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„Untersuchungen zur Wirkung synthetischer und nutritiver PPARα-Agonisten auf Stoffwechselwege in besonderen Stoffwechselsituationen im Nager“

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Abkürzungsverzeichnis

ACO	acyl-CoA oxidase
ACS	acyl-CoA synthase
BB	γ-Butyrobetain
BBD	γ-Butyrobetaindioxygenase
CACT	carnitine-acylcarnitine translocase
CLA	conjugated linoleic acid; konjugierte Linolsäure
CPT	carnitine palmitoyltransferase
CYP4A1	cytochrome P-450 A1
FABPpm	plasma membrane fatty acid binding protein
FAS	fatty acid synthase
FAT	fatty acid translocase
FATP	fatty acid transport protein
HODE	Hydroxyoctadecadiensäure
HPODE	Hydroxyperoxyoctadecadiensäure
HSL	hormone-sensitive lipase
HTMLA	3-Hydroxy-N-TML-Aldolase
LCAD	long-chain acyl-CoA dehydrogenase
LCFA	long-chain fatty acid ; langkettige Fettsäure
LDL	low density lipoprotein
LPL	lipoprotein lipase
MAT	ménage-à-trois
MCAD	medium-chain acyl-CoA dehydrogenase
MCFA	medium-chain fatty acid
M-CPT	muscle carnitine palmitoyltransferase
mRNA	messenger ribonucleic acid
NEFA	nonesterified fatty acids; unveresterte freie Fettsäuren
NF-κB	nuclear factor ´kappa-light-chain-enhauncer` of activated B-cells
OCTN	novel organic cation transporter
PGC	PPARγ coactivator
PPAR	peroxisome proliferator-activated receptor
PPRE	peroxisome proliferator response element
PUFA	poly unsatturated fatty acid; mehrfach-ungesättigte Fettsäure

ROS	reaktive Sauerstoffspezies
RXR	retinoic X-receptor
SLC22A	solute carrier 22A family
SREBP	sterol regulatory element-binding protein
TGZ	troglitazone
TMABA-DH	4-N-Trimethylaminobutyroaldehyddehydrogenase
TML	Trimethyllysin
TMLD	Trimethyllysindioxygenase
UCP	uncoupling protein
VLDL	very low density lipoprotein

1. Einleitung und Zielstellung

1.1 Peroxisome proliferator-activated receptors (PPARs): Definition und grundlegende Funktionen

PPARs sind liganden-aktivierte Transkriptionsfaktoren, die der Superfamilie der nukleären Hormonrezeptoren angehören und eine Peroxisomenproliferation im Nager nach Aktivierung durch Agonisten herbeiführen (Guan & Breyer *et al*., 2001; Desvergne & Wahli, 1999). Bisher wurden drei PPAR Isotypen in *Xenopus*, Hamster (Aperlo *et al*., 1995), Maus (Chen *et al*., 1993; Zhu *et al*., 1993; Kliewer *et al*., 1994), Ratte (Xing *et al*., 1995; Göttlicher *et al*., 1992) und dem Menschen (Schmidt *et al*., 1992; Sher *et al*., 1993, Green *et al*., 1995) identifiziert: PPARα (NR1C1), PPARβ/δ (NR1C2) und PPARγ (NR1C3). PPARα, der bisher in der Literatur am besten beschriebene Isotyp der PPARs, wurde durch Issemann und Green (1990) erstmals aus cDNA in Mausleberzellen kloniert und konnte bisher in den oben erwähnten Spezies sowie in neuesten Studien im Schwein und im Huhn nachgewiesen werden (Cheon *et al*., 2005; Luci *et al*., 2007; König *et al*., 2007). Die Aktivierung der PPARs erfolgt durch natürliche Liganden, wie freie unveresterte Fettsäuren (NEFA) aus dem weißen Fettgewebe oder der Nahrung, Fettsäurederivaten, Eicosanoiden, Komponenten oxidierter *low density lipoproteins* (LDL) und *very low density lipoproteins* (VLDL) sowie Linolsäurederivaten (Keller *et al*., 1993, Giguère, 1999; Bensinger & Tontonoz, 2008). Des Weiteren zählen eine Reihe synthetischer Pharmazeutika, welche in der Forschung und der Medizin ihre Verwendung finden, zu den Liganden der PPARs. Dazu gehören die in den 70er Jahren erstmals angewendeten lipidsenkenden Fibrate (Clofibrat und Fenofibrat) und insulinsensitivierende antidiabetische Pharmazeutika (Thiazolidinedione) (Berger *et al*., 1996). Nach erfolgter Aktivierung formen PPARs Heterodimere mit dem *retinoic-X receptor* (RXR) (Miyata *et al*., 1994) und binden an eine spezifische Region im Promotor von PPAR-Zielgenen, dem sogenannten *peroxisome proliferator response element* (PPRE). Die meisten PPREs sind Wiederholungen einer sechsstelligen Nukleotidsequenz, welche durch ein Basenpaar getrennt sind (AGGTCA) (Desvergne & Wahli, 1999; IJpenberg *et al*., 1997; Kliewer *et al*., 1992). Eine Ligandenbindung bewirkt eine Konformationsänderung im Protein, was eine Degeneration von Co-Repressoren und eine Verstärkung der Co-Aktivator-Komplexe nach sich zieht. Dieser Vorgang führt zu einer Aktivierung der Transkription von PPARα-Zielgenen und gleichzeitiger Regulation der Aktivität des Transkriptionsvorganges

(Abb. **1**). Das erste PPRE konnte in der Promotorregion des Gens für die *acyl-CoA oxidase* (ACO) gefunden werden (Dreyer *et al*., 1992; Tugwood *et al.*, 1992).

Die Aktivität der PPARs kann durch Co-Aktivatoren und Co-Repressoren, welche zwischen DNA-Bindung und Initialisierung der Transkription vermitteln, auf verschiedenen Wegen beeinflusst werden (**Abb. 1**). Eine Möglichkeit ist, dass Co-Aktivatoren unter physiologischen Stimuli und durch Liganden direkt mit den PPARs agieren und verstärkend auf den Transkriptionsvorgang wirken (Glass *et al*., 1997; Horwitz *et al*., 1996). Ein zweiter Einfluss auf die Aktivität der PPARs erfolgt Liganden-unabhängig über die Bindung des PPAR-RXR-Komplexes mit Co-Repressoren, wodurch die Transkription der Zielgene unterdrückt wird. Eine dritte, Liganden-abhängige Möglichkeit der Transkriptionsregulierung ist die Transrepression der Zielgene (Ruan *et al*., 2008). Neben dem Einfluss durch Liganden wird die Aktivität der PPARs, Liganden-abhängig oder -unabhängig, zudem über den Grad der posttranslationalen Phosphorylierung durch Kinasen modifiziert. Dies beeinflusst zusätzlich die Affinität der Liganden, die Stärke der DNA Bindung, die Verstärkung durch Co-Faktoren und die proteasomale Degradation (Diradourian *et al*., 2005) und somit die Transkription der Zielgene.

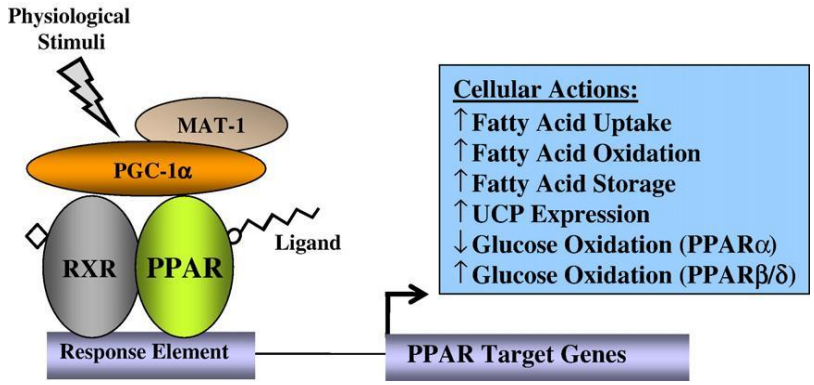

Abb. 1: PPAR-Aktivierung der Transkription von Zielgenen und Hauptfunktionen im Stoffwechsel (aus Madrazo *et al*., 2008). Die transkriptionelle Aktivität der PPARs wird durch die Bindung endogener Liganden und Co-Aktivatoren beeinflusst, wie dem PPARγ *coactivator* (PGC) 1α und dessen Regulator *ménage-à-trois-1* (MAT-1).

1.2 Die Rolle des PPARα im Lipidstoffwechsel

PPARα spielt eine entscheidende Rolle im Fettsäuremetabolismus und reguliert im Besonderen den Stoffwechsel im Fastenzustand (Kersten *et al*., 1999; Leone *et al.*, 1999). Fasten induziert einen erhöhten Glucagon- und Glucocorticoid-Spiegel im Plasma. Dies begünstigt die Hydrolyse von Triglyzeriden im Fettgewebe, wodurch sich die Konzentration an freien langkettigen Fettsäuren (LCFA) im Plasma erhöht. Auf die Aufnahme der LCFA über Fettsäuretransporter in die Zelle folgt die Steigerung der Expression und Aktivierung des PPARα (Escher *et al*., 2001). Die fundamentale Rolle des PPARα während des Fastens wird aus Studien mit PPARα-*knockout* Mäusen ersichtlich. Darin führte die Deletion des PPARα zu einer Störung des Fettsäurekatabolismus und infolge dessen zur Hypothermie, Hyperlipidämie und einer Fettleber, was mit einer Hypoketonämie und Hypoglycämie einherging. Die höchsten Genexpressionsraten des PPARα wurden bisher in Geweben mit einer hohen metabolischen Aktivität und somit einem hohen Energiebedarf ermittelt. In der ausgewachsenen Ratte konnten hohe Konzentrationen an *messenger ribonucleic acid* (mRNA) in der Leber, der Niere, dem Skelettmuskel, dem Herzen und dem braunem Fettgewebe nachgewiesen werden. Auch in der Retina, der Nebenniere und den Langerhansschen Inseln wurden signifikante Mengen PPARα mRNA gefunden (Braissant *et al*., 1996). In einigen Studien konnten geringere PPARα Level in der Leber des Menschen als in der Leber von Nagern beobachtet werden (Palmer *et al*., 1998). Überdies ist der PPARα im Herz, der Niere, dem Skelettmuskel, der Nierenrinde und dem Dickdarm im Menschen exprimiert. Eine hohe Expression von PPARα korreliert mit einer hohen Aktivität der peroxisomalen und mitochondrialen Fettsäureoxidation in einem Gewebe (Berger & Moller, 2002). Wie Studien mit PPARα-*knockout* Mäusen zeigen, wird durch den PPARα die Expression zahlreicher Gene des Lipidstoffwechsels beeinflusst (Leone *et al*., 1999). Dazu gehören Gene der mitochondrialen β-Oxidation, der peroxisomalen β-Oxidation, der zellulären Fettsäureaufnahme und -bindung sowie des Lipoproteinaufbaus und -transports und der Ketogenese (Aoyama *et al*., 1998; Peters *et al*., 1997; Motojima *et al*., 1998). Zur ersten Erkenntnis hinsichtlich der Funktion des Transkriptionsfaktors PPARα zählt die Regulierung der peroxisomalen β-Oxidation in der Leber, welche mit einer Proliferation der Peroxisomen einhergeht. Die ACO ist das limitierende Enzym der peroxisomalen β-Oxidation und gehört zum ersten identifizierten PPARα-Zielgen (Dreyer *et al.*, 1992). Ein weiterer wichtiger Teil der β-Oxidation ist das mitochondriale carnitinabhängige Transportsystem, welches den Transfer von Fettsäuren in das Mitochondrium vermittelt (Kerner & Hoppel, 2000). Ein

funktionelles PPRE konnte bereits in der Promotorregion der *carnitine palmitoyltransferase* (CPT) I und II gefunden werden (Mascaró *et al*., 1999; Napal *et al*., 2005).

Bevor Fettsäuren in die Zellen der Gewebe und danach in das Mitochondrium, dem Ort der β-Oxidation, gelangen, werden diese aus triglyzeridreichen Lipoproteinen im Plasma oder aus Triglyzeriden im Fettgewebe durch die *lipoprotein lipase* (LPL) und die *hormone-sensitive lipase* (HSL), welches weitere Zielgene des PPARα darstellen, abgespalten (Schoonjans *et al.*, 1996; Rakhshandehroo *et al*., 2007). Daraufhin erfolgt die Aufnahme der Fettsäuren über Fettsäuretransporter in die Zellen. Ein funktionelles PPRE konnte bereits in der Promotorregion des *fatty acid transport proteins* (FATP), des *fatty acid binding proteins* (FABP) und der *fatty acid translocase CD36* (FAT/CD36) gefunden werden (Frohnert *et al*., 1999; Issemann *et al*., 1992; Motojima *et al*., 1998). Weiterhin wurden bereits PPREs in der Promotorregion der *medium-chain acyl-CoA dehydrogenase* (MCAD) (Gulick *et al*., 1994) und der *long chain acyl-CoA dehydrogenase* (LCAD) identifiziert (Aoyama *et. al*., 1998). Unter direkter Kontrolle des PPARα steht die Transkription des *cytochrome P-450* A1 (CYP4A1), ein Protein, dessen Funktion in der ω-Hydroxylierung von Fettsäuren liegt und es dadurch eine wichtige Rolle in der Regulation der Phase I und Phase II Enzyme im Fremdstoffmetabolismus einnimmt (Aldrige *et al*., 1995; Fan *et al*., 2004).

Zu weiteren Funktionen des PPARα zählt die Regulation der zitterfreien Thermogenese. PPARα moduliert die Expression des *uncoupling protein* (UCP) 1 und 3 im Skelettmuskel (Boss *et al*., 1997; Gong *et al*., 1997; Brun *et al*., 1999; Barbera *et al*., 2001; Young *et al*., 2001). Überdies inhibiert PPARα in glatten Muskelzellen der Aorta, welche maßgeblich an der Entstehung von Plaques und somit der Atherosklerose beteiligt sind, die Bildung proinflammatorischer Moleküle (Interleukin 1, -6 und Prostaglandine) über eine Repression des Transkriptionsfaktors *nuclear factor ´kappa-light-chain-enhauncer´of activated B-cells* (NF-κB) (Staels *et al*., 1998). Des Weiteren wird der PPARα mit der Blutdruckregulation, Insulinsensitivisierung und der Kanzerogenese in Verbindung gebracht.

1.3 Adaptationsprozesse im Lipid- und Carnitinstoffwechsel während der Laktation

Zahlreiche Hinweise aus der wissenschaftlichen Literatur belegen, dass während der Laktation die Expression von Genen der Thermogenese, der Aufnahme und Oxidation von Fettsäuren im Skelettmuskel, sowie der Fettsäureoxidation und Ketogenese in der Leber vermindert ist (Pedraza *et al*., 2000; Pedraza *et al*., 2001; Xiao *et al*., 2004*b*; Whitelaw & Williamson, 1977). Die transkriptionelle Regulierung der Genexpression von Proteinen der

Thermogenese sowie von Enzymen der Fettsäureoxidation und -aufnahme ist PPARα-vermittelt (Mandard *et al*., 2004; Kersten *et al*., 1999; Leone *et al*., 1999; Barbera *et al*., 2001; Young *et al*., 2001). Die verminderte Expression von PPARα Zielgenen während der Laktation deutet auf eine reduzierte oder eingeschränkte Genexpression und/oder Transaktivierung des PPARα hin.

Die Laktation ist ein physiologischer Zustand, welcher durch einen beträchtlichen Anstieg des Energie- und Nährstoffbedarfs gekennzeichnet ist. Dies zeigt sich speziell in einem beachtlichen Anstieg der Futteraufnahme und der Mobilisierung von Energiespeichern, mit dem Ziel, Stoffwechselsubstrate für die Milchproduktion zur Aufzucht der Nachkommen einzusparen (Ota & Yokoyama, 1967). Zugleich entwickeln sich während der Laktation eine Reihe metabolischer Anpassungsprozesse um zusätzlich Energie- und Stoffwechselprodukte einzusparen (Smith & Grove, 2002; Brogan *et al*., 1999; Dewey, 1997). So konnte bereits in Studien mit Ratten und Mäusen eine Verminderung der Expression des UCP 1 und 3 im braunen Fettgewebe und des UCP3 im Skelettmuskel beobachtet werden (Pedraza *et al*., 2000; Pedraza *et al*., 2001; Xiao *et al*., 2004*a*). UCPs entkoppeln die oxidative Phosphorylierung und erhöhen die Permeabilität für Protonen durch die innere mitochondriale Membran, wobei der elektrochemische Protonengradient abgebaut wird. Dies führt zu einer Abnahme der oxidativen Energiegewinnung aus metabolischen Substraten und zu einer Steigerung der Thermogenese. Es wurde bereits von einer Reduktion der Expression von Proteinen der Fettsäureaufnahme, sowie der Fettsäureoxidation in Skelettmuskel und Leber während der Laktation berichtet (Xiao *et al*., 2004*b*; Zammit, 1980). Die Einsparung von Energie und metabolischen Substraten (z.B. Fettsäuren) während der Laktation, in Form einer Reduktion des Fettsäurekatabolismus, der oxidativen Energiegewinnung und der Thermogenese, verfolgt das Ziel die Milchproduktion sicherzustellen. Des Weiteren wird die Hydrolyse von Triglyzeriden im weißen Fettgewebe durch die HSL gesteigert, was nachfolgend zu einem Anstieg im Plasma und einer starken Umverteilung der NEFA zugunsten der Milchdrüse führt, welche dort von Fettsäuretransportern aufgenommen werden und für die Milchfettsynthese zur Verfügung stehen. Gleichzeitig ist die Aktivität der LPL, welche die Aufnahme von Fettsäuren in das Fettgewebe aus triglyzeridreichen Lipoproteinen, wie Chylomikronen und VLDL reguliert, während der Laktation herabgesetzt (Williamson, 1986).

Zu weiteren Adaptationsprozessen während der Trächtigkeit zählen zahlreiche hormonelle Veränderungen. Dazu gehört die Einstellung des Reproduktionszyklus, was zu einer verminderten Ausschüttung des luteinisierenden Hormons führt (Fox & Smith, 1984), sowie

die Umstellung der Verhaltensmuster und die Erhöhung der Oxytozin- und Prolaktin-Spiegel im Serum, welche beide eine bedeutende Stellung bei der Milchbildung und dem Milchfluss einnehmen. Hormone können möglicherweise auf molekularer Ebene modulierend in Stoffwechselprozesse eingreifen, indem sie Einfluss auf Transkriptionsfaktoren nehmen und stellen damit eine mögliche Ursache von Adaptationsprozessen während der Laktation dar. Überdies ist bekannt, dass sich die Expression der UCPs und die Thermogenese im braunen Fettgewebe laktierender Mäuse mit steigender Wurfgröße durch eine gesteigerte Milchleistung verringert, verglichen mit nicht-laktierenden Mäusen (Pedraza *et al*., 2001; Trayhurn *et al*., 1982; Isler *et al*., 1984). Dies wird auf eine Steigerung der Prolaktinausschüttung durch einen höheren Stimulus der säugenden Nachkommen zurückgeführt und könnte somit eine weitere Ursache der Adaptationsprozesse darstellen.

Aus jüngsten Studien ist bekannt, dass eine PPARα-Aktivierung zu einer erhöhten Expression von Proteinen der zellulären Aufnahme des Carnitins (*novel organic cation transporter* - OCTN) in Geweben mit einer hohen PPARα Expression führt (Luci *et al*., 2006; Ringseis *et al*., 2007*b*; Ringseis *et al*., 2008; van Vlies *et al*., 2007; Koch *et al*., 2008). Dies ist bedeutsam, da mit einer Aktivierung des PPARα eine Steigerung der Fettsäureoxidation und des carnitinabhängigen Transports aktivierter Fettsäuren in das Mitochodrium, dem Ort der β-Oxidation, einhergeht und sich daher der Carnitinverbrauch in den Gewebezellen erhöht. Somit muss eine ausreichende Carnitinversorgung der Zellen sichergestellt werden. In tierexperimentellen Studien konnte bereits eine verminderte Expression der CPT I beobachtet werden, was auf einen verminderten Transport von aktivierten Fettsäuren in das Mitochodrium während der Laktation schließen lässt (Xiao *et al*., 2004*a*; Zammit, 1980). Dies liefert möglicherweise einen Hinweis auf eine verminderte PPARα-Aktivierung, welche für die Herunterregulierung von hepatischen Enzymen der Carnitinsynthese und der OCTN verantwortlich sein könnte und damit zu verminderten Carnitinkonzentrationen in Plasma und Geweben während der Laktation führen kann.

Carnitin ist ein essentielles Molekül des intermediären Stoffwechsels. Dabei wird zwischen D-Carnitin und dem für den Stoffwechsel relevanteren bioaktiven L-Carnitin unterschieden. Letzteres stellt die hier untersuchte Form dar (L-3-Hydroxy-4-*N*-Trimethylaminobutyrat, Gulewitsch & Krimberg, 1905; **Abb. 2A**). Carnitin ist im Plasma und in den Geweben in Form von freiem Carnitin und Acetylcarnitin zu finden, wobei Acetylcarnitin die Speicherform des Carnitins darstellt. Carnitin ist beteiligt an einer Vielzahl physiologischer Stoffwechselprozesse und unabdingbar für die β-Oxidation im Mitochodrium (Bremer, 1962; Carter *et al*., 1995). Eine der bedeutendsten Aufgaben des Carnitins ist der Transport

langkettiger Fettsäuren durch die innere mitochondriale Membran in Form von Acylcarnitin in die mitochondriale Matrix (McGarry & Brown, 1997; Ramsay *et al*., 2001; **Abb. 2B**). Zunächst werden Fettsäuren durch die *acyl-CoA synthase* (ACS) in ihre CoA-Derivate umgewandelt und sind somit aktiv. Nachfolgend können diese Fettsäuren durch ein carnitin-abhängiges Transportsystem, bestehend aus 3 Proteinen, in die mitochondriale Matrix, dem Ort der β-Oxidation, gelangen: CPT I, *carnitine-acylcarnitine translocase* (CACT) in der inneren Mitochondrienmembran (Rubio-Godzalbo *et al*., 2004), und CPT II, durch welche erneut die Fettsäure CoA-Derivate erhalten werden (Pande, 1975). Folglich sind alle Gewebe, deren Energiequelle aus Fettsäuren besteht, auf Carnitin angewiesen.

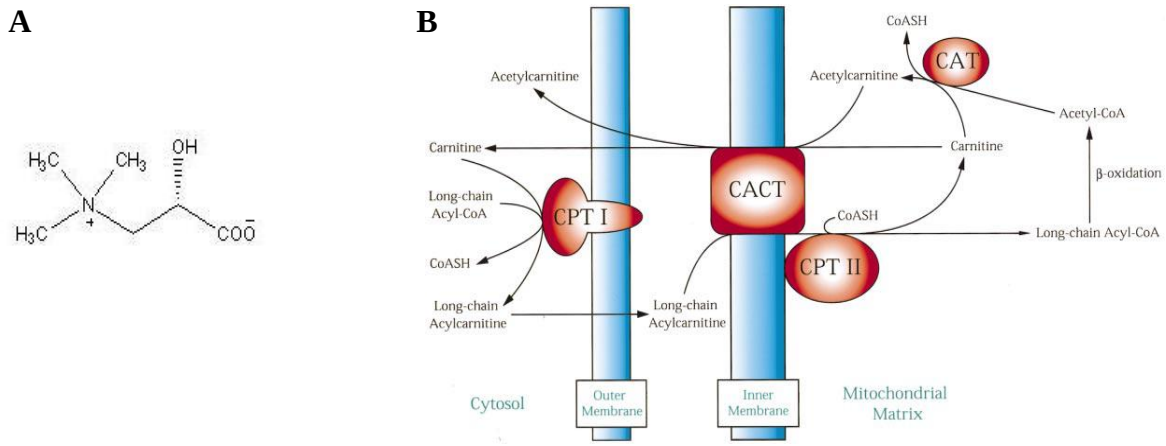

Abb. 2: Strukturformel L-Carnitin (**A),** Funktion des L-Carnitins beim Transport von langkettigen Fettsäuren durch die Mitochondrienmembran **(B)** (aus Vaz & Wanders, 2002).

Die Carnitinhomöostase wird durch einen ständigen Fluss zwischen moderater endogener Biosynthese, Zufuhr und Absorption aus der Nahrung sowie effektiver Reabsorption aufrecht erhalten (Rebouche & Seim, 1998; **Abb. 3**). Bis zum Produkt des γ-Butyrobetain (BB) ist die Biosynthese des Carnitins sowohl in der Leber als auch in Niere, Gehirn, Skelett- und Herzmuskel möglich. Andererseits ist die γ-Butyrobetaindioxygenase (BBD), welche den letzten Schritt der Carnitinsynthese katalysiert, lediglich in Leber, Gehirn und Niere des Menschen und der meisten anderen Spezies vorhanden. Somit sind Skelett- und auch Herzmuskel auf die Carnitinzufuhr über den Blutkreislauf angewiesen. Die Aminosäure Lysin fungiert als Grundgerüst des Carnitins. Diese ist über eine Peptidbindung in ein Protein eingebunden und wird posttranslational an der ε-Amino-Gruppe methyliert (Horne & Broquist, 1973). Durch hydrolytische Spaltung einer Peptidbindung innerhalb eines Proteins

kann dann Trimethyllysin (TML) freigesetzt werden. TML ist das erste Zwischenprodukt der Carnitinsynthese. Nach einer Hydroxylierung durch die TML-Dioxygenase (TMLD), einer Spaltung durch die 3-Hydroxy-*N*-TML-Aldolase (HTMLA) und einer Dehydrierung durch die 4-*N*-Trimethylaminobutyroaldehyddehydrogenase (TMABA-DH) entsteht BB. Carnitin wird anschließend durch die finale Hydroxylierungsreaktion des BB durch die BBD erhalten (Vaz & Wanders, 2002).

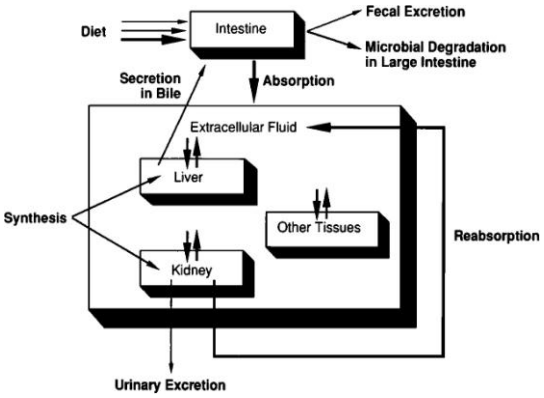

Abb. 3: Schematische Darstellung der Verteilung des L-Carnitins zwischen den Geweben (aus Rebouche & Seim, 1998)

Die Verteilung des Carnitins zwischen den Geweben und die intrazelluläre Carnitinhomöostase wird durch die OCTN kontrolliert, welche der *solute carrier 22A family* (SLC22A) angehören. Die wichtigsten Transporter der Carnitinhomöostase sind der Kationen-Carnitin Transporter OCTN1 (SLC22A4), der Na^+-Carnitin Cotransporter OCTN2 (SLC22A5) und der Na^+-unabhängige Transporter OCTN3 (Tamai *et al*., 1998; Tamai *et al*., 2000). Diese Transporter sind für die Absorption des Carnitins im Dünndarm, der Verteilung des Carnitins zwischen den Geweben sowie für die renale Reabsorption verantwortlich (Lahjouji *et al*., 2001). Sowohl OCTN1 als auch OCTN2 werden in zahlreichen Geweben exprimiert, wie etwa Niere, Darm, Skelettmuskel, Herz, Leber und Gehirn, in der Ratte und dem Menschen auch in der Plazenta (Wu *et al*., 1999; Wu *et al*., 2000; Tamai *et al*., 2000). Der OCTN3 dagegen wird in einigen wenigen Geweben exprimiert: vor allem in Hoden, Niere und Dünndarm (Durán *et al*., 2005; Tamai *et al*., 2000). Die weite Verbreitung des OCTN2 sowie die hohe Bindungsaffinität des Carnitins zu OCTN2 (Ohashi *et al*., 1999) deutet darauf hin, dass der OCTN2 die größte physiologische Bedeutung der drei OCTN aufweist. Dies wird dadurch bestätigt, dass angeborene und erworbene Defekte des OCTN2 zu einem primären oder sekundären systemischen Defizit des Carnitins führen können (Nezu

et al., 1999), was sich in einem erhöhten Verlust und einer verminderten Speicherung von Carnitin in den Geweben äußert (Longo *et al*., 2006).

Aufgrund der oben genannten verminderten Fettsäureoxidation, Fettsäureaufnahme und Thermogenese während der Laktation lag die Vermutung nahe, dass die Expression des PPARα während der Laktation vermindert ist. Ferner wird die Transkription der Gene der Synthese und Aufnahme des Carnitins über den PPARα gesteuert. Daher bestand im *ersten Komplex* der vorliegenden Arbeit das Ziel darin, (***1.1***) die Hypothese zu prüfen, dass die mRNA Konzentration des PPARα in der Leber der Ratten während der Laktation vermindert ist und dies zu einer reduzierten Expression von Enzymen der hepatischen Carnitinsynthese und der Carnitinaufnahme führt. Hierfür wurde ein Tierexperiment mit weiblichen nicht-laktierenden und laktierenden Sprague-Dawley Ratten durchgeführt. Dieses hat sich bereits in der Literatur als ein geeignetes Tiermodell bewährt um Stoffwechselvorgänge während der Laktation zu untersuchen (Brogan *et al*. 1999; Ringseis *et al*. 2004 und 2007*c*; Xiao *et al*. 2004*a* und 2004*b*). Um den Einfluss der Wurfgröße und somit der Milchleistung auf die Genexpression des PPARα und den entsprechenden Zielgenen in der Leber der laktierenden Ratten in die Untersuchungen einzubeziehen, wurde die Anzahl der Jungtiere je Muttertier angeglichen, woraus sich 3 Gruppen mit einer geringen, mittleren und hohen Anzahl an Jungtieren (4, 10 und 18) ergaben. Weiterhin wurden die Konzentrationen des Carnitins im Plasma und in zwei der wichtigsten Geweben des Carnitinstoffwechsels, der Leber und dem Skelettmuskel, bestimmt, um einen Zusammenhang zwischen Carnitinaufnahme, -synthese und -konzentration herzustellen.

Im Rahmen des *ersten Komplexes* sollte in einem zweiten Versuch (***1.2***) weiterhin geprüft werden, ob die Herunterregulierung des PPARα für die Vermittlung von Adaptations-prozessen, das heißt für die verminderten Expressionen von Genen der Fettsäureaufnahme und β-Oxidation in Geweben mit einer hohen Aktivität des Fettsäurekatabolismus, während der Laktation verantwortlich ist. Das Ziel war es, die bisher unbekannten biochemischen Mechanismen hinter den Adaptationsprozessen im laktierenden Nager aufzuklären. Hierfür wurde ein Versuch mit laktierenden PPARα-*knockout* Mäusen und entsprechenden Wildtyp-Mäusen durchgeführt, ein Modell, welches sich bereits bei Untersuchungen zu Regulationsmechanismen durch Transkriptionsfaktoren als geeignet erwies (Koch *et al*. 2008; Foreman *et al*., 2009; Peters *et al*., 2003; van Vlies *et al*., 2007). Nachfolgend sollte die Genexpression des PPARα und ausgewählter PPARα-Zielgene der Fettsäureaufnahme (FABPpm, FATP), Fettsäureoxidation (CPT I, Cyp4A10, MCAD) und Thermogenese (UCP3) in Leber und Skelettmuskel ermittelt werden. Durch Coaktivatoren wird die

transkriptionelle Aktivität des PPARα verstärkt. Deshalb stellten der PGC 1α und der PGC 1β, welche benötigt werden, um die maximale transkriptionelle Aktivität des PPARα zu erreichen (Yu & Reddy, 2007), einen weiteren wichtigen Teil der Untersuchungen dar. Ferner sollten die Expressionen der Gene der Fettsäuretransporter und der LPL in der Milchdrüse bestimmt werden sowie eine Messung der Konzentrationen der Triglyzeride im Plasma durchgeführt werden, um den Einfluss der Laktation auf die Aufnahme von Fettsäuren aus dem Plasma in die Milchdrüse zu prüfen. Weitere Details zu Material und Methodik sowie die ausführliche Beschreibung und Diskussion der Ergebnisse dieser Studie sind ersichtlich in:

*(**1.1**) Gutgesell A, Ringseis R, Brandsch C, Stangl GI, Hirche F, Eder K (2009) Peroxisome proliferator–activated receptor α and enzymes of carnitine biosynthesis in the liver are down-regulated during lactation in rats.* ***Metabolism Clinical and Experimental*** *58: 226-232.*

*(**1.2**) Gutgesell A, Ringseis R, Schmidt Eileen, Brandsch C, Stangl GI, Eder K (2009) Down-regulation of peroxisome proliferator-activated receptor α and its coactivators in liver and skeletal muscle mediates the metabolic adaptions during lactation in mice.* (online published in Journal of Molecular Endocrinology)

1.4 Die Regulation der *carnitine-acylcarnitine translocase* (CACT)

Der Transkriptionsfaktor PPARα wird in erheblichen Mengen in Geweben mit einer hohen Aktivität der Fettsäureoxidation exprimiert und vermittelt die Liganden-induzierte primäre Reaktion während des Fastenzustandes. Auf eine PPARα-Aktivierung folgt die Hochregulierung der Transkription einer Reihe von Genen des Fettsäurekatabolismus (Motojima *et al*., 1998; Mandard *et al*., 2004). Auch Gene des Carnitinstoffwechsels werden durch Aktivierung des PPARα hochreguliert (Luci *et al*., 2006; Ringseis *et al*., 2007*b*; Ringseis *et al*., 2008). Dies dient der Bereitstellung von Carnitin für den carnitinabhängigen Transport der LCFA in das Mitochondrium. In früheren Studien wurde bereits belegt, dass die CPT I und CPT II transkriptionell über den PPARα reguliert werden (Mascaro *et al*., 1999; Napal *et al*., 2005). Aus diesem Grund kann vermutet werden, dass die Regulation der CACT ebenso durch PPARα erfolgt, da diese eine elementare Rolle für den Import von Acylcarnitin in die mitochondriale Matrix spielt. Bisher wurde die CACT in der Literatur nicht als PPARα-Zielgen beschrieben. Daher leisten Untersuchungen zur Regulation der CACT durch PPARα einen weiteren wichtigen Beitrag zur Aufklärung der Funktion des PPARα als

Transkriptionsfaktor im Lipidstoffwechsel.

