Institut für Agrar- und Ernährungswissenschaften
(Direktor: Prof. Dr. R. Jahn)
der
Naturwissenschaftlichen Fakultät III
(Dekan: Prof. Dr. P. Wycisk)
der
Martin-Luther-Universität
Halle-Wittenberg

„Untersuchungen zum Einfluss verschiedener Nahrungsproteine auf den Lipidstoffwechsel“

Dissertation

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Diplom-Ernährungswissenschaftlerin
Julia Spielmann

Gutachter: Prof. Dr. habil. Klaus Eder
Prof. Dr. habil. Gabriele I. Stangl
Prof. Dr. habil. Gerald Rimbach

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Abkürzungsverzeichnis

ACC	Acetyl-CoA-Carboxylase
CYP4A1	Cytochrom P_{450}A1
CYP7A1	Cholesterin-7α-Hydroxylase
ER	endoplasmatisches Retikulum
FAS	*fatty acid synthase*
HDL	*high density lipoprotein*
HMG-CoA	3-Hydroxy-3-Methylglutaryl-Coenzym-A
Insig	*insulin-induced gene*
KHK	koronare Herzkrankheiten
LCAT	Lecithin-Cholesterin-Acyltransferase
LDL	*low density lipoprotein*
SCAP	SREBP *cleavage-activating protein*
SRE	*sterol response elements*
SREBP	*sterol regulatory element-binding protein*
VLDL	*very low density lipoprotein*

1. Einleitung

Atherosklerose ist die Hauptursache für koronare Herzkrankheiten (KHK), welche zu den häufigsten Todesursachen in der westlichen Bevölkerung gehören. Die Atherosklerose ist eine Systemerkrankung, während der es durch vielfältige Prozesse zur Ablagerung und Akkumulation von Cholesterin in Entzündungsherden der Gefäßwand kommt. Neben Übergewicht, Bluthochdruck, Bewegungsmangel, Diabetes mellitus, freien Radikalen z.B. durch Zigarettenrauch, erhöhten Homocystein-Konzentrationen im Plasma und der Kombination dieser Risikofaktoren spielen vor allem Imbalancen des Lipidstoffwechsels eine große Rolle bei der Entstehung von Atherosklerose. In epidemiologischen Studien konnte ein deutlicher Zusammenhang zwischen erhöhten Serum-Cholesterinspiegeln und KHK nachgewiesen werden (Law *et al.* 1994). Cholesterin wird im Blut in so genannten Lipoproteinen, makromolekularen Fett-Proteinkomplexen, transportiert. Der größte Teil des Cholesterins zirkuliert verpackt in *low density lipoproteins* (LDL-Cholesterin) im Körper. Erhöhte LDL-Cholesterin-Gehalte im Blut erhöhen das Risiko des Plaque-Wachstums. So sind erhöhte Plasmagehalte des LDL-Cholesterins ein wesentlicher Risikofaktor für die Entstehung der KHK (Adult Treatment Panel III 2002, Grundy *et al.* 2004). In vielen Studien konnte gezeigt werden, dass die Absenkung des LDL-Cholesterins das Risiko für KHK verringern kann (Baigent *et al.* 2005). Neben erhöhten Konzentrationen an LDL-Cholesterin können auch niedrige Gehalte von Cholesterin in den *high density lipoproteins* (HDL-Cholesterin) die Gefahr für KHK erhöhen (Vega und Grundy 1996, Adult Treatment Panel III 2002). Diese Makromoleküle, die in erster Linie in der Leber und im Darm synthetisiert werden, tragen dazu bei, Cholesterin aus dem Blut zu entfernen. Deshalb wird ein niedriger Gehalt an HDL-Cholesterin mit einem erhöhten Risiko für KHK in Zusammenhang gebracht.

Abgesehen vom Plasma-Cholesterinspiegel spielen auch erhöhte Triglyceridwerte eine Rolle bei der Entstehung von Atherosklerose. So besteht eine positive Beziehung zwischen den Triglyceridgehalten im Plasma und dem Risiko, an KHK zu erkranken (Kraus *et al.* 1998, Austin *et al.* 1998). Zudem erhöht sich das Risiko an KHK zu erkranken bei gleichzeitigem Auftreten von erhöhten Triglyceridwerten und niedrigen HDL-Cholesterin-Gehalten im Plasma (Adult Treatment Panel II, 1994).

Es ist deshalb von großem wissenschaftlichem Interesse, erhöhte Cholesterin- und Triglyceridkonzentrationen bei Patienten zu vermeiden und somit zu einer Verringerung des Risikos, an KHK zu erkranken, beizutragen. Dazu stehen bereits zahlreiche Medikamente wie z.B. Fibrate und Statine, welche zur Absenkung der Cholesterin- bzw. Triglyceridkonzentrationen im Plasma führen, zur Verfügung. Allerdings können auch

Veränderungen im Ernährungsverhalten der Patienten zur Risikominderung der KHK beitragen (Kris-Etherton *et al.* 1997). Im Jahr 1908 war Ignatowski einer der ersten, der Studien an Kaninchen durchführte, in denen der alleinige Einfluss der Ernährung auf die Entstehung der Atherosklerose untersucht wurde (Ignatowski 1908a, b, 1909). Er fand heraus, dass ausgewachsene Kaninchen, welchen Fleisch gefüttert wurde, und frisch entwöhnte Kaninchen, die Milch und Eigelb erhielten, Atherosklerose entwickelten. Auch in anderen Studien aus dieser Zeit wurde ein Zusammenhang zwischen der Verfütterung von tierischen Komponenten und der Entstehung von Atherosklerose beim Kaninchen beobachtet (Lubarsch 1909, Stuckey 1912, Saltykow 1913-1914, Steinbiss 1913). Nachdem im Jahr 1913 Anitschkow und Chalatow das hohe atherogene Potential von reinem Cholesterin entdeckten, verlor die Erforschung des Zusammenhangs von Nahrungsprotein und der Entstehung von Atherosklerose an Interesse.

In den 1970er Jahren beschäftigten sich Sirtori *et al.* erneut mit der Untersuchung von Sojaprotein und dessen Einfluss auf die Lipidkonzentration im Plasma von Menschen. In einer Studie an hypercholesterolämischen Probanden stellten Sirtori *et al.* (1977) fest, dass der Austausch von tierischem gegen pflanzliches Protein aus der Sojabohne in einer Diät zur deutlichen Absenkung der Cholesterinkonzentrationen im Plasma und LDL führte. In anderen Studien konnte sowohl ein hypocholesterolämischer (Sirtori *et al.* 1979, 1995, 1998, Anderson *et al.* 1995, Iritani *et al.* 1996, Sugiyama *et al.* 1996, Tovar *et al.* 2002, Koba *et al.* 2003) als auch ein hypotriglyceridämischer Effekt (Tovar *et al.* 2002, Ascencio *et al.* 2004, Brandsch *et al.* 2006) von Sojaprotein im Vergleich zu Casein in verschiedenen Modelltieren und am Menschen beobachtet werden.

Im Zuge einer weiteren Studie konnte auch ein anderes pflanzliches Protein, das Lupinenprotein, als lipidsenkendes Protein in hypercholesterolämischen Ratten identifiziert werden (Sirtori *et al.* 2004). Lupinen gehören wie die Sojabohne zur botanischen Familie der Leguminosen und weisen ebenso einen hohen Proteingehalt auf. Der Protein-Extrakt aus der Lupine senkte sowohl die Konzentration an Cholesterin als auch an Triglyceriden im Plasma der Ratten. Um den cholesterinsenkenden Effekt des Lupinenproteins erklären zu können, wurden im Rahmen dieser Studie auch Untersuchungen an HepG2-Zellen, einer humanen Leberzelllinie, durchgeführt. Es wurde festgestellt, dass Lupinenprotein zu einer erhöhten Aktivität des LDL-Rezeptors in den Leberzellen führt und dies wahrscheinlich der Grund für die verminderten Cholesterinkonzentrationen im Plasma der Tiere war. Die molekularen Mechanismen, über welche das Lupinenprotein seine triglyceridsenkende Wirkung ausübt, konnten jedoch im Rahmen dieser Studie nicht geklärt werden.

Die gelbe Erbse (*Pisum sativum L.)* ist eine weitere Pflanze, die reich an Protein ist und wie die Sojabohne und die Lupine zur Familie der Leguminosen gehört. Sojabohne, Lupine und Erbse weisen auch bezüglich ihrer Aminosäure-Zusammensetzung Ähnlichkeiten auf. Die Ähnlichkeit im Aminosäureprofil der Proteine aus Sojabohne, Lupine und Erbse lässt vermuten, dass Erbsenprotein eine ähnliche Wirkung auf den Lipidmetabolismus haben könnte wie Soja- oder Lupinenprotein. Bisher gibt es zwar einige Studien, die sich mit der Wirkung von ganzen Erbsen in einer Diät auf den Lipidstoffwechsel beschäftigt haben (Kingman *et al.* 1993, Martins *et al.* 2004), jedoch keine Studie, in der aufbereitetes Erbsenprotein verwendet wurde.

Hohe Gehalte der Aminosäure Cystein in Proteinen wird als Ursache für deren lipidsenkende Wirkung vermutet (Lin *et al.* 2007, Bettzieche *et al.* 2008a). Abgesehen von Sojaprotein weisen auch Kartoffel- und Fischprotein hohe Gehalte an Cystein auf. Bisher wurden jedoch nur zwei Studien an Ratten durchgeführt, die zeigen konnten, dass Kartoffelprotein im Vergleich zu Casein zur Absenkung des Cholesterinspiegels im Plasma führte (De Shrijver *et al.* 1990, Morita *et al.* 1997). In einer dieser Studien variierte allerdings die Futteraufnahme der Tiere während des Versuches, so dass ein Einfluss durch die verschiedenen Futtermengen auf die Plasmalipidkonzentrationen nicht ausgeschlossen werden kann (Morita *et al.* 1997).

Es ist allgemein anerkannt, dass pflanzliche Proteine im Vergleich zu tierischen Proteinen eine positive Wirkung auf den Lipidstoffwechsel haben. Dennoch beschäftigten sich verschiedene Studien mit dem Einfluss von Fischprotein auf den Lipidstoffwechsel (Kritchevsky *et al.* 1982, Zhang und Beynen 1993, Wergedahl *et al.* 2004, Ait Yahia *et al.* 2005). Der Einfluss von Fischprotein auf den Lipistoffwechsel wird in diesen Studien kontrovers diskutiert. In einige Studien an Ratten (Murata *et al.* 2004, Ait Yahia *et al.* 2005) und einem Versuch an Kaninchen (Bergeron *et al.* 1992) konnte nach der Verfütterung von Fischprotein eine Absenkung des Triglyceridspiegels in Plasma und VLDL im Vergleich zu Casein beobachtet werden. In anderen Studien an Ratten konnte jedoch kein Einfluss von Fischprotein auf den Triglyceridspiegel in Plasma und VLDL gefunden werden (Yahia *et al.* 2003, Wergedahl *et al.* 2004). Die Datenlage bezüglich des Einflusses von Kartoffel- und Fischprotein auf den Lipidstoffwechsel ist also insgesamt unbefriedigend und bedarf weiterer Untersuchungen. Die Mehrzahl der Versuche wurde am Modelltier Ratte durchgeführt, welche sich bezüglich ihres Lipoprotein-Metabolismus stark vom Menschen unterscheidet. Ein besser geeignetes Modelltier in Bezug auf die Übertragbarkeit der Ergebnisse auf den Menschen ist das Schwein (*Sus scrofa*), das aufgrund seiner Ähnlichkeit mit dem

menschlichen Lipidstoffwechsel weitgehend als Modelltier für Untersuchungen der Hypercholesterolämie und Atherosklerose anerkannt ist (Carey 1997, van Tol *et al.* 1991).

Obwohl die lipidsenkende Wirkung von pflanzlichen Proteinen schon sehr lange bekannt ist, herrscht große Uneinigkeit darüber, welche Komponenten der Protein-Isolate für die positive Wirkung auf den Lipidstoffwechsel verantwortlich sind. Es wurde vermutet, dass spezifische Peptide des Sojaproteins den LDL-Rezeptor in Leberzellen stimulieren und somit für die hypocholesterolämische Wirkung des Sojaproteins verantwortlich sind (Cho *et al.* 2007). In weiteren Studien wurde die Hypothese verfolgt, dass spezifische Peptide, wie z.B. die α und α` Untereinheiten des 7S Globulins im Sojaprotein (Lovati *et al.* 2000) und Conglutin γ im Lupinenprotein, die Wirkung dieser Proteine auf den Cholesterinstoffwechsel vermitteln (Sirtori *et al.* 2004). Zusätzlich könnten im Sojaprotein enthaltene Isoflavone und Ballaststoffe zur hypocholesterolämischen Wirkung beitragen (Lin *et al.* 2005, Gudbrandsen *et al.* 2005). In Proteinen, die aus Lupine, Erbse oder Fisch gewonnen wurden, sind Isoflavone und Ballaststoffe jedoch nur zu einem geringen Teil oder gar nicht vorhanden.

Neben Peptiden und antinutritiven Komponenten könnte, wie bereits erwähnt, auch die Aminosäure-Zusammensetzung eine Rolle bei der Wirkung von pflanzlichen Proteinen auf den Lipidstoffwechsel spielen. Kritchevsky *et al.* (1982) war einer der ersten, der die Hypothese aufstellte, dass die hypocholesterolämische Wirkung von Sojaprotein auf ein erhöhtes Arginin-zu-Lysin-Verhältnis im Vergleich zu Casein zurückzuführen ist. Auch Proteine aus der Lupine oder Erbse weisen ein hohes Verhältnis von Arginin zu Lysin auf. Es gibt bereits einige Studien, die zeigen, dass ein erhöhtes Arginin-zu-Lysin-Verhältnis in Proteinen zur Absenkung der Serum-Cholesterinkonzentration führt (Kritchevsky *et al.* 1982, Sugano *et al.* 1984). In einigen Studien wurden Sojaprotein-Isolat und Casein mit Lysin oder Arginin versetzt, um die Auswirkung eines veränderten Arginin-zu-Lysin-Verhältnisses auf die hypo- oder hyperlipidämische Wirkung dieser Proteine in Ratten zu demonstrieren (Vahouny *et al.* 1985, Rajamohan und Kurup 1990). Die Ergebnisse dieser Studien variierten jedoch sehr stark. So führte in einigen Studien die Supplementierung einer auf Sojaprotein-basierenden Diät mit Lysin zu hypercholesterolämischen Wirkungen und die Supplementierung einer Casein-basierenden Diät mit Arginin zu hypercholesterolämischen Wirkungen der Proteine (Kritchevsky *et al.* 1982, Kritchevsky *et al.* 1987, Rajamohan und Kurup 1990). Im Gegensatz dazu führte eine Supplementierung einer auf Casein basierenden Diät mit Arginin oder einer auf pflanzlichen Proteinen basierenden Diät mit Lysin zu nur kleinen oder gar keinen Effekten auf die Plasmalipidkonzentrationen (Huff und Carroll 1980, Katan *et al.* 1982, Park und Liepa 1982, Sugano *et al.* 1982, Gibney 1983). Es gibt bisher

jedoch noch keine Studie, in der die Effekte von Arginin und Lysin in Kombination getestet wurden. Dadurch konnte bisher auch nicht die mögliche Interaktion der beiden Aminosäuren untersucht werden. Zusätzlich wurde ein Großteil der Studien in den 1980er Jahren durchgeführt, wo noch sehr wenig über die Regulation des Lipid-Metabolismus bekannt war. Deshalb sind die Mechanismen, die der Wirkung von Arginin und Lysin und deren Verhältnis untereinander auf den Lipidstoffwechsel zu Grunde liegen, zum größten Teil noch unbekannt.

Die Stoffwechselwege, über welche pflanzliche Proteine und Fischprotein ihre positiven Wirkungen auf den Lipidstoffwechsel ausüben, sind weitgehend unbekannt. In einigen Studien zur lipidsenkenden Wirkung von Sojaprotein konnte jedoch festgestellt werden, dass Sojaprotein die Transkription der *sterol regulatory element-binding proteins* (SREBPs) beeinflusst (Tovar *et al.* 2002, Nagasawa *et al.* 2003, Ascencio *et al.* 2004, Tovar *et al.* 2005, Shukla et al. 2007). Es ist bekannt, dass der Metabolismus von Cholesterin und Fettsäuren in tierischen Zellen durch den kontrollierten Transport von SREBPs vom endoplasmatischen Retikulum (ER) zum Golgi-Apparat reguliert wird. SREBPs sind membrangebundene Transkriptionsfaktoren der *basic helix-loop-helix-leucine zipper*-Familie, welche die Transkription von mehr als 30 Genen, die in den Cholesterin-, Fettsäure-, Triglycerid- und Phospholipidmetabolismus involviert sind, steuern (Brown und Goldstein 1999, Osborne *et al.* 2000, Horton *et al.* 2003). Die drei bisher bekannten SREBP-Isoformen sind SREBP-1a, SREBP-1c und SREBP-2 (Goldstein *et al.* 2002). SREBP-1c aktiviert Gene, die an der Synthese von Fettsäuren und deren Einbau in Triglyceride und Phospholipide beteiligt sind (Horton *et al.* 1998). SREBP-1a aktiviert darüber hinaus auch Gene, welche in den Cholesterinstoffwechsel involviert sind. SREBP-2 wird vor allem in der Leber exprimiert und steuert die Transkription verschiedener Gene, wie z.B. der 3-Hydroxy-3-Methylglutaryl-Coenzym-A (HMG-CoA)-Reduktase und des LDL-Rezeptors, welche in die Cholesterinsynthese und -aufnahme involviert sind (Horton *et al.* 2003).

Das inaktive SREBP *precursor* Protein ist in der Membran des ER verankert und bindet direkt nach der Translation an das SREBP *cleavage-activating protein* (SCAP). SCAP ist ein polytopisches Protein, welches eine Transport- und eine sterolsensitive Funktion erfüllt. In Zellen, in denen die Sterolkonzentration abgesunken ist, eskortiert SCAP das SREBP in Vesikel, die sie zum Golgi-Apparat transportieren. Dort wird SREBP von zwei membrangebundenen Proteasen gespalten (Brown *et al.* 2000). Durch diese Spaltung wird das aminoterminale Ende des SREBP freigesetzt, welches im Nukleus an *sterol response elements* (SREs) in der *promoter/enhancer* Region der Zielgene bindet und so als Transkriptionsfaktor wirkt. Wenn die Sterolkonzentration der Zelle hoch ist, kommt es beim SCAP zu einer

Konformationsänderung (Brown *et al.* 2002), in deren Folge die Affinität zu den *insulin-induced genes* (Insig)-1 und -2 zunimmt. Insig-1 und -2 sind membrangebundene Proteine, die an SCAP binden und somit im weiteren Verlauf zu einem Verbleib des Insig-SCAP-SREBP-Komplexes im ER führen (Yang *et al.* 2002, Yabe *et al.* 2002). Als Resultat der Bindung des SCAP-SREBP-Komplexes an Insig-1 und Insig-2 im ER bei hohen Sterolkonzentrationen wird der Transport zum und die nachfolgende proteolytische Aktivierung der SREBPs im Golgi verhindert, was zur Reduktion des aktiven Transkriptionsfaktors im Zellkern und damit zur verringerten Synthese von Fettsäuren und Cholesterin führt.

Da SREBPs Schlüsselfaktoren der Triglycerid- bzw. Cholesterinsynthese sind, schien es uns als wahrscheinlich, dass Nahrungsproteine ihre positive Wirkung auf den Lipidstoffwechsel über diese Transkriptionsfaktoren und deren Zielgene vermitteln.

2. Zielstellung

Aufgrund der viel diskutierten positiven Eigenschaften von Proteinen auf den Lipidstoffwechsel sollten im Rahmen dieser Arbeit weitere Pflanzenproteine, wie z.B. Lupinenprotein, Erbsenprotein, Kartoffelprotein und Fischprotein, mit positiven Wirkungen auf den Lipidstoffwechsel untersucht werden. Weiterhin war es Ziel dieser Arbeit, die Stoffwechselwege, über welche Nahrungsproteine den Lipidstoffwechsel beeinflussen, zu analysieren. Hierzu wurden vor allem die beiden Transkriptionsfaktoren SREBP-1 und -2 und deren Zielgene im Lipidstoffwechsel betrachtet. Weitergehende Untersuchungen verfolgten das Ziel zu klären, ob die unterschiedliche Aminosäure-Zusammensetzung von Proteinen Ursache für deren positive Wirkung auf den Lipidstoffwechsel ist.

(1) Das Ziel der ersten Studie bestand darin, die Hypothese zu verfolgen, dass die beobachtete triglyceridsenkende Wirkung von Lupinenprotein bei Ratten über eine Hemmung des Transkriptionsfaktors SREBP-1c und somit durch eine verminderte Triglyceridsynthese verursacht wird. Dazu wurden Sprague-Dawley-Ratten mit einer Diät, die 200 g/kg Lupinenprotein enthielt, über einen Zeitraum von 22 Tagen gefüttert. Die Tiere der Kontrollgruppe wurden mit der gleichen Diät gefüttert, die jedoch statt Lupinenprotein Casein enthielt, welches als Vergleichsprotein diente. Am Ende des Versuches wurden die relativen mRNA-Konzentrationen des SREBP-1c und dessen Zielgen *fatty acid synthase* (FAS) und die Triglyceridgehalte in Leber, Plasma und Lipoproteinen bestimmt. Indem die relative mRNA-Konzentration der Lipoproteinlipase ermittelt wurde, konnte geklärt werden, ob die verminderten Triglyceridgehalte im Plasma der Ratten auf eine gesteigerte Lipolyse zurückzuführen sind. Weitere Details zu Materialien, sowie die detaillierte Beschreibung und Diskussion der Ergebnisse dieser Studie sind ersichtlich in:

JULIA SPIELMANN, ANJALI SHUKLA, CORINNA BRANDSCH, FRANK HIRCHE, GABRIELE I. STANGL, KLAUS EDER (2007) Dietary lupin protein lowers triglyceride concentrations in liver and plasma in rats by reducing hepatic gene expression of sterol regulatory element-binding protein-1c. ***Annals of Nutrition and Metabolism*** *51: 387-392.*

(2) Die gelbe Erbse (*Pisum sativum L.)* gehört wie die Sojabohne und die Lupine zur Familie der Leguminosen. Deshalb war es Ziel der zweiten Studie, die Hypothese zu verfolgen, dass aufbereitetes Protein aus der gelben Erbse einen ähnlichen Effekt auf den Lipidstoffwechsel von Ratten hat wie Soja- oder Lupinenprotein. Hierzu wurden Sprague-Dawley-Ratten mit einer Diät, die als Proteinquelle 200 g/kg Erbsenprotein enthielt, über einen Zeitraum von 16 Tagen gefüttert. Die Tiere der Kontrollgruppe erhielten die gleiche Diät, jedoch mit Casein als Proteinquelle. Am Ende des Versuches wurden die Konzentrationen von Triglyceriden und Cholesterin in Plasma, Lipoproteinen und Leber der Ratten bestimmt. Um eine mögliche cholesterinsenkende Wirkung des Erbsenproteins erklären zu können, wurden der Gallensäuregehalt im Kot der Tiere und die relative mRNA-Konzentration der Cholesterol-7α-Hydroxylase (CYP7A1), dem Schlüsselenzym in der Gallensäuresynthese, in der Leber bestimmt. Weiterhin wurde die relative mRNA-Konzentration des Transkriptionsfaktors SREBP-2 und dessen Zielgenen HMG-CoA-Reduktase und LDL-Rezeptor bestimmt. Weitere Details zu Materialien, sowie die detaillierte Beschreibung und Diskussion der Ergebnisse dieser Studie sind ersichtlich in:

JULIA SPIELMANN, GABRIELE I. STANGL, KLAUS EDER (2008) Dietary pea protein stimulates bile acid excretion and lowers hepatic cholesterol concentration in rats. ***Journal of Animal Physiology and Animal Nutrition*** *92: 683-693.*

(3) In einem dritten Versuch wurde die Hypothese verfolgt, dass die Wirkung von Kartoffelprotein und Fischprotein auf die Lipide im Plasma, den Lipoproteinen und der Leber im Modelltier Schwein ähnliche Wirkungen hat wie bei der Ratte. Schweine sind ein geeignetes Modell, um Hypercholesterolämie und Atherosklerose zu untersuchen, da sie im Hinblick auf diese Erkrankungen viele Ähnlichkeiten mit dem Menschen aufweisen. Die Tiere wurden mit einer Diät über einen Zeitraum von 21 Tagen gefüttert, die entweder 200 g/kg Kartoffelprotein, Fischprotein oder Casein, welches als Kontrollprotein diente, enthielt. Anschließend wurde die relative mRNA-Konzentration von Genen in der Leber bestimmt, die eine Rolle in der Cholesterinsynthese und –aufnahme und der Bildung von Gallensäuren spielen. Um den Einfluss der beiden Proteine auf den Triglyceridstoffwechsel zu erklären, wurden

die relativen mRNA-Konzentrationen von lipogenen Genen, wie z.B. FAS und Acetyl-CoA-Carboxylase (ACC) in Leber und Fettgewebe bestimmt. Weitere Details zu Materialien, sowie die detaillierte Beschreibung und Diskussion der Ergebnisse dieser Studie sind ersichtlich in:

JULIA SPIELMANN, HOLGER KLUGE, GABRIELE I. STANGL, KLAUS EDER (2008) Hypolipidaemic effects of potato protein and fish protein in pigs. ***Journal of Animal Physiology and Animal Nutrition*** *(Epub ahead of print).*

(4) Obwohl die hypocholesterolämische Wirkung von pflanzlichen Proteinen im Vergleich zu tierischen Proteinen schon relativ lange bekannt ist, konnte noch nicht geklärt werden, welche Komponenten der pflanzlichen Proteine die beobachteten Wirkungen auf den Cholesterinmetabolismus ausüben. Es gibt jedoch schon Vermutungen, dass hierbei die Aminosäure-Zusammensetzung der Proteine eine Rolle spielen könnte. Vor allem ein höheres Verhältnis von Arginin zu Lysin wurde als Ursache für die hypocholesterolämische Wirkung von pflanzlichen Proteinen in Betracht gezogen. Pflanzliche und tierische Proteine unterscheiden sich vor allem bezüglich ihres Verhältnisses von Arginin zu Lysin, wobei pflanzliche Proteine höhere Konzentrationen an Arginin und tierische Proteine höhere Konzentrationen an Lysin aufweisen. Bisher wurde jedoch noch keine Studie durchgeführt, in der die Bedeutung des Arginin-zu-Lysin-Verhältnisses in einer Diät isoliert betrachtet wurde. Entsprechend eines bifaktoriellen Designs wurden an Ratten in diesem Versuch Diäten mit drei verschiedenen Konzentrationen an Arginin (4,5; 9 oder 18 g pro kg Diät) und zwei verschiedenen Konzentrationen an Lysin (9 oder 18 g pro kg Diät) verfüttert. Da bekannt ist, dass die hypocholesterolämische Wirkung von Sojaprotein vor allem im hypercholesterolämischen Organismus zu beobachten ist, wurde der Versuch sowohl mit einer hypercholesterolämischen Diät, welche Cholesterin und Cholat enthielt, als auch einer normocholesterolämischen Diät durchgeführt. Anschließend wurden die Konzentrationen des Cholesterins im Plasma, den Lipoproteinen und der Leber bestimmt und die relativen mRNA-Konzentrationen von Genen, die im Cholesterinstoffwechsel eine Rolle spielen, ermittelt. Weitere Details zu Materialien,

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

JULIA SPIELMANN, ANNE NOATSCH, CORINNA BRANDSCH, GABRIELE I. STANGL, KLAUS EDER (2008) Effects of dietary arginine and lysine on concentrations of cholesterol in plasma and liver of rats. ***Annals of Nutrition and Metabolism*** *53: 223-233.*

3. Originalarbeiten

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Dietary Lupin Protein Lowers Triglyceride Concentrations in Liver and Plasma in Rats by Reducing Hepatic Gene Expression of Sterol Regulatory Element-Binding Protein-1c

Julia Spielmann Anjali Shukla Corinna Brandsch Frank Hirche
Gabriele I. Stangl Klaus Eder

Institute of Agricultural and Nutritional Sciences, Martin Luther University Halle-Wittenberg, Halle/Saale, Germany

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Lupin protein · Triglycerides · Sterol regulatory element-binding protein-1c

Abstract
Background: Recently, it has been shown that dietary lupin protein lowers plasma triglyceride concentrations in rats. In this study, we investigated the hypothesis that this effect is due to a downregulation of sterol regulatory element-binding protein (SREBP)-1c, a transcription factor that regulates the expression of lipogenic enzymes in the livers of rats. ***Methods:*** Two groups of 12 rats each were fed semisynthetic diets containing 200 g/kg of either casein (control group) or lupin protein from *Lupinus albus* for 22 days. ***Results:*** Rats fed the diet containing lupin protein had lower concentrations of triglycerides in the liver, plasma and VLDL + chylomicrons ($p < 0.05$). The concentration of protein in VLDL + chylomicrons was also lower in rats fed lupin protein than in rats fed casein ($p < 0.05$). The mRNA concentrations of SREBP-1c and fatty acid synthase in the liver were lower in rats fed lupin protein than in rats fed casein ($p < 0.05$). The mRNA concentrations of lipoprotein lipase in the liver did not differ between both groups of rats. ***Conclusion:*** This study confirms that a protein isolated from *L. albus* is strongly hypotriglyceridemic in rats. It is shown for the first time that this effect is at least in part due to a downregulation of SREBP-1c in the liver which in turn leads to a reduction in hepatic fatty acid synthesis.

