care and handling of laboratory animals and were approved by the council of Saxony-Anhalt. Mice in the treatment groups (wild-type mice, $n = 8$; PPARα$^{-/-}$ mice, $n = 8$) received 40 mg/kg WY 14,643 once daily 2 hrs after the beginning of the light cycle for 4 days. WY 14,643 was dissolved in DMSO and sunflower oil (50:50, v/v) at a final concentration of 8 mg/ml as described (26). The daily dose of WY 14,643 (in 0.12 ml) was given by gavage. Control animals (wild-type mice, $n = 8$; PPARα$^{-/-}$ mice, $n = 8$) were given the appropriate volume of the vehicle (DMSO and sunflower oil). All mice were fed a commercial, standard basal diet ("altromin 1324," Altromin GmbH, Lage, Germany) with a low carnitine concentration of 22 μmol/kg. According to the declaration of the manufacturer, each kilogram of this feed contains 11.9 MJ metabolizable energy (ME), 190 g crude protein, 60 g crude fiber, 40 g crude fat, and 70 g crude ash. To standardize food intake, the mice were fed restricted amounts (4 g daily). The daily intake of ME derived from the diet and oil was 49.6 kJ. This energy intake is about 20% more than the ME requirement for maintenance, which is approximately 41 kJ ME per day (27). Water was available *ad libitum* from nipple drinkers during the entire experiment. On day 4 of treatment, mice received the last dose of WY 14,643 or vehicle alone and 1 g of the diet and were killed 4 hrs later by decapitation under light anesthesia with diethyl ether. Blood was collected into ethylenediaminetetraacetic acid–containing tubes, and plasma was obtained by centrifugation (1100 *g*, 10 mins, 4°C) and stored at –20°C. Samples of liver, kidney, skeletal muscle, and testes for RNA isolation and for determination of carnitine concentration were snap-frozen in liquid nitrogen and stored at –80°C. The small intestine was rapidly excised and washed with 0.9% NaCl (w/v), and mucosal scrapings were obtained, snap-frozen, and stored at –80°C.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) Analysis. Total RNA was isolated from tissues by using TRIZOL reagent according to the manufacturer's protocol. The cDNA synthesis was carried out as described (28). The mRNA expression of genes was measured by real-time detection PCR using SYBR Green I and the Rotor Gene 2000 system (Corbett Research, Mortlake, Australia) as described (29). Target genes with characteristics of specific primers used are listed in Table 1. Annealing temperature for all primer pairs was 60°C; the only exception was those for β-actin, and their annealing temperature was 66°C. For determination of mRNA abundance, a threshold cycle (C_t) and amplification efficiency were obtained from each amplification curve by using the software RotorGene 4.6 (Corbett Research). Calculation of relative mRNA abundance was made by using the $\Delta\Delta C_t$ method as previously described (30). The housekeeping gene β-actin was used for normalization. The abundance of β-actin mRNA was not influenced by the treatment of mice with WY 14,643.

Carnitine Analysis. Concentrations of free carnitine,

Table 1. Characteristics of the Specific Primers Used for RT-PCR Analysis

Gene	Forward and reverse primers (5′ to 3′)	bp	NCBI GenBank
ACO	CAGGAAGAGCAAGGAAGTGG CCTTTCTGGCTGATCCCATA	189	NM_015729
β-actin	ACGGCCAGGTCATCACTATTG CACAGGATTCCATACCCAAGAAG	87	NM_007393
BBD	CCTAAAGGCAGAAGCAGTGG TCCGGTCAAATGTCAAATCA	200	BC019406
OCTN1	CCTGTTCTGTGTTCCCCTGT GGTTATGGTGGCAATGTTCC	232	AB016257
OCTN2	ACAGTATCCCGTTGGAGACG ACACCAGGTCCCACTCTGTC	213	AF110417
OCTN3	CTGGTGGTTCATCCCTGAGT CATAAATGTGGTGCGACTGG	188	NM_011396
TMLD	TTGGTGCCATACAACTTCCA CTGGCCAAGTGAAAAAGAGC	245	AY033513
TMABA-DH	AGCTGAAGACGGTGTGTGTG CTAATGACCCAAAGCCTGGA	154	NM_019993

acetyl carnitine, TML, and BB in plasma and tissues were determined by tandem mass spectrometry using deuterated carnitine-d_3 (Larodane Fine Chemicals, Malmö, Sweden) as an internal standard (31). Fifty milligrams of freeze-dried tissues were extracted with 0.5 ml methanol:water (2:1, v/v) by homogenization (Tissue Lyzer, Qiagen, Hilden, Germany), followed by sonification for 20 mins and incubation at 50°C for 30 mins in a shaker. After centrifugation (13,000 *g* for 10 mins) 20 μl of the supernatant were added to 100 μl methanol containing the internal standard, and the two were mixed, incubated for 10 mins, and centrifuged (13,000 *g*) for 10 mins. Plasma samples were handled at 4°C in the same manner as the supernatant after tissue extraction. The final supernatants were used for quantitation of the compounds by a 1100-er series high performance-liquid chromatography (HPLC) (Agilent Technologies, Waldbronn, Germany) equipped with a Kromasil 100 column (125 mm × 2 mm, 5-μm particle size, CS-Chromatographie Service, Langerwehe, Germany) and an API 2000 liquid chromatography-tandem mass spectrometry (LC-MS/MS)-System (Applied Biosystems, Darmstadt, Germany). The analytes were ionized by positive ion (5500 V) electrospray. As eluents, methanol and a methanol:water:acetonitrile:acetic acid mixture (100:90:9:1, v/v/v/v) were used.

Statistical Analysis. Data, including the factors treatment (WY 14,643 vs. control) and genotype (PPARα$^{-/-}$ vs. Wild-type) and the interactions between treatment and genotype, were subjected to analysis of variance (ANOVA) by using the Minitab Statistical Software (Minitab, State College, PA). When variances were heterogeneous, data were transformed into their logarithms before ANOVA. For statistically significant *F* values, individual means of the treatment groups were compared by Tukey's test. Means were considered significantly different at $P < 0.05$.

Results

Body and Liver Weights. Treatment with WY 14,643 did not influence the final body weights of the mice; there was also no effect of genotype and treatment × genotype interaction on final body weights (mean ± SD; $n =$ 8 per group; wild-type control, 25.1 ± 2.7 g; wild-type treated with WY 14,643, 25.2 ± 1.9 g; PPARα$^{-/-}$ control, 23.9 ± 3.1 g; PPARα$^{-/-}$ treated with WY 14,643, 23.2 ± 4.3 g). Liver weights of the mice were influenced by WY 14,643 treatment ($P < 0.05$) and genotype ($P < 0.05$), and there was a significant interaction between both factors ($P <$ 0.05). In PPARα$^{-/-}$ mice, liver weight was not influenced by treatment with WY 14,643; in wild-type mice, treatment with WY 14,643 significantly ($P < 0.05$) increased liver weight: the mean liver weight (±SD) per 100 g body weight was 4.02 ± 0.42 g for the wild-type control group ($n = 8$), 5.63 ± 0.35 g for the wild-type group treated with WY 14,643 ($n = 8$), 3.93 ± 0.22 g for the PPARα$^{-/-}$ control group ($n = 8$), and 3.98 ± 0.42 g for the PPARα$^{-/-}$ group treated with WY 14,643 ($n = 8$).

Abundance of ACO and OCTN mRNA in Tissues. To study the effect of WY 14,643 on the activation of PPARα, we determined the mRNA abundance of ACO, a PPARα target gene, in liver, skeletal muscle, kidney, testes, and small intestine. In the liver, kidney, and small intestine of wild-type mice, treatment with WY 14,643 increased the abundance of ACO mRNA ($P < 0.05$; Fig. 1). Untreated PPARα$^{-/-}$ mice had less ACO mRNA in their liver and small intestine than did untreated wild-type mice ($P < 0.05$); the abundance of ACO mRNA in skeletal muscle, testes, and kidney did not differ between these two groups of mice (Fig. 1). In PPARα$^{-/-}$ mice, treatment with WY 14,643 did not increase ACO mRNA abundance in any of the tissues analyzed (Fig. 1).

In wild-type mice, OCTN2 mRNA was increased by WY 14,643 treatment in the liver, skeletal muscle, kidney, and small intestine, whereas it remained unchanged in testes

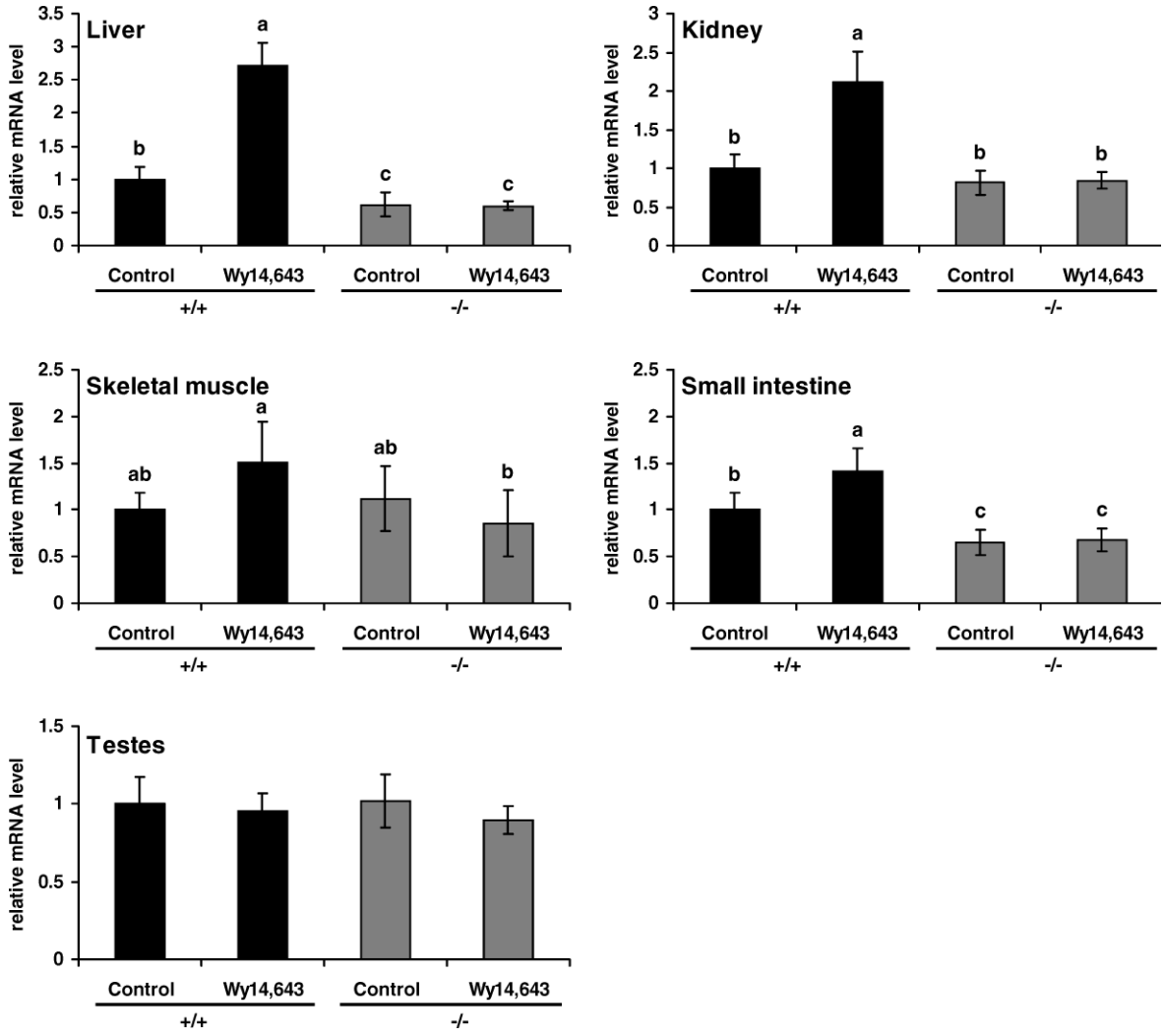

Figure 1. Effect of WY 14,643 on the relative ACO mRNA abundance in the liver, kidney, skeletal muscle, small intestine, and testes of wild-type (+/+) and PPARα$^{-/-}$ mice. Mice were treated orally with 40 mg/kg of WY 14,643 for 4 days. Control mice received the appropriate volume of the vehicle (sunflower oil and DMSO). Total RNA was extracted from tissues, and relative mRNA abundance was determined by real-time detection RT-PCR analysis using β-actin mRNA abundance for normalization. Values are the means ± SD ($n = 8$). Means with unlike letters differ ($P < 0.05$). The significance of factors for particular tissues was the following: liver, for treatment $P < 0.05$,for genotype $P < 0.05$, and for treatment × genotype $P < 0.05$; kidney, for treatment $P < 0.05$, for genotype $P < 0.05$, and for treatment × genotype $P < 0.05$; skeletal muscle, for treatment × genotype $P < 0.05$; small intestine, for treatment $P < 0.05$, for genotype $P < 0.05$, and for treatment × genotype $P < 0.05$. The P values for treatment and genotype in the skeletal muscle and the P values for treatment, genotype, and treatment × genotype in the testes did not reach significance.

(Fig. 2). OCTN2 mRNA in the liver, kidney, and small intestine was less in untreated PPARα$^{-/-}$ mice than in untreated wild-type mice ($P < 0.05$; Fig. 2). In PPARα$^{-/-}$ mice, treatment with WY 14,643 did not increase OCTN2 mRNA abundance in any of the tissues analyzed (Fig. 2).

The abundance of OCTN1 mRNA in the liver, skeletal muscle, kidney, small intestine, and testes was not influenced by WY 14,643 treatment in either genotype (Fig. 3). Moreover, OCTN1 mRNA abundance in all tissues was similar in both genotypes (Fig. 3).

OCTN3 mRNA abundance was determined in the testes, kidney, and small intestine. The abundance of OCTN3 mRNA in the kidney and testes did not differ between untreated wild-type and untreated PPARα$^{-/-}$ mice (Fig. 4). In contrast, the abundance of OCTN3 mRNA in the small intestine was less in untreated PPARα$^{-/-}$ mice than in untreated wild-type mice ($P < 0.05$; Fig. 4). In wild-type mice, expression of OCTN3 in the kidney and small intestine was increased by WY 14,643 ($P < 0.05$), whereas it remained unchanged in PPARα$^{-/-}$ mice (Fig. 4). Expression of OCTN3 in the testes was not altered by WY 14,643 treatment in either genotype (Fig. 4).

mRNA Abundance of Hepatic Enzymes Involved in Carnitine Biosynthesis. Untreated wild-type mice had more TMABA-DH mRNA in their liver than did untreated PPARα$^{-/-}$ mice ($P < 0.05$); the abundance of TMLD and BBD mRNAs did not differ between these two groups of mice (Fig. 5). In wild-type mice, treatment with

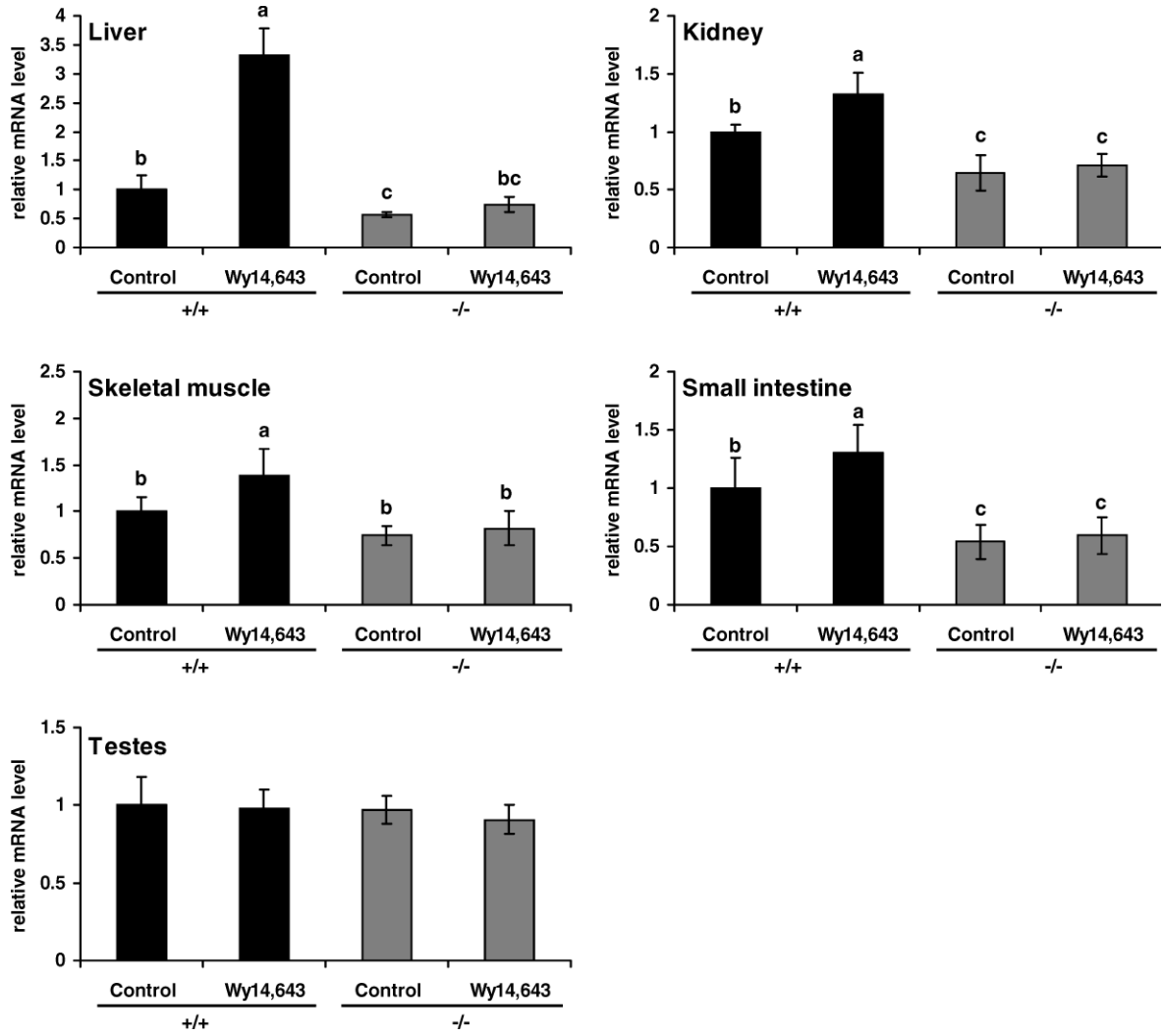

Figure 2. Effect of WY 14,643 on OCTN2 mRNA in the liver, kidney, skeletal muscle, small intestine, and testes of wild-type (+/+) and PPAR$\alpha^{-/-}$ mice. Mice were treated orally with 40 mg/kg of WY 14,643 for 4 days. Control mice received the appropriate volume of vehicle (sunflower oil and DMSO). Total RNA was extracted from tissues, and relative mRNA abundance was determined by real-time detection RT-PCR analysis using β-actin mRNA abundance for normalization. Values are the means ± SD ($n = 8$). Means with unlike letters differ ($P < 0.05$). The significance of factors for particular tissues was the following: liver, for treatment $P < 0.05$, for genotype $P < 0.05$, and for treatment × genotype $P < 0.05$; kidney, for treatment $P < 0.05$, for genotype $P < 0.05$, and for treatment × genotype $P < 0.05$; skeletal muscle, for treatment $P < 0.05$ and for genotype $P < 0.05$; small intestine, for treatment $P < 0.05$ and for genotype $P < 0.05$. The P values for treatment and for genotype in the testes and for treatment × genotype in the skeletal muscle, small intestine, and testes did not reach significance.

WY 14,643 increased the abundance of TMLD and BBD mRNA in the liver ($P < 0.05$), whereas the abundance of TMABA-DH mRNA remained unchanged (Fig. 5). In contrast, the mRNA abundance of all these enzymes in PPAR$\alpha^{-/-}$ mice was not influenced by WY 14,643 treatment (Fig. 5).

Concentrations of Carnitine, BB, and TML in Plasma and Tissues. Concentrations of carnitine, BB, and TML were determined in plasma, liver, kidney, skeletal muscle, and small intestine but not in testes (sufficient sample was not available from this tissue).

Wild-type mice treated with WY 14,643 had higher concentrations of free carnitine and acetyl carnitine in the liver and a higher concentration of free carnitine in skeletal muscle than did untreated wild-type mice. In the small intestine of wild-type mice, the concentration of free carnitine was increased by WY 14,643 treatment, whereas the concentration of acetyl carnitine was reduced ($P < 0.05$; Table 2). Concentrations of free carnitine and acetyl carnitine in the plasma and kidney of wild-type mice were reduced by WY 14,643 treatment. In untreated PPAR$\alpha^{-/-}$ mice, concentrations of free carnitine in plasma, liver, and kidney and those of acetyl carnitine in the liver and small intestine were lower than those in untreated wild-type mice ($P < 0.05$); concentrations of free carnitine in skeletal muscle and small intestine and concentrations of acetyl carnitine in plasma, kidney, and skeletal muscle were similar in those two groups (Table 2). Moreover, treatment of PPAR$\alpha^{-/-}$ mice with WY 14,643 did not cause any alteration in plasma and tissue carnitine concentrations.

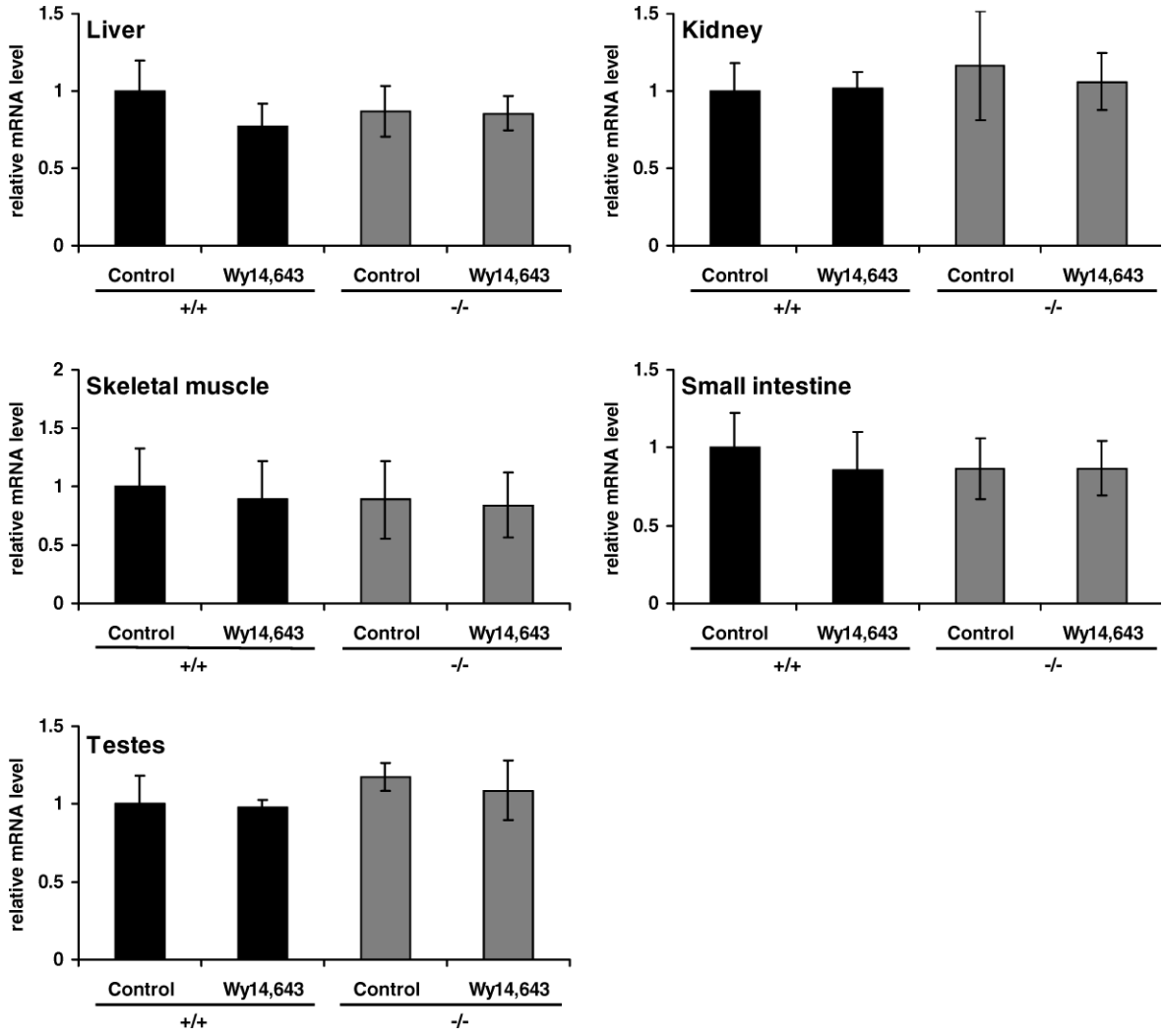

Figure 3. Effect of WY 14,643 on OCTN1 mRNA abundance in the liver, kidney, skeletal muscle, small intestine, and testes of wild-type (+/+) and PPARα$^{-/-}$ mice. Mice were treated orally with 40 mg/kg of WY 14,643 for 4 days. Control mice received the appropriate volume of vehicle (sunflower oil and DMSO). Total RNA was extracted from tissues, and relative mRNA abundance was determined by real-time detection RT-PCR analysis using β-actin mRNA abundance for normalization. Values are the means ± SD ($n = 8$). Means with unlike letters differ ($P < 0.05$). There was no significant effect of treatment, genotype, and treatment × genotype interaction in any tissue.

Wild-type mice treated with WY 14,643 had lower concentrations of BB, the precursor of carnitine, in plasma, liver, and kidney than did untreated wild-type mice ($P < 0.05$; Table 2). In contrast, BB concentrations in skeletal muscle and small intestine did not differ between treated and untreated wild-type mice (Table 2). In PPARα$^{-/-}$ mice, treatment with WY 14,643 did not change plasma and tissue BB concentrations (Table 2). In untreated PPARα$^{-/-}$ mice, concentrations of BB in the liver, kidney, and small intestine were similar to those in untreated wild-type mice; in contrast, BB concentrations in plasma and skeletal muscle were higher in untreated PPARα$^{-/-}$ mice than in untreated wild-type mice ($P < 0.05$; Table 2).

Concentrations of TML in plasma and tissues were not different between wild-type mice and PPARα$^{-/-}$ mice, and they were not influenced by WY 14,643 treatment (Table 2).

Discussion

To investigate the hypothesis that transcription of OCTNs is controlled by PPARα, we treated wild-type and PPARα$^{-/-}$ mice with WY 14,643. To demonstrate PPARα activation, we determined the mRNA abundance of ACO, a gene that possesses a PPAR response element (32). The finding that ACO mRNA was increased in the liver, kidney, and small intestine is therefore indirect proof of PPARα activation in these tissues of wild-type mice treated with WY 14,643. The fact that there was no upregulation of ACO in the testes and skeletal muscle by WY 14,643 may be due to the low expression of PPARα in these tissues (33). ACO mRNA abundance was not influenced in any tissue of PPARα$^{-/-}$ mice by WY 14,643; this result confirms that there was no activation of PPARα because of the lack of expression in those mice. Similarly, hepatomegaly indicative of peroxisome proliferation (34) was observed in wild-

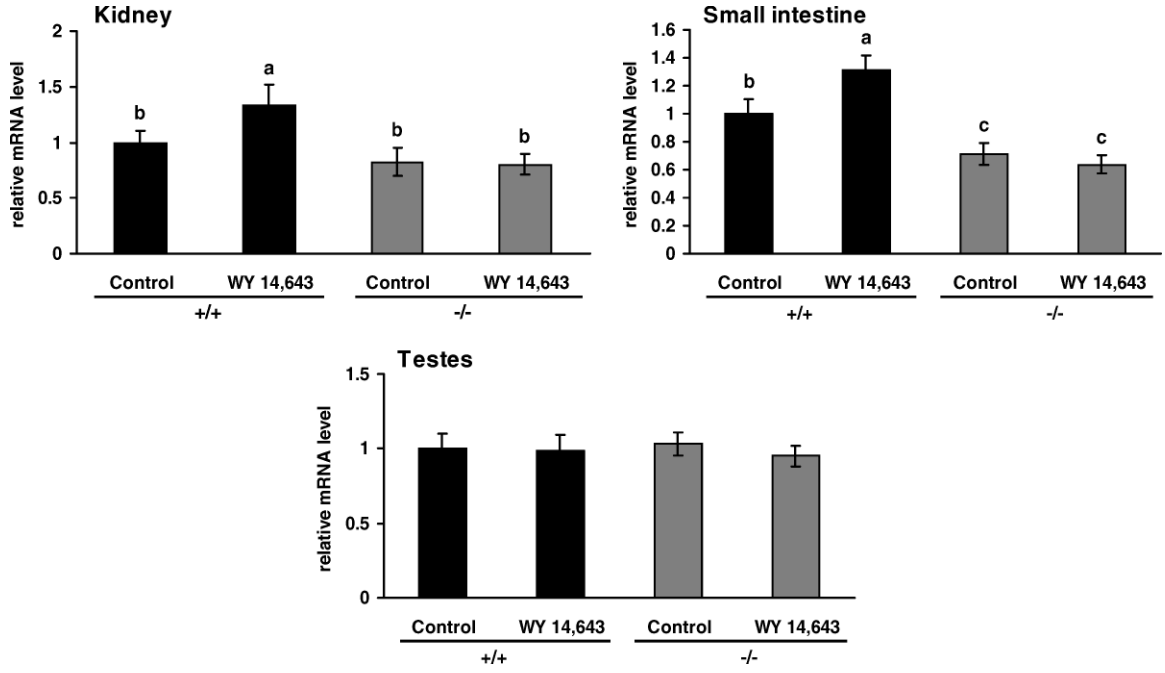

Figure 4. Effect of WY 14,643 on OCTN3 mRNA abundance in the kidney, small intestine, and testes of wild-type (+/+) and PPARα$^{-/-}$ mice. Mice were treated orally with 40 mg/kg of WY 14,643 for 4 days. Control mice received the appropriate volume of vehicle (sunflower oil and DMSO). Total RNA was extracted from tissues, and relative mRNA abundance was determined by real-time detection RT-PCR analysis using β-actin mRNA abundance for normalization. Values are the means ± SD ($n = 8$). Means with unlike letters differ ($P < 0.05$). The significance of factors for particular tissues was the following: kidney, for treatment $P < 0.05$, for genotype $P < 0.05$, and for treatment × genotype $P < 0.05$; and small intestine, for treatment $P < 0.05$, for genotype $P < 0.05$, and for treatment × genotype $P < 0.05$. The P values for treatment, genotype, and treatment × genotype in the testes did not reach significance.

type mice treated with WY 14,643 but not in PPARα$^{-/-}$ mice treated with WY 14,643.

The finding that treatment with WY 14,643 increased OCTN2 mRNA in the liver, muscle, kidney, and small intestine of wild-type mice but not of PPARα$^{-/-}$ mice demonstrates that transcriptional upregulation of OCTN2 is mediated by PPARα. The observation that OCTN2 was not upregulated in the testes may be due to the fact that WY 14,643 caused no activation of PPARα in this tissue as assessed by the response of ACO mRNA abundance. It is furthermore shown that OCTN3 mRNA in the kidney and small intestine is increased by WY 14,643 in wild-type mice but not in PPARα$^{-/-}$ mice; this result demonstrates that transcriptional upregulation of OCTN3 is also mediated by PPARα. The finding that OCTN3 was not upregulated in the testes of wild-type mice treated with WY 14,643 may be due to the lack of PPARα activation in that tissue. In contrast, OCTN1 was not upregulated in any tissue of wild-type mice; this finding indicates that its transcription is not influenced by PPARα activation. The observation that OCTN1 was not upregulated by WY 14,643 in wild-type mice is in accordance with our previous study in which treatment of Fao rat hepatoma cells with WY 14,643 did not influence OCTN1 mRNA abundance (18).

The present study moreover shows that WY 14,643 treatment upregulates the transcription of enzymes involved in hepatic biosynthesis, TMABA-DH and BBD, in wild-type mice but not in PPARα$^{-/-}$ mice. This result shows that transcriptional upregulation of enzymes involved in hepatic carnitine synthesis is also mediated by PPARα.