Das Ziel im z*weiten Komplex* dieser Arbeit bestand darin, die Regulation der Transkription der CACT durch den PPARα in der Leber zu prüfen. Zu diesem Zweck wurden zwei Versuche mit PPARα-*knockout*- und Wildtyp-Mäusen durchgeführt. Im ersten Experiment wurden die Mäuse für 48 Stunden gefastet, während im zweiten Experiment der selektive PPARα-Agonist WY-14,643 verabreicht wurde. Da einzelne PPARα-Zielgene ebenfalls durch andere PPAR-Isotypen reguliert werden (PPARγ, PPARδ; Cabrero *et al*., 2003), wurden außerdem Zellkulturversuche mit Leberzelllinien durchgeführt, welche mit unterschiedlichen Konzentrationen des selektiven PPARα-Agonisten WY-14,643, dem selektiven PPARγ-Agonisten Troglitazon und dem selektiven PPARδ-Agonisten GW0742 inkubiert wurden. Auf diesem Weg sollte der Effekt auf die Aktivierung der drei PPAR-Isotypen anhand der mRNA Konzentration der CACT beurteilt werden. Die humane Leberzelllinie HepG2 und die Rattenleberzelllinie FAO wurden für die Inkubationsversuche herangezogen. Aus der Literatur ist bekannt, dass sich HepG2-Zellen für Promotoraktivitätsstudien und Mutationsanalysen zur Identifikation neuer Zielgene von Transkriptionsfaktoren eignen (Yang *et al*., 2009; Takata *et al*., 2009; Nagasawa *et al*., 2009). Zudem wurden HepG2- und FAO-Zelllinien bereits für einige Expressionsanalysen verwendet (König & Eder, 2006; Kleiner *et al*., 2009). Weiterhin sollten Promotoraktivitätsstudien durchgeführt und DNA/Protein-Komplexe mittels Electrophoretic-Mobility Shift Assay sichtbar gemacht werden. Weitere Details zu Material und Methodik sowie die ausführliche Beschreibung und Diskussion der Ergebnisse dieser Studie sind ersichtlich in:

(2) *Gutgesell A, Wen G, König B, Koch A, Spielmann J, Stangl GI, Eder K, Ringseis R (2009) Mouse carnitine-acylcarnitine translocase (CACT) is transcriptionally regulated by PPARα and PPARδ in liver cells.* (online published in Biochimica et Biophysica Acta)

1.5 Einfluss von PPARα-Agonisten in der maternalen und fötalen Rattenleber während der Trächtigkeit

Oxidierte Fettsäuren werden aufgrund ihrer Fähigkeit eine PPARα-Aktivierung auszulösen den starken natürlichen PPARα-Aktivatoren zugeordnet (Chao *et al*., 2001; Sülzle *et al*., 2004; Ringseis *et al*., 2007*c*; Martin *et al*., 2000*b*; König & Eder, 2006; Echtay *et al*., 2002). Eine PPARα-Aktivierung führt sowohl zu einer Hypertrophie der Leber infolge einer Peroxisomenproliferation (Chao *et al*., 2005) als auch zu verminderten Triglyzerid-

konzentrationen in der Leber, im Plasma und in VLDLs von Ratten. Letzteres basiert auf einer verminderten Konzentration der mRNA und einer reduzierten Aktivität der *fatty acid synthase* (FAS) (Eder *et al*., 2003). In einigen tierexperimentellen Untersuchungen konnte bisher belegt werden, dass die Gabe des PPARα-Agonisten Clofibrat zu einer Erhöhung der mRNA der CYP4A1 (Simpson *et al*., 1996) und der Aktivität peroxisomaler Enzyme (Cibelli *et al*., 1988) sowie auch zu einer Vergrößerung der Peroxisomen (Wilson *et al*., 1991) in der fötalen Rattenleber während der Trächtigkeit führt. Dies weist auf einen möglichen transplazentaren Transport des maternal zugeführten PPARα-Agonisten und nachfolgender PPARα-Aktivierung in der fötalen Rattenleber hin. Ein transplazentarer Transport potenter PPARα-Agonisten in den fötalen Kreislauf könnte zu einer Reihe pathologischer Veränderungen führen. Unter anderem belegen Hinweise aus der wissenschaftlichen Literatur einen Anstieg der Expression von Protoonkogenen im Zellversuch, was auf den hepatokarzinogenen Effekt der Peroxisomenproliferatoren im Nager zurückzuführen ist (Miller *et al*., 1996; Ledwith *et al*., 1996). Bis heute sind aus der Literatur keinerlei Hinweise bekannt, dass die Gabe von oxidiertem Fett bereits während der Trächtigkeit zu einer PPARα-Aktivierung und Steigerung der Expression von PPARα-Zielgenen in der fötalen Rattenleber führt. In diesem Zusammenhang ist ebenfalls bisher unbekannt, ob Bestandteile des oxidierten Fettes in der Lage sind, die Plazenta zu passieren, da eine hohe Selektivität bezüglich des transplazentaren Transports von Fettsäuren aus der maternalen Nahrung besteht. So wird der Transport von LCFA, wie Arachidonsäure, auf Kosten anderer weniger wichtiger Fettsäuren bevorzugt (Herrera *et al*., 2006; Haggarty *et al*., 1999).

Im *dritten Komplex* dieser Arbeit sollte untersucht werden, ob die Fütterung von oxidiertem Fett während der Trächtigkeit bei Ratten eine PPARα-Aktivierung und eine Beeinflussung der relativen mRNA Konzentrationen von PPARα-Zielgenen in der Leber der Muttertiere und der Rattenföten hervorrufen kann und inwiefern dieser Einfluss auf den Fettsäurekatabolismus die Triglyzeridkonzentration in der Muttertier- und Fötenleber beeinträchtigt. In diesem Zusammenhang kann ebenfalls aufgedeckt werden, ob Komponenten oxidierter Fette in der Lage sind, die Plazentaschranke zu passieren und in den fötalen Kreislauf zu gelangen. Des Weiteren sollte die Wirkung von oxidiertem Fett auf die relativen mRNA Konzentrationen des Transkriptionsfaktors der Lipogenese *sterol regulatory element-binding protein* (SREBP) -1c und dessen Zielgen FAS geprüft werden, da bereits eine Reduzierung der Expression der FAS durch oxidiertes Fett nachgewiesen werden konnte (Eder *et al*., 2003). Dabei könnte die Fütterungsdauer des oxidierten Fettes den Effekt auf den Fettsäuremetabolismus in der fötalen Leber beeinflussen. Dementsprechend wurde ein Kurzzeit- und ein Langzeitversuch mit

weiblichen Sprague-Dawley Ratten durchgeführt. Während des Kurzzeitversuches wurde den Tieren der Behandlungsgruppe oxidiertes Fett mittels einer Schlundsonde an den Tagen 16 bis 21 der Laktation appliziert. Im Langzeitversuch erhielten die Ratten der Behandlungsgruppe in kontrollierter Fütterung semisynthetische Diäten mit oxidiertem Fett über den Verlauf der gesamten Trächtigkeit (1.- 21. Tag). Um mögliche ungünstige Effekte des oxidierten Fettes in der Leber der Muttertiere und Föten benennen zu können, wurden die mRNA Konzentrationen der Protoonkogene c-myc, c-jun und c-fos ermittelt. Weitere Details zu Material und Methodik sowie die ausführliche Beschreibung und Diskussion der Ergebnisse dieser Studie sind ersichtlich in:

*(**3**) Ringseis R, Gutgesell A, Dathe C, Brandsch C, Eder K (2007) Feeding oxidized fat during pregnancy up-regulates expression of PPARα-responsive genes in the liver of rat fetuses.* ***Lipids in Health and Disease*** *6: 6* (online published)

1.6 Der Einfluss durch *conjugated linoleic acids* (CLA) auf lipidstoffwechsel-relevante Parameter während der Laktation

Neben mehrfach ungesättigten Fettsäuren (PUFA), Eicosanoiden sowie Komponenten oxidierter LDL und VLDL zählen auch Derivate der Linolsäure, die konjugierten Linolsäuren (CLA), zu den natürlichen PPARα-Agonisten (Castrillo & Tontonoz, 2004). CLA, insbesondere die *c*9,*t*11-CLA, konnte bereits 1999 als PPARα-Aktivator und -Ligand in Ratten Hepatoma-Zelllinien beschrieben werden (Moya-Camarena *et al*., 1999). CLA sind Bestandteile tierischer Produkte von Wiederkäuern und hauptsächlich in Milch, Milchprodukten und Fleisch enthalten (de Deckere *et al*., 1999). Die Gruppe der CLA setzt sich aus verschiedenen Strukturisomeren der Linolsäure zusammen, welche durch konjugierte Doppelbindungen gekennzeichnet sind. In zahlreichen Studien konnten ihnen bereits eine Vielzahl biologischer Effekte in Menschen und Tieren zugeordnet werden (Martin *et al*., 2000*a*; Baumgard *et al*., 2001; Bhattacharya *et al*., 2006). Aus der Literatur ist zu entnehmen, dass die Gabe von PPARα-Aktivatoren während der Laktation zu einer Störung der Energiesparmechanismen und zu einer Beeinträchtigung der Milch(fett)synthese und Aufnahme von Fettsäuren in die Milchdrüse im laktierenden Organismus führt (Masters *et al*., 2002; Baumgard *et al*., 2002; Ringseis *et al*., 2004; Ringseis *et al*., 2007*c*). Besonders in experimentellen Studien mit laktierenden Mäusen, Ratten, Schafen, Kühen und Menschen konnte nach Gabe von CLA eine Verminderung der Triglyzeridgehalte in der Milch

beobachtet werden (Lin *et al*., 2004; Ringseis *et al*., 2004; Lock *et al*., 2006; Baumgard *et al*., 2001 und 2002; Masters *et al*., 2002). Dies wurde auf erhöhte Genexpressionen des PPARα und der ACO in der Leber laktierender Muttertiere zurückgeführt (Ringseis *et al*., 2004) und deutet auf eine erhöhte Fettsäureoxidation hin. Die reduzierten hepatischen Lipidkonzentrationen bestätigen diese Vermutung. Weiterhin konnte eine reduzierte Aktivität lipogener Enzyme in der Leber laktierender Ratten beobachtet werden, was zu einer verminderten *de-novo* Biosynthese von Fettsäuren führt (Lin *et al*., 2004). Dabei spielt die reduzierte Aktivität des SREBP-1c eine wichtige Rolle (Horton *et al*., 2002). Zudem konnte eine verminderte Aktivität der LPL und somit eine reduzierte Abspaltung von Fettsäuren aus triglyzeridreichen Lipoproteinen in der Milchdrüse ermittelt werden (Peterson *et. al*., 2004; Ringseis *et al*., 2004). Beides wurde, nach Gabe von CLA, durch eine verminderte Konzentration an *medium chain fatty acids* (MCFA) und LCFA und einer reduzierten Triglyzeridkonzentration in der Milch sichtbar. Bisher existieren keine Untersuchungen dazu, dass CLA einen Einfluss auf die Aufnahme von Fettsäuren durch Fettsäuretransporter in die Milchdrüse ausüben, welche beim Transport von Fettsäuren in die Milchdrüse, dem Ort der Milchfettsynthese, von hoher Bedeutung sind. Trotz einer stark erhöhten Futteraufnahme der Ratte während der Laktation (Ota & Yokoyama, 1967) liegt ständig eine negative Energiebilanz vor, besonders von Tag 12 bis Tag 14 Postpartum (Smith & Grove, 2002). Die Verwertung der Energiespeicher in Form von NEFA (Sampson & Jansen, 1984), welche aus dem weißen Fettgewebe in das Plasma abgegeben und von Fettsäuretransportern in die Milchdrüse zur Triglyzeridsynthese aufgenommen werden, nimmt daher eine wesentliche Stellung für die Milchfettsynthese ein. Daher leisten Untersuchungen der Fettsäuretransporter der Milchdrüse und NEFA-Konzentrationen im Plasma einen weiteren wichtigen Beitrag zur Ermittlung der Wirkung von CLA im Stoffwechsel während der Laktation.

Im *vierten Komplex* dieser Arbeit bestand das Ziel darin, den Einfluss von CLA auf die Konzentration der NEFA im Plasma und auf die Fettsäuretransporter in der Milchdrüse laktierender Ratten zu untersuchen. Es sollte geprüft werden, ob CLA einen Effekt auf die Aufnahme von NEFA aus dem Plasma in die Milchdrüse über eine Beeinflussung der Fettsäuretransporter der Milchdrüse ausüben, da dies bislang unbekannt ist. Aus diesem Grund erhielten laktierende weibliche Sprague-Dawley Ratten in der Behandlungsgruppe eine mit CLA-Öl angereicherte Versuchsdiät (Ringseis *et al*., 2004). Nachfolgend sollten in Plasmaproben Konzentrationsmessungen der NEFA und in den Gewebeproben der Milchdrüse Genexpressionsmessungen der relevanten Fettsäuretransporter (FAT/CD36, FATP und FABPpm) vorgenommen werden. Weitere Details zu Material und Methodik

sowie die ausführliche Beschreibung und Diskussion der Ergebnisse dieser Studie sind ersichtlich in:

*(**4**) Gutgesell A, Ringseis R, Eder K (2009) Short communication: Dietary conjugated linoleic acid down regulates fatty acid transporters in the mammary glands of lactating rats.* ***J. Dairy Sci****. 92: 1169-1173.*

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Peroxisome proliferator–activated receptor α and enzymes of carnitine biosynthesis in the liver are down-regulated during lactation in rats

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Abstract

This study investigated the hypothesis that lactation lowers gene expression of peroxisome proliferator–activated receptor (PPAR) α in the liver and that this leads to a down-regulation of hepatic enzymes involved in carnitine synthesis and novel organic cation transporters (OCTNs). Thirty-two pregnant female rats were divided into 4 groups. In the first group, all pups were removed, whereas in the other groups, litters were adjusted to sizes of 4, 10, or 18 pups per dam. Dams suckling their litters, irrespective of litter size, had lower relative messenger RNA concentrations of PPARα, various classic PPARα target genes involved in fatty acid catabolism, as well as enzymes involved in carnitine synthesis (trimethyllysine dioxygenase, 4-*N*-trimethylaminobutyraldehyde dehydrogenase, γ-butyrobetaine dioxygenase) and OCTN1 in the liver than dams whose litters were removed ($P < .05$). Moreover, dams suckling their litters had a reduced activity of γ-butyrobetaine dioxygenase in the liver and reduced concentrations of carnitine in plasma, liver, and muscle compared with dams without litters ($P < .05$). In conclusion, the present study demonstrates for the first time that lactation leads to a down-regulation of PPARα and genes involved in hepatic carnitine synthesis and uptake of carnitine (OCTN1) in the liver, irrespective of litter size. It is moreover suggested that down-regulation of PPARα in the liver may be a means to conserve energy and metabolic substrates for milk production in the mammary gland.

1. Introduction

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 [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. The released TML is further oxidized to γ-butyrobetaine (BB) by the action of TML dioxygenase (TMLD), 3-hydroxy-*N*-TML aldolase, and 4-*N*-trimethylaminobutyraldehyde dehydrogenase (TMABA-DH). BB is hydroxylated by BB dioxygenase (BBD) to form carnitine. The last reaction occurs in rats exclusively in liver and testes [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. The OCTNs are important for the absorption of carnitine in the small intestine, distribution of carnitine between tissues, and renal reabsorption of carnitine [7,8]. Three OCTNs have been identified so far: OCTN1, OCTN2, and OCTN3 [9-11]. OCTN1 and OCTN2 are expressed in several tissues such as kidney, intestine, skeletal muscle, heart, liver, and brain [11-13]. In contrast, OCTN3 is expressed exclusively in testes, kidney, and small intestine [11,14]. Because of its high binding affinity for carnitine and its wide expression, OCTN2 seems to be the most physiologically important carnitine transporter [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].

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Recently, it has been shown that the expression of hepatic enzymes of carnitine synthesis and OCTN is up-regulated by peroxisome proliferator–activated receptor (PPAR) α, a transcription factor belonging to the nuclear hormone receptor superfamily [15-18]. PPARα is highly expressed in tissues with high rates of fatty acid oxidation. It is activated by synthetic agonists such as fibrates or by natural agonists such as fatty acids or eicosanoids [19]. The physiologic role of PPARα lies in the mediation of metabolic responses to fasting. Upon activation, PPARα up-regulates genes involved in cellular fatty acid uptake and transport, mitochondrial and peroxisomal fatty acid oxidation, ketogenesis, and gluconeogenesis [20].

Lactation is a physiologic state characterized by a dramatic increase in the energy and nutrient requirement of the organism for milk production. This demand is usually met by an increased food intake and by the utilization of energy stores. In addition, several metabolic adaptations develop in the lactating organism aiming to conserve energy and metabolic substrates for milk production in the mammary gland [21-24]. Recently, it has been shown that down-regulation of uncoupling proteins (UCPs) 1 and 3 in brown adipose tissue and of UCP3 in skeletal muscle, leading to a decrease of metabolic fuel oxidation and thermogenesis, contributes to these metabolic adaptations during lactation [25-27]. Moreover, expression of enzymes involved in uptake and utilization of fatty acids in liver and skeletal muscle is reduced during lactation, an effect that helps spare fatty acids for milk production [28,29]. Uncoupling proteins 1 and 3 as well as enzymes involved in fatty acid oxidation are transcriptionally regulated by PPARα [20,30,31]. The finding that these genes are down-regulated during lactation suggests that gene expression and/or transactivation of PPARα is lowered during lactation, which, however, has not yet been investigated.

Under the assumption that PPARα is down-regulated during lactation, it is expected that the expression of genes involved in hepatic synthesis and cellular uptake of carnitine is reduced, too, because transcription of these genes is regulated by PPARα. The present study, therefore, aims to investigate the hypotheses that messenger RNA (mRNA) expression of PPARα in the liver is lowered in the lactating organism and that this leads to a down-regulation of hepatic enzymes involved in carnitine synthesis and OCTN, which in turn may lead to a reduction of plasma and tissue carnitine concentrations. For this end, we performed an experiment with lactating and nonlactating rats. It has been shown that expression of UCP [25] and thermogenesis in brown adipose tissue [24] decline with increasing litter sizes in rats as a result of increasing milk output. To investigate whether expression of PPARα and the respective PPARα target genes in the liver is also influenced by litter size, we adjusted litters to a low (4 pups), intermediate (10 pups), and high size (18 pups) as a way to influence the milk output by lactating dams.

2. Materials and methods

2.1. Animal experiment

Forty 11-week–old female rats were obtained from Charles River (Sulzfeld, Germany). They were kept in Macrolon cages (Uno Roestvaststaal, Zevenaar, The Netherlands) in a room maintained with controlled temperature (23°C ± 1°C), humidity (50%-60%), and lighting (6:00 AM to 6:00 PM). All the rats were mated by housing 1 male rat with 2 female rats. After pregnancy was ascertained by observation of sperm in the vaginal smears (only 32 of 40 rats), each rat was kept individually in 1 cage. At the day of parturition, designated as day 1 of lactation, rats and their litters were randomly assigned to 4 groups of 8 rats each. In the first group of dams, all pups were removed, whereas in the other groups, litters were adjusted to sizes of 4, 10, or 18 pups per dam. During the whole experiment, all the rats received a commercial standard rodent diet (Altromin, Lage, Germany) ad libitum. Food intake during the lactation was recorded. Water was available ad libitum from nipple drinkers during the whole experiment. At day 15 of lactation, the dams were killed by decapitation in the nonfasted state. Blood was collected from the opened neck into heparinized polyethylene tubes (Sarstedt, Nürnbrecht, Germany) by the use of heparinized plastic funnels. Liver, skeletal muscle, and mammary gland were quickly removed, snap-frozen in liquid nitrogen, and stored at −80°C pending analysis. Plasma was obtained by centrifugation of the blood (1100*g*, 10 minutes, 4°C) and stored at −20°C. All experimental procedures described followed established guidelines for the care and handling of laboratory animals [32] and were approved by the council of Saxony-Anhalt.

2.2. Lipid analysis

Plasma nonesterified fatty acid (NEFA) concentrations were determined using the enzymatic NEFA C kit from Wako Chemicals (Neuss, Germany, ref 99975406).

2.3. Analysis of carnitine, BB, and TML

Concentrations of free carnitine, acetyl carnitine, TML, and BB in plasma liver and skeletal muscle were determined by tandem mass spectrometry using deuterated carnitine-d_3 (Larodane Fine Chemicals, Malmö, Sweden) as internal standard [16]. Fifty milligrams of freeze-dried tissues was extracted with 0.5 mL methanol/water (2:1, vol/vol) by homogenization (Tissue Lyzer; Qiagen, Hilden, Germany), followed by sonification for 20 minutes and incubation at 50°C for 30 minutes in a shaker. After centrifugation (13 000*g*, 10 minutes), 20 μL of the supernatant was added with 100 μL methanol containing the internal standard, mixed, incubated for 10 minutes, and centrifuged (13 000*g*, 10 minutes). Plasma samples were handled at 4°C in the same manner as the supernatant after tissue extraction. The final supernatants were used for quantification of the compounds by a 1100-er series HPLC (Agilent Technologies, Waldbronn, Germany) equipped with a Kromasil 100 column (125 mm ×

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

Gene	Forward primer (from 5′ to 3′)	Reverse primer (5′ to 3′)	NCBI GenBank
ACO	CTTTCTTGCTTGCTTTCCTTCTCC	GCCGTTTCACCGCCTCGTA	J02752
BBD	ATTCTGCAAAAGCTCGGAAA	CTCCTTGGAGTCCTGCTCTG	NM_022629
CYP4A1	CAGAATGGAGAATGGGGACAGC	TGAGAAGGGCAGGAATGAGTGG	NM_14972
FABPpm	ACCATCCACTGCCGTCTTAC	CCCCGATGCGTAGGTATTCT	M18467
FAT/CD36	TCGTATGGTGTGCTGGACAT	GGCCCAGGAGCTTTATTTTC	L19658
FATP	GGTAGCAAATGCACCCTCAT	CTCCTGCTGTGATGTGAGGA	U89529
GAPDH	GCATGGCCTTCCGTGTTGC	GGGTGGTCCAGGGTTTCTTACTC	NM_017008
L-CPT I	GGAGACAGACACCATCCAACATA	AGGTGATGGACTTGTCAAACC	NM_031559
OCTN1	CCTCTCTGGCCTGATTGAAG	CTCCGCTGTGAAGACGTACA	NM_012930
OCTN2	AGCATTTGTCCTGGGAACAG	ACTCAGGGATGAACCACCAG	NM_022270
PPARα	CCCTCTCTCCAGCTTCCAGCCC	CCACAAGCGTCTTCTCAGCCATG	NM_013196
TMABA-DH	TTTGAGACTGAAGCCGAGGT	CACCGGGCTGACGTTATAGT	NM_022273
TMLD	GCCCTGTGGCATTCAAGTAT	GGTCCAACCCCTATCATGTG	AF374406

2 mm, 5-μm particle size; CS-Chromatographie Service, Langerwehe, Germany) and an API 2000 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, vol/vol/vol/vol) were used.

2.4. Activity of BBD

Activity of BBD in liver was determined as described previously in detail by van Vlies et al [6]. Homogenates from liver were prepared by homogenizing tissue in 10 mmol/L 3-morpholinepropanesulfonic acid buffer (pH 7.4) containing 0.9% (wt/vol) sodium chloride, 10% (wt/vol) glycerol, and 5 mmol/L dithiothreitol.

2.5. RNA isolation and real-time reverse transcriptase polymerase chain reaction

For the determination of mRNA expression levels of PPARα, cytochrome P450 A1 (CYP4A1), acyl–coenzyme A oxidase (ACO), liver-type carnitine palmitoyltransferase I (L-CPT I), fatty acid translocase (FAT/CD36), fatty acid transport protein (FATP), plasma membrane fatty acid

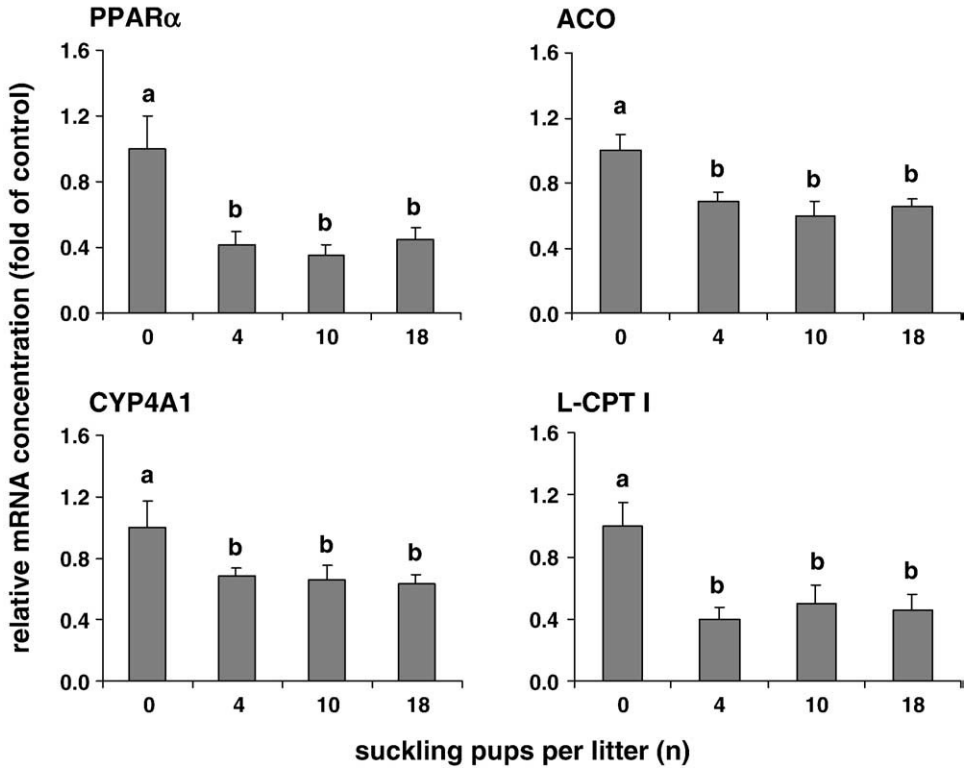

Fig. 1. Relative mRNA concentrations of PPARα, ACO, CYP4A1, and L-CPT I in the liver of lactating rats whose litters where either removed (group 0) or adjusted to 4 (group 4), 10 (group 10), and 18 (group 18) pups immediately after birth, at day 15 of lactation. Bars represent mean ± SE (n = 8 per group) and are expressed as fold of relative mRNA concentration of control (group 0). Bars without a common letter (a, b) differ ($P < .05$).

binding protein (FABPpm), OCTN1, OCTN2, TMABA-DH, TMLD, and BBD, total RNA was isolated from rat livers and rat mammary glands using Trizol reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocol. The RNA concentration and purity were estimated from the optical density at 260 and 280 nm, respectively. Complementary DNA synthesis and determination of relative mRNA concentrations were performed by real-time detection reverse transcriptase polymerase chain reaction as described previously [33]. Characteristics of gene-specific primers obtained from Operon (Köln, Germany) are shown in Table 1.

2.6. Statistical analysis

Statistical analysis of the data was performed using the Minitab Statistical Software (Minitab, State College, PA). Data were analyzed by 1-way analysis of variance. For significant F values, means were compared by Fisher multiple range test. In all experiments, means were considered significantly different for P less than .05. Values in the text are means ± SE.

3. Results

3.1. Food intake of dams and litter weights

Average daily food intake of the dams during the lactation phase (day 1 to day 15 of lactation) was influenced by the litter size. Dams whose litters were removed consumed less food per day than dams suckling their litters ($P < .05$). Dams with 4 pups consumed less food per day than dams with 10 or 18 pups ($P < .05$); daily food consumption of dams with 10 and 18 pups did not differ (0 pups per litter, 22 ± 1 g/d;

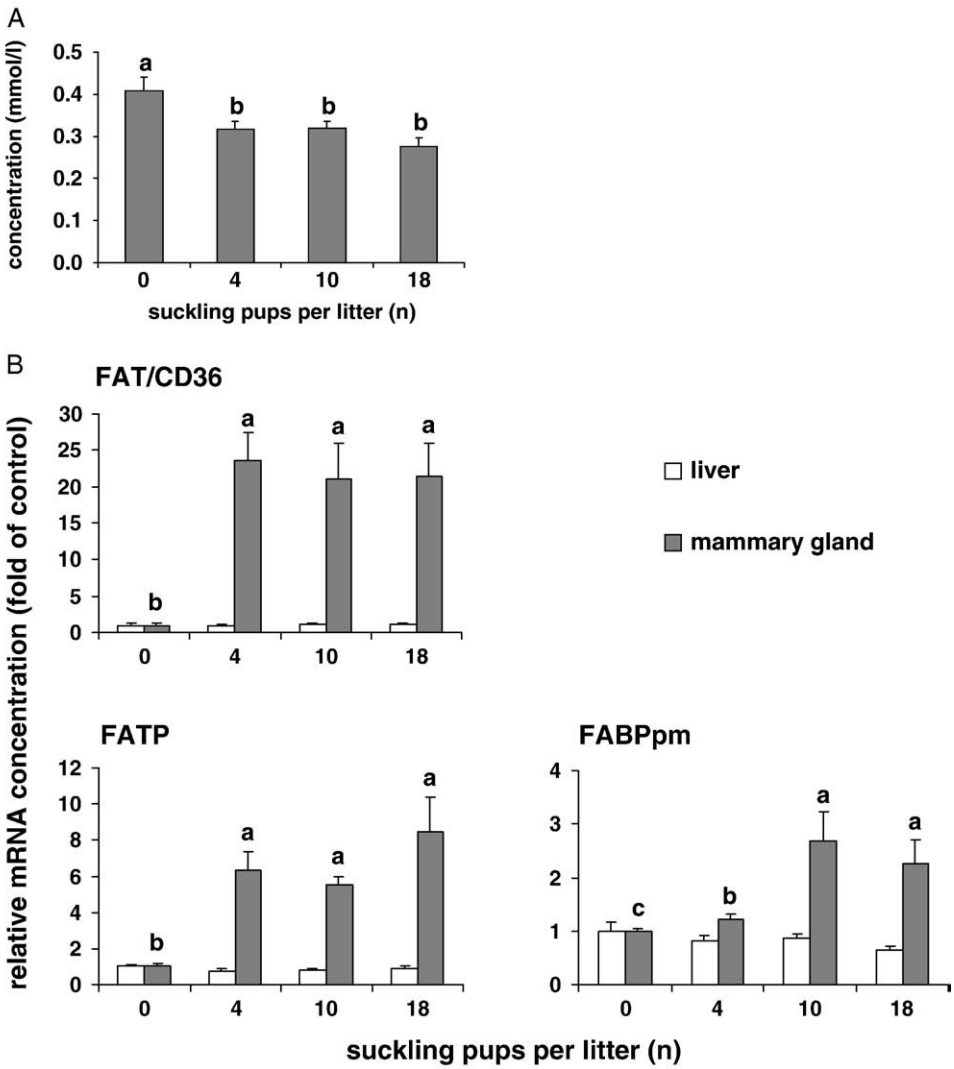

Fig. 2. A, Concentration of NEFA in the plasma of lactating rats whose litters where either removed (group 0) or adjusted to 4 (group 4), 10 (group 10), and 18 (group 18) pups immediately after birth, at day 15 of lactation. Bars without a common letter (a, b, c) differ ($P < .05$). B, Relative mRNA concentrations of FAT/CD36, FATP, and FABPpm in the liver and mammary gland of lactating rats whose litters where either removed (group 0) or adjusted to 4 (group 4), 10 (group 10), and 18 (group 18) pups immediately after birth, at day 15 of lactation. Bars represent mean ± SE (n = 8 per group) and are expressed as fold of relative mRNA concentration of control (group 0).

4 pups per litter, 38 ± 2 g/d; 10 pups per litter, 52 ± 1 g/d; 18 pups per litter, 55 ± 2 g/d). Final body weights of dams with different litter sizes did not differ (0 pups per litter, 317 ± 7 g; 4 pups per litter, 331 ± 12 g; 10 pups per litter, 331 ± 7 g; 18 pups per litter, 325 ± 9 g). Weights of whole

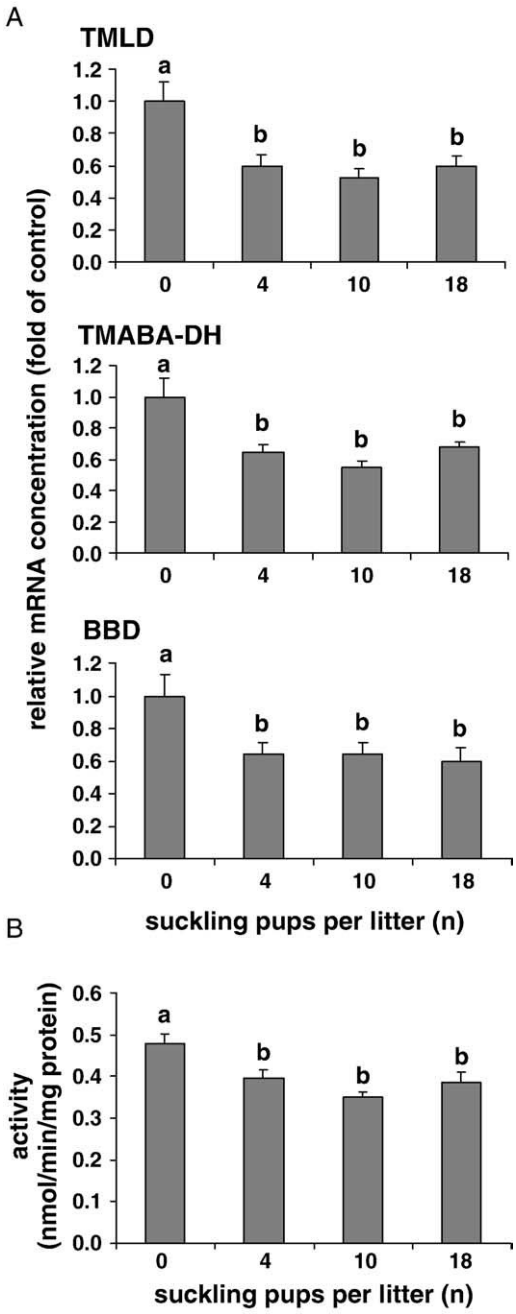

Fig. 3. A, Relative mRNA concentrations of TMLD, TMABA-DH, and BBD in the liver of lactating rats whose litters where either removed (group 0) or adjusted to 4 (group 4), 10 (group 10), and 18 (group 18) pups immediately after birth, at day 15 of lactation. Bars represent mean ± SE (n = 8 per group) and are expressed as fold of relative mRNA concentration of control group (group 0). B, Activity of BBD in the liver of lactating rats whose litters where either removed (group 0) or adjusted to 4 (group 4), 10 (group 10), and 18 (group 18) pups immediately after birth, at day 15 of lactation. Bars without a common letter (a, b) differ ($P < .05$).

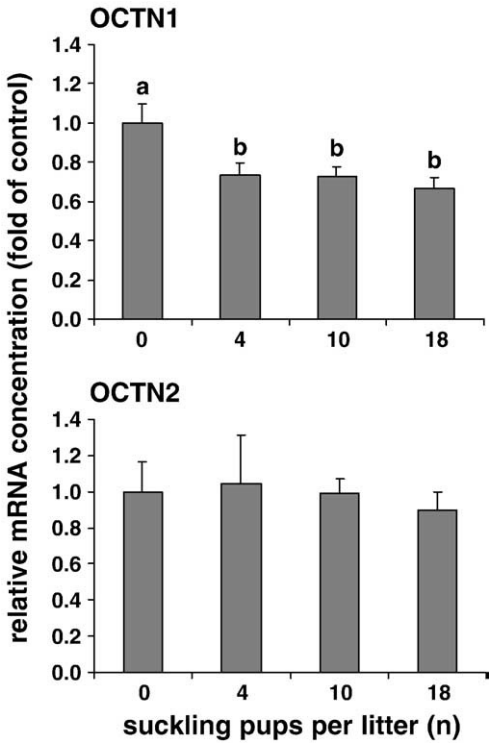

Fig. 4. Relative mRNA concentrations of OCTN1 and OCTN2 in the liver of lactating rats whose litters where either removed (group 0) or adjusted to 4 (group 4), 10 (group 10), and 18 (group 18) pups immediately after birth, at day 15 of lactation. Bars represent mean ± SE (n = 8 per group) and are expressed as fold of relative mRNA concentration of control (group 0). Bars without a common letter (a, b) differ ($P < .05$).

litters at day 15 were increased with increasing litter size (4 pups per litter, 148 ± 15 g per litter; 10 pups per litter, 304 ± 8 g per litter; 18 pups per litter, 376 ± 10 g per litter).

3.2. Expression of PPARα and target genes of PPARα in the liver

Dams with litters had lower relative mRNA concentrations of PPARα and its target genes (ACO, CYP4A1, L-CPT I) in the liver than dams whose litters were removed ($P < .05$, Fig. 1). No difference was observed in the relative mRNA concentrations of these genes between dams with 4, 10, or 18 pups, respectively (Fig. 1).

3.3. Concentrations of NEFA in plasma and expression of fatty acid transporters in liver and mammary gland

Plasma NEFA concentration was lower in dams with 4, 10, or 18 pups than in dams whose litters were removed. There was, however, no difference in plasma NEFA concentration between dams with 4, 10, or 18 pups (Fig. 2A). Dams with litters, irrespective of the litter size, had higher relative mRNA concentrations of fatty acid transporters (FAT/CD36, FATP, FABPpm) in the mammary gland than dams whose litters were removed ($P < .05$, Fig. 2B). In the liver, relative mRNA concentrations of fatty acid transporters did not differ between the 4 groups (Fig. 2B).