Introduction

It has been established that dietary proteins influence lipid metabolism in man and animals [1–4]. Most studies dealing with the effects of dietary proteins on lipid metabolism have focused on the effects of soy protein compared to casein. It has been reported many times that soy protein has hypocholesterolemic [2–8] and hypotriglyceridemic effects [8–10] in laboratory animals, pigs and humans when compared to casein. Lupins belong to the Fabaceae plant family and are related to other legumes including soy. Like soy beans, lupin seeds are comparatively high in protein, but they are lower in oil and isoflavones. Nowadays, four Mediterranean species of lupin *(Lupinus albus*, *L. angustifolius*, *L. luteus* and *L. mutabilis)* are cultivated for livestock and poultry feed and for human nutrition. These lupins are referred to as sweet lupins because they contain smaller amounts of toxic alkaloids than the bitter varieties. Sirtori et al. [11] have recently shown that dietary lupin protein extract lowers

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K. Eder
Institut für Agrar- und Ernährungswissenschaften
Martin-Luther-Universität Halle-Wittenberg, Emil-Abderhalden Strasse 26
DE–06108 Halle/Saale (Germany)
Tel. +49 345 552 2702, Fax +49 345 552 7124, E-Mail klaus.eder@landw.uni-halle.de

the concentrations of cholesterol and triglycerides in plasma of hypercholesterolemic rats. These authors also found that lupin protein increases the activity of the low density lipoprotein (LDL) receptor in HepG2 cells. This effect may be responsible for the reduced plasma cholesterol concentrations observed in rats fed lupin protein. In contrast, the molecular basis underlying the hypotriglyceridemic effect of lupin protein has, however, not yet been investigated.

Cellular lipid metabolism is regulated by sterol regulatory element-binding proteins (SREBPs). SREBPs belong to a large class of transcription factors containing basic helix-loop-helix-leucine zipper domains (bHLH-Zip) [for review, see 12] of which three isoforms have been characterized, SREBP-1a, 1c and 2. SREBP-1c, the predominant isoform in adult liver, preferentially activates genes required for fatty acid synthesis such as fatty acid synthase (FAS). SREBPs are synthesized as inactive precursors bound to the endoplasmatic reticulum membranes. For activation to occur they have to be cleaved by two resident proteases within the Golgi which sequentially cleave the SREBPs, release the amino terminal bHLH-Zip-containing domain from the membrane and allow it to translocate to the nucleus and activate transcription of their target genes.

Recently, it has been shown that soy protein reduces liver and plasma triglyceride concentrations by lowering hepatic fatty acid synthesis due to a downregulation of SREBP-1c [8–10, 13, 14]. This indicates that dietary proteins can alter SREBP-mediated transcription of genes involved in hepatic fatty acid synthesis. This prompted us to form the hypothesis that lupin protein influences hepatic triglyceride metabolism also by influencing gene expression or proteolytic activation of SREBP-1c. To test this hypothesis, we determined the mRNA concentrations of SREBP-1c and its target gene FAS, the key enzyme of fatty acid synthesis, in rats fed diets containing either lupin protein or casein which was used as a control protein. To investigate whether reduced plasma triglyceride concentrations could be due to an enhanced lipolysis, we also determined the mRNA concentration of lipoprotein lipase (LPL) in the liver.

Materials and Methods

Animals and Experimental Diets

Twenty-four male Sprague-Dawley rats (Charles River, Sulzfeld, Germany) with an initial body weight of 76 ± 5 g were randomly assigned to 2 groups of 12 rats each. All rats were kept individually in Macrolon cages in a room maintained at 22 ± 2°C and 50–60% relative humidity with lighting from 06.00 to 18.00 h. All experimental procedures described followed established guidelines for the care and handling of laboratory animals and were approved by the council of Saxony-Anhalt, Germany.

Table 1. Composition of the experimental diets (g/kg)

	Casein diet	Lupin protein diet
Protein (casein or lupin protein)	200	200
Corn starch	389.5	389.5
Sucrose	200	200
Lard	100	100
Cholesterol	0.5	0.5
Cellulose	50	40.6
Vitamin and mineral mixture	60	60
DL-Methionine	0	4.4
L-Lysine	0	5

All rats were fed a semisynthetic diet containing 200 g/kg of protein (either casein or a total protein extract from *L. albus*). The rats were fed a typical Western diet. The composition of the diets is shown in table 1. Due to its low native contents of methionine and lysine, the diet containing lupin protein was supplemented with 4.4 g DL-methionine and 5.0 g lysine/kg diet at the expense of cellulose. Vitamins and minerals were supplemented according to the recommendations of the American Institute of Nutrition for rat diets [15]. Diet intake was controlled and the amount of diet was slightly below that consumed ad libitum of similar diets by rats in preliminary studies. The amount of diet offered daily was increased continuously from 6.0 to 14.0 g. Water was freely available from nipple drinkers. The experimental diets were fed for 22 days.

Preparation and Characterization of the Dietary Proteins

Casein was obtained from Meggle (Wasserburg, Germany) and was not further processed. The defatted total protein extract of *L. albus* was obtained from the Fraunhofer Institute for Process Engineering and Packaging (Freising, Germany). The crude components of the dietary proteins were determined by the official methods of the Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten [16]. The dry matter content of casein and lupin protein was 909 and 939 g/kg, respectively; the concentration of protein in dry matter was 930 and 960 g/kg, respectively. The fat and fiber content was low in both proteins (fat content: 9 g/kg dry matter in casein, 11 g/kg dry matter in lupin protein; fiber content: 0.4 g/kg dry matter in casein, 0.3 g/kg dry matter in lupin protein). The fatty acid composition of total lipids was essentially identical in both diets. Major fatty acids determined by capillary gas chromatography of fatty acid methyl esters [17] were on average in both diets (in g/100 g total fatty acids): myristic acid (14:0), 1.6; palmitic acid (16:9), 25.3; palmitoleic acid (16:1), 2.7; stearic acid (18:0), 15.1; oleic acid (18:1), 39.9; linoleic acid (18:2n–6), 10.4; α-linolenic acid (18:3n–3), 0.9, and eicosenoic acid (20:1n–9), 0.9. To determine the amino acid concentrations in the diet, samples were oxidized and then hydrolyzed with 6 M HCl [16]. Separation and quantification of the amino acids were performed by ion exchange chromatography following post-

Table 2. Concentrations of amino acids in the experimental diets (g/kg diet)

	Casein diet	Lupin protein diet
Alanine	5.5	5.1
Arginine	6.3	18.3
Aspartic acid	13.2	19.6
Cysteine	0.8	2.0
Glutamic acid	42.2	42.8
Glycine	3.4	5.9
Histidine	6.4	4.2
Isoleucine	8.8	8.8
Leucine	17.7	14.3
Lysine	14.7	11.7
Methionine	5.3	5.0
Phenylalanine	9.7	7.3
Proline	20.2	7.4
Serine	10.8	9.5
Threonine	7.9	5.8
Tryptophan	2.3	0.8
Tyrosine	8.6	8.2
Valine	12.1	6.7

column derivatization in an amino acid analyzer (Biotronic LC 3000, Eppendorf, Hamburg, Germany). Tryptophan was determined by digesting the diet with barium hydroxide [18]. The tryptophan concentration in the diet was measured by reversed-phase high performance liquid chromatography [19]. The concentrations of amino acids in the two diets are shown in table 2.

Sample Collection

Rats were not fasted before killing by decapitation under light anesthesia with diethyl ether. Plasma was separated from heparinized whole blood by centrifugation at 1,500 *g* for 10 min at 4°C. Liver was excised, weighed and immediately snap frozen in liquid nitrogen. Aliquots of liver for RNA isolation were stored at –80°C; other samples were stored at –20°C. In the last 7 days of the experiment, feces were collected and weighed.

Plasma and Liver Lipids

Plasma lipoproteins very low density lipoproteins (VLDLs), low density lipoproteins (LDLs) and high density lipoproteins (HDLs) were separated by step-wise ultracentrifugation (900,000 *g* at 4°C for 1.5 h; Mikro-Ultrazentrifuge, Sorvall Products, Bad Homburg, Germany) by appropriate density cuts commonly used for the measurement of rat lipoproteins [20–22]. Plasma densities were adjusted by sodium chloride and potassium bromide and the lipoprotein fractions $\rho < 1.006$ kg/l (VLDL), 1.006 kg/l $< \rho <$ 1.063 kg/l (LDL) and $\rho > 1.063$ kg/l (HDL) were removed by suction. As the animals were not starved before killing, the VLDL fraction also contained chylomicrons. Therefore, we define this fraction as 'VLDL + chylomicrons'.

Lipids from liver were extracted with a mixture of n-hexane and isopropanol (3:2, v/v) [23]. For determination of the concentrations of lipids in liver, aliquots of the lipid extracts were dried and the lipids dissolved using Triton X-100 [24]. Concentrations of triglycerides in plasma, lipoproteins and liver were determined using enzymatic reagent kits (DiaSys Diagnostic Systems, Holzheim, Germany, Cat. No. 1.5760 99 90 314).

Relative mRNA Concentrations

Total RNA from liver was isolated by TRIZOL™ reagent according to the manufacture's protocol. cDNA synthesis was carried out as described [25].The relative mRNA quantities of SREBP-1c, FAS (EC 2.3.1.85) and LPL (EC 3.1.1.34) related to the reference gene glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) mRNA was measured by real-time detection polymerase chain reaction (PCR) using SYBR® Green I. Real-time detection PCR was performed with 1.25 U Taq DNA polymerase, 500 μm dNTPS and 26.7 pmol of the specific primers (Operon Biotechnologies, Cologne, Germany). The primer sequences used were as follows: 5′-GCA-TGG-CCT-TCC-GTG-TTC-C-3′ (forward) and 5′-GGG-TGG-TCC-AGG-GTT-TCT-TAC-TC-3′ (reverse) for rat GAPDH; 5′-GGA-GCC-ATG-GAT-TGC-ACA-TT-3′ (forward) and 5′-AGG-AAG-GCT-TCC-AGA-GAG-GA-3′ (reverse) for rat SREBP-1c; 5′-CCT-CCC-CTG-GTG-GCT-GCT-ACA-A-3′ (forward) and 5′-CCT-GGG-GTG-GGC-GGT-CTT-T-3′ (reverse) for rat FAS, and 5′-TCC-CAC-CAC-AAC-GAA-GTA-CA-3′ (forward) and 5′-TCA-GCC-AGG-GCA-TTA-TTT-TC-3′ (reverse) for rat LPL. To determine the mRNA concentration a threshold cycle (C_t) was obtained from each amplification curve using the $\Delta\Delta C_t$ method as previously described [26].

Statistics

Means of the two groups were compared by Student's t test. Means were considered to be significantly different at $p < 0.05$.

Results

Body and Liver Weights

Initial body weights of the rats were similar in both groups of rats (table 3). Although diet intake was similar in both groups (control group: 10.0 g/day; lupin group: 9.4 g/day), rats fed lupin protein gained less weight during the experimental period and had lower final body weights at the end of the experiment than rats fed casein ($p < 0.05$; table 3). Accordingly, the feed conversion (gain:feed) ratio was lower in rats fed lupin protein than in rats fed casein ($p < 0.05$; table 3). Rats fed the lupin protein, moreover, also had lower liver weights, both in absolute terms and relative to body weight ($p < 0.05$; table 3). The amount of feces excreted within the last 7 days of the experiment was not different between both groups of rats (table 3).

Concentrations of Triglycerides in Liver, Plasma and Lipoproteins

Rats fed the lupin protein had a lower concentration of triglycerides in plasma, VLDL + chylomicrons and in the liver than rats fed casein ($p < 0.05$; table 4). The concentration of protein in the triglyceride-rich fraction was

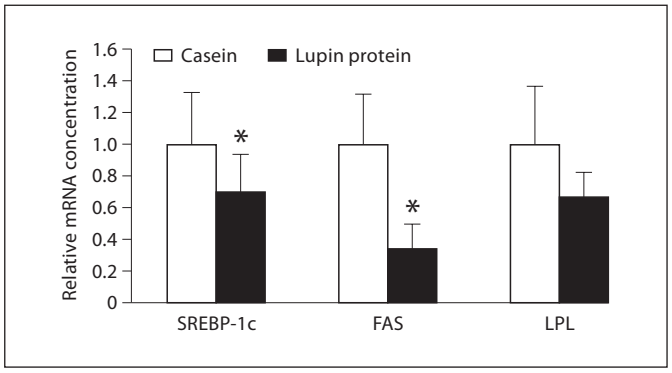

Fig. 1. Relative mRNA concentrations of sterol regulatory element-binding protein (SREBP)-1c, fatty acid synthase (FAS) and lipoprotein lipase (LPL) in the livers of male rats fed diets containing 200 g/kg of casein or lupin protein for 22 days. Values were related to the reference gene glyceraldehyde-3-phosphate-dehydrogenase (GAPDH). Each bar represents means ± SD (n = 12). * Different from rats fed the casein-based diet (expressed as 1) by the Student's t test: $p < 0.05$.

Table 3. Body and liver weights of rats fed a diet containing either casein or lupin protein

	Casein	Lupin protein
Body weight, g		
Day 1	77 ± 5	76 ± 5
Day 22	191 ± 6	153 ± 10*
Weight gain, g/day	5.45 ± 0.22	3.67 ± 0.45*
Feed conversion ratio, g weight gain/g feed	0.60 ± 0.03	0.38 ± 0.05*
Liver weight, g	8.62 ± 0.72	5.87 ± 1.08*
Liver weight:body weight, g/100g	4.51 ± 0.37	3.85 ± 0.74*
Amount of feces, g/7 days	7.62 ± 1.09	7.52 ± 1.07

Values are means ± SD for 12 rats in each group.
* Significantly different from the casein group: $p < 0.05$.

Table 4. Concentrations of triglycerides in the liver, plasma and lipoproteins of rats fed a diet containing either casein or lupin protein

	Casein	Lupin protein
Liver, μmol/g	10.3 ± 2.63	7.13 ± 2.17*
Plasma, mmol/l	2.35 ± 0.86	0.58 ± 0.26*
VLDL + chylomicrons, mmol/l	1.67 ± 0.58	0.32 ± 0.26*
LDL, mmol/l	0.32 ± 0.08	0.19 ± 0.05
HDL, mmol/l	0.12 ± 0.05	0.08 ± 0.03

Values are means ± SD of 12 rats in each group.
* Significantly different from the casein group: $p < 0.05$.

also lower in rats fed lupin protein than in rats fed casein (76 ± 33 vs. 414 ± 125 μg/ml, means ± SD, $p < 0.05$). The triglyceride concentrations in LDL and HDL did not differ between the 2 groups of rats (table 4).

Hepatic mRNA Concentrations of Genes Involved in Triglyceride Metabolism

Rats fed lupin protein had lower mRNA concentrations of SREBP-1 and FAS than rats fed casein ($p < 0.05$; fig. 1). The mRNA concentration of LPL in the liver was not different between both groups of rats (fig. 1).

Discussion

This study confirms the recent finding of Sirtori et al. [11] that lupin protein is able to lower the concentration of triglycerides in the plasma of rats. Our study, moreover, shows for the first time that dietary lupin protein also causes a strong reduction in the liver triglyceride concentration, and it suggests that these effects are at least partially induced by alterations in the expression of SREBP-1c in the liver of rats.

SREBP-1c is the key regulator of fatty acid and triglyceride synthesis in the liver. Increasing the nuclear concentration of SREBP-1c in the nucleus by either increasing its gene expression or its proteolytic activation leads to stimulation of the transcription of several genes involved in fatty acid synthesis and fatty acid esterification to lipids [12]. The present study shows that dietary lupin protein leads to reduced mRNA concentrations of SREBP-1c and FAS, one of the target genes of SREBP-1c, in the liver. Downregulation of the expression of FAS clearly indicates that there was less mature SREBP-1c in hepatic nuclei. Our study shows that this is at least in part due to a reduced gene expression of SREBP-1c, but the possibility that there was also an increased proteolytic activation of the immature SREBP-1c could not be excluded. The finding that the mRNA concentration of FAS, one of the key enzymes of hepatic fatty acid synthesis, was reduced clearly indicates that lupin protein suppressed hepatic fatty acid synthesis which may be at least partially responsible for the reduced triglyceride concentrations in the liver and plasma of rats fed lupin protein. A reduced hepatic synthesis of triglycerides leads to a reduction in the formation and secretion of VLDL. The finding that triglyceride and protein concentrations in the triglyceride-rich lipoprotein fraction were reduced in rats fed lupin protein suggests that these rats exhibited a reduced hepatic formation and secretion of VLDL. Interestingly, these effects are

similar to those observed in rats fed soy protein diets [8–10, 13, 14]. Therefore, it is possible that lupin protein also inhibited gene expression of SREBP-1c by an alteration in plasma concentrations of insulin and glucagon as observed in rats fed soy protein [9]. This, however, must be investigated in further studies. Soy and lupin belong to the same plant family, therefore it seems likely that their plant proteins are very similar and exert their effects on lipid metabolism via similar mechanisms.

The finding that the mRNA concentration of LPL, the key enzyme of lipolysis of triglyceride-rich lipoproteins, was not altered indicates that reduced concentrations of triglycerides were not caused by an enhanced lipolysis of triglyceride-rich lipoproteins.

In this study, rats fed the diets with lupin gained less body weight than rats fed casein, although both groups of rats had a similar diet intake during the experimental period. This may be due to the lower biological value of the lupin protein compared with casein. As lupin protein contains a very low concentration of methionine and lysine, we added both amino acids to the lupin diet to equalize their concentrations to those in the casein diet. Therefore, the possibility that the concentration of either methionine or lysine was limiting for the growth of rats fed the lupin protein diet can be ruled out. Comparison of the analyzed concentrations of amino acids in the lupin protein diet with estimates of indispensable amino acid requirement of rats by the National Research Council [27] suggests that the lupin protein supplied sufficient amounts of all the indispensable amino acids with the only exception of tryptophan. The tryptophan content of the lupin protein was 0.8 g/kg diet which is clearly below the estimated requirement of 2.0 g/kg for growing rats [27]. Therefore, we assume that tryptophan was the first limiting amino acid in lupin protein, and the low tryptophan content of the protein was probably responsible for the lower body weight gains of rats fed the lupin protein diet compared to rats fed the casein diet. We did not directly determine the digestibility of the nutrients but the finding that the amount of feces excreted was similar in both groups of rats suggests that the digestibility of the nutrients was not markedly, if at all, different between the 2 groups of rats. Under the assumption that the digestibility of the energy was not different, the reduced gain:feed ratio in rats fed the lupin protein indicates that these rats deposited less lean mass and more fat mass than rats fed casein. This indication is based on the fact that the deposition of 1 g fat mass requires much more energy than the accretion of 1 g lean mass. However, we have no experimental proof for this indication as we did not determine whole body composition.

It is still unknown which of the components of lupin protein isolate is responsible for the effects on lipid metabolism. Alterations in plasma amino acids mediated by differences in amino acid patterns of the dietary proteins could contribute to the effects observed on lipid metabolism. In studies dealing with the effects of soy protein on the lipid metabolism, it was shown that some of the effects were at least partially mediated by its low content of methionine [28–30]. We suggest that methionine might not be involved in the effects of soy protein on the lipid metabolism observed in this study because we adjusted methionine concentrations of both diets to a similar level. Whether differences in other amino acids, such as arginine, cysteine, tryptophan, proline or valine, could be responsible for the alterations in hepatic triglyceride metabolism in rats fed the lupin protein diet compared to rats fed the casein diet has to be investigated in further studies. The effects of lupin protein on lipid metabolism could also have been mediated in part by bioactive phytochemicals such as saponins and isoflavones. Isoflavones are thought to be the active components of soy protein and responsible for many of its beneficial effects on plasma lipids of man and animals [31, 32]. However, the isoflavone content of lupins is very low compared to soy. Therefore, it seems unlikely that the isoflavones might be responsible for the observed hypotriglyceridemic effect. We cannot exclude the possibility that the reduced body weight gain, probably associated with an increased fat deposition (as discussed above), was at least partially responsible for the reduced concentrations of triglycerides in the liver and plasma. Nevertheless, this option is unlikely as an increased availability of nutrients for fat deposition at the expense of protein synthesis will lead to an upregulation of SREBP-1c and its target genes involved in lipogenesis rather than to the downregulation of these genes observed in rats fed the lupin protein diet [12, 33].

In conclusion, this study shows that lupin protein causes a reduction in the triglyceride concentrations in the liver and plasma. These effects may be due, at least in part, to significant downregulation of SREBP-1 which in turn causes a reduction in hepatic fatty acid synthesis. The active components in lupin protein responsible for the beneficial effects on lipid metabolism remain to be elucidated.

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ORIGINAL ARTICLE

Dietary pea protein stimulates bile acid excretion and lowers hepatic cholesterol concentration in rats

J. Spielmann, G. I. Stangl and K. Eder

Institute of Agricultural and Nutritional Sciences, Martin-Luther-University of Halle-Wittenberg, Halle (Saale), Germany

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Correspondence
K. Eder, Institut für Agrar- und Ernährungswissenschaften, Martin-Luther-Universität Halle-Wittenberg, Emil-Abderhalden-Str. 26, D-06108 Halle (Saale), Germany.
Tel: +49 345 5522702; Fax +345 5527124;
E-mail: klaus.eder@landw.uni-halle.de

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Summary

It has been shown that some dietary plant proteins beneficially influence lipid metabolism in animals. The effect of pea protein in this respect however has not yet been investigated. Therefore, we studied the effect of purified pea protein on the lipid metabolism in rats. Twenty-four rats received diets with either 200 g/kg of casein or purified pea protein for 16 days. Concentrations of triacylglycerols in liver, plasma and lipoproteins did not differ between both groups of rats. However, rats fed the pea protein diet had a lower concentration of total cholesterol in the liver and the very low density lipoproteins (VLDL) fraction than rats fed the casein diet ($p < 0.05$); cholesterol concentration in plasma, low density lipoproteins (LDL) and high density lipoproteins (HDL) did not differ between both groups. Rats fed pea protein moreover had an increased mRNA concentration of cholesterol-7α-hydroxylase in the liver and an increased amount of bile acids excreted via faeces compared with rats fed casein ($p < 0.05$). Concomitantly, mRNA concentrations of sterol regulatory element-binding protein (SREBP)-2 and its target genes 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and LDL receptor in the liver were increased in rats fed pea protein ($p < 0.05$). The data of this study suggests that pea protein stimulates formation and excretion of bile acids, which leads to a reduced hepatic cholesterol concentration and a reduced secretion of cholesterol via VLDL. An increased gene expression of SREBP-2 and its target genes HMG-CoA reductase and LDL receptor may be a means to compensate for the increased loss of cholesterol for bile acid synthesis.

Introduction

It has been established that dietary proteins influence lipid metabolism in man and animals (Anderson et al., 1995; Sirtori et al., 1995, 1998; Sirtori and Lovati, 2001; Wergedahl et al., 2004; Brandsch et al., 2006; Gudbrandsen et al., 2006; Shukla et al., 2006). Most studies dealing with the effects of dietary proteins on the lipid metabolism have focused on the effects of soy protein compared to casein. It has been reported many times that soy protein has hypocholesterolemic (Sirtori et al., 1979, 1995, 1998; Anderson et al., 1995; Iritani et al., 1996; Sugiyama et al., 1996; Tovar et al., 2002; Koba et al., 2003) and hypotriglyceridemic (Tovar et al., 2002; Ascencio et al., 2004; Sirtori et al., 2004; Brandsch et al., 2006) effects in laboratory animals, pigs and humans when compared with casein. Recently, it has been shown that protein extracted from sweet lupins is also able to lower the concentrations of cholesterol and triacylglycerols in plasma of rats (Sirtori et al., 2004; Spielmann et al., 2007). Those studies confirm the hypolipidaemic potential of plant

proteins. The yellow pea (*Pisum sativum* L.) is another plant seed rich in protein which belongs, like soy-beans and lupins, to the botanic family of the legumes. Accordingly, there are some similarities in the amino acid profile of proteins from soy beans, lupins and peas. Like soy protein and lupin protein, pea protein has a low content of methionine and a high content of arginine. Moreover, in all these three proteins the arginine:lysine ratio is much higher than in casein. It has been suggested that the high arginine:lysine ratio may be responsible, at least in part, for the hypocholesterolemic effect of soy protein (Kritchevsky et al.,1982; Sugano et al., 1984; Vahouny et al., 1985; Gudbrandsen et al., 2006). These similarities in the amino acid profile give rise to the suspicion that pea protein could exert similar effects on the lipid metabolism as soy protein or lupin protein. To our knowledge, the effect of purified pea protein on the lipid metabolism has not yet been investigated. There are, however, a few studies which investigated the effect of whole peas on the lipid metabolism. In one of these studies a diet containing whole peas (*P. sativum*) led to increased levels of very low density lipoproteins (VLDL) + low density lipoproteins (LDL)-cholesterol and an increased faecal steroid excretion in pigs (Kingman et al., 1993). In another study, dietary raw peas lowered concentrations of cholesterol in plasma, LDL and liver of pigs fed a cholesterol-rich diet (Martins et al., 2004). As whole peas which contained considerable amounts of starch, non starch polysaccharides and plant sterols were used in those studies, it remains, however, unknown which of the ingredients of peas was responsible for the effects observed on lipid metabolism. Therefore, the present study aimed to investigate the effect of purified pea protein on the lipid metabolism of rats which were used as an animal model.

