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„Untersuchungen zur Wirkung einer PPARα-Aktivierung auf den Cholesterol- und Carnitinmetabolismus“

Dissertation

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Abkürzungsverzeichnis

ACO	Acyl-CoA-Oxidase
ATB^{0+}	Aminosäuretransporter B^{0+}
CPT	Carnitin-Palmitoyl-Transferase
Cyp4A1	Cytochrom P_{450} A1
γ-BB	γ-Butyrobetain
γ-BBD	γ-Butyrobetain Dioxygenase
HDL	Lipoproteine hoher Dichte
HMGCoA-R	Hydroxymetylglutaryl-CoA-Reduktase
Insig	*insulin-induced gene*
LDL	Lipoproteine geringer Dichte
OCTN	*organic cation transporters*
PPAR	*peroxisome proliferator-activated receptor*
PPRE	*PPAR-response-element*
SCAP	SREBP *cleavage-activating protein*
SREBP	s*terol regulatory element binding protein*
TMABA-DH	4-*N*-Trimethylaminobutyraldehyd-Dehydrogenase
TML	Trimethyllysin
TMLD	Trimethyllysin Dioxygenase
VLDL	Lipoproteine sehr geringer Dichte

1. Einleitung

Peroxisome proliferator-activated rezeptors (PPARs) sind Liganden-aktivierbare Transkriptionsfaktoren, welche zur Superfamilie der nukleären Rezeptoren gehören. Durch Isseman und Green (1990) wurde erstmals ein Rezeptor kloniert, welcher durch Clofibrat und andere Peroxisomenproliferatoren aktiviert und in der Folge als PPARα bezeichnet wurde. Die Gruppe der Fibrate, zu denen neben Clofibrat auch Fenofibrat, Ciprofibrat und das Fibrat-Analogon WY 14,643 gehören, stellen typische PPARα-Liganden dar und werden als hypolipidämische Medikamente eingesetzt (Staels *et al.*, 1998; Desvergne und Wahli, 1999; Peraza *et al.*, 2005). Dabei beruht deren Wirkung auf der Aktivierung des PPARα und dessen zentraler Bedeutung für den Lipid- und Lipoproteinmetabolismus. Die hypolipidämischen Effekte der Fibrate lassen sich zum einen mit einer erhöhten Hydrolyse von Plasmatriglyzeriden, einer Stimulation der zellulären Fettsäureaufnahme, der verminderten Synthese von Fettsäuren und Triglyzeriden und eine verringerten Synthese von Lipoproteinen sehr geringer Dichte (VLDL) erklären (Staels *et al.*, 1998). Der wesentliche Teil der hypolipidämischen Wirkung der Fibrate beruht allerdings auf der Steigerung der Expression lipolytischer Enzyme, wie der Acyl-CoA-Oxidase (ACO) und Cytochrom P_{450} A1 (Cyp4A1), welche in die peroxisomale β-Oxidation (ACO) beziehungsweise in die mikrosomale ω-Hydroxylierung (Cyp4A1) involviert sind und als typische PPARα-Zielgene gelten (Aldrige *et al.*, 1995; Varanasi *et al.*, 1998; Staels *et al.*, 1998). In der Promotorregion dieser Zielgene konnte ein *PPAR-response-element* (PPRE) nachgewiesen werden (Desvergne und Wahli, 1999). Über eine spezifische Bindung an diese PPREs steuert PPARα nach erfolgter Aktivierung und Heterodimerisierung mit dem 9-cis Retinoid-X-Rezeptor im Zellkern die Transkription zahlreicher weiterer Zielgene. Diese Gene kodieren für Enzyme, welche neben dem Fettsäurenkatabolismus auch in weitere Fettstoffwechselwege wie der zellulären Aufnahme und der Aktivierung von Fettsäuren, der Ketogenese und des Lipoproteinmetabolimus involviert sind (Lee *et al.*, 2003; Mandard *et al.*, 2004; Reddy und Sambasiva Rao, 2006). PPARα konnte durch Sher *et al*. (1993) in zahlreichen Spezies kloniert und charakterisiert werden und wird aufgrund seiner Bedeutung für den Fettstoffwechsel vor allem in Geweben mit einer hohen Fettsäureoxidationsrate wie der Leber, dem Herzen, der Niere, dem Muskel und der Dünndarmmukosa exprimiert (Braissant *et al.*, 1996; Auboeuf *et al.*, 1997; Desvergne und Wahli, 1999; Delerive *et al*., 2001; Berger und Moller, 2002; Van Raalte *et al.*, 2004). In der wissenschaftlichen Literatur sind zahlreiche natürliche Substanzen als potente PPARα-

Aktivatoren bekannt. Dazu zählen verschiedene langkettige Fettsäuren (Krey *et al.*, 1997; Kliewer *et al.*, 1997; Xu *et al.*, 1999; Kersten *et al.*, 1999; Pégorier *et al.*, 2004). So werden zum einen im Fasten vermehrt Fettsäuren freigesetzt, welche als Liganden des PPARα fungieren (Kersten *et al*., 1999; Leone *et al*., 1999). Es konnte aber ebenfalls gezeigt werden, dass auch über die Nahrung zugeführte langkettige Fettsäuren zu einer Aktivierung des PPARα führen können (Ren *et al*., 1996; Lin *et al*., 1999; Jump *et al*., 2005). Ebenso sind unterschiedliche primäre Oxidationsprodukte langkettiger Fettsäuren wie zum Beispiel die Hydroxy-Fettsäuren 9-Hydroxy-10,12-octadecadiensäure und 13-Hydroxy-9,11-octadecadiensäure, die Hydroperoxy-Fettsäure 13-Hydroperoxyoctadeca-9,11-diensäure und 8-Hydroxy-5,9,11,14-eicosatetraensäure, ein Metabolit der Arachidonsäure, als Einzelkomponenten oxidierter Fette in der wissenschaftlichen Literatur als potente PPARα-Liganden bekannt und imstande diesen zu aktivieren (Yu *et al.*, 1995; Forman *et al.*, 1997; Delerive *et al.*, 2000; Marx *et al.*, 2004; Mishra *et al*., 2004; König und Eder 2006). Auch zyklische Fettsäuremonomere, als sekundäre Lipid-peroxidationsprodukte, konnten als PPARα-Aktivatoren nachgewiesen werden (Martin *et al.*, 2000). Neben einer Aktivierung des PPARα durch isolierte Fettsäuren beziehungsweise deren primäre Oxidationsprodukte konnte in verschiedenen Studien auch bewiesen werden, dass, unabhängig von der Dauer und der Intensität der voran gegangenen Oxidation, oxidierte Fette selbst, als komplexes Gemisch, welches sich sowohl aus primären als auch aus sekundären Produkten zusammensetzt, in der Lage sind den PPARα zu aktivieren (Chao *et al.*, 2001; 2004; Sülzle *et al.*, 2004). Oxidierte Fette nehmen in der menschlichen Ernährung einen zunehmend höheren Stellenwert ein. Die heutige Ernährung in den Industrieländern beinhaltet ein steigendes Maß an Fertigprodukten, für die besonders hohe Gehalte an Fett kennzeichnend sind (Cohn, 2002). Die Erhitzung besonders fetthaltiger Fertigprodukte führt ebenso wie die durch den globalen Handel bedingten langen Lagerungsperioden zu erhöhten Konzentrationen an Lipid-peroxidationsprodukten (Kubow, 1992; Cohn, 2002). Die vermehrte Aufnahme oxidierter Fette wird mit der Pathogenese verschiedener Erkrankungen wie Arteriosklerose und Kolonkarzinomen in Verbindung gebracht (Yang *et al.*, 1998; Penumetcha *et al.*, 2000; Cohn, 2002). Die zunehmende Bedeutung oxidierter Fette in der Ernährung in Kombination mit ihrem Einfluss auf die Pathogenese unterschiedlicher Erkrankungen und ihrem bekannten Potential als PPARα-Aktivatoren machen sie zu einem interessanten Gegenstand der aktuellen Forschung. So existieren in der wissenschaftlichen Literatur bereits zahlreiche Untersuchungen, welche sich mit den Effekten einer Fütterung oxidierter

Fette auf den PPARα vermittelten Stoffwechsel beschäftigen. In verschiedenen Studien kam es nach der Verfütterung von oxidierten Fetten an unterschiedliche Modelltiere über eine Aktivierung des PPARα zu einer Reihe von Veränderungen im Lipidstoffwechsel (Eder, 1999; Eder und Stangl, 2000; Eder *et al.*, 2003a, 2003b).

Bei Miniaturschweinen konnte nach der Verfütterung von oxidierten Fetten eine Abnahme der Cholesterolkonzentrationen im Plasma, den Lipoproteinen geringer (LDL) und hoher Dichte (HDL) festgestellt werden (Eder und Stangl, 2000). In weiteren Versuchen führte eine Verfütterung oxidierter Fette an Ratten zu einer Verminderung der Cholesterol-konzentrationen in der Leber, dem Plasma und in den VLDL (Eder und Kirchgessner, 1998; Eder *et al.*, 2003a). Synthetische PPARα-Liganden führten in verschiedenen Humanstudien ebenfalls zu einer Verringerung der Konzentration an Cholesterol im Plasma (Sirtori *et al.*, 1983; Elisaf, 2002; Steals und Furchart, 2005).

Die Cholesterolkonzentration in der Zelle wird über einen *feedback* Mechanismus streng reguliert. Dieser ist durch das Zusammenspiel verschiedener Faktoren gekennzeichnet und wird über die Steuerung der Transkription von Genen realisiert, die in die Cholesterolsynthese und die -aufnahme involviert sind. Einen wichtigen Faktor stellt hierbei die Familie der *sterol regulatory element binding proteins* (SREBPs) dar. Diese wurden erstmal durch Briggs *et al*. (1993) und Wang *et al*. (1993) beschrieben und steuern die Transkription von mehr als 30 Genen, welche in den Cholesterol-, Fettsäure-, Triglyzerid- und Phospholipidmetabolismus involviert sind (Brown und Goldstein, 1999; Osborne, 2000; Horton *et al*., 2003). Von den 3 bekannten Isoformen SREBP-1a, SREBP-1c und SREBP-2 (McPherson und Gauthier, 2004; Weber *et al.*, 2004), steuert vor allem das SREBP-2, das in hohem Maße in der Leber exprimiert wird, die Transkription von verschiedenen Zielgenen wie der Hydroxymetylglutaryl-CoA-Reduktase (HMGCoA-R) und des LDL-Rezeptors, welche in die Cholesterolsynthese und die -aufnahme involviert sind (Horton *et al*., 2003). Die Synthese der SREBP-2 erfolgt zunächst als ein 120kDa großes *precursor* Protein im endoplasmatischen Retikulum, welche in einer inaktiven Form gebunden an das SREBP *cleavage-activating protein* (SCAP) vorliegt.

Bei einem Mangel an Sterolen gelangt der SREBP-SCAP-Komplex in den Golgi-Apparat, wo SREBP-2 proteolytisch gespalten wird und anschließend als reife Form im Zellkern über die Bindung an *sterol response elements* in der *promotor/enhancer* Region der Zielgene deren Transkription steuert (Brown und Goldstein, 1999; Espenshade *et al.*, 2002; Rawson, 2003; Anderson, 2003; McPherson und Gauthier, 2004; Espenshade und Hughes, 2007). In Gegenwart hoher Cholesterolkonzentrationen kommt es durch direkte Bindung

von Cholesterol an SCAP zu einer Konformationsänderung (Brown *et al.*, 2002; Radhakrishnan *et al.*, 2004), in deren Folge die Affinität des SCAP zu den ebenfalls membrangebundenen Proteinen *insulin-induced genes*-1 und -2 (Insig-1 beziehungsweise -2) steigt und es so zu einem Verbleib des Insig-SCAP-SREBP-Komplexes im endoplasmatischen Retikulum kommt (Yang *et al.*, 2002, Yabe *et al.*, 2002; Adams *et al.*, 2003). In der Folge werden der Transport zum Golgi-Apparat und die daran anschließende proteolytische Aktivierung des SREBP-2 unterbunden und somit die Transkription der Zielgene und folglich auch die Cholesterolsynthese verringert.

Insig-1 und -2 werden in den meisten Geweben des Körpers, in besonders hoher Menge aber in der Leber, exprimiert (Yang *et al.*, 2002; Yabe *et al.*, 2002). Insig-1 wird, im Gegensatz zu der des Insig-2, als obligatorisches SREBP-Zielgen nur in Gegenwart von aktivierten SREBP im Zellkern exprimiert, wodurch es zu einer *feedback*-Regulation kommt (Horton, 2002; Janowski 2002; Yabe *et al.*, 2002). Wird Insig-1 überexprimiert, ist es, wiederum im Unterschied zu Insig-2, in der Lage den SCAP-SREBP-Komplex auch ohne die Anwesenheit von Sterolen im endoplasmatischen Retikulum zurückzuhalten (Yang *et al.*, 2002; Yabe *et al.*, 2002). Die Aminosäuresequenzen der zwei Insig-Isoformen stimmen zu 59% überein. (Engelking *et al.*, 2004). Beiden Formen unterscheiden sich in ihrer Halbwertszeit und der Abhängigkeit ihres proteasomalen Abbaus von der vorherrschenden Cholesterolkonzentration (Lee und Ye, 2004; Gong *et al.*, 2006). Darüber hinaus kodiert das Insig-2-Gen für 2 Transkriptvarianten, welche sich in ihrem ersten, nicht kodierenden Exon unterschieden und von denen das leberspezifische Transkript Insig-2a, im Unterschied zu Insig-2b, durch Insulin reprimiert und beim Fasten induziert wird (Yabe *et al.*, 2003).

Der hier dargestellte Mechanismus der Regulation des Cholesterolstoffwechsels über das Zusammenspiel von SREBP-2 und Insig stellt einen interessanten Ansatzpunkt für einen möglichen regulatorischen Eingriff des PPARα dar. Dieser potentielle Zusammenhang wird bekräftigt durch Untersuchungen von Kast-Woelbern *et al.* (2004), bei denen ein erster Zusammenhang zwischen einer PPAR-Aktivierung und der Insig-vermittelten Prozessierung des SREBP nachgewiesen werden konnte. In dieser Studie wurde gezeigt, dass Insig-1 im weißen Fettgewebe von prädiabetischen Mäusen und in Präadipozyten durch den mit Rosiglitazon aktivierten PPARγ, welcher in früheren Studien neben PPARα und PPARβ/δ als weitere Isoform identifiziert werden konnte (Graves *et al.*, 1992; Dreyer *et al*., 1992; Schmidt *et al*., 1992), über ein PPRE reguliert wird. Die Aktivierung des PPARγ führte zum Anstieg der Insig-1 mRNA-Konzentration und zum gleichzeitigen

Absinken der relativen SREBP-1c mRNA-Konzentration. Im Hinblick auf diese interessanten Ergebnisse stellt sich die Frage, inwieweit PPARα in die Regulation der Insig-vermittelten Prozessierung des SREBP-2 involviert ist und sich so möglicherweise die verringerten Cholesterolkonzentrationen erklären lassen. Insgesamt konnte aber bislang kein molekularer Mechanismus als Erklärungsgrundlage für eine Verringerung der Cholesterolkonzentrationen nach erfolgter PPARα-Aktivierung gefunden werden. Insbesondere wurden die cholesterolsenkenden Effekte eines oxidierten Fettes im Zusammenhang mit dessen Potential als PPARα-Aktivator und im Hinblick auf die mögliche Regulation der Insig-abhängigen Prozessierung des SREBP-2 bislang nicht untersucht.