Table 2
Concentrations of free carnitine, acetylcarnitine, total carnitine, BB, and TML in plasma, liver, and skeletal muscle

	Litter size (pups per litter)			
	0	4	10	18
Plasma (μmol/L)				
Free carnitine	31.6 ± 2.2[a]	14.2 ± 1.0[b]	10.6 ± 0.8[b]	16.7 ± 1.2[b]
Acetyl carnitine	7.12 ± 0.62[a]	3.32 ± 0.34[b]	3.44 ± 0.43[b]	4.10 ± 0.37[b]
Total carnitine	40.0 ± 3.0[a]	17.8 ± 1.0[b]	14.3 ± 0.9[b]	21.2 ± 1.0[b]
TML	0.96 ± 0.06	0.95 ± 0.06	1.24 ± 0.10	1.19 ± 0.12
BB	0.39 ± 0.03	0.37 ± 0.03	0.36 ± 0.02	0.38 ± 0.04
Liver (nmol/g)				
Free carnitine	412 ± 36[a]	234 ± 26[b]	261 ± 16[b]	311 ± 14[b]
Acetyl carnitine	25.5 ± 2.6[a]	4.6 ± 0.2[b]	4.2 ± 1.0[b]	6.8 ± 1.5[b]
Total carnitine	441 ± 33[a]	245 ± 15[b]	278 ± 19[b]	320 ± 15[b]
TML	2.17 ± 0.17	2.36 ± 0.24	2.62 ± 0.14	2.15 ± 0.10
BB	4.98 ± 0.43[b]	4.89 ± 0.41[b]	4.90 ± 0.52[b]	8.36 ± 0.55[a]
Skeletal muscle (nmol/g)				
Free carnitine	430 ± 20[a]	342 ± 21[b]	260 ± 20[c]	322 ± 30[bc]
Acetyl carnitine	153 ± 8.0[a]	124 ± 6.8[b]	114 ± 9.3[b]	105 ± 7.6[b]
Total carnitine	585 ± 25[a]	468 ± 25[b]	375 ± 27[c]	429 ± 35[bc]
TML	12.7 ± 1.5	11.6 ± 0.6	13.5 ± 0.5	10.9 ± 0.5
BB	10.8 ± 0.4[b]	17.0 ± 0.9[a]	15.2 ± 0.6[a]	16.9 ± 0.7[a]

Data are means ± SE, n = 8 for each group. Means marked without a common superscript letter (a, b, c) differ ($P < .05$).

3.4. Expression of enzymes involved in hepatic carnitine synthesis and OCTN

Dams with litters, irrespective of litter size, had lower relative mRNA concentrations of TMLD, TMABA-DH, and BBD in the liver than dams whose litters were removed ($P < .05$, Fig. 3A). The activity of BBD in the liver was also lower in dams with litters compared with those whose litters were removed ($P < .05$, Fig. 3B). Relative mRNA concentration of OCTN1 was also reduced in dams with litters compared with those without litters ($P < .05$, Fig. 4). All these parameters, mRNA concentrations of enzymes involved in carnitine synthesis and of OCTN1 as well as activity of BBD, were however not different between dams with 4, 10, or 18 pups (Figs. 3A and 4). Relative mRNA concentration of OCTN2 in the liver did not differ between the 4 groups of rats (Fig. 4).

3.5. Carnitine concentrations in plasma and tissues

Dams with litters had lower concentrations of free carnitine, acetyl carnitine, and total carnitine in liver, skeletal muscle, and plasma than dams without litters ($P < .05$, Table 2). In liver and plasma, concentrations of total carnitine did not differ between dams nursing 4, 10, and 18 pups (Table 2). In skeletal muscle, concentration of free and total carnitine was lowest in dams nursing 10 pups ($P < .05$, Table 2). Dams nursing 18 pups did not differ in skeletal muscle free and total carnitine concentration from those nursing 4 or 10 pups (Table 2).

3.6. Concentrations of carnitine precursors in plasma and tissues

The concentration of TML in plasma, liver, and skeletal muscle did not differ between the 4 groups of rats (Table 2). Concentration of BB in plasma also did not differ between the 4 groups of rats. The BB concentration in skeletal muscle, however, was higher in dams with litters than in dams without litters ($P < .05$, Table 2). The BB concentration in liver was not different between dams with 4 or 10 pups and those whose litters were removed; in contrast, the hepatic BB concentration was higher in pups with 18 pups than in the other 3 groups ($P < .05$, Table 2).

4. Discussion

In this study, we tested the hypothesis that PPARα is down-regulated during lactation and that this leads to a down-regulation of enzymes involved in hepatic carnitine synthesis and OCTN. For this purpose, we compared lactating rats nursing 4, 10, or 18 pups with nonlactating rats whose litters were removed immediately after birth.

The data of this study confirm our hypothesis that lactation leads to a down-regulation of PPARα in the liver, some of its classic target genes (ACO, L-CPT-1, CYP4A1), as well as OCTN1 and enzymes involved in hepatic carnitine synthesis. Recently, it has been observed that gene expression of OCTN and of enzymes involved in hepatic carnitine synthesis is regulated by PPARα [15-18]. Although we have no direct proof for this, it is likely that down-regulation of OCTN1 and genes coding for enzymes involved in carnitine synthesis in the liver of lactating dams was due to the down-regulation of PPARα. In our previous experiments [15,16], OCTN2 was even more strongly up-regulated in rats by treatment with a PPARα agonist than OCTN1, suggesting that expression of OCTN2 is more sensitive toward PPARα activation than OCTN1. The finding that OCTN2, in contrast to OCTN1, was not down-regulated in response to the lowered expression of PPARα during lactation was, therefore, unexpected. It is, however, possible that baseline expression of PPARα caused already the minimum expression level of OCTN2. In this case, a reduction of the expression would not further down-regulate expression of OCTN2.

We found, moreover, that lactating rats have reduced carnitine concentrations in plasma, liver, and skeletal muscle compared with nonlactating female rats. Tissue carnitine concentrations are influenced by the rate of endogenous carnitine synthesis, the amount of carnitine absorbed from the diet, the distribution of carnitine within the body by carnitine transporters, the excretion of carnitine via the urine, and, in the lactating rat, additionally by the amount of carnitine excreted via the milk [34]. In the female rat, carnitine synthesis occurs exclusively in the liver [6]. BBD catalyzes the last step of synthesis in the liver [5]. However, it has been demonstrated in rats that the activity of BBD far exceeds that necessary to support the normal rate of carnitine synthesis [5]. Therefore, it is questionable whether the reduced activity of that enzyme, as observed in the liver of lactating rats, was responsible for the reduced plasma and tissue carnitine concentrations of lactating rats. It has been

shown that TML and its ability to penetrate to the site of TMLD are the rate-limiting factors for carnitine synthesis [35]. As TML concentration in the liver was unchanged, we assume that the availability of TML was not limiting for the carnitine synthesis during lactation.

In conclusion, the present study demonstrates for the first time that genes coding for enzymes involved in hepatic carnitine synthesis and OCTN1 are down-regulated in lactating rats. It is, moreover, shown that PPARα in the liver is down-regulated during lactation. As OCTN and enzymes of carnitine synthesis are up-regulated by PPARα, the down-regulation of PPARα may be the reason for the reduced gene expression of these proteins involved in whole-body carnitine synthesis. Down-regulation of PPARα, which also leads to an impairment of enzymes involved in hepatic β-oxidation, during lactation may be a means to conserve energy and metabolic substrates for milk production in the mammary gland.

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Down-regulation of peroxisome proliferator-activated receptor α and its coactivators in liver and skeletal muscle mediates the metabolic adaptations during lactation in mice

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Abstract
Previous studies have shown that genes involved in fatty acid uptake, fatty acid oxidation, and thermogenesis are down-regulated in liver and skeletal muscle of rats during lactation. However, biochemical mechanisms underlying these important metabolic adaptations during lactation have not yet been elucidated. As all these genes are transcriptionally regulated by peroxisome proliferator-activated receptor α (*Pparα*), we hypothesized that their down-regulation is mediated by a suppression of *Pparα* during lactation. In order to investigate this hypothesis, we performed an experiment with lactating and non-lactating *Pparα* knockout and corresponding wild-type mice. In wild-type mice, lactation led to a considerable down-regulation of *Pparα*, *Ppar* coactivators *Pgc1α* and *Pgc1β*, and *Pparα* target genes involved in fatty acid uptake, fatty acid oxidation, and thermogenesis in liver and skeletal muscle ($P < 0.05$). *Pparα* knockout mice had generally a lower expression of all these *Pparα* target genes in liver and skeletal muscle. In those mice, lactation did, however, not lower the expression of genes involved in fatty acid utilization and thermogenesis in liver and skeletal muscle. Expression levels of *Pparα* target genes in lactating wild-type mice were similar than in lactating or non-lactating *Pparα* knockout mice. In conclusion, the present findings suggest that down-regulation of *Pparα* and its coactivators in tissues with high rates of fatty acid catabolism is responsible for the reduced utilization of fatty acids in liver and skeletal muscle and the reduced thermogenesis occurring in the lactating animal, which aim to conserve energy and metabolic substrates for milk production in the mammary gland.

Introduction

Lactation is a physiological state characterized by a dramatic increase in the energy and nutrient requirement of the organism for milk production. This demand is usually met by a markedly increased food intake and by the utilization of energy stores. In addition, several metabolic adaptations develop in the lactating animal aiming to conserve energy and metabolic substrates for milk production in the mammary gland (Dewey 1997, Smith & Grove 2002, Williamson 1986, Trayhurn *et al.* 1982). Recently, it has been shown that down-regulation of uncoupling proteins (*Ucp*) 1 and 3 in brown adipose tissue and of *Ucp3* in skeletal muscle, leading to a decrease of metabolic fuel oxidation and thermogenesis, contributes to these metabolic adaptations during lactation (Trayhurn *et al.* 1982, Pedraza *et al.* 2000, Pedraza *et al.* 2001, Xiao *et al.* 2004a). Furthermore, expression of proteins involved in uptake and oxidation of fatty acids in skeletal muscle (Xiao *et al.* 2004b) as well as the rates of fatty acid oxidation and ketogenesis in the liver (Whitelaw & Williamson 1977) are reduced during lactation, effects that help to spare fatty acids for milk production in the mammary gland. Both, *Ucp* and proteins involved in fatty acid uptake and oxidation are transcriptionally regulated by peroxisome proliferator-activated receptor α (*Pparα*) (Mandard *et al.* 2004, Barbera *et al.* 2000, Bruns *et al.* 1999, Young *et al.* 2001). *Pparα* is a ligand-activated transcription factor, which is abundantly expressed in tissues with high rates of fatty acid oxidation, such as liver and skeletal muscle, and its physiologic role lies in the mediation of metabolic responses to fasting (Mandard *et al.* 2004, Schoonjans *et al.* 1997, Leone *et al.* 1999). Upon activation by either non-esterified fatty acids (NEFA) released from adipose tissue and taken up into tissues or exogenous ligands (diet-derived fatty acids or fibrates), *Pparα* up-regulates genes involved in all aspects of fatty acid catabolism including cellular fatty acid uptake and transport, mitochondrial and peroxisomal fatty acid oxidation as well as ketogenesis (Mandard *et al.* 2004). We have recently observed that lactating rats have reduced mRNA concentrations of *Pparα* and *Pparα* regulated genes involved in fatty acid utilization in the liver compared to non-lactating rats (Gutgesell *et al.* 2009). This finding suggested that down-regulation of genes involved in fatty acid uptake and oxidation as well as *Ucp* during lactation is mediated by suppression of *Pparα*.

The aim of the present study was to test the hypothesis that down-regulation of *Pparα* mediates the reduced expression of genes involved in fatty acid uptake and β-oxidation in tissues with high rates of fatty acid utilization such as liver and skeletal muscle, which favors the availability of fatty acids for milk triacylglycerol (TAG) production in the mammary gland. For that purpose, we performed an experiment with *Pparα* knockout and corresponding wild-type mice, and studied the influence of lactation on the expression of *Pparα* and selected *Pparα* responsive genes involved in fatty acid uptake (*Fabp*$_{pm}$, *Fatp*), fatty acid oxidation (*Cpt I*, *Cyp4a10*, *Mcad*), and thermogenesis (*Ucp3*) in liver and skeletal muscle, respectively. It has been shown that the transcriptional activity of *Ppar* is enhanced by several coactivators, including *Pparγ* coactivator (*Pgc*)*1α* and *1β*. These coactivators are required for the ability of *Ppar* to increase gene transcription to the maximum (Yu & Reddy 2007). In order to study whether lactation influences the expression of these coactivators, we also determined the expression of *Pgc1α* and *Pgc1β* in liver and skeletal muscle.

During lactation, an increased flow of NEFA, originating from hydrolysis of TAG by hormone-sensitive lipase (*Hsl*) in adipose tissue, into the mammary gland for milk production is observed, whereas uptake of fatty acids by lipoprotein lipase (*LPL*) from TAG-rich lipoproteins such as chylomicrons and very low-density lipoproteins (VLDL) into adipose tissue is reduced during lactation (Williamson 1986). The uptake of both, fatty acids released by *Lpl* from TAG-rich lipoproteins and albumin-bound NEFA in the plasma released from adipose tissue into the mammary gland is mediated by fatty acid transporters, and is an important source for milk TAG synthesis. To obtain information about alterations in the uptake of fatty acids into the mammary gland during lactation, we also determined expression

of fatty acid transporters and *Lpl* in the mammary gland as well as TAG concentrations in plasma.

Materials and methods

Animal experiment

The animal experiment was carried out with female *Pparα* knockout mice (129S4/SvJae-*Ppara*^{tm1Gonz}/J) and corresponding wild-type mice (129S1/SvImJ) obtained from Jackson Laboratory (Bar Harbor, ME, USA). They were kept in Macrolon cages in a room maintained with controlled temperature (23 ± 1°C), humidity (50-60%), and lighting (0600 to 1800 h). All animals were fed a commercial standard diet for rodents (Altromin GmbH, Lage, Germany). The standard diet was nutritionally adequate for lactating mice according to the recommendations of ASNS (Reeves et al. 1993), and contained 11.9 MJ/kg metabolizable energy. The composition of the standard diet was (g/kg diet): crude protein (19.0), crude fat (4.0), crude fibre (6.0), crude ash (7.0), nitrogen free extracts (53.0) (Altromin). Water was available *ad libitum* from nipple drinkers. All experimental procedures described followed established guidelines for the care and handling of laboratory animals (UFAW 1999, SfE) and were approved by the council of Saxony-Anhalt.

At 14 wk of age, the mice were mated by housing two female mice with one male mouse (129S1/SvImJ) for 6 d. At the day of parturition, designated as d 1 of lactation, wild-type mice (n = 10) and *Pparα* knockout mice (n = 14) were randomly assigned to 2 groups. In one group of the wild-type and *Pparα* knockout mice, all pups were removed ("without litter"), whereas in the other group litters were adjusted to 6 pups per dam ("with litter"). During pregnancy and lactation, female mice were kept individually in single cages, and diets fed ad libitum. Feed consumption was measured every day by determining the weight of the remaining diet at the day after feed administration. At d 15 of lactation, dams received the last dose of the diet at the beginning of the light cycle (0600 h) and were killed 4 h later at 1000 h by decapitation under light anesthesia with diethyl ether. Animals were killed in the non-fasted state to prevent fasting-induced *Pparα* activation.

Sample collection

Blood was collected into heparinized polyethylene tubes (Sarstedt, Nürnberg, Germany). Plasma was obtained by centrifugation of the blood (1100 X g; 10 min; 4°C) and stored at -20°C. Liver, mammary gland, and skeletal muscle were excised, immediately shock frozen with liquid nitrogen and stored at -80°C pending analysis.

Lipid analysis

Plasma TAG concentrations were measured using reagent kits obtained from Merck Eurolab (Darmstadt, Germany, Ref. 113009990314) according to the manufactors protocol.

RNA isolation and RT-PCR with real-time detection

Total RNA was isolated from the liver, mammary gland, and skeletal muscle (M. semitendinosus) using Trizol^{TM} reagent (Invitrogen, Karlsruhe, Germany) according to the manufactor´s protocol. RNA concentration and purity were estimated from the optical density at 260 and 280 nm (SpectraFluor Plus, Tecan, Crailsheim, Germany). Synthesis of cDNA and determination of mRNA abundance by RT-PCR with real-time detection (Rotorgene 2000, Corbett Research) using Sybr Green I was performed as recently described in detail (Ringseis *et al.* 2007*c*). Relative quantification of mRNA concentrations was performed using the $\Delta\Delta C_t$ method (Pfaffl 2001). Threshold cycle (C_t) values were obtained using Rotorgene Software 5.0 (Corbett Research). The housekeeping genes *Gapdh* (liver), *β-actin* (skeletal muscle), and *Cyp18* (mammary gland) were used for normalization. Different housekeeping genes were

Table 1. Characteristics of the primers used for RT-PCR analysis with real-time detection

Gene	Forward primer (from 5´to 3´)	Reverse primer (5´to 3´)	PCR product size	NCBI GenBank
β-actin	ACGGCCAGGTCATCACTATTG	CACAGGATTCCATACCCAAGAA	87	NM_007393
Cyp18	GTGGTCTTTGGGAAGGTGAA	TTACAGGACATTGCGAGCAG	210	NM_008907
Cyp4a10	TGAGGGAGAGCTGGAAAAGA	CTGTTGGTGATCAGGGTGTG	266	NM_010011
Fabp$_{pm}$	CCAGAAAGGGAAGGACATCA	GTCTCCAGTTCGCACTCCTC	132	NM_017399
Fas	TGGGTTCTAGCCAGCAGAGT	ACCACCAGAGACCGTTATGC	158	NM_007988
Fat/Cd36	GAGCAACTGGTGGATGGTTT	GCAGAATCAAGGGAGAGCAC	207	NM_007643
Fatp	TGCTTTGGTTTCTGGGACTT	GCTCTAGCCGAACACGAATC	156	NM_11977
Gapdh	AACGACCCCTTCATTGAC	TCCACGACATACTCAGCAC	191	XM_001476707
l-Cpt I	CCAGGCTACAGTGGGACATT	GAACTTGCCCATGTCCTTGT	209	NM_013495
Lpl	GGGCTCTGCCTGAGTTGTAG	AGAAATTTCGAAGGCCTGGT	157	NM_008509
Mcad	AGGTTTCAAGATCGCAATGG	CTCCTTGGTGCTCCACTAGC	152	NM_007382
m-Cpt I	GTCGCTTCTTCAAGGTCTGG	AAGAAAGCAGCACGTTCGAT	232	NM_009948
Pgc1α	AAACTTGCTAGCGGTCCTCA	TGTTGACAAATGCTCTTC	342	NM_008904
Pgc1β	AACCCAACCAGTCTCACAGG	TGCTGCTGTCCTCAAATACG	371	NM_133249
Pparα	CGGGAAAGACCAGCAACAAC	TGGCAGTGGAAGAATCG	137	NM_011144
Ucp3	CCACACTT CCTCCTGCTCTC	GTATAGGGCGCTCAAATGGA	235	NM_009464

used in the various tissues, because none of the housekeeping genes tested served as an appropriate reference gene in all tissues. The existence of a single PCR product of the expected length was guaranteed by melting curve analysis and 1% agarose gel electrophoresis. Relative mRNA concentrations are expressed as fold of mRNA concentration of the wild-type "without litter" group. Characteristics of the primers (Eurofins MWG Operon, Ebersberg, Germany) used for RT-PCR with real-time detection are shown in Tab. 1.

Statistics

Data were analyzed using the Minitab Statistical Software (Minitab Rel. 15, State College, PA, USA). Treatment effects were analyzed by two-way ANOVA with classification factors being litter, genotype and the interaction of litter and genotype. For significant *F*-values, means were compared by Fisher's multiple range test. Means were considered significantly different for $P < 0.05$. Data presented in the text are shown as means ± SEM.

Results

Food intake, body weights of dams and weights of litters

Dams with litters consumed more food per day than those without litters, irrespective of genotype (wild-type mice without litter: 2.0 ± 0.2 g/d; wild-type mice with litter: 7.7 ± 0.7 g/d; *Pparα* knockout mice without litter: 2.1 ± 0.2 g/d; *Pparα* knockout mice with litter: 7.9 ± 1.0 g/d; $P < 0.05$). Body weights of dams at day 1 of lactation did not differ between groups (wild-type mice without litter: 22.2 ± 0.6 g; wild-type mice with litter: 22.1 ± 0.9 g; *Pparα* knockout mice without litter: 22.7 ± 1.3 g; *Pparα* knockout mice with litter: 23.7 ± 2.2 g). However, body weights at day 15 of lactation were higher in dams with litters than in those without litters, irrespective of the genotype (wild-type mice without litter: 20.8 ± 0.6 g; wild-type mice with litter: 26.0 ± 0.7 g; *Pparα* knockout mice without litter: 20.1 ± 0.9 g; *Pparα* knockout mice with litter: 26.1 ± 2.1 g; $P < 0.05$). Litter weights of dams of both genotypes did not differ neither at d 1 nor at d 15 of lactation (d 1 of lactation: wild-type mice: 11.8 ± 1.0 g; *Pparα* knockout mice: 11.2 ± 1.1 g; d 15 of lactation: wild-type mice: 43.5 ± 2.1 g; *Pparα* knockout mice: 44.8 ± 2.2 g).

TAG concentrations in plasma

TAG concentrations in plasma were influenced by the factors litter and genotype ($P < 0.05$); mice with litters had lower concentrations of TAG in plasma than those without litters, irrespective of the genotype; *Pparα* knockout mice had higher TAG concentrations in plasma than wild-type mice (wild-type mice without litter: 0.61 ± 0.10 mmol/L, wild-type mice with litter: 0.39 ± 0.02 mmol/L, *Pparα* knockout mice without litter: 0.90 ± 0.10 mmol/L, *Pparα* knockout mice with litter: 0.69 ± 0.09 mmol/L).

Expression of *Pparα* in liver and skeletal muscle

Wild-type mice with litters had markedly lower relative mRNA concentrations of *Pparα* in liver and skeletal muscle than wild-type mice without litters ($P < 0.05$; **Fig. 1**).

Expression of *Pparα* target genes in the liver

Wild-type mice with litters had markedly lower relative mRNA concentrations of *l-Cpt I*, *Cyp4A10*, *Fatp* and *Fabp*$_{pm}$ in the liver than wild-type mice without litters ($P < 0.05$; Fig. 2). Relative mRNA concentrations of all these genes in the liver were lower in *Pparα* knockout mice with and without litters than in wild-type mice with litters ($P < 0.05$; **Fig. 2**); however, relative mRNA concentrations of these genes in the liver did not differ between *Pparα* knockout mice with litters and those without litters (**Fig. 2**).

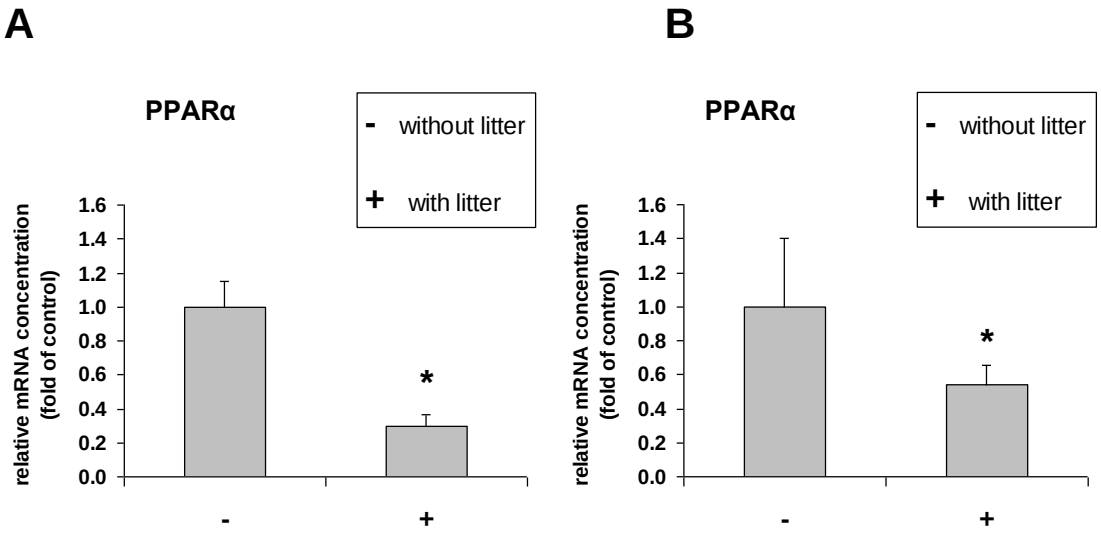

Fig. 1. Relative mRNA concentrations of *Pparα* in the liver (A) and skeletal muscle (B) of wild-type mice, whose litters where either removed (without litter) or adjusted to 6 pups/dam (with litter) immediately after birth, at day 15 of lactation. Bars represent means ± SEM for $n = 5$. Bars without a common letter (a, b) differ, $P < 0.05$.

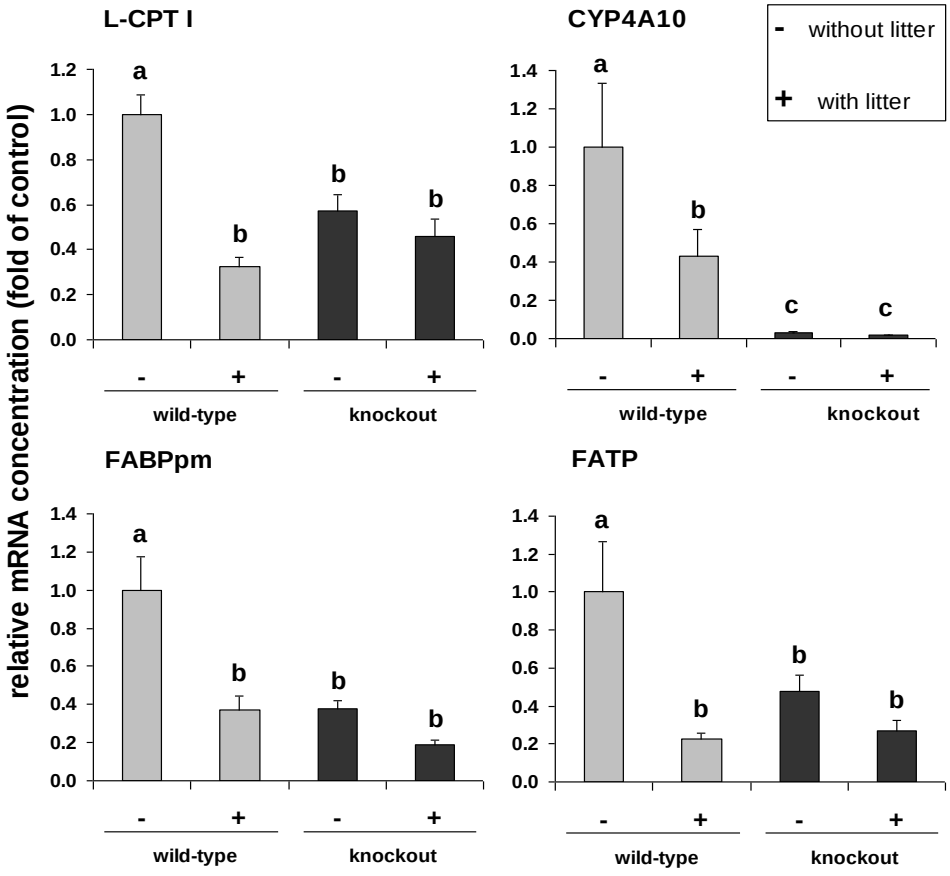

Fig. 2. Relative mRNA concentrations of the *Pparα* target genes *l-Cpt I*, *Cyp4a10*, *Fabppm* and *Fatp* in the liver of lactating wild-type and lactating *Pparα* knockout mice, whose litters where either removed (without litter) or adjusted to 6 pups/dam (with litter) immediately after birth, at day 15 of lactation. Bars represent means ± SEM for $n = 5$ in wild-type mice and $n = 7$ in *Pparα* knockout mice. Bars without a common letter (a, b, c) differ, $P < 0.05$. Significant effects ($P < 0.05$) from two-way ANOVA: *l-Cpt I*: litter, genotype, litter x genotype; *Cyp4a10*: litter, genotype, litter x genotype; *Fabppm*: litter, genotype, litter x genotype; *Fatp*: litter, genotype, litter x genotype.

Expression of *Pparα* target genes in skeletal muscle

In skeletal muscle, relative mRNA concentrations of *m-Cpt I*, *Mcad*, *Fatp* and *Ucp3* were lower in wild-type mice with litters compared to wild-type mice whose litters were removed ($P < 0.05$; **Fig. 3**). In contrast, relative mRNA concentrations of these genes did not differ between *Pparα* knockout mice with litters and *Pparα* knockout mice without litters (**Fig. 3**). The relative mRNA concentrations of *m-Cpt I*, *Mcad*, *Fatp* and *Ucp3* in skeletal muscle were lower in *Pparα* knockout mice with and without litters than in wild-type mice without litters ($P < 0.05$; **Fig. 3**). Unexpectedly, relative mRNA concentrations of *m-Cpt I* and *Mcad* in skeletal muscle in wild-type mice with litters were even lower than in *Pparα* knockout mice with and without litters ($P < 0.05$; **Fig. 3**). The relative mRNA concentration of *Fabp*$_{pm}$ in skeletal muscle did not differ between the four groups of mice (**Fig. 3**).

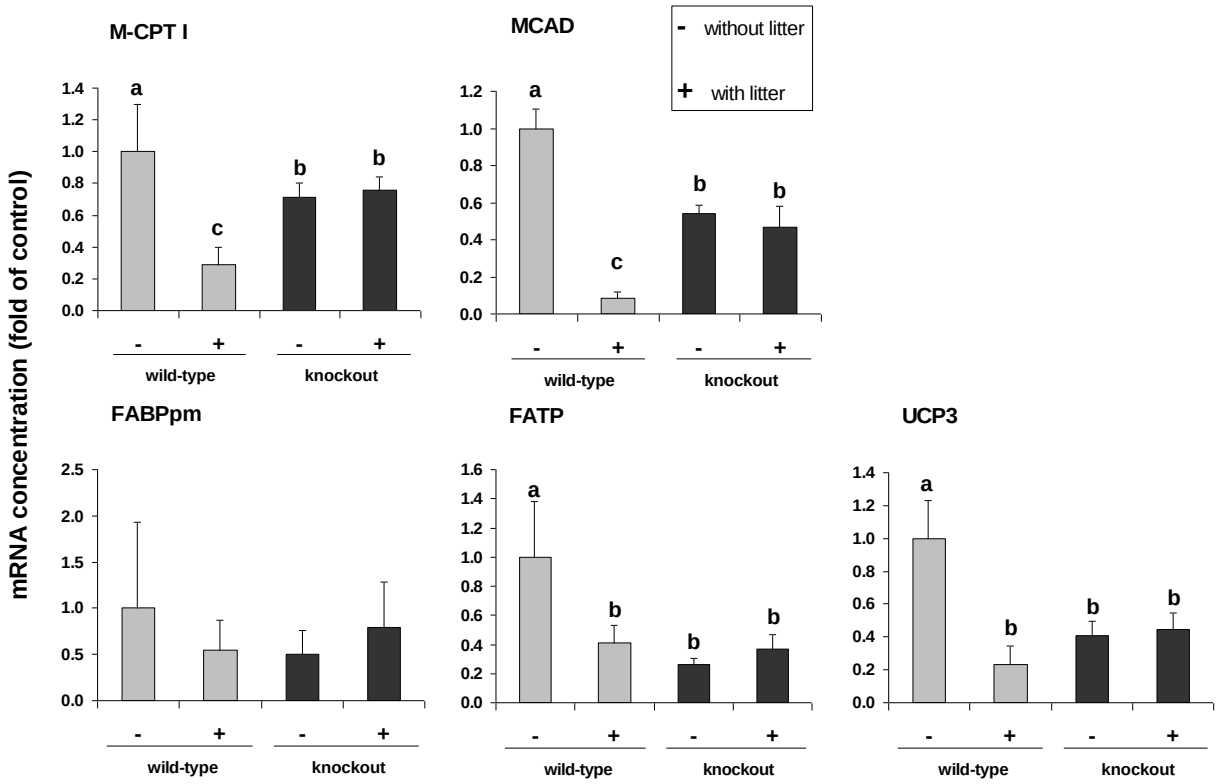

Fig. 3. Relative mRNA concentrations of the *Pparα* target genes *m-Cpt I*, *Mcad*, *Fabppm*, *Fatp*, and *Ucp3* in the skeletal muscle of lactating wild-type and lactating *Pparα* knockout mice, whose litters where either removed (without litter) or adjusted to 6 pups/dam (with litter) immediately after birth, at day 15 of lactation. Bars represent means ± SEM for $n = 5$ in wild-type mice and $n = 7$ in *Pparα* knockout mice. Bars without a common letter (a, b, c) differ, $P < 0.05$. Significant effects ($P < 0.05$) from two-way ANOVA: *m-Cpt I*: litter, litter x genotype; *Mcad*: litter, litter x genotype; *Fatp*: genotype, litter x genotype; *Ucp3*: litter, litter x genotype.

Expression of *Ppar* coactivators *Pgc1α* and *Pgc1β* in liver and skeletal muscle

In liver and skeletal muscle, relative mRNA concentrations of *Pgc1α* and *Pgc1β* were lower in wild-type mice with litters compared to wild-type mice whose litters were removed ($P < 0.05$; **Fig. 4**). In contrast, relative mRNA concentrations of *Pgc1α* and *Pgc1β* in liver and skeletal muscle did not differ between *Pparα* knockout mice with litters and *Pparα* knockout mice without litters (**Fig. 4**). However, whereas relative mRNA concentrations of *Pgc1α* and *Pgc1β* in liver were lower in *Pparα* knockout mice with and without litters than in wild-type mice without litters ($P < 0.05$; **Fig. 4**), relative mRNA concentrations of these genes in

skeletal muscle did not differ between wild-type mice without litters and *Pparα* knockout mice with and without litters (**Fig. 4**).

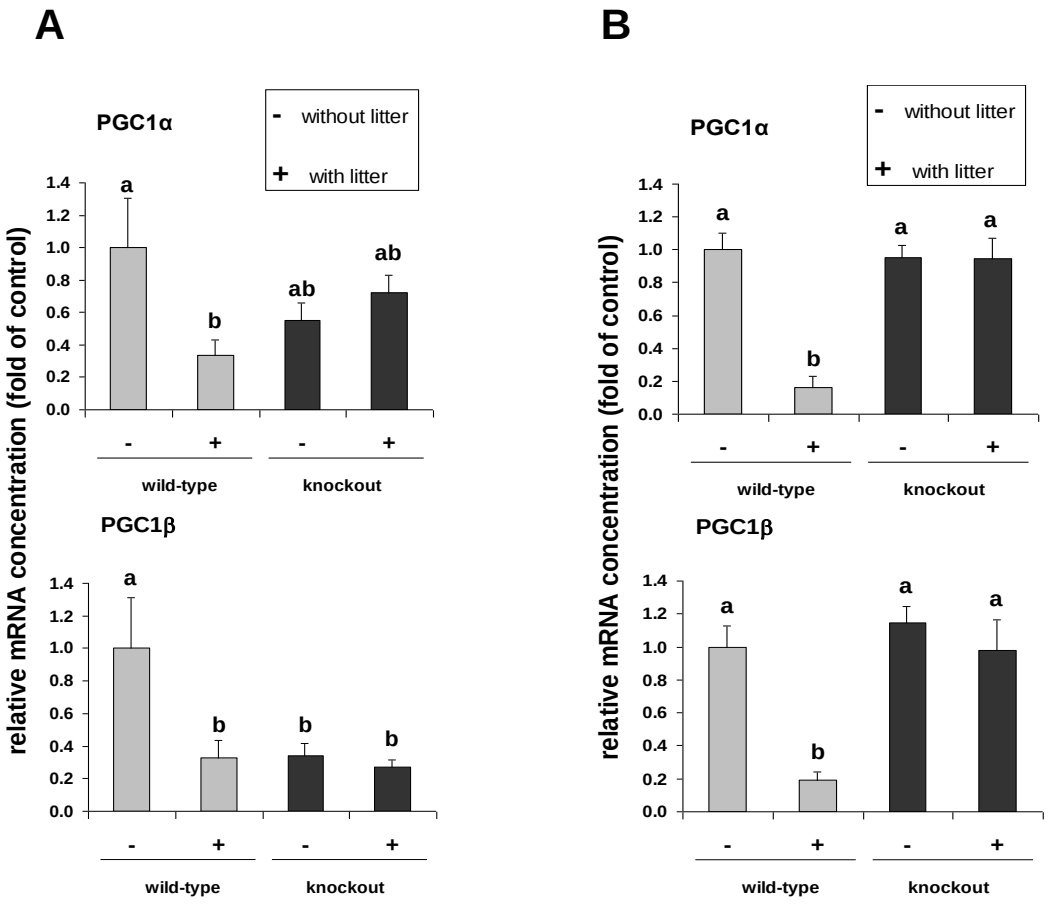

Fig. 4. Relative mRNA concentrations of *Pgc1α* and *Pgc1β* in liver (A) and skeletal muscle (B) of lactating wild-type and lactating *Pparα* knockout mice, whose litters where either removed (without litter) or adjusted to 6 pups/dam (with litter) immediately after birth, at day 15 of lactation. Bars represent means ± SEM for $n = 5$ in wild-type mice and $n = 7$ in *Pparα* knockout mice. Bars without a common letter (a, b, c) differ, $P < 0.05$. Significant effects ($P < 0.05$) from two-way ANOVA: *Pgc1α* liver: litter, litter x genotype; *Pgc1β* liver: litter, genotype, litter x genotype; *Pgc1α* skeletal muscle: litter, litter x genotype; *Pgc1β* skeletal muscle: litter, litter x genotype.