Materials and methods

Animals

Twenty-four male Sprague–Dawley rats supplied by Charles River (Sulzfeld, Germany) were randomly assigned to two groups of twelve rats each with an initial body weight of 113 (SD 10) g. All rats were housed individually in Macrolon cages whose floors were coated with wood shavings. The room was maintained at a temperature of 23 °C and 50–60% relative humidity with lighting from 06:00 to 18:00 hours. All the experimental procedures described followed established guidelines for the care and handling of laboratory animals and were approved by the council of Saxony-Anhalt, Germany.

Experimental diets

All rats were fed a semi-synthetic diet containing 200 g of either casein which was used as reference protein or pea protein per kg (Table 1). To equalize methionine concentrations of the diets, DL-methionine was supplemented at the expense of starch in a concentration of 2 g/kg diet in the casein diet and a concentration of 5.7 g/kg in the pea protein diet. Lard was used as type of dietary fat to mimic Western diets which are commonly rich in saturated fats. Vitamins and minerals were supplemented according to recommendations of the American Institute of Nutrition for rat diets (Reeves et al., 1993). During the experiment the food intake was controlled. The amount of food administered was slightly below that of similar diets consumed *ad libitum* by rats in preliminary unpublished studies. The amount of food offered daily was increased continuously from 11.0 to 15.0 g. Water was freely available from nipple drinkers. The experimental diets were fed for 16 day, a period which is long enough to induce changes in lipid metabolism by dietary proteins (Iritani et al., 1996; Sugiyama et al., 1997; Ascencio et al., 2004).

Preparation and characterization of the dietary proteins

A commercial available pea protein isolate (Pisane®; Georg Breuer GmbH Food Ingredients, Königstein, Germany) was used. As peas contain moderate amounts of isoflavones (Matscheski et al., 2006), the pea protein isolate was ethanol washed additionally as described by Fukui et al. (2004) to exclude possible effects of native isoflavones on the lipid metabolism. To ensure that all proteins used in this

Table 1 Composition of the experimental diets (g/kg)

Components	Casein diet	Pea protein diet
Casein	200	–
Pea protein	–	200
Sucrose	200	200
Lard	100	100
Cellulose	50	50
Corn starch	388	384.3
Vitamin and mineral mixture	60	60
DL-methionine	2	5.7

experiment are treated equally, casein (Nähr Kasein, Meggle, Wasserburg/Inn, Germany) was also underwent this procedure. The proteins were washed twice with 10 volumes of 60% ethanol by stirring for 2 h at room temperature. After washing the protein was filtered, air dried and grounded. After extracting the pea protein with ethanol, the content of genistein, analysed by HPLC (Sung et al., 2004), was 0.88 mg per kg protein. The crude components of the dietary proteins, determined by official methods (Naumann and Basler, 1993) are given in Table 2. Both proteins had low concentrations of crude fat; the concentration of crude fibre in the pea protein was also low. Major fatty acids in the fat of the pea protein were (in g/100 g total fatty acids) palmitic acid (10.4), stearic acid (2.7), oleic acid (19.2), linoleic acid (51.2) and α-linoleinic acid (10.3). However, due to the low fat concentration in both diets, differences in the fatty acid composition of total lipids between both diets were small. The pea protein diet had slightly higher proportions of linoleic acid and α-linolenic acid and slightly lower proportions of palmitic acid, stearic acid and oleic acid in the total lipid fraction than the casein diet (Table 3). The amino acid composition of both diets is shown in Table 4.

Table 2 Concentrations of crude nutrients in the dietary proteins (g/kg dry matter)

Nutrient	Casein	Pea protein
Crude protein	939	917
Crude fat	17	10
Crude fibre	<1	6
Crude ash	24	29

Table 3 Fatty acid composition of total lipids of the experimental diets (g/100 g total fatty acids)*

Fatty acid	Casein diet	Pea protein diet
12:0	0.13	0.12
14:0	1.57	1.55
16:0	25.3	25.0
16:1 (*n*−7 + *n*−9)	2.60	2.57
18:0	15.1	14.9
18:1 (*n*−9 + *n*−11)	39.8	39.4
18:2 (*n*−6)	10.4	11.2
18:3 (*n*−3)	0.88	1.20
20:0	0.25	0.25
20:1 (*n*−9)	0.89	0.88
20:2 (*n*−6)	0.45	0.44
20:4 (*n*−6)	0.12	0.12
22:5 (*n*−3)	0.15	0.16

*Fatty acids with proportions <0.1 g/100 g total fatty acids are not shown

Table 4 Concentrations of amino acids in the diet (g/kg diet)

Amino acid	Casein diet	Pea protein diet
Cysteine	0.7	1.4
Methionine	7.2	7.2
Aspartamic acid	13.8	20.8
Threonine	8.2	6.2
Serine	10.8	8.5
Glutamic acid	44.3	30.9
Proline	21.3	7.0
Glycine	3.4	6.7
Alanine	5.6	7.1
Valine	12.0	8.5
Isoleucine	9.1	7.7
Leucine	17.8	14.3
Tyrosine	9.3	5.6
Phenylalanine	9.6	9.2
Histidine	5.4	4.3
Lysine	14.8	12.3
Arginine	6.7	14.8
Tryptophan	2.2	1.4

Sample collection

Rats received 4 g of diet 4 h before they were killed by decapitation under light anaesthesia with diethyl ether. This last portion of food was given as food deprivation before killing leads to a significant down regulation of the genes involved in lipid metabolism which were considered in this study (Horton et al., 1998; Shimano et al., 1999). Blood was collected in heparinized polyethylene tubes. Plasma was separated from whole blood by centrifugation at 1500 ***g*** for 10 min at 4 °C. Liver was excised, weighed and immediately snap-frozen in liquid nitrogen. Aliquots of liver for RNA isolation were stored at −80 °C; other samples were stored at −20 °C. During the last 7 days of the experimental period, faeces were picked out manually from the wood shavings with tweezers. Faeces were dried, weighed and stored at −20 °C until analysis.

Analysis of amino acids and fatty acids in the diets

To determine the amino acid concentrations in the diet, samples were oxidized and then hydrolysed with 6 M-HCl (Naumann and Basler, 1993). Separation and quantification of the amino acids were performed by ion-exchange chromatography following post-column derivatization in an amino-acid analyser (Biotronic LC 3000; Eppendorf, Hamburg; Germany). Tryptophan was determined by digesting the diet with barium hydroxide (Fontaine et al.,

1998). The tryptophan concentration in the diet was measured by reversed-phase HPLC using a Agilent 1100 Series system (Agilent Technologies, Waldbronn, Germany). Separation was performed in a RP-18 column (LiChrospher 100, RP-18e, 5 μm, 125 × 4 mm^2; Agilent Technologies) by means of a gradient elution using a mixture of 0.01 M potassium dihydrogenphosphate solution and methanol (92:8, v/v) and methanol as solvent. Detection was by fluorescence (excitation wavelength: 280 nm, emission wavelength: 355 nm) (Eder et al., 2001). For determination of fatty acid composition of total lipids in the diets, lipids were extracted from the diet with a mixture of n-hexane and isopropanol (3:2, v/v, according to Hara and Radin, 1978). Total lipids of the extracts were methylated with trimethylsulfonium hydroxide. Fatty acid methyl esters were separated by a gas chromatograph (HP 5890, Hewlett Packard GmbH, Waldbronn, Germany), equipped with a polar capillary column (30 m FFAP, 0.53 mm I.D.; Macherey and Nagel, Düren, Germany), a flame ionization detector and an automatic on-column injector. Helium was used as carrier-gas with a flow of 5.4 ml/min. Fatty acid methyl esters were identified by comparing their retention times with those of individually purified standards (Brandsch et al., 2002).

Plasma and liver cholesterol and triacylglycerols

Plasma lipoproteins were separated by stepwise ultracentrifugation (Mikro-Ultrazentrifuge; Sorvall Products, Bad Homburg, Germany) at 900 000 × g at 4 °C for 1.5 h by appropriate density cuts commonly used for the measure of rat lipoproteins (Sparks et al., 1998; Giudetti et al., 2003; Sirtori et al., 2004). Plasma densities were adjusted by sodium chloride and potassium bromide and the lipoprotein fractions $\rho < 1.006$ kg/l (defined as VLDL), $1.006 < \rho < 1.063$ kg/l (defined as LDL) and $\rho > 1.063$ kg/l [defined as high density lipoproteins (HDL)] were removed by suction. Lipids were extracted from liver with a mixture of *n*-hexane and isopropanol (3:2, v/v, according to Hara and Radin, 1978). For determination of the concentration of cholesterol and triacylglycerols in the liver, aliquots of the lipid extracts were dried and the lipids dissolved using Triton X-100 (De Hoff et al., 1978). Concentrations of cholesterol and triacylglycerols in plasma and lipoproteins and those of liver were determined by spectrophotometry using an enzymatic reagent kit (Ecoline S+; DiaSys GmbH, Holzheim, Germany) and a Ultarspec 2000 UV/Visible spectrophotometer (Pharmacia Biotech, Freiburg, Germany).

Faecal bile acids

Bile acids were extracted from freeze-dried faeces with ethanol and NaOH (Dongowski et al., 2002). The total bile acid content of the faeces was determined by spectrophotometry using an enzymatic reagent kit (DiaSys Diagnostic Systems) and a Spectraflour Plus spectrophotometer (Tecan, Crailsheim, Germany).

Relative mRNA concentrations of hepatic genes

For analysis of gene expression, total RNA was isolated from frozen liver samples using TRIZOL™ (Sigma Aldrich, Steinheim, Germany) according to the manufacture's protocol. cDNA synthesis was carried out as described by König and Eder (2006). The mRNA concentrations of genes were measured by real-time detection PCR (Rotorgene 2000, Corbett Research, Mortlake, Australia) using SYBR® Green I (Sigma-Aldrich, Taufkirchen, Germany). Real-time detection PCR was performed with 1.25 U Taq DNA polymerase (Promega, Mannheim, Germany), 500 μM dNTPs and 26.7 pmol of the specific primers (Operon Biotechnologies, Cologne, Germany, Table 5). For determination of mRNA concentration a treshold cycle (C_t) was obtained from each amplification curve using the software RotorGene 4.6 (Corbett Research, Sydney, Australia). Calculation of the relative mRNA concentration was made using the $\Delta\Delta C_t$ method (Pfaffl, 2001). The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase was used for normalization.

Statistics

Mean of the two groups were compared by student's *t*-test. Mean were considered significantly different at $p < 0.05$.

Results

Body and liver weights

Food intake throughout the feeding period was the same for each rat in this experiment, averaging 13 g/day. In spite of this, rats fed pea protein had lower body weight gains than rats fed casein ($p < 0.05$; Table 6). Liver weight, both on absolute terms and expressed per 100 g body weight, was lower in rats fed pea protein than in rats fed casein ($p < 0.05$; Table 6).

Table 5 Characteristics of the specific primers used for reverse transcriptase polymerase chain reaction analysis

Gene	Forward and reversed primers	bp	Annealing temperature °C	CNCBI Genbank
CYP7A1	For: 5′-CAAGACGCACCTCGCTATCC-3′ Rev: 5′-CCGGCAGGTCATTCAGTTG-3′	206	60	EC1141317
GAPDH	For: 5′-GCATGGCCTTCCGTGTTCC-3′ Rev: 5′-GGGTGGTCCAGGGTTTCTTACTC-3′	337	60	BC059110
HMG-CoA reductase:	For: 5′-AAGGGGCGTGCAAAGACAATC-3′ Rev: 5′-ATACGGCACGGAAAGAACCATAGT-3′	406	57	BC064654
LDL receptor:	For: 5′-AGAACTGCGGGGCCGAAGACAC-3′ Rev: 5′-AAACCGCTGGGACATAGGCACTCA-3′	490	65	NM175762
SREBP-2	For: 5′ATCCGCCCACACTCACGCTCCTC-3′ Rev: 5′-GGCCGCATCCCTCGCACTG-3′	312	65	BC101902

CYP7A1, cholesterol 7α-hydroxylase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HMG-CoA R, 3-hydroxy-3-methylglutaryl-CoA, reductase; LDL receptor, low density lipoprotein-receptor; SREBP-2, sterol regulatory element-binding protein-2.

Table 6 Body and liver weights of rats fed a diet containing either casein or pea protein (n = 12/group)

	Casein		Pea protein	
	Mean	SD	Mean	SD
Body weight day 1 (g)	113	9	113	10
Body weight day 16 (g)	213	4	203*	8
Weight gain (g/day)	6.19	0.39	5.61*	0.45
Liver weight (g)	9.70	0.46	8.12*	0.67
Liver weight/body weight (g/100g)	4.56	0.21	3.99*	0.32

*Mean values were significantly different from those rats fed the casein based diet: $p < 0.05$.

Cholesterol and triacylglycerol concentrations in plasma and liver and excretion of bile acids via faeces

Concentrations of total cholesterol in plasma, LDL and HDL did not differ between the two groups of rats (Table 7). However, concentrations of cholesterol in VLDL was lower in rats fed pea protein than in rats fed casein ($p < 0.05$, Table 7). The cholesterol concentration in the liver was also lower in rats fed pea protein than in rats fed casein ($p < 0.05$; Table 7).

Concentrations of triacylglycerols in plasma, VLDL, LDL, HDL and liver did not differ between both groups of rats (Table 7). The amount of bile acids excreted via faeces was approximately twofold higher in rats fed pea protein compared to rats fed casein ($p < 0.05$; Table 7).

Relative mRNA concentrations of SREBP-2, HMG-CoA reductase, LDL receptor and cholesterol 7α-hydroxylase (CYP7A1) in the liver

Rats fed pea protein had higher relative mRNA concentrations of sterol regulatory element-binding protein (SREBP)-2, 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, LDL receptor and CYP7A1 in the liver than rats fed a diet containing casein ($p < 0.05$; Fig. 1).

Table 7 Concentrations of total cholesterol and triacylglycerols in liver, plasma and lipoproteins and excretion of bile acids via faeces in rats fed a diet containing either casein or pea protein (n = 12/group)

	Casein		Pea protein	
	Mean	SD	Mean	SD
Total cholesterol				
Liver (μmol/g)	6.71	0.68	5.77*	0.57
Plasma (mmol/l)	1.72	0.12	1.67	0.25
VLDL (mmol/l)	0.18	0.05	0.13*	0.05
LDL (mmol/l)	0.67	0.12	0.77	0.13
HDL (mmol/l)	1.06	0.10	1.12	0.13
Triacylglycerols				
Liver (μmol/g)	17.1	3.13	15.1	2.77
Plasma (mmol/l)	1.85	0.45	1.83	0.30
VLDL (mmol/l)	1.58	0.42	1.68	0.30
LDL (mmol/l)	0.22	0.05	0.23	0.10
HDL (mmol/l)	0.09	0.02	0.09	0.02
Bile acids in the faeces (μmol/week)	26.1	5.8	58.9*	14.4

*Mean values were significantly different from those rats fed the casein based diet: $p < 0.05$.

Discussion

In this study, we investigated the effect of purified pea protein on cholesterol and triacylglycerol concentrations in plasma, lipoproteins and liver of rats. The main findings were that pea protein lowers cholesterol concentrations in liver and VLDL but has no effect on triacylglycerol concentrations in plasma, liver and lipoproteins, in comparison to casein used as a reference protein. As an animal model, we used

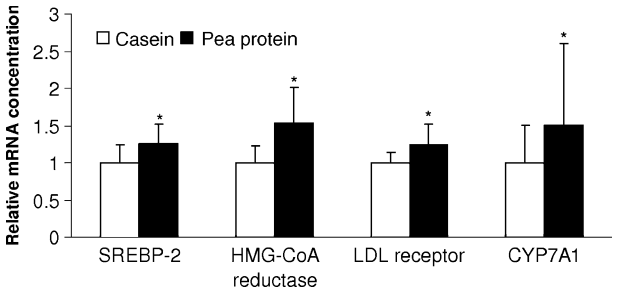

Fig. 1 Relative mRNA concentrations of sterol regulatory element-binding protein (SREBP)-2, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA)-reductase, LDL receptor and cholesterol 7α-hydroxylase (CYP7A1) in the liver of male rats fed diets containing 200 g/kg of casein or pea protein for 16 days. Values were related to the reference gene glyceraldehyde-3-phosphate-dehydrogenase (GAPDH). Each bar represents mean ± SD, $n = 12$. *Significantly different from rats fed the casein-based diet (=1.0), $p < 0.05$.

rats which are widely used to find out potential effects of proteins on lipid metabolism (e.g. Sirtori et al., 1984; Lovati et al., 1985; Vahouny et al., 1985; Madani et al., 1998; Brandsch et al., 2006; Shukla et al., 2006). To avoid a different food intake which could have interfering effects on the lipid metabolism, diet intake was controlled. Although diet intake was identical in both groups, the rats fed the pea protein diet had lower body weight gains than rats fed casein. This may be due to the lower biological value of the pea protein compared with casein. In the native pea protein, methionine is the first-limiting amino acid. However, as methionine amino acid was supplemented to both diets at a level of the requirement for growing rats (National Research Council 1995), the possibility that the concentration of either methionine was limiting for growth of the rats fed the pea protein diet can be ruled out. Comparison of the analysed concentrations of amino acids in the pea protein diet with estimates of indispensable amino acid requirement of rats (National Research Council 1995) suggests that the pea protein supplied sufficient amounts of all the indispensable amino acids with the only exception of tryptophan. The tryptophan content of the pea protein diet was 1.4 g/kg which is below the estimated requirement of 2.0 g/kg for growing rats (National Research Council 1995). Therefore, we assume that tryptophan was the first limiting amino acid in pea protein, and the low tryptophan content of the protein was probably responsible for the lower body weight gains of rats fed the pea protein diet compared to rats fed the casein diet. We can also not completely rule out the possibility that digestibility of amino acids in the pea protein diet was also lower than in the casein diet.

It is still a subject of controversy which components of plant protein are responsible for their effects on the lipid metabolism. Alterations of plasma amino acids mediated by differences in amino acid patterns of the dietary proteins could contribute to the effects on lipid metabolism observed. Some studies showed that the effect of soy protein on the lipid metabolism is at least partially mediated by its low content of methionine (Morita et al., 1996; Sugiyama et al., 1996, 1997; Shimada et al., 2003). We suggest that methionine might not be involved in the effects of pea protein on the cholesterol metabolism observed in this study because methionine concentrations of both diets were adjusted to an identical level. It has been suggested that dietary proteins with high arginine:lysine ratios act hypocholesterolemic (Kritchevsky et al., 1982; Sugano et al., 1984; Vahouny et al., 1985; Gudbrandsen et al., 2006) and we found that this ratio was distinctly higher in pea protein diet than in the casein diet (the arginine:lysine ratio was 0.45 for casein and 1.20 for pea protein). In a study of Alonso et al. (2001), raw and extracted pea seed meals strongly reduced serum total cholesterol, LDL and the HDL/cholesterol ratio. The authors of that study suggested that these effects could be due to a lower plasma lysine:arginine ratio in rats fed diets supplemented with pea meals. However, it should be noted that in the study the effect of pea meal on serum cholesterol concentration could have been induced, at least in part, by nutrients different from protein, i.e. non-starch polysaccharides which are well known to lower plasma cholesterol concentration by stimulation of fecal excretion of bile acids (Morgan et al., 1993). Although we did not observe a hypocholesterolemic effect of pea protein, it is possible that the effects of pea protein on cholesterol metabolism were at least in part mediated by the concentrations of arginine and lysine in the respective diets. It is also possible that the effects of plant proteins are caused by specific peptides. In soy protein, alpha and alpha' subunits from 7S soy globulin have been identified as peptides that may regulate cholesterol homeostasis in HepG2 cells (Lovati et al., 2000); in lupine protein, conglutin γ has been identified as a peptide which increases LDL receptor activity in HepG2 cells (Sirtori et al., 2004). Whether amino acids or peptides are actually responsible for the observed effects of pea protein on cholesterol metabolism in rats remains to be clarified in further studies. However, it seems not very likely that the remaining isoflavones in the ethanol-washed pea

protein isolate may contribute to the observations made, because their concentration is extremely low compared to the concentrations required for induction of hypolipidaemia (Ali et al., 2004; Mullen et al., 2004).

To find out possible explanations for the reduced cholesterol concentration in the liver of rats fed pea protein, we determined hepatic relative mRNA concentrations of CYP7A1, faecal excretion of bile acids and hepatic relative mRNA concentrations of SREBP-2 and its target genes HMG-CoA reductase and LDL receptor. These analyses show that pea protein stimulates the excretion of bile acids via faeces, an effect which is probably mediated by an up-regulation of CYP7A1, the key enzyme of hepatic bile acid synthesis (Vlahcevic et al., 1999). An increased loss of bile acids via faeces causes a reduction of hepatic cholesterol pool (Beynen, 1987) and this may be the explanation for the reduced hepatic cholesterol concentration observed in rats fed pea protein. Martins et al. (2004) have recently found that feeding of raw peas to rats stimulates excretion of bile acids via faeces. They suggested that this effect was induced by non-starch polysaccharides which are present in relatively high concentrations in peas. As the pea protein used in this study had a low concentration of crude fibre, it is suggested that the protein fraction contributes mainly to the stimulating effect of peas on bile acid excretion.

The present study shows that pea protein increases hepatic mRNA concentrations of SREBP-2 and its target genes HMG-CoA reductase and LDL receptor. It has been shown that gene expression and proteolytic activation of SREBP-2 in the liver is controlled by the concentration of sterols in the liver (Brown et al., 2002). After synthesis in membranes of the endoplasmic reticulum (ER), SREBPs form a complex with SREBP-cleavage activating protein (SCAP). When cells are depleted of cholesterol, SCAP escorts SREBPs from ER to Golgi. Within the Golgi, two resident proteases, site-1 protease and site-2 protease sequentially cleave the SREBPs, release the amino-terminal bHLH-Zip-containing domain from the membrane, and allow it to translocate to the nucleus and activate transcription of their target genes (Brown and Goldstein, 1999; Brown et al., 2000). In contrast, when concentration of sterols in the cell is high, the SREBP–SCAP complex is retained in the ER, and thus proteolytic activation of SREBP-2 and up-regulation of its target genes is prevented. Therefore, we assume that the up-regulation of SREBP-2 and its target genes HMG-CoA reductase and LDL receptor in the liver of rats fed pea protein was induced by an increased consumption of cholesterol for bile acid synthesis. Therefore, an increased hepatic cholesterol synthesis and an increased uptake of LDL into the liver can be regarded as compensatory means to control the cholesterol concentration in the liver.

In rats fed whole raw peas, an increased activity of HMG-CoA reductase in the liver was also found (Martins et al., 2004). Our study suggests that this effect may have been induced at least in part by the protein fraction of peas. Interestingly, similar effects with respect to hepatic cholesterol concentration, excretion of bile acids via faeces and activities of HMG-CoA reductase and CYP7A1 as those observed in this study in rats fed pea protein have recently been reported in rats fed highly purified soy bean protein (Madani et al., 1998). This suggests that pea protein and soy protein could affect cholesterol metabolism in rats in a similar way.

Analysis of cholesterol concentrations in lipoproteins of different densities showed that rats fed pea protein have a lower concentration of cholesterol in the VLDL fraction than rats fed casein. VLDL is the vehicle for the removal of cholesterol from the liver to the tissues. We suggest that the reduced cholesterol concentration in VLDL was due to an increased hepatic loss of cholesterol by formation and excretion of bile acids. It has been shown that stimulation of bile acid formation in the liver lowers incorporation of cholesterol into VLDL (Spady et al., 1998).

Although rats fed pea protein had a reduced concentration of cholesterol in the liver, they did not differ in their plasma cholesterol concentration from rats fed casein. A similar observation was made in another study dealing with the effect of purified pea protein on cholesterol metabolism (Jacques et al., 1986). In that study, purified pea protein did not change plasma total serum cholesterol concentration in comparison with various other animal and vegetable proteins in rats fed cholesterol-free or cholesterol enriched diets. In contrast, in another study, feeding a pea protein concentrate at 10% protein level lowered plasma cholesterol in rats in comparison with casein (Mayilvaganan et al., 2004). As the protein concentrate used in that study was not highly purified, it cannot be ruled out that the cholesterol lowering effect was caused by the protein or by other compounds. In the present study, rats fed pea protein moreover did not differ in the concentrations of cholesterol in LDL

and HDL from rats fed casein. This is in contrast to soy protein which causes a distinct reduction of LDL cholesterol in man and animals (Carroll and Kurowska, 1995). LDL receptor is responsible for uptake of cholesterol-carrying lipoprotein particles into cells and is a major regulator of circulating LDL cholesterol (Brown et al., 1981). In rats fed pea protein, relative mRNA concentration of the LDL receptor in the liver was up-regulated by 24%. However, it seems that this extent of up-regulation of LDL receptor was not sufficient to efficiently stimulate uptake of LDL into the liver and thereby lower LDL cholesterol concentrations.

Both proteins had low concentrations of native lipids. As the lipids originating from pea protein had a high concentration of linoleic acid and α-linoleic acid, proportions of these two fatty acids in total lipids were slightly higher and those of saturated fatty acids were slightly lower in the pea protein diet than in the casein diet. It has been shown that n-6 PUFA are able to stimulate cholesterol synthesis and bile acid excretion (Fernandez and West, 2005), effects which were observed in the pea protein diet. However, as differences in the proportion of linoleic acid in both diets were small (11.2 vs. 10.4 g/100 g total fatty acids), we assume that they were at least not mainly responsible for the alterations in cholesterol metabolism observed in rats fed the pea protein diet. N-3 PUFA influence mainly triacylglycerol metabolism, i.e. hepatic lipogenesis and VLDL secretion (Fernandez and West, 2005). As there were no differences in triacylglycerol concentrations in liver, plasma and VLDL between both groups of rats, the difference in the proportion of α-linolenic acid in both diets (1.20 vs. 0.88 g/100 g total fatty acids) was obviously insufficient to induce changes in hepatic triacylglycerol metabolism.