Neben den Veränderungen im Cholesterolstoffwechsel kam es bei Rattenversuchen nach der Gabe von oxidiertem Fett auch zu einer Verringerung der Triglyzeridkonzentrationen im Plasma, der Leber und den VLDL (Eder und Kirchgessner, 1998; Eder *et al.*, 2003a; Sülzle *et al.*, 2004). Der triglyzeridsenkende Effekt einer Liganden-induzierten PPARα-Aktivierung ist in der wissenschaftlichen Literatur sehr gut beschrieben (Mandard *et al*., 2004; Lee *et al*., 2003; Desvergne und Wahli, 1999) und stellt eine physiologisch wichtige Funktion des PPARα dar. Als so genannter Fastenrezeptor wird PPARα durch im Fastenzustand vermehrt freigesetzte Fettsäuren aktiviert (Kersten *et al*., 1999; Leone *et al*., 1999). Nach erfolgter Aktivierung kommt es zu einer gesteigerten Expression verschiedener Zielgene, wie der lipolytischen Enzyme ACO und Cyp4A1 (Moya-Camarena *et al.*, 1999; Chao *et al.*, 2001). PPARα sichert somit durch eine vermehrt stattfindende β-Oxidation in der Leber als Folge der Expressionssteigerung der typischen Enzyme im Fastenzustand die Energieversorgung und reguliert damit die *fasting response* des Körpers. Eine Aktivierung des PPARα führt außerdem zu einer Hochregulation der charakteristischen *downstream* Gene Carnitin-Palmitoyl-Transferase (CPT)-1 und CPT-2 (Brandt *et al*., 1998; Mascaro *et al*., 1998), welche für den Transport aktivierter Fettsäuren über die Mitochondrienmembran verantwortlich sind. CPT-1 und -2 benötigen Carnitin als Co-Faktor und tragen durch ihre zentrale Stellung für die β-Oxidation in Kombination als typische PPARα-Zielgene einen wesentlichen Teil zur Steuerung der *fasting response* des Körpers und zur Funktion des PPARα als Fastenrezeptor bei (Kramer *et al.*, 2003; Mandard *et al.*, 2004). Die Beteiligung des Carnitins am Transport langkettiger, aktivierter Fettsäuren vom Zytosol in die Mitochondrien stellt, neben weiterer Funktionen wie dem Transport von Produkten der β-Oxidation in die Mitochondrien zur Bereitstellung für den Zitratzyklus, der Funktion als Azetylpuffer zur Aufrechterhaltung des Azetyl-CoA/CoA-

Verhältnisses und der Speicherung von Energie in Form von Azetyl-Carnitin, dessen Hauptfunktion dar (McGarry und Brown, 1997; Brass, 2002; Steiber *et al.*, 2004). Diese zentrale Rolle des Carnitins im mitochondrialen Fettsäurestoffwechsel und die gleichzeitige Bedeutung des PPARα für diesen Stoffwechselweg, machen diese enge Verknüpfung zu einem spannenden Forschungsgebiet. Ein erhöhter Bedarf an Carnitin resultierend aus der Funktion als Co-Faktor für CPT-1 und CPT-2 und deren PPARα-abhängiger Hochregulation der Expression sollte zum einen durch eine erhöhte *de novo* Carnitinsynthese in der Leber und zum anderen durch einen vermehrte Aufnahme von Carnitin aus dem Plasma in die Leber abgedeckt werden. In verschiedenen Studien konnte ein erster Zusammenhang zwischen einer Fibratsupplementierung und dem Carnitin-stoffwechsel beobachtet werden. So zeigen bereits sehr frühe Studien, dass es bei Ratten nach einer Behandlung mit den PPARα-Liganden Clofibrat beziehungsweise Ciprofibrat zu einem Anstieg der Carnitinkonzentration in der Leber der Tiere kommt (Paul und Adibi, 1979; Bhuiyan *et al.*, 1988), welcher vermutlich mit dem parallel bei einer PPARα-Aktivierung einhergehenden erhöhten Bedarf an Carnitin als Co-Faktor für die CPTs zu begründen ist. Ebenso konnte auch im Fastenzustand, in dem PPARα durch endogen freigesetzte Fettsäuren in der Leber aktiviert wird (Kersten *et al.*, 1999), eine Erhöhung der Carnitinkonzentration in der Leber beobachtet werden (McGarry *et al.*, 1975; Slitt *et al.*, 2002). In einem Fastenversuch mit PPARα-*knockout*-Mäusen konnte bei diesen Tieren im Vergleich zu Wildtyp-Mäusen keine Erhöhung der hepatischen Carnitinkonzentration festgestellt werden und somit indirekt ein erster Zusammenhang zwischen einer PPARα-Aktivierung und der Konzentration von Carnitin in der Leber festgestellt werden (Hashimoto *et al*., 1999). Unklar ist zum jetzigen Zeitpunkt, ob diese erhöhten Carnitinkonzentrationen in der Leber die Folge einer vermehrten Aufnahme oder einer erhöhten Carnitinsynthese sind. Die Synthese des Carnitins aus den beiden Aminosäuren Lysin und Methionin deckt nur einen geringen Teil des Bedarfs und verläuft dabei über mehrere Zwischenschritte in verschiedenen Geweben (Hoppel und Davis, 1986). In einem letzten, reaktionsgeschwindigkeitsbestimmenden Schritt wird γ-Butyrobetain (γ-BB) durch die γ-Butyrobetain Dioxygenase (γ-BBD) zu Carnitin hydroxyliert. Im Gegensatz zu den vorangegangenen Syntheseschritten, findet diese letzte Reaktion ausschließlich in der Leber, der Niere und dem Gehirn statt, da die γ-BBD nur in diesen Geweben exprimiert wird (Vaz und Wanders, 2002). Da der Bedarf an Carnitin nur zu einem geringen Teil über die Biosynthese gedeckt wird, spielen die Aufnahme aus der Nahrung, sowie der intrazelluläre und der Transport zwischen den einzelnen Organen für die Aufrechterhaltung

der Carnitinhomöostase im Körper eine entscheidende Rolle. Weiterhin ist bislang unklar, welche Effekte synthetische und natürliche Liganden auf den Carnitinstoffwechsel haben. In einer Studie von Luci *et al*. (2006) gelang es erstmals einen positive Korrelation zwischen einer Aktivierung des PPARα durch die Behandlung von Ratten mit Clofibrat und einer vermehrten Expression der *organic cation transporters* (OCTN) in der Leber der Tiere festzustellen. Dieser Zusammenhang konnte in der gleichen Studie auch nach der Inkubation von Fao-Zellen mit WY 14,643 beobachtet werden. Somit konnte durch unsere Arbeitsgruppe ein erster Hinweis auf einen möglichen funktionellen Zusammenhang zwischen einer Aktivierung des PPARα und erhöhter Carnitinkonzentrationen in der Leber als Folge einer Behandlung mit Fibraten belegt werden. Die OCTNs spielen durch ihre Funktion als Carnitintransporter eine entscheidende Rolle in der Regulation der Gesamtkörper- und der intrazellulären Homöostase des Carnitins. Die regulatorische Bedeutung dieser Transporter für die Aufrechterhaltung des Carnitingleichgewichts wird durch die Entstehung eines primären oder sekundären systemischen Carnitinmangel verdeutlicht, welcher durch angeborene oder erworbene Defekte der OCTNs hervorgerufen wird (Tein, 2003). Diese Transporter, welche zu der *solute carrier* 22A Familie gehören, sind in der apikalen Zellmembran lokalisiert (Lahjouji *et al.*, 2001; Tein, 2003). Zum jetzigen Zeitpunkt sind die drei Vertreter OCTN1, OCTN2 und OCTN3 identifiziert worden (Tamai *et al.*, 1997; 1998; 2000; Wu *et al.*, 2000). Diese unterscheiden sich in der Abhängigkeit des Carnitintransports von Natrium, in ihrer Affinität zu Carnitin und anderen Kationen und in ihren Expressionsmustern (Tamai *et al*., 1998; Wu *et al*., 1999; Tamai *et al.,* 2000; Slitt *et al*., 2002; Grube *et al*., 2006; Koepsell *et al*., 2007). Aufgrund seiner hohen Affinität zu Carnitin und auch zu γ-BB und der weiten Verbreitung des OCTN2 in verschiedenen Geweben scheint dieser Vertreter der physiologisch bedeutendste zu sein und stellt ein attraktives Target für weitere Untersuchungen im Zusammenhang mit einer PPARα-Aktivierung dar.

Die Bedeutung des PPARα für die β-Oxidation über die Regulation der Transkription von zahlreichen Zielgenen ist in der wissenschaftlichen Literatur detailliert beschrieben (Schoonjans *et al*., 1996; Desvergne und Wahli, 1999; Latruffe *et al*., 2000). PPARα nimmt somit eine zentrale Stellung im Fettsäurekatabolismus ein. Gleichzeitig liefern die aus Voruntersuchungen bekannten Effekte eine interessante Grundlage zur weiteren Untersuchung des Einflusses einer PPARα-Aktivierung auf die verschiedenen Fettstoffwechselwege. Mechanistische Untersuchungen zur Ursache der bei den bereits

erwähnten Fütterungsversuchen festgestellten verringerten Cholesterolkonzentrationen im Zusammenhang mit einer parallel einhergehenden PPARα-Aktivierung fehlen bislang. Ebenso wurde bislang nicht untersucht, inwieweit oxidiertes Fett als PPARα-Aktivator den Insig-abhängigen Cholesterolstoffwechsel beeinflusst. Weiterhin fehlen in der wissenschaftlichen Literatur auch Studien zu systemischen Effekten einer Aktivierung des PPARα durch oxidiertes Fett im Hinblick auf die Beeinflussung der Expression des OCTN2 und deren Bedeutung für die Carnitinverteilung im Körper. Gleichzeitig bietet sich durch den Einsatz von PPARα-*knockout*-Mäusen eine interessante Möglichkeit, die Zusammenhänge zwischen einer Fibrat-induzierten Aktivierung des PPARα und der Expression der OCTNs weiter aufzuklären.

2. Zielstellung

Ziel dieser Arbeit war es zum einen, zu untersuchen, wie sich die aus Vorversuchen bekannten Verringerungen der Cholesterolkonzentrationen nach der Verfütterung oxidierter Fette erklären lassen. Das Fehlen funktioneller Studien der Insig-vermittelten Reifung des SREBP-2 im Zusammenhang mit einer Aktivierung des PPARα und der damit einhergehenden Beeinflussung des Cholesterolstoffwechsels bildete eine Grundlage für diese Arbeit. Um diese Zusammenhänge zu untersuchen, wurden zunächst Studien mit synthetischen PPARα-Liganden durchgeführt. Aufgrund der zunehmenden Bedeutung oxidierter Fette in der menschlichen Ernährung und deren bekanntem Potential als natürliche PPARα-Aktivatoren, wurde auch ein Versuch mit oxidiertem Fett im Hinblick auf dessen Wirkung auf die Insig-vermittelte Prozessierung des SREBP-2 durchgeführt.

Die interessanten Ergebnisse unserer Arbeitsgruppe bei der Untersuchung der Aktivierung des PPARα mit Clofibrat und der damit einhergehenden vermehrten Expression des OCTN2 stellten einen weiteren wichtigen Ausgangspunkt für diese Arbeit dar. Aufbauend auf diesen Ergebnissen, sollte die Rolle des PPARα, nach erfolgter Aktivierung mit einem oxidierten Fett als natürlichen Liganden, im Hinblick auf die Regulation der mRNA-Konzentrationen verschiedener Gene der Carnitinsynthese und insbesondere der OCTNs und deren Einfluss auf die Carnitinaufnahme und -verteilung bei Ratten untersucht werden. In Kombination mit den aus früheren Vorversuchen bekannten Erhöhungen der Carnitinkonzentration in der Leber von Ratten nach der Gabe von Fibraten beziehungsweise dem Fasten sollte in dieser Arbeit untersucht werden, ob für diese Veränderungen ein, aufgrund seiner hohen Carnitinaffinität vor allem über OCTN2 vermittelter, vermehrter Transport in Frage kommt. Auch hier existieren in der wissenschaftlichen Literatur noch keine funktionellen Studien, welche sich mit einem Einfluss einer durch oxidiertes Fett induzierten PPARα-Aktivierung auf die Expression der OCTNs und die damit verbundenen Veränderungen der Carnitinverteilung im Körper beschäftigen.

Die Studien wurden zum einen an Sprague-Dawley-Ratten durchgeführt, welche als geeignetes Modell zur Untersuchung der Wirkung von Fibraten beziehungsweise oxidiertem Fett bezogen auf die Funktion des PPARα in der wissenschaftlichen Literatur etabliert sind (Martin *et al.*, 1997; Nakatani *et al.*, 2002; Chao *et al.*, 2005; Ringseis *et al.*, 2007a; 2007b). Die für die *in vitro* Untersuchungen verwendete Rattenhepatozyten-Zelllinie Fao ist ebenfalls ein weit verbreitetes Modell zur Untersuchung der Wirkung von PPARα-Agonisten auf den

hepatischen Stoffwechsel (Brocard *et al.*, 1993; Duclos *et al.*, 1997; Poirier *et al.*, 1997; Vanden Heuvel *et al.*, 2003; Landrier *et al.*, 2004). In einer weiteren Untersuchung wurde der Effekt von WY 14,643 auf die Expression der OCTNs und der Gene der Carnitinsynthese bei PPARα-k*nockout*-Mäusen und den entsprechenden Wildtyp-Mäusen untersucht. Diese Mäuse sind in der wissenschaftlichen Literatur ein bewährtes Modell zur Untersuchung der Rolle des PPARα in verschiedenen Stoffwechselwegen (Kamijo *et al*., 2007; Xiao *et al*., 2006; Cuzzocrea *et al*., 2006; Knight *et al*., 2005; Yang und Gonzalez, 2004). Im Bezug auf diese Arbeit eignen sie sich hervorragend als *in vivo*-Modell, um den Einfluss des PPARα auf die Expression der OCTNs nach der Behandlung mit PPARα-Liganden zu untersuchen.

