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„Molekulare Wirkmechanismen von Carnitin und Regulation der Carnitinhomöostase beim Modell- und Nutztier“

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Inhaltsverzeichnis

Seite

Abbildungsverzeichnis **III**

Abkürzungsverzeichnis **IV**

1. Einleitung **1**

2. Zielstellung **9**

2.1 Untersuchungen zur Carnitinsynthese beim Schwein 9

2.2 Untersuchungen zu molekularen Wirkmechanismen von Carnitin beim Schwein 10

2.3 Untersuchungen zur Wirkung einer PPARα-Aktivierung auf die Carnitinhomöostase am Modelltier Maus 12

3. Originalarbeiten **14**

3.1 Studie 1: Activities of gamma-butyrobetaine dioxygenase and concentrations of carnitine in tissues of pigs 15

3.2 Studie 2: Effect of L-carnitine on the hepatic transcript profile in piglets as animal model 23

3.3 Studie 3: Dietary L-carnitine alters gene expression in skeletal muscle of piglets 33

3.4 Studie 4: Supplementation with L-carnitine downregulates genes of the ubiquitin proteasome system in the skeletal muscle and liver of piglets 44

3.5 Studie 5: Regular endurance exercise improves the diminished hepatic carnitine status in mice fed a high-fat diet 53

4. Diskussion **63**

4.1 Carnitinsynthese beim Schwein 63

4.2 Molekulare Wirkmechanismen von Carnitin beim Schwein 65

4.2.1 Transkriptionelle Veränderungen in der Leber 66

4.2.2 Transkriptionelle Veränderungen in der Muskulatur 72

4.3 Wirkungen einer PPARα-Aktivierung auf die Carnitinhomöostase am Modelltier Maus 81

5. Zusammenfassung **84**

6. Summary **87**

7. Literaturverzeichnis **90**

Erklärung

Lebenslauf

Danksagung

Abbildungsverzeichnis

Abbildung	**Titel**	**Seite**
Abb. 1	Hypothetisches Modell der molekularen Wirkmechanismen von Carnitin beim Schwein	80

Abkürzungsverzeichnis

ACADSB	*acyl-CoA dehydrogenase, short/branched chain*
ACSL3	*acyl-CoA synthetase, long-chain family member 3*
BB	γ-Butyrobetain
BBD	γ-Butyrobetaindioxygenase
C.elegans	*Caenorhabditis elegans*
CACT	Carnitin-Acylcarnitin-Translokase
CAT	Carnitinacyltransferase
CoA	Coenzym A
CPT	Carnitin-Palmitoyl-Transferase
CS	Carnitin-Shuttle
d	Tag
FATP	*fatty acid transport protein*
FBP2	Fructose-1,6-Bisphosphatase
FoxO	*Forkheadbox O*
GLUT	Glukosetransporter
GSK3β	Glykogensynthasekinase 3β
HTMLA	3-Hydroxy-*N*-TML-Aldolase
IGF	*Insulin-like growth factor*
IGF1R	IGF-1-Rezeptor
IGFBP	IGF-bindendes Protein
IRS	Insulinrezeptorsubstrat
KG	Körpergewicht
LM	Lebendmasse
M.	*Musculus*
mRNA	*messenger ribonucleic acid*

mTOR	*mammalian target of rapamycin*
MuRF1	*muscle RING-finger protein-1*
OCTN	*novel organic cation transporter*
PCD	primäre Carnitindefizienz
PCK1	Phosphoenolpyruvatcarboxykinase-1
PDHC	Pyruvatdehydrogenasekomplex
PDPK1	3´-Phosphoinositid-abhängige Proteinkinase
PGC1α	*PPARγ-co-activator α*
PH	Pleckstrin-Homologie
PI3K	Phosphoinositid 3-Kinase
PPAR	*peroxisome proliferator-activated receptor*
PPRE	*peroxisome proliferator response element*
RNA	*ribonucleic acid*
SLC	*solute carrier family*
SORBS1	*Sorbin and SH3 containing-1*
TAG	Triazylglyzerin
TMABA-DH	4-*N*-Trimethylaminobutyroaldehyd-Dehydrogenase
TML	Trimethyllysin
TMLD	Trimethyllysin-Dioxygenase
UE	Untereinheit
UPS	Ubiquitin-Proteasom-System