In conclusion the present findings suggest that the pea protein isolated from yellow peas stimulates the excretion of bile acids via faeces, which in turn leads to a reduction of the cholesterol concentration in the liver and lowers secretion of cholesterol via VLDL. It is also shown that gene expression of SREBP-2 and its target genes HMG-CoA reductase and LDL-receptor in the liver is up-regulated, probably to compensate for the increased loss of cholesterol for bile acid synthesis.

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ORIGINAL ARTICLE

Hypolipidaemic effects of potato protein and fish protein in pigs

J. Spielmann, H. Kluge, G. I. Stangl and K. Eder

Institute of Agricultural and Nutritional Sciences, Martin-Luther-University Halle-Wittenberg, Halle (Saale), Germany

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Correspondence
K. Eder, Institute of Agricultural and Nutritional Sciences, Martin-Luther-University Halle-Wittenberg, Emil-Abderhalden-Str, 26 06108 Halle (Saale), Germany. Tel: 49 345 5522702; Fax: 49 345 5527124; E-mail: klaus.eder@landw.uni-halle.de

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Summary

This study was performed to assess the effects of potato protein and fish protein on concentrations of lipids in plasma and lipoproteins and the expression of genes involved in lipid metabolism in pigs used as an animal model. Therefore, 27 young male pigs with an average body weight of 22 kg were fed diets supplemented with protein extracted from potatoes (containing 849 g protein/kg dry matter), Alaska Pollack fillet as a source of fish protein (containing 926 g crude protein/kg dry matter) or casein which was used as control, for 3 weeks. Diets were formulated to supply identical amounts of each protein to the pigs by the three protein sources, namely 116 g/day in first week and 150 g/day in the second and third week. Pigs fed potato protein had lower concentrations of cholesterol in plasma and LDL than pigs fed casein ($p < 0.05$); no effect was observed on concentrations of HDL cholesterol and triglycerides. Pigs fed fish protein had lower cholesterol concentrations in plasma, LDL and HDL, and lower triglyceride concentrations in triglyceride-rich lipoproteins than pigs fed casein ($p < 0.05$). mRNA concentrations of genes involved in bile acid synthesis and cholesterol uptake were higher in pigs fed fish protein than in pigs fed casein ($p < 0.05$); no effect on these genes was observed in pigs fed potato protein. Expression of genes involved in lipogenesis and fatty acid oxidation was not altered by fish protein. In conclusion, this study shows that fish protein and potato protein lower plasma cholesterol concentrations in pigs. The hypocholesterolaemic effect of fish protein might be in part caused by a stimulation of bile acid synthesis; the reason for the hypocholesterolaemic effect of potato protein requires further elucidation.

Introduction

It has been established that dietary proteins influence lipid metabolism in man and animals (Anderson et al., 1995; Sirtori et al., 1995, 1998; Iritani et al., 1996; Sirtori and Lovati, 2001; Koba et al., 2003). Most studies dealing with the effects of dietary proteins on the lipid metabolism, however, have focused on the effects of soy protein compared to casein. It has been reported many times that soy protein has hypocholesterolaemic (Anderson et al., 1995; Sirtori et al., 1995, 1998; Iritani et al., 1996; Sugiyama et al., 1996; Tovar et al., 2002; Koba et al., 2003) and hypotriglyceridaemic effects (Tovar et al., 2002; Ascencio et al., 2004; Shukla et al., 2007) in laboratory animals and humans when compared with casein.

It has been suggested that individual amino acids are involved in the hypolipidaemic effects of dietary proteins (De Schrijver, 1990; Ascencio et al., 2004; Shukla et al., 2007). High levels of cysteine and a high arginine:lysine ratio are supposed to be two factors which could at least in part be responsible for the lipid lowering effect (Kritchevsky et al., 1982; Salil and Rajamohan, 2001; Lin and Yin, 2007). Besides soy beans, proteins isolated from potatoes or from fish are two other protein sources which are characterized by high concentrations of cysteine and

arginine compared to casein. Thus, it is expected that potato protein and fish protein could exert also hypolipidaemic effects compared to casein. So far, only two rat studies have been published which show that potato protein lowers plasma cholesterol concentration compared to casein (De Schrijver, 1990; Morita et al., 1997). However, in one of these studies food intake differed between the experimental groups (Morita et al., 1997) which could have confounded with the effects of the protein on plasma lipid concentration. Moreover, the few studies dealing with the effect of fish protein on plasma lipids reveal conflicting results. Recent studies with rats (Murata et al., 2004; Ait Yahia et al., 2005), and a previous experiment with rabbits (Bergeron et al., 1992) have found a reduction of the concentration of triglycerides in plasma and very low density lipoprotein (VLDL) by fish protein treatment compared to casein treatment. Other experiments with rats, however, did not find any effect of fish protein on concentrations of triglycerides in plasma and VLDL (Yahia et al., 2003; Wergedahl et al., 2004).

The lack of consistent data concerning the effects of potato and fish protein on plasma lipids is one factor that requires further systematic studies, the other one is that there are certain disadvantages in the common animal model used for these studies. In most experiments, rats were used as animal model. However, as a matter of fact rats differ strongly in their lipoprotein metabolism from humans. For instance, their lipoprotein profile is completely different from humans with HDL being the major plasma cholesterol carrier in the rat, and they do not possess plasma cholesteryl ester transfer protein, a critical component of human reverse cholesterol transport. Therefore, response of plasma lipids to diet in rats is commonly different to that of humans (Fernandez and West, 2005).

The aim of this study, therefore, was to examine the effects of potato and fish protein on plasma lipid concentrations in pigs, a model well accepted to study hypercholesterolaemia and atherosclerosis because of their similarities to humans in these areas (Van Tol et al., 1991). Casein was used as reference protein. To elucidate possible mechanism by which the test proteins could act on lipid metabolism, we considered expression of hepatic genes involved in cholesterol synthesis and uptake and bile acid synthesis. Whole body cholesterol homeostasis is achieved by a balance of biosynthesis, storage, catabolism and transport of cholesterol in the liver (Weber et al., 2004). Biosynthesis of cholesterol and uptake of cholesterol into the liver by LDL is controlled by sterol regulatory element-binding protein (SREBP)-2 which regulates the transcription of genes encoding enzymes in the biosynthetic pathway of cholesterol such as 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and cholesterol uptake such as LDL receptor (Vallett et al., 1996; Horton et al., 2002). To clarify whether dietary proteins may influence the excretion of bile acids via faeces, we determined hepatic mRNA concentration of cholesterol 7α-hydroxylase (CYP7A1), the key enzyme in the synthesis of bile acids from cholesterol. mRNA concentrations of lipogenic genes such as fatty acid synthase (FAS) and acyl CoA carboxylase (ACC) and peroxisome proliferators-activated receptor (PPAR)α related genes involved in fatty acid oxidation such as acyl-CoA oxidase (ACO) and carnitine-palmitoly-transferase (CPT)-1a may help to elaborate possible mechanism by which the test proteins may influence the concentrations of circulating triglycerides.

Material and methods

All experimental procedures described followed established guidelines for the care and use of laboratory animals according to law on animal welfare and were approved by the local veterinary office (Halle/Saale, Germany).

Animals

Twenty-seven male 9-week-old crossbred pigs [(German Landrace × Large White) × Pietrain] with an average body weight of 22 (± 2, SD) kg were kept in a room under controlled conditions at 23 ± 2 °C and 55 ± 5% relative humidity with light from 06:00 to 18:00 hour. One day before the start of the experimental feeding period the pigs were weighed and randomly assigned to three groups of nine animals each.

Diets

All three groups received a basal diet (Table 1), which was supplemented with the respective protein source. Protein sources were either non-hydrolysed casein (Nähr Kasein, Meggle, Wasserburg/Inn, Germany; 919 g crude protein/kg DM), which was used as a control protein, potato protein (AVEBE, Veendam, the Netherlands; 849 g crude protein/kg DM) and Alaska Pollack fillet (aro, Goldhand Vertriebsgesellschaft mbH, Düsseldorf, Germany; 926 g crude protein/kg DM). Food intake was controlled to avoid

Table 1 Composition of the basal diet

Components	Basal diet (g/kg diet)
Wheat	580
Soy bean meal	200
Cellulose	50
Barley	100
Sunflower oil	30
Mono-calcium-phosphate	15
Mineral and vitamine premix	25

disturbing effects of potential differences in food intake. The amount of diet was slightly below that consumed *ad libitum* of similar diets by pigs in preliminary studies. During the first week (day 1 to day 7) each animal in the experiment received 666 g of basal diet/day, supplemented with the respective protein source in an amount to yield 116 g of crude protein/day (Table 2). As the pigs gained body weight and were able to consume more food, the amount of food administered was increased after the first week. In the second and the third week (day 8 to day 21) each animal in the experiment received 833 g of basal diet/day, supplemented with the respective protein source in an amount to yield 150 g of crude protein/day (Table 2). The fish diet was freshly prepared each day by adding raw fillet of Alaska Pollack to the basal diet. Diets were formulated to meet the requirement of nutrients according to National Research Council (1998). All three protein sources had relatively small concentrations of lipids (g/kg dry matter: casein, 1.4; potato protein, 22.8; fish protein: 18.2). To avoid confounding effects of fatty acids on lipid metabolism, the fatty acid composition of the dietary proteins was analysed and the differences in the amounts of n-3 polyunsaturated fatty acids (PUFA) between the proteins were adjusted by adding individual amounts of salmon oil (Caelo, Hilden, Germany) to the casein and potato protein diet (Table 2).

Table 2 Amounts of basal diet and valuable ingredients administered to pigs in feeding period 1 (day 1 to day 7) and feeding period 2 (day 8 to day 21)

	Casein diet		Potato protein diet		Fish protein diet	
Feeding period	1	2	1	2	1	2
Component (g/day)						
Basal diet	666	833	666	833	666	833
Casein	137	177	0	0	0	0
Potato protein	0	0	150	194	0	0
Alaska pollack fillet	0	0	0	0	893	1154
Salmon oil	6.25	7.75	6.25	7.75	0	0

The daily amount of food was administered once a day at 7.00 hours. To avoid differences in food intake, the diet intake was controlled. As the amount of diet administered was approximately 15% below that consumed *ad libitum* by pigs of a similar weight (as assessed in a previous study), the diet offered was completely consumed by all pigs during the experiment. All pigs had free access to water. The experimental diets were administered for 21 days.

Analysis of amino acids and fatty acids in the diets

To determine the amino acid concentrations in the diet, samples were oxidized and then hydrolysed with 6 M HCl (Naumann and Basler, 1993). Separation and quantification of the amino acids were performed by ion-exchange chromatography following post-column derivatization in an amino-acid analyser (Biotronic LC 3000; Eppendorf, Hamburg; Germany). Tryptophan was determined by digesting the diet with barium hydroxide (Fontaine et al., 1998). The tryptophan concentration in the diet was measured by reversed-phase HPLC (Eder et al., 2001). For determination of fatty acid composition of total lipids in the diets to calculate the amounts of fatty acids ingested per day, lipids were extracted from the diet with a mixture of *n*-hexane and isopropanol (3:2, v/v) (Hara and Radin, 1978). Lipids were transmethylated with trimethylsulfonium hydroxide and fatty acid methyl esters were separated by capillary gas chromatography (Brandsch et al., 2002).

Sample collection

At the end of the experimental period of 21 days, the pigs were captive-bolt stunned and exsanguinated. As it has shown that fasting causes a down-regulation of many genes involved in lipid metabolism (Shimano et al., 1999), each pig received its last food portion 3 h before killing. Blood was collected into heparinized polyethylene tubes. Plasma was obtained by centrifugation of the blood (1100 *g*; 10 min; 4 °C). Livers were removed, weighed and stored at −80 °C until analysis. Samples of the adipose tissue from the neck of the animals fed casein and fish protein were removed and stored at −80 °C until analysis.

Lipoprotein separation and lipid analyses

Plasma lipoproteins were separated by step-wise ultracentrifugation (900 000 *g* at 4 °C for 1.5 h;

Mikro-Ultrazentrifuge, Sorvall Products, Bad Homburg, Germany) using appropriate density cuts (VLDL, $\rho < 1.006$ kg/l; LDL, 1.006 kg/l $< \rho <$ 1.063 kg/l; HDL, $\rho > 1.063$ kg/l). As the animals were not fasted, the VLDL fraction was contaminated with chylomicrons. Therefore, the density fraction $\rho < 1.006$ kg/l is denominated 'VLDL + chylomicrons'. Plasma densities were adjusted by addition of sodium chloride and potassium bromide (Eder and Brandsch, 2002). Lipids from liver were extracted with a mixture of *n*-hexane and isopropanol (3:2, v/v) (Hara and Radin, 1978). For determination of the concentrations of lipids in liver, aliquots of the lipid extracts were dried and the lipids dissolved using Triton X-100 (De Hoff et al., 1978). Concentrations of cholesterol in plasma and lipoproteins and those of liver were determined using an enzymatic reagent kit (Cat.-No. 1.1300 99 90 314; DiaSys Diagnostic Systems, Holzheim, Germany). Using this kit, cholesterol esters are hydrolysed by cholesterol esterase and free cholesterol is subsequently oxidized to cholesterol-3-one in a reaction catalysed by cholesterol oxidase in which hydrogen peroxide is released. In the subsequent reaction catalysed by peroxidase, 4-aminoantipyrine and phenol form quinoneimine in the presence of hydrogen peroxide. Quinoneimine is used as the colorimetric indicator which is quantified at a wavelength of 500 nm using a spectrophotometer (Ultraspec 2000; Pharmacia Biotech, Freiburg, Germany).

Concentrations of triglycerides in plasma and lipoproteins and those of liver were also determined by an enzymatic reagent kit (Cat.-No.1.5760 99 90 314; DiaSys Diagnostic Systems). Using this kit, triglycerides are first hydrolysed to glycerol and free fatty acids by the action of lipoprotein lipase. Glycerol is then in the presence of adenosine triphosphate phosphorylated to glycerol-3-phosphate, which is thereafter oxidized to dihydroxyacetone phosphate by the action of glycerol-3-phosphate oxidase under release of hydrogen peroxide. In the subsequent reaction catalysed by peroxidase, quinoneimine is formed from aminoantipyrine and 4-chlorophenol in the presence of hydrogen peroxide. Quinoneimine is used as the colorimetric indicator which is quantified at a wavelength of 550 nm using a spectrophotometer (Ultraspec 2000; Pharmacia Biotech). For quantification of cholesterol and triglyceride concentrations, appropriate standards (HV calibrator; DiaSys Diagnostic Systems) were used.

RT-PCR analysis

Total RNA was isolated by the tissue lyser (Qiagen, Hilden, Germany) from frozen liver and adipose tissue samples using TRIZOL™ (Sigma-Aldrich, Steinheim, Germany) according to the manufacturer's protocol. cDNA synthesis was carried out as described recently (König and Eder, 2006). The mRNA concentrations of genes were measured by realtime detection PCR (Rotorgene 2000; Corbett Research, Mortlake, Australia) using SYBR® Green I (Sigma-Aldrich). Realtime detection PCR was performed with 1.25 U Taq DNA polymerase (Promega, Mannheim, Germany), 500 μM dNTPs and 26.7 pmol of the specific primers (Operon Biotechnologies, Cologne, Germany, Table 3). For determination of mRNA concentration a treshold cycle (C_t) was obtained from each amplification curve using the software RotorGene 4.6 (Corbett Research, Sydney, Australia). Calculation of the relative mRNA concentration was made using the $\Delta\Delta C_t$ method (Pfaffl, 2001). The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase was used for normalization.

Statistics

Data were statistically analysed with a one-way analysis of variance with dietary protein as factor using the Minitab Statistical Software (Minitab, State College, PA, USA). When there was an effect of the dietary protein ($p < 0.05$), means of the treatment groups (potato or fish protein treatment) were compared with the casein treatment by the Student's *t*-test. Means were considered significantly different at $p < 0.05$. Data in the text are given as means ± SD.

Results

Nutrient intake of the pigs

Daily intake of energy, crude protein, crude fat, crude ash and amino acids in the three treatments is shown in Table 4. Pigs fed the fish protein diet had a slightly lower energy intake than pigs fed the casein and the potato protein diet. The differences in the amino acid intake were caused by the different amino acid composition of the three protein sources. Among the indispensable amino acids the most marked differences were observed in the intake of phenylalanine + tyrosin which was lower in pigs fed the fish protein diet than in pigs fed casein, and the intake of threonine which was higher in pigs fed

Table 3 Characteristics of the specific primers used for reverse transcriptase polymerase chain reaction analysis

Gene	Forward primers (from 5′ to 3′)	Reverse primers (from 5′ to 3′)	Base pairs	Annealing temperature	NCBI GenBank
ACC	CTC CAG GAC AGC ACA GAT CA	GCC GAA ACA TCT CTG GGA TA	170	60 °C	AF175308
ACO	CTC GCA GAC CCA GAT GAA AT	TCC AAG CCT CGA AGA TGA GT	218	60 °C	AF185048
CPT-1	GCA TTT GTC CCA TCT TTC GT	GCA CTG GTC CTT CTG GGA TA	198	60 °C	AF288789.1
CYP7A1	TAT AGG GCA CGA TGC ACA GA	ACC TGA CCA GTT CCG AGA TG	200	60 °C	NM001005352
FAS	AGC CTA ACT CCT CGC TGC AAT	TCC TTG GAA CCG TCT GTG TTC	196	62 °C	EF589048
GAPDH	AGG GGC TCT CCA GAA CAT CAT CC	TCG CGT GCT CTT GCT GGG GTT GG	446	60 °C	AF017079
HMG-CoA reductase	GGT CAG GAT GCG GCA CAG AAC G	GCC CCA CGG TCC CGA TCT CTA	127	65 °C	S79678
LDL receptor	AGA ACT GGC GGC TGA AGA GCA TC	GAG GGG TAG GTG TAG CCG TCC TG	115	60 °C	S64272
SREBP-2	CGC TCG CGA ATC CTG CTG TG	GGT GCG GGT CCG TGT CGT G	103	65 °C	DQ0204761
PPARα	CAG CCT CCA GCC CCT CGT C	GCG GTC TCG GCA TCT TCT AGG	341	58 °C	NM00104426

ACC, acyl-coenzyme A carboxylase; ACO, acyl-coenzyme oxidase; CPT, carnitine-palmitoyl transferase; CYP7A1, cholesterol 7α-hydroxylase; FAS, fatty acid synthase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; PPARα, peroxisome proliferators-activated receptorα; SREBP-2, sterol regulatory element-binding protein-2.

Table 4 Daily nutrient intake of pigs fed diets containing casein, potato protein or fish protein in feeding period 1 (day 1 to day 7) and feeding period 2 (day 8 to day 21)

	Casein		Potato protein		Fish protein	
Feeding period	1	2	1	2	1	2
Nutrient intake (per day)						
ME (MJ)	11.4	14.3	11.4	14.4	10.9	13.7
Crude protein (g)	180	230	180	230	180	230
Crude fat (g)	34.2	42.7	37.1	46.4	30.0	37.6
Crude ash (g)	30.4	38.2	31.0	39.0	31.9	40.2
Indispensable amino acids (g)						
Arginine	7.1	10.3	9.0	11.3	11.3	14.5
Histidine	4.8	6.1	3.8	4.9	4.5	5.7
Isoleucine	7.4	9.4	8.2	10.3	7.2	9.2
Leucine	15.2	19.3	16.2	20.5	14.3	18.3
Lysine	11.6	14.7	11.7	14.8	13.2	17.0
Methionine	4.3	5.4	3.7	4.6	4.9	6.4
Methionine + cysteine	5.9	7.3	6.6	8.2	7.8	9.9
Phenylalanine	8.5	10.8	10.5	13.2	7.8	10.0
Phenylalanine + tyrosine	15.6	19.7	17.9	22.6	13.2	17.0
Threonine	7.0	8.8	9.0	11.2	7.6	9.7
Tyrosine	7.1	8.9	7.4	9.4	5.4	7.0
Tryptophan	2.1	2.7	2.1	2.7	2.0	2.5
Valine	9.5	11.9	9.9	12.5	8.3	10.6
Dispensable amino acids (g)						
Alanine	5.7	7.1	8.0	10.1	9.4	12.1
Aspartamic acid	11.1	14.1	17.7	22.4	15.4	19.9
Glutamic acid	43.2	54.7	30.1	37.7	37.9	48.2
Glycine	4.4	5.6	8.1	10.2	7.7	9.8
Proline	23.1	29.3	13.7	17.5	12.6	15.9
Serine	9.3	11.8	8.5	10.7	7.8	10.0

potato protein than in pigs fed casein. Among the dispensable amino acids, pigs fed potato protein and fish protein, respectively, had a higher intake of aspartamic acid, glycine, alanine and arginine and a lower intake of glutamic acid and proline than pigs fed casein. The daily intake of fatty acids is shown in Table 5. Due to the addition of salmon oil to the casein and potato protein diets, the intake of n-3 PUFA was similar in the three groups of pigs. The daily intake of saturated fatty acids, monounsaturated fatty acids and n-6 PUFA was also similar between the three groups of pigs.

Body and liver weights

Initial body weights and body weights at day 21 did not differ between the three groups of pigs (Table 6). Pigs fed the potato protein had higher liver weights, in absolute terms, and higher relative liver weights, expressed per kg of body weight, than the control pigs fed casein ($p < 0.05$, Table 6). Fish protein treatment did not influence the liver weights to the animals compared to casein treatment.

Concentrations of total cholesterol and triglycerides in plasma, lipoproteins and liver

Pigs fed potato protein had 10% lower cholesterol concentrations in plasma and 17% lower concentrations of LDL cholesterol than pigs fed casein ($p < 0.05$, Table 7). Concentrations of cholesterol in HDL and liver were not influenced by potato protein compared to casein. Pigs fed fish protein had 13% lower cholesterol concentrations in plasma, 15% lower cholesterol concentrations in LDL and 10% lower concentrations of cholesterol in HDL than pigs fed casein ($p < 0.05$, Table 7). Liver cholesterol concentration was not influenced by fish protein.

Concentrations of triglycerides in plasma, VLDL + chylomicrons and liver did not differ between pigs fed potato protein and pigs fed casein

Table 5 Daily fatty acid intake of pigs fed diets containing casein, potato protein or fish protein in feeding period 1 (day 1 to day 7) and feeding period 2 (day 8 to day 21)

	Casein		Potato protein		Fish protein	
Feeding period	1	2	1	2	1	2
Fatty acids (g/day)						
14:0	0.39	0.48	0.39	0.48	0.13	0.17
16:0	4.35	5.47	4.88	6.12	4.41	5.57
18:0	1.06	1.33	1.18	1.49	1.07	1.35
∑SFA	5.80	7.28	6.45	8.09	5.61	7.09
16:1	0.51	0.63	0.52	0.64	0.11	0.15
18:1	6.55	8.23	6.38	8.01	5.80	7.27
∑MUFA	7.06	8.86	6.90	8.65	5.91	7.42
18:2 (n-6)	13.9	17.4	14.2	17.9	12.6	15.7
20:4 (n-6)	0.08	0.09	0.08	0.09	0.05	0.06
∑n-6 PUFA	14.0	17.5	14.3	18.0	12.7	15.8
18:3 (n-3)	1.60	2.01	1.95	2.45	1.47	1.84
20:5 (n-3)	0.81	1.00	0.82	1.02	0.78	1.01
22:5 (n-3)	0.22	0.28	0.24	0.30	0.10	0.13
22:6 (n-3)	0.58	0.72	0.59	0.73	0.87	1.13
∑n-3 PUFA	3.21	4.01	3.60	4.50	3.22	4.11

MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids.

Table 6 Body and liver weights of pigs fed diets containing casein, potato protein or fish protein

Diet	Casein	Potato protein	Fish protein
Body weight, day 1 (kg)	22.1 ± 1.8	21.5 ± 2.2	21.7 ± 2.5
Body weight, day 21 (kg)	34.2 ± 2.6	35.0 ± 1.5	35.6 ± 2.5
Liver weight (g)	708 ± 62	823 ± 69*	758 ± 46
Liver weight/body weight (g/kg)	20.8 ± 2.0	23.6 ± 2.5*	21.4 ± 2.9

Mean values and standard deviations, n = 9/group.
*Significantly different from casein group ($p < 0.05$).

Table 7 Concentrations of total cholesterol and triglycerides in plasma, lipoproteins and liver of pigs fed diets containing casein, potato protein or fish protein

Diet	Casein	Potato protein	Fish protein
Total cholesterol			
Plasma (mmol/l)	2.07 ± 0.15	1.86 ± 0.12*	1.81 ± 0.16*
LDL (mmol/l)	1.15 ± 0.13	0.95 ± 0.13*	0.98 ± 0.13*
HDL (mmol/l)	0.90 ± 0.07	0.86 ± 0.07	0.81 ± 0.05*
Liver (μmol/g)	5.54 ± 1.21	5.86 ± 0.38	5.93 ± 0.77
Triglycerides			
Plasma (mmol/l)	0.45 ± 0.09	0.50 ± 0.09	0.33 ± 0.05*
VLDL + chylomicrons (mmol/l)	0.25 ± 0.08	0.27 ± 0.08	0.15 ± 0.05*
Liver (μmol/g)	7.17 ± 1.21	7.64 ± 0.72	7.09 ± 1.19

Mean values and standard deviations. n = 9/group.
*Significantly different from the casein group ($p < 0.05$).

(Table 7). Pigs fed fish protein had 27% lower triglyceride concentrations in plasma and 40% lower triglyceride concentrations in VLDL + chylomicrons than pigs fed casein ($p < 0.05$, Table 7). Triglyceride concentrations in liver did not differ between the two groups of pigs.

Relative mRNA concentrations of genes involved in hepatic cholesterol metabolism

Relative mRNA concentrations of SREBP-2, HMG-CoA reductase, LDL receptor and CYP7A1 in liver were not different between pigs fed potato protein and pigs fed casein (Fig. 1). Pigs fed fish protein had higher mRNA concentrations of SREBP-2 and its target genes HMG-CoA reductase and LDL receptor as well as higher mRNA concentrations of CYP7A1 in the liver than pigs fed casein ($p < 0.05$, Fig. 1).