(1) Das Ziel der ersten Studie (***Vers. 1***) bestand darin, zu untersuchen, über welchen Mechanismus eine Liganden-induzierte PPARα-Aktivierung den Cholesterol-metabolismus in der Ratten-Leber beeinflusst. Dazu wurde Sprague-Dawley-Ratten in der Behandlungsgruppe in Anlehnung an andere funktionelle Untersuchungen in Verbindung mit einer PPARα-Aktivierung bei Ratten (Lenart *et al.*, 1998; Newaz *et al.*, 2004; Léonard *et al.*, 2006) der synthetische PPARα-Ligand Clofibrat in einer ausreichenden Dosis von 250 mg/kg Körpergewicht oral verabreicht und nach Versuchsende die Genexpression und die nukleäre Konzentration des SREBP-2, sowie die Genexpression des Insig-1 und Insig-2a untersucht. Weiterhin wurde geprüft, inwieweit sich diese Behandlung der Ratten auf die relative mRNA-Konzentration von typischen SREBP-2 Zielgenen wie der HMGCoA Reduktase und des LDL-Rezeptors auswirkt. Zur weiteren Verifizierung der Daten, wurde die Rattenhepatozyten-Zelllinie Fao mit verschiedenen Konzentrationen (25-100 µM) von WY 14,643, einem weiteren selektiven PPARα-Agonisten, inkubiert und anschließend die Genexpression von Insig-1, -2a, SREBP-2 und der entsprechenden Zielgene, sowie die Cholesterolsynthese gemessen. Weitere Details zu Material und Methodik sowie die detaillierte Beschreibung und Diskussion der Ergebnisse dieser Studie sind ersichtlich in:

B. KÖNIG, A. KOCH, J. SPIELMANN, C. HILGENFELD, G. I. STANGL, K. EDER (2007) Activation of PPARα lowers synthesis and concentration of cholesterol by reduction of nuclear SREBP-2. ***Biochemical Pharmacology*** *73: 574-585.*

(2) In vorangegangenen Untersuchungen konnte gezeigt werden, dass oxidiertes Fett, ähnlich wie Fibrate, in der Lage ist den PPARα zu aktivieren (Chao *et al.*, 2001; 2004; Sülzle *et al.*, 2004). Parallel konnte in verschiedenen Studien eine Reduzierung der Cholesterolkonzentration in verschiedenen Geweben nach der Verfütterung eines oxidierten Fettes festgestellt werden (Eder und Kirchgessner, 1998; Eder, 1999; Eder und Stangl, 2000; Eder *et al.*, 2003a). Das Ziel dieser zweiten Studie (***Vers. 2a***) bestand nun darin, zu untersuchen, ob eine Gabe von oxidiertem Fett die Regulation des Cholesterolstoffwechsels in der Rattenleber in Analogie zu den Ergebnissen der ersten Studie auf eine ähnliche Weise beeinflusst und sich so die Daten aus der ersten Untersuchung reproduzieren lassen. Ferner sollte untersucht werden, ob sich die aus Voruntersuchungen mit oxidierten Fetten bekannte Verringerung der Cholesterolkonzentration nach der Verfütterung oxidierter Fette möglicherweise durch die Insig-abhängige Regulation des Cholesterolstoffwechsels erklären lässt. Dazu wurde Sprague-Dawley-Ratten oral ein moderat oxidiertes Fett verabreicht, welches über einen Zeitraum von 25 d bei 60°C oxidiert wurde und sich durch einen hohen Anteil an primären Lipid-peroxidationsprodukten auszeichnete. Dabei wurden 25% der täglichen Energiezufuhr durch oxidiertes beziehungsweise frisches Fett gedeckt. Nach Versuchsende wurden die Genexpression und die nukleäre Konzentration des SREBP-2 sowie die Genexpression typischer SREBP-2 Zielgene, wie der HMGCoA Reduktase und des LDL-Rezeptors, und des Insig-1 und Insig-2a untersucht. Weitere Einzelheiten zu Material und Methodik sowie der ausführlichen Beschreibung und Diskussion der Ergebnisse sind folgender Studie zu entnehmen:

A. KOCH, B. KÖNIG, J. SPIELMANN, A. LEITNER, G. I. STANGL, K. EDER (2007) Thermally Oxidized Oil Increases the Expression of Insulin-Induced Genes and Inhibits Activation of Sterol Regulatory Element-Binding Protein-2 in Rat Liver. ***Journal of nutrition*** *137: 2018-2023.*

(3) In weiteren Untersuchungen (***Vers. 2b***) wurde der Einfluss des oxidierten Fettes als PPARα-Aktivator auf den Carnitinstoffwechsel und -transport untersucht. Dazu wurden die aus ***Vers. 2a*** gewonnen Gewebeproben hinsichtlich der relativen

mRNA-Konzentrationen weiterer typischer PPARα-Zielgene untersucht und damit weitere Belege für eine PPARα-Aktivierung zu erhalten. Ein Schwerpunkt der anschließenden Untersuchungen lag darin, die Beeinflussung der Expression der OCTNs, insbesondere des OCTN2, in der Leber der Tiere in Folge einer durch das oxidierte Fett hervorgerufenen PPARα-Aktivierung zu untersuchen. Weiterhin wurde auch der Einfluss auf die relative mRNA-Konzentration von Genen der Carnitinbiosynthese untersucht. Gleichzeitig wurde auch die Konzentrationen an Carnitin in der Leber, dem Plasma, dem Herzen und des Skelettmuskels bestimmt. Da OCTN1 und OCTN2 genau wie PPARα auch im Darm exprimiert werden und OCTN2 vermutlich eine wichtige Rolle in der Absorption von Carnitin aus der Nahrung spielt, wurde auch die Genexpression von OCTN1 und OCTN2 im Dünndarm untersucht. Da Carnitin im Darm auch über Aminosäuretransporter B^{0+} (ATB^{0+}) transportiert werden kann (Kekuda *et al*., 1997; Sloan und Mager, 1999; Taylor, 2001; Nakanishi *et al.*, 2001), wurde auch dessen relative mRNA-Konzentration im Darm bestimmt. Weitere Einzelheiten der Untersuchungen im Hinblick auf Material und Methodik sowie die ausführliche Darstellung und Diskussion der Ergebnisse sind folgender Veröffentlichung zu entnehmen:

A. KOCH, B. KÖNIG, S. LUCI, G. I. STANGL, K. EDER (2007) Dietary oxidised fat up regulates the expression of organic cation transporters in liver and small intestine and alters carnitine concentrations in liver, muscle and plasma of rats. ***British Journal of Nutrition*** *98: 882-889.*

(4) In einem weiteren, zweifaktoriellen Versuch (***Vers. 3***) wurden PPARα-*knockout*-Mäuse beziehungsweise Wildtyp-Mäuse oral mit einer ausreichenden Dosis (40 mg/kg KG) WY 14,643 behandelt. Das WY 14,643 wurde dabei in Sonnenblumenöl/DMSO (50/50, v/v) gelöst. Die jeweiligen Kontrollgruppen erhielten eine entsprechende Menge Sonnenblumenöl/DMSO. Im Anschluss an diese Behandlung wurden die Veränderungen der relativen mRNA-Konzentrationen der Gene der Carnitinsynthese und des -transports untersucht. Ziel war es hierbei vor allem, zu untersuchen, ob die aus ***Vers. 2b*** und anderer Voruntersuchungen unserer Arbeitsgruppe bekannten Expressionssteigerungen des OCTN2 in Folge einer Aktivierung des PPARα im *knockout*-Modell nicht mehr nachzuweisen sind. Durch

diese Untersuchungen sollte ein eindeutiger Zusammenhang zwischen einer Aktivierung des PPARα und der Expression des OCTN2 nachgewiesen werden. Gleichzeitig wurde auch der Einfluss des PPARα auf weitere Gene des Carnitintransportes in verschiedenen Geweben untersucht. Um die Bedeutung einer Beeinflussung der OCTN-Genexpression auf die Carnitinverteilung zu untersuchen, wurden auch die Carnitinkonzentrationen in den verschiedenen Geweben analysiert. Zur weiteren Abschätzung des Einflusses der Carnitinsynthese auf erhöhte Carnitinkonzentrationen in der Leber wurden die mRNA-Konzentrationen der entsprechenden Enzyme, welche in die Synthese involviert sind, bestimmt. Weiterhin wurden die Konzentrationen an Trimethyllysin (TML) und γ-BB in der Leber gemessen, welche beide als Ausgangsmoleküle für die Carnitinsynthese dienen können. Weitere Details zu Material und Methodik sowie die ausführliche Beschreibung und Diskussion der Ergebnisse dieser Studie sind ersichtlich in:

A. KOCH, B. KÖNIG, G. I. STANGL, K. EDER (2008) PPARα mediates transcriptional upregulation of novel organic cation transporters (OCTN)-2 and -3 and enzymes involved in hepatic carnitine synthesis. ***Exp. Biol. Med. (Maywood)*** *233: 356-365.*

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

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ABSTRACT

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

1. Introduction

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

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

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

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

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

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

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

2. Material and methods

2.1. Materials

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

2.2. Cell culture

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

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

2.3. Animals, diets and sample collection

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

2.4. RT-PCR analysis

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

2.5. Immunoblot analysis

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

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

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

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

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

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

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

2.7. Determination of cholesterol synthesis

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

2.8. Statistical analysis

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

3. Results

3.1. Activation of PPARα in rat liver and Fao cells

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

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

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

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

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

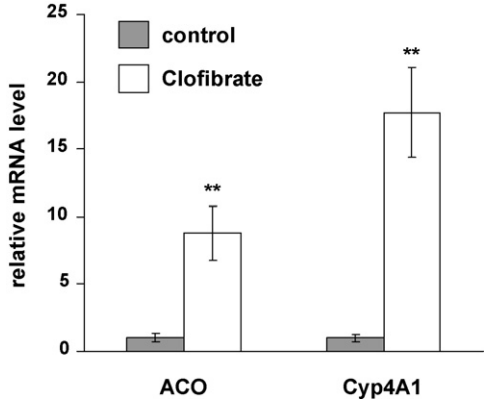

Fig. 1 – Effect of the PPARα agonist clofibrate on the mRNA concentration of PPARα downstream genes ACO and Cyp4A1 in rat liver. Rats were treated orally with 250 mg/kg of clofibrate for 4 days. Control animals obtained the appropriate volume of the vehicle sun flower oil. Total RNA was extracted from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

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

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

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

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

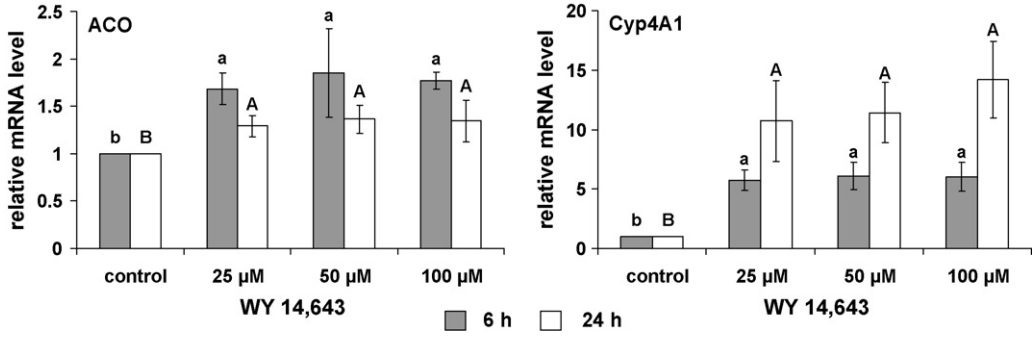

Fig. 2 – Effect of the PPARα agonist WY 14,643 on the mRNA concentration of PPARα downstream genes ACO and Cyp4A1 in Fao cells. Fao cells were grown in culture medium until subconfluent state. Medium was then changed to low-serum medium (0.5% fetal calf serum). After 16 h preincubation in this medium,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

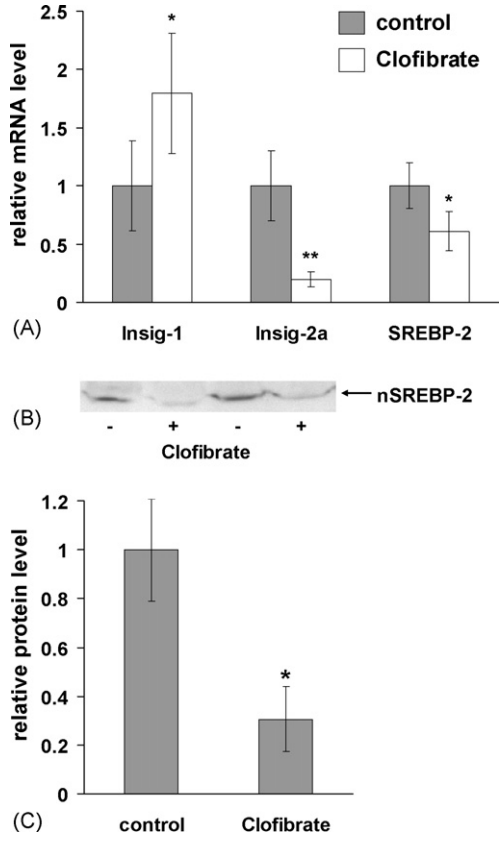

Fig. 3 – Effect of clofibrate treatment on Insig-1, Insig-2a and SREBP-2 in rat liver. Rats were treated orally with 250 mg/kg of clofibrate for 4 days. Control animals obtained the appropriate volume of the vehicle sun flower oil. (A) Total RNA was extracted from rat livers and Insig-1, Insig-2a and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

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

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

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

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

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

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

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

4. Discussion

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

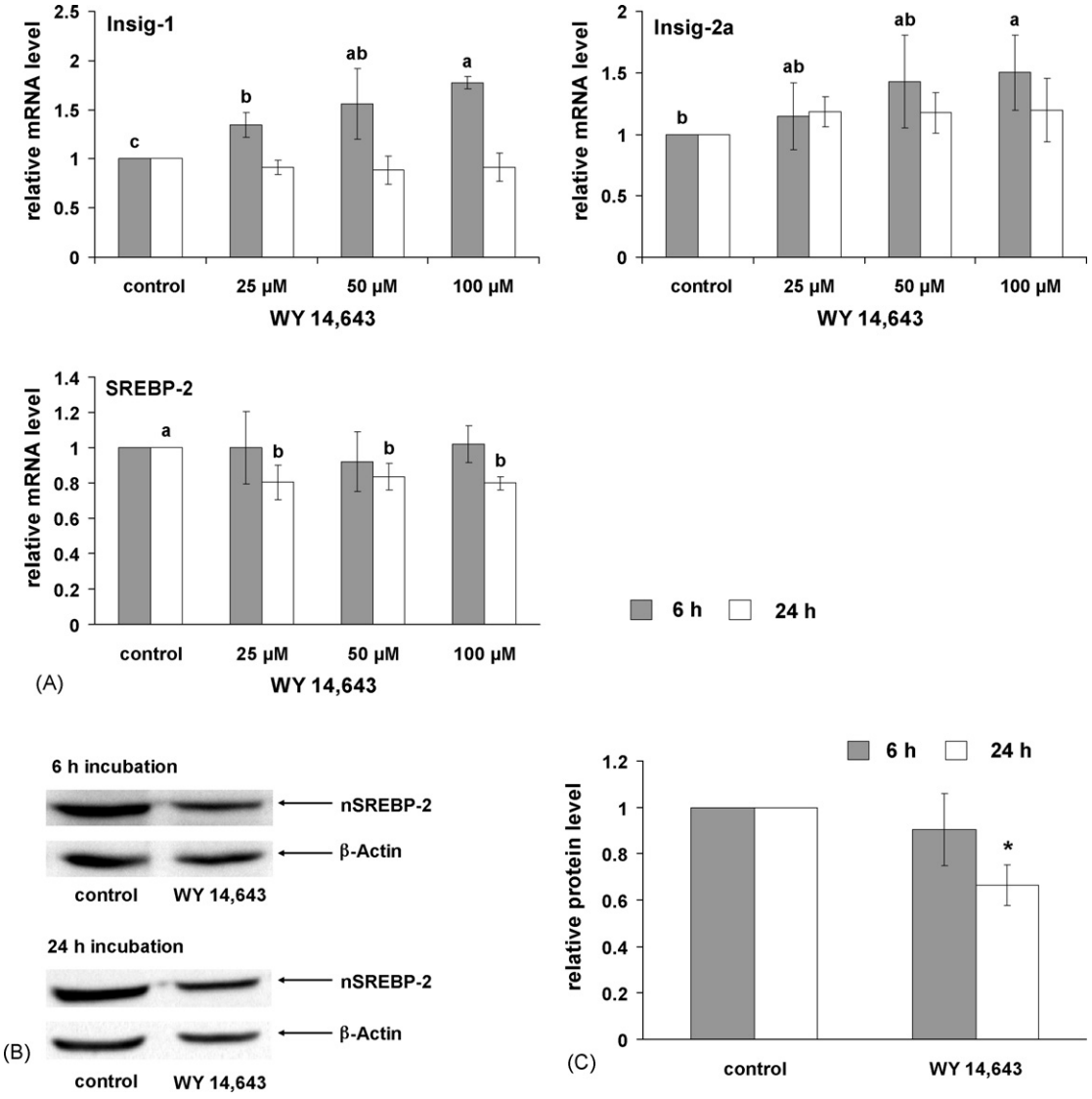

Fig. 4 – Effect of WY 14,643 on Insig-1, Insig-2a and SREBP-2 in Fao cells. Fao cells were grown in culture medium until subconfluent state. Medium was then changed to low-serum medium (0.5% fetal calf serum). After 16 h preincubation in this medium, cells were incubated with increasing concentrations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