1. Einleitung

L-Carnitin (L-3-hydroxy-4-*N*,*N*,*N*-trimethylaminobutyrat) zählt als Vertreter der Betaine zu den vitaminähnlichen Verbindungen. In der vorliegenden Arbeit ist mit dem Begriff Carnitin stets das stoffwechselaktive L-Carnitin gemeint. Aufgrund seiner zentralen Bedeutung im Fettsäurestoffwechsel stellt es einen lebensnotwendigen Bestandteil des Körpers dar. Carnitin geht aus den Aminosäuren Lysin und Methionin hervor, wobei Lysin das Kohlenstoffgrundgerüst liefert und Methionin in Form von S-Adenosylmethionin als Methylgruppendonor fungiert. Proteingebundenes Lysin wird zunächst posttranslational mithilfe spezifischer Methyltransferasen zu proteingebundenem N^6-Trimethyl-Lysin (TML) umgesetzt. Dieses Zwischenprodukt wird anschließend über drei weitere enzymatische Reaktionen, einer Hydroxylierung durch die Trimethyllysin-Dioxygenase (TMLD), einer anschließenden Spaltung durch die 3-Hydroxy-*N*-TML-Aldolase (HTMLA) und einer Dehydrierung durch die 4-*N*-Trimethylaminobutyroaldehyd-Dehydrogenase (TMABA-DH) zu γ-Butyrobetain (BB) umgesetzt. Viele Gewebe verfügen über die enzymatische Ausstattung BB zu synthetisieren und geben dieses anschließend in die Blutbahn ab. Die letzte Reaktion, die Hydroxylierung von BB zu Carnitin, wird durch das Enzym γ-Butyrobetaindioxygenase (BBD) katalysiert. Aufgrund des gewebespezifischen Vorkommens und der altersabhängigen Aktivität der BBD, ist nicht jede Spezies in allen Geweben und in jedem Altersabschnitt gleichermaßen zur vollständigen Carnitinsynthese befähigt. Die Identifizierung der Gewebe in welchen die BBD exprimiert wird, stellte Gegenstand zahlreicher Studien dar. So konnte gezeigt werden, dass beim Nager, wie Ratte, Maus und Meerschweinchen aber auch beim Hund, die Leber der Hauptsyntheseort des Carnitins ist (Vaz und Wanders, 2002). Beim Menschen konnten neben der Leber, dem zentralen Stoffwechselorgan, hohe Aktivitäten der BBD in der Niere und geringe Aktivitäten im Gehirn nachgewiesen werden (Vaz und Wanders, 2002; Rigault *et al.*, 2006). Weitere Spezies, wie Hamster, Kaninchen, Rhesusaffe und Katze sind, ähnlich wie der Mensch, einzig in der Niere und Leber zur abschließenden Hydroxylierung der Carnitinvorstufe BB befähigt (Englard und Carnicero, 1978). Die Forschung zur Synthese und Verteilung des Carnitins beim Schwein, als bedeutendes landwirtschaftliches Nutztier und Modelltier für den Mensch, ist aufgrund der elementaren Bedeutung des Carnitins im Energiestoffwechsel von großem Interesse. Allerdings sind bisher keine Untersuchungen beim Schwein bekannt, welche Rückschlüsse auf die Aufklärung des Carnitinmetabolismus hinsichtlich des Vorkommens und der Aktivität der BBD zulassen könnten.