The present study confirms results of recent studies (18, 19, 25, 35) in showing that treatment with PPARα agonists increases the carnitine concentration in the liver of rodents. The present study moreover shows that treatment with a PPARα agonist increases carnitine concentration in skeletal muscle, which serves as a carnitine storage site in the body. The reason for increased carnitine concentrations in tissue cannot be clarified by this study. The liver has a very high capacity to convert BB to carnitine. Therefore, the availability of the carnitine precursors TML and BB is considered to be rate-limiting for carnitine biosynthesis (36). Paul *et al*. (27) proposed that clofibrate treatment stimulates hepatic carnitine biosynthesis by increasing the availability of TML. In contrast to that study, TML concentrations in the liver and other tissues remained completely unchanged by treatment with WY 14,643. The finding that the concentration of BB in the liver of wild-type mice was reduced by WY 14,643 despite unchanged TML concentrations could however indicate that more BB was converted to carnitine in the liver of these animals. In the present study we did not determine protein concentrations or transport activities of OCTN2. However, the finding that the transcription of OCTN2 was strongly enhanced in the liver of wild-type mice treated with WY 14,643 suggests that increased

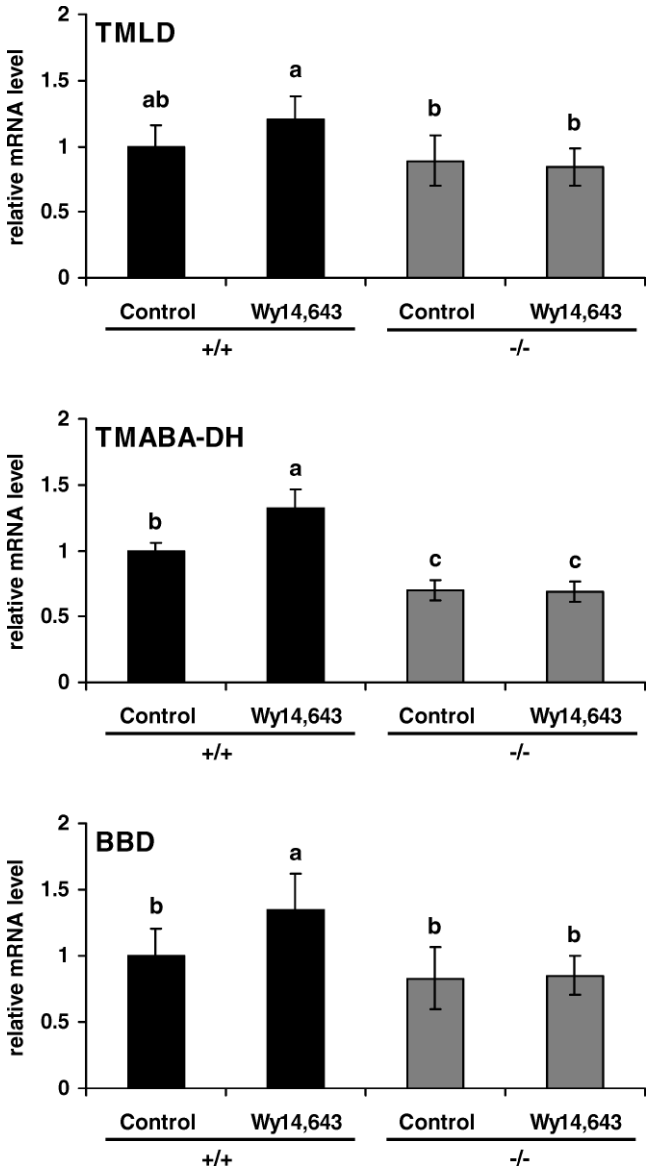

Figure 5. Effect of WY 14,643 on mRNA abundance of enzymes involved in carnitine biosynthesis in the liver of wild-type (+/+) and PPARα$^{-/-}$ mice. Mice were treated orally with 40 mg/kg of WY 14,643 for 4 days. Control mice received the appropriate volume of vehicle (sunflower oil and DMSO). Total RNA was extracted from the liver, and relative mRNA abundance was determined by real-time detection RT-PCR analysis using β-actin mRNA abundance for normalization. Values are the means ± SD ($n = 8$). Means with unlike letters differ ($P < 0.05$). The significance of factors for particular mRNAs was the following: TMLD, for genotype $P < 0.05$ and for treatment × genotype $P < 0.05$; TMABA-DH, for treatment $P < 0.05$, for genotype $P < 0.05$, and for treatment × genotype $P < 0.05$; BBD, for treatment $P < 0.05$, for genotype $P < 0.05$, and for treatment × genotype $P < 0.05$. The P value for treatment and TMLD mRNA abundance did not reach significance.

delivery of carnitine from blood to the liver may contribute to increased hepatic carnitine concentrations in these mice. As muscle is not able to produce carnitine (7), the increased carnitine concentration in skeletal muscle may be primarily the result of an increased uptake of carnitine from the blood by OCTN2. Reduced concentrations of carnitine in plasma of wild-type mice treated with WY 14,643 may be the result of increased uptake of carnitine into tissues. The reduced carnitine concentration in the kidney of wild-type mice treated with WY 14,643 cannot be explained by the data of this study. OCTNs in the kidney have the ability to reabsorb carnitine from urine (12). As OCTN2 and OCTN3 were upregulated in the kidney, it is assumed that reabsorption of carnitine in the kidney is stimulated by PPARα activation. However, as the tubular reabsorption rate of carnitine in humans and rodents is normally in excess of 90% (37–39), there is less potential for increasing the amount of carnitine reabsorbed from the tubules by PPARα activation. Therefore, increased expression of OCTNs in the kidney probably contributed less to increased tissue carnitine concentrations. Intestinal OCTN2 and OCTN3 are involved in the absorption of carnitine from the diet (17, 40). The observation that the abundance of these carnitine transporter mRNAs in the small intestine was increased in wild-type mice by WY 14,643 treatment suggests that dietary absorption of carnitine may have improved. However, because the carnitine concentration of the diet used in this study was very low, we assume that an increase in the rate of intestinal carnitine absorption should have had less effect on whole-body carnitine homeostasis in this study.

During the preparation of the revised version of this manuscript, a study by van Vlies *et al.* (41) was published, and in this study they also investigated the effect of WY 14,643 on activities of enzymes involved in hepatic carnitine synthesis, expression of OCTN2, and concentrations of TML, BB, and carnitine in plasma and various tissues of wild-type and PPARα$^{-/-}$ mice. Their study showed that WY 14,643 increases hepatic activity of BBD and OCTN2 mRNA abundance in the liver of wild-type mice but not of PPARα$^{-/-}$ mice. It furthermore revealed that carnitine concentrations in plasma, liver, kidney, and heart are increased by WY 14,643 in wild-type mice but not in PPARα$^{-/-}$ mice. The authors of that study concluded that WY 14,643 treatment increases carnitine concentrations by enhanced carnitine biosynthesis and enhanced import of carnitine into cells. In the study by van Vlies *et al.* (41), it was also shown that similar effects occur in rats during fasting. The findings of that study agree well with those of the present study and support the hypothesis that transcription of OCTN2 and enzymes of hepatic carnitine biosynthesis is regulated by PPARα.

The observed upregulation of OCTN2 in tissues due to activation of PPARα may be relevant not only to carnitine homeostasis but also to tissue distribution and intestinal absorption of other compounds. OCTN2 is polyspecific and is able to bind other monovalent cations and various drugs such as verapamil, spironolactone, and mildronate (15, 42–46). The effect of PPARα activation on such compounds therefore deserves further investigation.

In conclusion, the present study shows that transcriptional upregulation of OCTN2 and OCTN3 as well as hepatic enzymes of carnitine synthesis is mediated by

Table 2. Effect of WY 14,643 on Concentrations of Free Carnitine, Acetyl Carnitine, TML, and BB in Plasma, Liver, Kidney, Skeletal Muscle, and Small Intestine of Wild-Type (+/+) and PPARα$^{-/-}$ Mice[a]

	Control	WY 14,643	Control	WY 14,643
Treatment	+/+		−/−	
Plasma				
Free carnitine (μmol/l)[b,c]	48 ± 4[d]	38 ± 6[e]	29 ± 8[e,f]	26 ± 4[f]
Acetyl carnitine (μmol/l)[g]	25 ± 4	19 ± 3	19 ± 6	23 ± 5
TML (μmol/l)	11 ± 1	11 ± 1	12 ± 1	12 ± 1
BB (μmol/l)[b,c,g]	1.3 ± 0.3[e]	0.6 ± 0.2[f]	1.8 ± 0.2[d]	1.5 ± 0.2[d,e]
Liver				
Free carnitine (nmol/g)[b,c,g]	483 ± 57[e]	1202 ± 94[d]	229 ± 53[f]	254 ± 52[f]
Acetyl carnitine (nmol/g)[b,c,g]	3.6 ± 0.9[e]	11 ± 4[d]	2.0 ± 0.9[e]	1.4 ± 0.5[e]
TML (nmol/g)	5.7 ± 1.0	6.3 ± 0.8	5.3 ± 1.5	5.1 ± 1.7
BB (nmol/g)[b]	7.4 ± 1.9[d]	4.7 ± 1.5[e]	7.7 ± 1.8[d]	6.3 ± 0.9[d,e]
Kidney				
Free carnitine (nmol/g)[b,c,g]	498 ± 49[d]	371 ± 46[e]	279 ± 44[f]	299 ± 41[f]
Acetyl carnitine (nmol/g)[b,c,g]	20 ± 5[d]	14 ± 3[e]	16 ± 4[d,e]	16 ± 1[d,e]
TML (nmol/g)	14 ± 1	14 ± 2	15 ± 3	15 ± 3
BB (nmol/g)[b,c,g]	12 ± 2[d]	5.2 ± 0.8[e]	13 ± 2[d]	12 ± 1[d]
Skeletal muscle				
Free carnitine (nmol/g)[d]	134 ± 17[e]	160 ± 21[d]	128 ± 20[e]	130 ± 23[e]
Acetyl carnitine (nmol/g)[d]	80 ± 7[d,e]	86 ± 8[d]	68 ± 14[e]	66 ± 7[e]
TML (nmol/g)	21 ± 9	24 ± 6	34 ± 10	27 ± 8
BB (nmol/g)[d]	4.0 ± 0.5[e]	3.3 ± 0.5[e]	7.3 ± 1.2[d]	7.5 ± 1.1[d]
Small intestine				
Free carnitine (nmol/g)	93 ± 23	120 ± 22	90 ± 22	92 ± 23
Acetyl carnitine (nmol/g)[b,c,g]	18 ± 6[d]	8.9 ± 4.2[e]	9.5 ± 4.1[e]	9.1 ± 3.1[e]
TML (nmol/g)	28 ± 7	24 ± 8	23 ± 7	22 ± 7
BB (nmol/g)	29 ± 11	27 ± 8	34 ± 13	36 ± 16

[a] Values are the means ± SD (8 per group).
[b] Significant effect of treatment ($P < 0.05$).
[c] Significant effect of genotype ($P < 0.05$).
[d,e,f] Means with unlike letters differ ($P < 0.05$).
[g] Significant effect of treatment × genotype ($P < 0.05$).

PPARα in mice. Through regulation of these enzymes and transporters, PPARα is involved in the regulation of carnitine homeostasis.

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Dietary oxidised fat up regulates the expression of organic cation transporters in liver and small intestine and alters carnitine concentrations in liver, muscle and plasma of rats

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It has been shown that treatment of rats with clofibrate, a synthetic agonist of PPARα, increases mRNA concentration of organic cation transporters (OCTN)-1 and -2 and concentration of carnitine in the liver. Since oxidised fats have been demonstrated in rats to activate hepatic PPARα, we tested the hypothesis that they also up regulate OCTN. Eighteen rats were orally administered either sunflower-seed oil (control group) or an oxidised fat prepared by heating sunflower-seed oil, for 6 d. Rats administered the oxidised fat had higher mRNA concentrations of typical PPARα target genes such as acyl-CoA oxidase, cytochrome P450 4A1 and carnitine palmitoyltransferases-1A and -2 in liver and small intestine than control rats ($P<0·05$). Furthermore, rats treated with oxidised fat had higher hepatic mRNA concentrations of OCTN1 (1·5-fold) and OCTN2 (3·1-fold), a higher carnitine concentration in the liver and lower carnitine concentrations in plasma, gastrocnemius and heart muscle than control rats ($P<0·05$). Moreover, rats administered oxidised fat had a higher mRNA concentration of OCTN2 in small intestine (2·4-fold; $P<0·05$) than control rats. In conclusion, the present study shows that an oxidised fat causes an up regulation of OCTN in the liver and small intestine. An increased hepatic carnitine concentration in rats treated with the oxidised fat is probably at least in part due to an increased uptake of carnitine into the liver which in turn leads to reduced plasma and muscle carnitine concentrations. The present study supports the hypothesis that nutrients acting as PPARα agonists influence whole-body carnitine homeostasis.

Carnitine: Oxidised fat: Peroxisome proliferator-activated receptor-α: Organic cation transporters

Carnitine (L-3-hydroxy-4-*N*-*N*-*N*-trimethylaminobutyrate) is an essential metabolite that has a number of indispensable functions in intermediary metabolism. The most prominent function lies in its role in the transport of activated long-chain fatty acids from the cytosol to the mitochondrial matrix where β-oxidation takes place[1–3]. All tissues that use fatty acids as a fuel source require carnitine for normal function. Carnitine is derived from dietary sources and endogenous biosynthesis[4]. Carnitine biosynthesis involves a complex series of reactions involving several tissues[5]. Lysine provides the carbon backbone of carnitine. Lysine in protein peptide linkages undergoes methylation of the ϵ-amino group to yield trimethyllysine, which is released upon protein degradation. Muscle is the major source of trimethyllysine. The released trimethyllysine is further oxidised to butyrobetaine by the action of trimethyllysine dioxygenase, 3-hydroxy-*N*-trimethyllysine aldolase and 4-*N*-trimethylaminobutyraldehyde dehydrogenase. Butyrobetaine is hydroxylated by γ-butyrobetaine dioxygenase to form carnitine. The last reaction which is rate-limiting for carnitine synthesis occurs primarily in the liver and kidneys[6] (see Fig. 1).

Distribution of carnitine within the body and intracellular homeostasis of carnitine are controlled by organic cation transporters (OCTN) which belong to the solute carrier (SLC) 22A family, localised to the apical membrane of cells[7,8]. Three OCTN have been identified so far: OCTN1, OCTN2 and OCTN3[9–11]. OCTN are polyspecific; they transport several cations and L-carnitine[12,13]. Carnitine transport by OCTN1 and OCTN2 is Na dependent whereas that by OCTN3 is Na independent[11]. OCTN1 and OCTN2 are expressed in several tissues such as kidney, intestine, skeletal muscle, heart, liver and brain[11,14,15]. In contrast, OCTN3 is expressed exclusively in the testes and kidneys[11]. Among the three OCTN, OCTN3 has the highest specificity for carnitine; OCTN1 has the lowest one[11]. OCTN operate on the intestinal absorption and renal reabsorption of carnitine and play a major role in tissue distribution by catalysing the uptake of carnitine into body cells. Due to its high binding affinity for carnitine and its wide expression, OCTN2 seems to be the most physiologically important carnitine transporter. OCTN1 contributes less to carnitine transport than OCTN2 due to its low carnitine transport activity. OCTN3 may be important for carnitine uptake into the testes, and may contribute to the reabsorption of carnitine in the kidneys[11]. The fact that inborn or acquired defects of OCTN lead to primary or secondary systemic carnitine deficiency demonstrates their essential role in carnitine homeostasis[8].

Abbreviations: CPT, carnitine palmitoyltransferase; Cyp, cytochrome P450; OCTN, organic cation transporter.

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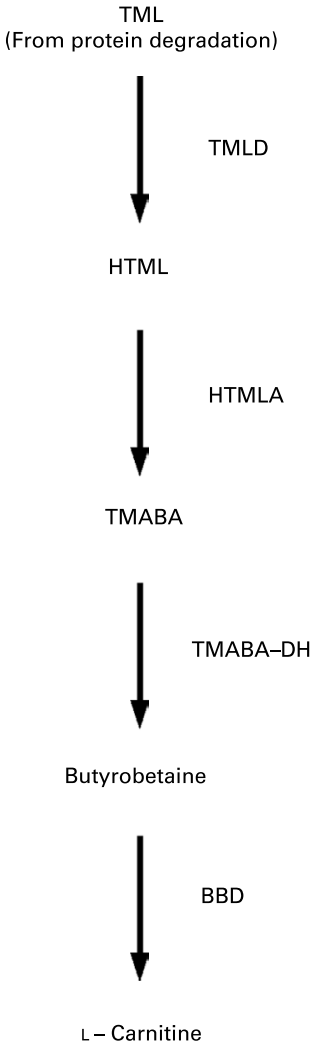

Fig. 1. Schematic diagram of carnitine biosynthesis from trimethyllysine (TML) (according to Vaz & Wanders[6]). TML is oxidised to butyrobetaine by trimethyllysine dioxygenase (TMLD), 3-hydroxy-*N*-trimethyllysine aldolase (HTMLA) and 4-*N*-trimethylaminobutyraldehyde dehydrogenase (TMABA-DH). In the last rate-limiting step, butyrobetaine is hydroxylated to L-carnitine by γ-butyrobetaine dioxygenase (BBD). HTML, 3-hydroxy-*N*-trimethyllysine; TMABA, 4-*N*-trimethylaminobutyraldehyde.

It has been shown previously that starvation or treatment of rats with clofibrate increases the concentration of carnitine in the liver[16–18]. Both starvation and clofibrate treatment lead to an activation of PPARα, a transcription factor belonging to the nuclear hormone receptor superfamily[19]. We have recently shown that activation of PPARα by clofibrate treatment causes an up regulation of OCTN1 and OCTN2 in rat liver[20]. These results strongly indicated that increased carnitine concentrations in livers of rats starved or treated with clofibrate were due to increased uptake of carnitine from blood into the liver. Indeed, plasma carnitine concentrations were reduced in rats treated with clofibrate which may be caused by an increased uptake into the liver[20].

In addition to synthetic agonists, several naturally occurring compounds are able to activate PPARα *in vivo*. Recently, we and others have shown that dietary oxidised fats prepared by the heating of vegetable oils activate hepatic PPARα in rats and pigs[21–25]. In the present study, we tested the hypothesis that oxidised fats are also able to up regulate the expression of OCTN (OCTN1, OCTN2) in the liver due to their ability to activate PPARα and thereby increase hepatic carnitine concentration. For this end, we performed an experiment with growing rats as an animal model, according to a previous study dealing with the effects of an oxidised oil on PPARα activation[24].

More than 95 % of the total carnitine in the body is localised in the muscle which serves as a carnitine storage[2]. When plasma carnitine concentrations are lowered, such as by treatment with pivalate, carnitine is mobilised from the muscle in order to normalise plasma carnitine concentrations[26]. Therefore, an increased uptake of carnitine from the blood into the liver by up regulation of hepatic OCTN should lead to a mobilisation of carnitine storage in the muscle. To investigate this, we also determined carnitine concentrations in skeletal muscle and heart of the rats.

OCTN1 and OCTN2 are also highly expressed in the intestine and particularly OCTN2 plays an important role in the absorption of L-carnitine from the diet[15,27,28]. As the small intestine also has a high expression of PPARα, it seems possible that an oxidised fat could increase the gene expression of OCTN also in the small intestine via an activation of PPARα. Besides OCTN, the amino acid transporter ATB^{0+} is involved in the intestinal absorption of carnitine from the diet[27,29]. In order to obtain information whether PPARα activation by synthetic or native agonists could influence intestinal carnitine absorption, we also determined mRNA concentration of ATB^{0+} in small intestine.

Materials and methods

Animal experiment

Male Sprague–Dawley rats, aged 5 weeks old, supplied by Charles River (Sulzfeld, Germany) with an average initial body weight of 115 (SD 14) g were randomly assigned to two groups of nine rats each. They were kept individually in Macrolon cages in a room controlled for temperature (22 ± 2°C), relative humidity (50–60 %) and light (12 h light–dark cycle). All experimental procedures described followed established guidelines for the care and handling of laboratory animals and were approved by the council of Saxony-Anhalt. The animals received either 2 ml fresh sunflower-seed oil (control group) or oxidised sunflower-seed oil (see Preparation of the oxidised fat) by oral administration once per d 2 h after the beginning of the light cycle. Afterwards, they obtained their daily food ration. All rats were fed a commercial standard basal diet (Altromin 1324; Altromin GmbH, Lage, Germany). Concentration of total carnitine in the basal diet was 22 μmol/kg. To standardise food intake, diet intake was controlled. Each rat in the experiment received 12 g diet/d. This amount of diet which is approximately 20 % below the amount of diet rats would consume *ad libitum* was completely ingested by all rats. Thus, the diet intake was identical in all the rats within this experiment. Water was available *ad libitum* from nipple drinkers during the whole experiment. At day 6 of treatment, rats received the last dose of fresh or oxidised fat and 9 g diet and were killed 4 h later by decapitation under light anaesthesia with diethyl ether. Blood was collected into heparinised polyethylene tubes. Liver, heart

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and gastrocnemius muscles were quickly removed, frozen with liquid N_2 and stored at −80°C pending further analysis. Plasma was obtained by centrifugation of the blood (1100 ***g***; 10 min; 4°C) and stored at −20°C. Liver samples for RNA isolation and lipid extraction were snap-frozen in liquid N_2 and stored at −80°C. The small intestine was rapidly excised, washed with cold 0·9 % NaCl (w/v) and mucosal scrapings were obtained from the jejunum (defined by length), snap-frozen and stored at −80°C for RNA extraction.

Preparation of the oxidised fat

The thermoxidised oil was prepared by heating sunflower-seed oil obtained from a local supermarket in an electric fryer (Saro Gastro-Products GmbH, Emmerich, Germany) for 25 d at 60°C. Throughout the heating process, air was continuously bubbled into the fat. The extent of lipid peroxidation was determined by assaying the peroxide value[30], concentration of thiobarbituric acid-reactive substances[31] and conjugated dienes[32], acid values[30], the percentage of total polar compounds[33] and the concentration of total carbonyls[34]. The oxidised fat had much higher concentrations of peroxides (126-fold), conjugated dienes (>2740-fold), thiobarbituric acid-reactive substances (12-fold), total carbonyls (33-fold), polar compounds (5-fold) and a higher acid value (15-fold) than the fresh fat (Table 1).

Carnitine analysis

Carnitine was determined as [^{3}H]acetyl-carnitine after the esterification of non-esterified carnitine by carnitine acyltransferase according to McGarry & Foster[35] with modifications proposed by Parvin & Pande[36] and Christiansen & Bremer[37]. Plasma samples were used directly for the determination of the total carnitine after alkaline hydrolysation as described for the tissue samples below. Tissue samples were freeze dried and milled. Then 100 mg liver or 50 mg muscle powder were sonificated in 5 ml water for 15 min. Samples were centrifuged (12 000 ***g***; 5 min) and non-esterified carnitine in the supernatant fraction was measured. For the determination of the total carnitine the samples were hydrolysed before the centrifugation. For this, 10 ml 0·2 M-potassium hydroxide were added, the samples were incubated at 30°C for 1 h and then neutralised by the addition of 0·2 M-HCl. Carnitine esterification was done in a final volume of 1 ml containing 0·1 M-HEPES (pH 7·4), 2 mM-*N*-ethylmaleimide, 1·25 mM-EDTA, 25 μM-[^{3}H]acetyl-CoA (29·4 MBq/mmol; GE Healthcare, Buckinghamshire, UK) and 1 U carnitine acyltransferase (Roche Diagnostic, Mannheim, Germany) for 30 min at room temperature. [^{3}H]acetyl-CoA not consumed by the reaction was bound to Dowex 1-X 8 and separated by centrifugation. Carnitine concentration was calculated using the radioactivity of the supernatant fraction measured in a liquid scintillation counter and corrected for non-specific radioactivity.

Table 1. Concentrations of various lipid oxidation products in the fats*

Oxidation product	Fresh fat	Oxidised fat
Peroxide value (mEq O_2/kg)	3·0	378·6
Conjugated dienes (mmol/kg)	<0·1	273·6
TBARS (mmol/kg)	1·1	13·1
Total carbonyls (mmol/kg)	2·9	96·9
Total polar compounds (%)	5·1	27·8
Acid value (g KOH/kg)	0·4	5·8

TBARS, thiobarbituric acid-reactive substances.
* Data are the results of single measurements.

Reverse transcriptase polymerase chain reaction analysis

Total RNA was isolated from rat livers and mucosa scrapings, respectively, by TRIZOL™ reagent (Sigma-Aldrich, Steinheim, Germany) according to the manufacturer's protocol. cDNA synthesis was carried out as described[38]. The mRNA concentration of genes was measured by real-time detection PCR using SYBR® Green I and the Rotor Gene 2000 system (Corbett Research, Mortlake, Australia). Real-time detection PCR was performed with 1·25 U Taq DNA polymerase (Promega, Mannheim, Germany), 500 μM-dNTP and 26·7 pmol of the specific primers (Operon Biotechnologies, Cologne, Germany; Table 2). Annealing temperature for all primers was 60°C. For determination of mRNA concentration a threshold cycle (C_t) and amplification efficiency was obtained from each amplification curve using the software RotorGene 4·6 (Corbett Research). Calculation of the relative mRNA concentration was made using the $\Delta\Delta C_t$ method as previously described[39]. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase was used for normalisation. mRNA concentration of glyceraldehyde-3-phosphate dehydrogenase was not influenced by the treatment of rats with oxidised fat.

Statistical analysis

Means of the treatment and control groups were compared by an unpaired *t* test using the Minitab Statistical Software (Minitab, State College, PA, USA). Differences with $P<0·05$ were considered to be significant.

Results

Final weights and body-weight gains of the rats

Final body weights of rats treated with the oxidised fat (133 (SD 14) g) were not significantly different from the control rats (144 (SD 14) g) (nine rats for each group). However, rats treated with the oxidised fat had a lower body-weight gain (17·5 (SD 6·4) g) over the feeding period than the control rats (29·7 (SD 4·5) g) (nine rats for each group; $P<0·05$).

mRNA concentrations of acyl-CoA oxidase, cytochrome P450-4A1, carnitine palmitoyltransferases-1A and -2, organic cation transporters-1 and -2 and enzymes involved in hepatic carnitine synthesis (trimethyllysine dioxygenase, 4-N-trimethylaminobutyraldehyde dehydrogenase and γ-butyrobetaine dioxygenase) in the liver

Rats treated with the oxidised fat had higher mRNA concentrations of acyl-CoA oxidase, cytochrome P450 (Cyp)-4A1, carnitine palmitoyltransferase (CPT)-2, OCTN1 and OCTN2 in the liver than control rats ($P<0·05$); mRNA concentration of CPT1A, however, was not different in the rats treated with oxidised fat from the control rats (Fig. 2). Rats treated with the oxidised fat had a higher mRNA concentration of trimethyllysine dioxygenase in the liver than control

Table 2. Characteristics of the primers used in reverse transcriptase polymerase chain reaction analysis

Gene	Forward primer (from 5′ to 3′)	Reverse primer (from 5′ to 3′)	bp	NCBI GenBank
ACO	CTTTCTTGCTTGCCTTCCTTCTCC	GCCGTTTCACCGCCTCGTA	415	NM_017340
ATB^{0+}	ATCCGGAAGCACTAGCTCAA	CCCAGTAAATTCCAGCCTGA	237	NM_001037544
BBD	ATTCTGCAAAAGCTCGGAAA	CTCCTTGGAGTCCTGCTCTG	183	NM_022629
Cyp4A1	CAGAATGGAGAATGGGGACAGC	TGAGAAGGGCAGGAATGAGTGG	460	NM_175837
CPT1A	GGAGACAGACACCATCCAACATA	AGGTGATGGACTTGTCAAACC	416	NM_031559
CPT2	TCCTCGATCAAGATGGGAAC	GATCCTTCATCGGGAAGTCA	237	NM_012930
GAPDH	GCATGGCCTTCCGTGTTCC	GGGTGGTCCAGGGTTTCTTACTC	337	BC059110
OCTN1	AGCATTTGTCCTGGGAACAG	ACTCAGGGATGAACCACCAG	200	NM_022270
OCTN2	CCTCTCTGGCCTGATTGAAG	CTCCGCTGTGAAGACGTACA	226	NM_012930
TMLD	GCCCTGTGGCATTCAAGTAT	GGTCCAACCCCTATCATGTG	201	AF374406
TMABA-DH	TTTGAGACTGAAGCCGAGGT	CACCGGGCTGACGTTATAGT	156	NM_022273

ACO, acyl-CoA oxidase; BBD, γ-butyrobetaine dioxygenase; Cyp, cytochrome P450; CPT, carnitine palmitoyltransferase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; OCTN, organic cation transporter; TMLD, trimethyllysine dioxygenase; TMABA-DH, 4-*N*-trimethylaminobutyraldehyde dehydrogenase.

rats ($P<0{\cdot}05$; Fig. 2). mRNA concentrations of 4-*N*-trimethylaminobutyraldehyde dehydrogenase and γ-butyrobetaine dioxygenase in the liver, however, did not differ between the two groups of rats (Fig. 2).

mRNA concentrations of acyl-CoA oxidase, cytochrome P450-4A1, carnitine palmitoyltransferases-1A and -2, organic cation transporters-1 and -2 and ATB^{0+} in the small intestine

Rats treated with oxidised fat had higher mRNA concentrations of acyl-CoA oxidase, Cyp4A1, CPT1A, CPT2 and OCTN2 ($P<0{\cdot}05$), and they tended to have a higher mRNA concentration of OCTN1 ($P=0{\cdot}066$) in the small intestine compared with control rats (Fig. 3). mRNA concentration of ATB^{0+} in the small intestine was reduced in the rats fed the oxidised fat compared with those fed the fresh fat ($P<0{\cdot}05$; Fig. 3).

Carnitine concentrations in liver, plasma and muscle

Rats treated with the oxidised fat had a higher carnitine concentration in the liver than control rats ($P<0{\cdot}05$, Fig. 4). Plasma carnitine concentration was lower in the rats treated with oxidised fat (18·8 (SD 3·1) μmol/l) than in the control group (28·4 (SD 4·3) μmol/l) (nine rats for each group; $P<0{\cdot}05$). Rats treated with oxidised fat also had lower carnitine concentrations in gastrocnemius and heart muscle than control rats ($P<0{\cdot}05$; Fig. 4).

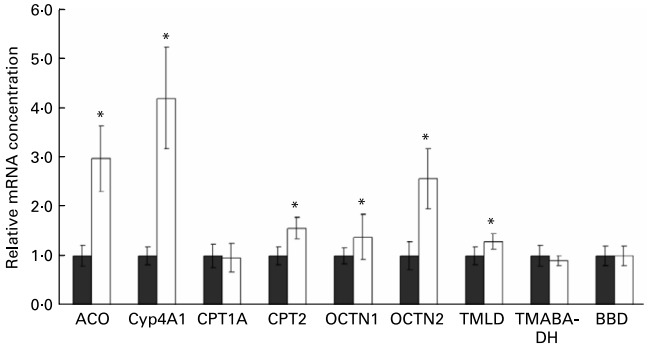

Fig. 2. Effect of an oxidised fat on the relative mRNA concentrations of acyl-CoA oxidase (ACO), cytochrome P450 (Cyp)-4A1, carnitine palmitoyltransferases (CPT)-1A and -2, organic cation transporters (OCTN)-1 and -2, trimethyllysine dioxygenase (TMLD), 4-*N*-trimethylaminobutyraldehyde dehydrogenase (TMABA-DH) and γ-butyrobetaine dioxygenase (BBD) in the liver of rats. Rats were treated orally with 2 ml oxidised fat (□) or fresh fat (■; control = 1·00) for 6 d. Total RNA was extracted from rat livers and mRNA concentrations were determined by real-time detection RT-PCR analysis using glyceraldehyde-3-phosphate dehydrogenase mRNA concentration for normalisation. Values are means, with standard deviations represented by vertical bars (n 9). *Mean value was significantly different from that of the control rats ($P<0{\cdot}05$).

Discussion

We have recently found that treatment with clofibrate causes a strong up regulation of OCTN2, and a less strong up regulation of OCTN1, in the liver of rats which was accompanied by an increased hepatic carnitine concentration[20]. This effect was probably caused by PPARα activation. In the present study, we investigated the hypothesis that oxidised fats are able to exert similar effects due to their ability to activate PPARα. Hydroxy- and hydroperoxy fatty acids such as hydroxyoctadecadienoic and hydroperoxyoctadecadienoic acid occurring in oxidised fats are very potent PPARα agonists[38,40,41]. These fatty acids are produced during the early stage of lipid peroxidation. Since they are unstable and decompose at high temperatures, fats treated at low temperature have much higher concentrations of these primary lipid

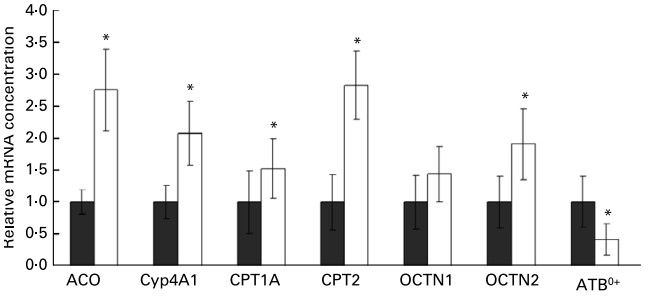

Fig. 3. Effect of an oxidised fat on the relative mRNA concentrations of acyl-CoA oxidase (ACO), cytochrome P450 (Cyp)-4A1, carnitine palmitoyltransferases (CPT)-1A and -2, organic cation transporters (OCTN)-1 and -2 and amino acid transporter ATB^{0+} in the small intestine of rats. Rats were treated orally with 2 ml oxidised fat (□) or fresh fat (■; control = 1·00) for 6 d. Total RNA was extracted from mucosal scrapings and mRNA concentrations were determined by real-time detection RT-PCR analysis using glyceraldehyde-3-phosphate dehydrogenase mRNA concentration for normalisation. Values are means, with standard deviations represented by vertical bars (n 9). *Mean value was significantly different from that of the control rats ($P<0{\cdot}05$).

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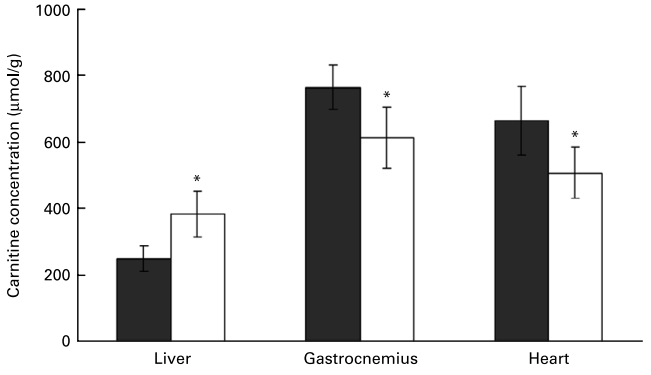

Fig. 4. Effect of an oxidised fat on the concentrations of total carnitine in liver, gastrocnemius and heart. Rats were treated orally with 2 ml oxidised fat (□) or fresh fat (■; control) for 6 d. Values are means, with standard deviations represented by vertical bars (n 9). *Mean value was significantly different from that of the control rats ($P<0{\cdot}05$).

peroxidation products than fats treated at high temperature[24]. This is the reason why we used a fat treated at a relatively low temperature for a long period. The high peroxide value and the high concentration of conjugated dienes indicate that this fat indeed had high concentrations of hydroxy- and hydroperoxy fatty acids which may be particularly responsible for the PPARα-activating effects of oxidised fats. To ensure that all rats obtained the same dose of oxidised fat, it was administered orally. The oxidised and fresh fat, respectively, accounted for about 25 % of total energy of the total daily feed. Since it was observed in a previous rat study that even short-term application of a PPARα agonist led to the typical changes known for PPARα activation such as up regulation of classical target genes involved in β-oxidation and reduction of TAG concentration[38], we decided to give the oxidised fat over a relatively short time of 6 d. It has been shown that the intake of oxidised fats could cause a reduction of the food intake in rats which could cause secondary effects which interact with the effect of treatment[42,43]. To ensure an identical food intake in both groups of rats, we used a controlled feeding system in which each rat consumed 12 g diet/d. This amount of diet is slightly below that that rats would consume *ad libitum* but in clear excess of that necessary to meet the maintenance energy requirement (which is approximately 6 g/d[44]) and ensures an adequate growth of the rats. It is known that fasting causes an activation of PPARα due to the release of NEFA from the adipose tissue[45]. To avoid PPARα activation due to an insufficient supply of energy, all the rats received their last portion of diet 4 h before decapitation. Therefore, we can exclude the possibility that PPARα was also activated in the control group fed the fresh fat. The finding that rats fed the oxidised fat gained less weight during the experimental period than those fed the fresh fat although both groups received an identical amount of diet indicates that the oxidised fat impaired the feed conversion ratio. This finding agrees with other reports which also showed that feeding of oxidised fats impairs the growth of rats[46–49]. We did not investigate the reason for this. Previous studies, however, have shown that oxidised fats lower the digestibility of nutrients[46,50] and this may be the reason for the reduced body-weight gains of the rats fed the oxidised fat observed in the present study. However, as rats fed the oxidised fat appeared quite normal, we assume that the oxidised fat did not cause general toxicity.