Expression of fatty acid transporters, *Lpl*, and fatty acid synthase in the mammary gland of mice

Wild-type mice with litters had markedly higher mRNA concentrations of *Fat*/*Cd36*, *Fatp*, *Lpl*, and fatty acid synthase (*Fas*) in the mammary gland than wild-type mice without litters ($P < 0.05$; Fig. 5). In *Pparα* knockout mice, the mRNA concentrations of these genes were also higher in the mammary gland of dams with litters than in those without litters ($P < 0.05$; Fig. 5).

Discussion

The aim of the present study was to test the hypothesis that down-regulation of *Pparα* in tissues with high rates of fatty acid catabolism (liver, skeletal muscle) is responsible for the metabolic adaptations occurring in the lactating animal, such as decreased fatty acid oxidation and diminished thermogenesis (Pedraza *et al.* 2000, Perdraza *et al.* 2001, Xiao *et al.* 2004a, Xiao *et al.* 2004b, Zammit 1980). Herein we could clearly demonstrate for the first time that down-regulation of *Pparα* and *Pparα* target genes involved in fatty acid uptake (*Fabp*$_{pm}$,

Fatp), fatty acid oxidation (*Cpt I*, *Cyp4a10*, *Mcad*), and thermogenesis (*Ucp3*) occurs only in tissues of lactating wild-type mice. In *Pparα* knockout mice, in contrast, lactation did not result in a reduced expression of *Pparα* and *Pparα* target genes in these tissues. These findings strongly indicate that the metabolic adaptations in the lactating animal (Pedraza *et al.* 2000, Perdraza *et al.* 2001, Xiao *et al.* 2004a, Xiao *et al.* 2004b, Zammit 1980) are mediated by the diminished expression of *Pparα* in liver and skeletal muscle. As expected, expression levels of *Pparα* target genes were markedly lower in liver and skeletal muscle of *Pparα* knockout mice than in those of non-lactating wild-type mice. In this regard it is, however, noteworthy that *Pparα* target genes, with the exception of *m-Cpt I* and *Mcad* in skeletal muscle, were expressed in tissues of *Pparα* knockout mice at levels comparable to those of lactating wild-type mice. This indicates that lactation in wild-type mice causes a similar reduction in the expression of *Pparα* target genes as disruption of *Pparα* expression.

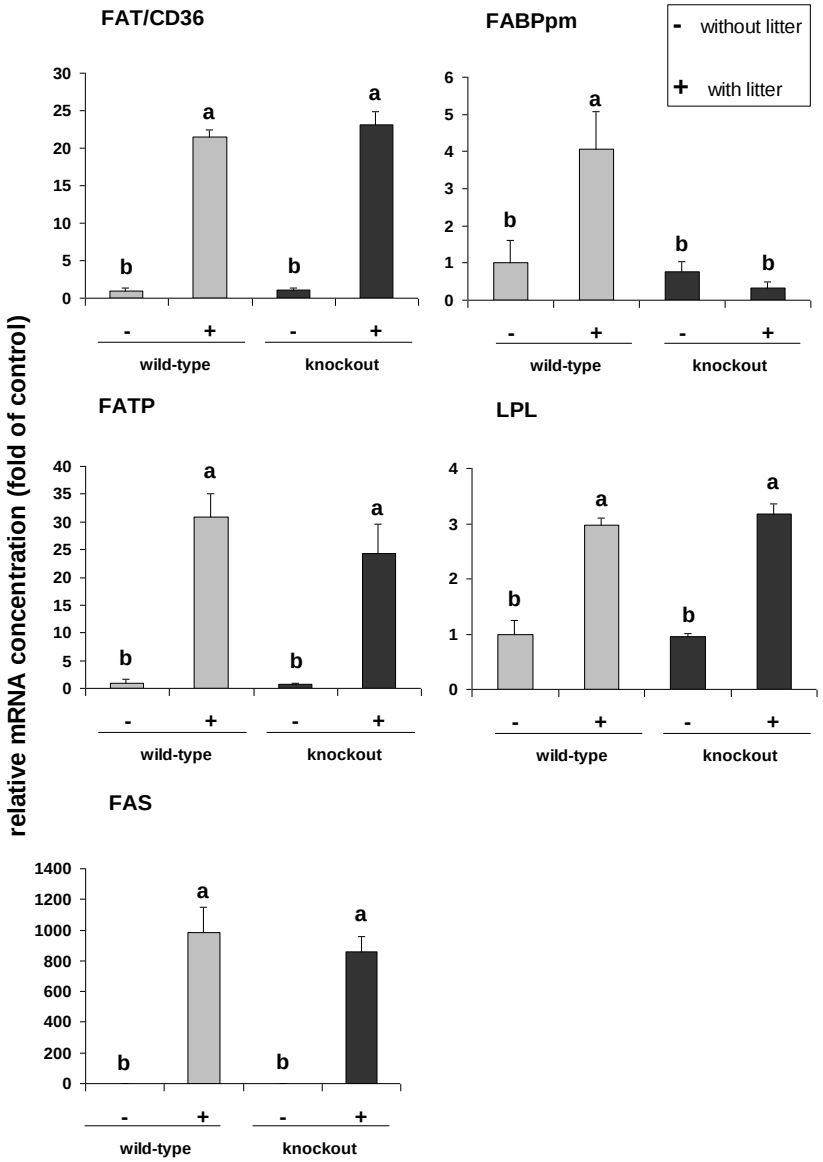

Fig. 5. Relative mRNA concentrations of *Fat/Cd36*, *Fatp*, *Lpl* and *Fas* in the mammary gland of lactating wild-type and lactating *Pparα* knockout mice, whose litters where either removed (without litter) or adjusted to 6 pups/dam (with litter) immediately after birth, at day 15 of lactation. Bars represent means ± SEM for $n = 5$ in wild-type mice and $n = 7$ in *Pparα* knockout mice. Bars without a common letter (a, b, c) differ, $P < 0.05$. Significant effects ($P < 0.05$) from two-way ANOVA: *Fat/Cd36*: litter; *Fabppm*: litter, litter x genotype; *Fatp*: litter; *Lpl*: litter.

Although *Pparα* is clearly an important transcription factor in regulating the expression of genes involved in fatty acid oxidation, the observation that the mRNA levels of *m-Cpt I* and *Mcad* in skeletal muscle were even stronger reduced by lactation than by the *Pparα* knockout implies that also other *Pparα*-independent mechanisms might be involved. As lactating wild-type mice had lower mRNA concentrations of *Pgc1α* and *Pgc1β*, which are required for maximal transcriptional activity of *Ppar* (Yu & Reddy 2007, Feingold *et al.* 2008), in liver and skeletal muscle than their non-lactating counterparts, we suggest that a reduced expression of *Ppar* coactivators could have also contributed to the decreased expression of *Pparα*-regulated genes in skeletal muscle and liver during lactation. In liver, the expression of both coactivators was lower in *Pparα* knockout than in wild-type mice indicating that *Pgc1α* and *Pgc1β* are regulated by *Pparα* in that tissue. However, in skeletal muscle, the expression of both coactivators did not differ between wild-type mice without litters and *Pparα* knockout mice with and without litters suggesting that *Pgc1α* and *Pgc1β* in skeletal muscle are regulated *Pparα*-independently. The finding that *Ppar* coactivators in skeletal muscle were not reduced in *Pparα* knockout mice compared to wild type mice could provide an explanation for the observation that some *Pparα*-regulated genes, such as *m-Cpt I* and *Mcad*, in skeletal muscle were only slightly reduced in knockout versus wild-type mice. Reduced mRNA concentrations of *Ppar* coactivators in skeletal muscle of lactating wild-type mice compared to lactating *Pparα* knockout mice could be responsible for the finding that the expression of some *Pparα* target genes such as *m-Cpt I* and *Mcad* in skeletal muscle was even lower in lactating wild-type than in knockout mice.

Besides down-regulation of fatty acid oxidation enzymes and fatty acid transporters in skeletal muscle, we observed down-regulation of *Ucp3* in skeletal muscle of lactating wild-type mice, which is consistent with recent observations in skeletal muscle and brown adipose tissue of lactating rats (Pedraza *et al.* 2000, Pedraza *et al.* 2001, Xiao *et al.* 2004*a*, Xiao *et al.* 2004*b*). Since *Ucp* function to uncouple the respiratory chain, and, thereby, increase heat production, down-regulation of *Ucp* in brown adipose tissue of rats during lactation (Pedraza *et al.* 2001, Xiao *et al.* 2004*a*, Xiao *et al.* 2004*b*) has been suggested to reflect the need to conserve energy during lactation through a decrease in non-shivering thermogenesis (Williamson 1986, Pedraza *et al.* 2000, Pedraza *et al.* 2001, Xiao *et al.* 2004*a*, Xiao *et al.* 2004*b*). The finding that *Ucp3* expression was reduced in skeletal muscle of lactating wild-type mice but not in lactating *Pparα* knockout mice again might indicate that the reduction of thermogenesis in skeletal muscle during lactation (Pedraza *et al.* 2000, Pedraza *et al.* 2001, Xiao *et al.* 2004*a*, Xiao *et al.* 2004*b*, Zammit 1980) is also mediated by the diminished expression of *Pparα*. Nevertheless, although *Ucp3* has been proposed to play an important role in regulating energy expenditure, and thermoregulation (Boss *et al.* 1997, Vidal-Puig *et al.* 1997), the exact physiological role of *Ucp3* is still elusive; i.e. observations from recent studies indicate that *Ucp3* function in skeletal muscle and heart is likely to be related to fatty acid catabolism (Muoio *et al.* 2002, Murray *et al.* 2005). In line with this assumption is the observation that *Ucp3* participates in mitochondrial antioxidant defense and serves as an "early response" to elevated ROS production and potential oxidative stress by increasing uncoupling respiration during prolonged exercise in rat skeletal muscle (Jiang *et al.* 2009). Therefore, future studies have to elucidate the exact physiological role of *Ucp3* down-regulation during lactation.

Based on the findings of this study and other studies in the literature (Trayhurn *et al.* 1982, Pedraza *et al.* 2000, Pedraza *et al.* 2001, Xiao *et al.* 2004a, Xiao *et al.* 2004b), we propose the model shown in Fig. 6, which suggests that down-regulation of *Pparα* and its coactivators in tissues with high rates of fatty acid utilization, such as liver, skeletal muscle and heart, and subsequently reduced utilization of fatty acids by these tissues during lactation mediates an increased flow of NEFA from white adipose tissues and TAG-rich lipoproteins into the mammary gland and, thus helps to spare energy and metabolic substrates for milk

production. The physiologically increased availability of fatty acids in the mammary gland during lactation (Dewey 1997, Smith & Grove 2002, Williamson 1986) is reflected by the marked up-regulation of fatty acid transporters (*Fat/Cd36* and *Fatp*) and *Lpl*, which mediate the uptake of albumin-bound NEFA and fatty acids released from TAG-rich lipoproteins, respectively, in the mammary gland and the reduced concentrations of NEFA (Pedraza *et al.* 2000) and TAG (Gutgesell *et al.* 2009) in plasma of lactating animals in both genotypes. In addition, expression of the lipogenic enzyme *Fas*, which is critical for *de novo*-fatty acid synthesis, was strongly increased in the mammary gland of lactating mice of both genotypes. This indicates that *Pparα* does not play an essential role for the uptake of fatty acids into the mammary gland, and that *de novo*-fatty acid synthesis in the mammary gland is similar in both genotypes. This indication was not unexpected as *Pparα* mRNA was only barely detectable in the mammary gland in the present study (data not shown) - a finding which largely confirms that from others (Rodriguez-Cruz *et al.* 2006, Gimble *et al.* 1998).

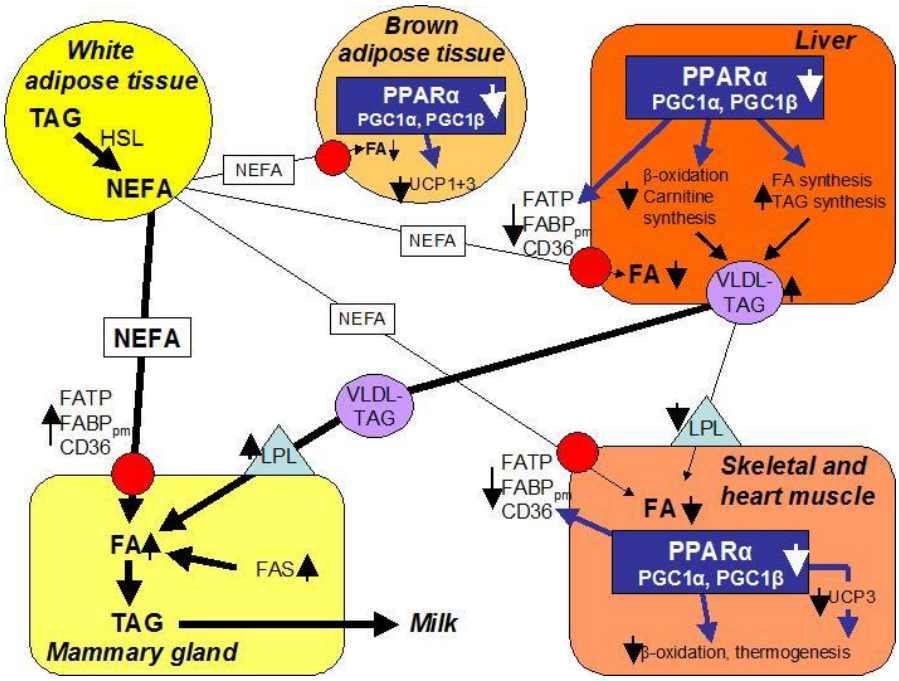

Fig. 6. Working hypothesis: Down-regulation of *Pparα* and its coactivators leads to a reduced uptake of fatty acids into tissues with high rates of β-oxidation, such as liver, skeletal muscle and heart, and to a reduced rate of β-oxidation in these tissues. Down-regulation of *Ucp*s in brown adipose tissue (*Ucp1* and *Ucp3*) and skeletal muscle (*Ucp3*) additionally diminishes thermogenesis and oxidation of fuels which are spared for milk synthesis. As recently shown, hepatic enzymes of carnitine synthesis are concomitantly also reduced during lactation which in turn leads to a reduced carnitine content in liver and skeletal muscle (Gutgesell *et al.* 2009). As carnitine is involved in β-oxidation by transferring fatty acids into the mitochondrion, inhibition of carnitine synthesis can be regarded also as a means to diminish fatty acid oxidation during lactation. On the other side, synthesis of fatty acids and TAG in the liver is enhanced during lactation, leading to an increased secretion of VLDL-TAG and an increased uptake of fatty acids from VLDL-TAG into the mammary gland due to the enhanced expression of *Lpl* (Grigor *et al.* 1982, Farid *et al.* 1978). Simultaneously, the uptake of NEFA derived mainly from lipolysis in white adipose tissue by fatty acid transporters into the mammary gland as well as *de novo*-fatty acid synthesis catalyzed by lipogenic enzymes including *Fas* in the mammary gland are increased, which also enhances the pool of fatty acids in the mammary gland available for milk TAG production.
Abbreviations used: *CD36/Fat*, CD36/fatty acid translocase; FA, fatty acid; *Fabppm*, plasma membrane-fatty acid binding protein; *Fas*, fatty acid synthase; *Fatp*, fatty acid transport protein; *Hsl*, hormone-sensitive lipase; *Lpl*, lipoprotein lipase; NEFA, non-esterified fatty acids; *Pgc1α/β*, PPARγ co-activator 1α/β; *Pparα*, peroxisome proliferator-activated receptor α; TAG, triacylglycerols; *Ucp1/3*, uncoupling protein 1/3; VLDL, very low-density lipoprotein.

Our proposed model is supported by the finding that activation of *Pparα* during lactation disturbs the normal metabolic adaptations during lactation. We have recently observed that activation of hepatic *Pparα* in the lactating rat by feeding a dietary oxidized fat, a potent activator of hepatic *Pparα* (Chao *et al.* 2001, Sülzle *et al.* 2004, Ringseis *et al.* 2007*b*), leads to an increased uptake of fatty acids into the liver and an enhanced β-oxidation in the liver, whereas uptake of fatty acids into the mammary gland by fatty acid transporters and *Lpl* was decreased which in turn led to a dramatically reduced milk TAG content and reduced weight gains of litters during suckling (Ringseis *et al.* 2007*a*). Similar observations regarding an impairment of lactation-induced energy-sparing mechanisms by the administration of *Pparα* activators during lactation have been reported from others (Pedraza *et al.* 2000).

In conclusion, the present study shows for the first time that down-regulation of *Pparα*, *Ppar* coactivators, and *Pparα* regulated genes, which are involved in fatty acid uptake, fatty acid oxidation, and thermogenesis, occurs only in tissues of lactating wild-type mice but not *Pparα* knockout mice. We postulate that down-regulation of *Pparα* and its coactivators in tissues with high rates of fatty acid catabolism is responsible for the reduced utilization of fatty acids in liver and skeletal muscle and the reduced thermogenesis occurring in the lactating animal, which aim to conserve energy and metabolic substrates for milk production in the mammary gland. The mechanism through which *Pparα* is down-regulated during lactation is currently unknown, but it may be speculated that hormonal changes associated with lactation, such as hyperprolactinemia or hypoleptinemia (Xiao *et al.* 2004a), but also changes in the levels of growth hormone or insulin might be causative. Therefore, further studies are necessary to identify the mechanisms behind the changes in *Pparα* expression during lactation.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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

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Key words: carnitine-acylcarnitine translocase (CACT), peroxisome proliferator-activated receptor α (PPARα), liver, fasting

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, 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. CONCLUSIONS: 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. GENERAL SIGNIFICANCE: 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, 10, 11, 12]. Upregulation of OCTN2 and enzymes involved in carnitine synthesis by 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 hours 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-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.

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 hours 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 hours 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 x 10^5 and 2 x 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 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 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 x 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 was 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 **Tab. 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-mCACT_1128, pGL4.10-mCACT_803, pGL4.10-mCACT_645, pGL4.10-mCACT_421, and pGL4.10-mCACT_170 were generated. The parental promoter construct pGL4.10-mCACT_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-mCACT_803 containing three PPRE (sequence spanning from –670 to +133), pGL4.10-mCACT_645 containing two PPRE (sequence spanning from –512 to +133), pGL4.10-mCACT_421 containing one PPRE (sequence spanning from –288 to +133) and mCACT_170 containing one PPRE (sequence spanning from –37 to +133) were PCR-amplified from the parental pGL4.10-mCACT_1128 promoter construct using specific 5´ primers flanking the putative PPRE (mCACT_170: 5´-ATACTCGAGAAGTAGACTTCAGGGCGGAA-3´, mCACT_421: 5´-ATACTCGAGCAAAGACAGGGACCTCTATG-3´, mCACT_645: 5´-

ATACTCGAGCTTCTGCCAGAAAGGTCCTT-3´, mCACT_803: 5´-ATACTCGAGGACTGATGTCCTCACAGTTC-3´) and one common 3´ primer (mCACT_HindIII: 5´-ATAAAGCTTGCTCAGTCTTCTGTCTGTCT-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-mCACT_1128mut, pGL4.10-mCACT_803mut, pGL4.10-mCACT_421mut, and pGL4.10-mCACT_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 primers (mCACT-PPREmut_forward: 5´-AGCGGCTCCGCCGACCATACACCTACATTCAGGTG-3´, mCACT-PPREmut_reverse: 5´-CACCTGAATGTAGGTGTATGGTCGGCGGAGCCGCT-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-mCACT_0 or positive control vector 3X 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-mPPARα 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, 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).

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

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´-GGCTCCGCCGACCATACACCTACATTCAGGTG-3´; for specific competition rACO-PPRE, 5´-TTCCCGAACGTGACCTTTGTCCTGGTCCC CTTTGATC-3´ and 5´-AAAGGGGACCAGGACAAAGGTCACGTTCGGGAAGATC-3´; for non-specific competition PPRE-cont: 5´-GACAGTGACTCTTGTGGGGATACTCTCT GACTCTACTCTTGAAGAATGATATATAC-3´ and 5´-ATATCATTCTTCAAGAGTAGA GTCAGAGAGTATCCCCACAAGAGTCACTGTCTGTT-3´. After annealing, 100 ng of double-stranded DNA probes were labelled with 0.05 mM DIG-ddUTP in 1X 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) polyacrylamid 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 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**).

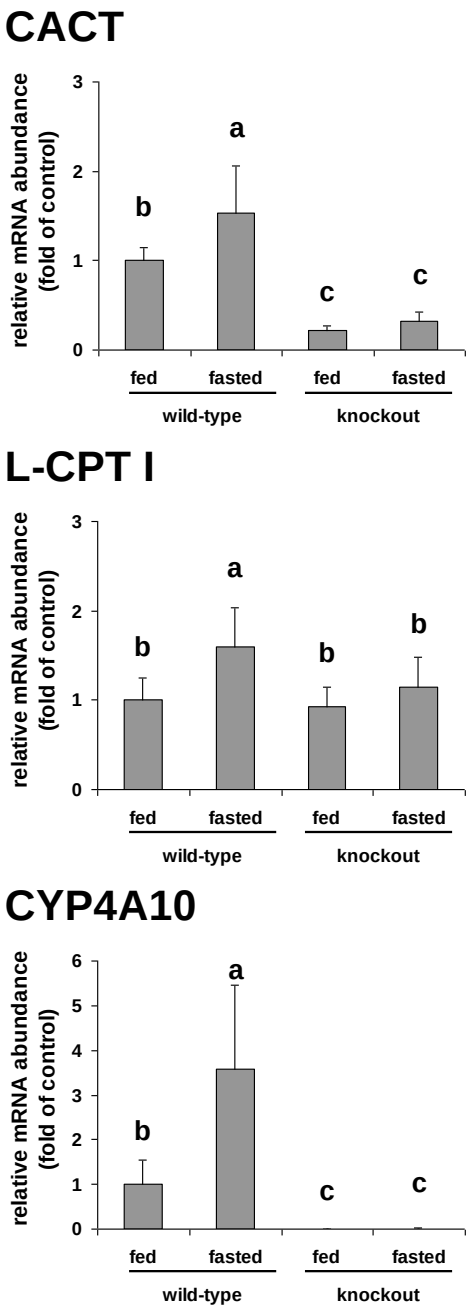

Fig. 1. Effect of fasting on mRNA abundances of carnitine-acylcarnitine translocase (CACT), liver-type carnitine-palmitoyltransferase I (L-CPT I), and cytochrome P450 4A10 (CYP4A10) in the liver of wild-type and PPARα knockout mice. Mice of both genotypes were either fasted for 48 h (fasted group) or 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 x genotype; L-CPT I: fasting, genotype; CYP4A10: fasting, genotype, fasting x genotype.

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 with 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**).

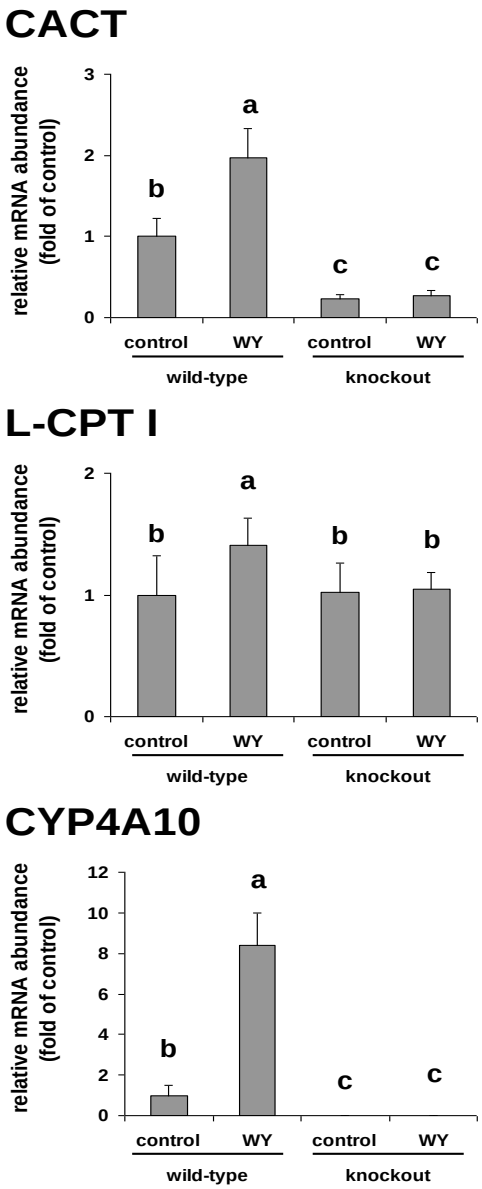

Fig. 2. Effect of WY-14,643 on mRNA abundances of carnitine-acylcarnitine translocase (CACT), liver-type carnitine-palmitoyltransferase I (L-CPT I), and cytochrome P450 4A10 (CYP4A10) in the liver of wild-type and PPARα knockout mice. Mice of both genotypes were treated orally for 4 days with either 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 x genotype; L-CPT I: WY-14,643; CYP4A10: WY-14,643, genotype, WY-14,643 x genotype.

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**).

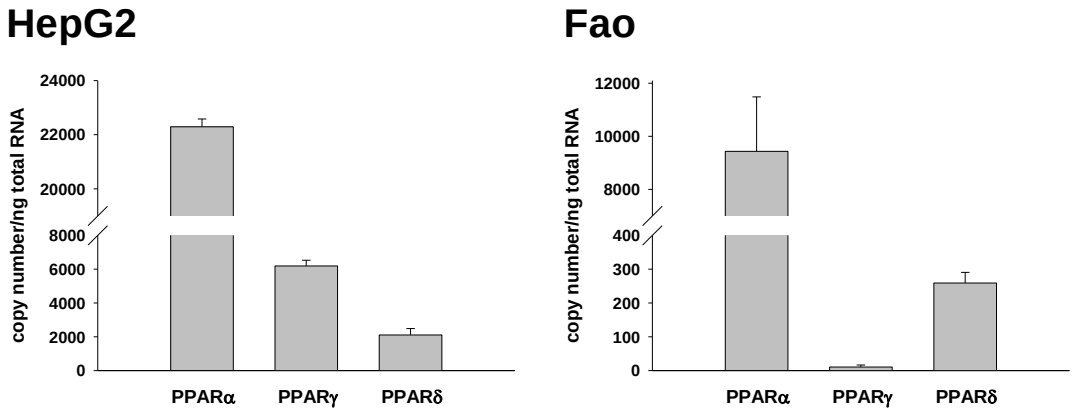

Fig. 3. Absolute mRNA abundances of PPARα, PPARγ and PPARδ in HepG2 cells, and Fao cells. Total RNA was extracted from cells and PPAR isotype-specific mRNA abundances were quantified by real-time detection RT-PCR analysis using standard curves generated with purified PCR products of human and rat, respectively,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

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**).

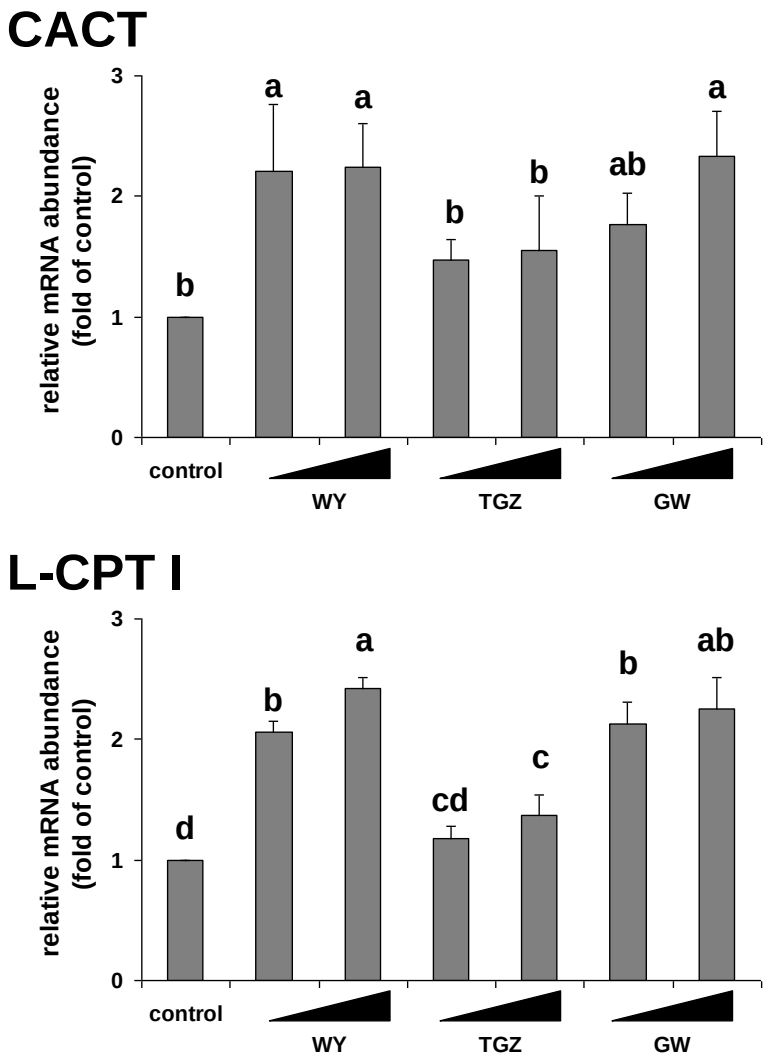

Fig. 4. Effect of PPARα agonist WY-14,643, PPARγ agonist TGZ, and PPARδ agonist GW0742 on the mRNA abundances of carnitine-acylcarnitine translocase (CACT), liver-type carnitine-palmitoyltransferase I (L-CPT I) in HepG2 cells. HepG2 cells were grown in culture medium until subconfluent state. Medium 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, $P < 0.05$.

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**).

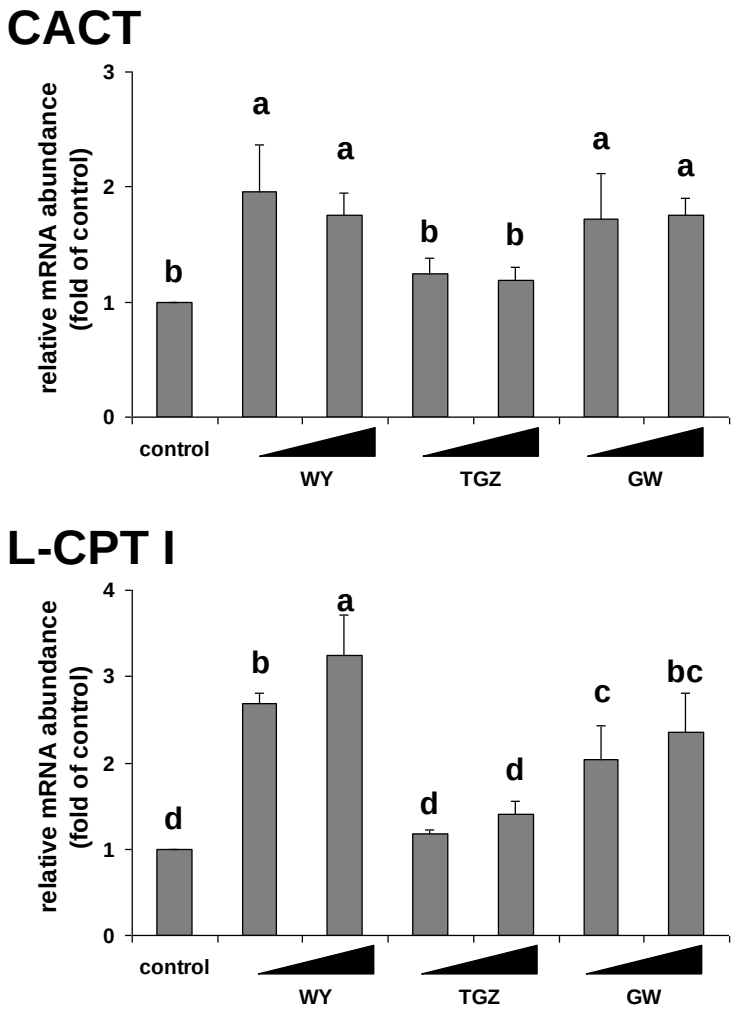

Fig. 5. Effect of PPARα agonist WY-14,643, PPARγ agonist TGZ, and PPARδ agonist GW0742 on the mRNA abundances of carnitine-acylcarnitine translocase (CACT), liver-type carnitine-palmitoyltransferase I (L-CPT I) in Fao cells. Fao cells were grown in culture medium until subconfluent state. Medium was 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, $P < 0.05$.

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-mCACT_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-mCACT_1128 construct indicative of activation of mouse CACT promoter ($P < 0.05$). In addition, co-transfection of mouse PPARα expression plasmid pCMX-mPPARα and mouse RXRα expression 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**).

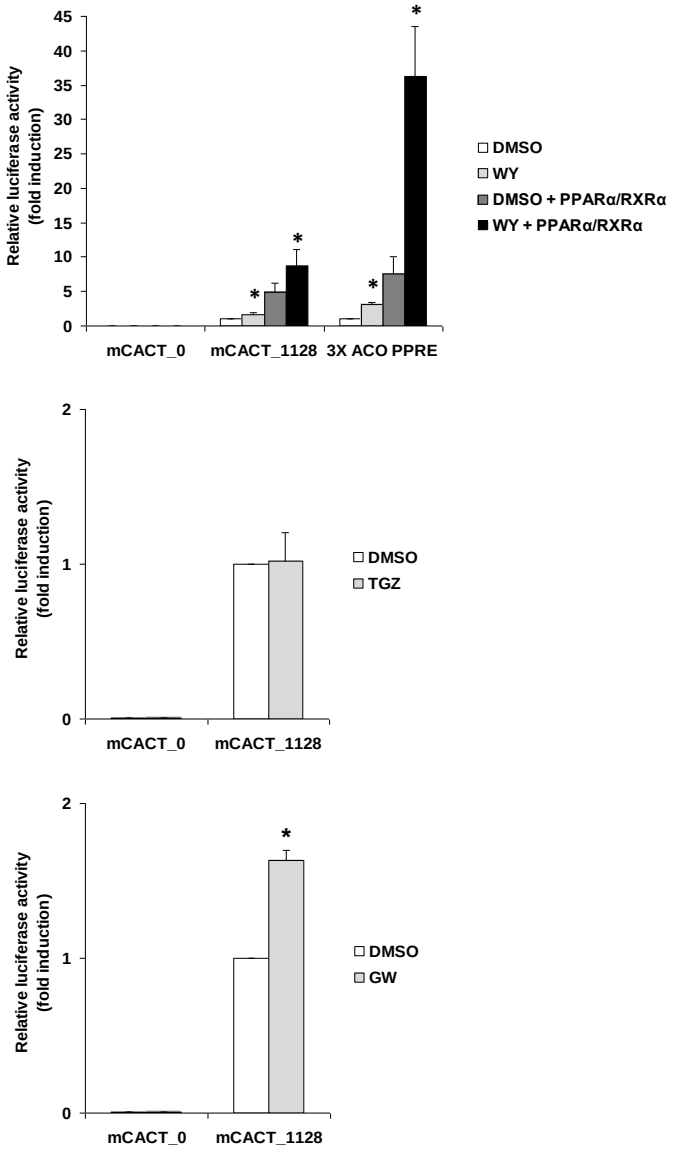

Fig. 6. Effect of PPARα agonist WY-14,643, PPARγ agonist TGZ, and PPARδ agonist GW0742 on the promoter activity of mouse carnitine-acylcarnitine translocase (CACT) in HepG2 cells. HepG2 cells were transiently transfected with either pGL4.10-mCACT_1128 construct containing four PPRE of mouse CACT promoter, negative control vector pGL4.10-mCACT_0 or positive control vector 3X 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 ($P < 0.05$).