Die Aufrechterhaltung des physiologischen Carnitingehaltes im menschlichen Organismus wird lediglich zu einem geringen Teil (25%) über die endogene Synthese gedeckt (Strijbis *et al.*, 2010). Eher liefern beim Menschen die Aufnahme über die Nahrung und der Transport von Carnitin zwischen den Geweben den entscheidenden Beitrag zur Aufrechterhaltung der Carnitinhomöostase im Körper. Die Absorption von Carnitin im Dünndarm, die renale Reabsorption und die Aufnahme in die Gewebe werden durch die Transportaktivität der *novel organic cation transporters* (OCTNs) gewährleistet. Von diesen Transportern, welche der Familie der *solute carrier family* (SLC) 22A angehören, sind bis zum jetzigen Zeitpunkt drei Isoformen (OCTN1, OCTN2, OCTN3) bekannt (Tamai *et al.*, 1997; 1998; 2000; Wu *et al.*, 1999). Der Na^+-Carnitin Cotransporter OCTN2 (SLC22A5), welcher beim Menschen vor allem in Niere, Darm, Skelettmuskel, Herz, Leber, Gehirn und Plazenta exprimiert wird, ist aufgrund seiner hohen Bindungsaffinität zu Carnitin aber auch zu BB der physiologisch bedeutendste Vertreter der Carnitintransporter (Tamai *et al.*, 1998; 2000; Wu *et al.*, 1999). Dies wird unter anderem bei der Ausprägung von angeborenen autosomal rezessiv vererbten Mutationen im codierenden Bereich des OCTN2-Gens deutlich. Diese Punktmutationen führen zu dem klinischen Bild eines primären Carnitindefizites (PCD), welches mit einer Störung der Fettsäureoxidation aufgrund eines erhöhten Verlustes von Carnitin über Darm und Niere und einer verminderten Speicherung in Form von Acetylcarnitin einhergeht (Burwinkel *et al.*, 1999; Wang *et al.*, 1999). Diese Patienten weisen stark verminderte Carnitinkonzentrationen in den Geweben auf, wobei hauptsächlich Herz- und Skelettmuskel sowie das zentrale Nervensystem betroffen sind (Erguven *et al.*, 2007). Der massiv beeinträchtigte Carnitinstatus äußert sich phänotypisch in der Entwicklung von Myopathien und/oder hypoketotischen hypoglykämischen Enzephalopathien (Erguven *et al.*, 2007). Folglich ist bei Diagnose der PCD eine Supplementierung von Carnitin auf Lebenszeit indiziert.

In einigen funktionellen Studien an Nager und Schwein wurde bereits die steigernde Wirkung eines fasteninduzierten Energiemangels auf die hepatische Carnitinsynthese und die vermehrte Aufnahme von Carnitin in die Gewebe bewiesen (van Vlies *et al.*, 2007; Ringseis *et al.*, 2009). In besonderen Stoffwechsellagen wie dem Fastenzustand, sind eine Hochregulation der lipolytischen Prozesse und eine erhöhte Energiebereitstellung aus dem Abbau von endogenen Fettsäuren zwingend erforderlich. Der Organismus ist in der Lage in fast allen Geweben Fettsäuren als Energiesubstrat zu nutzen. Eine Ausnahme stellen hierbei das Gehirn und die Erythrozyten dar, welche lediglich Glukose bzw. Ketonkörper energetisch nutzen können. Um Fettsäuren unter Energiegewinn in Form von ATP nutzen zu können,