The finding of increased mRNA concentrations of the typical PPARα downstream genes acyl-CoA oxidase, Cyp4A1, CPT1A and CPT2 (for a review, see Mandard *et al.*[45]) in liver and intestine indeed indicates that the oxidised fat caused an activation of PPARα in both liver and intestine of the rats. This indication agrees with recent studies in rats and pigs which also showed that intake of oxidised fats leads to an activation of PPARα in the liver[21–25].

The present study shows further that treatment of rats with an oxidised fat caused the same alterations as observed for clofibrate[20], namely increased hepatic mRNA concentrations of OCTN1 and OCTN2 and an increased hepatic carnitine concentration. Considering that a similar up regulation of OCTN1 and OCTN2 was observed in the liver of rats treated with the synthetic PPARα agonist clofibrate and in rat hepatoma cells treated with the more potent and selective PPARα agonist WY 14,643[20], we propose that the oxidised fat up regulated OCTN in the liver also by PPARα activation.

In rat liver, OCTN1 and OCTN2 are highly expressed[15]. Both of them are able to transport carnitine into the liver cell[51,52]. However, it has been shown that OCTN2 has a higher carnitine transport activity than OCTN1[11]. For that reason and as mRNA concentration of OCTN2 was more strongly increased by the oxidised fat than that of OCTN1, we assume that increased hepatic carnitine concentrations in rats treated with oxidised fat were caused mainly by an increased uptake of carnitine via OCTN2. Plasma carnitine concentrations are regulated by several events, namely intestinal absorption from the diet, renal excretion, endogenous synthesis in the liver and kidneys and movement of carnitine between plasma and tissues[53]. We have not studied the pharmacokinetics of carnitine but it seems plausible that reduced plasma concentrations of carnitine in rats fed the oxidised fat may at least in part be due to an enhanced uptake into the liver. We measured mRNA concentrations of OCTN only in liver and small intestine; however, it is possible that they were increased also in other tissues in rats fed the oxidised fat. Therefore, an increased uptake of carnitine into other tissues besides liver could also contribute to the reduced plasma carnitine concentrations. In the kidney, OCTN2 functions to reabsorb carnitine from the urine[13,54]. An up regulation of OCTN2 in kidney would be expected to reduce urinary excretion of carnitine which in turn results in an increased plasma carnitine concentration. However, the effect of oxidised fats on the gene expression of OCTN in those tissues and their consequences on whole-body carnitine homeostasis should be determined in future studies.

In the present study we also determined mRNA concentrations of various enzymes involved in hepatic carnitine biosynthesis in the liver which belongs like the kidney to the tissues being able to synthesise carnitine[6]. It was found that oxidised fat treatment led to a moderate up regulation of trimethyllysine dioxygenase while mRNA concentrations of 4-*N*-trimethylaminobutyraldehyde dehydrogenase and γ-butyrobetaine dioxygenase, the rate-limiting enzyme of carnitine biosynthesis[6], remained unchanged by the treatment. This finding shows that PPARα activation by the oxidised oil does not up regulate the gene expression of enzymes involved in hepatic carnitine synthesis. Nevertheless, it is

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possible that carnitine hepatic biosynthesis was increased in rats treated with oxidised fat. The liver has a high capacity to convert γ-butyrobetaine into carnitine[6]. As OCTN2 has a high affinity for γ-butyrobetaine[10,11] it is likely that an increased expression of OCTN2 may have led to an increased uptake of γ-butyrobetaine from plasma into the liver which in term may have stimulated synthesis of carnitine in the liver. This assumption, however, has to be proven in further studies.

Muscle contains more than 95 % of whole-body carnitine and serves as a carnitine storage[2]. When plasma carnitine concentrations are lowered, such as by treatment with pivalate, carnitine can be mobilised from the muscle in order to normalise plasma carnitine concentrations[26]. Therefore, we expected that a reduced plasma carnitine concentration may lead to a reduction of the carnitine concentration in muscle. The finding that the concentration of carnitine was reduced in gastrocnemius and heart muscle of rats treated with oxidised fat indeed suggests that carnitine might have been mobilised from muscle. In rats treated with clofibrate, a reduction of muscle carnitine concentration has also been found[20]. A reduced carnitine concentration in muscle could also be due to a reduced uptake of carnitine due to a decreased activity of OCTN, which, however, is unlikely with respect to the finding that OCTN in liver were up regulated in rats fed the oxidised fat. As muscle also has a high expression of PPARα, we expect that the expression of OCTN in muscle was increased rather than reduced by the dietary oxidised fat.

The present study further shows that a dietary oxidised fat leads to an up regulation of OCTN2 in the small intestine. As PPARα target genes (acyl-CoA oxidase, CYP4A1, CPT1a, CPT2) in the intestine were also up regulated in rats fed the oxidised fat, we assume that the increased expression of OCTN in intestine was also caused by activation of PPARα. As intestinal OCTN localised in the apical membrane of mucosa cells are able to transport carnitine from the diet into the cell[27,28], an increased expression of these transporters may enhance their capacity to absorb carnitine. However, as ATB^{0+}, another transporter involved in the intestinal absorption of carnitine[27], was down regulated in rats fed the oxidised fat, it is difficult to draw conclusions about the whole intestinal absorption of carnitine from the diet. Nevertheless, the observed up regulation of intestinal OCTN may be relevant because they are polyspecific and do not only transport carnitine from the intestinal lumen into the mucosa cell but are also able to bind various drugs such as verapamil, spironolactone or mildronate and other monovalent cations[14,28,55–58]. As oxidised fats increase the gene expression of OCTN in the small intestine, it is possible that these fats also increase the absorption of various drugs from the intestine.

The hypothesis that the up regulation of OCTN was caused by PPARα activation provides also an explanation for the observed increased hepatic carnitine concentrations in fasted rats[16,17]. During fasting, NEFA are liberated from adipose tissue and act as activators of PPARα when they have entered the liver. Activation of PPARα up regulates many genes involved in hepatic mitochondrial and peroxisomal β-oxidation of fatty acids to supply acetyl-CoA used for the generation of ATP via the citrate cycle and for the generation of ketone bodies, an important fuel for the brain during fasting[59,60]. These metabolic adaptations during fasting triggered by PPARα aim to minimise the use of protein and carbohydrates as fuel and allow mammals to survive long periods of energy deprivation. CPT are rate limiting for β-oxidation of fatty acids[61,62]. The up regulation of CPT, which is essential for the metabolic adaptations occurring during fasting, might increase the demand for carnitine in liver cells. We postulate that up regulation of OCTN by PPARα activation is a means to supply liver cells with sufficient carnitine required for the transport of excessive amounts of fatty acids into the mitochondrion, and therefore plays an important role in the adaptive response of liver metabolism to fasting.

In conclusion, the present study shows that an oxidised fat causes an up regulation of OCTN2 in the liver and small intestine of rats. As OCTN2 catalyses the uptake of carnitine into cells, these fats influence whole-body carnitine homeostasis. An increased hepatic carnitine concentration in rats treated with oxidised fat may be at least in part due to an increased uptake of carnitine from blood into the liver. Since OCTN2 binds not only carnitine but also various drugs, the possibility exists that increased OCTN2 expression in the small intestine may improve the absorption of various drugs.

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Monocarboxylate transporter (MCT)-1 is up-regulated by PPARα

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ABSTRACT

Peroxisome proliferator-activated receptor (PPAR)-α mediates an adaptive response to fasting by up-regulation of genes involved in fatty acid oxidation and ketone body synthesis. Ketone bodies are transferred in and out of cells by monocarboxylate transporter (MCT)-1. In this study we observed for the first time that activation of PPARα in rats by clofibrate treatment or fasting increased hepatic mRNA concentration of MCT1. In Fao rat hepatoma cells, incubation with the PPARα agonist WY 14,643 increased mRNA concentration of MCT1 whereas the PPARγ agonist troglitazone did not. To elucidate whether up-regulation of MCT1 is indeed mediated by PPARα we treated wild-type and PPARα-null mice with WY 14,643. In wild-type mice, treatment with WY 14,643 increased mRNA concentrations of MCT1 in liver, kidney and small intestine whereas no up-regulation was observed in PPARα-null mice.

1. Introduction

The ability of animals to survive food deprivation requires biochemical and physiological responses to the lack of food. The overall metabolic response to fasting operates at numerous levels, and peroxisome proliferator-activated receptor (PPAR)-α which belongs to the family of ligand-activated nuclear hormone receptors mediates an adaptive response to fasting. During fasting, fatty acids are liberated from adipose tissue, thereby increasing the concentration of free fatty acids in plasma. Fatty acids are taken up across the plasma membrane in tissues by fatty acid transporters and directly activate PPARα [1,2]. Thus, PPARα acts as a nutritional state sensor and stimulates the transcription of genes involved in fatty acid uptake through membranes, fatty acid binding in cells, peroxisomal and mitochondrial fatty acid oxidation, lipoprotein assembly and synthesis of ketone bodies [3]. The first rate-limiting step in ketogenesis is catalyzed by mitochondrial 3-hydroxy-3-methylglutaryl-CoA (mHMG-CoA) synthase, an enzyme that has a functional PPAR response element (PPRE) within its promoter [4]. During prolonged fasting, hepatic ketone body synthesis is enhanced, in concert with an increase of circulating ketone body concentration.

Ketone bodies are crucial energy substrates when glucose, the most important energy fuel, is reduced in some altered metabolic situations such as starvation [5,6] or in pathological conditions such as diabetes [7] and ischaemia [8,9]. Ketone bodies represent substantial energy substrates for the brain during fasting [10,11]. Monocarboxylate transporters (MCT) facilitate the transport of monocarboxylic acids such as ketone bodies across biological membranes. So far, 14 members of MCT have been identified but only MCT1-4 have been expressed in an active form and characterized as proton-linked MCT [12]. MCT have an important role in various tissues due to their fundamental role in metabolic homeostasis and their critical role in the transfer of monocarboxylates into and out of cells (reviewed in [13]).

MCT1 was found in almost all tissues including muscle, kidney, liver, heart, brain and intestine [13]. MCT1 encodes a protein with an apparent molecular mass of about 45 kDa [14]. MCT1 is also proposed to be the major regulator of bidirectional monocarboxylic acid transport between the brain and the blood [15]. Considering that PPARα mediates the adaptive response to fasting such as stimulation of ketone body synthesis we hypothesized that activation of PPARα also could cause an up-regulation of the ketone body transporter MCT1. In order to investigate this hypothesis, we first analyzed the effect of PPARα activation by the synthetic agonist clofibrate and by fasting, respectively, on mRNA concentration of MCT1 in the liver of rats. Furthermore, we stimulated rat hepatoma Fao cells with WY 14,643, another selective PPARα agonist, and compared the effects on MCT1 mRNA concentration with that of troglitazone, a selective agonist of PPARγ. Both experiments showed that PPARα might up-regulate MCT1 mRNA concentration. This raises the possibility that MCT1 might be a PPARα-regulated gene. To test this hypothesis, we performed an experiment with mice deficient in PPARα ($PPAR\alpha^{-/-}$ mice) which were treated like wild-type mice with WY 14,643 and determined mRNA concentrations of MCT1.

2. Materials and methods

2.1. Animal experiments

All experimental procedures described followed established guidelines for the care and handling of laboratory animals and were approved by the council of Saxony-Anhalt. Animals were kept individually in Macrolon cages in a room controlled for temperature

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(22±2 °C), relative humidity (50–60%) and light (12 h light/dark cycle). All animals were fed a commercial standard basal diet ("altromin 1324", Altromin GmbH, Lage, Germany). Water was available ad libitum from nipple drinkers during the whole experiment. For analysis of the effects of clofibrate [ethyl 2-(4-chlorophenoxy)-2-methylpropionate; Fluka Chemie GmbH, Buchs, Switzerland] treatment, male Sprague–Dawley rats (Charles River, Sulzfeld, Germany), with an average initial body weight of 366 g (±28; SD), were randomly assigned to two groups (n=8). The animals were treated with 250 mg/kg of clofibrate in 1 mL sunflower oil or with an equal volume of vehicle sunflower oil by gavage once a day 2 h after beginning of the light cycle. To standardize food intake, diets were fed daily in amounts of 18 g per day. At day 4 of treatment, animals received the last dose of clofibrate or vehicle alone and 9 g of the diet and were killed 4 h later. For analysis of the effects of fasting, female Sprague–Dawley rats, with an average initial body weight of 267 g (±32; SD) were randomly assigned to two groups (n=9). Control rats received the diet ad libitum. Rats of the fasting group received the diet ad libitum and were then fasted for 24 h before killing.

For analysis of the role of PPARα, we used male PPARα-null mice (129S4/SvJae-*Ppara*^{tm1Gonz}/J; PPARα$^{-/-}$) and corresponding wild-type control mice (129S1/SvImJ), purchased from the Jackson Laboratory (Bar Harbor, USA), 11–12 weeks of age with an average initial body weight of 24.3 g (±3.2; SD). Mice from both genotypes were randomly assigned to two groups. Mice of the treatment groups (wild-type mice, n=8; PPARα$^{-/-}$ mice, n=8) obtained 40 mg/kg WY 14,643 once a day 2 h after beginning of the light cycle by gavage for 4 days. WY 14,643 was dissolved in DMSO/sunflower oil (50/50, v/v) at a final concentration of 8 mg/mL as described [16]. Control animals (wild-type mice, n=8; PPARα$^{-/-}$ mice, n=8) obtained the appropriate volume of the vehicle DMSO/sunflower oil. To standardize food intake, diets were fed daily in amounts of 4 g per day. At day 4 of treatment, mice received the last dose of WY 14,643 or vehicle alone and 1 g of the diet and were killed 4 h later.

2.2. Sample collection

All animals were killed by decapitation under light anaesthesia with diethyl ether. Blood was collected into EDTA tubes and plasma was obtained by centrifugation (1100 *g*, 10 min, 4 °C) and stored at −20 °C. Tissue samples for RNA isolation were snap-frozen in liquid nitrogen and stored at −80 °C. The small intestine of mice was rapidly excised, washed with 0.9% NaCl (w/v) and mucosal scrapings were obtained, snap-frozen and stored at −80 °C.

2.3. Determination of non-esterified fatty acids (NEFA) and 3-hydroxybutyrate

The concentrations of NEFA and 3-hydroxybutyrate in plasma were determined using enzymatic reagent kits (NEFA, Wako Chemicals GmbH, Neuss, Germany; 3-hydroxybutyrate, R-Biopharm AG, Darmstadt, Germany).

2.4. Cell culture experiment

Fao rat hepatoma cells (ECACC, Salisbury, UK) were cultured in Ham-F12 medium supplemented with 10% FCS and 0.05 mg/mL gentamicin (Invitrogen, Karlsruhe, Germany). Cells were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. For experiments, Fao cells were seeded in 24-well culture plates at a density of $2.1 \cdot 10^5$ cells per well and used prior to reaching confluence (usually 3 days after seeding). The cells were preincubated with low-serum medium (0.5% FCS) for 16 h and then stimulated for 6 and 24 h with WY 14,643 and troglitazone (Sigma-Aldrich, Steinheim, Germany), respectively. WY 14,643 and troglitazone were added to the low-serum medium from stock solutions in DMSO. Final DMSO concentration did not exceed 0.1% (v/v). Cells treated with the appropriate vehicle concentration were used as a control. Cell viability of Fao cells was not reduced by 24 h incubation with WY 14,643 up to a concentration of 100 μM and with troglitazone up to a concentration of 20 μM as demonstrated by the MTT assay [17] (data not shown).

2.5. RT-PCR analysis

Total RNA was isolated from cells and tissue samples, respectively, by TRIZOL reagent (Sigma-Aldrich) according to the manufacturer's protocol. cDNA synthesis was carried out as described [18]. The mRNA concentration of genes was measured by realtime detection PCR using SYBR® Green I and the Rotor Gene 2000 system (Corbett Research, Mortlake, Australia). Realtime detection PCR was performed with 1.25 U Taq DNA polymerase (Promega, Mannheim, Germany), 500 μM dNTPs and 26.7 pmol of the specific primers (Operon Biotechnologies, Cologne, Germany; Table 1). For determination of mRNA concentration a threshold cycle (C_t) and amplification efficiency were obtained from each amplification curve using the software RotorGene 4.6 (Corbett Research). Calculation of the relative mRNA concentration was made using the amplification efficiencies and the C_t values [19]. The housekeeping gene β-actin was used for normalization.

2.6. Statistical analysis

For rat experiments and those with Fao cells, means of treatments and control were compared by Student's t-test using the Minitab Statistical Software (Minitab, State College, PA, USA). Differences with $p<0.05$ were considered to be significant. For experiments with PPARα$^{-/-}$ mice, means of the treatment and the control groups were analyzed by one-way ANOVA using the Minitab Statistical Software. For significant F values ($p<0.05$), means of the four groups were compared by Fisher's multiple range test. Differences with $p<0.05$ were considered to be significant.

Table 1
Characteristics of the specific primers used for RT-PCR analysis

Gene	Forward and reverse primers	bp	T_m (°C)	NCBI GenBank
β-Actin (rat)	5′ ATCGTGCGTGACATTAAAGAGAAG 3′ 5′ GGACAGTGAGGCCAGGATAGAG 3′	429	60	BC063166
MCT1 (rat)	5′ AAGCGGAGGAAAAGAAGAGG 3′ 5′ TAGACTAGGGGCCAGCAGAA 3′	217	60	NM_012716
mHMG-CoA synthase (rat)	5′ GGCCTTGGACCGATGCTATGC 3′ 5′ GGGAGGCCTTGGTTTTCTTGTTG 3′	323	58	BC083543
β-Actin (mouse)	5′ ACGGCCAGGTCATCACTATTG 3′ 5′ CACAGGATTCCATACCCAAGAA 3′	87	66	NM_007393
MCT1 (mouse)	5′ GTGACCATTGTGGAATGCTG 3′ 5′ CTCCGCTTTCTGTTCTTTGG 3′	186	60	AF058055
mHMG-CoA synthase (mouse)	5′ CCTCTGTGAATCCTGGGTGT 3′ 5′ CTGTGGGGAAAGATCTGCAT 3′	141	60	NM_008256

3. Results

3.1. Concentration of NEFA and 3-hydroxybutyrate in plasma of fasted rats

Rats fasted for 24 h had higher concentrations of NEFA in plasma than control rats (control: 0.39±0.07 mmol/L, fasted: 0.56±0.17 mmol/L, $p<0.05$). Concentration of 3-hydroxybutyrate in plasma was also higher in fasted rats than in control rats (control: 0.21±0.07 mmol/L, fasted: 0.47±0.09 mmol/L, $p<0.001$).

3.2. Effect of clofibrate treatment or fasting on mRNA concentrations of mHMG-CoA synthase and MCT1 in the liver of rats

In rats treated with the PPARα agonist clofibrate, mRNA concentration of the PPARα target gene mHMG-CoA synthase in liver was 3.6-fold higher than in control rats ($p<0.001$; Fig. 1A). mRNA concentration of MCT1 was 2.7-fold higher in the liver of clofibrate treated rats compared to that of control animals ($p<0.001$; Fig. 1A).

In rats fasted for 24 h, mRNA concentrations of mHMG-CoA synthase and MCT1 in the liver were 23% and 29%, respectively, higher than in rats fed ad libitum ($p<0.05$; Fig. 1B).

3.3. Effect of WY 14,643 or troglitazone on mRNA concentrations of mHMG-CoA synthase and MCT1 in Fao cells

We incubated Fao cells with increasing concentrations of the PPARα agonist WY 14,643 and the PPARγ agonist troglitazone, respectively, for 6 and 24 h. After 6 and 24 h of incubation, mRNA concentration of mHMG-CoA synthase was about 3-fold higher in cells treated with 25, 50 and 100 μM WY 14,643 than in control cells ($p<0.05$; Fig. 2). mRNA concentration of MCT1 was about 1.7-fold higher in cells treated with 25, 50 and 100 μM WY 14,643 for 6 h than in control cells ($p<0.05$; Fig. 2). After 24 h of incubation, mRNA concentration of MCT1 was about 2.2-fold higher in WY 14,643 treated than in control cells ($p<0.05$; Fig. 2). Incubation of Fao cells with 10 and 20 μM troglitazone for 6 and 24 h did not influence mRNA concentration of mHMG-CoA synthase (Fig. 2). After 6 h of incubation, MCT1 mRNA concentration was slightly increased (17%; $p<0.05$) in cells treated with 20 μM troglitazone compared to control cells but remained unchanged after 24 h of incubation (Fig. 2).

3.4. Effect of WY 14,643 on mRNA concentrations of mHMG-CoA synthase and MCT1 in PPARα-null mice

To study the effect of WY 14,643 on activation of PPARα, we determined mRNA concentration of mHMG-CoA synthase in the liver,

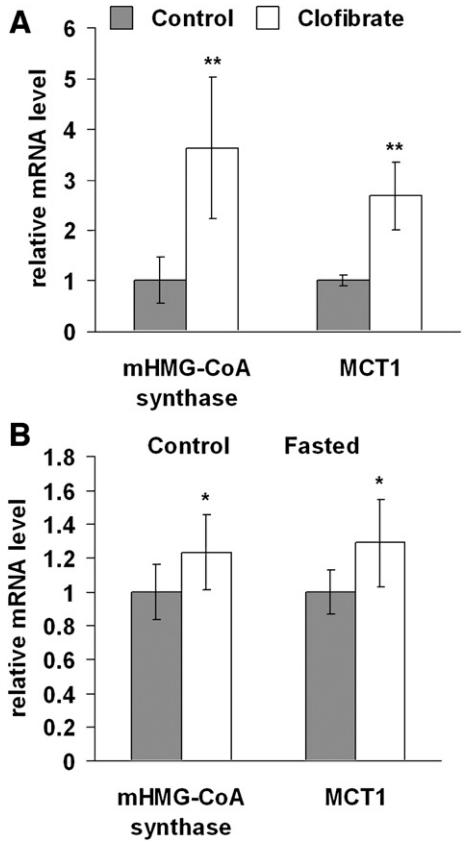

Fig. 1. Effect of the PPARα agonist clofibrate (A) or fasting (B) on the mRNA concentrations of mitochondrial 3-hydroxy-3-methylglutaryl-(mHMG)-CoA synthase and monocarboxylate transporter (MCT)-1 in rat liver. (A) Rats were treated orally with 250 mg/kg of clofibrate for 4 days. Control animals obtained the appropriate volume of the vehicle sunflower oil. (B) Rats were fasted for 24 h before killing, control animals received standard diet ad libitum. Total RNA was extracted from rat livers and mRNA concentrations were determined by realtime detection RT-PCR analysis using β-actin mRNA concentration for normalization. Values are means±SD [n=8 (clofibrate); n=9 (fasting)]. Symbols indicate significant difference from control rats (*p<0.05, **p<0.001).

kidney, heart and intestinal epithelium of mice. Treatment with WY 14,643 increased mRNA concentration of mHMG-CoA synthase in wild-type mice in the liver (5.4-fold), kidney (2.3-fold), heart (2.1-fold) and intestinal epithelium (3.5-fold; p<0.05, Fig. 3). Untreated PPARα$^{-/-}$ mice had a lower mRNA concentration of mHMG-CoA synthase in the liver, kidney and intestinal epithelium than untreated wild-type mice (p<0.05); mRNA concentration of mHMG-CoA synthase in the heart did not differ between these two groups of mice (Fig. 3). In PPARα$^{-/-}$ mice, treatment with WY 14,643 did not increase mHMG-CoA synthase mRNA concentration in any of the tissues analyzed (Fig. 3).

In wild-type mice, mRNA concentration of MCT1 was increased by WY 14,643 treatment in the liver (2.8-fold), kidney (1.5-fold) and intestinal epithelium (1.4-fold; p<0.05) whereas it remained unchanged in the heart (Fig. 4). mRNA concentration of MCT1 in all tissues examined did not differ between control wild-type mice and control PPARα$^{-/-}$ mice (Fig. 4). In PPARα$^{-/-}$ mice, treatment with WY 14,643 failed to increase MCT1 mRNA concentration in any of the tissues analyzed (Fig. 4).

4. Discussion

The adaptive response to fasting which includes an increased synthesis of ketone bodies is mediated by PPARα. This study was designed to elucidate whether MCT1 which is responsible for the transport of ketone bodies is also regulated by PPARα.

In fasted rats, mobilisation of triacylglycerols from adipose tissue caused an increase in the concentrations of NEFA in plasma which then activate PPARα in tissues [20]. Moreover, increased synthesis of ketone bodies upon PPARα activation induced by fasting was indicated by elevated concentration of 3-hydroxybutyrate in plasma of fasted rats. Activation of PPARα by both treatment with the synthetic agonist clofibrate and fasting for 24 h increased the mRNA concentration of mHMG-CoA synthase in the liver of rats. mHMG-CoA synthase has been identified as PPARα target gene [4] and the observation that expression of this gene was up-regulated in the livers of rats treated with clofibrate and in those of fasted rats indicated PPARα activation. In a comparable extent as mHMG-CoA synthase, also MCT1 mRNA was up-regulated in the livers of rats treated with clofibrate and rats fasted for 24 h, respectively. Thus we suggested that also MCT1 is regulated by PPARα at transcriptional level. The up-regulation of both mHMG-CoAS and MCT1 in fasted rats was not as strong as in clofibrate treated rats. In the fasting experiment, we used female rats whereas in the clofibrate experiment male rats were used. Female rats are less responsive to various effects of fibrates than male rats [21] but induction of PPARα mRNA by fasting is similar in male and female rats [22]. Nevertheless we cannot exclude that induction of MCT1 mRNA would be stronger in male rats.

To further analyze up-regulation of MCT1 by PPARα activation, we incubated rat hepatoma Fao cells with the more potent PPARα agonist WY 14,643 that exhibits a more strict PPAR subtype specificity than

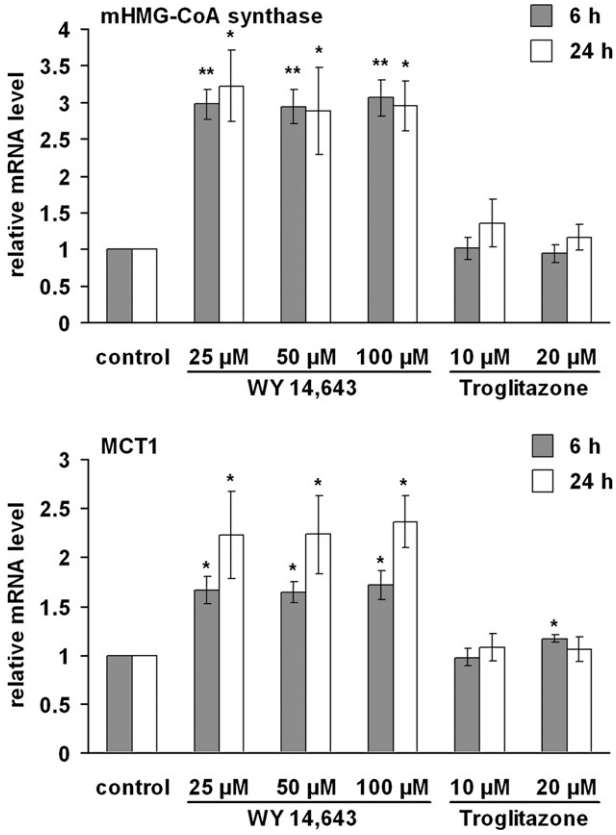

Fig. 2. Effect of the PPARα agonist WY 14,643 and the PPARγ agonist troglitazone on the mRNA concentrations of mitochondrial 3-hydroxy-3-methylglutaryl-(mHMG)-CoA synthase and monocarboxylate transporter (MCT)-1 in Fao cells. Fao cells were grown in culture medium until subconfluent state. Medium was then changed to low-serum medium (0.5% fetal calf serum). After 16 h preincubation in this medium, cells were incubated with increasing concentrations of WY 14,643 (25, 50 and 100 µM) or troglitazone (10 and 20 µM) for 6 and 24 h, respectively. Control cells were incubated with medium containing vehicle alone. Total RNA was extracted from cells and mRNA concentrations were determined by realtime detection RT-PCR analysis using β-actin mRNA concentration for normalization. Values are means±SD (n=3). The asterisks indicate significant differences from control cells (*p<0.05; **p<0.001).

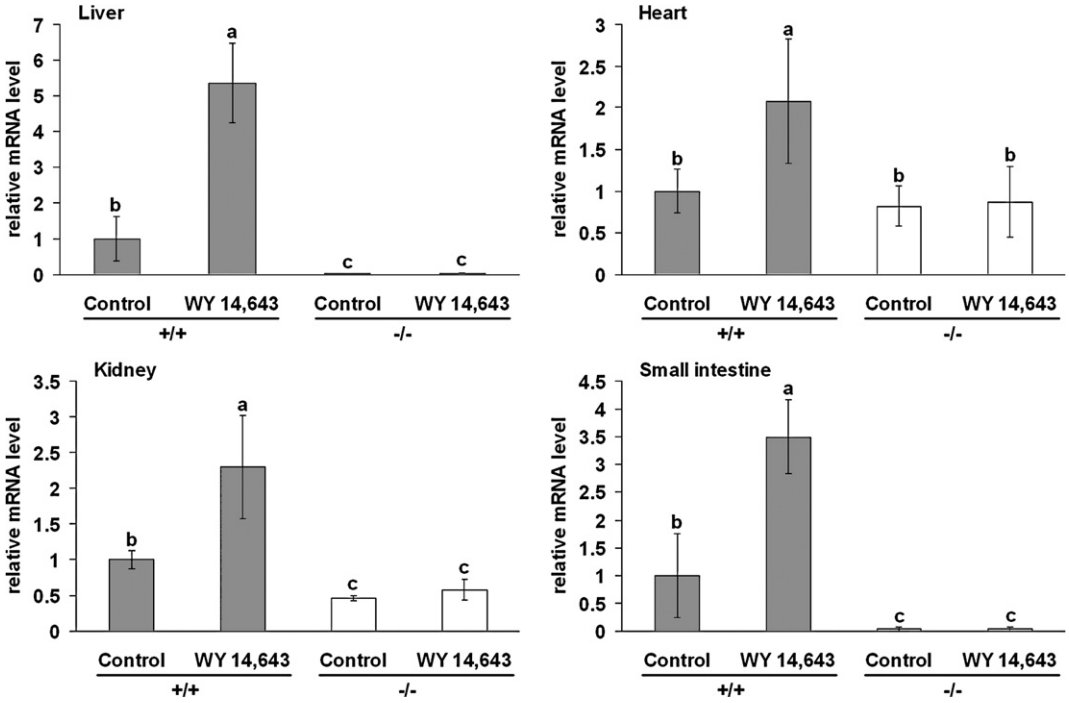

Fig. 3. Effect of WY 14,643 on mRNA concentration of mitochondrial 3-hydroxy-3-methylglutaryl-(mHMG)-CoA synthase in the liver, kidney, heart and intestinal epithelium of wild-type (+/+) and PPARα$^{-/-}$ mice. Mice were treated orally with 40 mg/kg of WY 14,643 for 4 days. Control mice obtained the appropriate volume of vehicle sunflower oil/DMSO. Total RNA was extracted from tissues and mRNA concentrations were determined by realtime detection RT-PCR analysis using β-actin mRNA concentration for normalization. Values are means ± SD (n = 8). Means with unlike letters differ, $p < 0.05$.

clofibrate [23,24] and compared the effects with that of troglitazone, an agonist of PPARγ [25]. Also in Fao cells, activation of PPARα by WY 14,643 was indicated by a comparable strong up-regulation of mHMG-CoA synthase after 6 and 24 h of incubation. In agreement with the results in rat liver, also MCT1 mRNA was up-regulated by WY 14,643, and the effect was stronger after 24 h than after 6 h of incubation. The reason why MCT1 up-regulation in contrast to mHMG-CoA synthase is stronger after 24 than after 6 h of incubation is unknown. A stronger up-regulation of the PPARα target gene cytochrome P450 4A1 after 24 than after 6 h incubation was also observed in Fao cells treated with WY 14,643 whereas mRNA concentration of acyl-CoA oxidase, another PPARα target gene, was more induced after 6 than after 24 h [18]. In

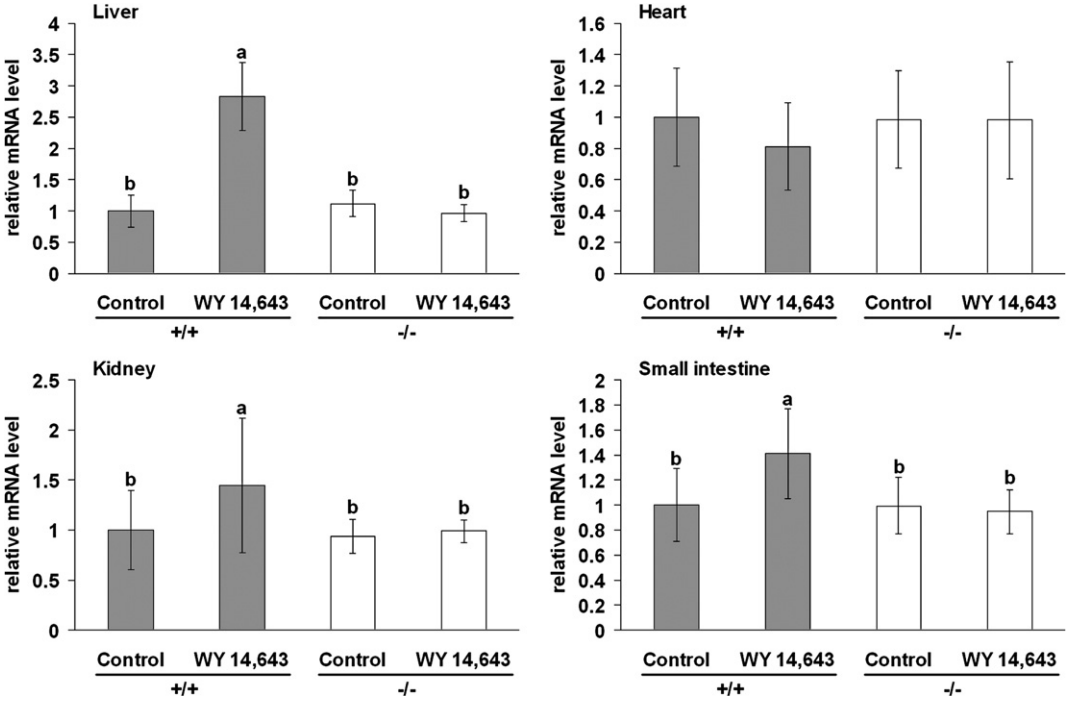

Fig. 4. Effect of WY 14,643 on mRNA concentration of monocarboxylate transporter (MCT)-1 in the liver, kidney, heart and intestinal epithelium of wild-type (+/+) and PPARα$^{-/-}$ mice. Mice were treated orally with 40 mg/kg of WY 14,643 for 4 days. Control mice obtained the appropriate volume of vehicle sunflower oil/DMSO. Total RNA was extracted from tissues and mRNA concentrations were determined by realtime detection RT-PCR analysis using β-actin mRNA concentration for normalization. Values are means ± SD (n = 8). Means with unlike letters differ, $p < 0.05$.

contrast to WY 14,643 treatment, both, mHMG-CoA synthase and MCT1 were not up-regulated by the PPARγ agonist troglitazone indicating that up-regulation of MCT1 is specific to PPARα.