3.10 Identification of functional PPREs 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-mCACT_803), two putative PPREs (construct pGL4.10-mCACT_645), and one putative PPRE (construct pGL4.10-mCACT_421 and construct pGL4.10-mCACT_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-mCACT_1128mut, pGL4.10-mCACT_803mut, pGL4.10-mCACT_421mut, pGL4.10-mCACT_170), each of which contained a targeted mutation within PPRE at position +45 to +57. As a consequence, induction of 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 whether 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 interfere with 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**).

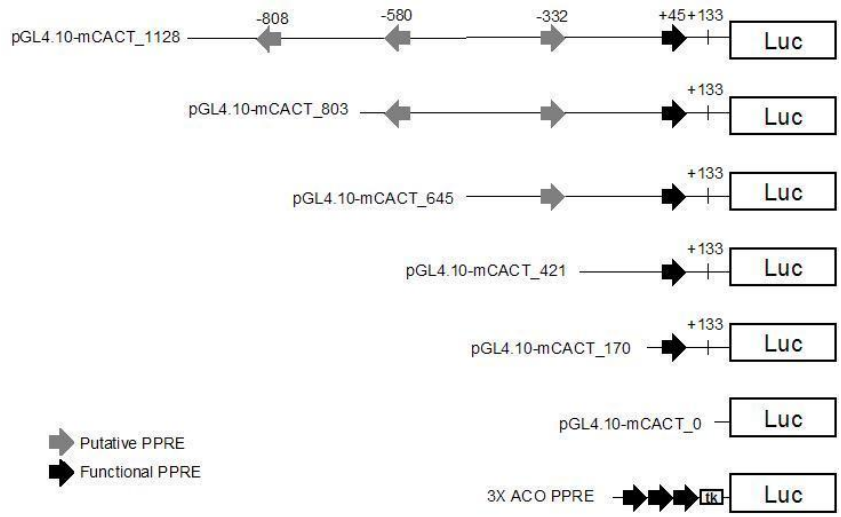

Fig. 7A. Identification of functional PPRE in mouse CACT promoter: Mouse CACT promoter constructs used. Numbers of the constructs (1128, 803, 645, 421, 170, 0) denote the 5´-deletion end point of the constructs.

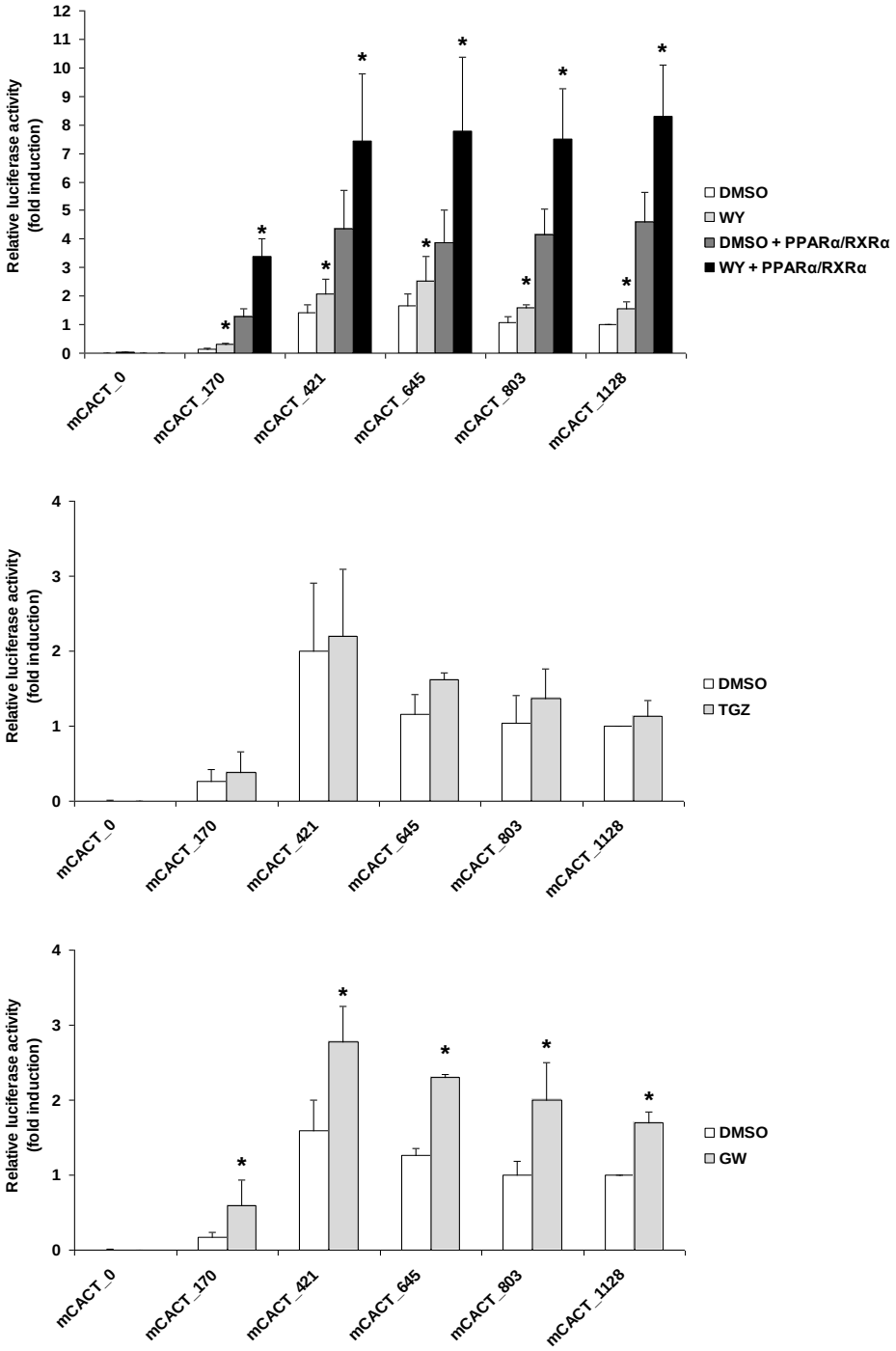

Fig. 7B. Identification of functional PPRE in mouse CACT promoter: Effect of PPARα agonist WY-14,643, PPARγ agonist TGZ, and PPARδ agonist GW0742 on the promoter activity of mouse carnitine-acylcarnitine translocase (CACT) in HepG2 cells. HepG2 cells were transiently transfected with either pGL4.10-mCACT 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 ($P < 0.05$).

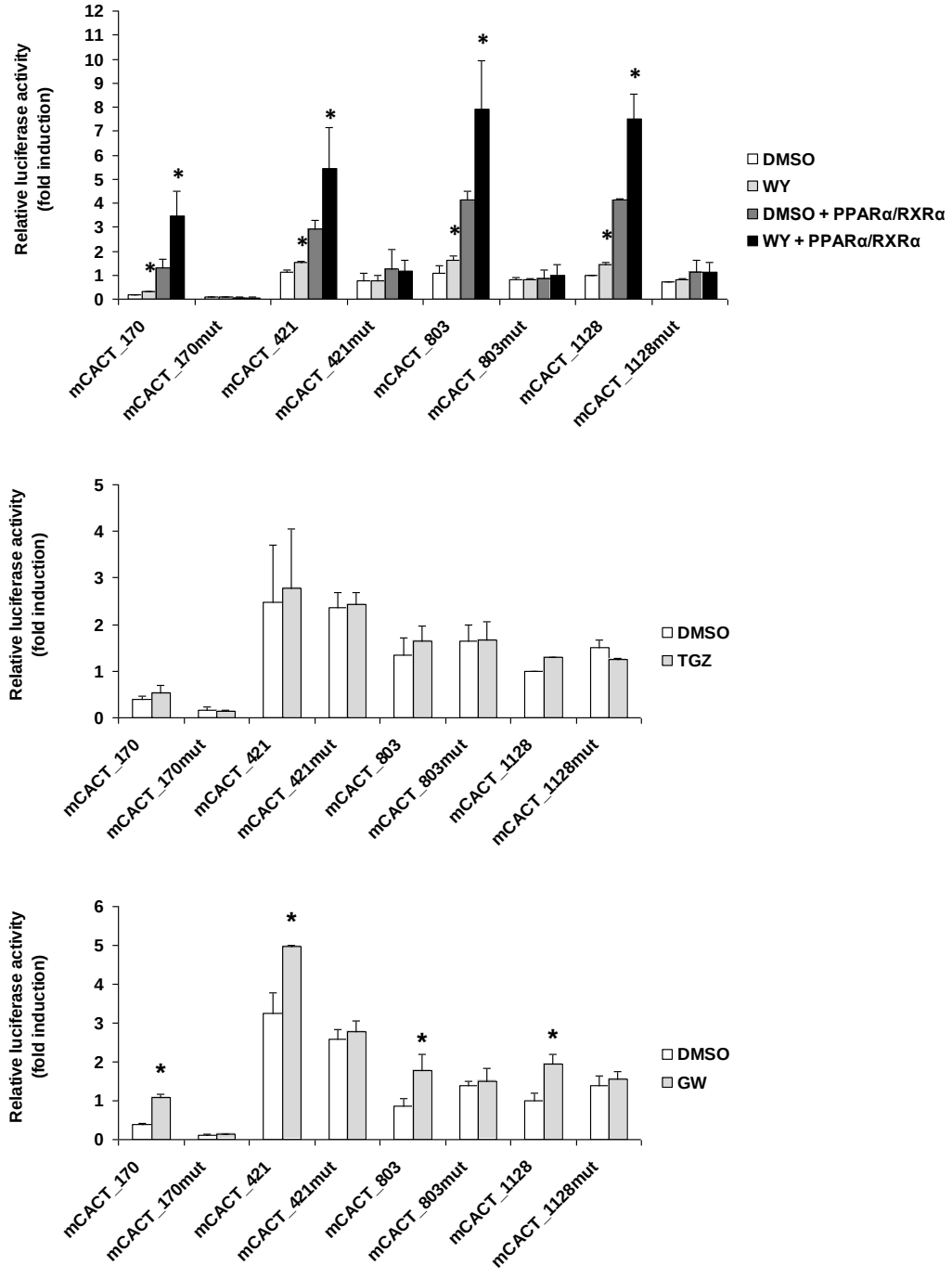

Fig. 7C. Identification of functional PPRE in mouse CACT promoter: Effect of PPARα agonist WY-14,643, PPARγ agonist TGZ, and PPARδ agonist GW0742 on the promoter activity of mouse carnitine-acylcarnitine translocase (CACT) in HepG2 cells. HepG2 cells were transiently transfected with either wild-type 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 ($P < 0.05$).

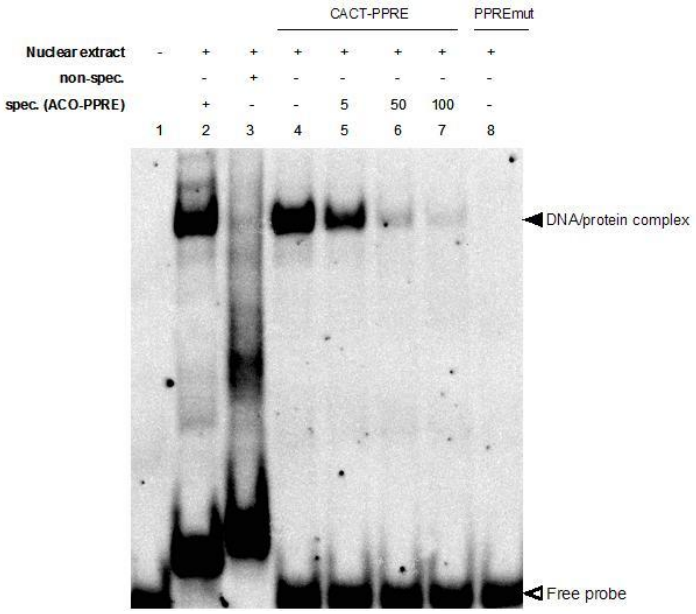

Fig. 7D. Identification of functional PPRE in mouse CACT promoter: Binding of the PPARα/RXRα heterodimer to the putative CACT PPRE (+45 to +57) as determined by gel shift assay. Aliquots of Fao nuclear extracts were incubated with DIG-labelled oligonucleotides corresponding to either the PPRE at position +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) polyacrylamid 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.

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Consensus PPRE                  AACTAGGTCAAAGGTCA
Human           GCCCCGCCCACCCAAAAGTGGGTGAAAGGTCGGCGGCGCCGGCACTGCAGC
Mouse           GCCCCGCCCACCTGAATGTAGGTGAAAGGTCGGCGGAGCCGCTCGGGCAGC
Rat             GCCCCGCCCACCTTAATGTAGGTGAAAGGTCGGTGTAGCCGCTTGGACAGC
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Fig. 7E. Identification of functional PPRE in mouse CACT promoter: 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.

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 hours 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 PPARs 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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Short paper

Feeding oxidized fat during pregnancy up-regulates expression of PPARα-responsive genes in the liver of rat fetuses

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Abstract

Background: Feeding oxidized fats causes activation of peroxisome proliferator-activated receptor α (PPARα) in the liver of rats. However, whether feeding oxidized fat during pregnancy also results in activation of PPARα in fetal liver is unknown. Thus, this study aimed to explore whether feeding oxidized fat during pregnancy causes a PPARα response in fetal liver. Two experiments with pregnant rats which were administered three different diets (control; oxidized fat; clofibrate as positive control) in a controlled feeding regimen during either late pregnancy (first experiment) or whole pregnancy (second experiment) were performed.

Results: In both experiments pregnant rats treated with oxidized fat or clofibrate had higher relative mRNA concentrations of the PPARα-responsive genes acyl-CoA oxidase (ACO), cytochrome P_{450} 4A1 (CYP4A1), L-type carnitin-palmitoyl transferase I (L-CPT I), medium-chain acyl-CoA dehydrogenase (MCAD), and long-chain acyl-CoA dehydrogenase (LCAD) in the liver than control rats ($P < 0.05$). In addition, in both experiments fetuses of the oxidized fat group and the clofibrate group also had markedly higher relative mRNA concentrations of ACO, CYP4A1, CPT I, MCAD, and LCAD in the liver than those of the control group ($P < 0.05$), whereas the relative mRNA concentrations of PPARα, SREBP-1c, and FAS did not differ between treatment groups. In the second experiment treatment with oxidized fat also reduced triacylglycerol concentrations in the livers of pregnant rats and fetuses ($P < 0.05$).

Conclusion: The present study demonstrates for the first time that components of oxidized fat with PPARα activating potential are able to induce a PPARα response in the liver of fetuses. Moreover, the present study shows that feeding oxidized fat during whole pregnancy, but not during late pregnancy, lowers triacylglycerol concentrations in fetal livers.

Background

In recent years the contribution of dietary oxidized fats to total energy intake has markedly increased in industrialized countries, mainly, due to the increasing consumption of fast food which contain significant amounts of these heated and processed fats. We and others have shown that

oxidized fats are strong activators of peroxisome proliferator-activated receptor α (PPARα) [1-3], a ligand-activated transcription factor that belongs to the family of nuclear receptors. PPARα is centrally involved in the regulation of lipid homeostasis in the liver and is essential for normal liver function [4]. Upon activation of PPARα by a ligand, which includes the fibrate class of hypolipidemic drugs such as clofibrate or fenofibrate, fatty acids, and eicosanoids, the transcription of genes containing a PPAR response element (PPRE) in its promoter region is induced. Typical PPARα-responsive genes in the liver, which are also up-regulated by oxidized fats and its components such as oxidized fatty acids (e.g. hydroperoxy-fatty acids, cyclic fatty acids) [1-3,5,6], include a wide array of genes that are involved in peroxisomal and mitochondrial fatty acid β-oxidation such as L-type carnitin-palmitoyl transferase I (L-CPT I), acyl-CoA oxidase (ACO), cytochrome P_{450} 4A1 (CYP4A1), medium-chain acyl-CoA dehydrogenase (MCAD), and long-chain acyl-CoA dehydrogenase (LCAD) [7,8]. PPARα activation by oxidized fat was also shown to cause liver enlargement due to peroxisome proliferation and reduce triacylglycerol concentrations in liver, plasma, and very low-density lipoproteins in rats [3,9,10]. In part, the triacylglycerol-lowering effect of oxidized fat in the rat is presumably also due to the observed reduction in mRNA concentrations and activities of lipogenic enzymes such as fatty acid synthase (FAS) [9].

During pregnancy treatment of rats with the pharmacological PPARα activator clofibrate has been shown to cause proliferation of peroxisomes [11,12], induction of peroxisomal enzyme activities [13], and induction of CYP4A1 in fetal liver [14] indicating that clofibrate is capable of activating PPARα transplacentally. In addition, pathological changes in newborn rats born to mothers treated with clofibrate during pregnancy [15], and an impaired fetal growth of fenofibrate-treated pregnant rats have been reported [16]. Moreover, in rat and mouse liver epithelial cells treatment with the PPARα agonist WY-14,643 caused up-regulation of proto-oncogenes [17-19], which has been attributed to the hepatocarcinogenic effect of peroxisome proliferators in rodents [18]. However, whether feeding oxidized fat to pregnant rats also results in activation of PPARα and up-regulation of PPARα target genes in fetal livers is unknown from the literature. Moreover, it is unknown whether components of oxidized fats such as oxidized fatty acids are able to substantially pass the placenta and enter the fetus, because it has been shown that the transplacental transport of fatty acids from the maternal diet is highly selective for individual fatty acids, e.g. long-chain polyunsaturated fatty acids such as docosahexaenoic acid and arachidonic acid are preferentially transported through the placenta at the expense of other less important fatty acids [20-22].

Therefore, the present study aimed to explore whether feeding oxidized fat during pregnancy causes a PPARα response in fetal liver as estimated by the up-regulation of typical PPARα-responsive genes such as ACO, L-CPT I, CYP4A1, MCAD, and LCAD and whether the induction of fatty acid catabolism might also affect fetal hepatic triacylglycerol concentrations. We also analyzed the mRNA abundance of the lipogenic transcription factor sterol regulatory-element binding protein (SREBP)-1c and its target gene FAS, because in previous studies administration of oxidized fats has also been demonstrated to reduce mRNA expression of lipogenic enzymes [9]. To address possible adverse effects of treatment with oxidized fat, we also determined the mRNA abundance of the proto-oncogenes c-myc, c-jun, and c-fos.

Since the duration of administration of oxidized fat during pregnancy might also influence the effect on lipid metabolism in the fetal liver, we performed two experiments which varied in the duration of administration of the oxidized fat. In the first experiment we investigated the effect of short-term administration (last 5 d of pregnancy) of oxidized fat on the PPARα response in maternal and fetal liver, whereas in the second experiment the effect of long-term administration (whole pregnancy) was studied.

Results

Fatty acid composition and concentrations of lipid peroxidation products in the experimental fats

The oxidized fat in the short-term experiment had lower proportions of polyunsaturated fatty acids (C18:2) but higher proportions of saturated (C16:0, C18:0) and monounsaturated (C18:1) fatty acids compared to the control fat due to the heat treatment of sunflower oil during preparation of the oxidized fat (Table 1). In the long-term experiment the proportions of C18:2 and C18:1 in the oxidized fat were similar to those in the control fat due to adjustment of fatty acid composition of the control fat, whereas the proportions of saturated fatty acids were lower in the oxidized fat compared to the control fat (Table 2). In both experiments the oxidized fat had higher concentrations of peroxides, TBARS, and conjugated dienes, and a higher acid value than the fat of the control group. The concentrations of lipid peroxidation products (peroxide value and conjugated dienes) in the control fat were higher in the long-term experiment than in the short-term experiment. This is probably due to the fact that fats in the long-term experiment were included into a semi-synthetic diet which contained pro-oxidant minerals such as iron or copper whereas fats in the short-term experiment were administered the rats directly by gastric tube.

Table 1: Characteristics of the experimental fats of the short-term experiment

Treatment group	Control	Clofibrate	Oxidized fat[1]
Fat	SFO[2]		oxidized SFO[2]
Major fatty acids, g/100 g total fatty acids			
C16:0	6.1		9.1
C18:0	3.4		5.3
C18:1 (n-9)	32.6		38.3
C18:2 (n-6)	56.6		44.7
Peroxidation products			
Peroxide value, mEq O_2/kg	3.0		379
Acid value, g KOH/kg	0.4		5.8
Conjugated dienes, mmol/kg	< 0.1		274
TBARS[2], mmol/kg	1.1		13.1

[1]Prepared by heating at a temperature of 60°C for 25 d.
[2]Abbreviations: SFO, sunflower oil; TBARS, thiobarbituric acid-reactive substances.

Final body weights and relative liver weights of pregnant rats

Body weight development was not affected by dietary treatment in both experiments due to the controlled feeding regimen applied. In the short-term experiment, final body weights of pregnant rats at d 21 of pregnancy did not differ between the three treatment groups (control, 300 ± 28 g; oxidized fat, 299 ± 24 g; clofibrate, 307 ± 17 g, mean ± SD). Pregnant rats treated with clofibrate or oxidized fat had higher relative liver weights than control rats (P < 0.05; control, 3.6 ± 0.2 g/100 g body weight; oxidized fat, 4.4 ± 0.3 g/100 g body weight; clofibrate, 4.9 ± 0.2 g/100 g body weight).

In the long-term experiment, final body weights of pregnant rats at d 21 of pregnancy were also not different between treatment groups (control, 342 ± 35 g; oxidized fat, 331 ± 40 g; clofibrate, 328 ± 18 g). Relative liver weights of pregnant rats treated with clofibrate or oxidized fat were also higher than those of control rats (P < 0.05; control, 3.4 ± 0.2 g/100 g body weight; oxidized fat, 4.4 ± 0.5 g/100 g body weight; clofibrate, 4.5 ± 0.3 g/100 g body weight).

Relative mRNA concentrations of PPARα and PPARα-responsive genes in livers of pregnant rats and fetuses

In the short-term experiment, relative mRNA concentrations of PPARα in the liver of pregnant rats and fetuses were not different between treatment groups (pregnant rats: 1.00 ± 0.49, control group; 1.02 ± 0.48, oxidized fat group; 1.26 ± 0.22, clofibrate group; P = 0.439; fetuses: 1.00 ± 0.29, control group; 1.29 ± 0.50, oxidized fat group; 1.15 ± 0.74, clofibrate group; P = 0.653). Pregnant rats treated with oxidized fat had 2.4-, 3.0-, 2.5- and 2.1-fold higher relative mRNA concentrations of ACO, CYP4A1, MCAD, and LCAD, respectively, in the liver than control rats (P < 0.05; Fig. 1). The relative mRNA concentration of L-CPT I did not differ between both groups of rats. Treatment of pregnant rats with clofibrate resulted in 4.8-, 11-, 1.6-, 2.5- and 2.4-fold higher relative mRNA concentrations of ACO, CYP4A1, L-CPT I, MCAD, and LCAD, respectively, in the liver compared to control treatment (P < 0.05). Fetuses of the oxidized fat group had 6.3-

Table 2: Characteristics of the experimental fats of the long-term experiment

Treatment group	Control	Clofibrate	Oxidized fat
Fat	SFO[2] : lard (54:46)		oxidized SFO[2]
Major fatty acids, g/100 g total fatty acids			
C16:0	14.9		10.2
C18:0	9.5		6.2
C18:1 (n-9)	28.9		34.4
C18:2 (n-6)	42.0		44.2
Peroxidation products			
Peroxide value, mEq O_2/kg	6.6		230
Conjugated dienes, mmol/kg	12.3		139
TBARS[2], mmol/kg	1.0		19

[1]Prepared by heating at a temperature of 60°C for 25 d.
[2]Abbreviations: SFO, sunflower oil; TBARS, thiobarbituric acid-reactive substances.

, 9.0-, 6.4-, 1.5- and 2.1-fold higher relative mRNA concentrations of ACO, CYP4A1, L-CPT I, MCAD, and LCAD respectively, in the liver than those of the control group ($P < 0.05$), whereas fetuses of the clofibrate group had 20-, 51-, 12-, 2.8- and 3.0-fold higher relative mRNA concentration of ACO, CYP4A1, L-CPT I, MCAD, and LCAD, respectively in the liver than those of the control group ($P < 0.05$).

In the long-term experiment, relative mRNA concentrations of PPARα in the liver of pregnant rats and fetuses also did not differ between treatment groups (pregnant rats: 1.00 ± 0.49, control group; 1.56 ± 0.99, oxidized fat group; 0.63 ± 0.29, clofibrate group; $P = 0.053$; fetuses: 1.00 ± 0.70, control group; 0.72 ± 0.20, oxidized fat group; 0.75 ± 0.31, clofibrate group; $P = 0.201$). Treatment of pregnant rats with oxidized fat resulted in 4.0-, 4.4-, 2.5-, 2.8- and 2.0-fold higher relative mRNA concentrations of ACO, CYP4A1, L-CPT I, MCAD, and LCAD, respectively, in the liver compared to control treatment ($P < 0.05$; Fig. 1). Pregnant rats treated with clofibrate had 4.8-, 8.3-, 2.0- and 1.9-fold higher relative mRNA concentrations of ACO, CYP4A1, MCAD, and LCAD, respectively, in the liver than control rats ($P < 0.05$), whereas the relative mRNA concentration of L-CPT I did not differ between both treatment groups. Fetuses of the oxidized fat group had 2.2-, 6.0-, 3.2- and 2.0-fold higher relative mRNA concentrations of ACO, CYP4A1, MCAD, and LCAD, respectively, in the liver than those of the control group ($P < 0.05$), whereas the relative mRNA concentration of L-CPT I did not differ between both treatment groups. Fetuses of the clofibrate group had 3.5-, 28-, 18- and 3.9-fold higher relative mRNA concentration of ACO, CYP4A1, L-CPT I, and LCAD, respectively, in the liver than those of the control group ($P < 0.05$), whereas the relative mRNA concentration of MCAD did not differ between both treatment groups.

Relative mRNA concentrations of SREBP-1c and FAS in livers of pregnant rats and fetuses

In the short-term experiment, treatment of pregnant rats with oxidized fat resulted in lower relative mRNA concentrations of FAS in the liver relative to control treatment ($P < 0.05$), whereas the relative mRNA concentration of SREBP-1c did not differ between these two groups (Fig. 2). Relative mRNA concentrations of SREBP-1c and FAS in the liver of pregnant rats treated with clofibrate did not differ from those of control rats. However, pregnant rats treated with clofibrate had higher relative mRNA concentrations of SREBP-1c in the liver than those fed oxidized fat ($P < 0.05$). In fetal livers relative mRNA concentrations of SREBP-1c and FAS did not differ between treatment groups.

In the long-term experiment, relative mRNA concentrations of SREBP-1c and FAS in the liver of pregnant rats and fetuses did not differ between the three treatment groups (Fig. 2).

Concentrations of triacylglycerols in livers of pregnant rats and fetuses

In the short-term experiment, concentrations of triacylglycerols in the liver of pregnant rats did not differ between the three treatment groups (Fig. 3). Fetuses of the clofibrate group had lower concentrations of triacylglycerols in the liver than those of the control and the oxidized fat group ($P < 0.05$); oxidized fat had no effect on the triaclyglycerol concentration in fetal livers compared to control.

In the long-term experiment, treatment of pregnant rats with oxidized fat or clofibrate resulted in lower concentrations of triacylglycerols in the liver compared to control treatment ($P < 0.05$). Fetuses of the oxidized fat group and the clofibrate group also had lower concentrations of triacylglycerols in the liver than those of the control group ($P < 0.05$).

Relative mRNA concentrations of c-myc, c-jun, and c-fos in livers of pregnant rats and fetuses

In the short-term experiment, relative mRNA concentrations of c-myc, c-jun, and c-fos in the liver of pregnant rats and fetuses did not differ between the three treatment groups (Fig. 4).

In the long-term experiment, treatment of pregnant rats with oxidized fat resulted in higher relative mRNA concentrations of c-myc, c-jun, and c-fos relative to control treatment ($P < 0.05$). In fetal livers relative mRNA concentrations of c-myc, c-jun, and c-fos did not differ between treatment groups.

Discussion

The present study demonstrates for the first time that components of oxidized fat with PPARα activating potential are able to induce a PPARα response in the liver of fetuses. Moreover, the present study shows that feeding oxidized fat during whole pregnancy, but not during late pregnancy, lowers triacylglycerol concentrations in fetal livers. Hydroxy- and hydroperoxy-fatty acids such as hydroxyoctadecadienoic acid (HODE) and hydroperoxyoctadecadienoic acid (HPODE) occurring in oxidized fats are very potent PPARα agonists [6,23,24]. These oxidized fatty acids are produced during the early stage of lipid peroxidation, and, due to their low thermodynamic stability, easily decompose at high temperatures. Thus, fats treated at low temperatures have markedly higher concentrations of these primary lipid peroxidation products than fats treated at high temperature [3]. Therefore, in order to provoke a significant PPARα activating effect of the oxidized

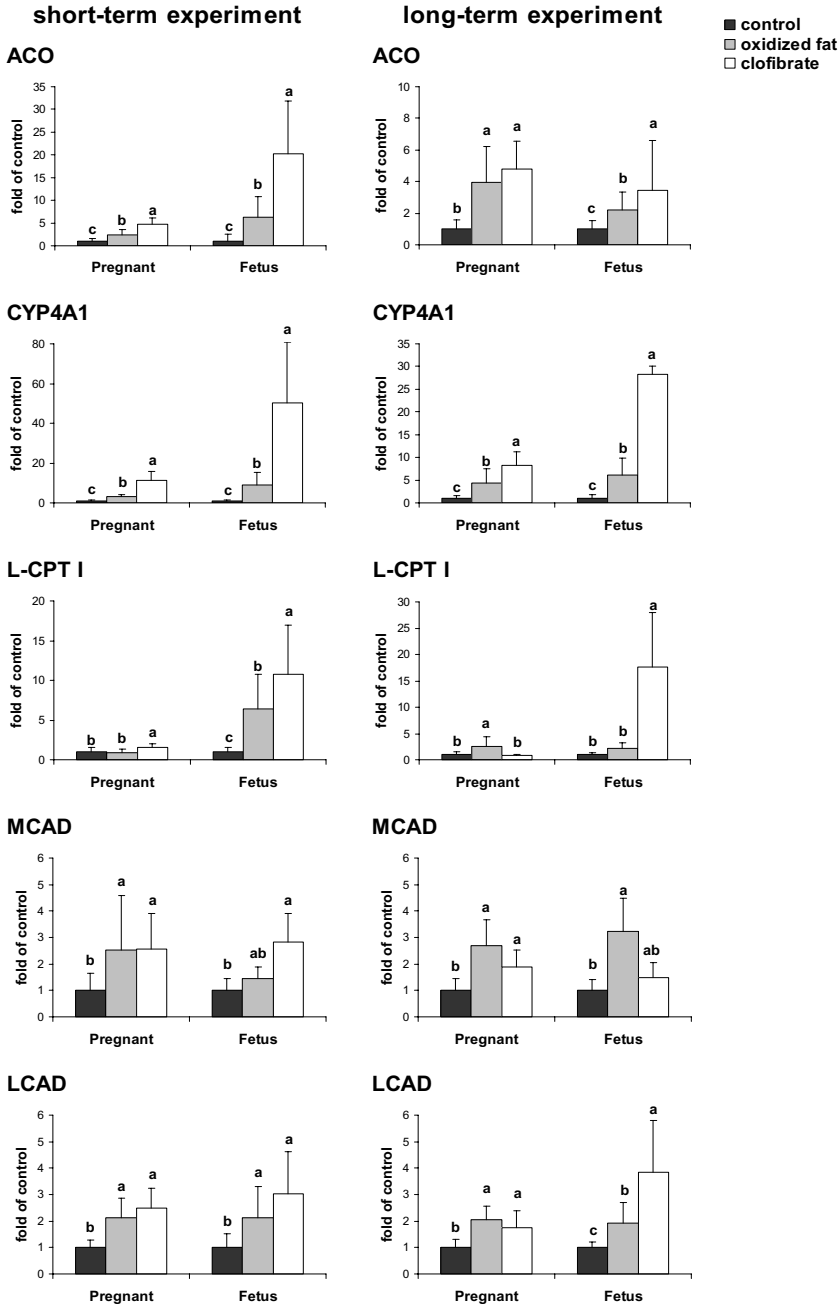

Figure 1

Effects of treatment on relative mRNA concentrations of PPARα-responsive genes in livers of pregnant rats and fetuses. Effect of short-term (d 16 – d 21 of pregnancy, left) and long-term (d 1 – d 21 of pregnancy, right) administration of three different diets (control, oxidized fat, clofibrate) during pregnancy on relative mRNA concentrations of ACO, CYP4A1, CPT I, MCAD, and LCAD in the liver of pregnant rats and fetuses at d 21 of pregnancy. Left, Bars represent mean ± SD (n = 9/group). Right, Bars represent mean ± SD (n = 12/group). Bars marked without a common superscript letter differ ($P < 0.05$). Results from one-way ANOVA (*P*-values): short-term experiment: ACO, $P = 0.0001$ (pregnant), $P = 0.001$ (fetus); CYP4A1, $P = 0.0001$ (pregnant), $P = 0.002$ (fetus); L-CPT I, $P = 0.013$ (pregnant), $P = 0.012$ (fetus); MCAD, $P = 0.042$ (pregnant), $P = 0.001$ (fetus); LCAD, $P = 0.001$ (pregnant), $P = 0.03$ (fetus); long-term experiment: ACO, $P = 0.0001$ (pregnant), $P = 0.015$ (fetus); CYP4A1, $P = 0.0001$ (pregnant), $P = 0.001$ (fetus); L-CPT I, $P = 0.016$ (pregnant), $P = 0.011$ (fetus); MCAD, $P = 0.001$ (pregnant), $P = 0.019$ (fetus); LCAD, $P = 0.001$ (pregnant), $P = 0.001$ (fetus).

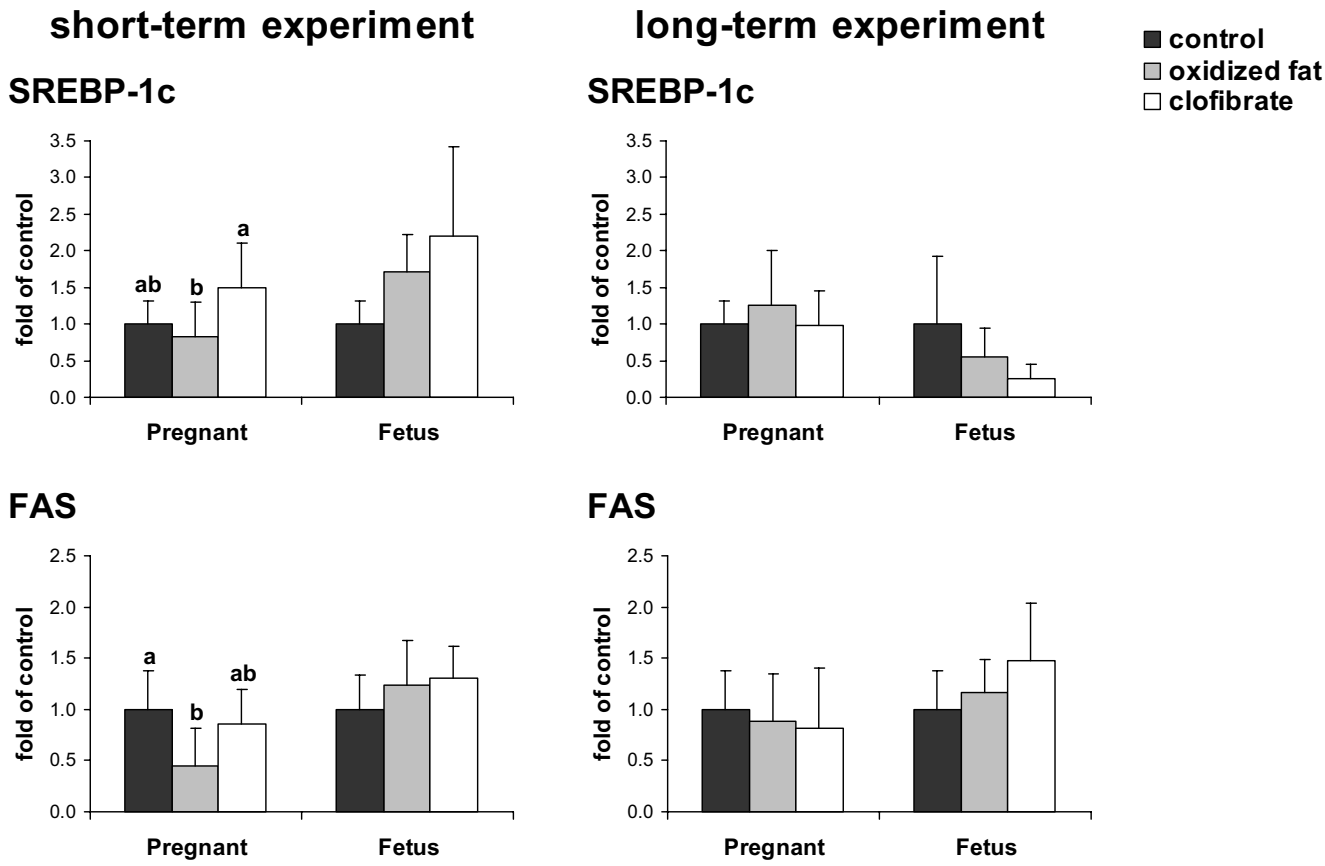

Figure 2
Effects of treatment on relative mRNA concentrations of SREBP-1c and FAS in livers of pregnant rats and fetuses. Effect of short-term (d 16 – d 21 of pregnancy, left) and long-term (d 1 – d 21 of pregnancy, right) administration of three different diets (control, oxidized fat, clofibrate) during pregnancy on relative mRNA concentrations of SREBP-1c and FAS in the liver of pregnant rats and fetuses at d 21 of pregnancy. Left, Bars represent mean ± SD (n = 9/group). Right, Bars represent mean ± SD (n = 12/group). Bars marked without a common superscript letter differ ($P < 0.05$). Results from one-way ANOVA (*P*-values): short-term experiment: SREBP-1c, $P = 0.041$ (pregnant), $P = 0.058$ (fetus); FAS, $P = 0.026$ (pregnant), $P = 0.425$ (fetus); long-term experiment: SREBP-1c, $P = 0.526$ (pregnant), $P = 0.189$ (fetus); FAS, $P = 0.716$ (pregnant), $P = 0.334$ (fetus).

fat, we decided to use a fat treated at a relatively low temperature for a long period. Although we did not determine the concentrations of oxidized fatty acids such as 13-HODE or 13-HPODE in the oxidized fat, the high peroxide value and the high concentration of conjugated dienes indicate that the oxidized fats used in both experiments presumably had high concentrations of hydroxy- and hydroperoxy-fatty acids which may be particularly responsible for the PPARα activating effect of oxidized fats. Nonetheless, other components of heated fats such as cyclic fatty acid monomers which also show a strong PPARα response [5] might be also causative for PPARα activation.