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

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

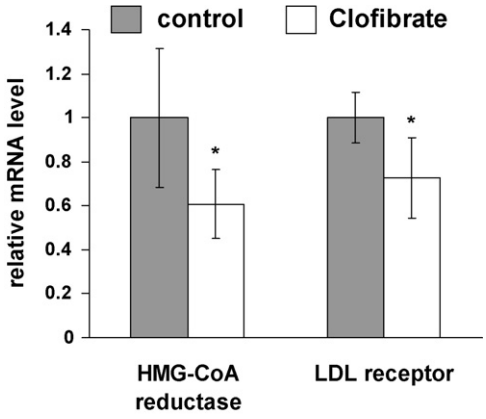

Fig. 5 – Effect of clofibrate treatment on the mRNA concentrations of HMG-CoA reductase and LDL receptor in rat liver. Rats were treated orally with 250 mg/kg of clofibrate for 4 days. Control animals obtained the appropriate volume of the vehicle sun flower oil. Total RNA was extracted from rat livers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

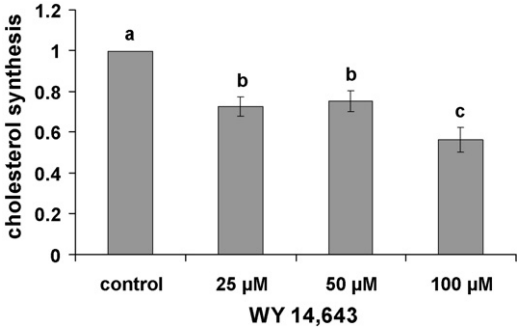

Fig. 7 – Effect of WY 14,643 on the relative cholesterol synthesis rate in Fao cells. Cells were pre-incubated for 22 h with different concentrations of WY 14,643 or with vehicle alone (control). Thereafter, cells were incubated for further 2 h with or without the indicated concentrations of WY 14,643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

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

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

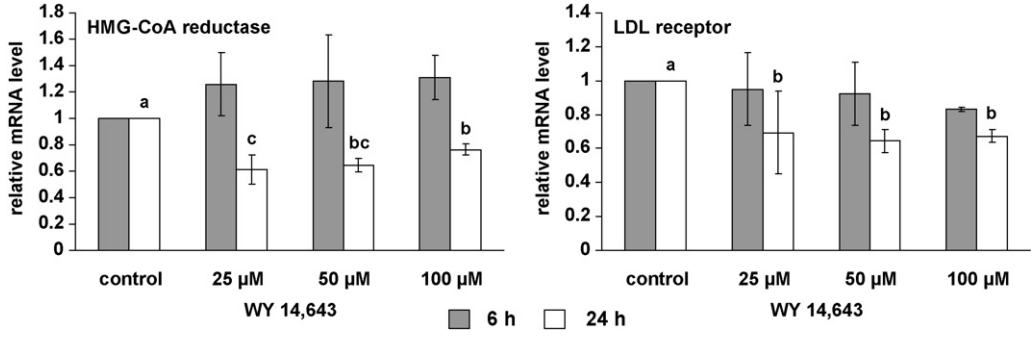

Fig. 6 – Effect of WY 14,643 on the mRNA concentration of HMG-CoA reductase and LDL receptor in Fao cells. Fao cells were grown in culture medium until subconfluent state. Medium was then changed to low-serum medium (0.5% fetal calf serum). After 16 h preincubation in this medium, cells were incubated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

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

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

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

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

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

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

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

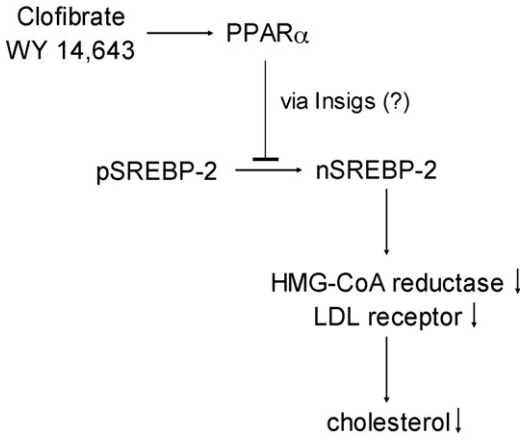

Fig. 8 – Schematic diagram of the proposed pathways leading to decreased cholesterol synthesis and concentration upon PPARα activation. PPARα activation by clofibrate or WY 14,643 reduces the amount of nuclear SREBP-2, probably via an upregulation of Insigs. In turn, this leads to a decreased expression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

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

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

Acknowledgment

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

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Abstract

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

Introduction

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

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

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

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

Materials and Methods

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

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

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

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

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

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

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

Results

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

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

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

TABLE 1 Characteristics of the experimental oils

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

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

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

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

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

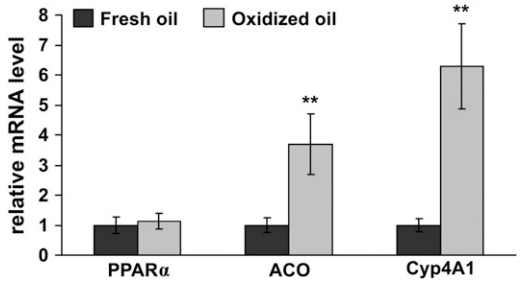

FIGURE 1 Relative mRNA concentrations of PPARα, ACO, and Cyp4A1 in rat livers treated with fresh or oxidized oil. Values are means ± SD, $n = 9$. **Significantly different from rats treated with fresh oil, $P < 0.001$.

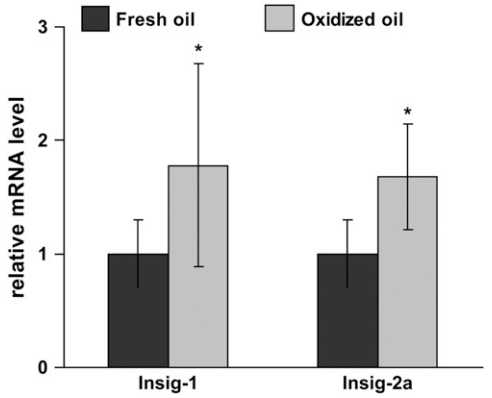

FIGURE 2 Relative mRNA concentrations of Insig-1 and Insig-2a in rat livers treated with fresh or oxidized oil. Values are means ± SD, $n = 9$. *Significantly different from rats treated with fresh oil, $P < 0.05$.

Discussion

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

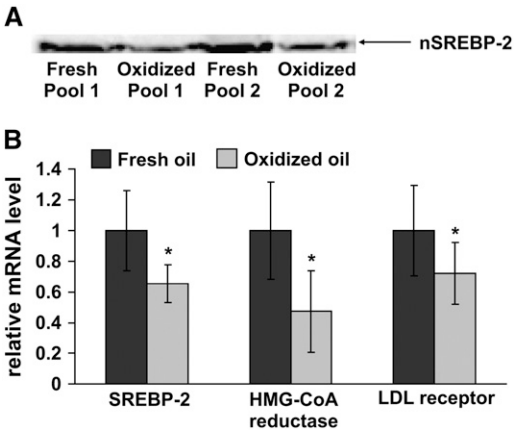

FIGURE 3 Effect of an oxidized oil on SREBP-2 and its target genes in the liver of rats. Concentration of nuclear SREBP-2 (~68 kDa) in the liver of rats treated with fresh or oxidized oil was determined by western blot (*A*). Liver nuclear extracts of 5 and 4 rats, respectively, from each group were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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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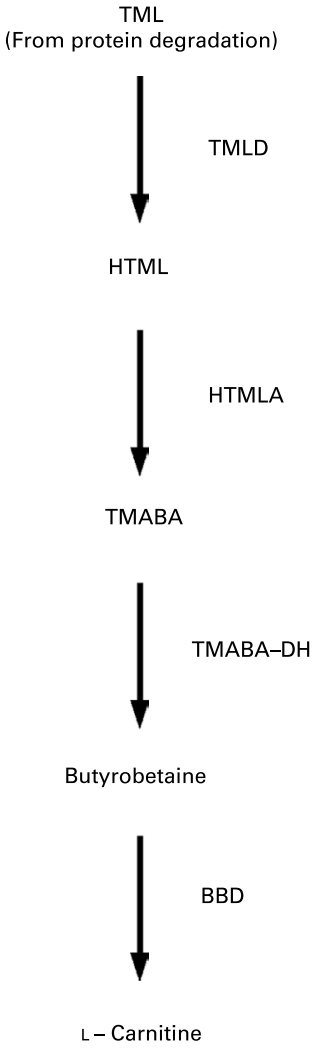

Fig. 1. Schematic diagram of carnitine biosynthesis from trimethyllysine (TML) (according to Vaz & Wanders[6]). TML is oxidised to butyrobetaine by trimethyllysine dioxygenase (TMLD), 3-hydroxy-*N*-trimethyllysine aldolase (HTMLA) and 4-*N*-trimethylaminobutyraldehyde dehydrogenase (TMABA-DH). In the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

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

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

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

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

Materials and methods

Animal experiment

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

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

Preparation of the oxidised fat

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

Carnitine analysis

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

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

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

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

Reverse transcriptase polymerase chain reaction analysis

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

Statistical analysis

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

Results

Final weights and body-weight gains of the rats

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

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

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

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

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

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

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

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

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

Carnitine concentrations in liver, plasma and muscle

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

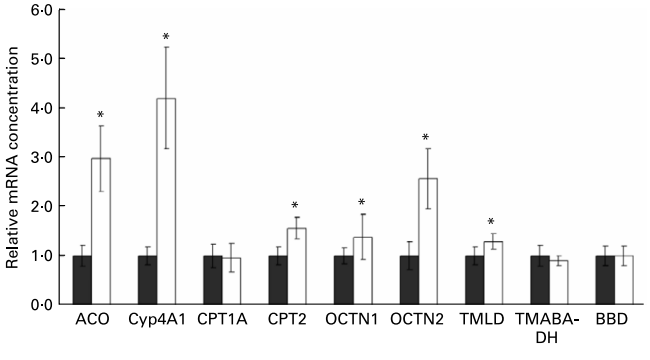

Fig. 2. Effect of an oxidised fat on the relative mRNA concentrations of acyl-CoA oxidase (ACO), cytochrome P450 (Cyp)-4A1, carnitine palmitoyltransferases (CPT)-1A and -2, organic cation transporters (OCTN)-1 and -2, trimethyllysine dioxygenase (TMLD), 4-*N*-trimethylaminobutyraldehyde dehydrogenase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

Discussion

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

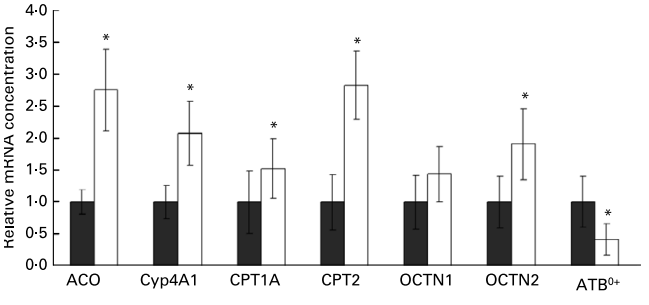

Fig. 3. Effect of an oxidised fat on the relative mRNA concentrations of acyl-CoA oxidase (ACO), cytochrome P450 (Cyp)-4A1, carnitine palmitoyltransferases (CPT)-1A and -2, organic cation transporters (OCTN)-1 and -2 and amino acid transporter ATB^{0+} in the small intestine of rats. Rats were treated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

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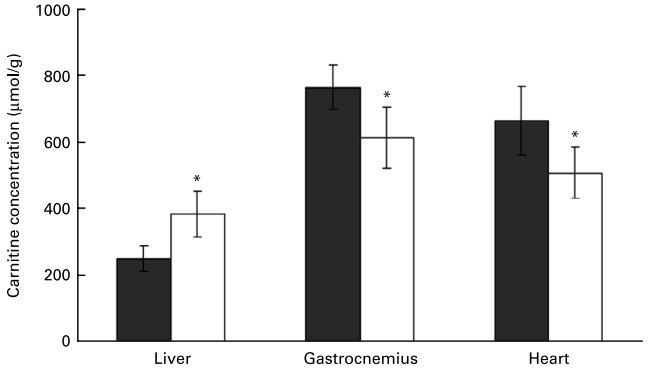

Fig. 4. Effect of an oxidised fat on the concentrations of total carnitine in liver, gastrocnemius and heart. Rats were treated orally with 2 ml oxidised fat (□) or fresh fat (■; control) for 6 d. Values are means, with standard deviations represented by vertical bars (n 9). *Mean value was significantly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

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

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

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

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

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

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

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

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

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

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

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

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

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

Introduction

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

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

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

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

Material and Methods

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

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

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

Carnitine Analysis. Concentrations of free carnitine,

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

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

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

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

Results

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

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

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

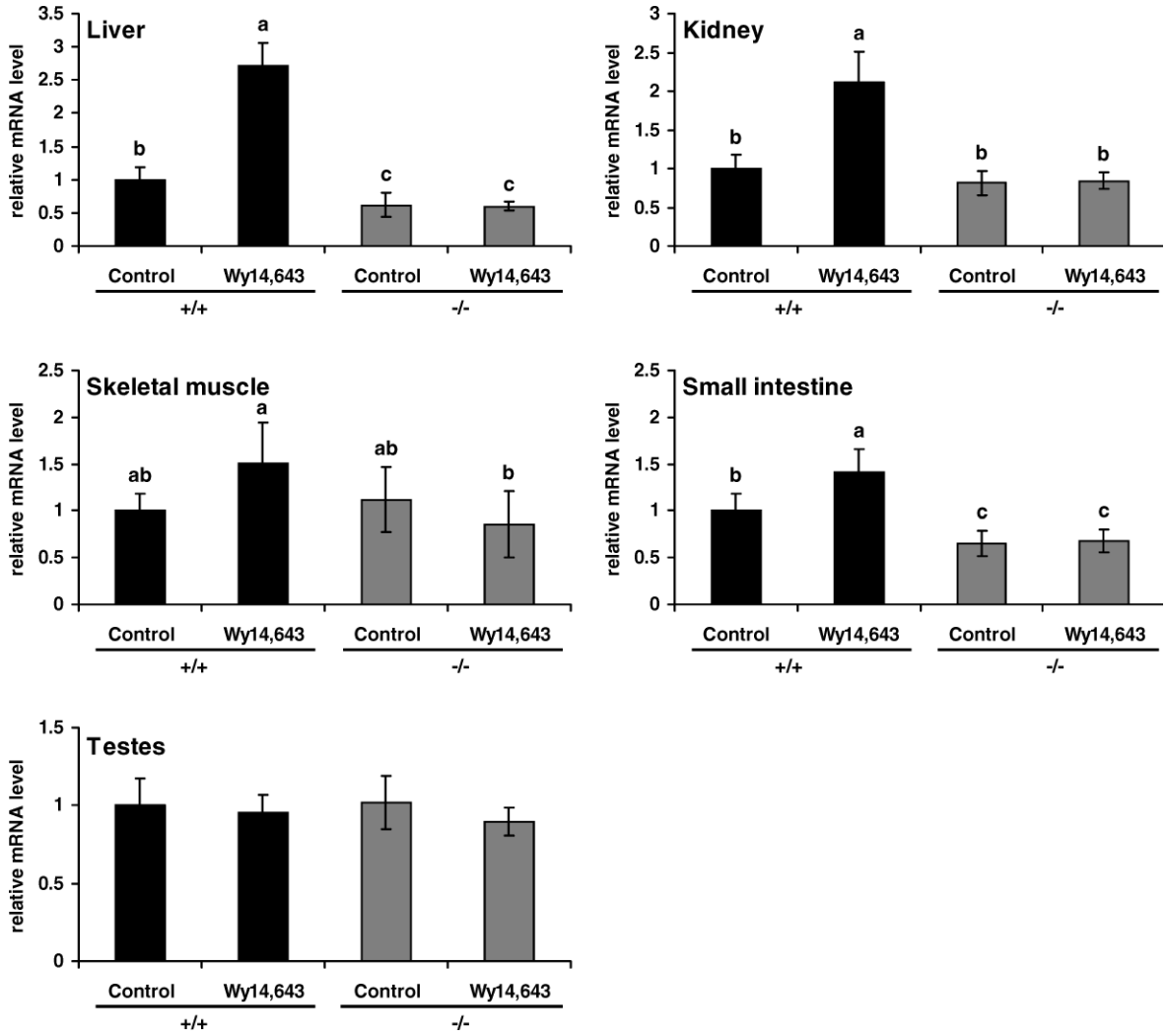

Figure 1. Effect of WY 14,643 on the relative ACO mRNA abundance in the liver, kidney, skeletal muscle, small intestine, and testes of wild-type (+/+) and PPARα$^{-/-}$ mice. Mice were treated orally with 40 mg/kg of WY 14,643 for 4 days. Control mice received the appropriate volume of the vehicle (sunflower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