To find out whether up-regulation of MCT1 is indeed mediated by PPARα we treated wild-type and PPARα$^{-/-}$ mice in this study with WY 14,643. To demonstrate PPARα activation, again we determined mRNA concentration of mHMG-CoA synthase which was up-regulated in all four tissues analyzed with the strongest effects in the liver and intestinal epithelium followed by the kidney and heart. These different responses to PPARα activation correlate well with the known tissue distribution of PPARα which is highest in the liver [26–28]. mHMG-CoA synthase mRNA concentration was not influenced in any tissue of PPARα$^{-/-}$ mice by WY 14,643 confirming that there was no activation of PPARα due to the lack of expression in those mice. In wild-type mice, treatment with WY 14,643 increased mRNA concentration of MCT1 in the liver, kidney and intestinal epithelium whereas no up-regulation was observed in PPARα$^{-/-}$ mice. Thus, this study demonstrates for the first time that PPARα is required for induction of MCT1. In this study, we did not determine protein levels of MCT1; nevertheless, the strong up-regulation of MCT1 mRNA concentration suggests that also protein levels of MCT1 are increased upon PPARα activation.

It has been demonstrated that MCT1 is expressed ubiquitously [13] but relatively little is known about its regulation. Regulation of MCT1 mRNA and protein levels in most tissues seems to be responsive to metabolic state of the tissues or the availability of substrate as metabolic fuel and may involve both transcriptional and post-transcriptional mechanisms (reviewed in [15]). For instance, in the colon and brain, MCT1 up-regulation is mediated by butyrate and ketone bodies, respectively [29,30]; regulation of the MCT1 promoter in human intestinal epithelial cells by butyrate involves the NF-κB pathway [31].

In contrast to the liver, kidney and intestinal epithelium, no up-regulation of MCT1 was observed in the heart of wild-type mice. Since activation of PPARα in the heart was comparable with that in the kidney as apparent from up-regulation of mHMG-CoA synthase, the lack of increase of MCT1 in the heart may reflect tissue-specific regulation of MCT1 by PPARα.

Regulation of gene transcription by PPARα is mediated via PPRE in the promoter of its target genes. While the data of this study clearly demonstrate that PPARα is required for up-regulation of MCT1 it remains to be established whether MCT1 indeed is a target gene of PPARα. We analyzed the 5'-flanking region of mouse MCT1 using the PPRE consensus sequence from literature [32] and found at least one putative PPRE at position −610 upstream of the transcription start site of the reported cDNA (NCBI GenBank: AF058055). To determine whether this putative PPRE is indeed functionally active has to be clarified in future experiments by electrophoretic mobility shift assays and reporter gene assays using different deleted or mutated constructs of the MCT1 proximal promoter.

In contrast to mHMG-CoA synthase, expression of MCT1 in control PPARα$^{-/-}$ mice was not lower than in control wild-type mice. This must not necessarily mean that the expression of MCT1 does not directly depend on PPARα since also several other PPARα target genes are expressed in PPARα$^{-/-}$ mice at a level comparable to wild-type mice [33,34].

Recent studies showed that fibroblast growth factor 21 is induced directly by PPARα in the liver and mediates the pleiotropic actions of PPARα. For instance, it stimulates ketogenesis by increasing the protein levels of mHMG-CoA synthase [35]. Thus, it is possible, that up-regulation of MCT1 upon PPARα activation is in part also mediated by a mechanism involving fibroblast growth factor 21.

It has been shown that both intestinal and brain MCT1 have an important role in the transport of exogenous or pharmacologically active compounds such as nicotinate, salicylate, valproate, atorvastatin or pravastatin (reviewed in [15,36]). Thus, the up-regulation of MCT1 by PPARα may also have implications for drug delivery.

In conclusion, this study shows for the first time that PPARα is involved in the regulation of MCT1. During fasting, up-regulation of MCT1 by PPARα may support the supply of cells with energy fuels.

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RESEARCH ARTICLE

Monocarboxylate transporter 1 and CD147 are up-regulated by natural and synthetic peroxisome proliferator-activated receptor α agonists in livers of rodents and pigs

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Monocarboxylate transporter (MCT)-1 mediates the transport of ketone bodies and other monocarboxylic acids across the plasma membrane. MCT1 is up-regulated by peroxisome proliferator-activated receptor (PPAR)-α, a transcription factor that mediates the adaptive response to fasting by up-regulation of genes involved in fatty acid oxidation and ketogenesis. Here, we show for the first time that MCT1 is up-regulated by dietary natural PPAR-α agonists. Both, an oxidized fat and conjugated linoleic acids increased MCT1 mRNA concentration in the liver of rats. Also, in the liver of pigs as non-proliferating species MCT1 was up-regulated upon PPAR-α activation by clofibrate, oxidized fat and fasting. Concomitant with up-regulation of MCT1, mRNA level of CD147 was increased in livers of rats and pigs. CD147 is a plasma membrane glycoprotein that is required for translocation and transport activity of MCT1. CD147 mRNA increase upon PPAR-α activation could not be observed in mice lacking PPAR-α, which also fail in up-regulation of MCT1 indicating a co-regulation of MCT1 and CD147. Analysis of the 5′-flanking region of mouse MCT1 gene by reporter gene assay revealed that promoter activity of mouse MCT1 was not induced by PPAR-α, indicating that the 5′-flanking region is not involved in MCT1 regulation by PPAR-α.

Keywords:
CD147 / Conjugated linoleic acids / Monocarboxylate transporter-1 / Oxidized fat / Peroxisome proliferator-activated receptor-α

1 Introduction

Monocarboxylate transporter (MCT)-1 is a member of the proton-linked MCT family, which is known to comprise at least 14 isoforms [1]. MCT are involved in the transport of monocarboxylates such as lactate, pyruvate and ketone bodies as well as short chain fatty acids and thus are important to energy metabolism, homeostasis and pH control in various tissues [2, 3]. Regulation of MCT has been demonstrated to occur *via* transcriptional, translational and post-transcriptional mechanisms that appear to be age- and tissue-dependent [4–6]. In colonic epithelium, both MCT1 mRNA and protein are up-regulated by butyrate *via* NF-κB-dependent regulation of MCT1 promoter [7, 8]. MCT1 is up-regulated during diet-induced ketosis and by lactate in rat brain and L6 cells, respectively [9, 10]. Furthermore, MCT1 is regulated by its association with the cell surface protein CD147, a widely expressed glycoprotein of the immunoglobulin supergene family [11–13]. The initial association of CD147 and MCT1 is required for the translocation of MCT1 to the plasma membrane [11]. In addition, the continued presence and correct conformation of CD147 are critical for transporter function of MCT1 [14].

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Abbreviations: CLA, conjugated linoleic acids; **MCT,** monocarboxylate transporter; **mHCS,** mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase; **PPAR,** peroxisome proliferator-activated receptor; **PPRE,** PPAR response element; **TBARS,** thiobarbituric acid substance

Recently we could show that MCT1 is up-regulated by peroxisome proliferator-activated receptor (PPAR)-α [15]. PPAR-α is a lipid-activated nuclear hormone receptor that acts as a nutritional state sensor in mammalian cells and mediates an adaptive response to fasting [16, 17]. Upon fasting, it stimulates transcription of a magnitude of genes involving those for synthesis of ketone bodies [18]. Mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase (mHCS) catalysing the first rate-limiting step in ketogenesis is regulated by direct binding of PPAR-α to a functional PPAR response element (PPRE) within its promoter [19].

We could demonstrate that MCT1 mRNA was up-regulated by both fasting and a synthetic PPAR-α agonist in rats and that this increase depends on the presence of PPAR-α since it was absent in mice lacking PPAR-α [15]. Thus, during fasting, up-regulation of MCT1 by PPAR-α may support the supply of cells with energy fuels. However, the mechanism of regulation of MCT1 by PPAR-α is still unknown.

Recently, we and others could demonstrate that PPAR-α is also activated by various naturally occurring lipids such as n-3 fatty acids, conjugated linoleic acids (CLAs) and oxidized fats [20–23]. It was shown that oxidized fats, which comprise a growing part of human nutrition in western countries [24], influence lipid metabolism in rats similar to synthetic PPAR-α agonists [25] and activate PPAR-α also in pigs as a member of non-proliferating species [26]. Non-proliferating species like human and pig have a lower expression of PPAR-α in liver and the response of many genes to PPAR-α activation is much weaker than in proliferating species like rodents [27, 28].

Thus, the aim of the present study was to investigate whether MCT1 is also up-regulated by natural PPAR-α agonists like oxidized fats and CLA. Therefore, we performed an experiment with rats that were orally administered a fresh fat, an oxidized fat, CLA or clofibrate as positive control. We determined expression of MCT1 in liver of rats by measurement of its mRNA concentration using RT-PCR. Taking into account the critical role of CD147 for MCT1 activity [14], we also determined CD147 mRNA concentration. To test whether MCT1 is also up-regulated in non-proliferating species, we conducted an experiment with pigs that were fed a fresh fat, an oxidized fat or clofibrate and analysed expression of MCT1 and CD147 in their livers. In a second experiment with pigs, we tested the effect of fasting on MCT1/CD147 expression in liver. To estimate PPAR-α activation, mRNA level of the PPAR-α target gene mHCS, the key enzyme of ketone body synthesis, was analysed in all experiments. In rat as well as pig experiments we observed an up-regulation of both MCT1 and CD147 upon PPAR-α activation. Treatment of wild-type mice and mice lacking PPAR-α (knockout) with the synthetic PPAR-α agonist WY 14 643 revealed that not only up-regulation of MCT1 but also that of CD147 is absent in PPAR-α knockout mice, indicating a co-regulation of these two genes. In an attempt to analyse the mechanism of PPAR-α-mediated up-regulation of MCT1, we performed promoter activation studies.

2 Materials and methods

2.1 Animals and diets

All experimental procedures described followed guidelines for the care and handling of laboratory animals and were approved by the regional council of Saxony-Anhalt.

2.1.1 Rat experiment

Male Sprague–Dawley rats supplied by Charles River (Sulzfeld, Germany) with an average initial body weight of 115 g (±25 g; SD), were randomly assigned to four groups of nine rats each. They were kept individually in Macrolon cages in a room controlled for temperature (22±2°C), relative humidity (50–60%) and light (12 h light/dark cycle). All rats were fed a commercial standard basal diet (''altromin 1324'', Altromin, Lage, Germany) containing 19% crude protein (herbal source, mainly soy), 4% crude fat, 6% crude fibre and 7% crude ash. To standardize food intake, the diets were fed daily in restricted amounts of 12 g *per* day, equivalent to an intake of 143 kJ metabolizable energy *per* day. Water was available *ad libitum* from nipple drinkers during the whole experiment. All rats were treated with 2 mL of the experimental fat by gavage once a day 2 h after the beginning of the light cycle. The first group (control group) received sunflower oil, the second group (oxidized fat group) oxidized sunflower oil, the third group (CLA group) CLA (BASF, Ludwigshafen Germany) and the fourth group (clofibrate group) 250 mg clofibrate (Fluka, Buchs, Switzerland) *per* kg body weight in 2 mL of sunflower oil.

The oxidized fat was prepared by heating sunflower oil for 25 days at 60°C. The extent of lipid peroxidation was determined by assaying the peroxide value, concentration of thiobarbituric acid substances (TBARS), concentration of conjugated dienes, concentration of total carbonyls, acid values and the percentage of total polar compounds as described earlier [25]. The concentrations of lipid peroxidation products were (oxidized *versus* fresh fat): peroxide value (379 *versus* 3 mEq O_2/kg), TBARS (13.1 *versus* 1.1 mmol/kg), conjugated dienes (274 *versus* <1 mmol/kg), total carbonyls (96.9 *versus* 2.9 mmol/kg), acid value (5.8 *versus* 0.4 g KOH/kg), and polar compounds (27.8 *versus* 5.1%). The CLA oil contained 60 g CLA isomers (29.3 g *c*9*t*11-CLA, 28.9 g *t*10*c*12-CLA, 1.4 g *c*10*c*12-CLA, 0.3 g *t*9*t*11-CLA) *per* 100 g CLA oil as analysed by Ag^+-HPLC-DAD [29]. At day 6 of treatment, rats received the last dose of the experimental fats and 9 g of the diet again 2 h after the beginning of the light cycle and were killed 4 h later by decapitation under light anaesthesia with diethyl ether.

2.1.2 Pig experiment

Twenty-seven male 8-wk-old pigs (German Landrace × Large White × Pietrain), obtained from a local breeder, were kept in a room under controlled temperature of 23 ± 2°C and 50–60% relative humidity with lighting from 06.00 to 18.00. After 1 wk of adaptation the pigs were weighed, randomly assigned to three groups of nine pigs each with mean body weights of 12.0 ± 1.1 kg (control group), 12.2 ± 0.9 kg (oxidized fat group) and 11.9 ± 0.6 kg (clofibrate group) and then fed the experimental diets. The diets were nutritionally adequate for growing pigs [30] and contained 14.4 MJ/kg metabolizable energy. The composition of the experimental diet was (g/kg diet): wheat (400), soy bean meal (230), wheat bran (150), barley (98.1), fat (90) and mineral premix (30) including L-lysine, L-threonine and DL-methionine. The first (control) group obtained fresh fat, the second group (oxidized fat group) oxidized fat and the third group (clofibrate group) fresh fat with additionally 5 g clofibrate *per* kg diet, which was freshly added to the diet each day.

The oxidized fat was sunflower oil that was heated at 200°C for 24 h. The fresh fat was a mixture of sunflower oil and palm oil in a ratio of 93:7. This ratio was chosen to equalize the fatty acid composition of the fresh fat with that of the oxidized fat, since the heating process caused a loss of PUFA. The major fatty acids in the fresh fat and the oxidized fat were (g/100 g total fatty acids): palmitic acid (16:0), 9.0 *versus* 6.8; stearic acid (18:0), 4.0 *versus* 4.2; oleic acid [18:1(n-9)], 24.3 *versus* 24.1; linoleic acid [18:2(n-6)], 60.0 *versus* 60.6; and α-linolenic acid [18:3(n-3)], 0.1 *versus* 0.1. Other fatty acids, including transfatty acids, were present only in traces (< 0.5 g/100 g fatty acids). The extent of lipid peroxidation in the fats after inclusion into the diets was estimated after extraction from the diets with a mixture of hexane and isopropanol according to the method of Hara and Radin [31]. The concentrations of lipid peroxidation products were (oxidized *versus* fresh fat): peroxide value (10.0 *versus* 2.5 mEq O_2/kg), TBARS (271 *versus* 9 μmol/kg), conjugated dienes (89.1 *versus* 22.7 mmol/kg), total carbonyls (24.5 *versus* 2.5 mmol/kg) and acid value (8.0 *versus* 1.6 g KOH/kg). To standardize food intake, each pig received daily 700 g of diet throughout the whole experiment. Water was provided by nipple drinking systems *ad libitum*. The experimental diets were fed for 28 days. After completing of the feeding period the pigs were fed with 300 g of the respective diets 4 h before killing with a captive bolt pistol.

In a second experiment, 20 male, 10-wk-old pigs received a nutritionally adequate diet [30]. One day before the start of the experiment, at an average body weight of 25 kg, the pigs were divided into two groups of ten animals each. At the day of the experiment, the first group (control group) received the diet *ad libitum* for the next 24 h. The second group received their last meal at 8 a.m. and was then fasted for the next 24 h. Pigs were then killed at the morning of the next day either in the fed status (control group) or after a 24-h fasting period (treatment group).

2.1.3 Mouse experiment

Female PPAR-α knockout mice (129S4/SvJae-*Ppara*^{tm1Gonz}/J) and corresponding wild-type control mice (129S1/SvImJ) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Mice from both genotypes with an average initial body weight of 24.3 g (± 3.2; SD) were randomly assigned to two groups and kept individually in Macrolon cages in a room controlled for temperature (22 ± 2°C), relative humidity (50–60%) and light (12-h light/dark cycle). Mice of the treatment groups (wild-type mice $n = 8$ and PPAR-α^{--}mice $n = 8$) obtained 40 mg/kg body weight WY 14 643 once a day 2 h after beginning of the light cycle by gavage for 4 days. WY 14 643 was dissolved in DMSO/sun flower oil (50/50 v/v) at a final concentration of 8 mg/mL as described earlier [32]. Control animals (wild-type mice $n = 8$ and PPAR-α^{--}mice $n = 8$) obtained the appropriate volume of the vehicle DMSO/sun flower oil. All mice were fed with a commercial standard basal diet (''altromin 1324''). To standardize food intake, the diets were fed daily in restricted amounts of 4 g *per* day. Water was available *ad libitum* from nipple drinkers during the whole experiment. At day 4 of treatment, mice received the last dose of WY 14 643 or vehicle alone and 1 g of the diet and were killed 4 h later by decapitation under light anaesthesia with diethyl ether.

2.2 Sample collection

After killing, the livers were excised and samples for RNA isolation were snap-frozen in liquid nitrogen and stored at −80°C.

2.3 RT-PCR analysis

Total RNA isolation from liver samples and cDNA synthesis were carried out as described earlier [20]. The mRNA expression of genes was measured by real-time detection PCR using SYBR® Green I and the Rotor Gene 2000 system (Corbett Research, Mortlake, Australia) as described earlier [15]. Primer pairs were designed using Primer3 software (http://frodo.wi.mit.edu/primer3/) and are listed in Table 1. For determination of mRNA concentration a threshold cycle and amplification efficiency were obtained from each amplification curve using the software RotorGene 4.6 (Corbett Research). Calculation of the relative mRNA concentration was made using the amplification efficiencies and the threshold cycle values [33]. The housekeeping gene β-actin was used for normalization.

2.4 Promoter reporter gene constructs

To identify putative PPRE in the mouse *MCT1* promoter, approximately 2.2 kb of the 5′-flanking region including

Table 1. Characteristics of the specific primers used for RT-PCR analysis

Gene	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)	PCR product size (bp)	T_m (°C)	NCBI GenBank
β-Actin (rat)	ATCGTGCGTGACATTAAAGAGAAG	GGACAGTGAGGCCAGGATAGAG	429	60	BC063166
CD147 (rat)	GGCACCATCGTAACCTCTGT	TCTTTCCCACCTTGATCCTG	265	60	BC059145
MCT1 (rat)	AAGCGGAGGAAAAGAAGAGG	TAGACTAGGGGGCCAGCAGAA	217	60	NM_012716
mHMG-CoA synthase (rat)	GGCCTTGGACCGATGCTATGC	GGGAGGCCTTGGTTTTCTTGTTG	323	58	BC083543
β-Actin (pig)	GACATCCGCAAGGACCTCTA	ACATCTGCTGGAAGGTGGAC	205	60	BC142413
CD147 (pig)	CCTCGGAGACCAAGACAGAG	TCATTCACGTGGTGTCCACT	289	60	EU404087
MCT1 (pig)	GGTGGAGGTCCTATCAGCAG	TCCTGCACGGTGTTACAGAA	122	60	NM_001128445
mHMG-CoA synthase (pig)	GGACCAAACAGACCTGGAGA	ATGGTCTCAGTGCCCACTTC	198	62	NM_214380
β-Actin (mouse)	ACGGCCAGGTCATCACTATTG	CACAGGATTCCATACCCAAGAA	87	66	NM_007393
CD147 (mouse)	ACTGGGGAAGAAGAGGCAAT	AACCAACACCAGGACCTCAG	246	60	BC010270
mHMG-CoA synthase (mouse)	CCTCTGTGAATCCTGGGTGT	CTGTGGGGAAAGATCTGCAT	141	60	NM_008256
MCT1 (mouse)	GTGACCATTGTGGAATGCTG	CTCCGCTTTCTGTTCTTTGG	186	60	AF058055

positions −2142 to +56 relative to the transcription start site (NCBI GenBank NT_039240) were analysed using NUBIScan [34]. Furthermore, the sequence of the first intron starting at position +169 relative to the transcription start site and including about 10 kb was analysed.

The mouse *MCT1* promoter/intron construct containing at least two putative PPRE was generated by PCR amplification of the mouse *MCT1* sequence from positions −1656 to +868 using mouse BAC clone RP23-208N5 (imaGene, Berlin, Germany). The promoter construct pGL4.10-m*MCT1*_−1654/+56 was generated by subcloning of the generated PCR product containing two adapters of SacI and XhoI site into the SacI and XhoI sites of the pGL4.10[luc2] basic reporter vector (encoding the synthetic Firefly luciferase reporter gene; Promega, Mannheim, Germany). The PCR primer sequences were as follows: 5′-atcgagctccgaactcagaaatctgcctg-3′ and 5′-tagctcgaggctccttggctgctgcac-3′. The reporter gene construct pGL4.23-m*MCT1*_+151/+868 containing sequences from the first intron including at least one putative PPRE was generated by subcloning of the generated PCR product containing two adapters of XhoI and HindIII site into the XhoI and HindIII sites of the pGL4.23[luc2/minP] reporter vector (containing a minimal promoter sequence and the synthetic Firefly luciferase reporter gene; Promega). The PCR primer sequences were as follows: 5′-atcctcgagcacacataacggttag-3′ and 5′-tagaagcttatgtactcggcaaagggatg-3′. After cloning, the integrity and fidelity of all m*MCT1* constructs were verified by DNA sequencing.

2.5 Transient transfection and dual luciferase assay

For promoter activation studies, HepG2 cells were used, which are an accepted model for studying the regulation of genes by PPAR-α [35–37]. HepG2 cells (about 70% confluent) were transiently transfected with either the generated MCT1 promoter constructs, negative control vector pGL4.10-m*MCT1*_0 and pGL4.23-m*MCT1*_0, respectively, or positive control vector 3 × ACO PPRE (containing three copies of consensus PPRE from the ACO promoter in front of a luciferase reporter gene; this vector was a generous gift from Dr. Sander Kersten, Nutrigenomics Consortium, Top Institute Food and Nutrition, Wageningen, The Netherlands), and pGL4.74[hRluc/TK] (encoding the Renilla luciferase reporter gene; Promega), which was used as an internal control reporter vector to normalize for differences in transfection efficiency, using FuGENE 6 transfection reagent (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol. In addition, co-transfections with mouse *PPAR*-α expression plasmid pCMX-m*PPAR*-α and mouse *RXR*-α expression plasmid pCMX-m*RXR*-α (both, generous gifts from R. M. Evans, Salk Institute for Biological Studies, San Diego, CA, USA) were carried out. Subsequently, cells were treated with either 100 μM WY 14 643 or vehicle (DMSO = control) for 24 h.

Afterwards the cells were washed with phosphate-buffered saline and lysed with passive lysis buffer (Promega). Luciferase activities were determined with the Dual-Luciferase Reporter Assay System from Promega according to the manufacturer's instructions using a Mithras LB940 luminometer (Berthold Technologies, Bad Wildbad, Germany) as described earlier [38].

2.6 Statistics

Data of all experiments were analysed using the Minitab Statistical Software (Minitab, State College, PA, USA). Treatment effects of rat and pig experiments were evaluated by one-way ANOVA. For significant F values ($p<0.05$), means of the treatments (fasting, clofibrate, oxidized fat, CLA) were compared pairwise with the control group by Student's t test. Treatment effects of mice experiment were analysed by two-way ANOVA with classification factors being treatment (WY 14 643), genotype and the interaction of treatment (WY 14 643) and genotype. In all experiments, means were considered significantly different for $p<0.05$.

3 Results

3.1 Effect of PPAR-α agonists on MCT1/CD147 expression in rat liver

mRNA concentration of the PPAR-α target gene mHCS was higher in rats fed oxidized fat, CLA or clofibrate compared with control (fresh fat) rats ($p<0.05$; Fig. 1), indicating activation of PPAR-α by all agonists tested. Furthermore, both MCT1 and CD147 mRNA concentrations were higher in the liver of rats fed oxidized fat, CLA or clofibrate compared with control rats ($p<0.05$; Fig. 1).

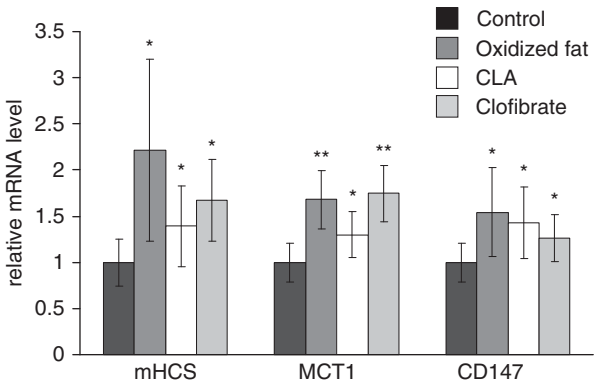

Figure 1. Effect of oxidized fat, CLA and clofibrate on the mRNA concentrations of mHCS, MCT1 and CD147 in rat liver. Rats were treated orally with fresh fat (control group), oxidized fat, CLA or 250 mg/kg of clofibrate for 6 days. Total RNA was extracted from rat livers and mRNA concentrations were determined by real-time detection RT-PCR analysis using β-actin mRNA concentration for normalization. Values are means ± SD ($n=9$). Symbols indicate significant differences from control rats (*$p<0.05$, **$p<0.001$).

3.2 Effect of PPAR-α agonists and fasting on MCT1/CD147 expression in pig liver

mHCS was up-regulated by both oxidized fat and clofibrate in the liver of pigs compared with pigs fed a fresh fat (control group), indicating PPAR-α activation ($p<0.05$; Fig. 2A). mRNA concentration of MCT1 was higher in the liver of pigs fed the oxidized fat or clofibrate than in the livers of control pigs ($p<0.05$; Fig. 2). CD147 mRNA level was higher in the liver of clofibrate-treated than in that of control pigs ($p<0.05$) but was not influenced by oxidized fat treatment (Fig. 2A). Fasting of pigs for 24 h led to a potent activation of PPAR-α in liver as indicated by a strong up-regulation of mHCS compared with non-fasted (control) pigs ($p<0.001$, Fig. 2B).

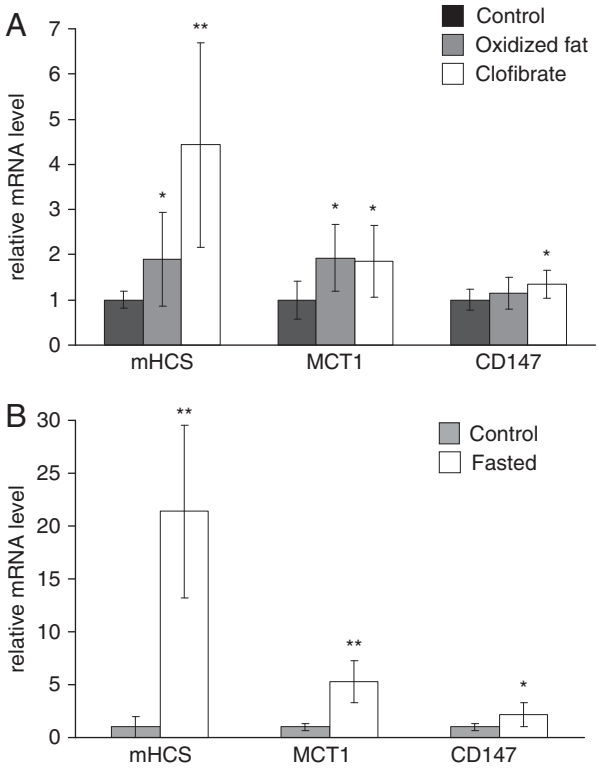

Figure 2. Effect of oxidized fat, clofibrate (A) and fasting (B) on the mRNA concentrations of mHCS, MCT1 and CD147 in pig liver. (A) Pigs were fed a diet containing fresh fat (control group), oxidized fat or 5 g/kg diet of clofibrate for 28 days. (B) Pigs were fasted for 24 h before killing; control animals received standard diet *ad libitum*. Total RNA was extracted from pig livers and mRNA concentrations were determined by real-time detection RT-PCR analysis using β-actin mRNA concentration for normalization. Values are means ± SD ($n=9$ (oxidized fat/clofibrate); $n=10$ (fasting)). Symbols indicate significant differences from control rats (*$p<0.05$, **$p<0.001$).

Furthermore, both, MCT1 ($p<0.001$) and CD147 mRNA ($p<0.05$) concentrations were higher in the liver of fasted than that of control pigs (Fig. 2B).

3.3 Effect of WY 14 643 on MCT1/CD147 expression in PPAR-α knockout mice

As already observed [15], both mHCS and MCT1 mRNA concentrations increased in wild-type mice treated with WY 14 643 but were unchanged in PPAR-α knockout mice ($p<0.05$; Fig. 3). Also, CD147 mRNA was higher in livers of wild-type mice treated with WY 14 643 compared with control mice, whereas this up-regulation was not observed in PPAR-α knockout mice ($p<0.05$; Fig. 3).

3.4 Analysis of MCT1 promoter activity

Sequence analysis of the 5′-flanking region and the first intron of mouse MCT1 using NUBIScan revealed putative PPRE at positions −622 to −615 and +437 to +449 relative to the transcription start site. To test whether mouse MCT1 promoter is activated by PPAR-α, we cloned the 5′-regulatory region of mouse MCT1 (sequence spanning from −1654 to +56) into a luciferase reporter vector, and transiently transfected HepG2 cells with the promoter reporter construct, which was named pGL4.10-m*MCT1*_−1654/+56. As shown in Fig. 4, luciferase activity of HepG2 cells transiently transfected with the pGL4.10-m*MCT1*_−1654/+56 construct was much higher than that of cells transfected with negative control vector pGL4.10-m*MCT1*_0, indicating strong transcriptional activity of mouse *MCT1* promoter. However, the PPAR-α ligand WY 14 643 did not increase the luciferase activity in HepG2 cells transiently transfected with the pGL4.10-m*MCT1*_−1654/+56 construct but that of HepG2 cells transiently transfected with the positive control vector 3 × ACO PPRE (Fig. 4). In addition, co-transfection of mouse *PPAR*-α expression plasmid pCMX-m*PPAR*-α and mouse *RXR*-α expression plasmid pCMX-m*RXR*-α did not increase transcriptional activity of mouse *MCT1* promoter in the absence or presence of WY 14 643. In contrast, luciferase activity of HepG2 cells transfected with the positive control vector 3 × ACO PPRE was strongly increased upon co-transfection of pCMX-m*PPAR*-α and pCMX-m*RXR*-α in the absence and presence of WY 14 643, indicating the functionality of the assay (Fig. 4). In a second trial, we cloned the first 700 bp of the first intron of mouse *MCT1* (sequence spanning from +151 to +868) into a luciferase reporter vector with minimal promoter. Transfection of HepG2 cells with the resulting plasmid pGL4.23-m*MCT1*_+151/+868 did not increase luciferase activity of the cells both, in the presence of WY 14 643 and upon co-transfection with expression plasmids for mouse *PPAR*-α and mouse *RXR*-α (data not shown).

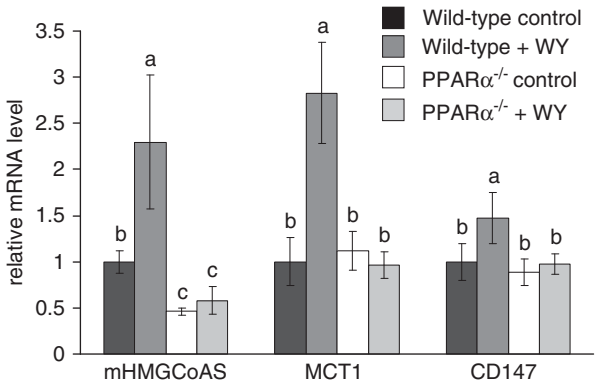

Figure 3. Effect of WY 14 643 on the mRNA concentrations of mHCS, MCT1 and CD147 in the liver of wild-type and PPAR-$\alpha^{-/-}$ (knockout) mice. Mice were treated orally with 40 mg/kg of WY 14 643 for 4 days. Control mice obtained the appropriate volume of vehicle sunflower oil/DMSO. Total RNA was extracted from livers and mRNA concentrations were determined by real-time detection RT-PCR analysis using β-actin mRNA concentration for normalization. Values are means ± SD ($n=8$). Means with unlike letters differ significantly ($p<0.05$). Significant effects ($p<0.05$) from two-way ANOVA: mHCS: genotype, WY 14 643, WY 14 643 × genotype; MCT1: genotype, WY 14 643, WY 14 643 × genotype; CD147: genotype, WY 14 643, WY 14 643 × genotype.

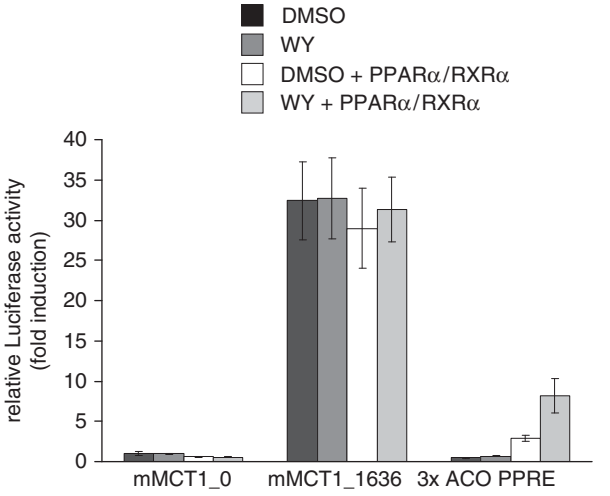

Figure 4. Effect of the PPAR-α agonist WY 14 643 on the promoter activity of mouse MCT1 in HepG2 cells. HepG2 cells were transiently transfected with either pGL4.10-m*MCT1*_−1654/+56 construct containing one putative PPRE of mouse *MCT1* promoter, negative control vector pGL4.10-m*MCT1*_0 or positive control vector 3 × ACO PPRE, and internal control vector pGL4.74. In addition, co-transfections with mouse *PPAR*-α expression plasmid pCMX-m*PPAR*-α and mouse *RXR*-α expression plasmid pCMX-m*RXR*-α were carried out. Subsequently, cells were treated with 100 μM WY 14 643 or vehicle (DMSO = control) for 24 h. Afterwards cells were lysed and luciferase activities were determined. Relative luciferase activity is shown as the ratio of *Firefly*/*Renilla* luciferase. One representative result from three independent experiments is presented; values are means ± SD from three replicates.