Relative mRNA concentrations of genes involved in fatty acid metabolism of pigs fed fish protein in liver and adipose tissue

As pigs fed fish protein had lower concentrations of triglycerides in plasma and VLDL + chylomicrons compared to pigs fed casein, we examined mRNA concentrations of ACC and FAS, genes involved in fatty acid synthesis in liver and adipose tissue, the most important tissues involved in *de novo* synthesis of fatty acids in the pig. We also determined mRNA concentrations of some genes involved in hepatic fatty acid oxidation such as PPARα, a transcription

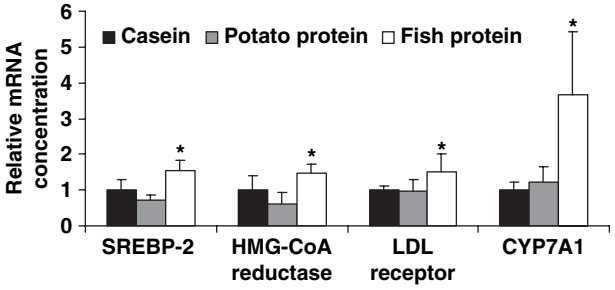

Fig. 1 Relative mRNA concentrations of sterol regulatory element-binding protein (SREBP)-2, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA)-reductase, LDL receptor and cholesterol 7α-hydroxylase (CYP7A1) in the liver of pigs fed diets containing casein, potato protein or fish protein for 21 days. Total RNA was extracted from liver and SREBP-2, HMG-CoA-reductase, LDL receptor and CYP7A1 mRNA concentrations were determined by realtime detection RT-PCR analysis using glyceraldehyde-3-phosphate-dehydrogenase mRNA concentration for normalization. Each bar represents means ± SD, n = 9. Data are expressed relative to mRNA concentrations of casein fed pigs (casein = 1). *Significantly different from the casein group ($p < 0.05$).

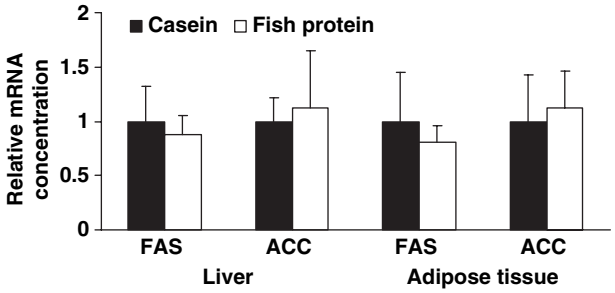

Fig. 2 Relative mRNA concentrations of fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC) in liver and adipose tissue of pigs fed a diet containing casein or fish protein for 21 days. Total RNA was extracted from liver and adipose tissue, respectively, and FAS and ACC mRNA concentrations were determined by realtime detection RT-PCR analysis using glyceraldehyde-3-phosphate-dehydrogenase mRNA concentration for normalization. Each bar represents means ± SD, $n = 9$. Data are expressed relative to mRNA concentrations of casein fed pigs (casein = 1).

factor that controls mitochondrial and peroxisomal β-oxidation of fatty acids, as well as ACO and CPT-1a which are target genes of PPARα.

The relative mRNA concentrations of FAS and ACC in liver and in adipose tissue of pigs fed fish protein did not differ from those of pigs fed casein (Fig. 2). Relative mRNA concentrations of PPARα (0.85 ± 0.18 vs. 1.00 ± 0.31) and ACO (0.85 ± 0.20 vs. 1.00 ± 0.18) in the liver did not differ between pigs fed fish protein and those fed casein; the relative mRNA concentration of CPT-1a in liver was lower in pigs fed fish protein than in pigs fed casein (0.65 ± 0.17 vs. 1.00 ± 0.37; $p < 0.05$).

Discussion

The aim of the present study was to investigate the effects of potato protein and fish protein on the concentrations of cholesterol and triglycerides in plasma and lipoproteins of pigs as an adequate animal model for human lipoprotein metabolism. The study shows that both protein sources act hypocholesterolaemic compared to casein by reducing LDL cholesterol concentration, and in the fish protein treatment also by reducing HDL cholesterol concentration. The observed effect of the proteins on plasma cholesterol are in accordance to data obtained from rats fed potato protein (De Schrijver, 1990; Morita et al., 1997) and from rats fed fish protein (Zhang and Beynen, 1993; Wergedahl et al., 2004; Ait Yahia et al., 2005; Shukla et al., 2006). Our gene expression data strongly suggest that the cholesterol-lowering effect of fish protein might be caused mainly by an up-regulation of CYP7A1, the initial and rate-limiting enzyme in conversion of cholesterol to 7α-hydroxylated bile acids (Vlahcevic et al., 1999; Chiang et al., 2001). An up-regulation of this enzyme leads to an increased secretion of bile acids from the liver into the small intestine and in turn to a reduced excretion of bile acids via faeces (Vlahcevic et al., 1999). An increased loss of bile acids via faeces causes a reduction of hepatic cholesterol pool (Beynen, 1987), which in turn may have stimulated gene expression of LDL receptor via an activation of SREBP-2. Gene expression and proteolytic activation of SREBP-2 in the liver are controlled by the concentration of sterols in the liver (Brown et al., 2002). After synthesis in membranes of the endoplasmatic reticulum (ER), SREBPs form a complex with SREBP-cleavage activating protein (SCAP). When cholesterol level in hepatocytes decreases SCAP escorts SREBPs from ER to Golgi. Within the Golgi, two resident proteases, site-1 protease and site-2 protease sequentially cleave the SREBPs, release the amino-terminal bHLH-Zip-containing domain from the membrane, and allow it to translocate to the nucleus and activate transcription of their target genes such as HMG-CoA reductase or LDL receptor (Brown and Goldstein, 1999; Brown et al., 2000). The observed up-regulation of hepatic LDL receptor leads to an increased uptake of LDL into the liver which may explain the reduced concentration of LDL cholesterol in pigs treated with fish protein. The HDL cholesterol-lowering effect observed in pigs treated with fish protein is in accordance with findings in hyperlipidaemic obese 'Zucker' rats fed fish protein from salmon (Wergedahl et al., 2004) and recent findings in rats fed fish protein derived from Alaska Pollack fillet (Shukla et al., 2006). HDL is the principle vehicle for removal of surplus cholesterol from the peripheral tissues for disposal in the liver. Several genes are involved in HDL metabolism, such as lecithin:cholesterol acyl transferase (LCAT), critical for cholesterol uptake and maturation of HDL-3 to HDL-2 (Genest et al., 1999), apo AI, a structural component of HDL, and SR-B1, responsible for the selective uptake of HDL. A recent study with rats has found that fish protein stimulates the conversion of HDL3 to HDL2, probably as a result of an enhanced activity of LCAT activity (Ait Yahia et al., 2005). This could be also an explanation for the observed HDL lowering effect of fish protein in this study.

However, the cholesterol-lowering effect of potato protein observed in this study could not be explained on the base of alterations in gene expression because neither SREBP-2 and its target genes

nor the expression of CYP7A1 was altered in pigs treated with potato protein compared to those treated with casein. However, this is in contrast to a previous study of Morita et al. (1997) who found an increased gene expression of CYP7A1 and an increased excretion of sterols via faeces in rats fed potato protein. Other possible mechanisms of the hypocholesterolaemic action of potato protein observed in this study with pigs could be an increased binding activity of LDL receptor, an increased transport of cholesterol into the bile acid or a reduced formation of LDL from VLDL.

The triglyceride-lowering effect of the fish protein found in this study is in agreement with some studies in rats, rabbits and pre-menopausal women in which fish protein also lowered plasma triglyceride concentration (Bergeron et al., 1992; Gascon et al., 1996; Shukla et al., 2006). A reduction of plasma triglyceride concentration could principally be caused by a diminished synthesis of triglycerides in the liver, a diminished secretion of triglycerides from the liver via VLDL, an increased catabolism of fatty acids or an increased hydrolysis of triglyceride rich lipoproteins by lipoprotein lipase (LPL). As mRNA concentrations of FAS and ACC, two key enzymes in the synthesis of triglycerides, remained unchanged, it is unlikely that fish protein lowered *de-novo* synthesis of triglycerides in the liver. As in pigs, in contrast to rats, a large part of triglyceride synthesis occurs in adipose tissue (Ding et al., 2000) we also determined the relative mRNA concentration of FAS and ACC in that tissue. However, there was no indication that fish protein alters fatty acid synthesis in adipose tissue. Moreover, we examined whether fish protein could have stimulated β-oxidation in liver or adipose tissue by an activation of PPARα, a nuclear protein that controls mitochondrial and peroxisomal β-oxidation (Desvergne and Wahli, 1999). As mRNA concentration of PPARα and ACO were not altered in both tissues and as mRNA concentration of CPT-1a in the liver was even reduced, it is suggested that fish protein did not stimulate β-oxidation by an activation of PPARα. It has been well established that n-3 PUFA exert hypotriglyceridaemic effects by down-regulation of lipogenic enzymes in the liver due to inhibition of gene expression of SREBP-1 and by stimulation of hepatic β-oxidation of fatty acids due to activation of PPARα (Price et al., 2000; Neschen et al., 2002). The finding that these effects were not observed in pigs is a clear indication that the effects of fish protein in this study were not confounded by native n-3 PUFA present in pollack fillet. In this study, we have not examined the possibility that plasma triglyceride concentrations could be lowered by an increased expression of lipoprotein lipase (LPL). LPL catalyses the hydrolysis of triglycerides in circulating chylomicrons and VLDL. Besides its classical function, LPL can perform a non-catalytic bridging function that allows it to bind simultaneously to both lipoproteins and specific cell surface proteins, including receptors for LDL and VLDL (reviewed by Goldberg, 1996). LPL is known as a target gene of liver X receptor (LXR) which in turn is also a positive regulator of CYP7A1 transcription (Chiang et al., 2001). The observed up-regulation of CYP7A1 observed in the pigs fed fish protein therefore could be caused by an increased LXR activity which in turn could have stimulated the expression of LPL and the clearance of plasma triglycerides.

Studies dealing with the effects of different dietary proteins from plant and animal sources on lipid metabolism suggest that specific amino acids could be responsible for the various effects observed (Kritchevsky et al., 1982; Morita et al., 1997; Kern et al., 2002; Wergedahl et al., 2004; Lin and Yin, 2007). While cysteine has been shown to lower hepatic biosynthesis of triglycerides and cholesterol (Lin and Yin, 2007), arginine is supposed to lower plasma cholesterol concentration by an increased conversion of cholesterol to bile acids (Salil and Rajamohan, 2001). Both, fish protein and potato protein have higher levels of cysteine and arginine than casein. Therefore, it is possible that a part of the effects observed in this study was induced by these amino acids.

In conclusion, this study shows that potato protein and fish protein had cholesterol-lowering effects compared to casein, suggesting possible beneficial effects of these proteins. While the cholesterol-lowering effect of potato protein could not be explained on the base of mRNA data, fish protein seems to exert its effect via an up-regulation of CYP7A1 and LDL receptor. The observed reduction of HDL cholesterol in pigs fed the Alaska Pollack fillet could possibly limit the beneficial effect of the fish protein. Fish proteins also exerted a triglyceride-lowering effect which seemed not to be caused by reduced lipogenesis or increased fatty acid oxidation.

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Effects of Various Dietary Arginine and Lysine Concentrations on Plasma and Liver Cholesterol Concentrations in Rats

Julia Spielmann Anne Noatsch Corinna Brandsch Gabriele I. Stangl
Klaus Eder

Institute of Agricultural and Nutritional Sciences, Martin Luther University Halle-Wittenberg, Halle/Saale, Germany

Key Words
Arginine · Lysine · Cholesterol · Liver · Lipoproteins

Abstract
Background: It has been hypothesized that the arginine:lysine ratio of dietary proteins influences cholesterol concentrations in plasma and liver of men and animals. This study was performed to test this hypothesis in rats by using diets with various concentrations of arginine and lysine, differing in their arginine:lysine ratios. ***Methods:*** Two experiments with growing rats were performed, some of which received diets containing 4.5, 9 or 18 g arginine/kg and 9 or 18 g lysine/kg, respectively, for a period of 21 days. In the first experiment, a cholesterol-free diet was used; in the second experiment, a diet supplemented with cholesterol and sodium cholate as hypercholesterolaemic compounds was used. ***Results:*** In experiment 1, increasing the arginine concentration lowered HDL and plasma cholesterol concentration; however, cholesterol concentrations in liver, LDL and VLDL remained unchanged. In experiment 2, increasing the arginine concentration lowered HDL cholesterol and increased liver cholesterol ($p < 0.05$); cholesterol concentrations in plasma, LDL and VLDL remained unchanged. The only effect of the dietary lysine concentration concerned the effect on VLDL and liver cholesterol concentration, which were both lower in rats fed the diets with 18 g lysine/kg than in those fed the diets with 9 g lysine/kg ($p < 0.05$). Varying the dietary arginine:lysine ratio between 0.25 and 2.0 had no influence on cholesterol concentration in LDL and VLDL in both experiments; HDL cholesterol concentration was lowered by increasing this ratio ($p < 0.05$). ***Conclusion:*** The present study does not support the hypothesis that an increase in the dietary arginine:lysine ratio causes hypocholesterolaemic effects in rats.

Introduction

It has been established that proteins isolated from plants have beneficial effects on the lipid metabolism in man and animals [1–5]. Most studies dealing with the effects of dietary protein sources on the lipid metabolism have focused on the effects of protein isolated from soybeans compared to casein. It has been reported many times that protein isolated from soybeans has hypocholesterolaemic effects in laboratory animals, pigs and humans when compared to casein [2, 5–7]. Recently, it has been found that proteins extracted from sweet lupins [8] and peas [9] as well as from fish as a source of a protein of animal origin [10–12] also have beneficial effects on the cholesterol metabolism of rats and pigs.

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Klaus Eder
Institute of Agricultural and Nutritional Sciences
Martin Luther University Halle-Wittenberg
Emil-Abderhalden-Strasse 26, DE–06108 Halle/Saale (Germany)
Tel. +49 345 552 2702, Fax +49 345 552 7124, E-Mail klaus.eder@landw.uni-halle.de

Although hypocholesterolaemic effects of proteins isolated from plants have been recognized for a long time, it is still a subject of controversy which components of the protein isolates are responsible for their effects on cholesterol metabolism. It has been suggested that specific peptides such as alpha and alpha′ subunits from 7S soy globulin in soy protein [13] or conglutin γ in lupin [8] could mediate the effects of the respective proteins on the cholesterol metabolism. A recent study showed that peptides from soy protein within a molecular weight range from 200 to 3,000 Da stimulate transcription of LDL receptor in liver cells, and thus could be responsible for the hypocholesterolaemic effect of soy protein [14]. In soy protein isolate, isoflavones or fibre could contribute to the hypocholesterolaemic effects [15, 16]. However, in other hypolipidaemic protein sources such as proteins extracted from lupins, peas or fish, isoflavones and fibre are present in much lower concentrations or are completely absent. Besides peptides and antinutritive components, the amino acid composition of proteins could also contribute to the effects on cholesterol metabolism observed. Kritchevsky et al. [17] were the first to hypothesize that the hypocholesterolaemic effect of soy protein isolate is due to its higher ratio of arginine:lysine compared to casein. There are indeed several studies which show an inverse correlation between the arginine:lysine ratio of the proteins in the diets and serum cholesterol levels [17, 18]. In few studies, soy protein isolate and casein were supplemented with either lysine or arginine, respectively, to demonstrate the impact of the arginine:lysine ratio for the hypo- or hyperlipidaemic effects of these proteins [19, 20]. The results of these studies, however, were variable. In few studies, supplementation of lysine to a soy protein-based diet indeed had a hypercholesterolaemic effect and supplementation of arginine to a casein-based diet a hypocholesterolaemic effect [17, 19, 21]. In contrast, in some other studies supplementation of either arginine to a casein-based diet or of lysine to a plant protein-based diet had less or even no effect on plasma lipid concentrations [22–26]. While in these studies either arginine or lysine were supplemented to diets, no study has been performed in which the effects of arginine and lysine were tested in combination. Therefore, the direct interaction between these amino acids on the lipid metabolism has not yet been investigated. Moreover, biochemical mechanisms underlying the effects of arginine or lysine on the lipid metabolism are largely unknown. The reason for this is that there was much less knowledge about the regulation of the lipid metabolism in the 1980s of the last century when most of these studies were performed than today.

The aim of the present study was to investigate the effect of different concentrations of arginine and lysine in the diet in combination on the concentrations of cholesterol in plasma, lipoproteins and liver and to explore the biochemical mechanisms underlying these effects. Normolipidaemic rats were used as an animal model in which hypocholesterolaemic effects of proteins from soybeans, lupins, peas or fish have already been demonstrated [e.g. 5, 8, 11, 27]. According to a bifactorial design, we used diets with three different arginine concentrations (4.5, 9 and 18 g/kg diet) and two different lysine concentrations (9 and 18 g/kg diet). Since previous studies have found that the hypocholesterolaemic effect of soy protein isolate is particularly evident in hypercholesterolaemic individuals, but is less consistently evident in normocholesterolaemic individuals [28], we assume that the effect of the arginine:lysine ratio on lipid metabolism could depend on the basal levels of circulating lipids. Hence, we tested the effects of various concentrations of lysine and arginine on lipid metabolism in rats fed a cholesterol-free diet or a diet containing cholesterol and cholate as hypercholesterolaemic compounds. In order to give an explanation for the effects observed, we determined relative mRNA concentrations of various genes involved in hepatic cholesterol metabolism.

Material and Methods

Animals

One hundred and twenty male Sprague-Dawley rats supplied by Charles River (Sulzfeld, Germany) were used for this study which consisted of two experiments. In the first experiment, 60 rats with an average body weight of 158 ± 15 g were divided into six groups; in the second experiment, 60 rats with an average body weight of 145 ± 8 g were divided into six groups. All the rats were housed individually in Macrolon cages whose floors were coated with wood shavings. The room was maintained at a temperature of 22°C, 50–60% relative humidity and a 12:12-hour light-dark cycle. All the experimental procedures described followed established guidelines for the care and handling of laboratory animals and were approved by the council of Saxony-Anhalt, Germany.

Experimental Diets

In the first experiment, a cholesterol-free basal diet was used (table 1). The diet used in the second experiment was similarly composed but contained additionally 10 g cholesterol and 5 g sodium cholate/kg as hypercholesterolaemic compounds (table 2). The addition of 10 g cholesterol and 5 g sodium cholate/kg at the expense of corn starch has been shown to efficiently increase liver and plasma cholesterol concentrations. Accordingly, such diets have been used to study the effects of nutrients on the lipid metabolism in hypercholesterolaemic rats [8, 9, 29]. We

Table 1. Composition of the diet used in experiment 1

	Arginine/lysine, g/kg					
	4.5/9	4.5/18	9/9	9/18	18/9	18/18
Components, g/kg diet						
Casein	120	120	120	120	120	120
Sucrose	229.0	217.7	224.5	213.1	215.4	204.1
Corn starch	440	440	440	440	440	440
Cellulose	50	50	50	50	50	50
Lard	90	90	90	90	90	90
Soybean oil	10	10	10	10	10	10
Vitamin and mineral mixture	51.3	51.3	51.3	51.3	51.3	51.3
L-Arginine	0.71	0.71	5.25	5.25	14.34	14.34
L-Lysine HCl	0.25	11.61	0.25	11.61	0.25	11.61
DL-Methionine	6.1	6.1	6.1	6.1	6.1	6.1
L-Threonine	1.5	1.5	1.5	1.5	1.5	1.5
L-Valine	0.2	0.2	0.2	0.2	0.2	0.2
L-Isoleucine	0.9	0.9	0.9	0.9	0.9	0.9
Analyzed concentrations of amino acids[1]*, g/kg diet*						
Arginine	4.8	4.8	9.5	9.8	19.1	19.4
Lysine	9.0	19.1	9.3	18.8	9.6	18.5

[1] Concentrations of other amino acids (g/kg diet): alanine 3.5, aspartic acid 8.2, cysteine 0.5, glutamic acid 27.8, glycine 2.1, histidine 3.5, isoleucine 6.3, leucine 10.3, methionine 9.4, phenylalanine 5.8, proline 14.0, serine 6.3, threonine 6.5, tyrosine 5.0, valine 7.6, tryptophan 1.3.

Table 2. Composition of the diet used in experiment 2

	Arginine/lysine, g/kg					
	4.5/9	4.5/18	9/9	9/18	18/9	18/18
Components, g/kg diet						
Casein	120	120	120	120	120	120
Sucrose	229.0	217.7	224.5	213.1	215.4	204.1
Corn starch	425	425	425	425	425	425
Cellulose	50	50	50	50	50	50
Lard	90	90	90	90	90	90
Soybean oil	10	10	10	10	10	10
Vitamin and mineral mixture	51.3	51.3	51.3	51.3	51.3	51.3
L-Arginine	0.71	0.71	5.25	5.25	14.34	14.34
L-Lysine HCl	0.25	11.61	0.25	11.61	0.25	11.61
Cholesterol	10	10	10	10	10	10
Sodium cholate	5	5	5	5	5	5
DL-Methionine	6.1	6.1	6.1	6.1	6.1	6.1
L-Threonine	1.5	1.5	1.5	1.5	1.5	1.5
L-Valine	0.2	0.2	0.2	0.2	0.2	0.2
L-Isoleucine	0.9	0.9	0.9	0.9	0.9	0.9
Analyzed concentrations of amino acids[1]*, g/kg diet*						
Arginine	4.7	4.9	9.5	9.7	20.2	20.5
Lysine	9.2	19.1	9.6	19.0	10.2	20.2

[1] Concentrations of other amino acids (g/kg diet): alanine 3.5, aspartic acid 8.6, cysteine 0.5, glutamic acid 25.8, glycine 2.5, histidine 3.5, isoleucine 6.8, leucine 10.6, methionine 9.2, phenylalanine 6.1, proline 11.9, serine 6.6, threonine 6.0, tyrosine 5.2, valine 7.5, tryptophan 1.3.

planned to study the effects of arginine and lysine over a wide range of concentrations in the diets, from the level of requirement until a moderate excess. Therefore, we used a basal diet with a relatively low concentration of protein, which contained 120 g of casein/kg diet as the sole source of protein. This protein source provided 3.8 g arginine/kg diet and 8.8 g lysine/kg diet. The experimental diets were supplemented individually with L-arginine and L-lysine HCl (both obtained from VWR International, Darmstadt, Germany) at the expense of sucrose to yield arginine concentrations of 4.5, 9 or 18 g/kg and lysine concentrations of 9 or 18 g/kg, according to a bifactorial design. The combination of those arginine and lysine concentrations resulted in dietary arginine:lysine ratios of 0.25, 0.5, 1.0 or 2.0. The lowest concentrations of both amino acids (4.5 g arginine/kg; 9 g lysine/kg) were still in accordance with the requirement of arginine (4.3 g/kg diet) and lysine (9.2 g/kg diet) for growing rats [30]. The analyzed concentrations of these amino acids were close to the planned concentrations (see tables 1, 2). Since the concentrations of the indispensable amino acids valine, methionine, isoleucine and threonine were below the recommendations, these amino acids were supplemented to the two basal diets in order to meet the requirements for growing rats [30] (tables 1, 2). Vitamins and minerals were supplemented according to recommendations for rat diets [30].

The food intake was controlled in order to avoid differences which could confound with the effects of arginine and lysine on cholesterol metabolism. In both experiments, each rat received 15 g of the diet per day throughout the whole experimental period. This amount of diet is slightly below that of similar diets consumed ad libitum by rats in preliminary unpublished studies. It is, however, in clear excess of the energy requirement for the maintenance of these rats [30]. Water was freely available from nipple drinkers. The experimental diets were fed for 21 days in both experiments, a period that has been shown to be long enough to induce changes in lipid metabolism by dietary proteins [3–5, 31].

Sample Collection

Rats received 8 g of the diet 4 h before they were killed by decapitation under light anaesthesia with diethyl ether. The last portion of food was given as food deprivation before killing leads to a significant downregulation of the genes involved in lipid metabolism which were considered in this study [32]. Plasma was obtained from whole blood by centrifugation at 1,500 *g* for 10 min at 4°C. The liver was excised, weighed and immediately snap-frozen in liquid nitrogen. Aliquots of liver for RNA isolation were stored at –80°C; other samples were stored at –20°C.

Table 3. Characteristics of the specific primers used for real-time RT-PCR analysis

Gene	Forward primers	Reversed primers	bp	Annealing temperature °C	NCBI GenBank
β-Actin	5′ATCGTGCGTGACATTAAAGAGAAG 3′	5′GGACAGTGAGGCCAGGATAGAG 3′	429	60	NM031144
Apo A1	5′CCTGGATGAATTCCAGGAGA 3′	5′TCGCTGTAGAGCCCAAACTT 3′	192	60	NM012738
HMG-CoA R	5′AAGGGGCGTGCAAAGACAATC 3′	5′ATACGGCACGGAAAGAACCATAGT 3′	406	57	BC064654
LCAT	5′AGCTGGCAGGACTGGTAGAGGAG 3′	5′CAGGGGGAAGTTGTGGTTATGC 3′	277	60	NM017024
LDL receptor	5′AGAACTGCGGGGCCGAAGACAC 3′	5′AAACCGCTGGGACATAGGCACTCA 3′	490	65	NM175762
CYP7A1	5′CAAGACGCACCTCGCTATCC 3′	5′CCGGCAGGTCATTCAGTTG 3′	206	60	EC1141317
SR-B1	5′GGTTTGGTGCGCCCCTGTTTCTC 3′	5′CGACGCCCTTGACGGATTTGATG 3′	198	60	NM031541

HMG-CoA R = HMG-CoA reductase.

Analysis of Amino Acids in Diets and Plasma

To determine the amino acid concentrations in the diet, samples were oxidized and then hydrolyzed with 6 M HCl. Separation and quantification of the amino acids were performed by ion-exchange chromatography following postcolumn derivatization in an amino acid analyzer (Biotronic LC 3000; Eppendorf, Hamburg, Germany). Tryptophan was determined by digesting the diet with barium hydroxide [33] followed by reversed-phase HPLC [34]. The concentrations of free arginine and lysine in the plasma of the rats were measured as isoindole derivatives by HPLC after precolumn derivatization [35]. Isoindole derivates were detected at an excitation wavelength of 337 nm and an emission wavelength of 454 nm.

Cholesterol in Plasma, Liver and Lipoproteins

Plasma lipoproteins were separated by stepwise ultracentrifugation (Mikro-Ultrazentrifuge; Sorvall Products, Bad Homburg, Germany) at 900,000 *g* at 4°C for 1.5 h by appropriate density cuts commonly used for measuring rat lipoproteins [8, 36, 37]. Plasma densities were adjusted by sodium chloride and potassium bromide and the lipoprotein fractions $\rho < 1.006$ kg/l (defined as VLDL), $1.006 < \rho < 1.063$ kg/l (defined as LDL) and $\rho > 1.063$ kg/l (defined as HDL) were removed by suction. Lipids were extracted from liver with a mixture of n-hexane and isopropanol (3:2, v/v) [38]. For determination of the concentration of cholesterol in the liver, aliquots of the lipid extracts were dried and the lipids were dissolved using Triton X-100 [39]. Concentrations of cholesterol in plasma and lipoproteins and those of liver were determined using an enzymatic reagent kit (DiaSys Diagnostic Systems, Holzheim, Germany; Cat. No. 1.1300 99 90 314). For quantification of cholesterol concentrations, appropriate standards (HV calibrator; DiaSys Diagnostic Systems) were used.