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

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

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

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

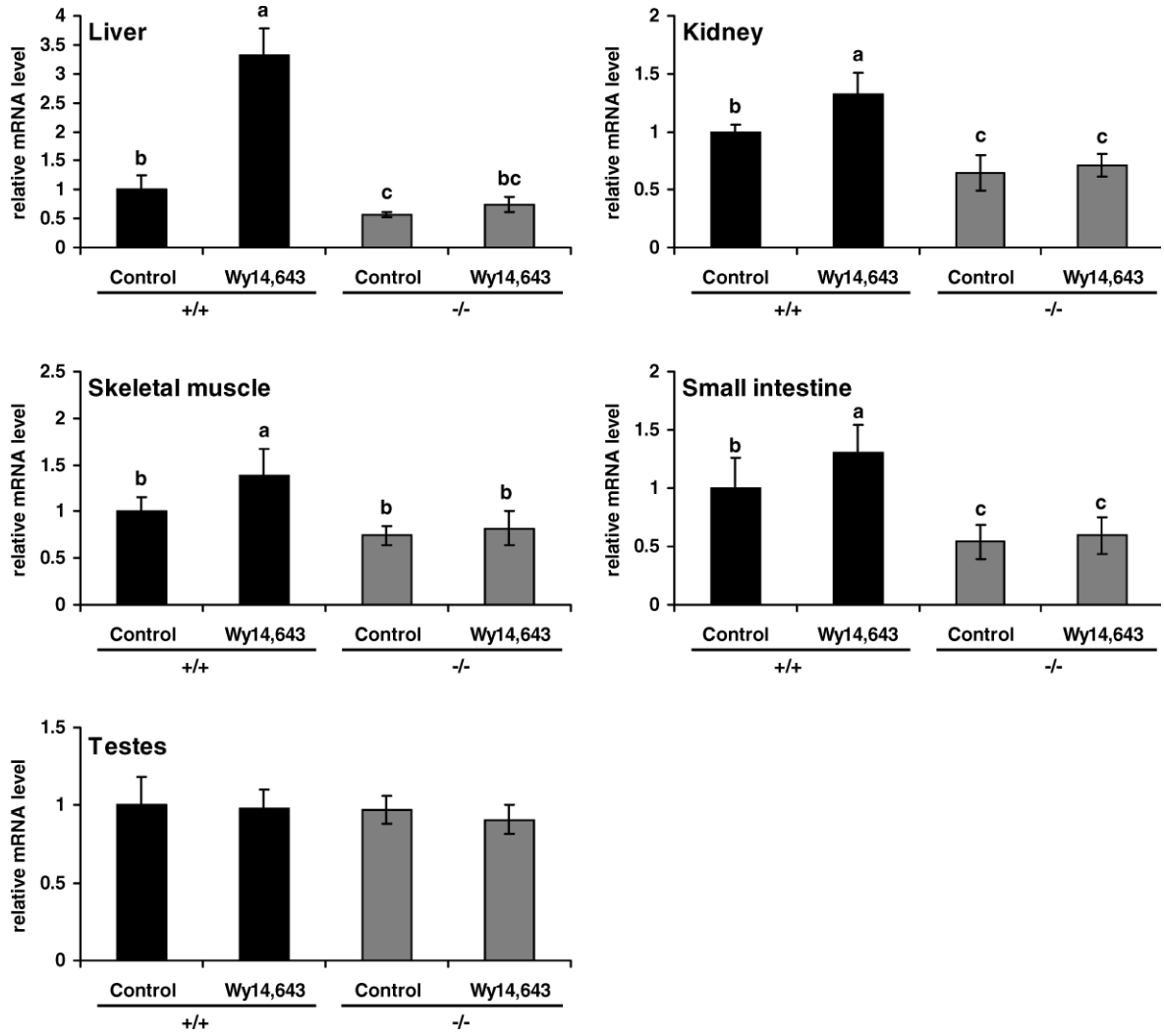

Figure 2. Effect of WY 14,643 on OCTN2 mRNA in the liver, kidney, skeletal muscle, small intestine, and testes of wild-type (+/+) and PPAR$\alpha^{-/-}$ mice. Mice were treated orally with 40 mg/kg of WY 14,643 for 4 days. Control mice received the appropriate volume of vehicle (sunflower oil and DMSO).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

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

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

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

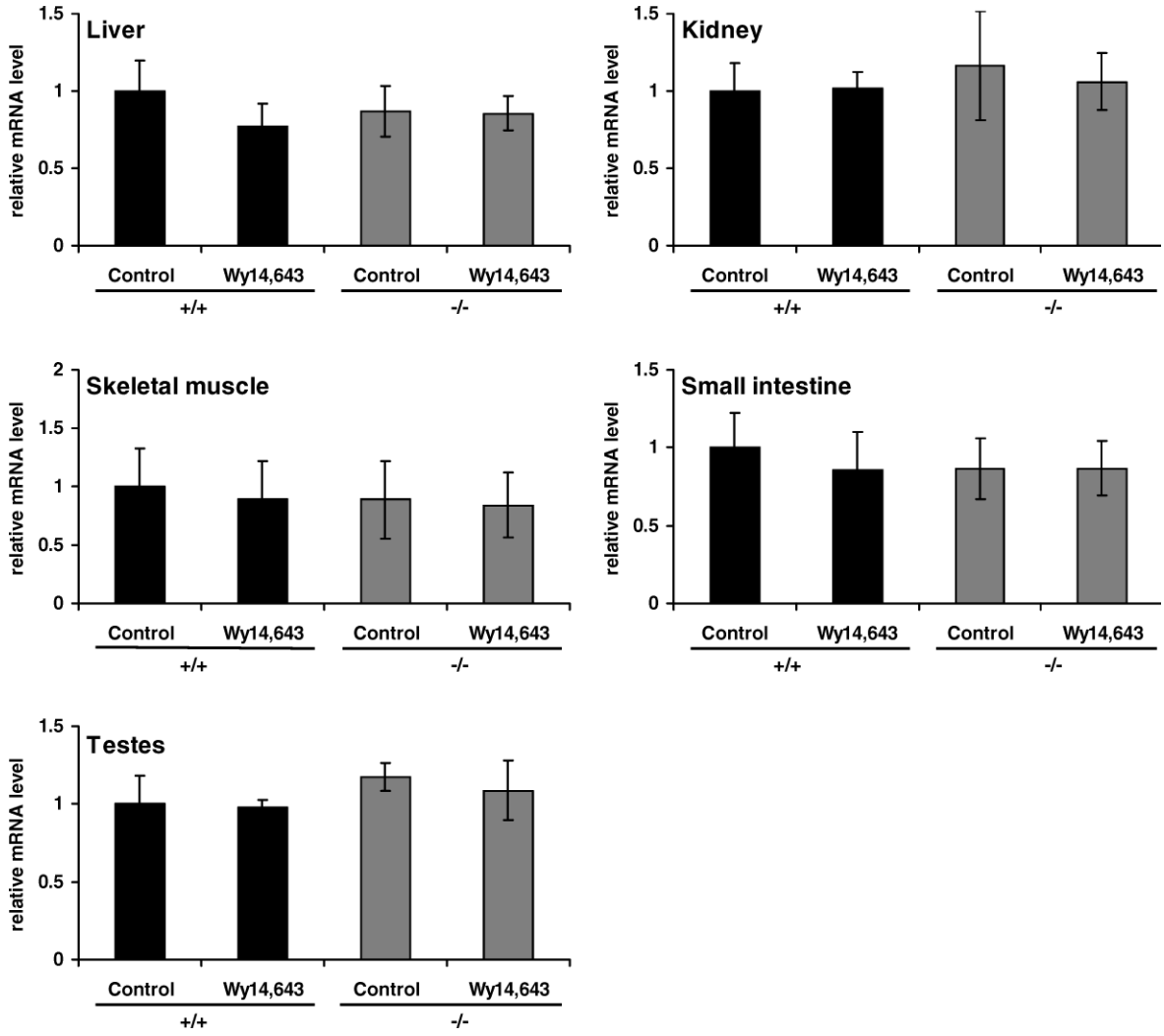

Figure 3. Effect of WY 14,643 on OCTN1 mRNA abundance in the liver, kidney, skeletal muscle, small intestine, and testes of wild-type (+/+) and PPARα$^{-/-}$ mice. Mice were treated orally with 40 mg/kg of WY 14,643 for 4 days. Control mice received the appropriate volume of vehicle (sunflower oil and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

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

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

Discussion

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

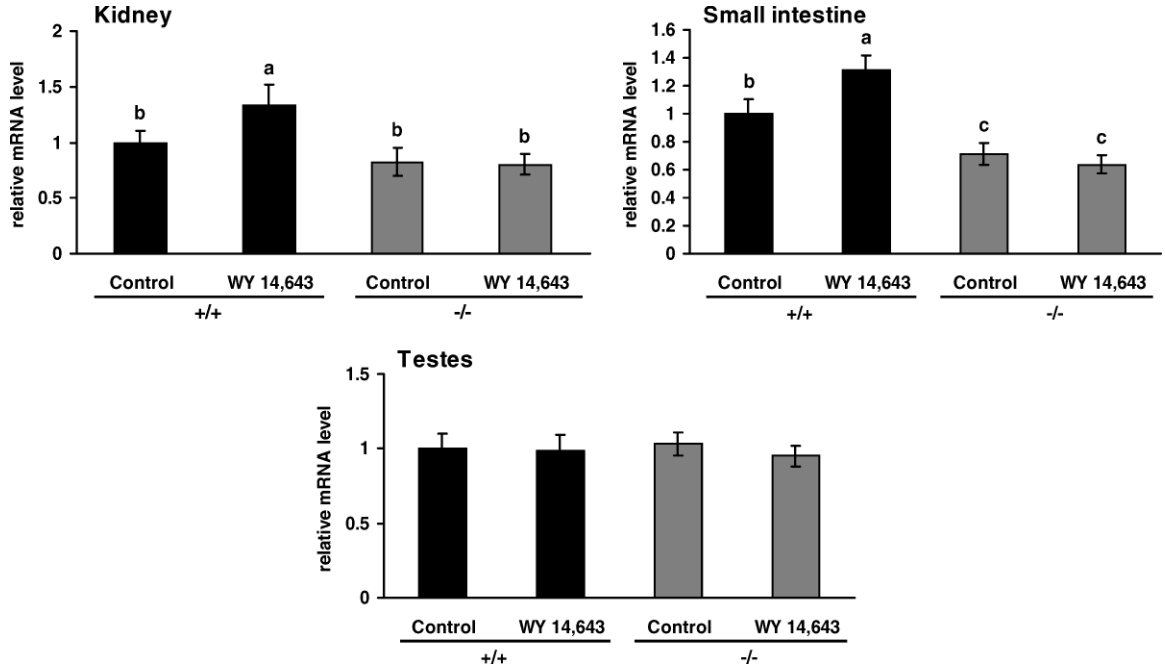

Figure 4. Effect of WY 14,643 on OCTN3 mRNA abundance in the kidney, small intestine, and testes of wild-type (+/+) and PPARα$^{-/-}$ mice. Mice were treated orally with 40 mg/kg of WY 14,643 for 4 days. Control mice received the appropriate volume of vehicle (sunflower oil and DMSO). Total RNA was extracted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

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

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

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

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

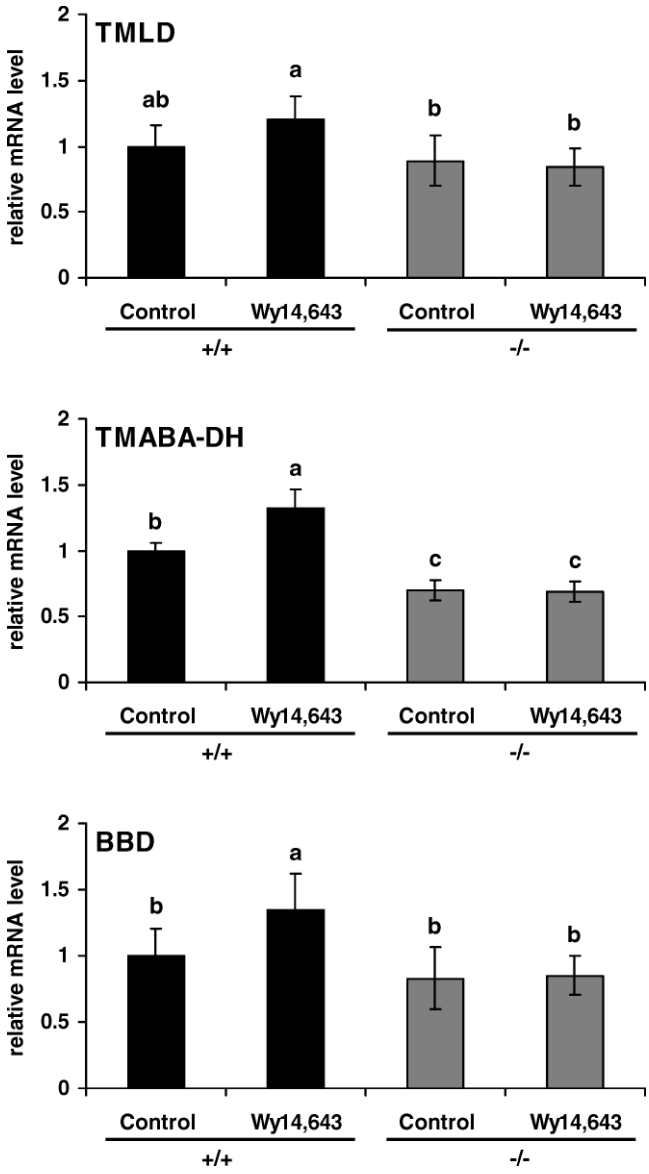

Figure 5. Effect of WY 14,643 on mRNA abundance of enzymes involved in carnitine biosynthesis in the liver of wild-type (+/+) and PPARα$^{-/-}$ mice. Mice were treated orally with 40 mg/kg of WY 14,643 for 4 days. Control mice received the appropriate volume of vehicle (sunflower oil and DMSO). Total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

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

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

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

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

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

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

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

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

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4. Diskussion

PPARα nimmt als Transkriptionsfaktor eine zentrale Stellung im Stoffwechsel ein und ist an der Regulation zahlreicher Stoffwechselprozesse wie dem Lipid- und Lipoprotein-metabolismus, der Glukosehomöostase, der Zellproliferation und inflammatorischer Prozesse beteiligt (Desvergne und Wahli, 1999; Chinetti *et al.*, 2000; Duval *et al.*, 2002; Mandard *et al.*, 2004; Lefebvre *et al.*, 2006; Zandbergen und Plutzky, 2007). Seine wesentlichen physiologischen Funktionen im Lipidstoffwechsel beruhen auf der Regulation der Expression von Enzymen welche in die unterschiedlichsten Fettstoffwechselwege, wie die zelluläre Aufnahme und Aktivierung von Fettsäuren, sowie die mitochondriale und peroxisomale β-Oxidation, die Ketogenese, die mikrosomale ω-Oxidation und den Lipoproteinmetabolimus involviert sind (Lee *et al.*, 2003; Mandard *et al.*, 2004; Reddy und Sambasiva Rao, 2006). Neben den in der wissenschaftlichen Literatur bekannten PPARα-abhängigen Regulations-mechanismen standen in dieser Arbeit neue Aspekte im Bezug auf die Funktion des PPARα im Hinblick auf die Regulation der Cholesterolsynthese und der Carnitinverteilung im Körper im Mittelpunkt. Sowohl Cholesterol als auch Carnitin besitzen zahlreiche physiologische Funktionen und sind von essentieller Bedeutung für eine normale Funktion des Körpers. Gleichzeitig unterliegt die Regulation der Konzentration beider Substanzen einem komplexen Mechanismus, welcher durch das Zusammenspiel verschiedener Faktoren gekennzeichnet ist.
Cholesterol und seine Stoffwechselprodukte haben eine große Bedeutung für das Wachstum und die Lebensfähigkeit der Zelle. Weiterhin dient Cholesterol dem Körper als Ausgangs-produkt für die verschiedensten Steroidhormone und Gallensäuren und ist zugleich wichtiger Bestandteil der Zellmembran. Eine Störung des Cholesterolmetabolismus führt zu verschiedenen Erkrankungen (Maxfield und Tabas, 2005). Eine durch angeborene genetische Störungen beziehungsweise durch falsche Ernährung induzierte Hypercholesterolämie ist zum Beispiel ein Hauptrisikofaktor für die Entstehung von arteriosklerotischen Herzerkrankungen (Chang *et al.*, 2006). Eine reguläre Synthese, sowie ein normal funktionierender Stoffwechsel des Cholesterols spielen daher für den Körper eine wichtige Rolle. Der Steuerung des Cholesterolstoffwechsels kommt also eine zentrale Rolle bei der Aufrechterhaltung normaler Körperfunktionen zu. Der Cholesterolmetabolismus wird in der Leberzelle, als dem Hauptort der Cholesterolsynthese, durch das komplizierte Zusammenspiel verschiedener Faktoren kontrolliert. Die Regulation der Cholesterolkonzentration erfolgt dabei durch einen *feedback* Mechanismus, welcher durch die Insigs, SCAP und des SREBP-2 reguliert wird (McPherson

und Gauthier, 2004; Goldstein *et al.*, 2006). SREBP-2 selbst steuert dabei die Transkription verschiedener Gene, welche in die Cholesterolsynthese beziehungsweise die -aufnahme involviert sind.