4 Discussion

Recently, we could demonstrate that MCT1, which is responsible for transport of ketone bodies and other monocarboxylic acids in and out of cells, is up-regulated by PPAR-α, which was activated by synthetic ligands and fasting, respectively, in rats and mice [15]. The present study was conducted to examine whether MCT1 expression is also influenced by dietary natural PPAR-α agonists. Here, we show that feeding of rats with both, an oxidized fat and CLA, increased MCT1 mRNA concentration in liver compared with control rats fed a fresh fat, in a comparable extent as observed for the synthetic PPAR-α agonist clofibrate. Furthermore, also mRNA concentration of mHCS was up-regulated in liver of rats upon treatment with oxidized fat and CLA, respectively, indicating PPAR-α activation. mHCS catalyses the first rate-limiting step in ketogenesis and has a functional PPRE within its promoter [19]. It is known that oxidized fatty acids and CLA are also able to activate PPAR-γ [39, 40], indicating that also PPAR-γ activation by oxidized fat and CLA may contribute to the observed induction of MCT1. Nevertheless, in our previous study we could show that the synthetic and selective PPAR-γ agonist troglitazone did not increase MCT1 mRNA in Fao rat hepatoma cells [15]. Thus, we conclude that up-regulation of MCT1 by CLA and oxidized fat is rather mediated by activation of PPAR-α than PPAR-γ. Therefore, the data of the present study indicate that the dietary PPAR-α agonists oxidized fat and CLA have similar effects on ketone body synthesis and transport than synthetic PPAR-α agonists in rats. This is in accordance with previous studies showing that dietary oxidized fats exert similar effects on cholesterol metabolism and carnitine homeostasis as synthetic PPAR-α agonists in rats [25, 41].

Since pigs as well as humans belong to the so-called non-proliferating species that response differently upon PPAR-α activation compared with proliferating species as rats and mice [27, 28], pigs represent a suitable model for humans to study the effects of PPAR-α activation. It has been already shown that clofibrate, oxidized fat as well as fasting activate PPAR-α in liver and other tissues of pigs [26, 27, 42]. This is consistent with the up-regulation of mHCS in the liver of pigs treated with clofibrate or oxidized fat as well as fasted pigs compared with control groups observed in this study. Concomitant with the raise in mHCS mRNA, also up-regulation of MCT1 was observed in the liver of pigs of the treatment groups compared with control pigs. Thus, not only in proliferating species like rats and mice but also in pigs as a model for non-proliferating species up-regulation of ketogenesis upon PPAR-α activation is accompanied by an up-regulation of ketone body transporter MCT1. Since MCT1 is also involved in the transport of pharmaceutical agents like γ-hydroxybutyrate, salicylic acid and statins [43] up-regulation of MCT1 upon dietary intake of oxidized fats or CLA may also have implications for drug delivery in humans.

The chaperone and transmembrane protein CD147 is essential for the correct membrane assembly and transporter function of MCT1 [11, 14]. Several studies demonstrated that up-regulation of MCT1 by different stimuli was accompanied by an up-regulation of its chaperone CD147 [44–46]. Furthermore, silencing studies showed that maturation and cell surface expression of CD147 depend on MCT1 expression [47, 48]. Also in our study, up-regulation of MCT1 was generally accompanied by an increase of CD147 mRNA level supporting that CD147 activity is regulated by MCT1. In accordance with that, CD147 up-regulation upon PPAR-α activation could not be observed in PPAR-α knockout mice, which also failed in up-regulation of MCT1. However, in other studies, maturation and surface expression but not mRNA level of CD147 were affected [45, 47], indicating differences in the regulatory mechanisms.

Transcriptional regulation of genes by PPAR is mediated by binding of activated PPAR/retinoid X receptor heterodimers to specific DNA sequences, called PPRE present in and around the promoter of PPAR target genes [49, 50]. Furthermore, several studies demonstrate functionally active PPRE in intronic sequences of PPAR-α target genes [51–53]. Whether or not the up-regulation of MCT1 is mediated by binding of PPAR-α to PPRE sequences within the *MCT1* gene is not known. *In silico* analysis of mouse MCT1 promoter and the first intron revealed at least to putative PPRE at positions −622 to −615 and +437 to +449 relative to the transcription start site with high homology to the consensus PPRE (AGGTCAAAGGTCA). To determine whether these putative PPRE are fuctional, we cloned the proximal promoter region and the respective part of the first intron, respectively, in luciferase reporter vectors. However, for both reporter gene constructs, no induction of promoter activity by the PPAR-α agonist WY 14 643, with or without co-expression of m*PPAR*-α/m*RXR*-α, could be observed. This indicates that mouse *MCT1* gene is probably not regulated by direct binding of PPAR-α/RXR-α to sequences in the 5′-regulatory region. Also, the putative PPRE in the analysed first intron seems not to be responsible for regulation of mouse *MCT1* by PPAR-α. We cannot exclude that other than the analysed sequences of mouse *MCT1* gene are responsible for its regulation by PPAR-α. On the other hand, an indirect regulation of MCT1 by PPAR-α by interference with other regulatory pathways or proteins that regulate MCT1 expression is possible. Substrate-induced up-regulation of MCT1 was demonstrated [4, 10]. However, since incubation of Fao cells with β-hydroxybutyrate and acetoacetate did only slightly increase MCT1 mRNA concentration (data not shown) other mechanisms than substrate induced activation of MCT1 by increased ketone body concentrations may account for the observed up-regulation of MCT1 by PPAR-α.

In conclusion, this study shows that natural PPAR-α agonists like constituents of oxidized fats and CLA up-regulate MCT1 in liver. Also, in pigs that belong as humans to the non-proliferating species, MCT1 was up-regulated upon PPAR-α activation by clofibrate, oxidized fat and fasting. Since MCT1 is involved in the transport of pharmaceutical agents, up-regulation of MCT1 by dietary PPAR-α

agonists may also have implications for drug delivery. We could also show that the chaperone CD147, which is essential for translocation and activity of MCT1, is co-induced with MCT1 also in a PPAR-α-dependent manner. However, the mechanism of up-regulation of MCT1 by PPAR-α seems not to involve a transcriptional action of PPAR-α on the 5′-flanking region of mouse *MCT1*.

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The authors have declared no conflict of interest.

5 References

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Research Article

Fasting Upregulates PPARα Target Genes in Brain and Influences Pituitary Hormone Expression in a PPARα Dependent Manner

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PPARα is a lipid-activable transcription factor that mediates the adaptive response to fasting. Recent data indicate an important role of brain PPARα in physiological functions. However, it has not yet been shown whether PPARα in brain can be activated in the fasting state. Here we demonstrate that fasting of rats increased mRNA concentrations of typical PPARα target genes implicated in β-oxidation of fatty acids (acyl-CoA oxidase, carnitine palmitoyltransferase-1, medium chain acyl-CoA dehydrogenase) and ketogenesis (mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase) in pituitary gland and partially also in frontal cortex and diencephalon compared to nonfasted animals. These data strongly indicate that fasting activates PPARα in brain and pituitary gland. Furthermore, pituitary prolactin and luteinizing hormone-β mRNA concentrations were increased upon fasting in wild-type mice but not in mice lacking PPARα. For proopiomelanocortin and thyrotropin-β, genotype-specific differences in pituitary mRNA concentrations were observed. Thus, PPARα seems to be involved in transcriptional regulation of pituitary hormones.

1. Introduction

The ability of animals to survive during food deprivation requires biochemical and physiological responses to the lack of food. The overall metabolic response to fasting operates at numerous levels, and peroxisome proliferator-activated receptor (PPAR)-α which belongs to the family of nuclear hormone receptors mediates an adaptive response to fasting. PPARα is directly activated by nonesterified fatty acids (NEFAs) [1, 2] which are liberated from adipose tissue into plasma by lipolytic stimuli and acts as a nutritional state sensor by stimulating the transcription of genes involved in fatty acid uptake through membranes, fatty acid binding in cells, peroxisomal and mitochondrial fatty acid oxidation, ketone body synthesis as well as glycogenolysis and gluconeogenesis [3]. All of these metabolic responses are crucial to shift the body fuel utilization from carbohydrates and fat in the fed state to almost exclusively fat and ketone bodies during fasting and to maintain blood glucose levels to provide tissues such as brain with sufficient amounts of glucose.

PPARα is expressed mostly in tissues with high rates of fatty acid oxidation and peroxisomal metabolism like brown adipose tissue, liver, kidney, and heart [3] but is also expressed in different regions of the brain [4]. Several studies exist that indicate an important role of brain PPARα in physiological functions like neuroprotection [5] and the control of whole-body glucose homeostasis during fasting [6]. Furthermore, specific upregulation of the prolactin gene in a pituitary cell line by PPARα was shown [7] indicating a possible role of PPARα in transcriptional regulation of pituitary hormone production.

Until now it is not known whether PPARα in brain is indeed activated during fasting. It was shown that administration of the PPARα agonist ciprofibrate upregulates typical PPARα target genes such as mitochondrial 3-hydroxy-3-methylglutaryl-(mHMG)-CoA synthase and, to a lesser extent, acyl-CoA oxidase (ACO) and medium

chain acyl-CoA dehydrogenase (MCAD; [8]) and increases the rate of fatty acid oxidation in brain homogenates [9].

Thus, this study was designed to test whether fasting would result in an activation of PPARα in brain of rats. PPARα activation was measured by determination of mRNA concentrations of the typical PPARα target genes such as ACO, carnitine palmitoyltransferase (CPT)-1, medium chain acyl-CoA dehydrogenase (MCAD), and mHMG-CoA synthase. A clofibrate-treated group was included to compare the effects of fasting with those of PPARα activation by a synthetic ligand. For analysis, we choose frontal cortex, part of the diencephalon, and the pituitary gland which are known to express PPARα in detectable amounts [4]. We could show a significant upregulation of PPARα target genes particularly in pituitary gland of rats. Considering that and the described regulation of prolactin gene by PPARα in a pituitary cell line [7], we analysed a possible role of brain PPARα in regulation of pituitary hormones during fasting. For that, we analysed mRNA concentrations of prolactin, proopiomelanocortin (POMC), luteinizing hormone (LH)-β, follicle-stimulating hormone (FSH)-β, growth hormone (GH) and thyrotropin (TSH)-β in pituitary gland of fasted and fed PPARα knockout and corresponding wild-type mice. Obtained data indicate an involvement of PPARα in transcriptional regulation of pituitary hormones.

2. Materials and Methods

2.1. Animal Experiments. Animals were kept individually in Macrolon cages in a room controlled for temperature ($22 \pm 2^{\circ}$C), relative humidity (50–60%), and light (12 hours light/dark cycle). All experimental procedures described followed established guidelines for the care and handling of laboratory animals and were approved by the council of Saxony-Anhalt. Male Sprague-Dawley rats, with an average initial body weight of 258 g ($\pm$17; SD), were randomly assigned to three groups ($n = 9$). All rats were fed a commercial standard basal diet ("altromin 1324," Altromin GmbH, Lage, Germany). To standardize food intake, the diets were fed daily in restricted amounts of 22 g per day. Water was available ad libitum from nipple drinkers during the whole experiment. The animals were treated with 250 mg/kg of clofibrate in 1 mL sunflower oil (clofibrate group) or with an equal volume of the vehicle sunflower oil (control group and fasting group) by gavage once a day 2 hours after beginning of the light cycle. Animals of the fasting group were fasted 36 hours before killing. During food deprivation, they obtained water instead of sunflower oil by gavage. At day 4 of treatment, animals received the last dose of clofibrate, sunflower oil, and water, respectively, and 7 g of the diet (except fasting group) and were killed 4 hours later by decapitation under light anaesthesia with diethyl ether. Blood was collected into heparinized polyethylene tubes. The brains were removed and dissected. For analysis, the pituitary gland, frontal cortex, and the ventral part of diencephalon were used. The liver was excised. Brain and liver samples for RNA isolation were snap-frozen in liquid nitrogen and stored at -80°C.

Female PPARα knockout mice (129S4/SvJae-*Ppara*^{tm1Gonz}/J) and corresponding wild-type control mice (129S1/SvImJ) were purchased from the Jackson Laboratory (Bar Harbor, USA). Mice from both genotypes with an average initial body weight of 27.4 g ($\pm$1.4; SD) were randomly assigned to two groups. Mice of the control groups (wild-type mice $n = 10$ and PPARα knockout mice $n = 10$) were fed ad libitum with a commercial standard basal diet (altromin 1324). Mice of the fasting groups (wild-type mice $n = 10$ and PPARα knockout mice $n = 10$) were fasted 48 hours before killing. Mice were killed by decapitation under light anaesthesia with diethyl ether. For RNA analysis, the liver and pituitary gland were excised, snap-frozen in liquid nitrogen, and stored at -80°C.

2.2. RT-PCR Analysis. Total RNA isolation from tissues and cDNA synthesis were carried out as described [10]. For RNA isolation from pituitary glands of mice, tissue samples of two animals were pooled. The mRNA expression of genes was measured by real-time detection PCR using SYBR Green I and the Rotor Gene 2000 system (Corbett Research, Mortlake, Australia) as described in [11]. Annealing temperature for all primer pairs (Operon Biotechnologies, Cologne, Germany; Table 1) was 60°C (exception: mouse mHMG-CoA synthase, TSHβ, 58°C; mouse β-actin, 66°C). For determination of mRNA concentration a threshold cycle (C_t) and amplification efficiency were obtained from each amplification curve using the software Rotor Gene 4.6 (Corbett Research). Calculation of the relative mRNA concentration was made using the amplification efficiencies and the C_t values [12]. The housekeeping gene β-actin was used for normalization.

2.3. Concentration of NEFA. The concentration of NEFA in plasma was determined using an enzymatic reagent kit (Wako Chemicals GmbH, Neuss, Germany).

2.4. Statistics. Data of all experiments were analyzed using the Minitab Statistical Software (Minitab, State College, PA, USA). Treatment effects of rat experiment were evaluated by one-way ANOVA. For significant F values ($P < .05$), means of the treatments (fasting, clofibrate) were compared pairwise with the control group by Student's t-test. Treatment effects of mice experiment were analyzed by two-way ANOVA with classification factors being treatment (fasting), genotype and the interaction of treatment (fasting) and genotype. In all experiments, means were considered significantly different for $P < .05$.

3. Results

3.1. Final Body Weights of Rats and Mice. Final body weight of rats was significantly influenced by fasting but not by clofibrate treatment (control: 265 $\pm$ 18 g; fasting: 241 $\pm$ 12 g; clofibrate treatment: 266 $\pm$ 17 g; mean $\pm$ SD, $n = 9$). Final body weights of fasted rats were lower than those

Table 1: Characteristics of the specific primers used for RT-PCR analysis.

Gene	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)	PCR product size (bp)	NCBI GenBank
β-Actin (rat)	ATCGTGCGTGACATTAAAGAGAAG	GGACAGTGAGGCCAGGATAGAG	429	BC063166
ACO (rat)	CTTTCTTGCTTGCCTTCCTTCTCC	GCCGTTTCACCGCCTCGTA	415	NM017340
CPT1A (rat)	GGAGACAGACACCATCCAACATA	AGGTGATGGACTTGTCAAACC	416	NM031559
CPT1C (rat)	CATCTCCAGCAAGCAATCAA	GATCCCCAATACCCCTGTCT	299	BC105882
LCAD (rat)	AAGGATTTATTAAGGGCAAGAAGC	GGAAGCGGAGGCGGAGTC	380	NM012849
MCAD (rat)	CAAGAGAGCCTGGGAACTTG	CCCCAAAGAATTTGCTTCAA	154	NM016986
Mitochondrial HMG-CoA synthase (rat)	GGCCTTGGACCGATGCTATGC	GGGAGGCCTTGGTTTTCTTGTTG	323	BC083543
PPARα (rat)	CCCTCTCTCCAGCTTCCAGCCC	CCACAAGCGTCTTCTCAGCCATG	555	NM013196
β-Actin (mouse)	ACGGCCAGGTCATCACTATTG	CACAGGATTCCATACCCAAGAAG	87	NM007393
ACO (mouse)	CAGGAAGAGCAAGGAAGTGG	CCTTTCTGGCTGATCCCATA	189	NM015729
LHβ (mouse)	GTCCCAGGACTCAACCAATG	GGGAGGGAGGGATGATTAGA	110	NM008497
Mitochondrial HMG-CoA synthase (mouse)	CCTCTGTGAATCCTGGGTGT	CTGTGGGGAAAGATCTGCAT	141	NM008256
POMC (mouse)	GGGTCCCTCCAATCTTGTTT	GCACCAGCTCCACACATCTA	137	NM008895
Prolactin (mouse)	CTCAGGCCATCTTGGAGAAG	TCGGAGAGAAGTCTGGCAGT	174	NM011164
FSHβ (mouse)	AGGGAGGAAAGGAAAGTGGA	AGCCAGCTTCATCAGCATTT	202	NM008045
GH (mouse)	ACGCGCTGCTCAAAAACTAT	GCTAGAAGGCACAGCTGCTT	120	NM008117
TSHβ (mouse)	TCAACACCACCATCTGTGCT	TCTGACAGCCTCGTGTATGC	239	NM009432

of nonfasted (control) rats ($P < .05$). Final body weights of mice were significantly influenced by fasting and the genotype (wild-type control, 26.4 ± 1.4 g; wild-type fasting, 23.5 ± 1.5 g; PPARα-knockout control, 27.8 ± 1.4 g; PPARα-knockout fasting, 24.4 ± 1.4 g; mean ± SD; $n = 10$). Final body weights of fasted mice were lower than those of non-fasted mice ($P < .05$); final body weights of PPARα-knockout mice were higher than those of wild-type mice ($P < .05$). The interaction of fasting and genotype had no effect on final body weights.

3.2. Concentrations of NEFA in Plasma of Rats. Fasted rats had higher concentrations of NEFA in plasma than control rats whereas those of clofibrate treated rats were unchanged (control: 234 ± 37 μmol/L; fasted: 676 ± 162; clofibrate: 216 ± 32 μmol/L; $n = 9$ for each group; $P < .05$).

3.3. mRNA Concentrations in Rat Brain and Pituitary Gland. To elucidate a possible activation of PPARα in rat brain by fasting, we measured mRNA concentrations of typical PPARα target genes in frontal cortex, diencephalon, and pituitary gland of fasted and non-fasted rats. mRNA concentration of ACO was higher in pituitary gland of fasted rats compared to control rats ($P < .05$; see Figure 1). Oral treatment of a third group of rats with the synthetic PPARα agonist clofibrate did not change mRNA concentration of ACO in all brain areas examined and pituitary gland compared to control rats (Figure 1).

Concentration of CPT1A mRNA, which represents the widely expressed liver isoform of CPT1 [13], was higher in pituitary gland of fasted rats compared to control rats ($P < .05$; see Figure 1). In clofibrate treated rats, CPT1A mRNA concentration was higher in frontal cortex compared to control rats ($P < .05$; Figure 1). mRNA concentration of CPT1C, the brain specific isoform of CPT1 [14], was higher in pituitary gland of fasted rats compared to control rats ($P < .05$; Figure 1). Clofibrate treatment did not change mRNA concentration of CPT1C in all brain areas examined and pituitary gland compared to control rats (Figure 1). MCAD mRNA concentration was higher in pituitary gland of fasted rats compared to control rats ($P < .05$; Figure 1). Clofibrate treatment did not change mRNA concentration of MCAD in all examined tissues (Figure 1). In fasted and clofibrate treated rats, mRNA concentrations of LCAD were higher in frontal cortex and pituitary gland than those in control rats ($P < .05$; Figure 1).

mHMG-CoA synthase mRNA concentration was higher in frontal cortex, diencephalon, and pituitary gland of fasted and clofibrate treated compared to control rats ($P < .05$; Figure 1).

Fasting tended to increase the mRNA concentration of PPARα in pituitary gland compared to nonfasting ($P = .073$; Figure 1). Clofibrate treatment did not alter mRNA concentration of PPARα in all brain areas examined and pituitary gland compared to control rats (Figure 1).

3.4. mRNA Concentrations in Rat Liver. In liver, mRNA concentrations of ACO, CPT1A, LCAD, and PPARα were higher in fasted than in control rats ($P < .05$; Figure 2). In the liver of clofibrate treated rats, mRNA concentrations

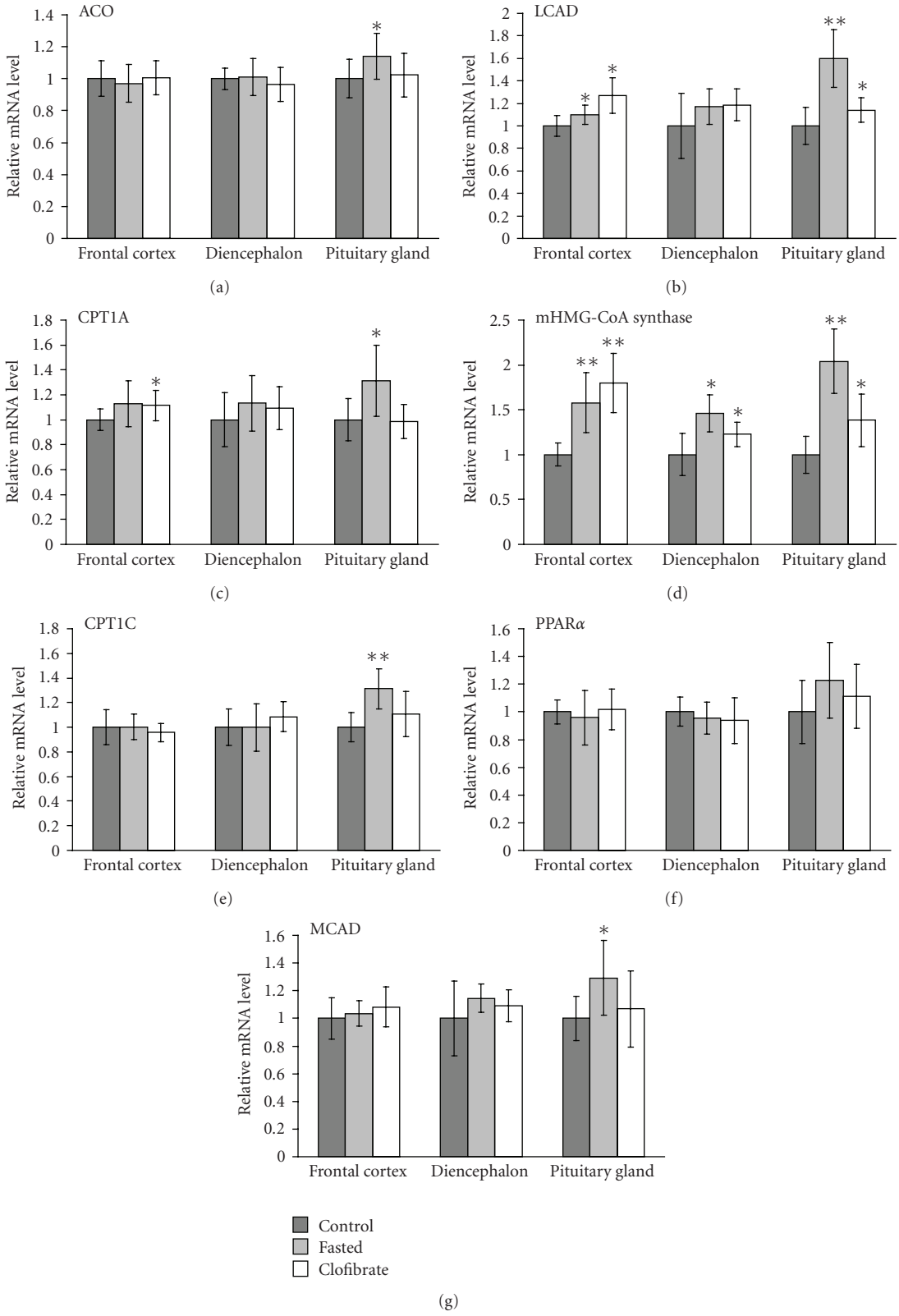

Figure 1: Effect of fasting or clofibrate treatment on mRNA concentration of acyl-CoA oxidase (ACO), carnitine palmitoyltransferases (CPT)-1A and -1C, medium chain acyl-CoA dehydrogenase (MCAD), long chain acyl-CoA dehydrogenase (LCAD), mitochondrial 3-hydroxy-3-methylglutaryl-(mHMG)-CoA synthase, and peroxisome proliferator-activated receptor (PPAR)-α in different brain areas of rats. All rats obtained a standard basal diet for 4 days. Animals of the fasting group were fasted 36 hours before killing. Rats of the clofibrate group were treated orally with 250 mg/kg of clofibrate per day. Values are means ± SD ($n = 9$). Symbols indicate significant difference from control rats ($^{*}P < .05$, $^{**}P < .001$).

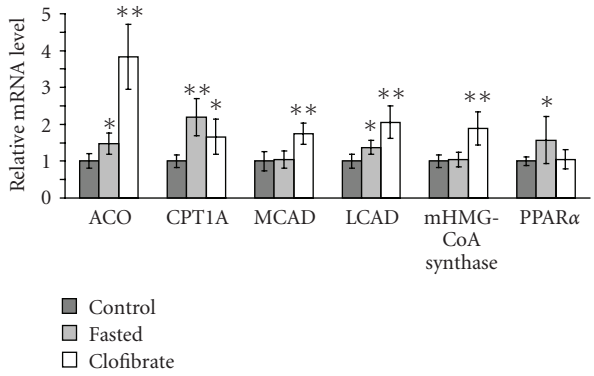

Figure 2: Effect of fasting or clofibrate treatment on mRNA concentration of acyl-CoA oxidase (ACO), carnitine palmitoyltransferases (CPT)-1A, medium chain acyl-CoA dehydrogenase (MCAD), long chain acyl-CoA dehydrogenase (LCAD), mitochondrial 3-hydroxy-3-methylglutaryl-(mHMG)-CoA synthase, and peroxisome proliferator-activated receptor (PPAR)-α in the liver of rats. All rats obtained a standard basal diet for 4 days. Animals of the fasting group were fasted 36 hours before killing. Rats of the clofibrate group were treated orally with 250 mg/kg of clofibrate per day. Values are means ± SD ($n = 9$). Symbols indicate significant difference from control rats ($^{*}P < .05$, $^{**}P < .001$).

of ACO, CPT1A, MCAD, LCAD and mHMG-CoA synthase were higher than in control rats ($P < .05$; Figure 2).

3.5. mRNA Concentrations in Pituitary Gland of PPARα Knockout and Wild-Type Mice. To elucidate a possible role of PPARα in expression of pituitary hormones, wild-type and PPARα knockout mice were fasted for 48 hours. In pituitary gland, the PPARα target gene ACO was not significantly increased in fasted mice compared to non-fasted mice of both genotypes (Figure 3). Fasting led to an increase of mHMG-CoA synthase mRNA concentration in pituitary gland of wild-type mice ($P < .05$; see Figure 3). Fasting did also increase mHMG-CoA synthase mRNA in PPARα knockout mice but this effect was less than that observed in wild-type mice ($P < .05$; Figure 3). For pituitary mHMG-CoA synthase, there was a tendency to interaction of genotype and fasting ($P = .090$). For comparison, mRNA levels of ACO and mHMG-CoA synthase were also measured in liver of mice. Liver ACO mRNA concentration was lower in PPARα knockout than in wild-type mice ($P < .05$) but was not increased upon fasting for 48 hours in both genotypes (Figure 3). Liver mHMG-CoA synthase was increased upon fasting in wild-type mice but not in PPARα knockout mice and was lower in PPARα knockout mice than in wild-type mice ($P < .05$; see Figure 3). There was a significant interaction of genotype and fasting for liver mHMG-CoA synthase mRNA ($P < .05$).

Next we analysed mRNA concentrations of prolactin, POMC, LHβ, FSHβ, GH, and TSHβ in pituitary gland of fasted and non-fasted wild-type and PPARα knockout mice. mRNA concentration of prolactin in pituitary gland was higher in fasted than in non-fasted wild-type mice ($P < .05$; see Figure 4). In PPARα knockout mice, fasting caused a decrease of prolactin mRNA concentration ($P < .05$; see Figure 4). Prolactin mRNA concentration of wild-type and PPARα knockout control (fed) mice did not differ. For prolactin mRNA concentration in pituitary gland, there was an interaction of genotype and fasting ($P < .05$).

mRNA concentration of POMC in pituitary gland was higher in fasted than in non-fasted mice of both genotypes ($P < .05$; see Figure 4). POMC mRNA concentration was higher in fasted PPARα knockout mice than in fasted wild-type mice ($P < .05$; see Figure 4). There was no difference in POMC mRNA levels between non-fasted groups of both genotypes.

Fasting led to an increase of mRNA concentration of LHβ in pituitary gland of wild-type ($P < .05$) mice but not of PPARα knockout mice (Figure 4). For LHβ mRNA concentration, there was a tendency to interaction of genotype and fasting ($P = .053$). FSHβ mRNA concentration in pituitary gland was higher in fasted than in non-fasted mice of both genotypes ($P < .05$; see Figure 4). For GH mRNA concentration, no significant differences were observed between all four groups of mice (Figure 4).

Fasting led to a significant decrease of TSHβ mRNA in pituitary gland of both wild-type and PPARα knockout mice ($P < .05$; Figure 4). Furthermore, TSHβ mRNA level was lower in PPARα knockout mice than in the corresponding group of wild-type mice ($P < .05$; see Figure 4).

4. Discussion

This study was designed to investigate the effect of fasting on the expression of PPARα target genes in rat brain. As expected, food deprivation for 36 hours led to a considerable loss of body weight of the rats. Mobilization of triacylglycerols from adipose tissue by fasting moreover caused an increase in the concentrations of NEFA in plasma whereas clofibrate treatment did not change plasma NEFA concentrations of the rats.

In this study, we could demonstrate for the first time that fasting of rats for 36 hours upregulates mRNA concentration of ACO, CPT1, MCAD, LCAD, and mHMG-CoA synthase in pituitary gland and, partially, also in frontal cortex and diencephalon of the brain. All enzymes are typical PPARα target genes or PPARα responsive genes (LCAD; reviewed in [3]), and the observed upregulation of these genes during fasting strongly indicates PPARα activation in brain during food deprivation. This finding supports the hypothesis of Knauf et al. [6] that PPARα activation in brain is vital during fasting.

It has been shown that PPARα is well expressed in various brain areas and that it is colocalized with ACO indicating that also in brain tissue peroxisomal β-oxidation is regulated by PPARα [4, 15]. Although it is generally thought that the liver supplies the brain with ketone bodies as a glucose-replacing fuel, for example, during fasting, it has been demonstrated that fatty acids are oxidized by the brain and isolated astrocytes [9, 16] and that cultured astrocytes are able to synthesize ketone bodies from fatty acids [16] assuming that astrocytes may provide neurons with ketone

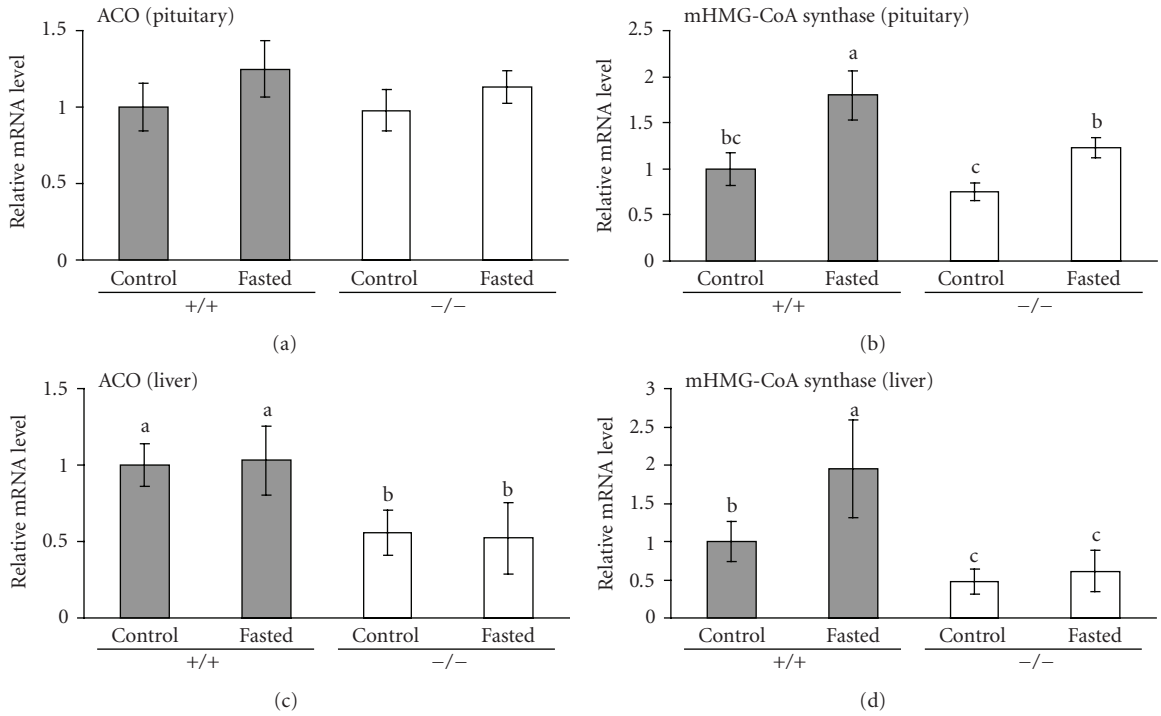

Figure 3: Effect of fasting on mRNA concentrations of acyl-CoA oxidase (ACO) and mitochondrial 3-hydroxy-3-methylglutaryl-(mHMG)-CoA synthase in pituitary gland and liver of wild-type (+/+) and PPARα knockout mice (−/−). Mice of both genotypes were either fasted for 48 hours (control group) or fed a standard rodent diet ad libitum for 48 hours (fed group). Livers and pituitary glands of mice were excised, pituitary glands from two animals were pooled, total RNA was extracted, and mRNA abundances were determined by real-time detection RT-PCR analysis using β-actin for normalization. Bars represent means ± SD (liver: $n = 10$; pituitary: $n = 5$). Means without a common letter differ, $P < .05$. Significant effects ($P < .05$) from two-way ANOVA: mHMG-CoA synthase (pituitary): genotype, fasting, fasting × genotype ($P = .09$); ACO (liver): genotype; mHMG-CoA synthase (liver): fasting, genotype, fasting × genotype.

bodies as a glucose-replacing fuel in vivo. This hypothesis is supported by our findings that genes involved in β-oxidation of fatty acids (ACO, CPT1, MCAD, LCAD) and mHMG-CoA synthase which is implicated in ketogenesis are upregulated upon fasting in rat brain and pituitary gland.

Among the PPARα target genes analysed in brain, mHMG-CoA synthase was upregulated strongest by fasting. Regarding the different brain areas tested, upregulation of PPARα responsive genes in general was stronger in pituitary gland than in frontal cortex and diencephalon. mHMG-CoA synthase and LCAD were the only PPARα responsive genes that were considerably upregulated by clofibrate treatment in brain, with the strongest effects in frontal cortex followed by pituitary gland and diencephalon. These distinct relative intensities of upregulation in the three brain areas observed by fasting and clofibrate treatment indicate that the different responses in frontal cortex, diencephalons, and pituitary gland upon fasting are probably not due to different expression levels of PPARα.