The lower concentrations of primary lipid peroxidation products (peroxides, conjugated dienes) in the oxidized fat used in the long-term experiment compared to that used in the short-term experiment are probably explained by the fact that in the long-term experiment the lipid peroxidation products in the oxidized fat were determined after inclusion into the diet. Primary lipid peroxidation products easily decompose and are partially degraded in the presence of other diet components such as metal ions (e.g. iron, copper) acting as catalysts.

Since unspecific effects might have been caused by a different fatty acid composition of the experimental fats (the heating process caused a loss of polyunsaturated fatty acids), we aimed at equalizing in particular the concentration of the polyunsaturated fatty acid C18:2 (n-6) in the fresh fat and the oxidized fat in the second experiment which lasted during the whole pregnancy. Although the

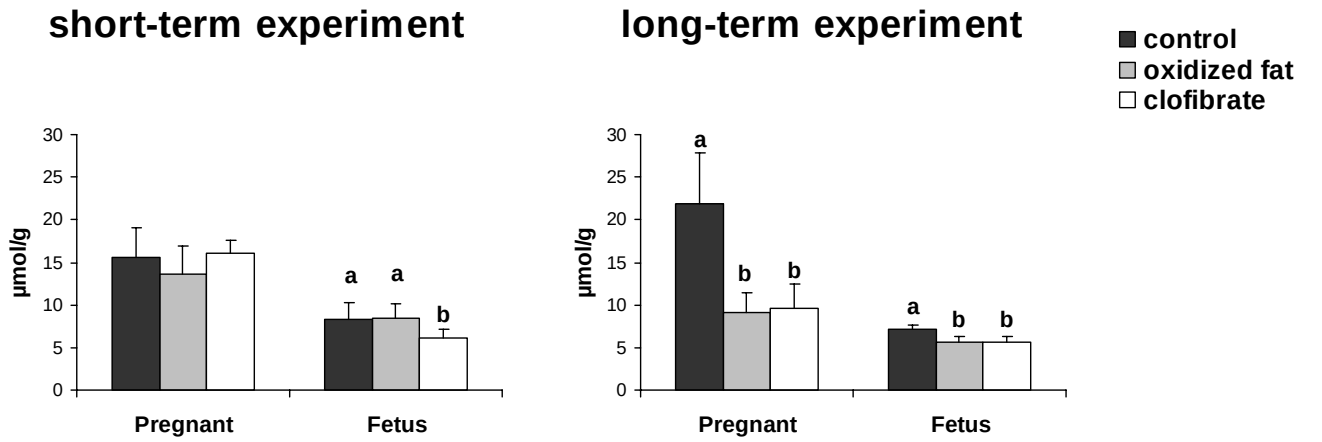

Figure 3

Effects of treatment on triacylglycerol concentrations in livers of pregnant rats and fetuses. Effect of short-term (d 16 – d 21 of pregnancy, left) and long-term (d 1 – d 21 of pregnancy, right) administration of three different diets (control, oxidized fat, clofibrate) during pregnancy on triacylglycerol concentrations in the liver of pregnant rats and fetuses at d 21 of pregnancy. Left, Bars represent mean ± SD (n = 9/group). Right, Bars represent mean ± SD (n = 12/group). Bars marked without a common superscript letter differ ($P < 0.05$). Results from one-way ANOVA (*P*-values): short-term experiment: $P = 0.239$ (pregnant), $P = 0.025$ (fetus); long-term experiment: $P = 0.0001$ (pregnant), $P = 0.045$ (fetus).

concentrations of the long-chain saturated fatty acids C16:0 and C18:0 were consequently decreased in the oxidized fat compared to the fresh fat, we think that these differences are not responsible for the differences in the PPARα response observed between the experimental groups, because saturated long chain-fatty acids were shown to bind and activate PPARα only very weakly compared to polyunsaturated fatty acids such as C18:2 (n-6) [25]. Therefore, we assume that the PPARα response to an oxidized fat might depend on the balance between unoxidized fatty acids with low PPARα transactivation activity and oxidized fatty acids with high PPARα transactivation activity because both types of fatty acids compete for the PPARα-ligand binding site.

A further consequence of the use of lard in the control fat was that the cholesterol content of the control and the clofibrate diet slightly differed from that of the oxidized fat diet. However, based on an average cholesterol concentration of about 80 mg per 100 g lard the control and the clofibrate diet contained less than 0.004% cholesterol. Therefore, we assume that the slight difference in the cholesterol concentration between the experimental diets is negligible, especially since no relation between PPARα activation and dietary cholesterol is known from the literature.

The present study clearly shows that feeding oxidized fat during pregnancy, similar to clofibrate, which was used as a positive control, causes not only a PPARα response in the liver of pregnant animals as shown by liver enlargement and up-regulation of PPARα-responsive genes but also in the liver of the fetuses. Although the induction of the PPARα response by oxidized fat in the fetal liver was not as pronounced as observed with clofibrate, the oxidized fat also caused a strong up-regulation of PPARα-target genes of up to 9-fold in the livers of fetuses which was even more pronounced than the effect of oxidized fat in the livers of pregnant rats. Therefore, these findings suggest that not only pharmacological PPARα activators but also components of oxidized fat are able to sufficiently pass the placenta and activate PPARα in the fetal liver. This finding is novel since the placental transfer of these components of oxidized fat with PPARα activating potential from the maternal diet to the fetus is unknown. Indeed, the transplacental transport of fatty acids is highly selective for individual fatty acids [20-22], but no data are available from the literature with respect to the placental passage of oxidized fatty acids which are presumably decisive for the PPARα activating effect of oxidized fat. Thus, the present results indicate that these critical components of oxidized fats are also sufficiently transported across the placenta. Moreover, the observation from the short-term experiment that up-regulation of mRNA expression of ACO, CYP4A1 and L-CPT I by oxidized fat in the fetal liver was even more pronounced than in the liver of pregnant rats indicates that components of oxidized fat responsible for PPARα activation are presumably transported through the placenta with high preference.

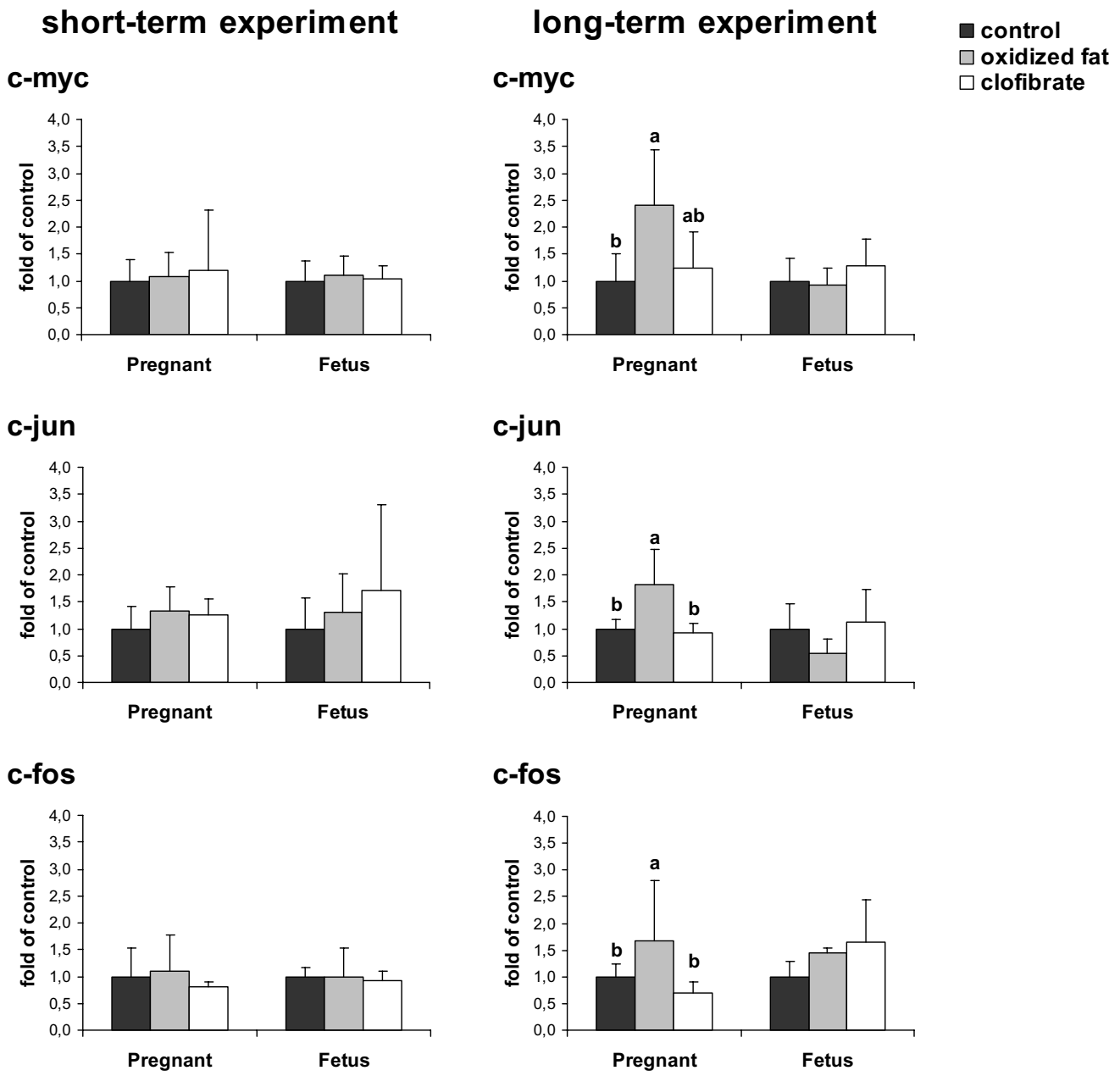

Figure 4

Effects of treatment on relative mRNA concentrations of proto-oncogenes in livers of pregnant rats and fetuses. Effect of short-term (d 16 – d 21 of pregnancy, left) and long-term (d 1 – d 21 of pregnancy, right) administration of three different diets (control, oxidized fat, clofibrate) during pregnancy on relative mRNA concentrations of c-myc, c-jun, and c-fos in the liver of pregnant rats and fetuses at d 21 of pregnancy. Left, Bars represent mean ± SD (n = 9/group). Right, Bars represent mean ± SD (n = 12/group). Bars marked without a common superscript letter differ ($P < 0.05$). Results from one-way ANOVA (*P*-values): short-term experiment: c-myc, $P = 0.880$ (pregnant), $P = 0.807$ (fetus); c-jun, $P = 0.362$ (pregnant), $P = 0.552$ (fetus); c-fos, $P = 0.669$ (pregnant), $P = 0.966$ (fetus); long-term experiment: c-myc, $P = 0.001$ (pregnant), $P = 0.420$ (fetus); c-jun, $P = 0.0001$ (pregnant), $P = 0.086$ (fetus); c-fos, $P = 0.048$ (pregnant), $P = 0.323$ (fetus).

Since activation of hepatic PPARα by clofibrate or oxidized fat has been demonstrated to enhance the fatty acid oxidation capacity in the liver and to lower hepatic triacylglycerol concentrations in non-pregnant rats [1,3,9], we also studied the effect of oxidized fat on the concentrations of triacylglycerols in the fetal liver. Indeed, we could demonstrate that feeding the oxidized fat during pregnancy also reduced the concentrations of triacylglycerols in the fetal liver indicating that the transplacental induction of PPARα responsive genes enhanced the peroxisomal and mitochondrial fatty acid oxidation capacity of the fetal liver. However, this effect has only been observed when the oxidized fat was fed during the whole pregnancy, but not when the oxidized fat was fed for the last 5 d of pregnancy only, although the PPARα responsive genes were markedly up-regulated in fetal livers of pregnant rats treated during either whole or late pregnancy. This suggests that short-term administration of oxidized fat to pregnant rats causes significant alterations on the gene expression level, which have no implications on the phenotypic level, e.g. triaclyglycerol concentrations. In contrast, short-term administration of clofibrate during pregnancy even revealed alterations on the phenotypic level as evidenced by reduced triacylglycerol concentrations in fetal livers. The latter might be attributed to the fact that clofibrate caused a more pronounced activation of PPARα in fetal livers due to a higher affinity for PPARα compared to oxidized fat leading to a marked induction of fatty acid oxidation and a significant lowering of triacylglycerol concentrations in fetal livers even after short-term exposure to clofibrate.

Interestingly, no effect of oxidized fat and even clofibrate on hepatic triacylglycerol concentrations following short-term treatment could be observed in pregnant rats, although clofibrate reduced triacylglycerol concentrations in fetal livers following short-term treatment. The failure of clofibrate or oxidized fat to reduce hepatic triacylglycerol concentrations in pregnant rats in the short-term experiment might be attributed to the fact that treatment was only performed during late pregnancy when significant alterations in lipid metabolism [e.g. hypertriacylglycerolemia as a consequence of enhanced adipose tissue lipolytic activity, enhanced liver production of VLDL particles, and decreased extrahepatic lipoprotein lipase activity [26-29]] occur in the pregnant animal. The enhanced arrival of free fatty acids, which also serve as ligands for PPARα, in the liver during late pregnancy could decrease the availability for the stronger PPARα activators clofibrate or components of oxidized fat with PPARα activating potential to their PPARα-ligand binding site. This in turn could reduce the capability of fibrates or oxidized fat to activate PPARα and consequently, its metabolic effects. This might be important since a substantial body of evidence suggests that not all alterations in gene transcription induced by pharmacological PPARα activators are also induced by fatty acids [30], e.g. some genes that contain a PPRE do not respond to fatty acids but to high-affinity PPARα activators [31-33]. Thus, administration of oxidized fat or clofibrate during late pregnancy might provoke differential effects on lipid metabolism than in virgin or early pregnant rats. The observation that treatment of rats with the triacylglycerol-lowering PPARα agonist fenofibrate during late pregnancy even increased triacylglycerol concentrations in plasma, whereas in virgin rats treatment with fenofibrate caused a reduction in plasma triacylglycerol concentrations [16], is probably supportive of this assumption.

Whether the effect observed with oxidized fat or clofibrate might have been also influenced by the metabolic state, e.g. fasting vs. non-fasting, cannot be answered with certainty. In the present study we decided to perform gene expression analysis of PPARα-responsive genes in the liver in the non-fasting state, because lipolysis of stored triacylglycerols in adipose tissue is strongly activated during fasting, resulting in a marked increase in plasma free fatty acid levels. These free fatty acids act as endogenous PPARα ligands and might have competed with the exogenous ligands (e.g. clofibrate, oxidized fatty acids or cyclic fatty acid monomers) for the PPARα-ligand binding site. Therefore, an altered ratio between endogenous free fatty acids and exogenous oxidized fatty acids as a consequence of the fasting state might have provoked a different PPARα response in the liver of pregnant rats and fetuses than observed in the non-fasting state. However, further studies are required to definitely resolve this question.

Only slight effects have been observed in the present study with respect to the lipogenic transcription factor SREBP-1c and its target gene FAS. Namely, oxidized fat caused a slight reduction in the mRNA abundance of FAS in the liver of pregnant rats, which is consistent with recent findings in non-pregnant rats [9]. However, no effect of oxidized fat has been observed on gene expression of lipogenic enzymes in fetal livers indicating that the reduced hepatic triacylglycerol concentrations in fetuses from pregnant rats treated with oxidized fat are probably largely due to an enhanced fatty acid oxidation capacity due to transplacental activation of PPARα. In part, the failure of oxidized fat on FAS gene expression in fetal livers might be explained by the fact that lipogenesis in fetal livers is generally very low and only increased during late pregnancy [34], and, therefore, probably does not respond to variations in the maternal diet.

Since pathological changes in newborn rats born to mothers treated with clofibrate during pregnancy have been reported [15], treatment with fenofibrate has been shown to impair fetal growth [16], and a link between induced

CYP4A1 expression, peroxisome proliferation, and carcinogenesis in rat livers has been described [35], we also addressed possible adverse effects of treatment with oxidized fat on fetal livers. With respect to the hepatocarcinogenic effect of peroxisome proliferators in rodents it has been suggested that enhanced DNA synthesis as a consequence of up-regulation of proto-oncogenes including c-fos, c-jun, and c-myc might be mechanistically involved [17-19]. In the present study an up-regulation of proto-oncogenes in the livers of pregnant rats has been observed in the long-term experiment, but not in the short-term experiment, indicating that short-term administration of oxidized fat has no impact on mRNA expression of proto-oncogenes. However, in fetal livers mRNA expression of proto-oncogenes was not affected by oxidized fat regardless of the duration of oxidized fat administration suggesting that the oxidized fat is uncritical with respect to hepatocarcinogenesis. Unexpectedly, treatment with clofibrate had no effect on proto-oncogene expression in pregnant rats and fetuses either in the short-term and the long-term experiment, although it has been reported that the high-affinity PPARα-ligand WY-14,643 strongly up-regulated various proto-oncogenes in rat and mouse liver epithelial cells [18,19]. This differential action of WY-14,643 and clofibrate on proto-oncogene mRNA expression cannot be explained at the moment and, therefore, requires further research activities.

Although oxidized fat had no effect on proto-oncogene expression in fetal livers in the present study, oxidized fats might be considered critically in view of inducing oxidative stress in different tissues as shown in recent studies [36,37]. In addition, specific components of oxidized fats such as cyclic fatty acid monomers, which are formed in substantial amounts during domestic frying of frozen foods in sunflower oil [38], are probably toxic, e.g. earlier studies reported that mice receiving cyclic fatty acid monomers as well as rat pups from mothers fed cyclic fatty acid monomers had a higher death rate [39-41].

Conclusion

In conclusion, the present study demonstrates for the first time that components of oxidized fat with PPARα activating potential contained in the maternal diet are able to induce a PPARα response in the liver of fetuses as evidenced by an up-regulation of PPARα target genes. In addition, the present study shows that feeding oxidized fat during whole pregnancy, but not during late pregnancy, lowers triacylglycerol concentrations in fetal livers, probably as a consequence of an enhanced peroxisomal and mitochondrial β-oxidation capacity. Although administration of oxidized fat during pregnancy had not impact on fetal proto-oncogene mRNA expression either after short-term or long-term administration, the observed pronounced transplacental PPARα activation by oxidized fat might be considered critically because of other recently reported adverse effects of treatment with PPARα activators during pregnancy [15,16]. Therefore, further research should be encouraged with respect to possible detrimental effects of oxidized fat on fetal development.

Methods

Animals

Two experiments were carried out with female Sprague-Dawley rats obtained from Charles River (Sulzfeld, Germany). At 11 wk of age, the rats were mated by housing one male rat with two female rats. D 1 of pregnancy was assigned upon observation of sperm in the vaginal smears, at which time rats were randomly assigned to the treatment groups. The short-term experiment was performed from d 16 to d 21 of pregnancy and included 27 pregnant rats with an initial body weight (d 16 of pregnancy) of 297 ± 26 (Mean ± SD) g, which were allotted to three groups of nine rats each. The long-term experiment was performed from d 1 to d 21 of pregnancy and included 36 rats with an initial body weight (d 1 of pregnancy) of 238 ± 27 g, which were allotted to three groups of twelve rats each. Pregnant rats were kept individually in Macrolon cages in a room maintained with controlled temperature (23 ± 1°C), humidity (50–60%), and lighting (0600 to 1800 h). All experimental procedures described followed established guidelines for the care and handling of laboratory animals [42] and were approved by the council of Saxony-Anhalt.

Diets

Short-term experiment

In the short-term experiment, rats received 2 mL of different experimental fats by gavage daily at 0800 h, and, additionally, fed a commercial standard rodent diet (Altromin, Lage, Germany). To standardize food intake, the diets were fed daily in controlled amounts of 16 g per d. The first group (control group) received sunflower oil, the second group (oxidized fat group) oxidized fat (see "preparation of the oxidized fat"), and the third group (clofibrate group) sunflower oil containing 75 mg clofibrate (Fluka, Buchs, Switzerland) equivalent to 250 mg clofibrate per kg body weight. The experimental fats were given for 5 d from d 16 of pregnancy to d 21 of pregnancy. The standard diet was completely consumed by all the rats. Thus, all the rats within this experiment consumed identical amounts of the food.

Long-term experiment

In the long-term experiment, semipurified diets, composed according to the recommendations of ASNS for rats during reproduction [43], were used. The diet consisted of (g/kg diet): casein, 200; cornstarch, 390; saccharose, 198; cellulose, 50; fat, 100; mineral mixture, 40; vitamin mix-

ture, 20; DL-methionine, 2. The type of fat was varied according to a one-factorial design. The first group (control group) received a mixture of sunflower oil and lard (54:46, w/w) was used. 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 polyunsaturated fatty acids, therefore, excluding that the treatment effects were caused by a different fatty acid composition of the experimental fats. The second group (oxidized fat group) received oxidized fat (see "preparation of the oxidized fat"). The third group (clofibrate group) received the same fat as in the control group, and clofibrate was added to the diet at a concentration of 5 g/kg. The vitamin E concentration of the diets was 50 mg α-tocopherol equivalents per kg diet. To adjust the vitamin E concentration of the diets, the native concentrations of tocopherols of the fats were analyzed. Based on the native concentrations of the fats, 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). Diets were prepared by mixing the dry components with the fat and water and subsequent freeze drying. The residual water content of the diet was below 5 g/100 g of diet. In preliminary experiments rats fed diets with clofibrate or oxidized fat ad libitum consumed their diets over a longer period than rats fed control diets, which consequently shortened the fasting period and which itself has a pronounced effect on PPARα-response. Therefore, we decided to administer food daily at 0800 h in controlled amounts to standardize intake and to ensure that rats from all treatment groups had a comparable fasting period. The amount of food administered was 20% less than the amounts of identical diets with fresh fats consumed ad libitum by rats in preliminary studies. The amount of food offered daily was increased continuously during the experiment from 14 g to 17 g. In this feeding system, the food offered was completely consumed by all the rats. Thus, all the rats within this experiment consumed identical amounts of food. The experimental diets were fed from d 1 of pregnancy to d 21 of pregnancy.

In both experiments, water was available *ad libitum* from nipple drinkers during the whole experiment.

Preparation of the oxidized fat

The oxidized fat was prepared by heating sunflower oil at a temperature of 60°C for 25 d. Sunflower oil was filled into a glass beaker and placed into a drying oven set at the intended temperature. Throughout the heating process, air was continuously bubbled through the fat at a flow rate of 650 ml/min. This treatment caused a loss of polyunsaturated fatty acids, and a complete loss of tocopherols and raised the concentrations of lipid peroxidation products in the fats. The extent of lipid peroxidation in the oxidized fat was estimated by assaying the peroxide value (POV) [44], acid value [44], concentration of thiobarbituric acid substances (TBARS) [45], and concentration of conjugated dienes [46]. To assess lipid peroxidation products in the oxidized fat after inclusion into the diet (long-term experiment), the fat was extracted from aliquots of the diets with a mixture of hexane and isopropanol (3:2, v/v) and analysed for peroxide value, concentration of conjugated dienes, and TBARS.

Sample collection

4 h after the final portion had been administered the rats were anesthetized with diethyl ether and killed by decapitation. The liver and fetuses were excised immediately, and frozen with liquid nitrogen. In addition, livers from three randomly taken fetuses per pregnant rat were excised, and frozen with liquid nitrogen. All samples were stored at -80°C pending analysis.

Lipid analysis

Lipids of maternal livers and pools of fetal livers were extracted with a mixture of hexane and isopropanol (3:2, v/v) [47]. Total cholesterol and triacylglycerol concentrations were determined using enzymatic reagent kits obtained from Merck Eurolab (Darmstadt, Germany). Prior to enzymatic measurement, lipids of the extract were dissolved in Triton X-100 as described by De Hoff et al. [48]. Fatty acid composition of experimental fats was determined by GC-FID analysis of fatty acid methyl esters (FAME) as described previously in detail [49].

RNA isolation and real-time RT-PCR

For the determination of mRNA expression levels of PPARα, CYP4A1, ACO, L-CPT I, MCAD, LCAD, SREBP-1c, FAS, c-myc, c-jun, and c-fos total RNA were isolated from maternal liver and fetal liver pools 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, respectively. cDNA synthesis and relative quantification of target gene mRNA compared to the housekeeping gene GAPDH mRNA was determined by real-time detection RT-PCR as described previously [50]. Sequences of gene-specific primers obtained from Operon (Köln, Germany) were as follows (NCBI GenBank; forward, reverse): GAPDH (NM_017008; 5'-GCA TGG CCT TCC GTG TTC C-3', 5'-GGG TGG TCC AGG GTT TCT TAC TC-3'), PPARα (NM_013196; 5'-CCC TCT CTC CAG CTT CCA GCC C-3', 5'-CCA CAA GCG TCT TCT CAG CCA TG-3'), CYP4A1 (M14972; 5'-CAG AAT GGA GAA TGG GGA CAG C-3', 5'-TGA GAA GGG CAG GAA TGA GTG G-3'), ACO (J02752; 5'-CTT TCT TGC TTG CCT TCC TTC TCC-3', 5'-GCC GTT TCA CCG CCT CGT A-3'), L-CPT I (NM_031559; 5'-GGA GAC AGA CAC CAT CCA ACA TA-3', 5'-AGG TGA TGG ACT TGT CAA ACC-3'), MCAD (NM_016986; 5'-CAA

GAG AGC CTG GGA ACT TG-3', 5'-CCC CAA AGA ATT TGC TTC AA-3'), LCAD (NM_012819; 5'-AAG GAT TTA AGG GCA AGA AGC-3', 5'-GGA AGC GGA GGC GGA GTC-3'), SREBP-1c (XM_213329; 5'-GGA GCC ATG GAT TGC ACA TT-3', 5'-AGG AAG GCT TCC AGA GAG GA-3'), FAS (NM_017332; 5'-AGG TGC TAG AGG CCC TGC TA-3', 5'-GTG CAC AGA CAC CTT CCC AT-3'), c-myc (NM_012603; 5'-CTG GAG TGA GAA GGG CTT TG-3', 5'-CAG CAG CTC GAA TTT CTT CC-3'), c-jun (NM_021835; 5'-ACC AAG AAT TCC GTG ACG AC-3', 5'-CAA GGT CAT GCT CTG CTT CA-3'), and c-fos (NM_022197; 5'-CAT CGG CAG AAG GGG CAA AGT AGA G-3', 5'-TGC CGG AAA CAA GAA GTC ATC AAA G-3').

Statistical analysis

Treatment effects were analyzed using one-way ANOVA. For significant *F*-values, means were compared by Fisher's multiple range test. Differences with $P < 0.05$ were considered significant.

List of abbreviations used

ACO, acyl-CoA oxidase; CYP4A1, cytochrome P_{450} 4A1; FAS, fatty acid synthase; LCAD, long-chain acyl-CoA dehydrogenase; L-CPT I, L-type carnitin-palmitoyl transferase I; MCAD, medium-chain acyl-CoA dehydrogenase; PPARα, peroxisome proliferator-activated receptor α; PPRE, PPAR response element; SREBP-1c, sterol regulatory-element binding protein-1c.

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

RR participated in the design of the study and in the interpretation of the results and prepared the manuscript.

AG and CD carried out the feeding experiments, quantification of lipid concentrations, and mRNA expression analysis.

CB participated in the design and coordination of the study, and interpretation of the results.

KE conceived of the study and its design, coordinated work, participated in the interpretation of the results, and helped to draft the manuscript.

All authors read and approved the final manuscript.

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Short communication: Dietary conjugated linoleic acid down-regulates fatty acid transporters in the mammary glands of lactating rats

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ABSTRACT

Recent studies indicated that reduction of milk triacylglycerol concentrations by dietary conjugated linoleic acid (CLA) involves an impairment of both de novo fatty acid synthesis and uptake of fatty acids from circulating triacylglycerol-rich lipoproteins into the mammary gland. However, nonesterified fatty acids (NEFA) in the plasma released from adipose tissue and taken up into the mammary gland by fatty acid transporters are a further important source of fatty acids available for milk triacylglycerol synthesis. Therefore, the aim of the present study was to investigate the effect of dietary CLA on plasma concentrations of NEFA and the expression of fatty acid transporters in the mammary glands of lactating rats fed either a CLA diet or a control diet. Dams fed diets with CLA had a greater concentration of NEFA in plasma than those fed the control diet. In addition, relative mRNA concentrations of fatty acid transporters (fatty acid translocase/CD36, fatty acid transport protein, and plasma membrane fatty acid binding protein) were about 45, 75, and 70% lower, respectively, in the mammary gland of dams fed diets with CLA compared with those fed the control diet. In conclusion, the present findings indicate that reduced uptake of circulating NEFA released from white adipose tissue into the mammary gland could also contribute to the reduction of milk triacylglycerol concentrations by dietary CLA in rats. The mechanism through which CLA inhibits expression of fatty acid transporters deserves further study.
Key words: conjugated linoleic acid, fatty acid transporters, lactation, mammary gland

Conjugated linoleic acid (**CLA**) is a collective term for a group of positional and geometric isomers of linoleic acid with conjugated double bonds. Conjugated linoleic acid is naturally found in significant amounts in milk, dairy products, and meat of ruminants (Steinhart et al., 2003). Several studies showed that dietary CLA exerts many biological effects in humans and animals (Martin et al., 2000; Baumgard et al., 2001; Masters et al., 2002; Terpstra et al., 2002; Toomey et al., 2006). For instance, in lactating rats (Ringseis et al., 2004; Hayashi et al., 2007), sheep (Lock et al., 2006), cows (Baumgard et al., 2001, 2002; Peterson et al., 2004; Harvatine and Bauman, 2006), and humans (Masters et al., 2002) dietary CLA causes a reduction in milk triacylglycerol concentration.

Milk triacylglycerol synthesis depends on the availability of fatty acids in the mammary gland, which are derived from 3 different sources. The first source represents de novo biosynthesis of fatty acids within the mammary gland by the activity of lipogenic enzymes. Medium-chain fatty acids with 8 to 14 carbon atoms are the main products of this process, which is controlled by the lipogenic transcription factor sterol regulatory element-binding protein (**SREBP**)-1c (Barber et al., 2003). Fatty acids released from triacylglycerol-rich lipoproteins by lipoprotein lipase and taken up into the mammary gland by fatty acid transporters are a second important source for milk triacylglycerol synthesis (Scow et al., 1977). Nonesterified fatty acids in the plasma released from adipose tissue by hormone-sensitive lipase and taken up into the mammary gland by fatty acid transporters are a third source of fatty acids available for milk triacylglycerol synthesis. Fatty acids with 16 carbon atoms and long-chain fatty acids with 18 to 22 carbon atoms, either saturated or unsaturated, largely reflect the second and third sources of fatty acids for milk triacylglycerol synthesis (Green et al., 1981; Ross et al., 1985). The contribution of NEFA to milk fat synthesis in lactating cows is especially important during the early lactation stage, because the capacity for feed intake is limited at the beginning of lactation and therefore adipose tissue depots, which have accumulated during pregnancy, are actively mobilized during this stage. In mid and late lactation NEFA are of minor importance for milk fat synthesis in dairy cows because feed intake is sufficient to provide enough substrates for milk fat synthesis and even to replenish lipid stores during this lactation stage. In contrast, NEFA

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are an important source for milk fat synthesis during the whole lactation in species with a high milk yield such as the rat, whose demand for lactation is so large that food intake is dramatically increased (up to 3- to 4-fold compared with nonlactating rats; Peterson and Baumgardt, 1971). In spite of the large increase in feed consumption, however, rats are generally in negative energy balance during lactation, in particular during peak lactation (d 12 to 14 postpartum), where mobilization of body fat and protein is greatest (Sampson and Janson, 1984). Consequently, rats usually lose weight during lactation regardless of the feeding regimen (e.g., ad libitum) or the energy content of the diet (Sainz et al., 1986).