Relative mRNA Concentrations of Hepatic Genes

For analysis of gene expression, total RNA was isolated from frozen liver samples using Trizol™ (Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocol. cDNA synthesis was carried out as described by König and Eder [40]. The mRNA concentrations of genes were measured by real-time detection PCR (Rotorgene 2000; Corbett Research, Mortlake, Australia) using SYBR® Green I (Sigma-Aldrich, Taufkirchen, Germany). Real-time detection PCR was performed with 1.25 U Taq DNA polymerase (Promega, Mannheim, Germany), 500 μM dNTPs and 26.7 pmol of the specific primers (Operon Biotechnologies, Cologne, Germany; table 3). For determination of mRNA concentration a threshold cycle (C_t) was obtained from each amplification curve using the software RotorGene 4.6 (Corbett Research). The relative mRNA concentration was calculated using the $\Delta\Delta C_t$ method [41]. The housekeeping gene β-actin was used for normalization.

Statistics

Statistical evaluation of data was performed with the Minitab Statistical Software (Minitab, State College, Pa., USA). Effects of dietary arginine and lysine concentrations and the interaction between these two factors were analyzed by bifactorial ANOVA. For statistically significant F values ($p < 0.05$), means of the main factors were compared by Fisher's multiple range test. For parameters that showed a significant interaction between dietary arginine and lysine concentration ($p < 0.05$), means of the treatment groups were compared by Fisher's multiple range test. Effects of the dietary arginine:lysine ratio were evaluated by one-factorial ANOVA, followed by Fisher's multiple range test in the case of statistically significant F values ($p < 0.05$). Means were considered significantly different at $p < 0.05$. Values in the text are given as means ± SD.

Results

Body Weights and Weight Gains

In both experiments, rats of all treatment groups showed growth rates which were in the physiological range for growing Sprague-Dawley rats in a body weight range between 150 and 300 g [30]. The final body weight of the rats at day 21 was not influenced by dietary arginine and dietary lysine concentrations in both experiments, and there was no interaction between these factors influencing final body weight. In experiment 1, the final average body weight of the rats in the six groups was 279

Table 4. Concentrations (μmol/l) of arginine and lysine in plasma of rats fed a cholesterol-free diet (experiment 1) or a diet supplemented with cholesterol and sodium cholate (experiment 2) with various concentrations of arginine (4.5, 9 or 18 g/kg) and lysine (9 or 18 g/kg)

Effect	Experiment 1		Experiment 2	
	arginine	lysine	arginine	lysine
Treatment groups (n = 10)				
Arginine/lysine, g/kg				
4.5/9	125 ± 45	657 ± 170	125 ± 23	400 ± 90^{B}
4.5/18	149 ± 57	1,215 ± 210	124 ± 28	1,010 ± 128^{A}
9/9	153 ± 60	607 ± 95	171 ± 37	429 ± 112^{B}
9/18	251 ± 141	1,243 ± 159	186 ± 34	979 ± 221^{A}
18/9	298 ± 114	555 ± 107	258 ± 38	384 ± 83^{B}
18/18	333 ± 63	1,173 ± 183	264 ± 52	711 ± 226^{C}
ANOVA, p				
Arginine	0.001	0.40	0.001	0.003
Lysine	0.04	0.001	0.50	0.001
Arginine × lysine	0.37	0.768	0.80	0.02
Main effects				
Arginine (n = 20), g/kg				
4.5	139 ± 52^{a}	936 ± 341	125 ± 25^{c}	687 ± 331^{a}
9	205 ± 118^{b}	925 ± 353	179 ± 36^{b}	687 ± 328^{a}
18	316 ± 91^{c}	864 ± 350	261 ± 44^{a}	538 ± 232^{b}
Lysine (n = 30), g/kg				
9	195 ± 108^{b}	608 ± 133^{b}	187 ± 65	403 ± 94^{b}
18	238 ± 119^{a}	1,209 ± 181^{a}	194 ± 69	892 ± 236^{a}

Values are means ± SD. Different superscript small letters (a, b, c) within one column indicate significant differences ($p < 0.05$) of means in main effects. Different superscript capital letters (A, B, C) within one column indicate significant differences ($p < 0.05$) of means in parameters with a significant interaction between arginine and lysine.

± 11 g (n = 60). Average daily body weight gain of the rats was 5.77 ± 0.48 g (n = 60). In experiment 2, the final average body weight of the rats in the six groups was 262 ± 7 g (n = 60). The average daily body weight gain of the rats was 5.57 ± 0.39 g (n = 60). The dietary arginine:lysine ratio had no influence on average daily body weight gains and final body weights of the rats (data not shown).

Concentrations of Arginine and Lysine in Plasma

Concentrations of arginine and lysine in plasma increased continuously with increasing concentrations of these amino acids in the diet ($p < 0.05$, table 4). There was, moreover, an interaction between lysine and arginine with respect to plasma lysine concentration in experiment 2 ($p < 0.05$). At higher arginine concentrations, the plasma lysine concentration increased less by increasing the dietary lysine concentration from 9 to 18 g/kg than at lower arginine concentration ($p < 0.05$). This interaction did, however, not occur in experiment 1.

Cholesterol Concentrations in Plasma, Lipoproteins and Liver

In experiment 1 in which a cholesterol-free diet was used, increasing the dietary arginine concentration from 4.5 to 9 or 18 g/kg caused a slight but significant reduction of plasma cholesterol concentration ($p < 0.05$, table 5). This effect was due to a decline of HDL cholesterol, whereas cholesterol concentrations in VLDL and LDL were not influenced by dietary arginine concentration (table 5). Liver cholesterol concentration was also not influenced by dietary arginine concentration (table 5). The dietary lysine concentration did not influence cholesterol concentration in plasma, LDL, HDL and liver (table 5). Increasing the dietary lysine concentration from 9 to 18 g/kg, however, lowered the VLDL cholesterol concentration ($p < 0.05$, table 5). Variation of the arginine:lysine ratio in the diet between 0.25 and 2.00 did not cause an alteration of the concentrations of cholesterol in LDL, VLDL and liver (table 5). Rats fed the diets with an arginine:lysine ratio of 1.00 had a significantly lower concentration of cholesterol in plasma and HDL than rats fed the diets with an arginine:lysine ratio of 0.25 or 0.50 ($p < 0.05$, table 5). Rats fed the diets with an arginine:lysine ratio of 2.00 did, however, not differ from rats fed diets with arginine:lysine ratios of 0.25, 0.50 or 1.00 in their plasma and HDL cholesterol concentrations (table 5).

In experiment 2 in which a diet supplemented with cholesterol and sodium cholate was used, concentrations of cholesterol in plasma, LDL and VLDL remained unchanged by dietary arginine and lysine concentrations (table 6). The arginine:lysine ratio in the diet did also not influence these parameters (table 6). Rats fed the diets containing 9 or 18 g arginine/kg had, however, a significantly lower concentration of cholesterol in HDL and a significantly higher concentration of cholesterol in the liver than those fed the diet with 4.5 g arginine/kg ($p < 0.05$, table 6). Rats fed the diet containing 18 g lysine/kg had a lower cholesterol concentration in the liver than those fed the diet with 9 g lysine/kg ($p < 0.05$, table 6). According to these observations, an increase in the dietary arginine:lysine ratio lowered HDL cholesterol concentration. Rats fed the diet with an arginine:lysine ratio of 0.25 had the highest HDL cholesterol concentration, those fed the diet with ratios of 1.0 or 2.0 had the lowest

Table 5. Concentrations of cholesterol in plasma, VLDL, LDL, HDL and liver of rats fed a cholesterol-free diet with various concentrations of arginine (4.5, 9 or 18 g/kg) and lysine (9 or 18 g/kg)

Effect	Plasma mmol/l	VLDL mmol/l	LDL mmol/l	HDL mmol/l	Liver μmol/g
Treatment groups (n = 10)					
Arginine/lysine, g/kg					
4.5/9	2.02 ± 0.43	0.22 ± 0.07	0.81 ± 0.24	0.99 ± 0.23	6.1 ± 1.5
4.5/18	1.84 ± 0.19	0.18 ± 0.06	0.72 ± 0.24	0.95 ± 0.07	5.9 ± 1.3
9/9	1.66 ± 0.30	0.23 ± 0.08	0.62 ± 0.17	0.81 ± 0.17	7.2 ± 1.1
9/18	1.76 ± 0.26	0.14 ± 0.06	0.74 ± 0.18	0.86 ± 0.10	6.4 ± 1.4
18/9	1.78 ± 0.26	0.16 ± 0.09	0.74 ± 0.21	0.88 ± 0.14	6.5 ± 1.4
18/18	1.57 ± 0.27	0.17 ± 0.05	0.64 ± 0.17	0.76 ± 0.10	6.7 ± 1.3
ANOVA, p					
Arginine	0.02	0.33	0.32	0.003	0.15
Lysine	0.23	0.03	0.64	0.30	0.46
Arginine × lysine	0.22	0.08	0.12	0.19	0.57
Main effects					
Arginine (n = 20), g/kg					
4.5	1.93 ± 0.34[a]	0.20 ± 0.07	0.77 ± 0.20	0.97 ± 0.17[a]	6.0 ± 1.4
9	1.71 ± 0.28[b]	0.19 ± 0.08	0.68 ± 0.18	0.83 ± 0.14[b]	6.8 ± 1.3
18	1.68 ± 0.28[b]	0.16 ± 0.07	0.69 ± 0.19	0.82 ± 0.13[b]	6.6 ± 1.3
Lysine (n = 30), g/kg					
9	1.82 ± 0.36	0.20 ± 0.08[a]	0.72 ± 0.22	0.89 ± 0.19	6.6 ± 1.4
18	1.73 ± 0.26	0.16 ± 0.06[b]	0.70 ± 0.16	0.86 ± 0.12	6.3 ± 1.3
Arginine:lysine ratio					
0.25 (n = 10)	1.84 ± 0.19[a, b]	0.18 ± 0.06	0.72 ± 0.14	0.95 ± 0.07[a]	5.9 ± 1.3
0.50 (n = 20)	1.90 ± 0.38[a]	0.18 ± 0.07	0.78 ± 0.21	0.93 ± 0.18[a]	6.3 ± 1.4
1.00 (n = 20)	1.62 ± 0.28[b]	0.20 ± 0.07	0.64 ± 0.16	0.79 ± 0.14[b]	6.9 ± 1.2
2.00 (n = 10)	1.78 ± 0.26[a, b]	0.16 ± 0.09	0.74 ± 0.21	0.88 ± 0.14[a, b]	6.5 ± 1.4

Values are means ± SD. Different superscript small letters (a, b, c) within one column indicate significant differences of means ($p < 0.05$).

HDL cholesterol concentrations (table 6). Rats fed the diet with an arginine:lysine ratio of 2.0, moreover, had a higher liver cholesterol concentration than those fed the diets with ratios of 0.25, 0.5 or 1.0.

In both experiments, there were no interactions between dietary arginine and dietary lysine concentration with respect to cholesterol concentrations in plasma, lipoproteins and liver (table 6).

Relative mRNA Concentrations of Hepatic Genes Involved in Cholesterol Metabolism

In rats of experiment 2, the expression of several genes involved in the metabolism of cholesterol was determined to find out possible explanations for the effects of arginine and lysine observed on cholesterol concentrations.

Increasing the dietary arginine concentration from 4.5 to 9 or 18 g/kg caused an increase of relative mRNA concentrations of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase), LDL receptor and scavenger receptor B1 (SR-B1) in the liver ($p < 0.05$, table 7). Relative mRNA concentrations of apolipoprotein A1 (apo A1), lecithin-cholesterol acyltransferase (LCAT) and cholesterol 7α-hydroxylase (CYP7A1) in the liver were not influenced by the dietary arginine concentration (table 7). Increasing the dietary lysine concentration from 9 to 18 g/kg caused an upregulation of CYP7A1 ($p < 0.05$, table 7). The relative mRNA concentrations of HMG-CoA reductase, LDL receptor, SR-B1, apo A1 and LCAT in the liver were not influenced by the dietary lysine concentration (table 7). With respect to mRNA concentrations of all the genes involved in cholesterol metabolism examined, no interactions between dietary arginine and dietary lysine concentrations were observed (table 7).

Table 6. Concentrations of cholesterol in plasma, VLDL, LDL, HDL and liver of rats fed a diet supplemented with cholesterol and sodium cholate with various concentrations of arginine (4.5, 9 or 18 g/kg) and lysine (9 or 18 g/kg)

Effect	Plasma mmol/l	VLDL mmol/l	LDL mmol/l	HDL mmol/l	Liver μmol/g
Treatment groups (n = 10)					
Arginine/lysine, g/kg					
4.5/9	5.23 ± 0.48	3.30 ± 0.35	1.40 ± 0.39	0.53 ± 0.11	158 ± 23
4.5/18	5.27 ± 0.84	2.90 ± 0.78	1.73 ± 0.37	0.61 ± 0.15	149 ± 18
9/9	5.45 ± 0.92	3.39 ± 0.78	1.62 ± 0.46	0.40 ± 0.03	178 ± 18
9/18	5.47 ± 0.81	3.49 ± 0.59	1.53 ± 0.48	0.40 ± 0.07	174 ± 20
18/9	5.48 ± 2.02	3.60 ± 1.34	1.47 ± 0.69	0.37 ± 0.05	196 ± 36
18/18	5.09 ± 1.65	3.38 ± 1.38	1.36 ± 0.52	0.35 ± 0.06	159 ± 31
ANOVA, p					
Arginine	0.88	0.44	0.55	0.001	0.007
Lysine	0.76	0.51	0.78	0.44	0.01
Arginine × lysine	0.85	0.74	0.36	0.16	0.09
Main effects					
Arginine (n = 20), g/kg					
4.5	5.25 ± 0.66	3.10 ± 0.62	1.57 ± 0.40	0.57 ± 0.13[a]	154 ± 21[b]
9	5.46 ± 0.84	3.44 ± 0.67	1.57 ± 0.46	0.40 ± 0.05[b]	176 ± 18[a]
18	5.29 ± 1.80	3.50 ± 1.32	1.42 ± 0.60	0.36 ± 0.05[b]	178 ± 38[a]
Lysine (n = 30), g/kg					
9	5.40 ± 1.30	3.44 ± 0.91	1.51 ± 0.53	0.44 ± 0.10	178 ± 30[a]
18	5.28 ± 1.14	3.28 ± 0.97	1.53 ± 0.47	0.45 ± 0.15	161 ± 25[b]
Arginine:lysine ratio					
0.25 (n = 10)	5.27 ± 0.84	2.90 ± 0.78	1.73 ± 0.38	0.61 ± 0.15[a]	150 ± 18[b]
0.50 (n = 20)	5.36 ± 0.67	3.40 ± 0.50	1.47 ± 0.43	0.47 ± 0.11[b]	166 ± 22[b]
1.00 (n = 20)	5.26 ± 1.33	3.39 ± 1.09	1.50 ± 0.50	0.37 ± 0.05[c]	169 ± 27[b]
2.00 (n = 10)	5.48 ± 2.02	3.60 ± 1.34	1.47 ± 0.69	0.37 ± 0.05[c]	196 ± 36[a]

Values are means ± SD. Different superscript small letters (a, b, c) within one column indicate significant differences of means ($p < 0.05$).

Increasing the dietary arginine:lysine ratio from 0.25 to 1.0 or 2.0 caused a significant elevation of HMG-CoA reductase mRNA concentration in the liver; increasing this ratio from 0.25 to 0.5, 1.0 or 2.0 caused a significant elevation of SR-B1 mRNA concentration in the liver ($p < 0.05$, table 7). Varying the arginine:lysine ratio in the diet between 0.25 and 2.00 did, however, not cause an alteration of the mRNA concentrations of LDL receptor, CYP7A1, apo A1 and LCAT in the liver (table 7).

Discussion

To our knowledge, this is the first study in which the effect of various concentrations in the diets of arginine and lysine in combination was tested on the concentrations of cholesterol in plasma, lipoproteins and liver of rats fed either a cholesterol-free diet or a diet supplemented with cholesterol and sodium cholate. It has been well established that supplementation of cholesterol and sodium cholate as hypercholesterolaemic compounds causes an increase in the concentration of cholesterol in plasma, VLDL and LDL, a reduction of cholesterol in HDL and a strong accumulation of cholesterol in the liver [5, 42, 43]. All these effects were observed in the rats fed the diet supplemented with cholesterol and sodium cholate of experiment 2 compared to those fed the cholesterol-free diet of experiment 1.

The lowest concentrations of dietary arginine and lysine were still in accordance with the amounts required for growing rats [30]. The finding that there was no influence of the diet on final body weights of the rats shows

Table 7. Relative mRNA concentrations of hepatic genes involved in cholesterol metabolism of rats fed a diet supplemented with cholesterol and sodium cholate with various concentrations of arginine (4.5, 9 or 18 g/kg) and lysine (9 or 18 g/kg)

Effect	HMG-CoA R	LDL receptor	CYP7A1	Apo A1	SR-B1	LCAT
Treatment group (n = 10)						
Arginine/lysine, g/kg						
4.5/9	1.00 ± 0.20	1.00 ± 0.16	1.00 ± 0.29	1.00 ± 0.26	1.00 ± 0.15	1.00 ± 0.15
4.5/18	0.98 ± 0.24	1.05 ± 0.31	1.25 ± 0.18	0.91 ± 0.24	0.94 ± 0.10	0.95 ± 0.18
9/9	1.30 ± 0.27	0.99 ± 0.35	0.98 ± 0.22	0.88 ± 0.30	1.12 ± 0.13	1.00 ± 0.23
9/18	1.19 ± 0.25	1.34 ± 0.26	1.04 ± 0.40	0.85 ± 0.15	1.15 ± 0.19	1.11 ± 0.14
18/9	1.29 ± 0.19	1.29 ± 0.23	0.84 ± 0.26	0.94 ± 0.12	1.13 ± 0.16	1.14 ± 0.21
18/18	1.26 ± 0.31	1.28 ± 0.34	1.18 ± 0.42	1.14 ± 0.48	1.28 ± 0.25	1.16 ± 0.35
ANOVA, p						
Arginine	0.004	0.03	0.48	0.17	0.001	0.07
Lysine	0.46	0.11	0.02	0.70	0.40	0.68
Arginine × lysine	0.83	0.17	0.42	0.24	0.20	0.58
Main effects						
Arginine (n = 20), g/kg						
4.5	1.00 ± 0.22[b]	1.00 ± 0.24[b]	1.00 ± 0.25	1.00 ± 0.26	1.00 ± 0.13[b]	1.00 ± 0.17
9	1.26 ± 0.27[a]	1.14 ± 0.34[a, b]	0.92 ± 0.29	0.91 ± 0.24	1.17 ± 0.17[a]	1.08 ± 0.20
18	1.28 ± 0.25[a]	1.25 ± 0.27[a]	0.91 ± 0.34	1.09 ± 0.38	1.24 ± 0.22[a]	1.18 ± 0.30
Lysine (n = 30), g/kg						
9	1.00 ± 0.22	1.00 ± 0.26	1.00 ± 0.28[b]	1.00 ± 0.25	1.00 ± 0.14	1.00 ± 0.19
18	0.95 ± 0.24	1.10 ± 0.29	1.19 ± 0.38[a]	1.03 ± 0.36	1.02 ± 0.21	1.03 ± 0.25
Arginine:lysine ratio						
0.25 (n = 10)	1.00 ± 0.25[b]	1.00 ± 0.30	1.00 ± 0.15	1.00 ± 0.26	1.00 ± 0.11[b]	1.00 ± 0.20
0.50 (n = 20)	1.11 ± 0.24[a, b]	1.12 ± 0.26	0.82 ± 0.27	1.01 ± 0.25	1.15 ± 0.20[a]	1.11 ± 0.16
1.00 (n = 20)	1.30 ± 0.29[a]	1.09 ± 0.35	0.86 ± 0.27	1.11 ± 0.45	1.27 ± 0.22[a]	1.15 ± 0.33
2.00 (n = 10)	1.30 ± 0.20[a]	1.23 ± 0.22	0.67 ± 0.21	1.03 ± 0.13	1.20 ± 0.16[a]	1.20 ± 0.22

Values are means ± SD. Different superscript small letters (a, b, c) within one column indicate significant differences of means ($p < 0.05$). HMG-CoA R = HMG-CoA reductase.

that these lower concentrations of arginine and lysine were indeed sufficient for maximum growth of these rats. It is also shown that the highest lysine and arginine concentrations which were 2- or 4-fold in excess of the requirement of these amino acids did obviously not lead to metabolic interactions that would lead to growth depression. Metabolic antagonism between lysine and arginine has been shown in several species [44]. In rats, a decrease in growth rate in rats fed a diet containing 5.1 g arginine/kg was observed when dietary lysine concentration was greater than 28 g/kg [45], a concentration which is clearly higher than the highest dietary lysine concentration of the present study. The ratios between arginine and lysine in the diets used in the present study ranged between 0.25 and 2.0 and thus covered those of plant proteins such as protein isolated from soybeans (1.1) [46], sweet lupines (1.5) [47], peas (1.2) [9] or proteins from animal origin such as fish protein (0.69) [48] or casein (0.45) [9, 47, 48]. As expected, plasma concentrations of arginine and lysine, respectively, rose with increasing dietary concentrations of these amino acids. Interestingly, the increase in plasma lysine concentration by lysine supplementation in experiment 2 was lowered by increasing the dietary arginine concentration. It is possible that this interaction is due to the competition of arginine and lysine at both intestinal and renal transporters [49].

Our study shows that dietary concentration of arginine does not have an effect on cholesterol concentrations in LDL and VLDL, irrespective of the cholesterolaemic status of the animals. However, we observed a significant decline of HDL cholesterol when dietary arginine concentration was elevated from 4.5 to 9 or 18 g/kg diet in both, rats fed a cholesterol-free diet or a diet supplemented with cholesterol and sodium cholate. In rats fed the

diet supplemented with the hypercholesterolaemic compounds, moreover, a slight but significant elevation of liver cholesterol was observed in those fed the diets with 9 or 18 g arginine/kg compared to those fed the diet with 4.5 g arginine/kg. HDL is the principal vehicle for the removal of cholesterol from peripheral tissues for disposal in the liver. Several genes are involved in HDL metabolism such as LCAT, critical for cholesterol uptake and maturation of HDL-3 to HDL-2 [50], apo A1, a structural component of HDL, and SR-B1, which is responsible for selective uptake of HDL. The finding of an increased relative mRNA concentration of SR-B1 in the liver of rats fed the diets with 9 or 18 g arginine/kg opens the possibility that HDL cholesterol was reduced in these rats due to an increased uptake of HDL into the liver. The finding that dietary arginine lowers HDL cholesterol concentration agrees with another study which suggested that a reduced activity of LCAT could be involved in the reduced HDL cholesterol concentration observed in rats fed a casein-based diet supplemented with arginine [24]. This suggestion is, however, in disagreement with our study which did not detect an effect of dietary arginine on hepatic LCAT expression, although we are aware of the fact that expression data do not necessarily reflect activity levels. To find out possible explanations for the increase of hepatic cholesterol by arginine in the rats fed the diet supplemented with cholesterol and sodium cholate, we determined the relative mRNA concentrations of HMG-CoA reductase and LDL receptor. We found that relative mRNA concentrations of HMG-CoA reductase and LDL receptor were increased in the rats fed arginine concentrations of 9 or 18 g/kg diet compared to those fed 4.5 g/kg diet. This suggests that the higher concentrations of cholesterol in the liver of rats fed the higher arginine concentrations could be, at least in part, caused by an increased synthesis of cholesterol in the liver and an increased uptake of LDL cholesterol from blood into the liver. The finding that the mRNA concentration of CYP7A1, the key enzyme of hepatic bile acid synthesis, was not influenced by dietary arginine concentration suggests that arginine did not increase hepatic cholesterol concentration by an impairment of bile acid synthesis.

The dietary lysine concentration had even less effect on cholesterol concentrations in plasma, lipoproteins and liver than the dietary arginine concentration. The only effect of an increase in the dietary lysine concentration from 9 to 18 g/kg was a moderate reduction of cholesterol concentration in VLDL of normocholesterolaemic rats and a slight reduction of liver cholesterol concentration in hypercholesterolaemic rats. The slightly increased gene expression of CYP7A1 in rats fed 18 g lysine/kg compared to rats fed 9 g/kg suggests that the reduction of the cholesterol concentration in liver of the rats with the high supply of dietary lysine could be due to an increased formation of bile acids in the liver. This suggestion, however, has to be proved in further studies.

According to the bifactorial design of this study, we had the possibility to test interactions between dietary arginine and lysine concentrations. An interesting observation was that there were no interactions between both amino acids with respect to cholesterol concentrations and genes involved in hepatic cholesterol metabolism. This shows that there was obviously no effect of metabolic interactions of these two amino acids on cholesterol metabolism.

Feeding proteins extracted from plants has been repeatedly shown to lower concentrations of cholesterol in plasma, VLDL and liver in hypercholesterolaemic as well as in normocholesterolaemic rats [e.g. 1–6, 8–10]. It has been hypothesized that effects of these proteins on cholesterol metabolism, i.e. cholesterol concentrations in plasma and lipoproteins, could be mediated by a higher arginine:lysine ratio compared to proteins of animal origin [22, 24–26]. The present study clearly shows that the variation of the dietary arginine:lysine ratio over a wide range (0.25–2.0) does not influence cholesterol concentrations in LDL and VLDL in rats fed either a cholesterol-free diet or a diet supplemented with hypercholesterolaemic compounds. It has been shown several times that effects of amino acids or proteins on the lipid metabolism of rats manifest within a short period, usually within 2 weeks or even earlier [51–53]. Hence, we can exclude the possibility that the feeding period of 3 weeks in the present experiments was too short to observe potential effects of the dietary arginine:lysine ratio on LDL and VLDL cholesterol concentrations. It has, moreover, been shown that increasing the arginine:lysine ratio even lowers HDL cholesterol and increases liver cholesterol concentration. Therefore, the present study does not support the hypothesis that the hypocholesterolaemic effects of plant proteins (reduction of cholesterol concentration in LDL, VLDL and liver) are mediated by the higher arginine:lysine ratio compared to casein.