In general, the effect of fasting on PPARα responsive genes in brain was much stronger than that of clofibrate treatment. In contrast, in the liver, upregulation of most of these genes was much stronger in clofibrate treated than in fasted rats. This discrepancy may reflect the different access of PPARα agonists to the brain. It has been demonstrated that fibrates can access the brain but that they cross the blood-brain barrier slowly [9, 17]. During fasting, hydrolysis of triacylglycerols in adipose tissue is stimulated leading to increased concentrations of plasma NEFA which then activate PPARα in the liver. We suggest that the observed activation of PPARα in brain by fasting was accomplished by the strong increase in NEFA concentration in plasma of fasted rats. It has been demonstrated that the access of circulating NEFA to the central nervous system is generally proportional to their plasma concentration [18]. Thus, although both, clofibrate, and NEFA are potent PPARα agonists [19], the PPARα activation by NEFA released upon fasting is stronger than that of orally administered clofibrate.

NEFA that are released upon fasting also upregulate PPARα transcription in the liver [20], as could be also observed in rats fasted for 36 hours in this study. In brain, PPARα was slightly but not significantly ($P = .073$) upregulated only in pituitary gland which corresponds well with the upregulation pattern of its target genes which was also the strongest in this area. Clofibrate treatment of rats did not change mRNA concentration of PPARα which is in agreement with other studies showing that PPARα activation by fibrates does not necessarily upregulate expression of PPARα [21, 22].

In the liver of rats, there was weak or even no upregulation of PPARα responsive genes after 36 hours of fasting. This is consistent with data showing that mRNA concentrations of

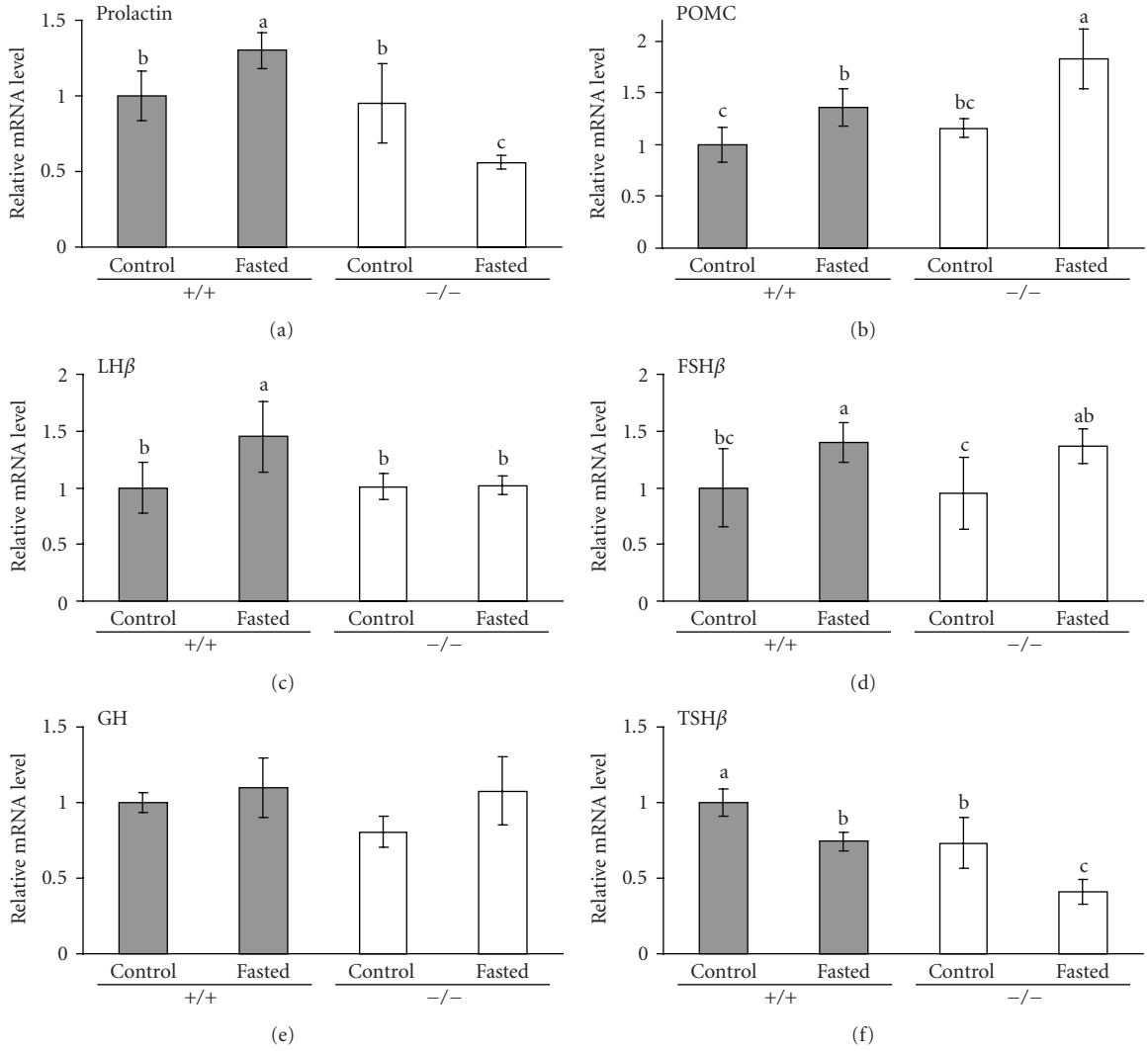

FIGURE 4: Effect of fasting on mRNA concentrations of prolactin, proopiomelanocortin (POMC), luteinizing hormone (LH)-β, follicle-stimulating hormone (FSH)-β, growth hormone (GH), and thyrotropin (TSH)-β in pituitary gland of wild-type (+/+) and PPARα knockout mice (−/−). Mice of both genotypes were either fasted for 48 hours (control group) or fed a standard rodent diet ad libitum for 48 hours (control group). Pituitary glands of mice were excised, pooled from two animals, total RNA was extracted, and mRNA abundances were determined by real-time detection RT-PCR analysis using β-actin for normalization. Bars represent means ± SD ($n = 5$). Means without a common letter differ, $P < .05$. Significant effects ($P < .05$) from two-way ANOVA: prolactin: genotype, fasting × genotype; POMC: fasting, genotype; LHβ: fasting, fasting × genotype ($P = .053$); FSHβ: fasting; TSHβ: fasting, genotype.

PPARα target genes in the liver of rats are upregulated after 24 hours of fasting, but decrease after longer fasting times to control levels [23]. In a preliminary experiment, we analysed expression of PPARα target genes in brain of rats fasted for 24 hours. Interestingly, upregulation of PPARα target genes in brain after 24 hours of fasting was much weaker than after 36 hours of fasting (data not shown) indicating that activation of PPARα in brain is delayed compared to that in liver. Further experiments regarding this time dependency of PPARα activation in brain and liver may be helpful to explain this phenomenon.

As already mentioned, the prolactin gene is activated by PPARα as demonstrated in a rat pituitary tumor cell line and in reporter gene assays [7]. Thus, our results with fasted rats prompted us to investigate whether PPARα activation upon fasting is involved in regulation of hormone production in pituitary gland. Fasting led to an upregulation of mHMG-CoA synthase in pituitary gland of wild-type mice indicating PPARα activation. However, also in PPARα knockout mice mRNA concentration of mHMG-CoA synthase was increased upon fasting but not as strong as in wild-type mice. In contrast, the upregulation of mHMG-CoA synthase in livers of wild-type mice upon fasting could not be observed in the livers of PPARα knockout mice indicating the PPARα-dependent regulation of mHMG-CoA synthase in liver. Thus, additional mechanisms seem to be involved in upregulation of mHMG-CoA synthase mRNA in pituitary gland upon fasting. It has been shown that in skeletal muscle PPARβ/δ can compensate the lack of PPARα in regulation of fatty acid homeostasis during starvation [24]. Since PPARβ/δ

is also ubiquitously expressed in brain [4] it is possible that it can compensate at least in part the lack of PPARα mediated upregulation of mHMG-CoA synthase in brain of PPARα knockout mice upon fasting. The fact that no upregulation of ACO was observed in liver and pituitary gland of mice fasted for 48 hours is consistent with the marginal upregulation of ACO observed in rats after 36 hours of fasting and is attributed to the increased fasting time [23].

Analysis of mRNA concentrations of several genes of pituitary hormones in wild-type and PPARα knockout mice revealed that prolactin gene mRNA was upregulated 1.3-fold in wild-type mice upon fasting but was downregulated about 50% in fasted PPARα knockout mice. Thus, our data demonstrate for the first time that the prolactin gene is transcriptionally regulated by PPARα in vivo and support the findings of Tolon et al. [7]. They demonstrated that the rat prolactin promoter is stimulated by PPARα by a mechanism different from that commonly described for PPARα target genes involving interaction of the PPARα/RXRα heterodimer complex with a PPRE [7].

Besides prolactin, also for the LHβ mRNA a genotype-dependent response to fasting was observed. The 1.5-fold upreguation of LHβ mRNA concentration by fasting in wild-type mice was not observed in PPARα knockout mice (P value for interaction of treatment (fasting) and genotype: .053). This indicates that PPARα may also be involved in regulation of LHβ expression in pituitary gland during fasting. Fasting also led to about 1.4-fold increases in pituitary FSHβ mRNA concentration; however this effect was observed both in wild-type and in PPARα knockout mice indicating that PPARα is not involved in regulation of FSHβ mRNA during fasting in mice.

POMC mRNA concentration was higher in pituitary gland of PPARα knockout than of wild-type mice and it was induced in both genotypes upon fasting. POMC, a multifunctional precursor protein of a number of bioactive peptides, is produced both in pituitary gland and hypothalamus. Interestingly, recently it was described that in the fed state hypothalamic POMC mRNA concentration is also higher in PPARα knockout than in wild-type mice; however it was downregulated upon 24 hours of fasting in both genotypes [6]. This different behaviour of POMC mRNA upon fasting may result from the fact that POMC expression in hypothalamus and pituitary is controlled by independent sets of enhancers [25].

During fasting, mRNA concentration of TSHβ was reduced in pituitary gland of both wild-type and PPARα knockout mice. Downregulation of TSHβ is part of a series of changes in the hypothalamic-pituitary-thyroid axis [26]. Interestingly, TSHβ mRNA concentration was lower in mice lacking PPARα than in wild-type mice indicating a role for PPARα in regulation of TSHβ that should be analysed in future studies.

5. Conclusions

In conclusion, the data of the present study show for the first time that fasting of rats upregulates typical PPARα target genes in frontal cortex, diencephalon, and pituitary gland. This strongly indicates that free fatty acids released upon energy restriction from adipose tissue activate PPARα not only in liver but also in brain. Besides a possible role of brain PPARα in regulation of ketone body synthesis it seems to be also involved in control of pituitary hormone production. Thus, regarding the multitude of genes regulated by PPARα, not only PPARα activation in liver, but also activation of PPARα in brain seems to be an important step in adoption to fasting.

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3 Zusammenfassende Diskussion

In den nachfolgenden Abschnitten werden die wichtigsten Ergebnisse der eigenen Arbeiten (**A1-A14**) zusammenfassend dargestellt und deren Relevanz im übergeordneten Kontext diskutiert. Auf eine detaillierte Diskussion der Ergebnisse sei an dieser Stelle verzichtet. Hierfür wird auf die entsprechenden Originalarbeiten verwiesen. Ebenso sind Hintergrund bzw. Zielstellung, Material und Methoden sowie die ausführliche Beschreibung der Ergebnisse den entsprechenden Originalarbeiten zu entnehmen.

3.1 Beeinflussung des SREBP-abhängigen Lipidstoffwechsels durch PPARα

Die günstige Beeinflussung des Lipidstoffwechsels durch Fibrate über eine Aktivierung des PPARα stellt die Grundlage für ihren erfolgreichen Einsatz als Therapeutikum von Fettstoffwechselstörungen dar. Dabei basieren die triglyzeridsenkenden Effekte der Fibrate hauptsächlich auf einer direkten Beeinflussung der Lipolyse und des Fettsäurekatabolismus durch PPARα [94]. Sowohl Fibrate als auch natürliche PPARα-Agonisten, wie PUFA und oxidierte Fette, bewirken außerdem eine Verringerung der Cholesterolkonzentrationen in Plasma und Leber, allerdings weniger effektiv als Statine [26-29]. Studien mit PPARα-*knockout*-Mäusen deuten darauf hin, dass PPARα in die Regulation der hepatischen Cholesterolsynthese involviert ist [95-97]. Der zentrale Regulator der Cholesterol- und Triglyzeridsynthese bei Säugern sind die *sterol regulatory element–binding proteins* (SREBP). Deshalb wurde untersucht, inwiefern eine PPARα-Aktivierung regulatorisch die SREBP-abhängige Cholesterol- und Triglyzeridsynthese beeinflusst. Die Rolle von natürlichen nutritiven PPARα-Agonisten wurde dabei ebenso betrachtet wie der Einfluss der Spezies.

3.1.1 PPARα-abhängige Regulation von Insig

SREBP sind eine Familie von Transkriptionsfaktoren, die die Expression von Genen der Lipidsynthese und -aufnahme kontrollieren und damit gemeinsam mit PPAR eine zentrale Stellung in der Aufrechterhaltung der Lipidhomöostase innehaben [98]. Die Isoform SREBP-1c reguliert dabei Gene der Fettsäure- und Triglyzeridsynthese, während SREBP-2 Gene der Cholesterolsynthese und –aufnahme kontrolliert. SREBP-1a reguliert sowohl Gene der Fettsäure- und Triglyzeridsynthese als auch Gene der Cholesterolsynthese [99]. Die Aktivität der SREBP wird in Abhängigkeit von der Sterolkonzentration in der Zelle durch einen Mechanismus, der die proteolytische Abspaltung der transkriptionell aktiven Domäne aus einem Vorläuferprotein nach Transport aus der ER-Membran in den Golgi beinhaltet, reguliert [99-101]. Der zentrale Regulator, der die Reifung der SREBP in Abhängigkeit von verschiedenen zellulären Signalen kontrolliert, sind die *insulin-induced genes* (Insig)-1 und -2

[102, 103]. Ihre komplexe und unterschiedliche Regulation [103-107] erlaubt die Kontrolle der Lipidsynthese über einen großen Sterolkonzentrationsbereich und bei verschiedenen Insulin-Konzentrationen.

Unsere eigenen Untersuchungen (**A1**) zeigten, dass die beobachteten verringerten Cholesterolkonzentrationen in Plasma und Leber der Ratte bei Aktivierung des PPARα durch Clofibrat auf einer Verringerung des nuklearen SREBP-2 beruhen, die durch eine Erhöhung der Expression des Insig-1 zustande kommt (**A1**). Infolge dessen kommt es zu einer verminderten Expression der SREBP-Zielgene HMG-CoA-Reduktase und *low density lipoprotein* (LDL)-Rezeptor und somit zu einer verringerten Cholesterolsynthese und -aufnahme. Damit konnte ein neuartiger Zusammenhang zwischen PPARα und dem Cholesterolstoffwechsel aufgezeigt werden. Den Mediator für die Beeinflussung des Cholesterolstoffwechsels durch PPARα stellt dabei Insig-1 dar. Dieses neuartige Zusammenspiel zwischen PPARα und SREBP-2 kann auch ein möglicher Erklärungsansatz für die bei PPARα-*knockout*-Mäusen beobachteten erhöhten Cholesterolkonzentrationen und die fehlende Abhängigkeit der hepatischen Expression von Genen der Cholesterolsynthese vom tageszeitlichen Rhythmus sein [95-97].

Sowohl unsere eigenen Daten aus *in vitro*-Studien mit Rattenhepatoma-Zellen (**A1**) als auch Literaturdaten sprechen für eine direkte transkriptionelle Aktivierung des Insig-1-Genes durch PPARα. Es konnte bereits gezeigt werden, dass das humane Insig-1-Gen über ein PPRE in der 5'-flankierenden Region sowohl durch PPARγ [108] als auch durch PPARβ/δ [109] reguliert wird. In eigenen Untersuchungen konnten wir ebenfalls eine Aktivierung des Maus-Insig-1-Promotors durch PPARα feststellen, eine Zuordnung dieses Effektes zu einem funktionell aktiven PPRE steht jedoch noch aus (unveröffentlichte Daten).

Die Regulation des Insig-1-Genes durch PPARγ bzw. PPARβ/δ scheint vor allem die SREBP-1-abhängige Lipogenese zu beeinflussen. Durch eine Überexpression des PPARβ/δ in übergewichtigen diabetischen *db/db*-Mäusen konnten über eine Insig-1-Induktion die SREBP-1-Aktivierung und die Expression von Genen der Fettsäuresynthese gehemmt und dadurch die Ausprägung einer Fettleber gemildert werden [109]. Die Regulation des Insig-1-Genes durch PPARγ beeinflusst vermutlich die SREBP-1c-abhängige Lipogenese im Fettgewebe von *db/db*-Mäusen [108]. Unsere eigenen Untersuchungen (**A2**) konnten zeigen, dass die Regulation des Insig-1 durch PPARα nicht nur die SREBP-2-abhängige Cholesterolsynthese, sondern auch die durch SREBP-1c regulierte Fettsäure- und Triglyzeridsynthese in Rattenhepatoma-Zellen beeinflusst. Eine Induktion von Insig-1 durch den synthetischen PPARα-Agonisten WY 14,643 führte in Fao-Zellen zu verminderten nukleären Konzentrationen des SREBP-1 und verringerten Expressionen von Schlüsselenzymen der Fettsäure- und Triglyzeridsynthese, was sich letztlich in einer

verminderten Syntheserate von Triglyzeriden und verringerten Triglyzeridkonzentrationen manifestierte (**A2**). Damit konnte erstmalig gezeigt werden, dass der triglyzeridreduzierende Effekt von Fibraten zum Teil auf einer Verminderung der Synthese von Fettsäuren und Triglyzeriden beruht, die auf eine Inhibierung der Reifung des SREBP-1 über eine Induktion des Insig-1 zurückzuführen ist. Eine Hemmung der SREBP-1c-Aktivität durch PPARα-Aktivierung wurde auch über eine Inhibierung der Aktivität des *liver X receptor* (LXR) beschrieben [110], welcher nach Bindung von spezifischen LXR-Agonisten die Transkription des SREBP-1c Vorläuferproteins aktiviert [111-113]. PPARα vermindert dabei durch Konkurrenz um den gemeinsamen Heterodimerpartner RXRα die Bildung eines funktionellen LXRα/RXRα-Komplexes [110]. Dieser Effekt konnte allerdings nur bei gleichzeitiger LXRα-Aktivierung beobachtet werden, so dass seine Relevanz für eine Fibratwirkung unklar ist. Eine inhibitorische Wirkung der PPAR auf die Gentranskription, welche auch als Transrepression bezeichnet wird, stellt neben der Transaktivierung einen zweiten grundlegenden Mechanismus PPAR-vermittelter Effekte in Zellen dar. Diese Transrepression erfolgt über die Verhinderung der Bindung von Transkriptionsfaktoren (z.B. *nuclear factor kappaB*, *activator protein*-1) durch Protein-Protein-Wechselwirkungen oder durch Bindung für die Gentranskription essenzieller Cofaktoren und ist die Grundlage für die beobachtete entzündungshemmende Wirkung der PPAR [12, 114-116]. Die in den eigenen Arbeiten beschriebene Inhibierung der SREBP-Aktivität durch eine Aktivierung des PPARα über Insig stellt somit einen weiteren Mechanismus dar, der zur Inhibierung der Gentranskription durch PPARα führt.

In Übereinstimmung mit der beschriebenen Regulation des Insig-1-Genes durch PPARγ [108] konnten wir in unseren Untersuchungen zeigen, dass der synthetische PPARγ-Agonist Troglitazon ebenfalls über eine Induktion von Insig zu einer Inhibierung des SREBP-1c führte (**A2**). Synthetische PPARγ-Agonisten, so genannte Thiazolidindione, werden als Therapeutika zur Verminderung der Insulin-Resistenz eingesetzt [117]. Sie haben ebenso wie Fibrate triglyzeridsenkende Eigenschaften [118, 119], die unter anderem auf erhöhte Lipoproteinlipase-Aktivitäten zurückzuführen sind [119, 120]. Die von uns gezeigte Inhibierung der Triglyzeridsynthese in Fao-Zellen über eine Hemmung der SREBP-1c-Aktivität in Folge einer PPARγ-Aktivierung stellt somit einen weiteren Mechanismus dar, der der triglyzeridsenkenden Wirkung der Thiazolidindione zugrunde liegt.

Sowohl bei PPARα- als auch PPARγ-Aktivierung konnte in Fao-Zellen zusätzlich zur erhöhten Expression von Insig-1 eine Induktion des Insig-2a festgestellt werden (**A2**). Insig-2a ist eine leberspezifische Isoform des Insig-2, die durch Insulin reprimiert wird [105]. Die insulinabhängige Regulation der SREBP-1c-Aktivität über Insig-2a stellt vermutlich eine Möglichkeit zur Stimulation der Fettsäuresynthese bei hohen Cholesterolkonzentrationen dar

[105]. Eine besondere Rolle des Insig-2a für die SREBP-1c-Regulation wird auch durch die Daten unserer Untersuchungen unterstützt. Diese zeigten, dass bei einer PPARγ-Aktivierung ein besonders starker Anstieg des Insig-2a auftrat, der sich in einer – im Vergleich zur WY 14,643-Behandlung - schnelleren Absenkung der SREBP-1c-Aktivität widerspiegelte (**A2**). Somit lassen unsere Ergebnisse vermuten, dass nicht nur Insig-1, sondern auch Insig-2a transkriptionell durch PPARα und PPARγ reguliert wird. Weitere Hinweise dazu finden sich auch in einer Studie zur Regulation des Insig-2a-Genes über ein funktionelles Vitamin D *response element*, in der gleichzeitig die Regulation des Insig-2a-Promotors durch PPARα und den Thyroidhormonrezeptor als unveröffentlichte Daten erwähnt wird [121]. Diese komplexen Regulationsmechanismen verdeutlichen die Schlüsselstellung des Insig-2a in der Regulation der Lipidhomöostase.

Zusammenfassend konnten wir in unseren eigenen Untersuchungen einen neuartigen Regulationsmechanismus aufzeigen, über den PPARα und auch PPARγ in die Lipidhomöostase über eine Inhibierung der Aktivität der SREBP eingreifen. Auch wenn eindeutige Beweise noch ausstehen, scheint die Induktion von Insig-1 dabei vor allem für die Beeinflussung der Cholesterolsynthese über SREBP-2 und die des Insig-2a für die Inhibierung der Fettsäure- und Triglyzeridsynthese über SREBP-1c entscheidend zu sein, was auch mit den Hinweisen aus Literaturdaten übereinstimmt [102, 103].

Unklar bleibt, welche Relevanz die Regulation der Insig durch PPARα generell für die Regulation der SREBP-Aktivität in Säugern, abseits einer „künstlich“ herbeigeführten PPARα-Aktivierung, z. B. durch Fibrate, besitzt. Entsprechend ihren unterschiedlichen Funktionen in der Regulation von Cholesterol- und Fettsäuremetabolismus weisen SREBP-2/1a und SREBP-1c unterschiedliche Regulationsmechanismen auf. Der Gehalt an nuklearem SREBP-2 und SREBP-1a wird vor allem über die Sterolkonzentration reguliert, während die entsprechenden Vorläuferproteine meist konstitutiv exprimiert werden [122]. Die tageszeitlich unterschiedliche Expression der HMG-CoA-Reduktase, welche entgegengesetzt zum tageszeitlichen Rhythmus der Expression des PPARα verläuft und in PPARα-*knockout*-Mäusen nicht auftritt [96, 97], könnte demnach über die Beeinflussung des Insig-1 durch PPARα vermittelt sein. Dafür spricht auch, dass die SREBP-2 mRNA-Konzentration unabhängig vom Tagesrhythmus ist [97]. Ebenso könnte die Wechselwirkung von PPARα und Insig auch zur Reduktion der Cholesterolsynthese im Hungerzustand beitragen, welche offenbar durch eine verminderte Expression der HMG-CoA-Reduktase, aber nicht des SREBP-2, zustande kommt [97]. Im Gegensatz zu SREBP-2 unterliegt SREBP-1c einer sehr komplexen Regulation durch Veränderungen in der Aufnahme von Kohlehydraten und die Insulin-Konzentration [123]. Neben der bereits erwähnten Regulation der SREBP-1c-Transkription durch LXR-Agonisten [111-113] stellt Insulin einen

Schlüsselregulator der SREBP-1c-Transkription dar [124-129]. PUFA verringern die Menge an nuklearem SREBP-1c durch Inhibition der Transkription, vermindertes Prozessing und eine verringerte mRNA-Stabilität [130, 131]. Schließlich wird auch der proteasomale Abbau des nuklearen SREBP-1c durch Insulin und PUFA beeinflusst [132, 133]. Die Mechanismen, über die PUFA die proteolytische Aktivierung des SREBP-1 inhibieren, sind weitgehend unklar, beinhalten aber vermutlich eine Beeinflussung des Sphingolipid-Metabolismus [131, 134-136]. Es ist denkbar, dass die PPARα-Aktivierung durch PUFA und die nachfolgende Induktion der Insig ebenfalls zur beobachteten Inhibierung der SREBP-1-Proteolyse durch PUFA beiträgt.

3.1.2 Natürliche PPARα-Agonisten und speziesspezifische Unterschiede

3.1.2.1 Untersuchungen beim Schwein und in HepG2-Zellen

Verschiedene Studien belegen, dass oxidierte Fette, die über die Nahrung aufgenommen werden, vielfältige physiologische Wirkungen, wie zum Beispiel die Induktion von oxidativem Stress, Beeinträchtigungen der Glukosetoleranz und der Schilddrüsenfunktion sowie proatherogene Wirkungen, hervorrufen [137-143]. Darüber hinaus beeinflussen oxidierte Fette aber auch den Lipidstoffwechsel günstig, in dem sie die Plasmatriglyzeride absenken [27, 89, 144-146]. Dies wird auf die Aktivierung des hepatischen PPARα und die damit gesteigerte peroxisomale und mitochondriale β-Oxidation zurückgeführt, wodurch die Synthese und Sekretion triglyzeridreicher Lipoproteine absinkt [89, 90, 147, 148]. In eigenen Untersuchungen konnten wir nun auch die Mechanismen, die den verringerten Cholesterolkonzentrationen in Plasma und Leber von Ratten, die mit oxidierten Fetten gefüttert wurden [27], zugrunde liegen, aufklären (**A3**). Demnach führt auch oxidiertes Fett zu einer Inhibierung der SREBP-2-abhängigen Cholesterolsynthese– und aufnahme, indem es die Expression von Insig-1 und auch Insig-2a erhöht. Ausgehend von den Übereinstimmungen in den Effekten von Clofibrat (**A1**) und oxidiertem Fett (**A3**) vermuten wir, dass die Induktion von Insig-1 ebenfalls durch eine Aktivierung des PPARα durch die Bestandteile des oxidierten Fettes erfolgte. Interessanterweise konnte im Gegensatz zu den mit Clofibrat behandelten Ratten (**A1**), aber in Übereinstimmung mit den Daten aus Fao-Zellen (**A1, A2**), bei oxidiertem Fett auch eine Erhöhung des Insig-2a beobachtet werden, was die Hypothese stützt, dass Insig-2a ebenso wie Insig-1 durch PPARα reguliert wird. Übereinstimmend damit konnten wir in Fao-Zellen ebenfalls eine Induktion von Insig-1 und Insig-2a bei Behandlung der Zellen mit 9-Hydroxy-10,12-Octadecadiensäure, einem Oxidationsprodukt der Linolsäure und damit einem Hauptbestandteil oxidierter Fette, feststellen (unveröffentlichte Daten). Insgesamt zeigen unsere Daten, dass Fibrate und oxidierte Fette den Cholesterolstoffwechsel bei Ratten in vergleichbarer Weise modulieren

und die beobachteten cholesterolsenkenden Effekte zumindest zum Teil auf einer durch PPARα-Aktivierung verminderten SREBP-2-abhängigen Cholesterolsynthese beruhen. Es ist wahrscheinlich, dass noch andere Mechanismen als der hier beschriebene zu den beobachteten verringerten Cholesterolkonzentrationen bei Gabe von Fibraten oder oxidierten Fetten beitragen. So konnte bereits gezeigt werden, dass PPARα-Agonisten die Plasma-Cholesterolkonzentration über eine Verringerung der Cholesterolabsorption im Darm erniedrigen [149-152]. Hierbei spielt offenbar eine PPARα-abhängige Regulation spezifischer Cholesteroltransporter bzw. -exporter eine entscheidende Rolle [149, 153]. Inwiefern diese Mechanismen auch für die Wirkung oxidierter Fette relevant sind, bleibt zu untersuchen.

Da oxidierte Fette in der menschlichen Ernährung, vor allem in den Industrieländern, eine wachsende Rolle spielen [81], ist es von grundlegendem Interesse, inwiefern die in Ratten beobachteten Mechanismen auch für den Menschen relevant sind. Allerdings können die bei Ratten erhaltenen Daten nicht direkt auf den Menschen übertragen werden, da sich Ratten bzw. Mäuse und der Mensch hinsichtlich ihrer Reaktion auf eine PPARα-Aktivierung deutlich voneinander unterscheiden. Das Schwein, das wie der Mensch zu den so genannten nicht-proliferierenden Spezies gehört, stellt dagegen ein gutes Modelltier für den Menschen dar. Die Expression des PPARα in der Leber ist vergleichbar mit der des Menschen und etwa fünf- bis zehnfach geringer als in der Leber der Ratte [63, 67]. Verschiedene Studien, die sich mit der Wirkung von Clofibrat im Schweinemodell beschäftigten, konnten in Analogie zum Menschen eine Verringerung der Plasmakonzentrationen an Triglyzeriden und Cholesterol feststellen [67, 154]. Dabei kommt es in der Leber und auch im Fettgewebe zu einer Induktion von Genen der peroxisomalen und mitochondrialen Fettsäureoxidation, die allerdings schwächer ist als bei Ratten oder Mäusen [67, 155-157]. Diese Steigerung der Fettsäureoxidation ist vermutlich die Ursache für die beobachteten verringerten Triglyzeridgehalte und auf eine Aktivierung des PPARα zurückzuführen [67]. Die Tatsache, dass vergleichbare Effekte in der Leber von Schweinen auch im Hungerzustand auftraten [155, 158], unterstreicht das Vorhandensein eines funktionellen PPARα in Schweinen als Vertreter der nicht-proliferierenden Spezies. In unseren eigenen Untersuchungen konnten wir nun zeigen, dass auch die Fütterung eines oxidierten Fettes zu einer Aktivierung des PPARα in der Leber von Schweinen führt (**A4**). Wie bereits für Clofibrat beobachtet [67], waren die Effekte aber deutlich geringer als die Änderungen, die in der Leber von Ratten auftreten. Für die Versuche wurde ein Fett verwendet, das unter gewöhnlichen Frittierbedingungen behandelt wurde und dessen Oxidationsgrad eher gering war (**A4**), vor allem verglichen mit dem im Rattenversuch verwendeten Fett (**A3**). Somit kann vermutet werden, dass oxidierte Nahrungsfette vergleichbare Effekte beim Menschen hervorrufen können.

Während die durch oxidiertes Fett induzierten Änderungen der Expression der Gene der β-Oxidation in den Lebern der Schweine gering waren, konnte ein deutlicher Anstieg der Expression der mHMG-CoA-Synthase, verbunden mit einer Erhöhung der Konzentration an 3-Hydroxybutyrat im Plasma der Schweine, beobachtet werden (**A4**). Dies steht in Übereinstimmung mit verschiedenen Studien, die die Wirkungen von synthetischen PPARα-Agonisten auf die Expression typischer responsiver Gene in humanen Leberkarzinomzellen (HepG2) untersuchten, um speziesspezifische Unterschiede zu analysieren. Nur in wenigen Studien konnte eine schwache Induktion der Acyl-CoA-Oxidase, dem Markerenzym der Peroxisomenproliferation, dessen Expression durch PPARα reguliert wird [62], durch PPARα-Agonisten beobachtet werden [159-161]. In der Mehrzahl der Studien konnte allerdings keine Beeinflussung der Acyl-CoA-Oxidase-Expression in HepG2-Zellen festgestellt werden [162-166]. Dagegen war die mHMG-CoA-Synthase, die in die Ketonkörpersynthese involviert ist, auch in HepG2-Zellen nach Behandlung mit synthetischen PPARα-Agonisten erhöht [163-165]. Diese unterschiedlich starke Beeinflussung PPARα-abhängiger Gene in HepG2-Zellen konnten wir in eigenen Untersuchungen auch für eine oxidierte Fettsäure feststellen (**A5**). 13-Hydroperoxy-9,11-Octadecadiensäure (13-HPODE) stellt das primäre Oxidationsprodukt der Linolsäure und damit einen Hauptbestandteil oxidierter Nahrungsfette dar. Während die Expression von Genen der peroxisomalen und mitochondrialen β-Oxidation in HepG2-Zellen durch 13-HPODE nicht beeinflusst wurde, konnte eine geringe Induktion der mHMG-CoA-Synthase in 13-HPODE-behandelten HepG2-Zellen beobachtet werden (**A5**). In Übereinstimmung mit der in Ratten beobachteten deutlichen Induktion von Genen der peroxisomalen und mitochondrialen β-Oxidation führte eine Inkubation mit 13-HPODE auch in Fao-Zellen zu einer Induktion der entsprechenden Gene (**A5**). Diese Ergebnisse konnten zum einen erstmals zeigen, dass 13-HPODE in der Lage ist, den PPARα zu aktivieren und damit neben anderen bereits bekannten Komponenten [76, 77, 80] mit verantwortlich ist für den PPARα-aktivierenden Effekt von oxidierten Fetten. Zum anderen konnten wir mit den vergleichenden Untersuchungen in Fao- und HepG2-Zellen die speziesspezifischen Unterschiede, die bereits für synthetische Agonisten beschrieben wurden, auch für die PPARα-aktivierende Wirkung von oxidierten Fettsäuren aufzeigen.