müssen diese zunächst mittels eines komplexen Shuttlesystems in das Mitochondrium, dem Ort der β-Oxidation, gelangen. Carnitin vermittelt hierbei als Carriermolekül den Übertritt mittel- bis langkettiger aktivierter Fettsäuren (> C10) über die äußere und innere Mitochondrienmembran. Zu diesem Zweck werden die Coenzym-A-Ester langkettiger Fettsäuren durch die Carnitin-Palmitoyl-Transferase 1 (CPT-1) an der äußeren Mitochondrienmembran umverestert. Dabei wird die Acylgruppe von der β-Hydroxylgruppe des Carnitins unter Bildung von Acylcarnitin übernommen und Coenzym A (CoA) freigesetzt. Der Transport des Acylcarnitins in den Matrix-Raum erfolgt über einen spezifischen Antiporter, die Carnitin-Acylcarnitin-Translokase (CACT). In diesem Zellkompartiment katalysiert die Carnitin-Palmitoyl-Transferase 2 (CPT-2) die Reveresterung der Fettsäure mit CoA. Der Antiporter CACT befördert das frei gewordene Carnitin zurück, wo es erneut zur Verfügung steht (McGarry und Brown, 1997). Ein vermehrter Transport von Fettsäuren in die Mitochondrien, um anschließend energetisch genutzt werden zu können, stellt einen wichtigen Anpassungsprozess des Organismus an den Fastenzustand dar. Die Beobachtung einer gesteigerten hepatischen Carnitinsynthese und -aufnahme während der Nahrungskarenz ist auf die Tatsache zurückzuführen, dass sowohl die Expression von Enzymen der Carnitinsynthese in der Leber als auch die Aufnahme von Carnitin aus dem Plasma in die extrahepatischen Gewebe der transkriptionellen Kontrolle spezifischer Kernrezeptoren unterliegen. Durch jüngste Untersuchungen unserer Arbeitsgruppe konnte bewiesen werden, dass sowohl die Enzyme der Carnitinsynthese BBD und TMABA-DH, als auch der OCTN2, Zielgene des *peroxisome proliferator-activated receptor α* (PPARα) darstellen (van Vlies *et al.*, 2007; Koch *et al.*, 2008; Ringseis und Eder, 2009; Wen *et al.*, 2010; 2011). PPARα zählt zur Familie der nukleären Hormonrezeptoren und nimmt eine zentrale Rolle im Energie- und Lipidstoffwechsel ein, wobei er im Besonderen bei der Anpassung des fastenden Organismus an die verminderte Energiezufuhr von Bedeutung ist (Desvergne und Wahli, 1999). Diverse Liganden, natürlicher und synthetischer Art, sind in der Lage PPARα in unterschiedlichem Maße zu binden und somit zu aktivieren. So stellen sowohl endogen aus dem weißen Fettgewebe freigesetzte unveresterte Fettsäuren, langkettige mehrfach ungesättigte Fettsäuren, sowie konjugierte Linolsäuren aus der Nahrung, als auch synthetische Pharmazeutika wie die hypolipidämisch wirksamen Fibrate (Clofibrat und Fenofibrat) potente PPARα-Aktivatoren dar (Krey *et al.*, 1997; Staels *et al.*, 1998; Desvergne und Wahli, 1999; Kersten *et al.*, 1999; Lin *et al.*, 1999; Moya-Camarena *et al.*, 1999; Xu *et al.*, 1999). Nach erfolgreicher ligandeninduzierter Aktivierung folgt anschließend die Heterodimerisierung des PPARα mit dem *retinoic-X-receptor* (RXR). Daraufhin gelangt

dieser Komplex aus dem Zytosol in den Zellkern und kann dort an sogenannte *peroxisome proliferator response elements* (PPREs) in der Promotorregion oder im Bereich der Introns von PPARα-Zielgenen binden (Schoonjans *et al.*, 1997; Desvergne und Wahli, 1999; Qi *et al.*, 2000; Tan *et al.*, 2005). Über die spezifische Bindung des aktivierten PPARα an die PPREs erfolgt die transkriptionelle Steuerung zahlreicher Gene, die für Enzyme kodieren, welche vorrangig bei der zellulären Fettsäureaufnahme, dem intrazellulären Fettsäuretransport, dem mitochondrialen Fettsäuretransport, der mitochondrialen und peroxisomalen Fettsäureoxidation sowie der Ketogenese, Glukoneogenese und dem Lipoproteinmetabolismus beteiligt sind (Brandt *et al.*, 1998; Mascaró *et al.*, 1998; Kersten *et al.*, 1999; Mandard *et al.*, 2004). Daher ist es physiologisch sinnvoll, dass PPARα vorwiegend in Geweben mit hoher Fettsäureoxidationsrate, wie Leber, Niere, Herz, Muskel und Dünndarmmukosa exprimiert wird (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). Nager, wie Ratte und Maus aber auch das Schwein sind in der Lage den erhöhten Bedarf an Carnitin, bedingt durch eine PPARα-induzierte Hochregulierung des Fettsäureimportes in die Mitochondrien, durch eine gesteigerte Aufnahme von Carnitin in die extrahepatischen Gewebe zu decken. Darüber hinaus konnte in zahlreichen Studien gezeigt werden, dass mit Ausnahme der Ratte, eine PPARα-Aktivierung zu gesteigerten Carnitinsyntheseraten beim Säuger führt (McGarry *et al.*, 1975; Brass und Hoppel, 1978; Paul und Adibi, 1979; Paul *et al.*, 1986; Luci *et al.*, 2006; van Vlies *et al.*, 2007; Koch *et al.*, 2007; 2008; Ringseis *et al.*, 2007; 2008; 2009; Maeda *et al.*, 2008). In der Literatur gibt es bis heute keinerlei Hinweise, ob auch die durch körperliche Belastung, wie beispielsweise Ausdauertraining, endogen freigesetzten Fettsäuren, ähnlich wie bei Nahrungskarenz, zu einer PPARα-vermittelten Hochregulierung der Carnitinsynthese und -aufnahme führen.