Carnitin ist eine wasserlösliche Substanz, die bereits im Jahr 1905 entdeckt wurde (Gulewitsch und Krimberg, 1905). Seither konnten für Carnitin wichtige Funktionen für einen normalen Intermediärstoffwechsel sowie in der zellulären Physiologie nachgewiesen werden. Alle Gewebe die Fettsäuren zur Energiegewinnung nutzen benötigen Carnitin. Die zentrale Rolle des Carnitins für den Energiestoffwechsel besteht darin, dass es durch seine Beteiligung am Transport von aktivierten Fettsäuren in die Mitochondrien deren Abbau über die β-Oxidation ermöglicht (McGarry und Brown, 1997; Rebouche und Seim, 1998; Brass, 2002; Steiber *et al.*, 2004). Über 75% der Gesamtkonzentration an Carnitin im Körper wird durch die Nahrung zugeführt (Steiber *et al.*, 2004), wobei die Aufnahme über die Nahrung mit der Plasmakonzentration positiv korreliert (Lennon *et al.*, 1986). Während weniger als 1% des Carnitinpools im Plasma vorliegt (Bertoli *et al.*, 1981; Moorthy *et al.*, 1983; Angelini *et al.*, 1992), findet man über 90% der Gesamtkonzentration an Carnitin des Körpers im Skelettmuskel, welcher als Carnitinspeicher fungiert (Scholte und De Jonge, 1987; Rebouche, 1992; Brass, 2002). In den meisten anderen Geweben des Körpers ist die Carnitin-konzentration im Verhältnis zur Plasmakonzentration ebenfalls wesentlich höher und wird durch einen steten Transport des Carnitins aufrechterhalten (Evans und Fornasini, 2003). Die ausgewogene Homöostase des Carnitins in verschiedenen Geweben des Körpers als auch intrazellulär ist von großer Bedeutung für die Aufrechterhaltung einer normalen Körper-funktion und wird durch Membrantransporter wie die OCTNs gesteuert (Koepsell *et al.*, 2007). Die Entstehung eines primären und sekundären systemischen Carnitinmangels durch einen angeborenen oder erworbenen Defekt dieser Transporter verdeutlicht nochmals deren Bedeutung für eine reguläre Carnitinhomöostase (Nezu *et al.*, 1999). Zahlreiche Untersuchungen deuten darauf hin, dass PPARα eine zentrale Stellung in der Regulation der Cholesterol- beziehungsweise Carnitinkonzentration einnehmen könnte.

Wirkung einer PPARα-Aktivierung auf den Cholesterolmetabolismus

In verschiedenen Studien wurde gezeigt, dass eine Behandlung von Ratten zu einer Verringerung der Cholesterolkonzentrationen in der Leber, dem Plasma und den Lipoproteinen der Tiere führte (Sülzle *et al.*, 2004; Eder *et al.*, 2003a; Eder und Kirchgessner, 1998). Ähnliche Effekte konnten auch bei Miniaturschweinen beobachtet werden, bei denen die Gabe von oxidiertem Fett zu einer Abnahme der Cholesterolkonzentrationen im Plasma,

den LDL und HDL führte (Eder und Stangl, 2000). Ebenso sind Fibrate für ihre cholesterolsenkenden Effekte in der wissenschaftlichen Literatur bekannt (Sirtori *et al*., 1993; Elisaf, 2002). Mechanistische Untersuchungen dieser Effekte im Zusammenhang mit einer Aktivierung des PPARα fehlen bislang. Ein Ziel dieser Arbeit war es daher, zu untersuchen inwieweit synthetische PPARα-Liganden, wie Clofibrat und WY 14,643, den Cholesterol-stoffwechsel vor allem über eine vermehrte Expression der Insigs in Rattenleberzellen beeinflussen. Im Ergebnis konnte im Rahmen dieser Arbeit nicht nur eine Aktivierung des PPARα, sondern ebenso eine erhöhte mRNA-Konzentration des Insig-1 in Kombination mit einer verringerten Menge an nukleären SREBP-2 festgestellt werden (***Vers. 1***). Gleichzeitig führte die Behandlung der Ratten mit Clofibrat im Vergleich zur Kontrollgruppe zu einer Verringerung der Cholesterolkonzentrationen in der Leber, dem Plasma und den Lipoproteinen der Tiere. In den Fao-Zellen gelang es, parallel zur Aktivierung des PPARα nach der Inkubation mit WY 14,643, auch eine erhöhte mRNA-Konzentration von Insig-1 und -2a festzustellen. Ferner konnte nach der Inkubation der Fao-Zellen mit WY 14,643 eine Verringerung der Cholesterolsynthese festgestellt werden (***Vers. 1***). Insgesamt liefern diese *in vivo* und *in vitro* Daten völlig neuartige und wichtige Hinweise auf einen denkbaren Mechanismus bei dem eine PPARα-Aktivierung über eine erhöhte Expression der Insigs, insbesondere des Insig-1, und der möglicherweise daraus resultierenden Verringerung der SREBP-2-Reifung zu einer Abnahme der Menge an nukleären SREBP-2 führt. Dies führt wahrscheinlich in der weiteren Folge zu einer verminderten Expression der Zielgene HMGCoA-R und LDL-Rezeptor und so zu einer verringerten Cholesterolsynthese beziehungsweise -aufnahme. Interessanter Weise konnten diese Effekte sowohl nach der Gabe von Clofibrat in der Rattenleber als auch nach der Inkubation der Fao-Zellen mit WY 14,643 festgestellt werden, welches im Vergleich zu Clofibrat ein wesentlich stärkerer und selektiverer PPARα-Aktivator ist (Krey *et al.*, 1997; Forman *et al.*, 1997) und in zahlreichen *in vitro* Studien zur Untersuchung PPARα-abhängiger Effekte eingesetzt wird (Ammerschlaeger *et al.*, 2004; Lee *et al.*, 2004; Vanden Heuvel *et al.*, 2003; Fiedler *et al.*, 2001). Die Tatsache das zwei verschiedene PPARα-Liganden in zwei unterschiedlichen Systemen ähnliche Effekte im Hinblick auf die Insig-vermittelte Regulation der SREBP-2-Prozessierung liefern, stützt die Annahme, dass PPARα in die Regulation dieses Mechanismus involviert ist. Gestützt wird diese These durch weitere Studien in denen die Fütterung von Clofibrat beziehungsweise Fenofibrat zu einer Abnahme der Serumcholesterol-konzentration (Berndt *et al.*, 1978) und zu einer SREBP-2-vermittelten verringerten Expression der HMGCoA-R und des LDL-Rezeptors (Guo *et al.*, 2001) führte. In anderen

Untersuchungen, welche sich mit der Wirkung von Clofibrat beschäftigen, konnte ebenfalls sowohl in Ratten als auch in Hepatozytenzellen eine verringerte Cholesterolsynthese in Kombination mit einer verminderten Aktivität der HMGCoA-R beobachtet werden (Berndt *et al.*, 1978; Hayashi und Takahata, 1991; Hashimoto *et al.,* 1998, 2000; Shiota *et al.*, 2003). Im Bezug auf einen möglichen Einfluss des PPARα auf diesen Stoffwechselweg muss man allerdings bedenken, das in der wissenschaftlichen Literatur auch gegensätzliche Studien existieren, in welchen verschiedene PPARα-Agonisten zu gegenteiligen Effekten auf die Cholesterolsynthese und die Expression der HMGCoA-R führen (Roglans *et al.*, 2001; Shiota *et al.*, 2003; Le Jossic-Corcos *et al.*, 2004; Knight *et al.*, 2005). Neben synthetischen Liganden wird PPARα auch durch zahlreiche natürliche Liganden aktiviert (Yu *et al.*, 1995; Forman *et al.*, 1997; Krey *et al.*, 1997; Kliewer *et al.*, 1997; Xu *et al.*, 1999; Kersten *et al.*, 1999; Delerive *et al.*, 2000; Pégorier *et al.*, 2004; Marx *et al.*, 2004; Mishra *et al*., 2004; König und Eder 2006). Die Aktivierung durch Fettsäuren, welche zum Beispiel beim Fasten vermehrt freigesetzt werden, stellt einen zentralen Punkt der physiologischen Funktion des PPARα dar. Nach erfolgter Aktivierung stellt PPARα über eine Induktion der β-Oxidation durch eine vermehrte Expression verschiedener Schlüsselgene die Energieversorgung des Körpers sicher und reguliert so die *fasting response* des Körpers. Die PPARα-abhängige Regulation der Expression von Enzymen der β-Oxidation konnte auch nach der Gabe von oxidiertem Fett beobachtet werden (Sülzle *et al*., 2004; Chao *et al*., 2004). Im Rahmen dieser Arbeit stellte sich die Frage, inwieweit oxidiertes Fett darüber hinaus auch den Cholesterolstoffwechsel in ähnlicher Weise wie Clofibrat beziehungsweise WY 14,643 beeinflusst. Bei den in ***Vers. 2a*** durchgeführten Untersuchungen mit oxidiertem Fett, als natürlicher Aktivator des PPARα, konnte ebenfalls eine erhöhte mRNA-Konzentration von Insig-1 und -2a in der Leber der Tiere festgestellt werden. Parallel konnte, ebenso analog zu ***Vers. 1***, auch eine Verringerung der Menge an nukleären SREBP-2 in der Leber der mit oxidiertem Fett behandelten Ratten festgestellt werden. Diese Abnahme beruht in beiden Versuchen vermutlich auf dem Insig-vermittelten Zurückhalten des SREBP-SCAP-Komplexes im endoplasmatischen Retikulum und der daraus resultierenden Abnahme der proteolytischen Aktivierung des SREBP-2. In der weiteren Folge führt die Verringerung der Menge an nukleären SREBP-2 zu einer verminderten Expression der HMGCoA-R, dem reaktionsgeschwindigkeitsbestimmenden Enzym der Cholesterolsynthese, und des LDL-Rezeptors, wie sie in beiden Studien nachgewiesen werden konnte. Die beobachteten verringerten Cholesterolkonzentrationen in der Leber und im Plasma der Tiere, welche mit dem oxidierten Fett beziehungsweise Clofibrat behandelt wurden, sind dann wahrscheinlich

das Ergebnis einer verringerten Synthese. Darüber hinaus kommt für die verminderten Cholesterolkonzentrationen in der Leber auch eine verringerte Aufnahme von LDL in die Leberzellen in Betracht. Das eingesetzte Fett wurde über einen relativ langen Zeitraum bei niedriger Temperatur oxidiert und zeichnet sich durch einen hohen Gehalt an primären Lipidperoxidationsprodukten aus, welche aufgrund ihrer Instabilität bei zu hohen Temperaturen in sekundäre Produkte zerfallen würden (Kubow, 1992). Diese primären Oxidationsprodukte, wie sie indirekt über eine hohe Peroxidzahl und eine hohe Konzentration an konjugierten Dienen im Versuchsfett nachgewiesen werden konnten, gelten in der wissenschaftlichen Literatur als potente PPARα-Aktivatoren (Delerive *et al.*, 2000; Mishra *et al.*, 2004; König und Eder, 2006). Man muss jedoch beachten, dass verschiedene Hydroxy- und Hydroperoxy-Fettsäuren als Bestandteile oxidierter Fette ebenfalls an PPARγ binden und diesen aktivieren können (Krey *et al.*, 1997; Nagy *et al.*, 1998). Aufgrund dieser Tatsache kann man nicht völlig ausschließen, dass die hier beobachteten Effekte eine Aktivierung des PPARγ beruhen. Untersuchungen unserer Arbeitsgruppe ergaben zudem, dass die Inkubation der humanen Hepatozyten-Zelllinie HepG2 ebenfalls zu einer Verringerung der Menge an nukleären SREBP-2 und einer Abnahme der Cholesterolsynthese führte (Klopotek *et al.*, 2006). Man muss allerdings zugleich bedenken, dass PPARγ im Vergleich zu PPARα nur in sehr geringem Umfang in der Leber exprimiert wird (Escher *et al.*, 2001). Untersuchungen von Kast-Woelbern *et al.* (2004) zeigten erstmals einen Zusammenhang zwischen einer PPAR-Aktivierung und der Insig-abhängigen Beeinflussung der SREBP-Prozessierung. Dabei gelang es nach Aktivierung mittels Rosiglitazon eine direkte Bindung des PPARγ über ein PPRE in der Promotorregion des Insig-1 nachzuweisen. Die Tatsache, dass durch Kast-Woelbern *et al*. (2004) ein PPRE in der Promotorregion des Insig-1 nachgewiesen werden konnte, lässt spekulieren, dass die Regulation der Insig-Expression unmittelbar über PPARα gesteuert wird. Untermauert wird dieser potentielle Zusammenhang dadurch, dass parallel mit den erhöhten mRNA-Konzentrationen der Insigs im Rahmen der vorliegenden Arbeit auch eine Aktivierung des PPARα nach Inkubation der Fao-Zellen mit WY 14,643 beziehungsweise nach der Behandlung der Ratten mit Clofibrat (***Vers. 1***) nachgewiesen werden konnte. In verschiedenen Studien konnte bei Schweinen nach der Gabe des PPARα-Liganden Clofibrat zwar indirekt eine schwache Aktivierung des PPARα über die leicht erhöhte Expression verschiedener Zielgene festgestellt werden (Cheon *et al*., 2005; Luci *et al*., 2007a). Gleichzeitig führte bei Untersuchungen in unserer Arbeitsgruppe die Gabe von Clofibrat aber zu keiner Veränderung der Expression von Insig-1, -2a sowie SREBP-2 und dessen Zielgene HMGCoA-R und LDL-Rezeptor in der Leber der Tiere (Luci *et al.*, 2007a).