In Übereinstimmung mit den beobachteten Expressionsänderungen der Gene der β-Oxidation waren in Fao-Zellen auch die Konzentrationen an zellulären und sekretierten Triglyzeriden durch Inkubation mit 13-HPODE verringert. Dagegen traten in HepG2-Zellen keine Änderungen der Triglyzeridkonzentrationen auf (**A5**). Auch bei Schweinen führte die Verfütterung eines oxidierten Fettes nicht zu Änderungen der Triglyzerid- und Cholesterolkonzentrationen in Leber, Plasma und Lipoproteinen (**A4**). Das oxidierte Fett führte zu einer Aktivierung des PPARα in den Lebern der Schweine, was mit der Zunahme des relativen Lebergewichtes, einer Zunahme der Anzahl der Peroxisomen, einer moderaten

Induktion typischer PPARα-Zielgene und der Stimulation der Ketogenese belegt werden konnte. Allerdings reichte die geringe Induktion der Gene der β-Oxidation offenbar nicht aus, um signifikante Reduktionen der Triglyzeridkonzentrationen zu bewirken (**A4**). In Schweinen, die mit Clofibrat behandelt wurden, konnte dagegen eine deutliche Abnahme der Triglyzerid- und Cholesterolkonzentration in Plasma und Lipoproteinen beobachtet werden [67, 154]. Im Gegensatz zu dem inhibierenden Effekt, der für eine PPARα-Aktivierung auf die Insig/SREBP-regulierte Lipogenese in Ratten und Fao-Zellen gezeigt werden konnte (**A1, A2**), bewirkte Clofibrat bei Schweinen keine Änderung der Expression von Insig, SREBP-1 und -2 und der entsprechenden SREBP-Zielgene und der Cholesterol- und Triglyzeridkonzentrationen in der Leber [67]. Die stark reduzierten Cholesterol-konzentrationen im Plasma und den Lipoproteinen der Schweine nach Clofibratbehandlung konnten somit nicht mit einer verringerten Cholesterolsynthese in der Leber begründet werden und bedürfen weiterer Klärung [67]. Die Aufnahme von oxidiertem Fett führte bei Schweinen, wiederum im Gegensatz zu den bei Ratten beobachteten Effekten, zu einer Induktion von SREBP-1 und -2 und relevanter Zielgene der Fettsäure- und Cholesterolsynthese in der Leber und teilweise dem Dünndarm, was eine Stimulierung der Lipogenese vermuten lässt (**A4**). Möglicherweise erfolgte die Induktion von Genen der Fettsäuresynthese über einen SREBP-1-Anstieg kompensatorisch, um der β-Oxidation von Fettsäuren entgegen zu wirken. Dies könnte auch eine weitere Erklärungsmöglichkeit für die unveränderten Triglyzeridkonzentrationen in Leber und Plasma der Schweine, die oxidiertes Fett erhielten, sein. Inwiefern die beobachteten stimulierenden Effekte auf SREBP-1 und -2 auf eine Aktivierung des PPARα durch das oxidierte Fett zurückzuführen sind, bleibt ungeklärt. Die in Ratten und Fao-Zellen aufgezeigten Zusammenhänge zwischen einer PPARα-Aktivierung und der Regulation der SREBP-abhängigen Lipogenese (**A1, A2, A3**) konnten somit weder bei Clofibratbehandlung [67] noch bei Verfütterung eines moderat oxidierten Fettes in Schweinen (**A4**) nachvollzogen werden. Die fehlende Induktion der Insig sowohl in unserem Versuch (**A4**) als auch in der Clofibratstudie [67] kann zum einen auf der schwächeren Aktivierung des PPARα beim Schwein als nicht-proliferierende Spezies beruhen. Diese Hypothese wird durch die Tatsache gestützt, dass die Induktion der Insig durch PPARα-Aktivierung auch bei der Ratte mit Faktor 2 deutlich schwächer ist als die der „klassischen“ PPARα-Zielgene (Faktor 10 bis 20) [**A1, A3;** 167-169]. In Anbetracht der komplexen Regulation der Insig, zum Beispiel durch Insulin und SREBP selbst [103-107], kann aber auch nicht ausgeschlossen werden, dass Effekte des aktivierten PPARα auf die Insig durch andere Regulationsmechanismen überlagert wurden.

Es muss angemerkt werden, dass die beschriebenen Effekte einer Aktivierung des PPARα durch Fibrate in Bezug auf die SREBP auch innerhalb der proliferierenden Spezies inhomogen sind und nicht immer mit den hier beschriebenen Zusammenhängen

übereinstimmen. So wurden für Ratten und kultivierte Hepatozyten sowohl inhibierende [170-174] als auch fördernde Effekte [171, 175, 176] von Fibraten auf Cholesterolsynthese und -konzentrationen beschrieben. Bei Mäusen führte WY 14,643 zwar zu einer Reduktion der Cholesterolsynthese und zu reduzierten Cholesterolkonzentrationen in der Leber – ein Effekt, der bei PPARα-*knockout*-Mäusen nicht auftrat - die Expression der HMG-CoA-Reduktase korrelierte aber nicht damit [169]. In einer weiteren Studie bewirkte die Inkubation einer Maus-Hepatomazelllinie mit Fenofibrat eine Induktion der SREBP-2-Reifung und eine erhöhte HMG-CoA-Reduktase-Expression [177]. Auch für SREBP-1c werden bei Mäusen induzierende Effekte durch Fibrate, verbunden mit einem Anstieg der Fettsäuresynthese, berichtet, die bei PPARα-*knockout*-Mäusen nicht auftreten [149, 169]. Schließlich wurden bei Mäusen für Fibrate in Abhängigkeit von der Dosis sowohl fördende als auch hemmende Effekte auf SREBP-1c und seine Zielgene beschrieben, wobei eine geringe Dosis, die den therapeutischen Dosen beim Menschen entsprach, zu einer Inhibierung des SREBP-1c führte [178].

Die vorliegenden Daten zur Wirkung eines oxidierten Fettes beim Schwein (**A4**) lassen zwar den Schluss zu, dass auch beim Menschen eine Aktivierung des PPARα beim Verzehr von Nahrungsmitteln, die oxidierte Fette enthalten, in Betracht gezogen werden sollte. Inwiefern die bei Ratten beobachtete Beeinflussung der SREBP-regulierten Lipogenese durch eine Induktion der Insig im Rahmen der PPARα-Aktivierung auch relevant für den Menschen ist, kann aus den vorhandenen Daten allerdings nicht abgeschätzt werden.

3.1.2.2 Untersuchungen bei Legehennen

Im Vergleich zu Säugern zeichnen sich Legehennen durch eine hohe Rate der hepatischen Synthese von Triglyzeriden, Phospholipiden und Cholesterol aus. Diese spielt eine entscheidende Rolle bei der Bereitstellung von Lipiden für die Eibildung [72]. Die in der Leber synthetisierten Lipide werden in triglyzeridreiche Lipoproteine eingebaut und ins Blut sekretiert. Daher enthält das Plasma von Hennen besonders hohe Triglyzeridkonzentrationen, die vor allem in den VLDL lokalisiert sind [179]. Diese VLDL werden an spezifische Oozytenrezeptoren gebunden und dann in sich entwickelnde Eigelbfollikel eingelagert [72]. Sowohl prinzipiell zum Verständnis der Regulation des Lipidstoffwechsels bei der Legehenne als auch in Hinblick auf die Relevanz von Hühnereiern als Nahrungsmittel in der menschlichen Ernährung erscheinen Untersuchungen zur Regulation des Lipidstoffwechsels der Legehenne durch PPARα, speziell seine Beeinflussung durch Futterzusätze, als interessant. Verschiedene Studien existieren bereits zur Anwendung von pharmakologischen Substanzen, wie zum Beispiel Statinen, zur Senkung der Cholesterolkonzentrationen im Eigelb [180-183]. In einer Studie wurde dabei

auch der Effekt eines Fibrates auf Lipidkonzentrationen in Plasma und Eigelb untersucht [183]. Studien zu den molekularen Wirkungen von PPARα-Agonisten in Legehennen existieren dagegen noch nicht. Die Expression des PPARα bei Hühnern mit einer ähnlichen Gewebeverteilung wie bei Säugern wurde bereits beschrieben [184, 185]. In unseren eigenen Arbeiten (**A6**) konnten wir nun erstmals zeigen, dass Clofibrat auch bei Legehennen zu einer starken Aktivierung des hepatischen PPARα führte, was sich in Übereinstimmung mit den Effekten bei Säugern in einer starken Reduktion der Triglyzeride in Plasma, Leber und VLDL und der Induktion typischer PPARα-Zielgene der β-Oxidation und Lipolyse in der Leber zeigte. Fibrate haben demzufolge nicht nur bei Säugern, sondern auch bei Legehennen triglyzeridsenkende Eigenschaften, welche offenbar auf eine Aktivierung des hepatischen PPARα zurückzuführen sind. In Übereinstimmung damit konnte auch in der früheren Studie zur Wirkung eines Fibrates bei Legehennen eine Absenkung der Plasmatriglyzeride beobachtet werden [183]. Zwei jüngere Studien, die die molekularen Wirkungen von Fenofibrat und Dehydroepiandrosteron auf Primärkulturen von Hühnerembryo-Hepatozyten bzw. bei Broilern untersuchten, konnten unsere Daten zur Aktivierbarkeit des PPARα bei Hühnern ebenfalls bestätigen [186, 187].

Neben einer gesteigerten Fettsäureoxidation führte Clofibrat auch zur verminderten Expression der Fettsäuresynthase (FAS), so dass die verringerten Triglyzeridkonzentrationen nicht nur auf eine erhöhte Lipolyse und β-Oxidation, sondern offenbar auch auf eine verminderte Lipogenese zurückzuführen sind (**A6**). Inwiefern hierbei der in Fao-Zellen beobachtete Mechanismus einer Inhibierung des SREBP-1 über die PPARα-vermittelte Induktion von Insig (**A2**) eine Rolle spielt, ist unklar. Mit großer Wahrscheinlichkeit erfolgte die Inhibierung der FAS-Expression vor allem über die stark verringerten Östrogenkonzentrationen im Plasma der Clofibrat-behandelten Hennen (**A6**), da bekannt ist, dass die Triglyzeridsynthese bei Vögeln stark durch Östrogen stimuliert wird [188, 189]. Niedrige Östrogenkonzentrationen und eine stark verminderte Futteraufnahme waren vermutlich auch die Ursachen dafür, dass Clofibrat im Laufe der Versuchsdauer von 5 Wochen zu einer Einstellung der Eiproduktion bei den Hennen führte (**A6**). Das Phänomen der Reduktion der Futteraufnahme [190] und seine PPARα-Abhängigkeit [191] wurden auch für verschiedene PPARα-Agonisten bei Säugern beschrieben. Neben einer verringerten Fettsäuresynthese verursachte Clofibrat in der Leber der Hennen offenbar auch eine Absenkung der Cholesterolsynthese und –aufnahme über eine Absenkung des SREBP-2 und einer verminderte Expression seiner Zielgene HMG-CoA-Reduktase und LDL-Rezeptor, was sich in verringerten VLDL-Cholesterolkonzentrationen im Plasma widerspiegelte (**A6**). Die hepatische Synthese von Cholesterol und die Aufnahme von LDL in die Leber wurden vermutlich verringert, da aufgrund der eingestellten Eiproduktion weniger Cholesterol für die VLDL-Assemblierung und -Sekretion benötigt wurde. Auch hier kann aus den bisher

vorliegenden Daten ein Einfluss der PPARα-Aktivierung über Insig nicht abgeleitet werden. Die Insig-1-Expression in der Leber der Clofibrat-behandelten Hennen war in Übereinstimmung mit der beschriebenen Feedback-Regulation durch SREBP-2 [103] verringert, während Insig-2 unverändert blieb (**A6**).

Schlussfolgernd aus diesen Untersuchungen kann gesagt werden, dass die Wirkungen von Clofibrat bei der Legehenne prinzipiell mit denen beim Säuger übereinstimmen, was die Aktivierung des PPARα, die Induktion von Genen der β-Oxidation und Lipolyse und die Absenkung von Triglyzerid- und Cholesterolkonzentrationen einschließt. Wie bei Ratten konnte auch bei der Henne eine verringerte Expression von SREBP-2 und seinen Zielgenen beobachtet werden; inwiefern die bei der Ratte beobachteten Mechanismen (**A1**) dabei eine Rolle spielen, ist ungeklärt. Die drastischen Effekte von Clofibrat auf Futteraufnahme, Östrogenkonzentration und Eiproduktion sind vermutlich auf die hohen Konzentrationen an Clofibrat zurückzuführen, die sich an bei Ratten und Schweinen verwendeten Mengen orientierten. In der bereits erwähnten Studie zur Wirkung eines Fibrates bei Legehennen wurden bei Verwendung einer deutlich geringeren Menge Fibrat über 12 Wochen keine derartigen Effekte beschrieben [183]. Letztlich zeigen diese starken Effekte in unseren Untersuchungen aber auch die zentrale Rolle des PPARα in der Regulation der Lipidhomöostase der Legehenne hinsichtlich der hohen Lipidsyntheseleistung, die für die Eiproduktion entscheidend ist.

Im Zuge der Bemühungen zum Design von sogenannten „*functional foods*“, welche aufgrund ihrer Zusammensetzung gesundheitsfördernde Wirkungen für den Menschen aufweisen, spielt auch die Untersuchung von Futterzusätzen wie Fischöl oder CLA bei Legehennen eine große Rolle. Dies basiert auf den gesundheitsfördernden Eigenschaften, die für n-3 PUFA als Hauptbestandteil des Fischöls und für CLA gezeigt wurden. So weisen n-3 PUFA aus Fischöl triglyzeridsenkende, antiatherosklerotische und antiarrhythmische Eigenschaften auf [192-194], während für CLA antikarzinogene, antiatherogene, immunmodulatorische und antioxidative Eigenschaften sowie eine Reduktion des Körperfettes beschrieben wurden [195-199]. Zahlreiche Studien beschäftigten sich deshalb mit dem Versuch, die Konzentrationen an n-3 PUFA und CLA in Hühnereiern über eine Anreicherung des Futters mit diesen Fettsäuren zu erhöhen [200-206]. Die molekularen Wirkungen, die bei Fütterung von Fischöl bzw. CLA in Legehennen auftreten, wurden dagegen noch nicht untersucht. In Säugern basieren die triglyzeridsenkenden Eigenschaften von langkettigen n-3 PUFA auf einer Aktivierung des PPARα und somit erhöhter mitochondrialer und peroxisomaler β-Oxidation sowie einer verminderten Transkription des SREBP-1, was zur Repression lipogener Enzyme und damit zur Inhibierung der Neusynthese von Fettsäuren führt [207-210]. CLA aktivieren ebenfalls den PPARα [78, 79], führen aber eher zu einer Induktion der

Transkription von SREBP-1 und SREBP-2 in Leberzellen von Säugern und damit zu einer erhöhten Neusynthese von Fettsäuren und Cholesterol [211, 212]. In unseren eigenen Untersuchungen (**A7**) konnten wir nun zeigen, dass Fischöl auch in der Leber von Legehennen zu einer Aktivierung des PPARα führt, was sich in moderat erhöhten Expressionen von Genen der peroxisomalen und mitochondrialen β-Oxidation zeigte. Auch die bei Säugern beschriebene Absenkung der Plasmatriglyzeride durch Fischöl konnte bei den Hennen beobachtet werden (**A7**). Entgegen dem beschriebenen Mechanismus bei Säugern, der eine Repression der *de novo*-Fettsäuresynthese über die Hemmung der Transkription des SREBP-1 beinhaltet [207-210], scheint Fischöl bei Legehennen nicht die *de novo*-Fettsäuresynthese zu hemmen, wie anhand der mRNA-Konzentrationen relevanter Gene festgestellt werden konnte (**A7**). Insgesamt sind die für n-3 PUFA beschriebenen Einflüsse auf den Triglyzeridstoffwechsel komplex und umfassen neben einer Beeinflussung von PPAR und SREBP-1c auch die Aktivierung oder auch Hemmung verschiedener anderer nuklearer Rezeptoren wie LXR, *hepatocyte nuclear factor*-4 und *carbohydrate responsive element-binding protein* [3, 4, 213-215]. Ein derartiger vielschichtiger Mechanismus der Wirkung von Fischöl ist demzufolge auch für Legehennen in Betracht zu ziehen. In Übereinstimmung mit den Effekten von Clofibrat und oxidiertem Fett bei der Ratte (**A1, A3**) und der Wirkung von Clofibrat bei Legehennen (**A6**) führte auch Fischöl über eine Verminderung des nuklearen SREBP-2 zur verringerten Expression der HMG-CoA-Reduktase in der Leber und zu reduzierten Cholesterolkonzentrationen im Plasma der Hennen (**A7**). Diese Parallelen lassen vermuten, dass die Verminderung des nuklearen SREBP-2 ebenfalls auf eine Aktivierung des PPARα durch Fischöl zurückzuführen sein könnte. Andere bzw. zusätzliche Mechanismen, wie zum Beispiel eine Beeinflussung der Cholesterolausscheidung durch Fischöl, sind denkbar. So führte Fischöl bei Mäusen vermutlich durch eine Induktion des LXR zu einer verstärkten Transkription der Cholesterol-7α-Hydroxylase, dem Schlüsselenzym der Gallensäurensynthese, und somit zu einer erhöhten Cholesterolausscheidung [216].

Wie PUFA sind auch CLA in der Lage, den PPARα in der Leber von Ratten zu aktivieren [78, 79]. Dagegen konnte bei Legehennen anhand der Expression typischer PPARα-Zielgene keine PPARα-Aktivierung durch CLA in der Leber festgestellt werden (**A7**). Möglicherweise beruht dieser Unterschied auf der unterschiedlich hohen PPARα-Expression zwischen beiden Spezies [61, 69]. Die für CLA beschriebenen Effekte auf den Lipidmetabolismus von Säugern sind nicht einheitlich; so werden sowohl verringerte, unveränderte als auch erhöhte Cholesterolkonzentrationen nach CLA-Gabe beschrieben [217-220]. Bei Legehennen führte CLA-Fütterung zu erhöhten Cholesterolkonzentrationen in Leber und Plasma, wobei die erhöhten Cholesterolkonzentrationen in der Leber vermutlich auf die erhöhte Expression des LDL-Rezeptors zurückzuführen sind (**A7**). Auch in HepG2-Zellen kann bei Inkubation mit

CLA, speziell dem *trans*-10, *cis*-12-Isomer, eine Induktion des LDL-Rezeptors, vermittelt über SREBP-2, beobachtet werden [212]. Bei Legehennen scheint allerdings ein anderer, SREBP-2-unabhängiger Mechanismus vorzuliegen (**A7**). Bei Säugern wird die LDL-Rezeptor-Expression neben SREBP-2 und SREBP-1a noch durch weitere Transkriptionsfaktoren wie *specificity protein*-1, *activator protein*-1 und LXR [221-223] und zum anderen über den LXR-vermittelten verstärkten Proteinabbau [224] reguliert. CLA bewirkten auch eine Erhöhung der Triglyzeridkonzentration in der Leber der Legehennen (**A7**), was die Daten aus zwei früheren Studien mit Legehennen bestätigte [204, 225]. Unsere Untersuchungen konnten allerdings keinen Mechanismus für diesen Effekt aufzeigen (**A7**). Inwiefern die erhöhten Leberlipide bei Legehennen auf den bei Mäusen beobachteten Mechanismus zurückzuführen sind, bei dem *trans*-10, *cis*-12 CLA zu einer Hyperinsulinämie und nachfolgender Steatose führt [226, 227], bleibt zu untersuchen.

Die erhöhten Lipidkonzentrationen in den Lebern der CLA-behandelten Legehennen spiegelten sich auch in den Triglyzerid- und Cholesterolkonzentrationen im Eigelb wider, welche ebenfalls erhöht waren (**A7**). Die Effekte einer CLA-Behandlung auf die Eilipide in anderen Studien variieren, allerdings fehlen dort in der Regel vergleichende Werte zu den entsprechenden Konzentrationen in den Lebern der Hennen [225, 228, 229]. Unsere Untersuchungen lassen vermuten, dass aufgrund der erhöhten Lipidkonzentrationen in der Leber die Sekretion von VLDL aus der Leber und deren Aufnahme in die Oozyten erhöht waren, wodurch die Lipidkonzentrationen im Eigelb anstiegen (**A7**). Die durch die PPARα-Aktivierung bedingten verringerten Plasmatriglyzeride bei Fischöl-gefütterten Legehennen resultierten dagegen, vermutlich über eine verminderte VLDL-Sekretion und –Aufnahme in die Oozyten, in verringerten Triglyzeridkonzentration im Eigelb (**A7**).

Zusammenfassend kann eingeschätzt werden, dass synthetische und natürliche PPARα-Agonisten auch bei Legehennen Effekte auf den Lipidmetabolismus bewirken, die zum Teil den bei Säugern beobachteten Wirkungen vergleichbar sind. Die zugrunde liegenden Mechanismen sind aus den derzeitigen Versuchen aber nur zu vermuten bzw. in einigen Aspekten völlig unklar. Insbesondere hinsichtlich der bei Ratten beobachteten Wechselwirkung von PPAR und SREBP über eine PPAR-vermittelte Induktion von Insig kann über vergleichbare Zusammenhänge bei Legehennen nur spekuliert werden. Dies trifft ebenfalls auf unsere Untersuchungen beim Schwein (**A4**) zu. Weiterführende, vor allem Zellassay-basierende Experimente sind hier notwendig, um einzelne Regulations-mechanismen isoliert betrachten zu können und somit die im Tiermodell beobachteten Veränderungen auf molekularer Ebene erklären zu können.

3.2 Regulation der Anpassung an Hunger durch PPARα

PPAR traten erstmals vor ca. 280 Millionen Jahren auf, als sich die Evolutionslinie der Fische und Säugetiere trennte, um einen molekularen Mediator für die Anpassung an zeitweisen Nahrungsmangel bereitzustellen [230]. Zur Sicherung der Energiebereitstellung im Hungerzustand stimuliert PPARα Gene der Fettsäureoxidation, Ketogenese und Glukoneogenese [23] und ermöglicht Säugern so auch das Überleben von langen Hungerperioden durch Schutz der Protein- und Kohlenhydratreserven. Die Bedeutung des PPARα in der Verwaltung der Energieressourcen wird beim anhaltenden Fasten von PPARα-*knockout*-Mäusen sichtbar, die eine Hypoglykämie, eine Hypoketonämie und eine Fettleber entwickeln [30]. Die in diesem Kapitel vorgestellten Untersuchungen beschäftigen sich mit der Regulation von Genen, die eine entscheidende Rolle in den durch PPARα gesteuerten Stoffwechselwegen im Rahmen einer Anpassung an Energiemangelzustände inne haben, über deren Regulation durch PPARα bisher jedoch nichts bekannt ist. Dies betrifft zum einen die β-Oxidation von Fettsäuren in den Mitochondrien und zum anderen die Ketogenese. Desweiteren wurden Untersuchungen zur Rolle des PPARα im Gehirn durchgeführt.

3.2.1 Regulation der CACT

Derzeit sind ca. 80-100 Gene bekannt, deren Expression durch PPARα reguliert wird. Für eine Vielzahl von ihnen konnte bereits ein funktionelles PPRE beschrieben werden [23]. Darunter sind zahlreiche Gene, die in die peroxisomale und mitochondriale β-Oxidation involviert sind. In Energiemangelzuständen werden in den Peroxisomen vor allem langkettige und sehr langkettige Fettsäuren und in den Mitochondrien kurze, mittlere und hauptsächlich langkettige Fettsäuren oxidiert. Dabei werden die langkettigen Fettsäuren in aktivierter Form als Acylcarnitin in die mitochondriale Matrix transportiert. Für viele Enzyme und Proteine, die in die Bereitstellung der Fettsäuren für die mitochondriale β-Oxidation involviert sind, wurde bereits eine Regulation der entsprechenden Gene durch PPARα gezeigt [33-35, 231, 232]. In unseren eigenen Untersuchungen (**A8**) konnten wir nun zeigen, dass auch die CACT, welche in die innere Mitochondrienmembran eingelagert ist und den Transport des Acylcarnitins in die mitochondriale Matrix im Austausch gegen freies Carnitin katalysiert [233-235], durch PPARα reguliert wird. Sowohl Nahrungsentzug für 24 h als auch die Gabe des spezifischen PPARα-Agonisten WY 14,643 führte bei Wildtyp-Mäusen zu einer erhöhten Expression der CACT in der Leber, welche bei PPARα-*knockout*-Mäusen nicht auftrat (**A8**). Dabei war die Amplitude der Expressionssteigerung vergleichbar mit der Induktion der CPT-I, welche den vorgelagerten Schritt in der Bereitstellung der Fettsäuren für die mitochondriale β-Oxidation, die Übertragung des Carnitinrestes auf Acyl-CoA, katalysiert und ebenfalls direkt durch PPARα reguliert wird [34, 35]. Eine Induktion der CACT bei PPARα-

Aktivierung scheint somit zu gewährleisten, dass die erhöhte Menge an aktivierten Fettsäuren tatsächlich zur β-Oxidation und somit zur Energiebereitstellung in die Mitochondrien gelangt. Sowohl Reportergenassays als auch Gelshiftexperimente zeigten, dass die Regulation der CACT der Maus durch PPARα über die Bindung an ein funktionelles PPRE im 5'-untranslatierten Bereich des CACT-Genes erfolgt (**A8**). Die kritische Rolle dieses identifizierten PPRE für die Regulation der CACT durch PPARα wird durch die Tatsache unterstützt, dass der entsprechende Sequenzbereich auch in den 5'-regulatorischen Bereichen der CACT-Gene des Menschen und der Ratte hoch konserviert ist. In Übereinstimmung damit konnte auch eine vergleichbar starke Induktion der CACT durch WY 14,643 in Hepatomazellen der Ratte (Fao) und des Menschen (HepG2) gezeigt werden (**A8**). Das lässt vermuten, dass die CACT auch in Ratten und Menschen und somit sowohl in proliferierenden als auch nicht-proliferierenden Spezies durch PPARα reguliert wird, was den Stellenwert einer CACT-Induktion im Rahmen einer PPARα-Aktivierung zur Sicherung der Energiebereitstellung unterstreicht. Desweiteren bestätigt es die bisherigen Erkenntnisse, dass sich die speziesspezifischen Unterschiede vor allem auf die Induktion einer Hepatomegalie bzw. von Lebertumoren beschränken, während die durch PPARα vermittelten lipidsenkenden Effekte speziesunabhängig sind [59, 60].

Die Induktion der CACT durch einen spezifischen synthetischen Agonisten des PPARβ/δ in Fao und HepG2-Zellen sowie die Stimulation des Maus-CACT-Promotors durch diesen Agonisten im Reportergenassay zeigen weiterhin, dass CACT nicht nur durch PPARα, sondern vermutlich auch durch PPARβ/δ reguliert wird (**A8**). Die Regulation von PPAR-abhängigen Genen durch mehrere PPAR-Isotypen ist bekannt, so wird zum Beispiel auch die CPT-I sowohl durch PPARα als auch PPARβ/δ reguliert [236, 237]. PPARβ/δ weist eine hohe Expression in Geweben mit Relevanz für den Lipidstoffwechsel, wie Dünndarm, Herz, Muskel, Leber und Fettgewebe, auf [238, 239]. Vor allem im Skelettmuskel, wo die Expression des PPARβ/δ ca. 10- bis 50-mal höher ist als die von PPARα und –γ [239, 240], spielt PPARβ/δ eine bedeutende Rolle in der Regulation der Fettsäureoxidation im Fasten [241]. Aber auch in anderen Geweben wie Leber und Fettgewebe scheint PPARβ/δ für die Regulation der Umschaltung auf andere Energiequellen in physiologischen Zuständen wie Hunger oder körperliche Anstrengung entscheidend zu sein [242-244]. Somit stimmt die von uns beobachtete Induktion der CACT durch PPARβ/δ-Aktivierung mit der kritischen Rolle des PPARβ/δ in der Regulation des Fettsäurekatabolismus überein. Dagegen konnten wir keine Induktion der CACT durch den PPARγ-Agonisten Troglitazon feststellen (**A8**), was mit der fehlenden Regulation der CPT-I durch PPARγ [236, 237] übereinstimmt und die geringere Bedeutung des PPARγ im Fettsäurekatabolismus widerspiegelt. Stattdessen spielt PPARγ, der in Leberzellen nur gering exprimiert wird, eine bedeutende Rolle in der Steuerung der

Expression von Genen, die in die Adipogenese und die Triglyzeridspeicherung in Fettzellen involviert sind [245, 246].

Insgesamt zeigen unsere Daten zur Regulation der CACT durch PPARα, dass offenbar nicht nur bestimmte geschwindigkeitsbestimmende Schritte wie die von der CPT-I katalysierte Reaktion, sondern alle molekularen Ereignisse, die in die Bereitstellung von langkettigen Fettsäuren zur mitochondrialen β-Oxidation involviert sind, einer direkten Regulation durch PPARα unterliegen. Dies verdeutlicht einmal mehr die entscheidende regulatorische Rolle des PPARα in der Anpassung des Organismus an Energiemangelsituationen.

3.2.2 Regulation des Carnitinstoffwechsels

Da langkettige Fettsäuren nur in aktivierter Form als Acylcarnitin vom Cytosol in die mitochondriale Matrix transportiert werden können, stellt Carnitin einen unverzichtbaren Metabolit für die mitochondriale β-Oxidation dar [36-38]. Daneben hat Carnitin noch andere wichtige Funktionen inne, wie zum Beispiel beim Transfer von Produkten der peroxisomalen β-Oxidation zu den Mitochondrien zur Oxidation im Zitratzyklus, bei der Modulation des Acyl-CoA/CoA-Verhältnisses und bei der Speicherung von Energie in Form von Acetylcarnitin [36-38]. Somit stellt die Regulation der Carnitinhomöostase einen wichtigen Aspekt bei der Verwaltung der Energieressourcen des Körpers und der Anpassung an Energiemangelzustände dar. Bereits vor ca. 30 Jahren wurde beobachtet, dass sowohl Fasten als auch die Gabe von Clofibrat zu erhöhten Carnitinkonzentrationen in der Leber führen [42-45], was eine Rolle des PPARα in der Regulation des Carnitinstoffwechsels vermuten lässt. Mögliche Regulationsstellen sind dabei zum einen die körpereigene Carnitin-Biosynthese, die hauptsächlich in der Leber stattfindet, zum anderen die Aufnahme von Carnitin aus der Nahrung und seine Verteilung im Körper [39]. Letzteres wird durch die Membrantransporter OCTN-1, OCTN-2 und OCTN-3 kontrolliert, welche zur *solute carrier* 22A-Familie gehören und sowohl in der Plasmamembran als auch in der mitochondrialen Membran der Zellen lokalisiert sind [247-249]. Während OCTN-1 und OCTN-2 in verschiedenen Geweben wie Niere, Darm, Skelettmuskel, Herz, Leber und Gehirn exprimiert werden, ist die Expression von OCTN-3 auf Hoden, Niere und Darm beschränkt [249-252]. Die essenzielle Rolle der OCTN in der Carnitinhomöostase zeigt sich unter anderem darin, dass angeborene oder erworbene Defekte der OCTN zu primärer bzw. sekundärer Carnitindefizienz mit schwerwiegenden Folgen wie z.B. Kardiomyopathy führen [41]. In unseren eigenen Untersuchungen (**A9**) konnten wir nun erstmals zeigen, dass die erhöhten Carnitinkonzentrationen in der Leber, die bei Gabe von Clofibrat beobachtet wurden, zumindest teilweise auf einer Induktion des OCTN-2 in der Leber basieren. Dabei führt die erhöhte Expression des OCTN-2 zu einem verstärkten Transport von Carnitin aus dem

Plasma in die Leber, was die Carnitinkonzentration in der Leber erhöht, während die Plasma-Carnitinkonzentration sinkt (**A9**). Vergleichbare Ergebnisse wurden später auch bei fastenden Ratten erhalten [253], so dass vermutet werden kann, dass die beobachteten Effekte auf einer Aktivierung des PPARα beruhen. In einer Studie mit PPARα-*knockout*-Mäusen konnten wir nachweisen, dass die Induktion des OCTN-2 dabei tatsächlich durch PPARα vermittelt wird (**A10**). Dabei konnte bei Mäusen nicht nur in der Leber, sondern auch in Niere, Skelettmuskel und Dünndarm eine PPARα-abhängige Induktion des OCTN-2 durch WY 14,643 beobachtet werden (**A10**). In einer anderen Studie konnte gezeigt werden, dass nicht nur eine Aktivierung des PPARα durch synthetische Agonisten, sondern auch Fasten zu einer PPARα-abhängigen Induktion des OCTN-2 in der Leber von Mäusen führt [254]. Diese Daten lassen vermuten, dass OCTN-2 direkt transkriptionell durch PPARα reguliert wird. Erste Hinweise auf mögliche regulatorische Sequenzen lieferte eine Studie, die eine Aktivierung des proximalen Promotors des OCTN-2-Genes der Ratte durch PPARα zeigte, welche allerdings gering war im Vergleich zu bekannten PPARα-Zielgenen [255]. Eine weitere Arbeit konnte nun mit Hilfe von Reportergen- und Gelshiftassays die Regulation des OCTN-2-Genes der Maus über ein PPRE, welches im ersten Intron des Genes lokalisiert ist, nachweisen [256]. Bei Mäusen konnte außerdem eine Induktion des OCTN-3 durch WY 14,643 in den Nierentubuli und Dünndarm beobachtet werden, welche ebenfalls vom Vorhandensein des PPARα abhing (**A10**). Somit ist zu vermuten, dass die Transkription von OCTN-3, der eine besondere Bedeutung für die Rückabsorption von Carnitin in der Niere hat [249], ebenfalls durch PPARα reguliert wird. Für OCTN-1 wurde zwar eine Induktion in der Leber von Ratten durch Clofibratgabe gefunden (**A9**), bei Mäusen konnte dagegen keine Beeinflussung der OCTN-1-Expression in Leber, Niere, Muskel, Dünndarm oder Hoden beobachtet werden (**A10**). Somit basiert die Induktion des OCTN-1 durch Clofibrat vermutlich auf einem PPARα-unabhängigen Mechanismus.