Several studies have shown that the reduced milk triacylglycerol concentrations by dietary CLA were accompanied by a diminished activation of SREBP-1c and reduced expression and activity of lipogenic enzymes and lipoprotein lipase in the mammary gland (Baumgard et al., 2002; Peterson et al., 2004; Ringseis et al., 2004; Harvatine and Bauman, 2006). These findings clearly indicate that dietary CLA reduces milk triacylglycerol concentrations by impairment of both de novo fatty acid synthesis and uptake of fatty acids from circulating triacylglycerol-rich lipoproteins into the mammary gland. However, whether the reduced concentrations of long-chain fatty acids and triacylglycerols in the milk by dietary CLA are also the consequence of diminished uptake of NEFA by fatty acid transporters from plasma into the lactating mammary gland has not been investigated yet. Therefore, the aim of the present study was to investigate the effect of dietary CLA on plasma concentrations of NEFA and the expression of the most important fatty acid transporters, fatty acid translocase/CD36 (**FAT/CD36**), fatty acid transport protein (**FATP**), and plasma membrane fatty acid binding protein (**FABPpm**), in the mammary gland of lactating rats. For this purpose, we used samples of a recently performed feeding experiment with lactating rats that were fed either a CLA diet or a control diet containing sunflower oil (**SFO**; Ringseis et al., 2004). In this experiment, the rats of the CLA group had a 46% lower milk fat content and markedly lower absolute concentrations of medium-chain fatty acids, fatty acids with 16 carbon atoms, and long-chain fatty acids in the milk at d 10 postpartum [C8 to C14: CLA group: 56 ± 13 mmol/L, n = 8, SFO group: 149 ± 6 mmol/L, n = 10, means ± SEM, $P < 0.05$; C16: CLA group: 47 ± 9 mmol/L, n = 8, SFO group: 85 ± 4 mmol/L, n = 10, means ± SEM, $P < 0.05$; C18 to C22: CLA group: 99 ± 17 mmol/L, n = 8, SFO group: 140 ± 8 mmol/L, n = 10, means ± SEM, $P < 0.05$, (Ringseis et al., 2004)]. Relative proportions of medium-chain fatty acids in the milk were also lower in the CLA group than in the SFO group (CLA group: 17.6 ± 1.6 g/100 g of fatty acids, n = 8, SFO group: 28.5 ± 2.2 g/100 g of fatty acids, n = 10, means ± SEM, $P < 0.05$), whereas proportions of C16:0 and long-chain fatty acids did not significantly differ between the groups (C16:0: CLA group: 23.6 ± 0.5 g/100 g of fatty acids, n = 8, SFO group: 22.8 ± 0.8 g/100 g of fatty acids, n = 10, means ± SEM; C18 to C22: CLA group: 49.0 ± 3.2 g/100 g of fatty acids, n = 8, SFO group: 41.2 ± 1.5 g/100 g of fatty acids, n = 10, means ± SEM). The rats, at a mean BW of 64 ± 1 g (mean ± SEM), were randomly assigned to 2 groups (n = 12) and fed the diets with 30 g/kg diet of either sunflower oil (SFO group) or CLA oil (CLA group). The fatty acid compositions of total lipids of SFO and the CLA oil were similar except for the concentrations of 18:2 n-6 and CLA. The CLA oil contained 54 g of CLA isomers/100 g of CLA oil, whereas the CLA concentration in the SFO was <0.1 g per 100 g of total fatty acids. The CLA isomer distribution of the CLA oil was as follows (g/100 g of total CLA): *trans* (***t***)-10 *cis* (***c***)-12 (18.5), *c*11*t*13 (15.8), *c*9*t*11 (15.6), *t*8*c*10 (14.9), *t*10*t*12 (5.61), *t*9*t*11 (5.41), *t*7*t*9 (3.12), *c*13*t*15 and *t*13*c*15 (2.85), *t*11*t*13 (2.75), *t*8*t*10 (2.63), *c*10*c*12 (2.58), *t*12*t*14 (2.22), *c*9*c*11 (2.08), *c*11*c*13 (1.99), *c*8*c*9 (1.30), *c*12*t*13 and *t*12*c*13 (0.97), *t*7*c*9 (0.79), *t*11*c*13 (0.62), *t*6*t*8 (0.18), and *t*13*t*15 (0.13). At 11 wk of age, the rats were paired with adult male Sprague-Dawley rats for 6 d. At the day of parturition, designated as d 1 of lactation, litters were weighed and then adjusted to 10 pups per dam without differentiation of sex. The experimental diets were fed for a total of 13 wk, starting at 5 wk of age. During growth and pregnancy, the rats were fed identical amounts of the experimental diets, increasing from 7 to 19 g/d, except for wk 11. In wk 11, when the rats were paired with the male rats, they had free access to the experimental diets. Throughout the period of lactation, the rats also had free access to the experimental diets; however, daily food intake during lactation did not differ between groups [CLA group: 33.7 ± 1.4 g/d, n = 8; SFO group: 34.9 ± 0.9 g/d, n = 10, means ± SEM (Ringseis et al., 2004)]. Based on an energy content of the diets of 17.5 MJ/kg of diet, the daily food intake during lactation was 590 ± 25 kJ/d in the CLA group (n = 8) and 611 ± 16 kJ/d in the SFO group (n = 10), means ± SEM. Plasma and samples of mammary gland were obtained on d 17 of lactation, when the dams were anesthetized with diethyl ether and killed by decapitation. Day 17 of lactation in rats corresponds to the late lactation stage where milk yield is slowly declining because pups suckle less milk. During these final days of lactation, the energy and nutrient demands of the pups are partially met by the consumption of solid food. Further details regarding animals, diets, feeding regimen, and sample collec-

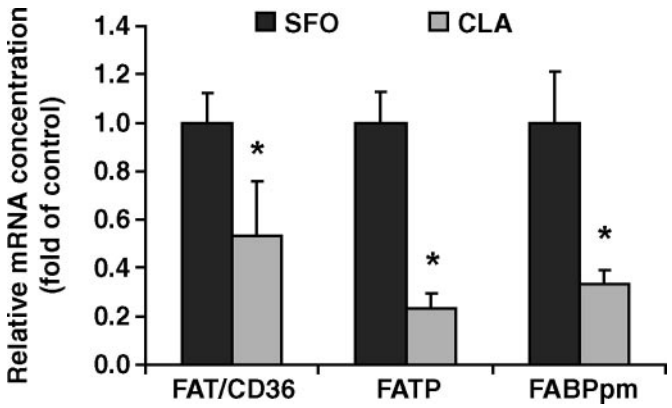

Figure 1. Relative mRNA concentrations of fatty acid transporters, fatty acid translocase/CD36 (FAT/CD36), fatty acid transport protein (FATP), and plasma membrane fatty acid binding protein (FABPpm), in the mammary gland of lactating rats fed diets with either conjugated linoleic acid (CLA) or sunflower oil (SFO). Bars represent mean ± SEM for n = 10 (SFO) and n = 8 (CLA) rats, respectively, and are expressed as fold changes of relative mRNA concentration in CLA group compared with the SFO group. *Different from rats fed diets with SFO, $P < 0.05$.

tion can be found in our recent article (Ringseis et al., 2004). Plasma NEFA concentrations were determined using the enzymatic NEFA C kit from Wako Chemicals (Neuss, Germany, Ref. 99975406). For the determination of mRNA expression levels of FAT/CD36, FATP, FABPpm, and GAPDH as internal standard for normalization, total RNA was isolated from mammary glands using TrizolP reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocol. Glyceraldehyde-3-phosphate dehydrogenase served as an appropriate reference gene in this experiment because the cycle threshold (CT) values of GAPDH did not differ between treatment groups. Concentration and purity of RNA were estimated from the optical density at 260 and 280 nm, respectively. Synthesis of cDNA and relative quantification of target-gene mRNA compared with housekeeping-gene mRNA was determined by real-time detection PCR as described previously (Ringseis et al., 2007). Relative expression ratios are expressed as fold changes of mRNA abundance in the CLA group compared with the SFO group. Sequences of gene-specific primers obtained from Operon (Köln, Germany) were as recently published (Ringseis et al., 2007). Treatment effects were analyzed using one-way ANOVA. For significant F-values, means were compared by Fisher's multiple range test. Differences with $P < 0.05$ were considered significant.

Figure 1 shows the effect of treatment on relative mRNA concentrations of fatty acid transporters in the mammary gland. Relative mRNA concentrations of FAT/CD36, FATP, and FABPpm were about 45, 75, and 70% lower, respectively, in the mammary glands of dams fed diets with CLA than in those fed diets with SFO ($P < 0.05$). Analysis of NEFA concentrations in plasma revealed that dams fed diets with CLA had a greater concentration of NEFA in plasma than those fed diets with SFO (CLA group: 440 ± 34 μEq/L, n = 8, SFO group: 330 ± 10 μEq/L, n = 10, means ± SEM, $P < 0.05$).

The present study shows for the first time that fatty acid transporters, which mediate cellular uptake of albumin-bound NEFA originating from hydrolysis of triacylglycerols by hormone-sensitive lipase in white adipose tissue and subsequent release into the circulation, are markedly down-regulated, as evidenced by decreased transcript levels in the mammary glands of dams fed CLA. Although down-regulation of fatty acid transporters was not proven on the protein level, we suggest that the transport capacity of the mammary gland for circulating NEFA is probably impaired in lactating rats by dietary treatment with CLA. Indeed, the finding that plasma NEFA concentrations were significantly greater in dams fed the CLA diet than in those fed the control diet is supportive of this assumption. Nevertheless, this finding in rats is in contrast to the observation in dairy cows that plasma NEFA levels are not altered by treatment with CLA (Perfield et al., 2002). Although we cannot explain this discrepancy, we are confident that the increased plasma NEFA concentrations in the CLA group are not the result of a decreased energy intake leading to an increased release of NEFA from adipose tissue and, subsequently, a reduced BW. This assumption is based on the finding that feed and energy intake as well as BW development during lactation did not differ between rats of the CLA and SFO groups (Ringseis et al., 2004). In connection with our recent observations that the concentrations of fatty acids with 16 carbon atoms and long-chain fatty acids with 18 to 22 carbon atoms in the milk, which originate both from the diet and white adipose tissue, were reduced by dietary CLA, we suggest that dietary CLA impairs the uptake of those fatty acids into the mammary gland. This may contribute to a reduced availability of fatty acids in the mammary gland, which in turn leads to a reduced milk triacylglycerol synthesis.

The CLA oil used in the present study contained a large number of CLA isomers. Among them, *t*10*c*12-CLA, *c*11*t*13-CLA, *c*9*t*11-CLA, and *t*8*c*10-CLA were the major CLA contributing to about 65 g/100 g of total CLA. The remaining CLA identified consisted of minor isomers in *trans*/*cis*-, *cis*/*trans*-, *trans*/*trans*-, and *cis*/*cis*-configurations. The *t*10*c*12-CLA isomer has been shown to be responsible for the reduction in milk fat synthesis in dairy cows and lactating sheep (Baumgard et al., 2002; Peterson et al., 2004; Lock et al.,

2006). However, the effects of minor CLA isomers on metabolism in animals are largely unknown. Therefore, because of the large number of CLA isomers contained in the CLA supplement, it is not known which CLA isomers were mainly responsible for the down-regulation of fatty acid transporters in the mammary gland of rats by the CLA supplement.

Whether down-regulation of fatty acid transporters in mammary tissue might also contribute to milk fat reduction by CLA in lactating cows is unknown. However, expression of fatty acid transporters in different tissues including mammary gland of cows has been reported (Ordovás et al., 2006; Bionaz and Loor, 2008a,b). Interestingly, expression of FAT/CD36 in mammary tissue of lactating cows largely increases during lactation, reaching a peak at about 6 wk postpartum, which corresponds to peak lactation in cows, and declines from then until weaning (Bionaz and Loor, 2008a). This pattern of FAT/CD36 expression during lactation is similar to the lactation course of daily yield of fatty acids with 16 and more carbon atoms, which originate from uptake from plasma, in the milk of dairy cows (Bionaz and Loor, 2008a); that is, daily yield of these fatty acids also peaks within a few weeks postpartum and markedly declines toward the end of lactation. This suggests that uptake of fatty acids from plasma plays an important role for milk fat synthesis during early and peak lactation in dairy cows. In contrast, daily yield of fatty acids synthesized de novo in cow milk predominates after 4 wk postpartum (Bionaz and Loor, 2008a), suggesting that fatty acid synthesis in mammary tissue plays a major role for milk fat synthesis during mid and late lactation in lactating cows. Therefore, it can be speculated that a potential impairment of fatty acid transport from plasma into mammary tissue by dietary CLA in lactating cows might significantly contribute to milk fat reduction only when CLA is administered during early lactation. However, the finding that CLA is less effective for milk fat reduction in early lactation than in mid or late lactation (Perfield et al., 2002) suggests that possible impairment of fatty acid uptake by CLA has only a minor effect on milk fat reduction in dairy cows. However, this assumption has to be proven in future studies.

The mechanism of the action of CLA on the expression of fatty acid transporters in the mammary gland cannot be resolved from the present study. It is known that regulation of fatty acid transporters includes transcriptional regulation through peroxisome proliferator-activated receptors (**PPAR**)-α and γ (Motojima et al., 1998; Frohnert et al., 1999). However, a central role of these transcription factors in the regulation of fatty acid transporter gene expression by CLA in the mammary gland is unlikely, because both PPARα and PPARγ show only negligible expression in the lactating mammary glands of rodents (Gimble et al., 1998; Rodriguez-Cruz et al., 2006). Other ways of regulating expression of these genes by CLA might be conceivable, because the promoter region of fatty acid transporter genes contains other regulatory elements including insulin responsive elements (Hui et al., 1998). Therefore, further studies are warranted to elucidate the mechanism by which CLA affect gene expression of fatty acid transporters.

In conclusion, our present findings in connection with recent observations (Baumgard et al., 2001, 2002; Ringseis et al., 2004) strongly indicate that reduced uptake of circulating NEFA released from white adipose tissue into the mammary gland could also contribute to the reduction of milk triacylglycerol concentrations by dietary CLA in rats. Whether this applies to other species such as cows and sheep has to be demonstrated in future studies. The mechanism through which CLA inhibits expression of fatty acid transporters deserves further studies.

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3. Diskussion

3.1 Funktionen des PPARα während der Laktation

Der zentrale Schwerpunkt im ersten Komplex der vorliegenden Arbeit lag auf der Ermittlung der Rolle des PPARα im Lipidstoffwechsel während der Laktation. Hierbei sollte der Frage nachgegangen werden, ob der Transkriptionsfaktor PPARα metabolische Anpassungsprozesse im Lipidstoffwechsel laktierender Nager reguliert. Dabei bildeten Beobachtungen aus einigen vorrangegangenen Studien den Ansatzpunkt zu den hier durchgeführten Untersuchungen. Aus der wissenschaftlichen Literatur war bereits bekannt, dass sowohl die Fettsäureoxidation in Leber und Skelettmuskel, das heißt in Geweben mit einer hohen Aktivität des Fettsäurekatabolismus sowie die Thermogenese im Skelettmuskel und im braunen Fettgewebe während der Laktation vermindert sind (Pedraza *et al*., 2000; Pedraza *et al*., 2001, Xiao *et al*., 2004*a* und 2004*b*). Aufgrund dieser Beobachtungen lag die Vermutung nahe, dass Adaptationsprozesse während der Laktation möglicherweise auf einer verringerten Aktivität des PPARα basieren.

3.1.1 Molekulare Ursachen von Adaptationsprozessen im Stoffwechsel während der Laktation beim Nager

Im Rahmen des ersten Komplexes wurde in einem ersten Versuch untersucht, ob die hepatische mRNA Konzentration des PPARα während der Laktation vermindert ist und dies zu einer Herunterregulierung der Expression von Genen der Carnitinsynthese und der OCTN führt, was in reduzierten Plasma- und Gewebekonzentrationen von Carnitin deutlich werden könnte. Dazu wurden die Genexpression in der Leber laktierender Sprague-Dawley Ratten mit unterschiedlicher Wurfgröße (4, 10 oder 18) mit den Genexpressionen nicht-laktierender Ratten verglichen. In einem zweiten Versuch sollte die Rolle des PPARα als Mittler von Adaptationsprozessen im Lipidstoffwechsel während der Laktation anhand eines Versuchsmodells mit laktierenden und nicht-laktierenden PPARα-*knockout* Mäusen sowie laktierenden und nicht-laktierenden Wildtyp-Mäusen untersucht werden. Im ersten Komplex der vorliegenden Arbeit konnte zum ersten Mal gezeigt werden, dass die Expressionen des PPARα, seiner Coaktivatoren und von PPARα-regulierten Genen des Fettsäurekatabolismus, der Thermogenese, der Fettsäureaufnahme sowie der Carnitinaufnahme und der Carnitinsynthese in Geweben mit einer hohen Aktivität des Fettsäurekatabolismus, wie Leber

und Skelettmuskel, in Mäusen und Ratten während der Laktation deutlich herunterreguliert sind. Diese Ergebnisse weisen darauf hin, dass eine verminderte PPARα-Aktivierung zu einer Reduktion der Fettsäureaufnahme und Fettsäureoxidation sowie der Thermogenese in Leber und Skelettmuskel im laktierenden Nager führt. Aus vorherigen Studien war bereits bekannt, dass Gene der Carnitinaufnahme und -synthese transkriptionell über den PPARα reguliert werden (Luci *et al*., 2006; Ringseis *et al*., 2007*b*; van Vlies *et al*., 2007). Daher kann auch eine verminderte Carnitinaufnahme und -synthese in der Leber der laktierenden Ratten auf eine Hemmung der PPARα-Expression durch die Laktation zurückgeführt werden. Es konnte allerdings kein Unterschied hinsichtlich der Expression des PPARα und der PPARα-Zielgene zwischen den laktierenden Ratten mit unterschiedlicher Wurfgröße ermittelt werden. Dies lässt darauf schließen, dass die Herunterregulierung des PPARα unabhängig von der Wurfgröße ist. Im ersten Komplex dieser Arbeit konnten erstmals wichtige Regulationsmechanismen hinter den Adaptationsprozessen während der Laktation aufgeklärt werden. Es wurde gezeigt, dass der PPARα für die Vermittlung metabolischer Adaptationsprozesse hinsichtlich der Fettsäureverwertung und Energieeinsparung während der Laktation im Nager verantwortlich ist. Dies ist ein wichtiger Mechanismus im besonderen physiologischen Zustand der Laktation, um die Versorgung der Nachkommen sicherstellen zu können und mit Hilfe von Adaptationsprozessen Energie und metabolische Substrate, wie Fettsäuren, für die Milchsynthese in der Milchdrüse laktierender Ratten und Mäuse einzusparen (Pedraza *et al*., 2000 und 2001; Xiao *et al*., 2004*a* und 2004*b*).

Es ist erwähnenswert, dass in den untersuchten Geweben der PPARα-*knockout* Mäuse die Laktation nicht zu einer verminderten Expression des PPARα und der PPARα-Zielgene führte. Zusätzlich waren die relativen mRNA Konzentrationen des PPARα und dessen Zielgene deutlich geringer in Leber und Skelettmuskel der PPARα-*knockout* Mäuse im Vergleich zu den nicht-laktierenden Wildtyp-Mäusen. Von besonderer Bedeutung ist in diesem Zusammenhang, dass der PPARα und einige PPARα-Zielgene in den PPARα-*knockout* Mäusen auf ähnlicher Höhe exprimiert wurden wie in laktierenden Wildtyp-Mäusen, mit Ausnahme der M-CPT I und MCAD im Skelettmuskel. Dies weist darauf hin, dass durch die Laktation ein Effekt bei Wildtyp-Mäusen hervorgerufen wird, der mit einer Deletion des PPARα vergleichbar ist.

Der Transkriptionsfaktor PPARα ist maßgeblich für die Regulation der Fettsäureoxidation. Dennoch waren die mRNA-Level der M-CPT I und der MCAD im Skelettmuskel der Wildtyp-Mäuse während der Laktation sehr viel stärker vermindert, als in den PPARα-*knockout* Mäusen. Dies gibt einen Hinweis auf PPARα-unabhängige Regulations-

mechanismen. Durch Coaktivatoren des PPARα werden PPAR-spezifische Gentranskriptionsprozesse verstärkt. Zu diesen Coaktivatoren zählen der PGC-1 α und der PGC-1 β (Vega *et al*., 2000; Yu & Reddy, 2007). Die verminderte Expression des PGC-1α und β trug vermutlich dazu bei, dass die relativen mRNA Konzentrationen der PPARα-Zielgene in den laktierenden Wildtyp-Mäusen geringer waren, als die der nicht-laktierenden. Daher könnte die gesteigerte Expression von PGC-1α und β zu einer geringeren Senkung der M-CPT I und MCAD im Skelettmuskel der PPARα-*knockout* Mäuse im Vergleich zu den laktierenden Wildtyp-Mäusen geführt haben. Dies liefert eine mögliche Erklärung für die höhere Expression der M-CPT I und der MCAD im Skelettmuskel der PPARα-*knockout* Mäuse als in den laktierenden Wildtyp-Mäusen. Des Weiteren ist aus kürzlich durchgeführten Studien bekannt, dass in PPARα-*knockout* Mäusen einige PPARα-Zielgene in vergleichbarer Höhe exprimiert werden wie in Wildtyp-Mäusen (Zandbergen *et al*., 2005; Stienstra *et al*., 2007).

Eine PPARα-Aktivierung führt in der Ratte zu einer stärkeren Hochregulierung des OCTN2 im Vergleich zum OCTN1 (Luci *et al*., 2006; Ringseis *et al*., 2007*b*; Ringseis *et al*., 2008; Tein, 2003). Allerdings konnte die daraufhin vermutete PPARα-induzierte Herunterregulierung des OCTN2 in der Leber der laktierenden Ratten nicht beobachtet werden. Im Vergleich dazu war die Expression des OCTN1 stark vermindert. Dies kann vermutlich darauf zurückgeführt werden, dass die ohnehin schon basale Expression des PPARα während der Laktation nur eine minimale Expression des OCTN2 hervorruft. Daraufhin führt eine Herunterregulierung des PPARα keine weitere Reduktion der mRNA Konzentration des OCTN2 herbei. Die verminderte Expression der Carnitintransporter in der Leber ist sinnvoll, da während der Laktation die Fettsäureoxidation geringer ist (Rudolph *et al*., 2007) und somit der Transport von Carnitin in die Leberzellen in den Hintergrund tritt.

In der vorliegenden Arbeit konnten reduzierte Carnitinkonzentrationen in der Leber der laktierenden Ratten beobachtet werden. Während der Laktation ist die Konzentration an Carnitin in der Zelle abhängig von der Aufnahme des Carnitins über die Nahrung, der endogenen Synthese von Carnitin, der Verteilung mittels der Carnitintransporter, der Ausscheidung mit dem Urin und dem über die Milch abgegebenen Anteil an Carnitin (Rebouche, 2004). Somit wurden im Rahmen des ersten Versuches innerhalb des ersten Komplexes die Carnitinkonzentrationen in Plasma und Skelettmuskel sowie die mRNA Konzentrationen der Enzyme der Carnitinsynthese ermittelt. Aus der wissenschaftlichen Literatur ist bekannt, dass Fasten und die Gabe von PPARα-Agonisten zu einer Aktivierung des PPARα und einem damit induzierten Anstieg der Carnitinsynthese, des Carnitintransports über den OCTN2 und somit zu einer Konzentrationserhöhung des Carnitins in der Leber führt

(van Vlies *et al*., 2007). In dieser Arbeit konnte erstmals gezeigt werden, dass die Laktation in der Ratte zu einer reduzierten Expression von Enzymen der Carnitinsynthese in der Leber und damit auch zu einer verminderten Carnitinkonzentration in Leber und Skelettmuskel führte. Weiterhin war die Aktivität der BBD, welche den letzten Schritt der Carnitinsynthese reguliert, in der Leber während der Laktation vermindert (Vaz *et al*., 2002). Diese könnte zur Verminderung der Plasma- und Gewebekonzentrationen des Carnitins während der Laktation beigetragen haben. Die unveränderten Konzentrationen von TML in den Geweben und im Plasma deuten darauf hin, dass nicht TML den limitierenden Faktor in den laktierenden Ratten darstellte (Rebouche *et al*., 1986). Möglicherweise war die verringerte Expression der Synthesegene des Carnitins und die reduzierte Aktivität der BBD verantwortlich für die verminderten Carnitinkonzentrationen in den Geweben während der Laktation. Die verminderte Carnitinkonzentration in Leber und Skelettmuskel, die reduzierte Aktivität der Carnitineigensynthese in der Leber, dem einzigen Syntheseort in der weiblichen Ratte (van Vlies *et al*., 2006), und die verminderte Expression des OCTN1 und der CPT I in der Leber laktierender Ratten dient der Einsparung und Bereitstellung von Carnitin zugunsten der Milchbildung in der Milchdrüse der Muttertiere, um die Versorgung der Jungtiere sicherzustellen.

Während der Laktation ist die Thermogenese verringert (Trayhurn, 1983), was mit einer Verminderung des UCP1 und UCP3 im braunen Fettgewebe, sowie des UCP3 im Skelettmuskel in Verbindung gebracht wird. Wie in anderen Studien bereits gezeigt (Pedraza *et al*., 2000; Xiao *et al*., 2004*a*), waren auch im ersten Komplex der vorliegenden Arbeit die relativen mRNA Konzentrationen des UCP3 im Skelettmuskel der Wildtyp-Mäuse während der Laktation vermindert. Dieser Effekt konnte im Skelettmuskel der laktierenden PPARα-*knockout* Mäuse nicht beobachtet werden. Die reduzierte Expression der UCPs führt aufgrund ihrer Rolle im Stoffwechsel zu einer Reduktion der zitterfreien Thermogenese und somit zu einer Energieeinsparung für die Milchsynthese während der Laktation. Die zitterfreie Thermogenese im braunen Fettgewebe zählt nicht allein zu den Funktionen des UCP3. Inzwischen werden eine Reihe weiterer Funktionen im Stoffwechsel diskutiert. Dazu gehört der Transport von NEFA aus dem Mitochondrium, um eine Akkumulation von NEFA in der mitochondrialen Matrix zu verhindern (Schrauwen *et al*., 2001). Ferner reduziert UCP3 das Membranpotential der Mitochondrien, was zu einer Milderung der reaktiven Sauerstoffspezies (ROS) führt (Echtai *et al*., 2002). Weiterhin konnte in Studien mit UCP3 überexprimierenden Mäusen ein massiv reduziertes Körpergewicht und Anzeichen auf eine gesteigerte Glucoseoxidation und Insulin-Sensitivität beobachtet werden (Clapham *et al*.,

2000; Costford *et al*., 2006). Die verminderte Expression des UCP3 während der Laktation verfolgt ebenfalls den Zweck, metabolische Substrate und Energie einzusparen, um diese für die Milchsynthese in der Milchdrüse bereitzustellen.

Im Hinblick auf die verminderte Expression des UCP3 im Skelettmuskel der laktierenden Wildtyp-Mäuse und das Ausbleiben dieses Effektes im Skelettmuskel der PPARα-*knockout* Mäuse, kann vermutet werden, dass zugrundeliegende biochemische Regulations-mechanismen hinsichtlich der Energieeinsparung im Rahmen der Adaptationsprozesse zugunsten der Milchsynthese während der Laktation durch PPARα reguliert werden. Es ist bereits bekannt, dass die transkriptionelle Regulation des UCP3 durch den PPARα erfolgt (Barbera *et al*., 2001; Young *et al*. 2001). Ferner konnte in einer Studie mit laktierenden Ratten ein Einfluss auf UCP3 in Abhängigkeit der Wurfgröße beobachtet werden (Isler *et al*., 1984). Es ist bekannt, dass sich mit steigender Jungtierzahl die Prolaktinspiegel im Blutplasma der laktierenden Muttertiere erhöhen. Prolaktin ist ein Hormon aus der Adenohypophyse, welches während der Laktation eine bedeutende Rolle spielt und zu dessen wichtigsten Aufgaben die Steigerung des Milchflusses infolge einer vermehrten Ausschüttung nach Stimulierung durch säugende Jungtiere zählt (Freeman *et al*., 2000; Grosvenor & Whitworth 1974; Ben-Jonathan *et al*., 2006; Flint *et al*., 2003; Guyette *et al*., 1979). Daher ist es möglich, dass mit steigender Wurfgröße die Prolaktinkonzentrationen im Blutplasma der Muttertiere während der Laktation proportional ansteigen. Im ersten Versuch der vorliegenden Arbeit konnte allerdings kein Unterschied hinsichtlich der relativen mRNA Konzentrationen des UCP3 zwischen den Gruppen der laktierenden Ratten mit unterschiedlichen Würfen beobachtet werden. Allerdings beeinflusst möglicherweise die erhöhte Prolaktinsekretion während der Laktation die Aktivität des Transkriptionsfaktors PPARα, was zu einer Verringerung der Fettsäureoxidation, der Aufnahme von Fettsäuren in die Gewebe, der zitterfreien Thermogenese, der Carnitinsynthese und Gewebekonzentrationen an Carnitin, im relativen Vergleich zu Muttertieren ohne Wurf, führt. Prolaktin bindet an den Prolaktinrezeptor, welcher in Leber und Skelettmuskel ebenso exprimiert ist, wie in der Milchdrüse (Nagano & Kelly, 1994). Es ist möglich, dass Prolaktin dann über verschiedene Signalwege und Proteinkinasen (MAP Kinase, PI3 Kinase) auf den PPARα in Leber und Skelettmuskel Einfluss nehmen kann.

Aus den Beobachtungen der im Rahmen dieser Arbeit durchgeführten Versuche in Komplex 1 und Beobachtungen aus weiteren Studien (Trayhurn *et al*., 1983; Pedraza *et al*., 2000 und 2001; Xiao *et al*., 2004*a* und 2004*b*) ergab sich ein physiologisches Modell, welches darauf hindeutet, dass die Herunterregulierung des PPARα und dessen Coaktivatoren in Geweben

mit einer hohen Aktivität des Fettsäurekatabolismus und der damit zusammenhängenden reduzierten Fettsäureverwertung in diesen Geweben zu einem erhöhten Fluss von Fettsäuren aus den Speichergeweben, wie dem weißen Fettgewebe, und aus triglyzeridreichen Lipoproteinen zugunsten der Milchdrüse führt. Dies ist ein energie- und substratsparender Mechanismus zugunsten der Milchsynthese in der Milchdrüse, welcher während der Laktation von besonderer Bedeutung ist. Gleichzeitig bedeutet dies, dass vermehrt NEFA in die Milchdrüse aufgenommen werden können, um dort für die Triglyzeridsynthese zur Verfügung zu stehen (Dewey, 1997; Smith & Grove, 2002). Besonders in laktierenden Ratten ist die Verwertung von NEFA aus den Speichergeweben für die Triglyzeridsynthese in der Milchdrüse von großer Bedeutung, da trotz hoher Futteraufnahme eine negative Energiebilanz herrscht. Der erhöhte Bedarf an Fettsäuren in der Milchdrüse wird durch die erhöhte Expression der LPL in der Milchdrüse laktierender Mäuse in Versuch 2 des ersten Komplexes deutlich. Eine gesteigerte Aktivität der LPL führt zu einer erhöhten Aufnahme von Fettsäuren aus triglyzeridreichen Lipoproteinen im Plasma der laktierenden Mäuse. Dies konnte in der vorliegenden Arbeit sowie in zwei anderen Studien beobachtet werden (Ramírez *et al*., 1983; Ringseis *et al*., 2004). Weiterhin sind die Fettsäuretransporter FAT/CD36, FABPpm und FATP in der Milchdrüse während der Laktation deutlich höher exprimiert als in nicht-laktierenden Mäusen und Ratten, was ebenfalls eine gesteigerte Aufnahme von Fettsäuren in die Milchdrüse ermöglicht und zu einer Reduzierung der NEFA- (Pedraza *et al*., 2000) sowie Triglyzeridkonzentrationen, wie in Versuch 1 des ersten Komplexes beobachtet, im Plasma der laktierenden Nager führt. Zusätzlich wurde in der vorliegenden Arbeit eine gesteigerte Expression der FAS in der Milchdrüse der Nager beider Genotypen während der Laktation ermittelt, was zu einer gesteigerten Milchfettsynthese während der Laktation beiträgt (Rudolph *et al*., 2003). Die Regulation der Fettsäuretransporter, der LPL und der FAS stellt jedoch einen PPARα-unabhängigen Prozess dar, was dadurch ersichtlich wurde, dass die Expressionen dieser Gene gleichermaßen, sowohl bei den laktierenden Wildtyp-Mäusen als auch bei den laktierenden PPARα-*knockout* Mäusen, in der Milchdrüse erhöht waren. Diese Ergebnisse stimmen mit Hinweisen aus der Literatur überein, welche ebenfalls über eine kaum messbare mRNA Konzentration von PPARα in der Milchdrüse berichten konnten (Gimble *et al*., 1998; Rodriguez-Cruz *et al*., 2006).

3.2 Die Regulation der CACT durch PPARα

Während des Fastens werden vermehrt Fettsäuren in das Mitochondrium transportiert um mittels Fettsäureoxidation Energie zu gewinnen. Die im Fastenzustand freigesetzten Fettsäuren aus den Energiespeichern induzieren nach Aufnahme in die Gewebezellen eine Aktivierung des Transkriptionsfaktors PPARα. Zu den Aufgaben des PPARα zählt die Regulation metabolischer Anpassungsprozesse während des Fastens, wie die Steigerung der Fettsäureoxidation. In einer Reihe von Studien wurde die CPT I, deren Aufgabe in der Veresterung von Acyl-CoA mit Carnitin als Voraussetzung für den Transport in die mitochondriale Matrix liegt, bereits als ein PPARα-Zielgen beschrieben (Mascaro *et al*., 1999; Napal *et al*., 2005). In der vorliegenden Arbeit wurde die Hypothese geprüft, ob die CACT ebenfalls zu den Zielgenen des PPARα zählt.

Im Rahmen der in Komplex 2 der vorliegenden Arbeit durchgeführten Untersuchungen konnte zum ersten Mal gezeigt werden, dass die Promotoraktivität und die Transkription der CACT auf eine Induktion durch den PPARα und den PPARδ zurückgeführt werden kann. Die CACT sowie die bereits bekannten PPARα-Zielgene CYP4A10 und CPT I konnten durch WY-14,643, einem starken PPARα-Aktivator und Peroxisomen-Proliferator (Suga, 2004), und durch das Fasten in der Leber der Wildtyp-Mäuse hochreguliert werden. Dieser Effekt hingegen konnte in der Leber der PPARα-*knockout* Mäuse nicht beobachtet werden. Zusätzlich waren CPT I und CACT durch WY-14,643 in einer humanen Leberzelllinie und einer Rattenleberzelllinie hochreguliert. Dabei war die CACT auf gleicher Höhe exprimiert wie bereits bekannte PPARα-Zielgene. Die Transkriptionsfaktoren PPARα und PPARδ sind für die Regulierung zahlreicher Gene mit teilweise übergreifenden Funktionen im Lipidstoffwechsel verantwortlich (Gilde *et al*., 2003; Marx *et al*., 2004). Da der PPARδ die Regulierung von Genen der β-Oxidation im Skelettmuskel vermittelt (Holst *et al*., 2003), konnte, wie vorher vermutet, ebenso eine Hochregulierung der CACT durch den PPARδ-Aktivator GW0742 im Zellversuch beobachtet werden. PPARγ, der dritte bekannte PPAR Isotyp, wird hauptsächlich im weißen Fettgewebe exprimiert. Folglich zählen zu den Hauptfunktionen des PPARγ die Adipozytendifferenzierung und Fettspeicherung (Tontonoz *et al*., 1994; Rosen *et al*., 1999; Linford *et al*., 2007; Braissant *et al*., 1996). Auf Grund dessen lag die Vermutung nahe, dass PPARγ nicht an der Regulation der CACT beteiligt ist. Die fehlende Hochregulierung der CACT nach Inkubation der HepG2-Zellen mit Troglitazon bestätigte diese Vermutung. Mittels Promotoraktivitätsstudien im zweiten Komplex dieser Arbeit konnte zum ersten Mal ein funktionelles, durch PPARα und PPARδ aktivierbares

PPRE für die CACT der Maus in einer bestimmten Promotorregion beschrieben werden (Tan *et al*., 2005; Qi *et al*., 2000). Aufgrund dieser Beobachtungen kann gesagt werden, dass eine Aktivierung des PPARα und des PPARδ neben der bereits bekannten Hochregulierung der CPT I und CPT II ebenso eine Hochregulierung der CACT herbeiführen kann. Demnach kann auch eine gesteigerte Expression der CACT zu einem erhöhten Transport von Acylcarnitin in die mitochondriale Matrix und zu einer Steigerung der β-Oxidation während des Fastens führen.

3.3 Effekte von Komponenten oxidierter Fette mit PPARα aktivierbarem Potential während der Trächtigkeit

Die Gabe von starken natürlichen PPARα-Agonisten, CLA und oxidierten Fettsäuren führt zu massiven Störungen der Adaptationsprozesse während der Laktation (Ringseis *et al*., 2007*c*). Es stellt sich die Frage, ob bereits während der Trächtigkeit ein Einfluss auf fötale PPARα-abhängige Gentranskriptionsprozesse durch potentiell pathologisch wirkende PPARα-Agonisten, wie oxidierten Fettsäuren, erfolgt (Chao *et al*., 2004 und 2005). Im dritten Komplex der vorliegenden Arbeit konnte zum ersten Mal gezeigt werden, dass Komponenten von oxidiertem Fett sowohl im Muttertier eine PPARα-Aktivierung hervorrufen als auch über die Plazentaschranke in den Fötus gelangen und dort einen PPARα-induzierten Einfluss auf den Lipidstoffwechsel nehmen können. Dieser Effekt konnte bereits in einigen Studien mit trächtigen Ratten durch Clofibrat beobachtet werden (Stefanini *et al*., 1989; Wilson *et al*., 1991; Cibelli *et al*., 1988; Simson *et al*., 1996). In der vorliegenden Arbeit führte oxidiertes Fett und Clofibrat sowohl in der Leber der Muttertiere als auch in der Leber der Föten zu einer Steigerung des Fettsäurekatabolismus. Dies zog im Langzeitversuch des dritten Komplexes dieser Arbeit reduzierte Triglyzeridkonzentration in der fötalen und maternalen Leber nach sich. Zudem wurde im Kurzzeitversuch eine verminderte Fettsäuresynthese in der Leber der trächtigen Ratten beobachtet, welche durch Fütterung von oxidiertem Fett hervorgerufen wurde. Dies ging bereits aus einer Studie mit nicht-trächtigen Ratten hervor (Eder *et al*., 2003). Beim Fötus scheint durch die Gabe von oxidiertem Fett über die gesamte Trächtigkeit und über einen kurzen Zeitraum am Ende der Trächtigkeit vorrangig eine erhöhte Fettsäureoxidation für die reduzierten Triglyzeridkonzentrationen in der Leber verantwortlich zu sein, da keine Verminderung der FAS beobachtet werden konnte.