Ein Grund für diese Unterschiede liegt vermutlich darin, dass das Schwein genau wie der Mensch, im Unterschied zur Ratte ein so genannter *„non-proliferator“* ist (Yu *et al.*, 2001; Pfeffer *et al.*, 2005) und verschiedene PPARα-Zielgene Gene mit einer wesentlich geringen Intensität auf eine PPARα-Aktivierung reagieren (Eacho *et al.*, 1986). Diese verringerte Expression konnte in verschiedenen Studien nach der Aktivierung des PPARα durch Liganden in der Leber von Schweinen und Menschen im Vergleich zur Ratte nachgewiesen werden (Holden und Tugwood, 1999; Lawrence *et al.*, 2001). Weiterhin wird PPARα in der Leber von Ratten im Vergleich zu Schweinen wesentlich stärker exprimiert (Luci *et al.*, 2007a) und führt nach erfolgter Aktivierung nicht nur zu einer Induktion zahlreicher Zielgene, sondern auch zu einer Peroxisomenproliferation (Peters *et al.*, 2005). Im Rahmen der vorliegenden Arbeit konnte bei den Ratten sowohl eine PPARα-Aktivierung nach der Gabe des Clofibrates als auch nach der Behandlung mit oxidiertem Fett beobachtet werden. In Übereinstimmung mit anderen Studien gelang dies auch nach einer relativ kurzen Versuchsdauer (Ringseis *et al.*, 2007a). Dabei wurde die Aktivierung in beiden Untersuchungen indirekt über verschiedene Marker nachgewiesen. In beiden Versuchen (***Vers. 1*** und ***2a/b***) konnte eine Zunahme der durchschnittlichen relativen Lebergewichte der mit Clofibrat beziehungsweise oxidiertem Fett behandelten Ratten festgestellt werden, welche auf eine PPARα-Aktivierung zurückzuführen ist. Im Zusammenhang mit einer Liganden-induzierten PPARα-Aktivierung kommt es zu einer Peroxisomenproliferation, in deren Folge die Hyperplasie und Hypertrophie der Hepatozyten zu einer Erhöhung der relativen Lebergewichte führen (Rao und Reddy, 1987; Takagi *et al.*, 1992; Bentley *et al.*, 1993; Lee *et al.*, 1995; Baker *et al.*, 2004). Weiterhin führte die orale Gabe von Clofibrat beziehungsweise des oxidierten Fettes im Ergebnis zu einer drastischen Erhöhung der relativen mRNA-Konzentrationen der ACO und des Cyp4A1. Ähnliche Effekte konnten auch nach der Inkubation der Fao-Zellen mit WY 14,643 festgestellt werden. ACO und Cyp4A1 sind typische PPARα-Zielgene, welche in der wissenschaftlichen Literatur über den Anstieg ihrer mRNA-Konzentrationen als Marker für eine PPARα-Aktivierung etabliert sind (Zhou *et al.*, 1998; Desvergne und Wahli, 1999; Chao *et al.*, 2001; König und Eder, 2006). Beide Enzyme sind in die peroxisomale β-Oxidation (ACO) beziehungsweise in die mikrosomale ω-Hydroxylierung (Cyp4A1) und damit in den Fettsäurekatabolismus involviert (Aldrige *et al.*, 1995; Varanasi *et al.*, 1998; Staels *et al.*, 1998). Als Folge der PPARα-induzierten Erhöhung der Expression dieser lipolytischer Enzyme konnte auch eine verminderte Konzentration an Triglyzeriden in der Leber, im Plasma und in den VLDL festgestellt werden (***Vers. 1***). Auch die Inkubation der Fao-Zellen mit WY 14,643 führte zu einer Verminderung der

Konzentration an intrazellulären und sekretierten Triglyzeriden, welche ebenfalls auf eine PPARα-Aktivierung schließen lassen. Die Beobachtungen aus den *in vivo* Versuchen beruhen aber auch auf weiteren hypolipidämischen Effekten einer PPARα-Aktivierung, wie der Hydrolyse von Plasmatriglyzeriden oder der Stimulation der zellulären Fettsäureaufnahme (Steals *et al.*, 1998). Der wesentlichste Teil des Fettsäurekatabolismus und damit vermutlich der Hauptgrund für die verringerten Triglyzeridkonzentrationen verläuft allerdings über die mitochondriale β-Oxidation.

Wirkung einer PPARα-Aktivierung auf den Carnitinmetabolismus

Der reaktionsgeschwindigkeitsbestimmende Schritt der mitochondrialen β-Oxidation wird über die Funktion der CPTs gesteuert, welche aktivierte Fettsäuren vom Zytosol in die Mitochondrienmatrix transportieren und diese so der β-Oxidation zur Verfügung stellen. CPT-1 und CPT-2 wurden bereits in früheren Studien als PPARα-Zielgene identifiziert (Brandt *et al.*, 1998; Mascaro *et al.*, 1998; Hashimoto *et al.*, 1999). Analog zu den Ergebnissen von Luci *et al.* (2006) konnte auch nach der Behandlung der Ratten mit oxidiertem Fett eine erhöhte mRNA-Konzentration der CPTs in der Leber und auch im Darm der Tiere nachgewiesen werden (***Vers. 2b***). Beide CPTs benötigen als Co-Faktor Carnitin. Eine vermehrte Expression dieser Enzyme als direkte Folge einer PPARα-Aktivierung sollte gleichzeitig den Bedarf an Carnitin steigern. Verschiedene Voruntersuchungen zeigten, dass eine durch Fibrate beziehungsweise Fasten induzierte PPARα-Akivierung zu einer Erhöhung der Carnitinkonzentrationen in der Leber führte (Paul und Adibi, 1979; McGarry *et al.*, 1975; Brass und Hoppel, 1978; Paul *et al.* 1986; Slitt *et al.*, 2002). Diese Erhöhung kann entweder das Resultat einer vermehrten Synthese oder einer erhöhten Aufnahme von Carnitin in die Leber sein. In Untersuchungen unserer Arbeitsgruppe konnte nach einer Gabe von Clofibrat parallel mit einer nachgewiesenen PPARα-Aktivierung erstmals eine Erhöhung der mRNA-Konzentrationen von OCTN1 und OCTN2 in der Leber der Ratten nachgewiesen werden (Luci *et al*., 2006). Gleichzeitig konnte in dieser Studie keine Veränderung der mRNA-Konzentration von Genen festgestellt werden, welche für Enzyme der Carnitinsynthese wie die Trimethyllysin Dioxygenase (TMLD), 4-*N*-Trimethylaminobutyraldehyd-Dehydrogenase (TMABA-DH) und die γ-Butyrobetain Dioxygenase (γ-BBD) kodieren. Luci *et al*. (2006) spekuliert aufgrund der Daten dieser Untersuchung, dass die ebenfalls in dieser Studie beobachteten erhöhten Carnitinkonzentrationen in der Leber der Ratten, welche im Vergleich zur Kontrollgruppe mit Clofibrat behandelt wurden, durch eine vermehrte Aufnahme des Carnitins über die OCTNs aus dem Plasma in die Leberzellen und nicht durch eine vermehrte

Synthese zu erklären sind. Im zweiten Teil dieser Arbeit (***Vers. 2b*** und ***3***) wurde nun untersucht, inwieweit eine Aktivierung des PPARα durch oxidiertes Fett die relative mRNA-Konzentration der OCTNs und somit den durch die OCTNs vermittelten Carnitintransport beeinflusst. Parallel dazu wurde auch der Einfluss einer durch oxidiertes Fett induzierte PPARα-Aktivierung auf die Expression der an der Carnitinsynthese beteiligten Gene untersucht. Aufgrund der zunehmenden Bedeutung oxidierter Fette in der Ernährung von Menschen aus den westlichen Industrieländern (Kubow, 1992; Cohn, 2002) und ihrem bekannten Potential als PPARα-Aktivatoren (Sülzle *et al*., 2004), stellte sich die Frage, inwieweit ein oxidiertes Fett die Expression des OCTN2 und weiterer Gene analog zu den Ergebnisse von Luci *et al*. (2006) beeinflusst. Zur weiteren Bewertung des direkten Einflusses einer PPARα-Aktivierung auf die Expression dieser Gene, wurde auch ein Versuch mit PPARα-*knockout*-Mäusen durchgeführt. Aufgrund der hohen Affinität gegenüber Carnitin und der weiten Verbreitung in verschiedenen Geweben ist OCTN2 der physiologisch bedeutendste Vertreter der Carnitintransporter. Der Schwerpunkt dieser Untersuchungen lag daher, neben der Wirkung einer PPARα-Aktivierung auf die Expression weiterer Transporter und Gene der Carnitinsynthese, vor allem auf dem Effekt einer solchen Aktivierung auf der Expression des OCTN2. Die Behandlung der Ratten mit dem oxidierten Fett führte parallel zur Aktivierung des PPARα auch zu einem Anstieg der mRNA-Konzentration des OCTN1 und OCTN2 in der Leber der Tiere. Ebenso konnte eine verminderte Carnitinkonzentration in der Leber der Ratten, welche mit oxidiertem Fett gefüttert wurden im Vergleich zur Kontrollgruppe festgestellt werden. Diese Daten decken sich mit den Ergebnissen von Luci *et al*. (2006), in dem sowohl die Gabe von Clofibrat, als auch die Inkubation von Fao-Zellen mit WY 14,643 zu einem Anstieg der mRNA-Konzentrationen des OCTN2 führte. Man kann daher vermuten, dass oxidiertes Fett die OCTN-Expression ebenfalls über eine Aktivierung des PPARα steuert. Ein weiteres interessantes Ziel dieser Untersuchung bestand in der Aufklärung der Wirkung einer PPARα-Aktivierung auf die OCTN-Expression im Darm. Neben PPARα werden OCTN1 und OCTN2 auch im Dünndarm stark exprimiert, wo besonders OCTN2 eine wichtige Rolle bei der intestinalen Absorption des Carnitins aus der Nahrung spielt (Taylor, 2001; Slitt *et al*., 2002; Duran *et al*., 2002; Elimrani *et al.*, 2003; Kato *et al.*, 2006). In der vorliegenden Arbeit konnte eine im Fall des OCTN2 signifikante und bei OCTN1 tendenzielle Erhöhung der relativen mRNA-Konzentrationen im Dünndarm der mit oxidiertem Fett behandelten Ratten im Vergleich zur Kontrollgruppe festgestellt werden. Parallel mit der über die erhöhte Expression der *downstream* Gene festgestellten Aktivierung des PPARα im Darm, kann man davon ausgehen, dass diese erhöhte Expression ebenfalls

über PPARα gesteuert wird. Diese These wird durch eine weitere Untersuchung erhärtet. Ringseis *et al*. (2007c) konnte zeigen, dass auch die Gabe von Clofibrat an Ratten zu einer signifikant erhöhten mRNA-Konzentration des OCTN2 im Duodenum und Jejunum der Tiere führte. OCTN2 wird auch im Dünndarm beziehungsweise im Kolon des Menschen hoch exprimiert (Kim *et al.*, 2007; Meier *et al*., 2007). Darüber hinaus sind OCTNs polyspezifisch und neben Carnitin ebenfalls in der Lage verschiedene Medikamente zu binden und so die Absorption dieser Substanzen zu steuern (Wu *et al.*, 1999; Koepsell und Endou, 2004; Lahjouji *et al.*, 2004; Grube *et al*., 2006; Hirano *et al*., 2006; Kato *et al*., 2006; Srinivas *et al*., 2007). Auch in Mäusen konnte durch Hirai *et al*. (2007) eine positive Regulation der Expression von OCTN2 nach der Behandlung mit WY 14,643 festgestellt werden. Im Rahmen der vorliegenden Arbeit wurden ebenfalls Untersuchungen mit Mäusen durchgeführt. Ziel war es hierbei zu untersuchen, ob die in den Voruntersuchungen von Luci *et al*. (2006) beziehungsweise auch in dieser Arbeit beobachteten erhöhten mRNA-Konzentrationen von OCTN2 in Folge einer Behandlung mit PPARα-Aktivatoren im PPARα-*knockout*-Modell nicht mehr nachzuweisen sind. Mit Hilfe dieses Modells sollte dadurch eine direkte Beeinflussung der Expression über PPARα nachgewiesen werden. Durch eine vermehrte Expression der ACO, deren Expression als bekanntes PPARα-Zielgen über ein PPRE reguliert wird (Tugwood *et al*., 1992), nach der Behandlung der Wildtyp-Mäuse mit WY 14,643 konnte indirekt eine PPARα-Aktivierung nachgewiesen werden. Im Ergebnis konnte in dieser Studie (***Vers. 3***) auch ein Anstieg der OCTN2 mRNA-Konzentrationen in verschiedenen Geweben, unter anderem in der Leber und dem Dünndarm, der Wildtyp-Mäuse nach der Behandlung mit WY 14,643 festgestellt werden. Gleichzeitig konnte dieser Effekt in den PPARα-*knockout*-Mäusen nicht mehr nachgewiesen werden. Ebenso konnten in den PPARα-*knockout*-Mäusen im Gegensatz zu den Wildtyp-Mäusen keine Veränderungen in den Carnitinkonzentration beobachtet werden. Insgesamt liefern die Daten dieser Studie interessante Ergebnisse im Bezug auf die PPARα-abhängige Regulation der Expression des OCTN2. In einer jüngst veröffentlichten Studie konnte van Flies *et al*. (2007) ebenfalls zeigen, dass die Behandlung mit WY 14,643 bei PPARα-*knockout*-Mäusen keinen Effekt auf die OCTN2 mRNA-Konzentrationen in der Leber der Tiere hatte. Interessanter Weise führte die Behandlung der PPARα-*knockout*-Mäusen mit WY 14,643 im Rahmen der vorliegenden Arbeit auch zu einer offensichtlich PPARα-abhängigen Erhöhung der mRNA-Konzentrationen verschiedener Gene der Carnitinsynthese. Während die Behandlung der Wildtyp-Mäuse mit WY 14,643 zu einer Erhöhung der mRNA-Konzentrationen dieser Gene führte, konnten bei den PPARα-*knockout*-Mäusen keine Effekte beobachtet werden. Die

genaue Ursache für die in der Leber festgestellten erhöhten Carnitinkonzentrationen lässt sich anhand der Ergebnisse von ***Vers. 3*** nicht eindeutig klären. In weiteren Untersuchungen unserer Arbeitsgruppe konnte durch Luci *et al*. (2006) keine Beeinflussung der mRNA-Konzentrationen dieser Gene nach der Behandlung von Ratten mit Clofibrat festgestellt werden. Dies deckt sich mit den Ergebnissen dieser Arbeit, wonach die Gabe von oxidiertem Fett ebenfalls keine Veränderungen der mRNA-Konzentrationen von Genen der Carnitinsynthese hervorruft (***Vers. 2b***). Dagegen konnte in einer weiteren Studie unserer Gruppe (Ringseis *et al.*, 2007c) im Gegensatz zur vorliegenden Arbeit kürzlich eine erhöhte mRNA-Konzentration der Synthesegene TMLD und TMABA-DH, welche den ersten Schritt der Carnitinsynthese vom Trimethyllysin zum γ-BB steuern, in der Leber von mit Clofibrat behandelten Ratten feststellen. Die mRNA-Konzentration der γ-BBD blieb dagegen unbeeinflusst (Ringseis *et al.*, 2007a). In einer weiteren aktuellen Studie an Mäusen, führte sowohl die Behandlung mit WY 14,643 als auch das Fasten der Mäuse zu einer Erhöhung der Aktivität der γ-BBD, dem entscheidenden Enzym der Carnitinbiosynthese, in der Leber der Tiere (van Vlies *et al.*, 2007). Im Hinblick auf den Einfluss des PPARα auf die Regulation der Expression der Gene der Carnitinsynthese scheint es dringend notwendig zu sein, aufgrund der kontroversen Datenlage weitere funktionelle Untersuchungen durchzuführen.