Bereits frühere Studien mit Ratten deuteten an, dass die erhöhten Carnitinkonzentrationen in der Leber bei Clofibratbehandlung auch auf eine erhöhte hepatische Carnitinsynthese zurückzuführen sind [44, 45]. Die Carnitin-Biosynthese ist ein komplexer Prozess, bei dem proteingebundenes Lysin das Kohlenstoff-Rückgrat für Carnitin liefert, welches nach Methylierung unter Beteiligung von S-Adenosylmethionin als Trimethyllysin (TML) beim Proteinabbau freigesetzt wird. Unter Beteiligung der Enzyme Trimethyllysin-Dioxygenase, 3-Hydroxy-N-Trimethyllysin-Aldolase und 4-N-Trimethylaminobutyraldehyd-Dehydrogenase wird TML zu γ-Butyrobetain oxidiert, welches durch die γ-Butyrobetain-Dioxygenase (BBD) schließlich zu Carnitin hydroxyliert wird. Dieser letzte Schritt der Carnitinsynthese findet beim Menschen vorrangig in Leber und Nieren statt [39, 257, 258]. Es wurde vermutet, dass die Steigerung der Carnitinsynthese durch Clofibrat auf einer erhöhten Verfügbarkeit des TML, welches den geschwindigkeitsbestimmenden Parameter der Carnitinsynthese darstellt [259],

beruht. Die erhöhten TML-Konzentrationen in der Leber von Clofibrat-behandelten [44, 45, 260] als auch gefasteten Ratten [253, 261] resultieren dabei vermutlich aus der gesteigerten Proteolyse bei Clofibratbehandlung bzw. beim Fasten [262], welche zur erhöhten TML-Freisetzung führt [257, 263]. TML wiederum wird zu γ-Butyrobetain umgesetzt und in der Leber durch die hohe Aktivität der BBD schnell zu Carnitin umgewandelt [264]. Die Induktion des OCTN-2 bei Aktivierung des PPARα durch spezifische Agonisten oder Fasten fördert dabei vermutlich die Carnitinsynthese zusätzlich durch einen erhöhten Transport von γ-Butyrobetain in die Leber, da OCTN-2 eine hohe Kapazität für den Transport von γ-Butyrobetain aufweist [249, 265]. In unseren eigenen Studien (**A10**) konnten wir nun zeigen, dass die Carnitin-Biosynthese bei PPARα-Aktivierung auch durch die transkriptionelle Induktion von Enzymen der Carnitinsynthese (Trimethyllysin-Dioxygenase, 4-N-Trimethylaminobutyraldehyd-Dehydrogenase und BBD) in der Leber gesteigert wird und dass diese Induktion nur bei Wildtyp-Mäusen, jedoch nicht in der Leber von PPARα-*knockout*-Mäusen auftritt. Somit ist zu vermuten, dass PPARα auch die Transkription der Enzyme der Carnitin-Biosynthese reguliert. Diese Vermutung wird durch eine andere Studie gestützt, in der gezeigt werden konnte, dass nicht nur die Expression, sondern auch die Aktivität der BBD durch PPARα-Agonisten bzw. Fasten in PPARα-abhängiger Weise induziert wurde [254]. Schließlich konnten beim Vergleich der Promotorsequenzen der BBD-Gene von Maus, Ratte und Mensch bereits konservierte putative PPRE-Sequenzen identifiziert werden, deren Funktionalität jedoch noch nachgewiesen werden muss [254]. Ebenso bleibt der Mechanismus der möglichen transkriptionellen Regulation der Trimethyllysin-Dioxygenase und der 4-N-Trimethylaminobutyraldehyd-Dehydrogenase durch PPARα (**A10**) noch zu klären. Insgesamt zeigen unsere Daten gemeinsam mit Daten aus anderen Studien, dass PPARα die Carnitinsynthese zum einen durch die gesteigerte Expression und Aktivität der beteiligten Enzyme, zum anderen durch die Bereitstellung von biosynthetischen Vorläufermolekülen beeinflusst. Letzteres wird auch durch eine weitere Studie belegt, in der gezeigt werden konnte, dass Plasma und Gewebe von PPARα-*knockout*-Mäusen deutlich geringere Konzentrationen an Methionin und α-Ketoglutarat, welche als Methylgruppendonator bzw. als Kofaktor für Trimethyllysin-Dioxygenase und BBD fungieren, aufweisen [266].

Die in Ratten und Mäusen erhaltenen Daten zur Beeinflussung der Carnitinhomöostase durch PPARα dürften auch Relevanz für den Menschen haben, wie vergleichende Studien mit Schweinen als Modelltier für nicht-proliferierende Spezies zeigten. Sowohl Clofibrat-Behandlung als auch Fasten führten zur Induktion von OCTN-2 und zur Steigerung der Carnitinsynthese in der Leber von Schweinen [158, 267]. Somit scheint die Regulation der Carnitinhomöostase durch PPARα ein allgemeingültiger Mechanismus zu sein, der die essenzielle Rolle des PPARα als zentraler Mediator der Anpassung des Organismus an

Hungerzustände unterstreicht. Die durch PPARα gesteuerte Erhöhung der Rate der mitochondrialen β-Oxidation von Fettsäuren zur Synthese von ATP und Ketonkörpern resultiert auch in einem erhöhten Bedarf an Carnitin, welches zur Aktivierung der langkettigen Fettsäuren zum Transport in die mitochondriale Matrix notwendig ist. Die Induktion von OCTN-2 und Enzymen der Carnitin-Biosynthese durch PPARα im Hungerzustand stellt somit einen Mechanismus dar, um die Abdeckung des erhöhten Bedarfs an Carnitin in der Leber und anderen Geweben zu sichern.

Aufgrund des bekannten Potenzials nutritiver natürlicher PPARα-Agonisten zur Beeinflussung PPARα-abhängiger Stoffwechselvorgänge über eine Aktivierung des PPARα (**A4**) ist auch eine Beeinflussung des Carnitinstoffwechsels durch derartige Nahrungsbestandteile denkbar. In einer Studie mit Ratten (**A11**) konnten wir zeigen, dass ein oxidiertes Fett den Carnitinstoffwechsel ähnlich beeinflusst wie synthetische PPARα-Agonisten oder Fasten und sowohl zur Induktion des OCTN-2 als auch eines Enzyms der Carnitinsynthese und damit zu veränderten Carnitinkonzentrationen im Plasma und verschiedenen Geweben führt. In Hinblick auf die Tatsache, dass Carnitintransport und -synthese auch beim Schwein einer Regulation durch PPARα unterliegen [158, 267], erscheinen diese Erkenntnisse (**A11**) auch für die menschliche Ernährung relevant. Besonders interessant in diesem Zusammenhang ist die beobachtete Induktion des OCTN-2 durch oxidiertes Fett im Dünndarm der Ratten (**A11**). Auch bei Wildtyp-Mäusen, aber nicht bei PPARα-*knockout*-Mäusen (**A10**), sowie beim Schwein [267] konnte eine Induktion des OCTN-2 im Dünndarm durch synthetische PPARα-Agonisten gezeigt werden. Da der OCTN-2 im Dünndarm, welcher in der apikalen Membran der Mucosazellen lokalisiert ist, auch in die Aufnahme von Carnitin aus der Nahrung in die Zellen involviert ist [268, 269], ist eine erhöhte Aufnahme von Nahrungscarnitin im Dünndarm bei gleichzeitiger Aufnahme von oxidiertem Fett denkbar. Ein weiterer wichtiger Aspekt resultiert aus der Polyspezifität der OCTN, welche nicht nur Carnitin, sondern auch verschiedene pharmakologische Substanzen wie Verapamil, Spironolacton, Mildronat und andere monovalente Kationen transportieren [250, 269-273]. Somit ist es denkbar, dass die Induktion des OCTN-2 durch Aktivierung des PPARα durch oxidierte Nahrungsfette oder die Gabe von Fibraten zur Therapie von Hyperlipidämien auch die Absorption verschiedener Pharmaka im Darm verstärkt sowie ihre Gewebeverteilung beeinflusst.

3.2.3 Regulation des MCT1

Als Sofortreaktion werden im Hungerzustand zunächst die Glykogenspeicher der Leber mobilisiert, um den Blutglukosespiegel aufrecht zu erhalten. Da die Glykogenreserven begrenzt sind, werden zusätzlich gespeicherte Triglyzeride aus dem Fettgewebe als Glyzerol

und freie Fettsäuren freigesetzt. Während Glyzerol in der Leber für die Glukoneogenese verwendet wird, werden die Fettsäuren in der Leber oxidiert und u.a. für die Synthese von Ketonkörpern eingesetzt. Über die Zirkulation gelangen die Ketonkörper dann zu den peripheren Geweben. Über die Regulation der Expression des Schlüsselenzyms der Ketonkörpersynthese, mHMG-CoA-Synthase, durch PPARα [46, 47] wird die Ketogenese direkt mit der Induktion der β-Oxidation im Hungerzustand verknüpft. In unseren eigenen Untersuchungen (**A12**) konnten wir nun zeigen, dass auch der Transport der Ketonkörper über Membranen durch PPARα reguliert wird. MCT1, der bisher am besten charakterisierte und ubiquitär verbreitete Transporter aus der Familie der MCT, ist in apikalen und basolateralen Membranen lokalisiert und transportiert Ketonkörper, Laktat und Pyruvat im Austausch gegen ein Proton [48]. MCT1 wird sowohl in Rattenhepatomazellen als auch in den Lebern von Ratten und Mäusen bei Aktivierung des PPARα durch synthetische Agonisten oder Fasten induziert (**A12**). Wie in Untersuchungen mit Wildtyp- und PPARα-*knockout*-Mäusen gezeigt werden konnte, ist die Induktion des MCT1 nicht nur in der Leber, sondern auch in der Niere und im Dünndarm vom Vorhandensein des PPARα abhängig (**A12**). Somit steuert PPARα sowohl die Synthese von Ketonkörpern über die Regulation der mHMG-CoA-Synthase-Expression als auch deren Transport aus den Leberzellen und in die Gewebe über eine vermehrte Expression des MCT1 und gewährleistet damit, dass die neu synthetisierten Ketonkörper in die Zirkulation gelangen und effizient in die Zellen der peripheren Gewebe zur Energieversorgung aufgenommen werden können. Im Gegensatz zu Leber, Niere und Dünndarm konnten wir keine Beeinflussung der MCT1-Expression durch WY 14,643 im Herzen der Wildtyp-Mäuse feststellen (**A12**). Diese gewebespezifischen Unterschiede in der Regulation sind vermutlich durch Überschneidungen in der Funktion der verschiedenen bisher charakterisierten MCT-Isoformen bedingt. So werden im Herzen zum Beispiel auch die Isoformen MCT2 und MCT4 exprimiert [49, 274-277], von denen zumindest MCT4 ebenfalls Ketonkörper transportiert [278]. Auch zahlreiche andere der bisher identifizierten 14 MCT-Isoformen, die bisher allerdings nicht funktionell charakterisiert wurden, werden im Herzen exprimiert und könnten in den Transport der Ketonkörper im Hungerzustand involviert sein.

Die mit Hilfe von PPARα-*knockout*-Mäusen bestätigte PPARα-abhängige Induktion des MCT1 lässt vermuten, dass MCT1 ebenso wie die mHMG-CoA-Synthase direkt über Bindung des PPARα an ein PPRE in regulatorischen Bereichen des MCT1-Genes aktiviert wird. Eine *in silico*-Analyse relevanter Sequenzbereiche des MCT1-Genes der Maus zeigte mindestens zwei putative PPRE im 5'-regulatorischen Bereich und im ersten Intron, die eine hohe Übereinstimmung mit der PPRE-Consensus-Sequenz aufweisen. In einem Reportergenassay konnte jedoch weder für die Promotorregion noch für den relevanten Intronbereich eine Aktivierung durch WY 14,643 bzw. durch co-exprimierten PPARα/RXRα

festgestellt werden (**A13**). Inwiefern andere als die bereits von uns untersuchten Sequenzbereiche, sowohl im 5'-flankierenden Bereich als auch weitere Intronsequenzen, für die Aktivierung durch PPARα verantwortlich sind, bleibt zu untersuchen. Möglicherweise erfolgt die Aktivierung des MCT1 durch PPARα aber auch nicht über eine direkte Bindung des PPARα an regulatorische Sequenzen im MCT1-Gen. So wurde bereits eine Substrat-abhängige Induktion des MCT1 gezeigt [279, 280]. Wir konnten allerdings bei der direkten Inkubation von Fao-Zellen mit Ketonkörpern nur eine geringe Erhöhung der MCT1-mRNA-Konzentration feststellen (**A13**), so dass die MCT1-Induktion durch PPARα wahrscheinlich nicht über die erhöhten Ketonkörperkonzentrationen vermittelt wird. Ein weiterer, erst vor kurzem identifizierter zentraler Regulator der Anpassung des Stoffwechsels an Hunger ist der *fibroblast growth factor* (FGF)-21, der in der Leber synthetisiert und dann sekretiert wird und als eine Art Hormon fungiert. Während des Fastens sowie durch spezifische PPARα-Agonisten wird der hepatische FGF21 über einen PPARα-abhängigen Mechanismus, der die direkte Bindung an ein PPRE im FGF21-Gen beinhaltet, induziert [281-283]. Die Induktion des FGF21 ist dabei für die normale Aktivierung von Lipolyse, hepatischer Lipidoxidation, Ketogenese und Glukoneogenese notwendig [281, 282, 284]. FGF21 fungiert somit als ein Mediator der PPARα-vermittelten Effekte und scheint vor allem bei langanhaltenden Fastenzuständen wichtig zu sein [281, 282, 284, 285]. Die Stimulation der Ketogenese durch FGF21 erfolgt dabei u.a. durch eine Erhöhung der Proteinkonzentration der mHMG-CoA-Synthase [282], was einen zusätzlichen Mechanismus zur direkten transkriptionellen Regulation der mHMG-CoA-Synthase-Expression durch PPARα [46, 47] darstellt. So ist es denkbar, dass die Induktion des MCT1 durch PPARα zumindest teilweise durch FGF21 vermittelt wird. Weiterführende Untersuchungen sind notwendig, um die molekularen Mechanismen der PPARα-vermittelten Induktion des MCT1 und eine mögliche Rolle des FGF21 aufzuklären.

In unseren Untersuchungen zur Beeinflussung des Cholesterol- und Carnitinstoffwechsels durch PPARα konnten wir bereits zeigen, dass natürliche nutritive PPARα-Agonisten vergleichbare molekulare Effekte wie die synthetischen PPARα-Agonisten hervorrufen (**A3, A11**). In Übereinstimmung damit konnten wir ebenfalls eine Induktion sowohl der mHMG-CoA-Synthase als auch des MCT1 in der Leber von Ratten durch Gabe eines oxidierten Fettes bzw. von CLA beobachten (**A13**). Wir vermuten auch hier, dass dieser Effekt durch die Aktivierung des PPARα durch Bestandteile des oxidierten Fettes bzw. der CLA vermittelt wird. Eine Vermittlung des Effektes über eine Aktivierung des PPARγ durch die oxidierten Fettsäuren bzw. CLA [74, 286] konnten wir aufgrund unserer Untersuchungen in Fao-Zellen (**A12**) dagegen als sehr unwahrscheinlich einstufen.

Auch in der Leber von Schweinen kam es bei PPARα-Aktivierung durch Fasten bzw. die Gabe von Clofibrat oder eines oxidierten Fettes zu einer Induktion des MCT1 in der Leber (**A13**). Ebenso war die mHMG-CoA-Synthase-Expression in der Leber erhöht (**A13**). Somit kann man schlussfolgern, dass nicht nur bei Ratten und Mäusen als Mitglieder der proliferierenden Spezies, sondern auch bei Schweinen, die als Mitglieder der nicht-proliferierenden Spezies als Modelltier für den Menschen fungieren, die Induktion der Ketogenese bei PPARα-Aktivierung von einer Induktion des Ketonkörpertransporters MCT1 begleitet wird. Die MCT1-Induktion durch PPARα scheint somit einen allgemeinen Mechanismus darzustellen, der die Versorgung der Gewebe mit Ketonkörpern als Energiequelle im Hungerzustand sicherstellt. Diese Hypothese eines speziesübergreifenden Mechanismus wird auch durch unsere Beobachtung gestützt, dass sowohl bei Ratten und Mäusen als auch beim Schwein gleichzeitig mit der Induktion des MCT1 eine erhöhte Expression des CD147 zu verzeichnen war (**A13**). Das Chaperon CD147 ist ein Transmembranprotein, das für die korrekte Membranassemblierung und die Transporterfunktion des MCT1 essenziell ist [287-289]. Einige Studien konnten bereits zeigen, dass die Induktion des MCT1 durch verschiedene Stimuli von einer Induktion seines Chaperons CD147 begleitet wurde [290-292] und dass Reifung und Zelloberflächen-expression des CD147 von der Expression des MCT1 abhängen [293, 294]. Die gleichzeitige Induktion von MCT1 und CD147 in unseren Studien und die Tatsache, dass in PPARα-*knockout*-Mäusen neben der fehlenden Induktion des MCT1 auch keine Expressionssteigerung des CD147 zu beobachten war (**A13**), unterstützen somit die Literaturdaten zur Regulation der CD147-Aktivität durch MCT1.

Die Ergebnisse unserer Studien (**A12, A13**) zur Regulation des MCT1 durch PPARα haben allerdings nicht nur Bedeutung für das Verständnis der molekularen Prozesse, die der Anpassung der Organismen an Hungersituationen unterliegen. MCT1 transportiert neben den bereits erwähnten Monocarboxylaten wie Pyruvat, Laktat und Ketonkörpern auch Pharmaka wie γ-Hydroxybutyrat (Narkosemittel und Partydroge), Salicylate und Statine [295-299]. So wurde zum Beispiel die Transportmöglichkeit über MCT1 im Dünndarm in das Design von Gabapentin-Prodrugs mit einbezogen, um die Bioverfügbarkeit von Gabapentin zu erhöhen [300, 301]. Somit kann eine Aktivierung des PPARα bei therapeutischer Gabe von Fibraten oder durch Aufnahme von oxidierten Nahrungsfetten bzw. CLA nicht nur über die Induktion des OCTN-2 (**A10, A11**), sondern auch über die Induktion des MCT1 Auswirkungen auf die Aufnahme und den Transport von Medikamenten beim Menschen haben.

3.2.4 Rolle des PPARα im Gehirn

Neben den umfangreichen Funktionen des PPARα in Geweben mit hohen Raten der Fettsäureoxidation gibt es immer mehr Hinweise darauf, dass PPARα auch eine Rolle bei verschiedenen Stoffwechselvorgängen im Gehirn spielt. Hierfür sprechen zum Beispiel die beobachteten präventiven und akuten neuroprotektiven Eigenschaften von PPARα-Agonisten bei zerebraler Ischämie [302-305] oder bei verschiedenen neurodegenerativen Erkrankungen wie Morbus Alzheimer, Morbus Parkinson oder Multipler Sklerose [306-309]. Diese Effekte beruhen hauptsächlich auf den antioxidativen und antiinflammatorischen Eigenschaften des PPARα, wie zum Beispiel der Induktion und Aktivierung antioxidativer Enzyme wie Superoxiddismutase und Glutathionperoxidase bzw. der Inhibierung von Synthese und Ausschüttung von Cytokinen oder inflammatorischen Mediatoren wie Cyclo-Oxygenase 2 und Adhäsionsproteinen. Daneben scheint der PPARα im Gehirn auch eine zentrale Rolle in der Regulation der Glukosehomöostase des gesamten Körpers im Hungerzustand [55] und in der Regulation der Futteraufnahme [310] zu spielen. Mit unseren Untersuchungen (**A14**) konnten wir erstmals zeigen, dass im Hungerzustand tatsächlich PPARα-abhängige Gene, die in die peroxisomale und mitochondriale β-Oxidation von Fettsäuren und in die Ketogenese involviert sind, in verschiedenen Gehirnbereichen und in der Hypophyse von Ratten induziert werden. Somit wird im Hungerzustand offenbar nicht nur der PPARα in der Leber, sondern auch der des Gehirns aktiviert. Diese Aktivierung scheint allerdings zeitlich verzögert im Vergleich zu der in der Leber aufzutreten (**A14**). Inwiefern die im Hungerzustand erhöhten Konzentrationen an zirkulierenden freien Fettsäuren, die sich auch im Gehirn widerspiegeln [311], für die Aktivierung des PPARα im Gehirn und in der Hypophyse verantwortlich sind, ist bisher unklar. Neuere Untersuchungen zeigten überraschenderweise, dass nicht die vom Fettgewebe im Hungerzustand freigesetzten Fettsäuren den PPARα der Leber aktivieren, sondern nur solche, die unter Beteiligung der FAS neu synthetisiert wurden oder über die Diät neu zugeführt wurden [312, 313]. Ein Verlust der FAS im Hypothalamus beeinträchtigt ebenfalls die Expression PPARα-abhängiger Gene im Gehirn [310]. Als ein möglicher physiologisch relevanter endogener PPARα-Ligand in der Leber, dessen Synthese vom Vorhandensein der FAS abhängig ist, wurde vor kurzem ein spezielles Phospholipid identifiziert [314]. Inwiefern dieser endogene Ligand auch für die von uns beobachtete Aktivierung des PPARα im Gehirn und in der Hypophyse im Hungerzustand (**A14**) relevant ist, bleibt unklar. In Betracht gezogen werden muss auch eine mögliche Rolle des FGF21, der vor allem bei langanhaltenden Hungerzuständen in der Leber durch PPARα induziert und als eine Art Hormon in die Zirkulation abgegeben wird und als Mediator der PPARα-vermittelten Effekte fungiert [281-285]. Es konnte bereits gezeigt werden, dass intaktes FGF21 die Bluthirnschranke, wahrscheinlich auf dem Wege der Diffusion, passieren kann [315]. Da FGF21 auch Torpor stimuliert [282], einen kurzen Winterschlaf-ähnlichen

Zustand der regulierten Hypothermie bei kleinen Säugern zur Energieeinsparung bei Futtermangel, ist eine Wirkung von FGF21 im Gehirn durchaus denkbar.

Allgemein wird angenommen, dass das Gehirn im Hungerzustand auf die in der Leber synthetisierten Ketonkörper als Glukoseersatz zurückgreifen muss. Allerdings konnte auch gezeigt werden, dass Fettsäuren sowohl im Gehirn als auch in isolierten Astrozyten oxidiert werden können [316, 317] und dass kultivierte Astrozyten in der Lage sind, Ketonkörper aus Fettsäuren zu synthetisieren und so vermutlich Neuronen mit Ketonkörpern versorgen können [317]. Desweiteren werden typische PPARα-Zielgene, die in die β-Oxidation von Fettsäuren und die Ketogenese involviert sind, auch bei oraler Gabe von Fibraten im Gehirn induziert [318; **A14**]. Die Tatsache, dass diese Gene auch im Hungerzustand im Gehirn und in der Hypophyse induziert werden (**A14**), unterstützt damit die Hypothese, dass das Gehirn auch zur Eigensynthese von Ketonkörpern fähig ist, um seinen Energiebedarf bei Glukosemangel, z.B. im Hungerzustand, zu decken.

Von den in unseren Untersuchungen betrachteten Gehirnbereichen wurde die stärkste Aktivierung des PPARα in der Hypophyse festgestellt (**A14**). In einer früheren Studie konnte bereits mit Hilfe eines Hypophysen-Zellmodells gezeigt werden, dass die Transkription des Prolaktin-Gens spezifisch durch PPARα induziert wird [54]. In unseren eigenen Untersuchungen (**A14**) konnten wir nun mit Hilfe des PPARα-*knockout*-Mausmodells erstmals zeigen, dass die Expression des Prolaktin-Genes tatsächlich im Hungerzustand in der Hypophyse von Wildtyp-Mäusen induziert wird und dass dieser Effekt genotypspezifisch ist und somit bei PPARα-*knockout*-Mäusen nicht auftritt. Auch für ein anderes Hypophysenhormon, das Luteinisierende Hormon (LH), konnte eine solche PPARα-abhängige Induktion im Hungerzustand bei den Mäusen beobachtet werden (**A14**). Schließlich konnten wir auch für die Hypophysenhormone Proopiomelanocortin und Thyreoidea-stimulierendes Hormon (TSH) genotypspezifische Unterschiede in der Expression feststellen (**A14**). Somit scheint der PPARα im Gehirn nicht nur für z. B. die Ketogenese und die Regulation der Glukosehomöostase wichtig zu sein, sondern ist offenbar auch in die Kontrolle der Hormonproduktion in der Hypophyse involviert. Die Mechanismen, die den beobachteten Effekten auf die Hormonexpression zugrunde liegen, sind allerdings noch unklar. Für die transkriptionelle Regulation des Prolaktin-Genes durch PPARα wurde ein Mechanismus diskutiert, der nicht die klassische Interaktion eines PPARα/RXRα-Heterodimerkomplexes mit einem PPRE beinhaltet. Stattdessen scheint PPARα mit einem weiteren, Hypophysen-spezifischen Transkriptionsfaktor (GHF-1/Pit-1) zu interagieren, der die Expression des Prolaktin-Genes reguliert [54]. GHF-1/Pit-1 ist auch in die Regulation der Expression anderer Hypophysenhormone wie TSH und *growth hormone* involviert [319-321], so dass die beschriebene Beeinflussung der GHF-1/Pit-1-Aktivität durch

PPARα [54] zum Beispiel auch eine Rolle bei den beobachteten speziesspezifischen Unterschieden der TSH-Expression bei Wildtyp- und PPARα-*knockout*-Mäusen (**A14**) spielen könnte.

Insgesamt konnten wir mit unseren Studien (**A14**) erstmals zeigen, dass PPARα offenbar auch im Gehirn und in der Hypophyse im Hungerzustand aktiviert wird, was Literaturdaten zur essenziellen Rolle des gehirnlokalisierten PPARα in der Regulation der Glukosehomöostase des gesamten Körpers im Hungerzustand [55] unterstützt. Ebenso liefern unsere Untersuchungen weitere Hinweise auf eine mögliche Fähigkeit des Gehirns, Ketonkörper als Glukoseersatz selbst zu synthetisieren. Schließlich scheint PPARα auch in die Regulation der Hormonexpression in der Hypophyse im Hungerzustand involviert zu sein. Somit scheint nicht nur die Aktivierung des PPARα der Leber, sondern auch die Aktivierung des PPARα in Gehirn und Hypophyse ein wichtiger Schritt in der Anpassung des Körpers an Energiemangelzustände zu sein.

4 Zusammenfassung

Die in der vorliegenden Arbeit vorgestellten Untersuchungen beschäftigten sich schwerpunktmäßig mit der Aufklärung neuer Zusammenhänge hinsichtlich der Regulation des Lipid- und Carnitinstoffwechsels durch PPARα. Aufgrund der existierenden speziesspezifischen Unterschiede bezüglich einer PPARα-Aktivierung wurden verschiedene Spezies in die Untersuchungen einbezogen. Außerdem wurde die Wirkung von natürlichen nutritiven PPARα-Agonisten auf die untersuchten Stoffwechselwege betrachtet.

In Bezug auf die Frage, welche Mechanismen den verringerten Cholesterol- und Triglyzeridkonzentrationen in der Leber von Ratten nach Clofibratgabe unterliegen, konnte gezeigt werden, dass die verringerten Cholesterolkonzentrationen auf einer Inhibierung der Ausreifung des SREBP-2 und daraus resultierenden verminderten Transkriptmengen der HMG-CoA-Reduktase und des LDL-Rezeptors beruhen. Dies führt zu einer verringerten Synthese und Aufnahme von Cholesterol in die Leber und in Ratten-Hepatomazellen. Die verminderte nukleare Konzentration des SREBP-2 wird dabei durch Induktion des Insig-1 bei Aktivierung des PPARα hervorgerufen. Weiterhin konnte gezeigt werden, dass vergleichbare Mechanismen, nämlich die Inhibierung der Reifung des SREBP-1c durch Induktion von Insig-1 und Insig-2a, zu einer Verringerung der Triglyzeridsynthese bei Aktivierung sowohl des PPARα als auch des PPARγ führen. Somit konnte ein neuartiger Zusammenhang zwischen PPARα und dem SREBP-abhängigen Lipidstoffwechsel aufgezeigt werden, dessen Bindeglied die Regulation der Insig durch PPARα ist.

Bezüglich der Frage, ob auch natürliche PPARα-Agonisten wie oxidierte Fette vergleichbare Änderungen des Lipidstoffwechsels wie Clofibrat bewirken, und inwiefern speziesspezifische Unterschiede bei der Wirkung von PPARα-Agonisten auf den Lipidstoffwechsel existieren, wurden *in vitro*-Studien mit Hepatomazellen der Ratte und des Menschen und *in vivo*-Studien mit Ratten, Schweinen und Legehennen durchgeführt. Es konnte gezeigt werden, dass auch eine Aktivierung des PPARα durch oxidierte Fette in der Leber von Ratten zu einer Induktion der Insig, zur Inhibierung der SREBP-2-abhängigen Cholesterolsynthese und –aufnahme und damit zu verringerten Cholesterolkonzentrationen in Leber und Plasma führte. Bei Schweinen bewirkte ein oxidiertes Fett eine leichte Aktivierung des PPARα in der Leber, die Triglyzerid- und Cholesterolkonzentrationen blieben jedoch unverändert. SREBP-1 und -2 und relevante Zielgene der Fettsäure- und Cholesterolsynthese in Leber und Darm waren erhöht, ebenso das mHMG-CoA-Synthase-Transkript und die Konzentration an 3-Hydroxybutyrat im Plasma. In Übereinstimmung mit den unterschiedlich starken Effekten eines oxidierten Fettes in proliferierenden und nicht-proliferierenden Spezies führte die Inkubation von Hepatomazellen der Ratte (Fao) und des Menschen (HepG2) mit der

oxidierten Fettsäure 13-HPODE zu einer deutlichen Aktivierung des PPARα in Fao-Zellen, jedoch nicht in HepG2-Zellen. Bei Legehennen kam es durch Fütterung von Clofibrat zu einer starken Aktivierung des hepatischen PPARα, zu einer stark verminderten Futteraufnahme, verringerten Östrogenkonzentrationen im Plasma und einer Einstellung der Eiproduktion. SREBP-2 und seine Zielgene sowie FAS als auch die Cholesterol- und Triglyzeridkonzentrationen in Leber und Plasma waren vermindert. Fischöl als ein weiterer nutritiver PPARα-Agonist führte in Legehennen zu einer moderaten Aktivierung des hepatischen PPARα und zu einer Absenkung der Plasmatriglyzeride. Die Expression der Gene der Fettsäuresynthese in der Leber der Legehennen wurde durch Fischöl nicht beeinflusst. Das nukleare SREBP-2 und das HMG-CoA-Reduktase-Transkript sowie die Plasma-Cholesterolkonzentrationen waren vermindert. CLA führte zu keiner Aktivierung des PPARα in der Leber von Legehennen, auch die Plasmatriglyzeride wurden nicht beeinflusst. CLA-Fütterung erhöhte die Cholesterol- und Triglyzeridkonzentrationen in der Leber und die Cholesterolkonzentrationen im Plasma der Hennen. Nutritive PPARα-Agonisten weisen demnach umfangreiche Wirkungen auf den SREBP-abhängigen Lipidmetabolismus in Modelltieren auf, wobei sowohl Gemeinsamkeiten als auch Unterschiede in den Effekten in verschiedenen Spezies zu beobachten sind.

Die Untersuchungen zur Frage, ob die CACT durch PPARα reguliert wird, ergaben, dass CACT sowohl durch den PPARα-Agonisten WY 14,643 als auch durch Nahrungsentzug bei Wildtyp-Mäusen induziert wird, während bei PPARα-*knockout*-Mäusen kein derartiger Effekt zu beobachten ist. Somit wird CACT tatsächlich durch PPARα reguliert. Dabei konnte für das CACT-Gen der Maus gezeigt werden, dass die transkriptionelle Regulation durch Bindung des PPARα an ein funktionelles PPRE im 5'-untranslatierten Bereich des CACT-Genes erfolgt. Eine Induktion der CACT durch WY 14,643 konnte auch in Fao- und HepG2-Zellen gezeigt werden. Desweiteren führte die Aktivierung des PPARβ/δ ebenso zu einer erhöhten Expression der CACT in Fao- und HepG2-Zellen und zu einer Stimulation des Maus-CACT-Promotors, was eine Regulation der CACT auch durch PPARβ/δ vermuten lässt.

Hinsichtlich der Frage, welche Wirkmechanismen den erhöhten Carnitin-Konzentrationen in der Leber nach Clofibratgabe bzw. Fasten zugrunde liegen, ließ sich zeigen, dass PPARα sowohl Gene der Carnitin-Biosynthese als auch des Carnitintransportes reguliert. Dabei induziert PPARα einerseits den Carnitintransporter OCTN-2, was zu erhöhten Carnitinkonzentrationen in der Leber führt. Andererseits erhöht PPARα die Carnitinsynthese in der Leber über die Induktion der Carnitinsynthesegene Trimethyllysin-Dioxygenase, 4-N-Trimethylaminobutyraldehyd-Dehydrogenase und BBD. Die essentielle Rolle des PPARα für die Regulation des OCTN-2 sowie des OCTN-3 und der Gene der Carnitinsynthese konnte dabei in Versuchen mit Wildtyp- und PPARα-*knockout*-Mäusen demonstriert werden. In

Bezug auf die Frage, ob vergleichbare Effekte auch mit natürlichen PPARα-Agonisten hervorgerufen werden können, konnte gezeigt werden, dass ein oxidiertes Fett den Carnitinstoffwechsel in Ratten ähnlich beeinflusst wie synthetische PPARα-Agonisten oder Hungern. Dies zeigte sich in einer Induktion des OCTN-2 in Leber und Darm der Ratten ebenso wie der Induktion eines Enzyms der Carnitinsynthese sowie in erhöhten Carnitinkonzentrationen in der Leber.

Bezüglich der Frage, ob MCT1 ebenfalls zu den PPARα-regulierten Genen zählt, konnten wir zeigen, dass MCT1 sowohl in Rattenhepatomazellen als auch in den Lebern von Ratten und Mäusen bei Aktivierung des PPARα durch synthetische Agonisten oder Fasten induziert wird. Diese Induktion ist vom Vorhandensein des PPARα abhängig, wie in Versuchen mit PPARα-*knockout*-Mäusen demonstriert werden konnte. Somit kann geschlussfolgert werden, dass MCT1 tatsächlich durch PPARα reguliert wird. Hinsichtlich der Frage, ob vergleichbare Effekte in nicht-proliferierenden Spezies und bei Verwendung nutritiver PPARα-Agonisten beobachtet werden können, konnten wir zeigen, dass oxidierte Fette und CLA in der Leber von Ratten über eine Aktivierung des PPARα ebenfalls zur Induktion des MCT1 führen. Auch in der Leber von Schweinen wurde eine Erhöhung des MCT1 durch Fütterung von Clofibrat und oxidiertem Fett bzw. durch Fasten bewirkt, so dass davon ausgegangen werden kann, dass die Regulation des MCT1 durch PPARα einen speziesübergreifenden Mechanismus darstellt.

In Bezug auf die Frage, ob es während des Fastens zu einer Aktivierung des PPARα im Gehirn kommt, konnte eine Induktion von PPARα-regulierten Genen, die in die β-Oxidation von Fettsäuren und die Synthese von Ketonkörpern involviert sind, im Gehirn und in der Hypophyse von Ratten nach 36 h Fasten gezeigt werden. Damit ist davon auszugehen, dass der PPARα im Gehirn und in der Hypophyse tatsächlich im Hungerzustand aktiviert wird. Zur Beantwortung der Frage, ob PPARα in die Regulation der Expression von Hypophysenhormonen involviert ist, wurde die Expression relevanter Gene in der Hypophyse von Wildtyp- und PPARα-*knockout*-Mäusen im Hungerzustand untersucht. Dabei konnten eine PPARα-abhängige Induktion von Prolaktin und LH im Hungerzustand und genotypspezifische Unterschiede der Expression von Proopiomelanocortin und TSH gezeigt werden.

Insgesamt zeigen die in dieser Arbeit vorgestellten Untersuchungen eine Vielzahl neuer PPARα-vermittelter Regulationsmechanismen auf, welche die zentrale Stellung dieses Transkriptionsfaktors in der Regulation des Lipidstoffwechsels und der Anpassung des Organismus an Energiemangelzustände unterstreichen. Darüber hinaus sind diese Erkenntnisse auch für die Betrachtung der Wirkungen von nutritiven PPARα-Agonisten beim Menschen relevant.

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Eidesstattliche Erklärung

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