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4. Diskussion

Es ist schon seit langer Zeit bekannt, dass Nahrungsproteine einen Einfluss auf den Lipidstoffwechsel in Mensch und Tier haben (Anderson *et al.* 1995, Sirtori *et al.* 1998, Sirtori und Lovati 2001). In einem Großteil der Studien, die sich mit dem Einfluss von Nahrungsproteinen auf den Lipidstoffwechsel beschäftigt haben, wurde aus der Sojabohne isoliertes Protein verwendet und dessen Wirkung mit Casein verglichen. Es gibt somit zahlreiche Studien, die sowohl die hypocholesterolämische (Anderson *et al.* 1995, Sirtori *et al.* 1995, Sugiyama *et al.* 1996, Iritani *et al.* 1996, Sirtori *et al.* 1998, Tovar *et al.* 2002, Koba *et al.* 2003) als auch eine hypotriglyceridämische (Tovar *et al.* 2002, Ascencio *et al.* 2004, Shukla *et al.* 2007) Wirkung von Sojaprotein beschreiben. Im Zusammenhang mit den positiven Wirkungen der Sojabohne auf den Lipidstoffwechsel wird davon ausgegangen, dass der Konsum von Sojabohnen zu einer Abnahme des Risikos an KHK zu erkranken beiträgt (Yamori 2006, Carlson *et al.* 2008). So ist bekannt, dass in asiatischen Ländern, wie z.B. Japan, wo die Sojabohne und Sojaprodukte in großen Mengen verzehrt werden, das Risiko an KHK zu erkranken niedriger ist als in Europa oder den USA (Yamori 2006). Auf der Suche nach Inhaltsstoffen der Sojabohne, welche zur positiven Wirkung auf den Lipistoffwechsel beitragen, wurden u.a. Isoflavone identifiziert (Cassidy *et al.* 1995, Pelletier *et al.* 1995, Anthony *et al.* 1996, Balmir *et al.* 1996, Clarkson *et al.* 1998). Isoflavone, sogenannte Phytoöstrogene, zählen zu den sekundären Pflanzeninhaltsstoffen und haben eine hormonähnliche Wirkung. Vor allem Menschen, die in Gegenden der Welt leben, wo das Risiko an KHK zu erkranken relativ niedrig ist, weisen hohe Plasmaspiegel dieser Substanzen auf (Yamori 2006). Zu den Phytoöstrogenen in Soja zählen Genistein, Daidzein und Glycetein.

Es gibt jedoch auch Studien, die darauf hinweisen, dass isoliertes Sojaprotein mit sehr geringen Gehalten an Isoflavonen ebenfalls zur lipidsenkenden Wirkung im Plasma beiträgt (Tovar-Palacio *et al.* 1998, Adams *et al.* 2002, Jenkins *et al.* 2002, Lichtenstein *et al.* 2002).

Übergeordnetes Ziel der vorliegenden Arbeit war es, weitere Nahrungsproteine, welche einen positiven Effekt auf den Lipidstoffwechsel unabhängig von Isoflavonen ausüben, zu identifizieren. Da bisher auch noch nicht ausreichend geklärt werden konnte, über welche Stoffwechselwege Nahrungsproteine Einfluss auf den Lipidstoffwechsel nehmen, war ein weiteres Ziel dieser Arbeit, die zugrunde liegenden Wirkmechanismen zu charakterisieren.

4.1. Der Einfluss von Nahrungsproteinen auf den Cholesterinstoffwechsel

Es gibt bereits zahlreiche Studien, die zeigen konnten, dass Sojaprotein (Sirtori *et al.* 1979, 1995, 1998, Anderson *et al.* 1995, Iritani *et al.* 1996, Sugiyama *et al.* 1996, Tovar *et al.* 2002, Koba *et al.* 2003) und Lupinenprotein (Sirtori *et al.* 2004, Bettzieche *et al.* 2008b, c) im Vergleich zu Casein hypocholesterolämische Wirkungen in verschiedenen Modelltieren aufweisen. Es sind daneben Studien bekannt, die den Einfluss von Erbsen auf den Lipidstoffwechsel untersucht haben (Kingman *et al.* 2003, Martins *et al.* 2004). Erbsen gehören wie die Sojabohne und die Lupine zur botanischen Familie der Leguminosen. Es wird deshalb vermutet, dass auch die Verfütterung von Erbsen eine hypocholesterolämische Wirkung haben könnte. So beobachteten Martins *et al.* (2004) nach der Verfütterung einer cholesterinreichen Diät mit rohen Erbsen eine Absenkung der Cholesterinkonzentration in Plasma, LDL und Leber von Schweinen. In einer anderen Studie führte eine Diät mit ganzen Erbsen bei Schweinen zu erhöhten Konzentrationen an Cholesterin in den VLDL und LDL und einer erhöhten fäkalen Ausscheidung von Steroiden (Kingman *et al.* 1993). Welche Komponenten der Erbse zu den Wirkungen auf den Lipidstoffwechsel beiträgt, ist jedoch bisher unbekannt gewesen.

Unsere Untersuchungen an Ratten zeigten, dass die Verfütterung einer Diät mit isoliertem Erbsenprotein im Vergleich zu Casein zu einer Absenkung der Cholesterinkonzentrationen in Leber und VLDL führte (**A2**). Gleichzeitig war die Ausscheidung von Gallensäuren über den Kot stark erhöht, was über die CYP7A1, das Schlüsselenzym der hepatischen Gallensäure-Synthese (Vlahcevic *et al.* 1999), vermittelt wurde. Da eine erhöhter Verlust von Gallensäuren über den Kot zur Abnahme des hepatischen Cholesterin-Pools in der Leber führt, könnte dies die Erklärung für die verminderten Cholesterinkonzentrationen in der Leber der mit Erbsenprotein gefütterten Ratten sein. Auch Martins *et al.* (2004) beobachteten nach der Verfütterung von rohen Erbsen an Ratten eine erhöhte Ausscheidung von Gallensäuren über den Kot. Sie vermuteten jedoch, dass dieser Effekt auf die Nicht-Stärke-Polysaccharide, die in rohen Erbsen reichlich vorkommen, zurückzuführen ist. Unseren Untersuchungen zufolge ist dieser Effekt jedoch offensichtlich zumindest teilweise auf die Proteinfraktion in der Erbse zurückzuführen, da das in unserer Studie verwendete Erbsenprotein nur einen sehr geringen Gehalt an Rohfaser enthielt.

Neben einer erhöhten Ausscheidung von Gallensäuren mit dem Kot konnten wir in unseren Untersuchungen eine erhöhte Expression des Transkriptionsfaktors SREBP-2 und dessen Zielgenen HMG-CoA-Reduktase, einem Schlüsselenzym der Cholesterinbiosynthese,

und LDL-Rezeptor, verantwortlich für die Aufnahme von Cholesterin in die Leber, in der Leber der mit Erbsenprotein gefütterten Ratten beobachten. Es ist bekannt, dass die proteolytische Aktivierung und Genexpression des SREBP-2 in der Leber durch die hepatische Cholesterinkonzentration gesteuert wird (Brown *et al.* 2002). Wir gehen deshalb davon aus, dass es durch den Verlust von Gallensäuren über den Kot und der damit verminderten Cholesterinkonzentration in der Leber der Ratten nach der Verfütterung von Erbsenprotein kompensatorisch zu einer Hochregulierung von SREBP-2 und damit zu einer gesteigerten Cholesterinsynthese und einer vermehrten Aufnahme von Cholesterin in die Leber kam. Auch Martins *et al.* (2004) konnten nach der Verfütterung von rohen Erbsen eine gesteigerte Aktivität der HMG-CoA-Reduktase in der Leber beobachten. Wir nehmen an, dass dieser Effekt zumindest zum Teil auf die Proteinfraktion des Erbsenproteins zurückzuführen ist. Interessanterweise wurde in einer Studie, in der hoch aufbereitetes Sojaprotein an Ratten verfüttert wurde, bezüglich der Cholesterinkonzentrationen in der Leber, der Ausscheidung von Gallensäuren mit dem Kot und den Aktivitäten der HMG-CoA-Reduktase und der CYP7A1 ähnliche Beobachtungen gemacht wie in unserer Studie mit Erbsenprotein. Deshalb vermuten wir, dass Erbsenprotein auf eine ähnliche Weise auf den Cholesterinstoffwechsel in Ratten wirkt wie Sojaprotein.

In unseren Untersuchungen konnten wir trotz der verminderten Cholesterinkonzentrationen in der Leber der mit Erbsenprotein gefütterten Ratten keine Veränderungen bezüglich der Cholesterinkonzentrationen in Plasma, LDL oder HDL beobachten. Ähnliche Beobachtungen machten Jacques *et al.* (1986) in einer Studie an Ratten. Auch sie konnten keinen Effekt der Diät mit aufbereitetem Erbsenprotein auf die Plasma-Cholesterinkonzentration im Vergleich zu anderen tierischen und pflanzlichen Proteinen beobachten. In einer anderen Studie jedoch führte eine Diät mit Erbsenprotein im Vergleich zu Casein zur Absenkung des Plasma-Cholesterins in Ratten (Mayilvaganan *et al.* 2004). In dieser Studie wurde jedoch kein hoch aufbereitetes Erbsenprotein verwendet, so dass nicht ausgeschlossen werden kann, dass andere Komponenten, wie z.B. Rohfaser im Erbsenprotein, zur hypocholesterolämischen Wirkung beitrugen.

Im Gegensatz zum Sojaprotein, das sowohl in Human- als auch Tierstudien zu einer deutlichen Absenkung der Cholesterinkonzentration in den LDL führte, konnten wir in unseren Untersuchungen keinen Effekt des Erbsenproteins auf die Cholesterinkonzentration in den LDL beobachten. Der LDL-Rezeptor vermittelt die Aufnahme von cholesterinhaltigen Lipoproteinen in die Zelle und ist somit der wichtigste Regulator für das zirkulierende LDL-Cholesterin (Brown *et al.* 1981). Obwohl in unseren Untersuchungen die relativen mRNA-

Konzentrationen des LDL-Rezeptors in der Leber erhöht waren, war dieser Anstieg wahrscheinlich nicht groß genug, um die Aufnahme von LDL in die Leber zu stimulieren und somit die Konzentration von Cholesterin in den LDL zu verringern.

Zusammenfassend zeigen unsere Untersuchungen zum ersten Mal, dass aus der gelben Erbse gewonnenes Erbsenprotein bei Ratten die Ausscheidung von Gallensäuren über den Kot stimuliert, was zum Abfall des Cholesteringehalts in der Leber führt. Weiterhin konnte gezeigt werden, dass Erbsenprotein, ähnlich wie Sojaprotein, zu einer gesteigerten Expression des Transkriptionsfaktors SREBP-2 und seiner Zielgene HMG-CoA-Reduktase und LDL-Rezeptor führt. Dies ist vermutlich ein kompensatorischer Prozess, um den gesteigerten Verlust von Cholesterin über die Gallensäuren auszugleichen.

Ähnliche Befunde wie in unserer Studie zum Erbsenprotein an Ratten konnten wir in unserem Fütterungsversuch mit Fischprotein aus Alaska-Seelachs-Filet und Kartoffelprotein bei Schweinen erheben (**A3**). Beide Proteine hatten eine hypocholesterolämische Wirkung auf das Plasma der Schweine, wobei Kartoffelprotein vor allem zu einer Absenkung des LDL-Cholesterins führte und Fischprotein sowohl die LDL-, als auch die HDL-Cholesterin-Konzentrationen verringerte. Diese Wirkungen stimmen überein mit Studien an Ratten, welche mit Kartoffelprotein (Morita *et al.* 1997, De Schrijver *et al.* 1990) oder Fischprotein (Zhang und Beynen 1993, Wergedahl *et al.* 2004, Ait Yahia *et al.* 2005, Shukla *et al.* 2006) gefüttert wurden.

Die Verfütterung von Fischprotein an Schweine führte in unseren Untersuchungen, ähnlich wie bei der Verfütterung von Erbsenprotein an Ratten, zu einer gesteigerten Expression des Enzyms CYP7A1, was wiederum zu einem gesteigerten Verlust von Cholesterin über die Gallensäuren im Kot führte. Dieser vermehrte Verlust von Gallensäuren über den Kot führte zur Abnahme des hepatischen Cholesterin-Pools, was wiederum zur gesteigerten Expression von SREBP-2 und LDL-Rezeptors in der Leber führte. Die beobachteten erhöhten relativen mRNA-Konzentrationen des LDL-Rezeptors in der Leber führten zur verstärkten Aufnahme von cholesterinreichen LDL-Partikeln in die Leber und erklären somit auch die verringerten LDL-Cholesterin-Konzentrationen in den mit Fischprotein gefütterten Schweinen im Vergleich zum Casein (**A3**). Der von uns beobachtete HDL-Cholesterin-senkende Effekt des Fischproteins konnte auch in Studien bestätigt werden, in denen Ratten Fischprotein aus Lachs (Wergedahl *et al.* 2004) oder Alaska-Seelachs-Filet (Shukla *et al.* 2006) erhielten. In einer weiteren Studie konnte gezeigt werden, dass Fischprotein die Reifung von HDL-3 zu HDL-2 stimuliert und dies wahrscheinlich durch eine

gesteigerte Aktiviät der Lecithin-Cholesterin-Acyltransferase (LCAT) realisiert wird, welche eine wichtige Rolle in der Aufnahme von Cholesterin in die HDL spielt und somit für den reversen Cholesterintransport aus extrahepatischem Gewebe von Bedeutung ist (Genest *et al.* 1999).

In unseren Untersuchungen zum Kartoffelprotein bei Schweinen konnten wir, im Gegensatz zu einer Studie von Morita *et al.* (1997), keine Steigerung der Expression der CYP7A1 in der Leber beobachten. Auch die Expression von SREBP-2 und seiner Zielgene war im Vergleich zu den mit Casein gefütterten Schweinen unverändert. Es bleibt somit ungeklärt, über welche Mechanismen Kartoffelprotein seine hypocholesterolämische Wirkung ausübt.

Die bisher existierenden Daten zum Einfluss von Nahrungsproteinen auf den Cholesterinstoffwechsel zeigen, dass vor allem Proteine aus Leguminosen (Sojabohne, Lupine und Erbse) hypocholesterolämische Wirkungen haben. Diese werden offenbar zum großen Teil durch eine Hochregulierung der CYP7A1 und damit über eine vermehrte Bildung von Gallensäuren und anschließende Ausscheidung über den Kot realisiert. Gegenregulatorisch kommt es dabei zu einer verstärkten Expression des Transkriptionsfaktors SREBP-2, welcher wiederum Gene, die für Proteine der Cholesterinsynthese und –aufnahme codieren, aktiviert. Dem gleichen Mechanismus unterliegt offenbar die hypocholesterolämische Wirkung von Fischprotein beim Modelltier Schwein. Kartoffelprotein scheint hingegen seine hypocholesterolämische Wirkung über andere Stoffwechselwege zu vermitteln, was weiterer Untersuchungen bedarf.

Obwohl die hypocholesterolämische Wirkung von pflanzlichen und einigen tierischen Nahrungsproteinen seit langem bekannt ist, konnte immer noch nicht geklärt werden, welche Bestandteile der Proteine zu dieser Wirkung beitragen. Es wird vermutet, dass spezifische Peptide, wie z.B. die α`und α-Untereinheiten des 7S Globulins im Sojaprotein (Lovati *et al.* 2000) oder Conglutin γ in Lupinenprotein (Sirtori *et al.* 2004), die Wirkung dieser Proteine auf den Lipistoffwechsel vermitteln. In einer weiteren Studie wurde die Hypothese verfolgt, dass spezifische Peptide des Sojaproteins den LDL-Rezeptor in Leberzellen stimulieren und somit für die hypocholesterolämische Wirkung des Sojaproteins verantwortlich sind (Cho *et al.* 2007). Bezüglich der Aktivierung der CYP7A1 durch Nahrungsproteine gibt es bereits Untersuchungen, die sich damit beschäftigen, ob spezifische Peptide aus Casein und Sojaprotein an der Aktivierung dieses Enzyms und damit an der vermehrten Gallensäureausscheidung beteiligt sind (Nass *et al.* 2008). Im Sojaprotein könnten Isoflavone

oder Rohfaser zur hypocholesterolämischen Wirkung beitragen (Lin *et al.* 2005, Gudbrandsen *et al.* 2005). Weil jedoch in Proteinen aus Lupinen, Erbsen oder Fisch der Gehalt an Isoflavonen oder Rohfaser nur sehr gering ist oder diese Bestandteile gar nicht vorkommen, müssen andere Komponenten in diesen Proteinen zur hypocholesterolämischen Wirkung beitragen.

Neben spezifischen Peptiden könnte auch die verschiedene Aminosäure-Zusammensetzung von Proteinen für die hypolipidämischen Wirkungen eine Rolle spielen. So konnten Huff *et al.* (1977) zeigen, dass eine synthetische Diät mit dem gleichen Aminosäuremuster wie Sojaprotein im Vergleich zu einer Diät mit dem gleichen Aminosäuremuster wie Casein bei Kaninchen zur Absenkung der Cholesterinkonzentrationen führte. Es wurde gezeigt, dass Methionin und Lysin hypercholesterolämische Wirkungen haben (Kritchevsky *et al.* 1982, Sugiyama und Muramatsu 1990, Kurowska und Carroll 1994). Dennoch verschwand die hypocholesterolämische Wirkung von Sojaprotein, welches arm an Methionin ist, nicht, wenn der Gehalt an Methionin in der Diät dem der Casein-Diät angeglichen wurde (Kern *et al.* 2002, Shukla *et al.* 2006). Auch in unseren Versuchen mit Lupinenprotein (**A1**) und Erbsenprotein (**A2**), welche von Natur aus arm an Methionin sind, verlor sich der hypolipidämische Effekt dieser Proteine durch eine Supplementierung der Diäten mit Methionin nicht.

Es gibt auch Hinweise, dass hohe Gehalte an Cystein in einer Diät hypocholesterolämische Wirkungen haben (Sautier *et al.* 1986, Sugiyama und Muramatsu 1990, Zhang und Beynen 1993). Sowohl Fisch- und Kartoffelprotein (**A3**), als auch Lupinen- (**A1**) und Erbsenprotein (**A2**) weisen höhere Gehalte an Cystein auf als Casein, welches in unseren Untersuchungen als Kontrollprotein diente. Die beobachteten hypolipidämischen Wirkungen dieser Proteine könnten also zum Teil auf die höheren Konzentrationen an Cystein in diesen Proteinen zurückzuführen sein. Untersuchungen unserer Arbeitsgruppe zum Einfluss von Cystein auf die Expression von SREBP-1c und dessen Zielgen FAS in HepG2-Zellen zeigten zudem eine Hemmung dieser Gene (Bettzieche *et al.* 2008a), was auch den hypotriglyceridämischen Effekt von Lupinenprotein (**A1**) in unseren Untersuchungen erklären könnte. Allerdings hatte Cystein in dieser Studie (Bettzieche *et al.* 2008a) keinen Einfluss auf die Expression von SREBP-2 und den Zielgenen HMG-CoA-Reduktase und LDL-Rezeptor, so dass Cystein keinen Einfluss auf die Cholesterinbiosynthese und -aufnahme zu haben scheint.

Arginin und Lysin sind weitere Aminosäuren, deren Einfluss auf den Lipidstoffwechsel diskutiert wird. Dabei soll Arginin die Plasma-Cholesterinkonzentrationen

vor allem über den vermehrten Einbau von Cholesterin in Gallensäuren senken (Salil und Rajamohan 2001). Diäten mit einem Überschuss an Lysin hingegen führten zu einer Akkumulation von Lipiden in der Leber (Hevia und Visek 1980c, Hevia *et al.* 1980a, b). Entscheidender für die hypolipidämische Wirkung dieser beiden Aminosäuren scheint jedoch das Verhältnis beider Aminosäuren zueinander zu sein. So ist bekannt, dass pflanzliche Proteine mit einem höheren Gehalt an Arginin ein höheres Arginin zu Lysin Verhältnis aufweisen als tierische Proteine, die einen höheren Gehalt an Lysin haben. Kritchevsky *et al.* (1982) war einer der ersten, der vermutete, dass der hypocholesterolämische Effekt von Sojaprotein-Isolat auf sein höheres Verhältnis von Arginin zu Lysin im Vergleich zu Casein zurückzuführen ist. Es gibt einige Studien, die eine umgekehrte Proportionalität zwischen dem Verhältnis von Arginin zu Lysin in den Protein-Diäten und dem Plasma-Cholesterinspiegel nachweisen konnten (Kritchevsky *et al.* 1982, Sugano *et al.* 1984). In verschiedenen Studien wurden Sojaproteinisolat und Casein mit Lysin bzw. Arginin supplementiert, um den Einfluss des veränderten Verhältnisses zwischen Arginin und Lysin auf die hypo- bzw. hyperlipidämische Wirkung dieser Proteine zu demonstrieren (Vahouny *et al.* 1985, Rajamohan und Kurup 1990). Die Ergebnisse dieser Studien waren jedoch sehr unterschiedlich. So führte die Supplementierung einer Sojaproteindiät mit Lysin in einigen Studien zu einer hypercholesterolämischen und die Supplementierung einer Caseindiät mit Arginin zu einer hypocholesterolämischen Wirkung (Kritchevsky *et al.* 1982, Kritchevsky *et al.* 1987, Rajamohan und Kurup 1990). In anderen Studien fand sich dagegen nach der Supplementierung einer auf Casein basierenden Diät mit Arginin oder der Supplementierung einer auf pflanzlichen Proteinen basierenden Diät mit Lysin kein oder nur ein marginaler Effekt auf die Plasma-Lipidkonzentrationen (Huff *et al.* 1980, Katan *et al.* 1982, Park und Liepa 1982, Sugano *et al.* 1982, Gibney *et al.* 1983). In diesen Studien wurden die Diäten stets entweder mit Arginin oder mit Lysin supplementiert. In unserer Studie (**A4**) untersuchten wir die Wirkung von Arginin und Lysin in Kombination und somit die Interaktion dieser beiden Aminosäuren. In unserem Versuch verwendeten wir Diäten mit drei verschiedenen Argininkonzentrationen und zwei verschiedenen Lysinkonzentrationen, so dass der Versuch einem bifaktoriellen Design entsprach. Da bekannt ist, dass die hypocholesterolämische Wirkung von Sojaprotein vor allem in hypercholesterolämischen und weniger in normocholesterolämischen Individuen zu beobachten ist (Carroll 1991), verwendeten wir in unserem Versuch sowohl eine cholesterinfreie Diät, als auch eine Diät, der Cholesterin und Cholat zugesetzt wurden.

Unsere Untersuchungen zeigten, dass eine steigende Konzentration von Arginin in der Diät (4,5, 9 und 18 g/kg Diät), unabhängig vom cholesterolämischen Status der Ratten, keinen Einfluss auf die Cholesterinkonzentrationen in LDL und VLDL hat. Ebenfalls unabhängig vom cholesterolämischen Status konnten wir jedoch mit steigender Argininkonzentration in der Diät einen Abfall der Cholesterinkonzentration in den HDL beobachten. Zusätzlich fand sich in der Leber der Tiere, welche die hypercholesterolämische Diät erhielten, mit steigender Argininkonzentration ein leichter Anstieg im Cholesteringehalt. Als Ursache hierfür kommt eine gesteigerte Aufnahme von HDL-Cholesterin in die Leber in Frage, da auch eine gesteigerte Expression des *scavenger receptor* B1 in der Leber, welcher für die selektive Aufnahme von HDL verantwortlich ist, beobachtete wurde. Die cholesterinsenkende Wirkung von Arginin auf die HDL wurde bereits in einer anderen Studie in Ratten beobachtet (Park und Liepa 1982), jedoch vermuteten die Autoren, dass dies auf eine verminderte Aktivität der LCAT zurückzuführen ist. Die LCAT wird in der Leber produziert und an das Blut abgegeben. Dort befindet sie sich an der Oberfläche der HDL, bildet Cholesterinester aus extrahepatisch aufgenommenem Cholesterin und Lecithin und trägt somit zum reversen Cholesterintransport bei. In unserem Versuch konnten wir jedoch keine verminderte Expression der LCAT in der Leber bei verschiedenen Dosierungen von Arginin in der Diät beobachten, so dass dies nicht als Ursache für die verminderten Cholesterinkonzentrationen in den HDL in Frage kommt. Ursache für die mit steigender Argininkonzentration in der Diät beobachteten erhöhten Gehalte an Cholesterin in der Leber der Tiere war zum Teil die gesteigerte Expression der HMG-CoA-Reduktase und des LDL-Rezeptors. Somit ist der Grund für sie erhöhten Cholesterin-Gehalte in der Leber wahrscheinlich eine erhöhte Synthese und Aufnahme von Cholesterin in die Leber. Bezüglich der Expression der CYP7A1 in der Leber konnten wir mit steigender Argininkonzentration in der Diät keine Veränderung feststellen, so dass eine verminderte Synthese von Gallensäuren in der Leber nicht als Ursache für die erhöhten Cholesterinkonzentrationen in der Leber in Betracht kommt.

Die verschiedenen Konzentrationen von Lysin (9 und 18 g/kg Diät) in der Diät zeigten sogar noch weniger Wirkung auf die Cholesterinkonzentrationen in Plasma, Lipoproteinen und Leber. Es konnte mit steigender Lysinkonzentration in der normocholesterolämischen Diät nur eine leichter Abfall der Cholesterinkonzentration in den VLDL der Ratten beobachtet werden. Zusätzlich kam es in den Tieren, welche die hypercholesterolämische Diät mit der höchsten Lysinkonzentration erhielten, zu einem leichten Abfall der Cholesterinkonzentration in der Leber. Ursache hierfür könnte die mit steigender Lysinkonzentration in der Diät leicht

erhöhte Expression der CYP7A1 in der Leber und damit der vermehrte Einbau von Cholesterin in Gallensäuren sein.

Durch das bifaktorielle Design unserer Studie hatten wir die Möglichkeit, die Interaktion zwischen der Arginin- und Lysinkonzentration in der Diät zu untersuchen. Es ist jedoch erstaunlich, dass wir in unserem Versuch keine solche Interaktion dieser beiden Aminosäuren bezüglich der Cholesterinkonzentrationen und der Expression von Genen des hepatischen Cholesterinstoffwechsels beobachten konnten. Das bedeutet, dass es offensichtlich abweichend von bisherigen Vermutungen keinen Zusammenhang zwischen einer metabolischen Interaktion von Arginin und Lysin in einer Diät und dem Cholesterinstoffwechsel gibt.

Die cholesterinsenkende Wirkung von pflanzlichen Proteinen auf Plasma, VLDL und Leber wurde sowohl in hyper- (Anderson *et al.* 1995, Sirtori *et al.* 1998, Sirtori *et al.* 2001, Brandsch *et al.* 2006, Shukla *et al.* 2007, Bettzieche *et al.* 2008b) als auch in normocholesterolämischen Tieren (Sirtori *et al.* 2004, **A2**, **A3**) gezeigt. Es wurde vermutet, dass die hypocholesterolämische Wirkung von pflanzlichen Proteinen auf das höhere Verhältnis von Arginin zu Lysin dieser Proteine im Vergleich zu tierischen Proteinen zurückzuführen ist (Huff und Carroll 1980, Park und Liepa 1982, Sugano *et al.* 1982, Gibney 1983). In unserer Studie (**A4**) konnten wir zeigen, dass hyper- oder normocholesterolämische Diäten mit verschiedenen Verhältnissen zwischen den Aminosäuren Arginin und Lysin (0,25 bis 2,0) keinen Einfluss auf die Cholesterinkonzentrationen in LDL oder VLDL ausüben. Es konnte jedoch gezeigt werden, dass mit einem höheren Verhältnis zwischen Arginin und Lysin die Cholesterinkonzentrationen in den HDL sogar verringert und in der Leber erhöht waren. Deshalb sind wir der Ansicht, dass die hypocholesterolämische Wirkung von pflanzlichen Proteinen im Vergleich zu Casein nicht auf das höhere Verhältnis zwischen Arginin und Lysin zurückzuführen ist.