Schlussfolgerungen und Ausblick

Insgesamt liefern die Daten dieser Arbeit völlig neue Erkenntnisse im Bezug auf den regulatorischen Einfluss des PPARα auf die Expression der Insigs und OCTNs und die damit verbundene Steuerung des Cholesterolstoffwechsels und der Carnitinverteilung. Die Ergebnisse aus ***Vers. 1*** und ***2a*** zeigen einen möglichen neuen Zusammenhang zwischen einer Aktivierung des PPARα durch synthetische und natürliche Liganden und der Verringerung der Cholesterolkonzentrationen über eine vermehrte Expression von Insig-1 und -2a. Diese Daten legen zum jetzigen Zeitpunkt den Schluss nahe, dass die Regulation der Insig-Expression über PPARα reguliert wird und liefern eine mögliche Erklärung für die aus Voruntersuchungen bekannten cholesterolsenkenden Effekte verschiedener PPARα-Liganden (Sülzle *et al*., 2004; Eder *et al.*, 2003a; Elisaf, 2002; Eder und Stangl, 2000; Eder und Kirchgessner, 1998; Sirtori *et al*., 1993). Da zahlreiche Bestandteile eines oxidierten Fettes auch an PPARγ binden und diesen aktivieren können, wären zukünftige Untersuchungen mit PPARα-*knockout*-Mäusen sinnvoll, um den PPARα-abhängigen Anteil an den bei den Ratten beobachteten Veränderungen nach der Gabe des oxidierten Fettes abschätzen zu können. Weiterhin bleibt zu klären, ob die beobachteten Zusammenhänge zwischen einer durch

Fibrate oder oxidiertes Fett induzierten PPARα-Aktivierung und der vermehrten Expression der Insigs direkt über ein funktionelles PPRE in der Promotorregion gesteuert werden. Daneben wären weitere Studien mit PPARα-*knockout*-Mäusen im Bezug auf die Wirkung synthetischer Liganden auf die Regulation der Cholesterolkonzentration von großer Bedeutung zur weiteren Bewertung des Einflusses des PPARα auf diesen Stoffwechselweg. Die Daten aus ***Vers. 2b*** liefern in Übereinstimmung mit anderen Untersuchungen (Luci *et al*., 2006) bezüglich der Expression von OCTN1 und vor allem OCTN2 wichtige Hinweise zum Einfluss einer PPARα-Aktivierung auf die Expression dieser Gene in der Leber von Ratten. Diese Indizien konnten durch den Versuch mit PPARα-*knockout*-Mäusen (***Vers. 3***) untermauert werden, indem sehr anschaulich belegt werden konnte, dass die Gabe von WY 14,643 die Expression des OCTN2 in der Leber dieser Mäuse nicht verändert. Dadurch gelang es einen eindeutigen Zusammenhang zwischen der Aktivierung des PPARα und der Expression des OCTN2 zu zeigen. Gleichzeitig konnte in diesem Versuch eine vermehrte Expression des OCTN2 im Dünndarm der Wildtyp-Mäuse beobachtet werden. Auch nach der Behandlung der Ratten mit oxidiertem Fett konnte ein positiver Effekt auf die mRNA-Konzentrationen des OCTN2 im Darm der Tiere beobachtet werden. Möglicherweise ist eine Aktivierung des PPARα durch synthetische und natürliche Liganden im Darm und eine wahrscheinlich daraus resultierende vermehrte Expression der OCTNs für eine erhöhte Absorption verschiedener Medikamente aus dem Darmlumen von großer Bedeutung und sollte in weiteren Studien näher untersucht werden. Auch hier würden künftige Untersuchungen zur Wirkung eines oxidierten Fettes als natürlicher PPARα-Aktivator auf die Expression der OCTNs in PPARα-*knockout*-Mäusen wissenschaftlich wertvolle Daten liefern. Zu untersuchen, inwieweit diese an Ratten und Mäusen beobachteten Effekte auf den Cholesterol- und Carnitinstoffwechsel auch im Hinblick auf die Spezies-spezifischen Unterschiede im Bezug auf PPARα-abhängige Mechanismen auch für den Menschen zutreffen, bleibt weiteren Studien vorbehalten.

5. Zusammenfassung

Cholesterol und seine Metabolite sind für das Wachstum und die Lebensfähigkeit der Zelle von großer Bedeutung. Eine Störung des Cholesterolmetabolismus führt zu verschiedenen Erkrankungen. Daher sind eine reguläre Synthese und ein funktionierender Stoffwechsel des Cholesterols für die normale Funktion der Zelle unerlässlich. Carnitin ist ein multifunktionaler Bestandteil der Zelle und spielt eine Schlüsselrolle im Transport von aktivierten langkettigen Fettsäuren vom Zytosol in die Mitochondrienmatrix, wo die β-Oxidation stattfindet. Jedes Gewebe, welches Fettsäuren als Hauptenergiequelle nutzt, ist auf Carnitin angewiesen. Darüber hinaus ist eine intakte Carnitinhomöostase von großer Bedeutung für Aufrechterhaltung der normalen Körperfunktionen. In verschieden Studien konnte gezeigt werden, dass unterschiedliche PPARα-Liganden die Konzentration an Cholesterol und Carnitin in der Leber von Ratten und Menschen beeinflussen. Die Behandlung von Ratten mit verschiedenen PPARα-Liganden führte zu herabgesetzten Cholesterolkonzentrationen in der Leber, dem Plasma und den Lipoproteinen (VLDL). Die zu Grunde liegenden molekularen Mechanismen sind bislang unbekannt und wurden in dieser Arbeit untersucht. Die zelluläre Cholesterolkonzentration wird durch einen komplexen Mechanismus kontrolliert, an dem Insig, SCAP und SREBP-2 beteiligt sind. Das Zurückhalten des SCAP-SREBP-2-Komplexes im endoplasmatischen Retikulum wird durch die integralen Membranproteine Insig-1 und -2a vermittelt. In einer ersten Studie (***Vers. 1***) wurde der Effekt einer mit Clofibrat und WY 14,643 induzierten PPARα-Aktivierung auf den Cholesterolstoffwechsel in Rattenhepatozyten-Zellen untersucht. Aufgrund der zunehmenden Bedeutung oxidierter Fette in der menschlichen Ernährung in den Industrieländern und deren bekanntem Potential als PPARα-Aktivator, wurden auch die Effekte eines oxidierten Fettes auf die Expression verschiedener Schlüsselgene des Cholesterolmetabolismus untersucht (***Vers. 2a***). In beiden Versuchen konnte eine Aktivierung des PPARα festgestellt werden. Darüber hinaus konnte in der vorliegenden Arbeit erstmals gezeigt werden, dass die Behandlung von Ratten mit einem synthetischen PPARα-Liganden zu einer erhöhten mRNA-Konzentration von Insig-1 in der Leber führte (***Vers. 1***). Weiterhin führte die Inkubation der Rattenhepatozyten-Zelllinie Fao mit WY 14,643 für 6 h ebenfalls zu einem Anstieg der mRNA-Konzentrationen des Insig-1 und -2a. Die gleichen Ergebnisse konnten in der Leber von Ratten auch erstmalig durch die Gabe eines oxidierten Fettes festgestellt werden (***Vers. 2a***). In beiden Versuchen kam es zu einer Verringerung der Menge an nuklearem SREBP-2 in der Leber und der Cholesterol-

konzentrationen in der Leber, den Lipoproteinen und im Plasma. Zusammenfassend deuten die Daten von ***Vers. 1*** und ***2a*** stark darauf hin, dass eine PPARα-Aktivierung die Cholesterolkonzentration über eine Verringerung der Menge an nuklearem SREBP-2, resultierend aus einer erhöhten Expression der Insigs, senkt. Die Ergebnisse dieser Versuche liefern interessante Einblicke in die komplexe Regulation des Cholesterolstoffwechsels und zeigen einen neuen Zusammenhang zwischen einer PPARα-Aktivierung und der Regulation der Regulation der Cholesterolsynthese und -aufnahme. Weiterführende Untersuchungen sollten klären, ob eine Liganden-induzierte Aktivierung des PPARα die Expression des Insig-1 beziehungsweise -2a direkt über ein PPRE in der Promotorregion steuert. Neben diesen neuen Aspekten des Einflusses einer PPARα-Aktivierung auf den Cholesterolstoffwechsel konnte in verschiedenen Versuchen auch gezeigt werden, dass die Behandlung von Ratten mit Fibraten, einer Klasse von hypolipidämischen Medikamenten, zu einer Erhöhung der Carnitinkonzentration in der Leber führt. Wir konnten bereits in zeigen, dass Clofibrat die Expression von OCTN1 und OCTN2 in der Rattenleber steigert. Die Verteilung des Carnitins im Körper und die intrazelluläre Homöostase werden über diese membranständigen Carnitintransporter gesteuert. Das Ziel von ***Vers. 2b*** war es, die Rolle eines oxidierten Fettes als natürlicher PPARα-Aktivator auf die Expression dieser Transporter und weiterer Gene der Carnitinsynthese zu untersuchen. Um die Hypothese zu prüfen, ob die Expression der OCTNs direkt über PPARα vermittelt wird, wurde ein weiterer Versuch mit PPARα-*knockout*-Mäusen durchgeführt (***Vers. 3***). Die vorliegende Arbeit zeigt, dass die Behandlung von Ratten mit oxidiertem Fett zu einer starken Erhöhung der OCTN2-Expression in der Leber führte. Außerdem deuten die Daten aus ***Vers. 3*** stark darauf hin, dass die Expression des OCTN2 direkt durch PPARα gesteuert wird. Da OCTN2 der physiologisch bedeutendste Vertreter zu sein scheint, ist eine PPARα-abhängige vermehrte Expression dieses Transporters von herausragender Bedeutung für die Carnitinhomöostase des Körpers. In ***Vers. 2b*** konnte auch gezeigt werden, dass oxidiertes Fett die Expression von Genen der Carnitinbiosynthese in Ratten nicht beeinflusst. Allerdings zeigen die Ergebnisse aus ***Vers. 3*** einen direkten Einfluss des PPARα auf die Expression dieser Gene. Aufgrund dieser unterschiedlichen Ergebnisse sollten weitere Untersuchungen die Rolle des PPARα auf die Expression von Enzymen der Carnitinbiosynthese und deren Einfluss auf die Carnitinhomöostase im gesamten Körper untersuchen. Ebenso sollten weitere Versuche zur Funktion der PPARα-induzierten Steigerung der Expression weiterer Transporter, wie des OCTN3, und deren Bedeutung für die Carnitinhomöostase

durchgeführt werden. Zusammenfassend zeigt die vorliegende Arbeit neue Aspekte zur regulatorischen Funktion des PPARα auf den Cholesterol- und Carnitinstoffwechsel und einen neuen Zusammenhang zwischen einer PPARα-Aktivierung und der Expression von Schlüsselgenen dieser Stoffwechselwege. Darüber hinaus stimmen die Ergebnisse dieser Arbeit mit anderen Studien überein, bei welchen nach der Behandlung mit verschiedenen PPARα-Liganden erniedrigte Cholesterol- und erhöhte Carnitinkonzentrationen in der Leber festgestellt wurden. Zusätzlich bieten die gegenwärtigen Daten eine mögliche Erklärung für die molekularen Mechanismen hinter diesen Effekten.

5. Summary

Cholesterol and its metabolites are very important for cell growth and viability. Dysfunction of cholesterol metabolism results in different diseases. Therefore, regular synthesis and metabolism of cholesterol is essential for normal cell function. Carnitine is a multifunctional component of mammalian cells and plays a key role in the transport of activated long-chain fatty acids from the cytosol to the mitochondrial matrix where β-oxidation takes place. All tissues that use fatty acids as a fuel require carnitine for normal function. In addition, carnitine homeostasis is very important for the maintenance of normal body functions. In different studies it has been shown that several PPARα ligands influence liver cholesterol and carnitine concentrations in rats and humans. Treatments of rats with different PPARα ligands lead to lower cholesterol concentrations in liver, plasma and lipoproteins (VLDL). The underlying molecular mechanisms are still unknown and were investigated in this work. Cellular cholesterol concentration is regulated by a complex mechanism involving Insig, SCAP and SREBP. Retention of the SCAP/SREBP complex in the ER is mediated by the integral membrane proteins Insig-1 and -2a. In a first study (***study 1***), the effect of a ligand induced PPARα activation via clofibrate and WY 14,643 on the cholesterol metabolism in rat hepatoma cells was observed. Due to the increasing proportion of oxidized fat in human nutrition in industrialized countries and their known potential as PPARα activators the effects of oxidized fat on the expression of key genes of cholesterol metabolism were analyzed (***study 2a***). PPARα activation was detected in both experiments. Moreover, in the present work it was demonstrated for the first time that treatment of rats with a synthetic PPARα ligand resulted in the enhanced mRNA concentrations of Insig-1 in the liver (***study 1***). Furthermore, incubation of rat hepatoma cell line Fao with WY 14,643 for 6 h also leads to an increase of Insig-1 and -2a mRNA concentrations. The same results were observed in the liver of rats after the treatment with oxidized fat (***study 2a***). In both studies, the concentration of nuclear SREBP-2 in the liver and cholesterol concentrations in the liver, lipoproteins, and plasma were reduced. In conclusion, the data of ***study 1*** and ***2a*** strongly suggest that PPARα activation lowers the cholesterol concentration by reducing the abundance of nuclear SREBP-2, probably via an upregulation of the Insig expression. The results of the above studies give important insights in the complex regulation of cholesterol metabolism and demonstrate a novel link between PPARα activation and cholesterol synthesis and uptake. In the following studies it will be the aim if the ligand induced activation of PPARα directly controls the

expression of Insig-1 and -2a by binding to a PPRE in the promoter region. In addition to these new aspects about the influence of PPARα activation on cholesterol metabolism, several studies showed that the treatment of rats with fibrates, a class of hypolipidemic drugs, resulted in an increase of liver carnitine concentrations. We have already shown that clofibrate enhances the expression of OCTN1 and OCTN2 in rat liver. Carnitine distribution in the body and intracellular homeostasis are controlled by these membrane carnitine transporters. The aim of ***study 2b*** was to investigate the role of oxidized fat as a natural activator of PPARα in the expression of these transporters and genes of the carnitine synthesis. The next study (***study 3***) with PPARα-knockout-mice had been carried out to prove the hypothesis that the expression of OCTNs is directly mediated by PPARα. The present work shows that treatment of rats with oxidized fat results in an increase of OCTN2 expression in the liver. Also, the data of ***study 3*** strongly suggest that the expression of OCTN2 is directly controlled by PPARα. Since OCTN2 appears to be the main carnitine transporter, its enhanced expression via an activation of PPARα has a pronounced effect on the whole body carnitine homeostasis. It has already been shown in ***study 2b*** that oxidized fat did not affect the expression of genes of carnitine biosynthesis in rats. However, the results of ***study 3*** showed and direct impact of PPARα on the expression of these genes in WY 14,643 treated PPARα-knockout-mice. Because of these different results further studies should be conducted to investigate the role of PPARα in the expression of enzymes of carnitine biosyntheses and their influence on the whole body carnitine homeostasis. Moreover, further studies should be performed to test the function of PPARα induced expression of other transporters, such as OCTN3, and their impact on the carnitine homeostasis. Taken together, this work shows novel aspects of the regulatory function of PPARα in the cholesterol and carnitine metabolism and demonstrates a new correlation between PPARα activation and expression of the key genes of these metabolic pathways. Moreover, the results of this work are in line with other studies reporting decreased cholesterol and increased carnitine levels in the liver upon treatments with different PPARα ligands. In addition, the present data also provide possible explanation of the molecular mechanisms behind these effects.

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Eidesstattliche Erklärung

Hiermit versichere ich an Eides Statt, dass ich die eingereichte Dissertation ***„Untersuchungen zur Wirkung einer PPARα-Aktivierung auf den Cholesterol- und Carnitinmetabolismus“*** 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), den

_____

Alexander Koch

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