Die Ergebnisse der vorliegenden Studie deuten darauf hin, dass im oxidierten Fett enthaltene Lipidperoxidationsprodukte plazentagängig sind und zeigen, dass diese in der Lage sind in

den fötalen Kreislauf zu gelangen. Untersuchungen dazu sind in der Literatur bisher nicht zu finden. Bereits bekannt ist allerdings, dass der Übergang von Nährstoffen aus dem maternalen Kreislauf über die Plazentaschranke ein hochselektiver Vorgang ist (Schneider, 1991), insbesondere hinsichtlich dem Transport von Fettsäuren (Campbell *et al*., 1998; Herrera *et al*., 2006). Während der Trächtigkeit waren die Effekte von Komponenten oxidierter Fette auf die Expressionen von Genen des Fettsäurekatabolismus in der maternalen Leber weniger ausgeprägt als PPARα-induzierte Effekte in der fötalen Leber. Diese Ergebnisse weisen auf einen begünstigten Transport von oxidierten Fettsäuren und weiteren Lipidperoxidations-produkten in den fötalen Kreislauf hin, was möglicherweise eine PPARα-Antwort in der fötalen Leber während der Trächtigkeit auslöste.

Des Weiteren konnte bereits in der Leber der Föten ein beachtlicher Anstieg von Genen der β-Oxidation ermittelt werden, was auf eine PPARα-Aktivierung durch Komponenten von oxidiertem Fett zurückgeführt werden konnte (Sülzle *et al*., 2004; Ringseis *et al*., 2007*a*). Die Hydroxyoctadecadiensäure (HODE) und Hydroxyperoxyoctadecadiensäure (HPODE) sind im Vergleich zu anderen oxidierten Fettsäuren besonders starke PPARα-Aktivatoren (König & Eder, 2006; Delerive *et al*., 2000). Anhand der Fettkennzahlen ist erkennbar, dass diese zu großen Teilen in den Versuchsfetten enthalten waren. In einer anderen Studie konnten weitere Komponenten von oxidiertem Fett mit PPARα-aktiverbarem Potential identifiziert werden (Martin *et al*., 2000*b*). Somit kann geschlussfolgert werden, dass oxidierte Fettsäuren sowie andere Komponenten von oxidiertem Fett (Romero *et al*., 2006) über die Plazentaschranke gelangen und dort möglicherweise eine PPARα-Aktivierung auslösen können.

In Komplex 3 der vorliegenden Arbeit konnte beobachtet werden, dass der Einfluss des oxidierten Fettes auf Gene des Fettsäurekatabolismus von der Dauer der Aufnahme und der Art der Verabreichung abhängig ist. Dementsprechend führte die Gabe von oxidiertem Fett über einen längeren Zeitraum zu verminderten Triglyzeridkonzentrationen in der Leber trächtiger Ratten und deren Föten. Eine verminderte Triglyzeridkonzentration in der Leber von Ratten nach langer Fütterung von oxidiertem Fett konnte bereits in einer anderen Studie beobachtet werden (Sülzle *et al*., 2004). Im Kurzzeitversuch dagegen führte allein die Fütterung von Clofibrat zu einer verminderten Triglyzeridkonzentration in den Fötenlebern. Zusätzlich konnte auf Genexpressionsebene ein deutlich stärkerer Effekt durch Clofibrat als durch oxidiertes Fett im Kurzzeitversuch beobachtet werden. Die geringere Fütterungsdauer von oxidiertem Fett war möglicherweise die Ursache für das Ausbleiben des Effektes auf phenotypischer Ebene im Kurzzeitversuch.

PPARα-Aktivatoren sind Peroxisomenproliferatoren mit einer hohen Affinität zu PPARα und einer hepatokarzinogenen Wirkung (Reddy & Rao, 1989; Reddy & Rao, 1977). Im Langzeitversuch führte die Fütterung oxidierter Fettsäuren zu einer erhöhten Expression der Protoonkogene in den Lebern der trächtigen Muttertiere im Vergleich zur Kontrollgruppe. Daraus lässt sich schließen, dass bereits beschriebene pathologische Effekte der PPARα-Aktivatoren nach der Verabreichung über einen langen Zeitraum in den Lebern der Muttertiere hervorgerufen werden können. In der vorliegenden Arbeit hingegen weisen die unveränderten hepatischen mRNA Konzentrationen der Protoonkogene im Kurz- und im Langzeitversuch darauf hin, dass die hepatokarzinogene Wirkung des oxidierten Fettes im Fötus ausbleibt. Im Gegensatz dazu konnte in einer Studie beobachtet werden, dass der PPARα-Aktivator Fenofibrat in trächtigen Ratten ein verzögertes Wachstum von Föten hervorrufen kann (Soria *et al*., 2002).

Oxidiertes Fett sollte als Bestandteil der Nahrung trächtiger und laktierender Nager kritisch betrachtet werden. Sowohl in den Untersuchungen während der Trächtigkeit in der vorliegenden Arbeit, als auch während der Laktation in einer anderen Studie (Ringseis *et al*., 2007*c*) konnten problematische Einflüsse auf Adaptationsprozesse in den Muttertieren durch Komponenten oxidierter Fette beobachtet werden. Die Gabe von oxidiertem Fett während der Trächtigkeit führte zu einer gesteigerten Fettsäureverwertung im Muttertier und im Fötus, was in verminderten Triglyzeridkonzentration in der Leber der trächtigen Ratten sichtbar wurde. Ferner zog die Gabe von oxidiertem Fett während der Laktation reduzierte Triglyzeridkonzentration in der Milch laktierender Ratten nach sich, was auf einer verminderten Expression von Fettsäuretransportern und LPL in der Milchdrüse basierte (Ringseis *et al*., 2007*c*). Weiterhin erfolgte während der Trächtigkeit möglicherweise ein Übergang von Komponenten oxidierter Fette in den fötalen Kreislauf mit anschließender PPARα-Aktivierung. Verminderte Triglyzeridkonzentrationen in der Leber von Föten und in der Milch laktierender Ratten können möglicherweise ein verzögertes Wachstum und eine höhere Sterblichkeitsrate der Jungtiere nach sich ziehen (Ringseis *et al*., 2004; Soria *et al*., 2002).

3.4 Effekte von *conjugated linoleic acids* (CLA) während der Laktation

Es ist bereits bekannt, dass die Gabe von CLA zu verminderten Triglyzeridkonzentrationen in der Milch laktierender Ratten, Kühe, Schafe und Menschen führt (Ringseis *et al*., 2004; Hayashi *et al*., 2007; Baumgard *et al*., 2001 und 2002; Havartine & Baumann 2006; Lock *et al*., 2006; Masters *et al*., 2002). Zudem konnte bereits ermitteln werden, dass dieser Effekt auf

einer reduzierten de-*novo* Fettsäuresynthese sowie Aufnahme von Fettsäuren aus zirkulierenden triglyzeridreichen Lipoproteinen in die Milchdrüse laktierender Spezies basiert. Auf diesen Beobachtungen basiert die Fragestellung, ob möglicherweise eine verminderte Aufnahme von NEFA aus dem Plasma über eine Beeinflussung der Fettsäuretransporter der Milchdrüse ebenso zu den verringerten LCFA- und Triglyzeridkonzentrationen in der Milch laktierender Ratten beiträgt. In Komplex 4 konnte zum ersten Mal gezeigt werden, dass die Fettsäuretransporter FAT/CD36, FATP und FABPpm, zu deren Aufgabe die zelluläre Aufnahme albumingebundener NEFA aus dem Plasma in die Milchdrüse zählt, auf transkriptioneller Ebene vermindert sind, verglichen mit den Expressionen der Fettsäuretransporter in der Milchdrüse der laktierenden Kontrollratten. Dies bestätigt die Annahme, dass CLA die Genexpressionen der Fettsäuretransporter vermindern und dies gemeinsam mit der verminderten Aktivität des SREBP-1c sowie der verringerten Expression und Aktivität der LPL zu beträchtlich reduzierten Konzentrationen an NEFA im Plasma sowie LCFA und Triglyzeriden in der Milch laktierender Ratten führt (Ringseis *et al*., 2004; Hayashi *et al*., 2007).

In laktierenden Ratten besteht trotz beachtlicher Energieaufnahme eine stete negative Energiebilanz (Smith & Grove, 2002; Peterson & Baumgard, 1971). Auf Grund dessen leisten die aus dem weißen Fettgewebe in das Blutplasma abgegebenen NEFA einen wichtigen Beitrag zur Sicherung der Milchfettsynthese in der Milchdrüse. Allerdings konnte in Komplex 4 eine nach CLA-Gabe deutlich erhöhte NEFA-Plasmakonzentration verglichen mit der Kontrollgruppe während der Laktation beobachtet werden. Vermutlich ist dies eine Folge der verminderten Kapazität der Milchdrüse NEFA aus dem Plasma aufzunehmen. Zusätzlich führte die Gabe von CLA zu einer erhöhten Aktivität der *hormone-sensitive lipase* (HSL) im weißen Fettgewebe, was eine gesteigerte Freisetzung von NEFA nach sich zieht und die Konzentration im Plasma erhöht. Im Gegensatz dazu waren im ersten Komplex die NEFA-Konzentrationen im Plasma der laktierenden Ratten und Mäuse infolge der verringerten Aktivität des PPARα während der Laktation vermindert. Es kann ausgeschlossen werden, dass eine abweichende Nahrungsaufnahme der CLA-Ratten von denen der Kontrollratten zu einer erhöhten NEFA-Konzentration im Plasma führte, denn die Futteraufnahme der CLA-Gruppe entsprach der Futteraufnahme der Kontrollgruppe (Ringseis *et al*., 2004). In Kühen ist der Beitrag zur Milchfettbildung durch NEFA aus dem weißen Fettgewebe lediglich zu Beginn der Laktation von Bedeutung, was im Verlauf der Laktationsphasen abnimmt. Die Futteraufnahme laktierender Kühe ist ausreichend, um geleerte Speicher wieder aufzufüllen und Substrate zur Milchfettbildung bereitzustellen. Daher ist es nicht verwunderlich, dass

durch CLA-Fütterung die Konzentrationen der NEFA im Plasma von Milchkühen nicht beeinflusst wird (Perfield *et al*., 2002; Castañeda-Gutiérrez *et al*., 2007).

Die Fettsäuren für die Milchfettsynthese in der Milchdrüse stammen aus unterschiedlichen Quellen. Die *medium chain fatty acids* (MCFA) (C8-C14) werden durch die SREBP-1c gesteuerte de *novo*-Fettsäuresynthese in der Milchdrüse gebildet (Barber *et al*., 2003). Aus der Nahrung stammen vorwiegend die LCFA (C16; C18-C22). Diese gelangen entweder in Form von triglyzeridreichen Lipoproteinen über das Plasma zur Milchdrüse oder werden aus den Speichergeweben freigesetzt und erreichen in Form von albumingebundenen NEFA die Milchdrüse, wo sie über Fettsäuretransporter aufgenommen werden. Die NEFA und LCFA aus den Lipoproteinen gelangen dann nach Abspaltung über die Fettsäuretransporter in die Milchdrüsenzellen (Ringseis *et al*., 2004; Green *et al*., 1981; Ross *et al*., 1985; Scow *et al*., 1977). Durch die Fütterung der CLA war der absolute Anteil der MCFA und LCFA in der Milch der Ratten während der Laktation deutlich vermindert, was auf eine reduzierte de *novo*-Biosynthese von Fettsäuren und Fettsäureaufnahme aus triglyzeridreichen Lipoproteinen durch die LPL sowie, wie in Komplex 4 gezeigt, auf eine Verminderung der Fettsäuretransporter (FAT/CD36, FABPpm und FATP) zurückgeführt werden kann. Der genaue Mechanismus, auf welchem Weg die CLA Einfluss auf die Fettsäuretransporter in der Milchdrüse nehmen, ist bisher unbekannt und bedarf weiterer Untersuchungen.

4. Zusammenfassung

Die Laktation ist ein physiologischer Zustand, welcher durch einen dramatischen Anstieg des Energie- und Nährstoffbedarfs infolge der Milchproduktion charakterisiert ist. Zu einem Teil wird die erhöhte Nachfrage über eine gesteigerte Nahrungsaufnahme und Mobilisation metabolischer Substrate aus den Energiespeichern gedeckt. Des Weiteren entwickeln sich während der Laktation verschiedene Anpassungsprozesse im Stoffwechsel, mit dem Ziel, Energie und metabolische Substrate zugunsten der Milchbildung in der Milchdrüse einzusparen. In Studien mit laktierenden Ratten konnte bereits eine reduzierte zitterfreie Thermogenese im braunen Fettgewebe und im Skelettmuskel ermittelt werden. Des Weiteren ist bekannt, dass die Fettsäureaufnahme und -oxidation im Skelettmuskel sowie die Fettsäureoxidation und Ketonkörperproduktion in der Leber während der Laktation vermindert ist. Bisher fehlen jedoch Kenntnisse zu molekularen Ursachen dieser Anpassungsprozesse. Der *peroxisome proliferator-activated receptor α* (PPARα) vermittelt die transkriptionelle Regulation von Proteinen der Thermogenese, der Fettsäureaufnahme und -oxidation. Die physiologische Funktion des PPARα liegt in der Regulation des Stoffwechsels im Fastenzustand. Daher führt eine Liganden-induzierte PPARα-Aktivierung zu einer Hochregulierung einer Vielzahl von Genen des Fettsäurekatabolismus, einschließlich der zellulären Fettsäureaufnahme und dem Fettsäuretransport, der mitochondrialen und peroxisomale Fettsäureoxidation sowie der Ketogenese. Darüber hinaus führte die Gabe von starken PPARα-Aktivatoren, wie Fibraten, während der Laktation zur Beeinträchtigung der Energiesparmechanismen. Aufgrund dessen lag die Vermutung nahe, dass eine reduzierte PPARα-Aktivierung die Ursache für die verminderte Expression von Genen der Fettsäureaufnahme und -oxidation sowie des UCP während der Laktation darstellt. Diese Zusammenhänge sollten im *ersten Komplex* dieser Arbeit untersucht werden.

Im *ersten Komplex* dieser Arbeit sollte anhand eines Versuches mit Ratten die Hypothese geprüft werden, dass die Expression des PPARα während der Laktation vermindert ist und dies zu einer Herunterregulierung hepatischer Enzyme der Carnitinsynthese und der *novel organic cation transporter* (OCTN) führt. Dazu wurden am ersten Tag der Laktation 32 Ratten sowie deren Würfe in 4 Gruppen zu je 8 Ratten eingeteilt und die Wurfgrößen auf 0, 4, 10 und 18 Jungtiere je Muttertier angeglichen. Die relativen *messenger ribonucleic acid* (mRNA) Konzentrationen des PPARα, einiger PPARα-regulierter Gene des Fettsäurekatabolismus sowie von Enzymen der Carnitinsynthese (*Trimethyllysindioxygenase* (TMLD), *4-N-Trimethylaminobutyroaldehyddehydrogenase* (TMABA-DH), *γ-Butyrobetain-*

dioxygenase (BBD), und der OCTN1 waren in der Leber laktierendender Ratten tatsächlich geringer, unabhängig von der Wurfgröße, als in nicht-laktierenden Ratten. Ferner führte die Laktation zu einer reduzierten Aktivität der hepatischen BBD und zu geringeren Carnitinkonzentrationen in Plasma, Leber und Skelettmuskel der laktierenden Muttertiere verglichen mit den nicht-laktierenden Muttertieren. In der vorliegenden Arbeit konnte zum ersten Mal gezeigt werden, dass die Laktation, unabhängig von der Wurfgröße, zu einer Herunterregulierung des PPARα und PPARα-regulierter Gene der Carnitinsynthese und Carnitinaufnahme in der Leber führt. Diese Beobachtungen weisen darauf hin, dass der PPARα an der Regulation von Adaptationsprozessen während der Laktation beteiligt ist.

Im *ersten Komplex* der vorliegenden Arbeit sollte weiterhin geprüft werden, ob die verminderten Expressionen von Genen der Fettsäureaufnahme und der β-Oxidation in Geweben mit einer hohen Aktivität des Fettsäurekatabolismus, wie Leber und Skelettmuskel, im Rahmen der Adaptationsprozesse über den Transkriptionsfaktor PPARα während der Laktation vermittelt werden. Hierfür wurde ein Versuch mit weiblichen laktierenden und nicht-laktierenden PPARα-*knockout* Mäusen und entsprechenden weiblichen laktierenden und nicht-laktierenden Wildtyp-Mäusen durchgeführt. Bei den Wildtyp-Mäusen führte die Laktation zu einer erheblichen Verminderung der Expression des PPARα, der PPAR Coaktivatoren PPARγ *coactivator* (PGC) 1α und 1β sowie von PPARα-Zielgenen der Fettsäureaufnahme, Fettsäureoxidation und Thermogenese in Leber und Skelettmuskel. Die Laktation führte in den PPARα-*knockout* Mäusen nicht zu einer verminderten Expression der Gene der Fettsäureaufnahme und Fettsäureoxidation sowie der Thermogenese in Leber und Skelettmuskel. Zusätzlich waren die mRNA-Konzentrationen aller untersuchten PPARα-Zielgene in Leber und Skelettmuskel der PPARα-*knockout* Mäuse niedriger als in den nicht-laktierenden Wildtyp-Mäusen. Die mRNA-Level der PPARα-Zielgene der laktierenden und nicht-laktierenden PPARα-*knockout* Mäuse befanden sich auf gleicher Höhe wie die der laktierenden Wildtyp-Mäuse. Weiterhin konnte sowohl bei den laktierenden PPARα-*knockout* Mäusen als auch bei den laktierenden Wildtyp-Mäusen eine Genotyp-unabhängige gesteigerte Expression der *fatty acid translocase* CD36 (FAT/CD36), des *fatty acid transport protein* (FATP), der *lipoprotein lipase* (LPL) und der *fatty acid synthase* (FAS) im Vergleich zu den nicht-laktierenden Mäusen in der Milchdrüse beobachtet werden. In der vorliegenden Arbeit konnte zum ersten Mal gezeigt werden, dass die Herunterregulierung des PPARα und dessen Coaktivatoren in Geweben mit einer hohen Aktivität des Fettsäurekatabolismus für die verminderte Fettsäureverwertung in Leber und Skelettmuskel sowie für die reduzierte Thermogenese im Skelettmuskel verantwortlich ist. Diese Anpassungsprozesse im

Stoffwechsel laktierender Mäuse verfolgen das Ziel, Energie und metabolische Substrate einzusparen, um diese für die Milchproduktion in der Milchdrüse während der Laktation bereitzustellen.

PPARα fungiert als Vermittler der primären adaptativen Antwort während des Fastens indem die Expression einer Reihe von Genen des Fettsäurekatabolismus gesteigert wird. Dazu zählen ebenso die Gene des carnitinabhängigen Transportsytems, welches für den Transfer aktivierter Fettsäuren in die mitochondriale Matrix verantwortlich ist. Da die *carnitine-acylcarnitine translocase* (CACT) einen Teil dieses Systems darstellt, sollte im *zweiten Komplex* der vorliegenden Arbeit geprüft werden, ob der PPARα ebenso für die Regulierung dieses Enzymes in der Leber verantwortlich ist. Hierfür wurden zwei Versuche mit PPARα-*knockout* Mäusen und Wildtyp-Mäusen, welche entweder vor dem Schlachten für 48 Stunden in einen Fastenzustand versetzt oder mit dem PPARα-Agonist WY-14,643 behandelt wurden, durchgeführt. Da einige PPARα-Zielgene ebenso durch andere PPAR Isotypen reguliert werden (PPARγ und PPARδ), welche teilweise überschneidende biologische Funktionen haben, wurden zusätzlich Zellversuche mit kultivierten Leberzellen (HepG2) realisiert. Die Leberzelllinien wurden mit dem PPARα-Agonisten WY-14,643 behandelt und dieser Effekt auf die Expression der CACT mit den Effekten des PPARγ-Agonisten Troglitazon (TGZ) und dem PPARδ-Agonisten GW0742 auf die HepG2-Zellen verglichen. Weiterhin wurden CACT-Promotoraktivitätsstudien und elektrophoretische Analysen durchgeführt. Fasten und die Behandlung mit dem PPARα-Agonist WY-14,643 führte zu einer Erhöhung der hepatischen relativen mRNA Konzentrationen der CACT in Wildtyp-Mäusen, jedoch nicht in PPARα-*knockout* Mäusen. In Zellversuchen konnten nach der Inkubation mit WY-14,643 und dem PPARδ-Agonist GW0742 erhöhte relative mRNA Konzentrationen der CACT in Leberzellen ermittelt werden. Die Inkubation mit dem PPARγ Agonisten TGZ führte nicht zu einer erhöhten Expression der CACT. Ferner zeigten Reporter Assays die Aktivierbarkeit des Maus CACT Promotors durch WY-14,643 und GW0742, allerdings nicht durch TGZ. Darüber hinaus konnte durch Analysen des Promotors und der 5´-UTR ein funktionelles PPRE in der 5´-UTR der Maus-CACT nachgewiesen werden. Die Ergebnisse zeigen, dass PPARα und PPARδ, die beide eine bedeutende Rolle für die Regulation der Fettsäureoxidation während des Fastens einnehmen, neben der CPT I und CPT II ebenso eine Hochregulierung der CACT hervorrufen können. Dadurch erhöht sich der Transport aktivierter Fettsäuren in die mitochondriale Matrix, was eine gesteigerte β-Oxidation während des Fastens nach sich zieht.

Einen weiteren Untersuchungsgegenstand stellte der Einfluss natürlicher PPARα-Aktivatoren während Trächtigkeit und Laktation im Nager dar. Komponenten von oxidiertem Fett, wie

oxidierte Fettsäuren, rufen eine PPARα-Aktivierung in der Leber von Ratten hervor. Im *dritten Komplex* dieser Arbeit sollte daher geprüft werden, ob die Fütterung von oxidiertem Fett bereits während der Trächtigkeit eine PPARα-Aktivierung in der fötalen Rattenleber hervorruft. Dazu wurden ein Lang- und ein Kuzzeitversuch mit trächtigen Ratten durchgeführt, welche in jeweils 3 Gruppen eingeteilt wurden (Kontrolle, Clofibrat, oxidiertes Fett). Im Kurzzeitversuch wurde über einen kurzen Zeitraum am Ende der Trächtigkeit oxidiertes Fett verabreicht und im Langzeitversuch über die gesamte Trächtigkeit mit einer semisynthetischen Diät gefüttert, worin oxidiertes Fett enthalten war. Clofibrat und oxidiertes Fett führte in beiden Versuchen zu einer PPARα-Aktivierung und erhöhten relativen mRNA Konzentrationen der *acyl-CoA oxidase* (ACO), *cytochrome* P-450 A1 (CYP4A1), *carnitine palmitoyltransferase* (CPT) I, *medium-chain acyl-CoA dehydrogenase* (MCAD) und *long-chain acyl-CoA dehydrogenase* (LCAD) in der Leber der trächtigen Ratten verglichen mit den mRNA Konzentrationen der trächtigen Kontrollratten. In beiden Versuchen waren die relativen mRNA Konzentrationen von ACO, Cyp4A1, CPT I, MCAD und LCAD in den Lebern der Föten der Clofibratgruppe und der mit oxidiertem Fett gefütterten Gruppe höher als in der Kontrollgruppe, während sich die relativen mRNA Konzentrationen von PPARα, *sterol regulatory element-binding protein* (SREBP) 1c und *fatty acid synthase* (FAS) zwischen den Behandlungsgruppen nicht unterschieden. Im Langzeitversuch führte die Fütterung von oxidiertem Fett zu verminderten Triglyzeridkonzentrationen in der Leber der Muttertiere und Föten. Im *dritten Komplex* dieser Arbeit konnte zum ersten Mal gezeigt werden, dass bereits während der Trächtigkeit in oxidiertem Fett enthaltene Fettsäuren mit PPARα-aktivierendem Potential aus der Nahrung der Muttertiere eine Aktivierung des PPARα in der fötalen Leber hervorrufen können.

In verschiedenen tierexperimentellen Untersuchungen konnte beobachtet werden, dass die Gabe von *conjugated linoleic acids* (CLA) in der Milch laktierender Spezies zu reduzierten Triglyzeridkonzentration führte, was auf eine verminderte de-*novo* Fettsäuresynthese in der Milchdrüse und eine reduzierte Aufnahme von Fettsäuren aus triglyzeridreichen Lipoproteinen in die Milchdrüse zurückgeführt wurde. In der vorliegenden Arbeit sollte in einem *vierten Komplex* geprüft werden, ob die durch CLA-Fütterung hervorgerufenen verminderten Triglyzeridkonzentrationen in der Milch laktierender Spezies ebenso die Folge einer reduzierten Aufnahme von *nonesterified fatty acids* (NEFA) aus dem Plasma über die Fettsäuretransporter in die Milchdrüse sind. Hierfür wurden 24 weibliche Ratten in 2 Gruppen eingeteilt, welche entweder eine CLA-angereicherte Diät mit einer CLA-Konzentration von 1,47g/100g Diät oder eine Kontrolldiät mit Sonnenblumenöl erhielten. Die Fütterung von

CLA führte zu einer deutlich erhöhten NEFA-Konzentration im Plasma der laktierenden Ratten. Zusätzlich war die Expression der Fettsäuretransporter, FAT/CD36, FATP und *plasma membrane fatty acid binding protein* (FABPpm) geringer in der Milchdrüse der laktierenden CLA-Ratten verglichen mit den laktierenden Ratten der Kontrollgruppe. Diese Beobachtungen zeigen, dass CLA die Aufnahme der im Plasma zirkulierenden NEFA aus dem weißen Fettgewebe in die Milchdrüse reduzieren und dies vermutlich zu den verminderten Triglyzeridkonzentrationen in der Milch von Ratten während der Laktation beiträgt.

Zusammenfassend kann gesagt werden, dass in der vorliegenden Arbeit neue Einflüsse des Transkriptionsfaktors PPARα auf Stoffwechselprozesse während der Laktation im Nager gezeigt werden konnten. Die Beobachtungen in dieser Arbeit lassen darauf schließen, dass PPARα die Verantwortung für Adaptationsprozesse im Lipid- und Energiestoffwechsel im Nager während der Laktation übernimmt und somit einen beträchtlichen Beitrag zur Einsparung metabolischer Substrate und Energie für die Milchsynthese in der Milchdrüse leistet. Weiterhin konnte gezeigt werden, dass eine Hochregulierung der CACT durch den PPARα erfolgt und zu einer Erhöhung des Imports von Acylcarnitin in die mitochondriale Matrix während des Fastens führen kann. Des Weiteren konnten in der vorliegenden Arbeit neue ungünstige Wirkmechanismen natürlicher PPARα-Agonisten, wie CLA und oxidierten Fettsäuren, während der Trächtigkeit und Laktation ermittelt werden.

5. Summary

Lactation is a physiological state characterized by an organism's dramatic increase of energy and nutrient requirements for milk production. This demand is met by a markedly increased food intake and by mobilisation of metabolic substrates from energy stores, e.g. within white adipose tissue. In addition, several metabolic adaptions develop in the lactating animal to conserve energy and metabolic substrates for milk production in the mammary gland. It has been shown that thermogenesis in brown adipose tissue and sceletal muscle as well as the fatty acid oxidation in sceletal muscle and rates of fatty acid oxidation and ketogenesis in the liver are reduced during lactation. However, the molecular regulation of these metabolic adaptions is unknown. Proteins of thermogenesis and fatty acid uptake and fatty acid oxidation are transcriptionally regulated by *peroxisome proliferator activated receptor* α (PPARα). The physiologic function of the PPARα lies in the metabolic response to fasting. Thus, a ligand-induced PPARα activation leads to an up-regulation of genes involved in all aspects of fatty acid catabolism including cellular fatty acid uptake and transport, mitochondrial and peroxisomal fatty acid oxidation as well as ketogenesis. Moreover, an impairment of lactation-induced energy-sparing mechanisms by administration of the PPARα-activators such as fibrates have been observed in a recent study. These findings suggested that down-regulation of genes involved in fatty acid uptake and oxidation as well as UCP is mediated by suppression of PPARα during lactation. This assumption should be examined in a part of this work.

Therefore 32 rats and their litters were assigned to 4 groups of 8 rats each at day one of lactation and litters were adjusted to sizes of 0, 4, 10 and 18 pubs per dam. *Complex 1* aimes to investigate the hypothesis that the *messenger ribonucleic acid* (mRNA) expression of PPARα in the liver is lowered during lactation and that this leads to a down-regulation of hepatic genes of carnitine synthesis and *novel organic cation transporters* (OCTN). Dams suckling their litters, irrespective of litter size, had lower relative mRNA concentrations of PPARα, some PPARα target genes involved in fatty acid catabolism, as well as enzymes of carnitine synthesis *(trimethyllysine dioxygenase* (TMLD), *4-N-trimethylaminobutyroaldehyde dehydrogenase* (TMABA DH), *γ-butyrobetaine dioxygenase* (BBD)), and OCTN 1 in the liver compared to those whose litters were removed. Furthermore, lactation led to a reduced activity of the hepatic BBD and reduced concentrations of carnitine in plasma, liver and skeletal muscle in dams with litters compared to those whose litters where removed. This study shows for the first time that lactation, irrespective of litter size, leads to a down-regulation of PPARα and PPARα-regulated genes involved in carnitine synthesis and

carnitine uptake in the liver. These observations suggest that PPARα is involved in the regulation of the metabolic adaptions during lactation.

Therefor in the *first complex* the hypothesis should be tested that down-regulation of PPARα mediates the reduced expression of genes involved in fatty acid uptake and β-oxidation in tissues with high rates of fatty acid utilization such as liver and skeletal muscle, which favor the availibiliy of fatty acids for milk triacylglycerol synthesis in the mammary gland. Therefor an experiment with female lactating and non-lactating PPARα *knockout* mice and corresponding female wild-type mice was carried out. In wild-type mice, lactation led to a down-regulation of PPARα, PPAR coactivators PPARγ coactivator (PGC) 1α and 1β, and PPARα target genes involved in fatty acid uptake, fatty acid oxidation, and thermogenesis in the liver and skeletal muscle. Lactation did not lower the expression of genes involved in fatty acid utilization and thermogenesis in liver and skeletal muscle. In addition PPARα *knockout* mice had generally lower expression of all these PPARα target genes in liver and skeletal muscle. The expression levels of PPARα target genes in lactating wild-type mice were similar to those of the lactating or non-lactating PPARα *knockout* mice. Furthermore lactation led to an increased expression of *fatty acid translocase CD36* (FAT/CD36), *fatty acid transport protein* (FATP), *lipoprotein lipase* (LPL) and *fatty acid the synthase* (FAS) in the mammary gland of both genotypes wild-type mice and PPARα knockout mice. These findings suggest that down-regulation of PPARα and its coactivators in tissues with high rates of fatty acid catabolism is responsible for the reduced utilization of fatty acids in liver and skeletal muscle and for the reduced thermogenesis in the skeletal muscle which aim to conserve energy and metabolic substrates for milk production in the mammary gland during lactation.

PPARα acts as the primary mediator of the adaptive response to fasting by stimulating the transcription of a large number of genes involved in fatty acid catabolism. The *carnitine acylcarnitine translocase* (CACT) mediates the import of activated fatty acids into the mitochondrial matrix where β-oxidation takes place. In *complex 2* it should be investigated, wether CACT is also regulated by PPARα in the liver. Therefore two experiments with PPARα *knockout* mice and wild-type mice which either were fasted for 48 hours or treated with WY-14,643 were carried out. Several PPARα target genes are also regulated by other PPAR isotypes (PPARγ and PPARδ), which have partially overlapping biological functions. Thus experiments with cultured liver cells which were treated with WY-14,643 were performed and compared the effect on CACT expression with that troglitazone (TGZ), a agonist of PPARγ, and GW0742, a selective agonist of PPARδ. Furthermore, CACT promotor activation studies and gel shift assays were performed. Fasting and treatment with the PPARα

agonist WY-14,643 led to an increase of the hepatic relative mRNA concentration of CACT in wildtype-mice, but not in PPARα *knockout* mice. Cell culture experiments revealed that CACT mRNA abundance was higher in liver cells treated with either WY-14,643 or the PPARδ agonist GW0742, but not with PPARγ agonist troglitazone (TGZ) than in control cells. Furthermore, reporter assays demonstrated the activation of mouse CACT promoter by WY-14,643 and GW0742, but not by TGZ. Moreover, further analyses of CACT promotor and 5´-UTR revealed one functional PPRE in the 5´-UTR of mouse CACT. The upregulation of CACT by PPARα and PPARδ, which are both important for the regulation of fatty acid oxidation during fasting, may increase the import of acylcarnitine into the mitochondrial matrix during fasting.

The influence of natural PPARα activators during pregnancy and lactation represented a further object of investigation. Oxidized fat causes an activation of PPARα in the liver of rats. *Complex 3* aimed to explore whether feeding oxidized fat during pregnancy causes a PPARα activation in fetal rat liver. Thus, a long-term and a short-term experiment with pregnant rats was carried out. The rats were divided in 3 groups (control, clofibrat, oxidized fat) with a controlled feeding regimen during either the late stages of pregnancy (short-term experiment) or during the whole pregnancy (long-term experiment). In both experiments pregnant rats treated with oxidized fat or clofibrate had higher relative mRNA concentrations of the PPARα-responsive genes *acyl-CoA oxidase* (ACO), *cytochrome P-450 A1* (CYP4A1), *carnitine palmitoyltransferase* (CPT) I, *medium chain acyl CoA dehydrogenase* (MCAD) and *long chain acyl CoA dehydrogenase* (LCAD) in the liver than control rats. Furthermore, in both experiments relative mRNA concentrations of ACO, Cyp4A1, CPTI, MCAD and LCAD in fetal rat livers were higher in the treatment groups of clofibrate and oxidized fat than in the control group, whereas the relative mRNA concentrations of PPARα, *sterol regulatory regulatory element-binding protein* (SREBP) 1c and *fatty acid synthase* (FAS) did not differ between the treatment groups and the control group. In the long-term experiment treatment with oxidized fat reduced triacylglycerol concentrations in the livers of pregant rats and fetuses. In conclusion the study shows for the first time that components of oxidized fat with PPARα activating potential are able to induce a PPARα response in the liver of fetuses.

In several studies dietary *conjugated lioleic acids* (CLA) led to a reduction of triacylglycerol concentration in the milk of humans and animals, which was mediated trough the decreased de novo fatty acid synthesis in the mammary gland and the reduced uptake of fatty acids from triacylglycerol-rich lipoproteins into the mammary gland. Whether the reduced concentrations of triacylglycerols in the milk by dietary CLA are also a consequence of decreased uptake of

nonesterified fatty acids (NEFA) by fatty acid transporters from plasma into the lactating mammary gland has not been investigated and should be observed in *complex 4*. Therefor 24 female rats were divided into 2 groups, which received either a CLA enriched diet with a concentration of 1,47g CLA/100g diet or a control diet containing sunflower oil. Dams fed diets with CLA had a greater concentration of NEFA in the plasma than those fed the control diet. The relative mRNA concentrations of the fatty acid transporters CD36/FAT, FATP and *plasma membrane fatty acid binding protein* (FABPpm) were lower in the mammary glands of lactating dams fed diets with CLA compared to those fed the control diet. These findings indicate that dietary CLA reduces the uptake of NEFA released from white adipose tissue in the mammary gland and this could contribute to the reduction of the triacylglycerol concentrations in the milk in lactating rats.

In summary the present investigations show new effects of the transcription factor PPARα on metabolic processes in rodents during lactation. The observations in this work suggest that PPARα mediates the metabolic adaptations in the lipid and energy metabolism and that this aims to conserve energy and metabolic substrates for milk production in the mammary gland in rodents during lactation. Further, upregulation of CACT by PPARα may increase the import of acylcarnitine into the mitochondrial matrix during fasting, has been investigated. Moreover, new unfavorable effects of natural PPARα agonists like CLA and oxidized fatty acids were determined during pregnancy and lactation.

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Anke Gutgesell

Erklärung

Hiermit versichere ich an Eides Statt, dass ich die eingereichte Dissertation: *„Untersuchungen zur Wirkung synthetischer und nutritiver PPARα-Agonisten auf Stoffwechselwege in besonderen Stoffwechselsituationen im Nager“* selbstständig angefertigt und diese nicht bereits für eine Promotion oder ähnliche Zwecke an einer Universität eingereicht habe. Des Weiteren versichere ich, dass ich die zur Erstellung der Dissertationsschrift verwendeten wissenschaftlichen Arbeiten und Hilfsmittel genau und vollständig angegeben habe.

Zudem erkläre ich, dass keine Strafverfahren gegen mich anhängig sind.

Halle (Saale), den 07.12.2009

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Anke Gutgesell

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