4.2. Der Einfluss von Nahrungsproteinen auf den Triglyceridstoffwechsel

Neben zahlreichen Studien zur Wirkung von Sojaprotein auf den Lipidstoffwechsel gibt es nur wenige Studien, die die Wirkung von Lupinenprotein auf den Lipidstoffwechsel untersuchten (Bettzieche *et al.* 2008b, c, Sirtori *et al.* 2004). Die Lupine gehört wie die Sojabohne zur botanischen Familie der Leguminosen, weist einen hohen Proteingehalt auf und hat im Gegensatz zur Sojabohne nur einen niedrigen Gehalt an Isoflavonen. Lupinenprotein stellt damit ein ideales Protein dar, um die isoflavonunabhängige Wirkung

von pflanzlichen Proteinen auf den Lipidstoffwechsel zu untersuchen. Sirtori *et al.* (2004) konnten kürzlich zeigen, dass ein Protein-Extrakt aus der Lupine zur Absenkung des Cholesterinspiegels im Plasma von hypercholesterolämischen Ratten führte. Sie führten diese Wirkung auf eine erhöhte Aktivität des LDL-Rezeptors zurück, nachdem sie beobachtet hatten, dass mit Lupinenprotein behandelte HepG2-Zellen eine erhöhte Aktivität dieses Rezeptors aufwiesen. Zusätzlich wurde eine Absenkung des Plasma-Triglyceridspiegels in den mit Lupinenprotein gefütterten hypercholesterolämischen Ratten beobachtet. Bisher konnte jedoch noch nicht geklärt werden, über welche Stoffwechselwege diese hypotrygliceridämische Wirkung des Lupinenproteins vermittelt wird.

Ziel der ersten Studie **(A1)** war es deshalb, die hypotriglyceridämische Wirkung von Lupinenprotein im Rahmen eines Fütterungsversuches an Ratten im Vergleich zu Casein näher zu untersuchen. Die zelluläre Triglyceridsynthese wird hauptsächlich über den Transkriptionsfaktor SREBP-1c reguliert, welcher vor allem Gene aktiviert, die für die Fettsäuresynthese benötigt werden. In einigen Studien konnte bereits gezeigt werden, dass die hypotriglyceridämische Wirkung von Sojaprotein auf eine verminderte Triglyceridsynthese zurückzuführen ist, die auf einer Hemmumg des SREBP-1c basierte (Tovar *et al.* 2002, Nagasawa *et al.* 2003, Ascencio *et al.* 2004, Tovar *et al.* 2005, Shukla *et al.* 2007). Diese Erkenntnisse ließen vermuten, dass auch Lupinenprotein seine hypotriglyceridämische Wirkung über die Hemmung des Transkriptionsfaktors SREBP-1c und somit über eine verminderte Triglyceridsynthese ausüben könnte. Unsere Untersuchungen (**A1**) zeigten, dass die Verfütterung von Lupinenprotein an Ratten im Vergleich zu Casein als Kontrollprotein tatsächlich zu einer verminderten mRNA-Konzentration des SREBP-1c in der Leber und verminderten Triglyceridkonzentrationen in Leber und Plasma führte. Auch die relative mRNA-Konzentration der FAS, welche ein wichtiges Zielgen des SREBP-1c ist und als eines der Schlüsselenzyme in der Fettsäuresynthese gilt, war vermindert. Ähnliche Beobachtungen wurden in einer Studie an Ratten gemacht, die eine Diät mit 5 % Total-Protein-Extrakt aus der Lupinenart Vitabor, einer blauen Lupine, erhielten (Bettzieche *et al.* 2008c). Auch hier konnte die hypotriglyceridämische Wirkung des Proteins auf die über SREBP-1c regulierte verminderte Fettsäuresynthese zurückgeführt werden. In einer weiteren Studie, in welcher Ratten, wie in unserem Versuch, Protein aus der weißen Lupine in einer hypercholesterolämischen Diät erhielten, konnte der hypotriglyceridämische Effekt ebenfalls auf eine durch die Hemmung von SREBP-1c verminderte Fettsäuresynthese zurückgeführt werden (Bettzieche *et al.* 2008b). Der hypotriglyceridämische Effekt des Lupinenproteins

scheint also zumindest zum Teil auf eine Hemmung des SREBP-1c zurückzuführen zu sein, was wiederum zu einer verminderten Fettsäuresynthese in der Leber führte.

Verminderte Triglyceridkonzentrationen im Plasma können aber auch auf eine gesteigerte Lipolyse triglyceridreicher Lipoproteine zurückzuführen sein, was durch erhöhte mRNA-Konzentrationen der LPL, des Schlüsselenzyms der Lipolyse, in der Leber gekennzeichnet wäre. Bettzieche *et al.* (2008c) konnten in ihrer Studie zur Wirkung von blauen Lupinen in einer hypercholesterolämischen Diät eine gesteigerte Expression der LPL in der Leber der Ratten nachweisen und schlussfolgerten, dass der hypotriglyceridämische Effekt der blauen Lupine auch auf eine gesteigerte Lipolyse der Triglyceride zurückzuführen ist. In unseren Untersuchungen konnten wir im Gegensatz dazu keine erhöhten mRNA-Konzentrationen der LPL in der Leber der mit Lupinenprotein gefütterten Tiere finden und gehen deshalb davon aus, dass die weiße Lupine im Gegensatz zur blauen Lupine nicht zu einer gesteigerten Lipolyse triglyceridreicher Lipoproteine führt.

In früheren Studien an Ratten konnte auch für tierische Proteine, wie z.B. Protein aus Schweinefleisch (Brandsch *et al.* 2006) oder Ei-Albumin (Sugiyama *et al.* 1996) ein triglyceridsenkender Effekt im Vergleich zu Casein nachgewiesen werden. Fischprotein ist ein weiteres tierisches Nahrungsprotein, dessen triglyceridsenkender Effekt auf das Plasma bereits in Ratten, Kaninchen und premenopausalen Frauen aufgezeigt werden konnte (Bergeron *et al.* 1992, Gascon *et al.* 1996, Shukla *et al.* 2006). So wurde in Studien an Ratten (Murata *et al.* 2004, Ait Yahia *et al.* 2005) und an Kaninchen (Bergeron *et al.* 1992) durch die Verfütterung von Fischprotein eine Verminderung der Triglyceridkonzentrationen in Plasma und VLDL erreicht. Es gibt jedoch auch Studien an Ratten, die keinen Effekt von Fischprotein auf die Triglyceridkonzentrationen in Plasma und VLDL beobachteten (Yahia *et al.* 2003, Wergedahl *et al.* 2004). Die meisten Studien zum Einfluss des Fischproteins auf den Lipidstoffwechsel wurden allerdings an Ratten durchgeführt, die sich jedoch bezüglich ihres Lipoproteinmetabolismus stark vom Menschen unterscheiden (Fernandez und West 2005). Ziel unserer Untersuchungen **(A3)** war es deshalb, die Wirkung von Fischprotein auf den Triglyceridstoffwechsel am Modelltier Schwein (*Sus scrofa)* im Vergleich zu Casein zu untersuchen, da das Schwein aufgrund der Ähnlichkeiten zum Lipoproteinstoffwechsel des Menschen ein anerkanntes Modell für Untersuchungen zur Atherosklerose ist (Van Tol *et al.* 1991). Wir konnten mit unseren Untersuchungen zeigen, dass die Aufnahme von Fischprotein in einer Diät auch bei Schweinen zu einem deutlichen Abfall der Triglyceridkonzentrationen im Plasma (- 27%) und in den VLDL (- 40%) führt (**A3**). Die Analyse der relativen mRNA-

Konzentrationen der FAS und der ACC, zweier Enzyme, die in der Fettsäuresynthese eine Schlüsselrolle einnehmen, in der Leber und im Fettgewebe ergab jedoch keine signifikanten Änderungen im Vergleich zu den mit Casein gefütterten Tieren.

Zusammenfassend kann festgestellt werden, dass sowohl Lupinenprotein als auch Fischprotein hypotriglyceridämische Wirkungen in der Ratte bzw. im Schwein haben. Lupinenprotein aus der weißen Lupine übt diese Wirkung vor allem über die Hemmung des Transkriptionsfaktors SREBP1c und dessen Zielgen FAS und somit über eine verminderte Fettsäuresynthese aus. Die Mechanismen, über welche Fischprotein im Schwein seine hypotriglyceridämische Wirkung vermittelt, bleiben ungeklärt und bedürfen weiterer Untersuchungen.

4.3. Schlussfolgerungen und Ausblick

Die Untersuchungen im Rahmen dieser Arbeit bestätigen bisherige Studien, in denen gezeigt wurde, dass Nahrungsproteine einen Einfluss auf den Lipidstoffwechsel haben.

So konnte ein möglicher Mechanismus, über welchen Nahrungsproteine ihre cholesterinsenkende Wirkung ausüben, identifiziert werden. Sowohl in der Studie zur Wirkung von Erbsenprotein, als auch in der zur Wirkung von Fischprotein konnten erhöhte mRNA-Konzentrationen der CYP7A1 in der Leber der mit diesen Proteinen gefütterten Tiere beobachtet werden (**A2**, **A3**). Im Versuch zum Erbsenprotein resultierte die erhöhte mRNA-Konzentration der CYP7A1 dann auch in einer vermehrten Ausscheidung von Gallensäuren über den Kot. Vermutlich gegenregulatorisch zu den Verlusten von Cholesterin über die Ausscheidung von Gallensäuren der Tiere wurden in beiden Studien erhöhte mRNA-Konzentrationen des Transkriptionsfaktors SREBP-2 und dessen Zielgenen HMG-CoA-Reduktase und LDL-Rezeptor gefunden. Mit der erhöhten Expression dieser Gene kam es vermutlich zu einer gesteigerten Cholesterinbiosynthese und –aufnahme in die Leber, um den Verlust von Cholesterin über die Gallensäuren auszugleichen. Ähnliche Beobachtungen bezüglich CYP7A1, SREBP-2, HMG-CoA-Reduktase und LDL-Rezeptor konnten auch in einer Studie zur hypocholesterolämischen Wirkung von Sojaprotein gemacht werden, so dass vermutet werden kann, dass es sich hierbei um einen grundlegenden Mechanismus der hypocholesterolämischen Wirkung von Nahrungsproteinen handelt. Bezüglich der CYP7A1 gibt es bereits Untersuchungen, die sich damit beschäftigten, ob spezifische Peptide an der Aktivierung dieses Enzyms und damit an der vermehrten Gallensäureausscheidung beteiligt sind (Nass *et al.* 2008). Es konnten jedoch in dieser Studie im Sojaprotein keine aktiven

Peptide identifiziert werden, die die Promotoraktivität des CYP7A1 in einem Reportergen-Assay in HepG2-Zellen beeinflussten. Untersuchungen zur Rolle spezifischer Aminosäuren bei den beobachteten positiven Wirkungen von Nahrungsproteinen auf den Lipidstoffwechsel kommen zu unterschiedlichen Ergebnissen. Pflanzliche Proteine weisen im Gegensatz zu tierischen Proteinen einen höheren Gehalt an Arginin und einen niedrigeren Gehalt an Lysin auf. Da die hypocholesterolämische Wirkung vor allem für pflanzliche Proteine beobachtet wurde, ist ein erhöhtes Arginin-zu-Lysin-Verhältnis als Ursache für die hypocholesterolämische Wirkung von pflanzlichen Proteinen eine vieldiskutierte Theorie. Allerdings konnten wir mit unseren Untersuchungen an Ratten zeigen, dass ein hohes Arginin-zu-Lysin-Verhältnis in einer Diät nicht die Ursache für die cholesterinsenkende Wirkung von pflanzlichen Proteinen sein kann, da die Ratten, die eine Diät mit einem höherem Verhältnis zwischen Arginin und Lysin erhielten, sogar erhöhte Cholesterinkonzentrationen in der Leber und verminderte Cholesterinkonzentrationen in den HDL aufwiesen (**A4**). Auch die relative mRNA-Konzentration der CYP7A1 in der Leber der Ratten war mit steigendem Verhältnis von Arginin zu Lysin in der Diät unverändert. Es bleibt demzufolge offen, welche Bestandteile von Proteinen zur cholesterinsenkenden Wirkung beitragen.

Neben der hypocholesterolämischen wurde in dieser Arbeit auch die hypotriglyceridämische Wirkung von Nahrungsproteinen untersucht. So konnten die Ergebnisse unserer Analysen zur hypotriglyceridämischen Wirkung von Lupinenprotein (**A1**) dazu beitragen, den Mechanismus, über welchen diese Wirkung vermittelt wird, aufzuklären. Die Verfütterung von Lupinenprotein an Ratten führte in diesem Versuch über eine Hemmung des Transkriptionsfaktors SREBP-1c und verminderte relative mRNA-Konzentrationen der FAS vermutlich zur verminderten Fettsäuresynthese. Wir konnten auch zeigen (**A3**), dass Fischprotein hypotriglyceridämische Wirkungen im Schwein aufweist. Allerdings scheint diese Wirkung nicht wie beim Lupinenprotein über eine Hemmung von SREBP-1c und damit der Fettsäuresynthese vermittelt zu sein. Da sich Mensch und Schwein bezüglich ihres Lipidstoffwechsels sehr ähnlich sind, kann vermutet werden, dass Fischprotein auch in Menschen zur Absenkung der Triglyceridkonzentrationen im Plasma beiträgt. Ob diese Theorie zutrifft und welche Mechanismen der hypotriglyceridämischen Wirkung von Fischprotein im Schwein zu Grunde liegen, muss in weiterführenden Studien untersucht werden.

Zusammenfassend kann festgestellt werden, dass Nahrungsproteine sowohl hypocholesterolämische als auch hypotriglyceridämische Wirkungen haben und somit durchaus zur Minderung des Risikos für Atherosklerose und KHK beitragen können.

5. Zusammenfassung

Koronare Herzkrankheiten stellen die häufigste Todesursache innerhalb der westlichen Bevölkerung dar. Ursache hierfür ist das vermehrte Auftreten der Atherosklerose. Einen großen Risikofaktor für die Entstehung von Atherosklerose stellen Imbalancen im Lipidstoffwechsel dar. So führen vor allem erhöhte Cholesterinkonzentrationen in den LDL und verminderte Cholesterinkonzentrationen in den HDL zu einem Anstieg des Risikos, an Atherosklerose zu erkranken. Auch erhöhte Triglyceridkonzentrationen im Plasma konnten als Risikofaktor identifiziert werden. Zudem erhöht sich das Risiko für Atherosklerose noch bei gleichzeitigem Auftreten von niedrigen HDL-Cholesteringehalten und erhöhten Triglyceridwerten im Plasma. Die positive Beeinflussung dieser Parameter steht deshalb im Fokus der Ernährungsforschung.

Es konnte bereits in zahlreichen Studien gezeigt werden, dass Sojaprotein sowohl hypocholesterolämische als auch hypotriglyceridämische Wirkungen in Tier und Mensch hat. Ausgehend von diesen Beobachtungen wurde nach weiteren Nahrungsproteinen gesucht, welche positive Wirkungen auf den Lipidstoffwechsel ausüben. Hierbei wurde Protein aus der Lupine als ein vielversprechendes Nahrungsprotein im Hinblick auf seine hypocholesterolämische Wirkung identifiziert. Unsere Untersuchungen zeigten (**A1**), dass Lupinenprotein außerdem hypotriglyceridämische Wirkungen bei der Ratte hat und diese über die Hemmung des Transkriptionsfaktors SREBP-1c in der Leber vermittelt werden, was wiederum zur Verminderung der relativen mRNA-Konzentration der FAS in der Leber, einem Schlüsselenzym der Fettsäuresynthese, führt.

In einer anschließenden Studie (**A2**) wurde untersucht, ob auch Protein aus der Erbse, welche wie die Sojabohne und die Lupine zur Familie der Leguminosen gehört, hypocholesterolämische Wirkungen bei der Ratte hat. Es zeigte sich, dass die Verfütterung von Erbsenprotein an Ratten über eine vermehrte Ausscheidung von Cholesterin mit den Gallensäuren zur Absenkung des Cholesteringehaltes in der Leber führte. Gleichzeitig führte Erbsenprotein gegenregulatorisch zu den Verlusten im Cholesterin-Pool der Leber zu einer vermehrten Synthese und Aufnahme von Cholesterin in die Leber, was über die erhöhten mRNA-Konzentrationen des Transkriptionsfaktors SREBP-2 und seiner Zielgene HMG-CoA-Reduktase und LDL-Rezeptor vermittelt wird.

Da bekannt ist, dass auch Fischprotein hypocholesterolämische Wirkungen bei der Ratte hat, wurde ein dritter Versuch durchgeführt, der sich mit der Wirkung von Fischprotein auf den Fettstoffwechsel im Schwein beschäftigte (**A3**). Schweine sind dem Menschen

bezüglich ihres Lipidstoffwechsels sehr ähnlich und deshalb ein beliebtes Modelltier zur Untersuchung von Hypercholesterolämie und Atherosklerose. Unsere Untersuchungen zeigten übereinstimmend mit den Studien an Ratten eine hypocholesterolämische Wirkung des Fischproteins beim Schwein. Ähnlich wie beim Erbsenprotein wird die hypocholesterolämische Wirkung des Fischproteins durch erhöhte relative mRNA-Konzentrationen der CYP7A1 und damit über vermehrte Bildung von Gallensäuren verursacht. Auch durch das Fischprotein wurden vermutlich gegenregulatorisch die relativen mRNA-Konzentrationen von SREBP-2 und seiner Zielgene erhöht, um den verminderten Cholesterinpool in der Leber wieder aufzufüllen. Ähnliche Beobachtungen wurden schon für die cholesterinsenkende Wirkung von Soja- und Lupinenprotein gemacht, so dass man vermuten kann, dass Proteine aus Leguminosen und Fisch ihre cholesterinsenkende Wirkung auf ähnliche Weise vermitteln. Im gleichen Versuch konnten wir auch eine hypocholesterolämische Wirkung von Kartoffelprotein nachweisen. Wir vermuten jedoch, dass Kartoffelprotein nicht zur vermehrten Ausscheidung von Gallensäuren führte, da die relative mRNA-Konzentration der CYP7A1 unverändert war. Auch die relative mRNA-Konzentration von SREBP-2 und seinen Zielgenen war nicht verändert, so dass die hypocholesterolämische Wirkung von Kartoffelprotein vermutlich über andere Stoffwechselwege reguliert wird.

Der vierte Versuch (**A4**) sollte zur Aufklärung beitragen, welche Bestandteile der Proteine ursächlich für die hypocholesterolämische Wirkung von Nahrungsproteinen sind. Es wird vermutet, dass die unterschiedliche Aminosäure-Zusammensetzung der Proteine hierbei eine große Rolle spielt. Da pflanzliche Proteine mehr Arginin und tierische Proteine mehr Lysin aufweisen, wird angenommen, dass das höhere Verhältnis von Arginin zu Lysin zueinander ausschlaggebend für die hypocholesterolämische Wirkung von pflanzlichen Proteinen ist. Deshalb wurde ein Versuch mit bifaktoriellem Design durchgeführt, in welchem Ratten sowohl normo- als auch hypercholesterolämische Diäten mit verschiedenen Konzentrationen an Arginin und Lysin verabreicht wurde. Es konnte gezeigt werden, dass die Diäten mit einem höheren Verhältnis zwischen Arginin und Lysin im Vergleich zu den Diäten mit dem niedrigeren Verhältnis keinen Einfluss auf die Cholesterinkonzentration in den VLDL oder LDL hatten und in den HDL zu geringeren und in der Leber zu erhöhten Cholesterinkonzentrationen führten. Wir vermuten deshalb, dass die hypocholesterolämische Wirkung von pflanzlichen Proteinen nicht durch ihr höheres Verhältnis von Arginin zu Lysin verursacht wird. Aus diesem Grund sind weitere Untersuchungen notwendig, um die

Bestandteile von Proteinen und die Mechanismen, über welche sie ihre hypolipidämische Wirkung ausüben, zu identifizieren.

Zusammenfassend konnten wir mit unseren Untersuchungen zeigen, dass auch Proteine aus Lupine, Erbse, Kartoffel und Fisch hypolipidämische Wirkungen in Ratten und Schweinen haben. Wir konnten Stoffwechselwege identifizieren, über die diese Wirkungen zumindest zum Teil vermittelt werden. Es bleibt jedoch offen, welche Komponenten der Proteine für die beobachtete positive Beeinflussung des Lipidstoffwechsels verantwortlich sind.

5. Summary

Coronary heart diseases represent the most common cause of death in western population. This is caused by an increased incidence of atheroclerosis. Major risk factors for atherosclerosis are imbalances of the lipid metabolism. Thus, increased cholesterol concentrations in LDL and decreased cholesterol concentrations in HDL lead to a higher risk to develop atherosclerosis. Also increased concentrations of triacylglycerols in plasma represent risk factors for atherosclerosis. Furthermore, the risk to develop atherosclerosis raises even more by the concomitant incidence of low cholesterol concentrations in HDL and high triacylglycerol concentrations in plasma. Therefore, the positive interference of these parameters is in the focus of nutrition research.

It has been already shown in many studies that soy protein acts hypocholesterolemic and hypotriglyceridemic in man and animals. Based on this fact, we searched for other alimentary proteins with positive effects on lipid metabolism. At this, protein derived from lupine was identified as promising alimentary protein regarding its positive effects on lipid metabolism. Our analyses (**A1**) showed that lupine protein has additionally hypotriglyceridemic effects in rats, which were mediated through a down-regulation of the transcription factor SREBP-1c, which in turn led to decreased mRNA concentrations of FAS, one of the key enzymes of fatty acid synthesis.

In a second study (**A2**) we tested, whether protein derived from pea, which belongs like soy and lupine to the botanical family of the legumes, has also hypocholesterolemic effects in rats. We could show that pea protein leads to an increased excretion of cholesterol via bile acids and thus results in decreased cholesterol concentrations in the liver of rats. For compensation of the decreased cholesterol pool in the liver, the synthesis and uptake of cholesterol in the liver was increased in rats fed pea protein which was mediated by elevated relative mRNA-concentrations of the transcription factor SREBP-2 and its target genes HMG-CoA reductase and LDL-receptor.

Since it is known that also fish protein exhibits hypocholesterolemic effects in rats, a third study (**A3**) was conducted analysing the effects of fish protein in pigs. Pigs are a well accepted model to study hypercholesterolemia and atherosclerosis because their lipid metabolism is similar to humans. In accordance with our data obtained with rats, this study showed that fish protein also acts hypocholesterolemic in pigs. Similar to pea protein, the hypocholesterolemic effect of fish protein was mediated by increased relative mRNA concentrations of CYP7A1 in the liver and therefore by an increased formation of bile acids.

As soy and lupin protein, protein from fish also led to increased relative mRNA concentrations of SREBP-2 and its target genes, probably to compensate the lost cholesterol via bile acids. Thus we suppose that proteins derived from legumes and fish mediate their cholesterol lowering effect by the same mechanism. In the same study (**A3)** we also demonstrated a hypocholesterolemic effect of potato protein. However, relative mRNA-concentration of CYP7A1 was unchanged indicating that excretion of bile acids was not altered by potato protein. Furthermore the relative mRNA concentrations of SREBP-2 and its target genes remained unchanged. Thus, the hypocholesterolemic effect of potato protein is probably mediated by other mechanisms than that observed for proteins from soy, lupine, pea and fish.

The fourth study (**A4**) was conducted to find out which components of the alimentary proteins led to the observed hypocholesterolemic effects. It is assumed that differences in amino acid composition of the proteins may be relevant. Since plant proteins contain larger amounts of arginine and animal proteins more lysine, it is supposed, that a higher arginine:lysine ratio is a crucial factor for the hypocholesterolemic effect of plant proteins. Therefore, according to a bifactorial design, we conducted a study on rats in which we used normo- and hypercholesterolemic diets with different arginine and lysine concentrations. We showed that diets with a high arginine:lysine ratio, compared to those with a lower arginine:lysine ratio, had no influence on the cholesterol concentrations in VLDL and LDL and even led to lower concentrations of cholesterol in HDL and to higher concentrations of cholesterol in the liver. Hence, we suppose, that the hypocholesterolemic effect of plant proteins is not due to their higher arginine:lysine ratio. Therefore further studies are necessary to identify components of proteins and mechanisms by which they mediate their hypolipidemic effects.

Taken together, our analyses show that also proteins derived from lupine, pea, potato and fish have hypolipidaemic effects in rats and pigs. We could also identify metabolic pathways which are involved in this hypolipidemic action. Nevertheless, it still remains unexplained which components of the proteins are responsible for the observed positive effects on lipid metabolism.

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Kurz-Lebenslauf

Persönliche Daten

Name	Julia Spielmann
Geburtstag	07. März 1981
Geburtsort	Merseburg
Familienstand	ledig
Staatsangehörigkeit	deutsch

Schulausbildung

1999	Allgemeine Hochschulreife, Landesgymnasium Latina August Herrmann Francke

Praktikum

1999 bis 2000	kunsthandwerkliches Praktikum im Werkbund Werkstatt Nürnberg

Hochschullaufbahn

2000 bis 2006	Studium der Ernährungswissenschaft Martin-Luther-Universität Halle-Wittenberg
24. Januar 2006	Abschluss Diplom-Ernährungswissenschaftlerin
2006 bis 2008	Promotionsstudium der Ernährungswissenschaft an der Martin-Luther-Universität Halle- Wittenberg

Halle (Saale),

_____

Julia Spielmann

Eidesstattliche Erklärung

Hiermit versichere ich an Eides Statt, dass ich die eingereichte Dissertation ***„Untersuchungen zum Einfluss verschiedener Nahrungsproteine auf den Lipidstoffwechsel“*** selbstständig angefertigt und diese nicht bereits für eine Promotion oder ähnliche Zwecke an einer anderen Universität eingereicht habe. Weiterhin versichere ich, dass ich die zur Erstellung dieser Dissertationsschrift verwendeten wissenschaftlichen Arbeiten und Hilfsmittel genau und vollständig angegeben habe.

Des Weiteren erkläre ich, dass keine Strafverfahren gegen mich anhängig sind.

Halle (Saale),

_____

Julia Spielmann

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