Neben seiner elementaren Funktion im Fettstoffwechsel bei der Vermittlung des mitochondrialen Fettsäuretransportes, scheint Carnitin auch einen bedeutenden Einfluss auf die Regulation des Glukose- und Insulinstoffwechsels zu haben. Diese Beobachtungen sind Ergebnis neuester Forschungsarbeiten, in denen gezeigt werden konnte, dass Carnitin die Glukosetoleranz und Insulinsensitivität beeinflussen kann. Besonders bei einer beeinträchtigten Insulinsensitivität, hervorgerufen durch eine zu hohe Nahrungszufuhr und damit verbundenem Übergewicht oder *Diabetes Mellitus* Typ II, ist dieser Aspekt von entscheidender Bedeutung. In neueren Studien wurde beobachtet, dass chronischer metabolischer Stress, ausgelöst durch Übergewicht oder Alterung, zur Entwicklung

mitochondrialer Dysfunktion und Insulinresistenz beiträgt. Die Autoren vermuten, dass diese Stoffwechselstörungen auf einen Carnitinmangel zurückzuführen sind, da sich gezeigt hat, dass Carnitingaben zu einer Verbesserung dieser Stoffwechselimbalanzen führten (An *et al.*, 2004; Koves *et al.*, 2008; Noland *et al.*, 2009). In der Literatur werden mehrere Mechanismen diskutiert, über welche Carnitin seine günstigen Wirkungen auf den Glukose- und Insulinstoffwechsel vermittelt. Die Funktion des Carnitins, die Zelle vor einer Akkumulierung langkettiger AcylCoAs zu schützen und somit der Inhibierung von Enzymaktivitäten und der drohenden Entwicklung einer Insulinresistenz entgegenzuwirken, ist in der Literatur bereits detailliert beschrieben worden (McGarry und Brown, 1997; Kerner und Hoppel, 2000; Steiber *et al.*, 2004). Der Pool an verfügbarem CoA, sowohl im Zytosol als auch in der Mitochondrienmembran, ist unerlässlich für viele metabolische Prozesse und Stoffwechselvorgänge wie dem Citratzyklus, der Ketogenese und der Glukoneogenese (Bieber, 1988; Rebouche, 1992). Carnitin verringert aufgrund seiner Funktion als Cofaktor der CACT und Carnitinacyltransferase (CAT) das intramitochondriale AcetylCoA/CoA-Verhältnis um das 10 - 20-fache und gewinnt daher in der Humanmedizin immer mehr an Bedeutung (Lysiak *et al.*, 1988; Broderick *et al.*, 1992; Ramsay und Zammit, 2004). In der Literatur werden weitere Mechanismen, über welche Carnitin seine positiven Wirkungen auf die Glukosetoleranz vermittelt, diskutiert. So wird vermutet, dass die orale Gabe von Acetylcarnitin bei Patienten mit *Diabetes mellitus* Typ II zu einer Modulation der Energiesubstratnutzung zugunsten der Kohlenhydrate aufgrund einer veränderten Expression von glykolytischen und glukoneogenetischen Enzymen in der Leber beiträgt (Rugenetti *et al.*, 2009). Darüber hinaus konnte die Arbeitsgruppe Hotta *et al.* (1996) mithilfe des homozygoten *Juvenile Visceral Steatosis* Maus Modells zeigen, dass die carnitindefizienten Tiere verminderte hepatische Expressionen glykolytischer Enzyme, jedoch gesteigerte Expressionsraten des glukoneogenetischen Enzyms Phosphoenolpyruvatcarboxykinase-1 (PCK1) aufweisen. Des Weiteren existieren in der Literatur Hinweise auf einen insulinähnlichen Effekt von Carnitin und einer damit verbundenen Verbesserung der Glukosetoleranz beim stoffwechselgesunden Menschen (De Gaetano *et al.*, 1999; Galloway *et al.*, 2011). Aufgrund der Beobachtungen aus den vorangegangenen Untersuchungen wäre eine carnitinvermittelte Beeinflussung des Insulinstoffwechsels auf Ebene des Insulinrezeptors oder auf Post-Rezeptor-Ebene, welche die intrazelluläre Insulinsignalkaskade einschließt, denkbar. Ferner zeigten Untersuchungen beim Tier und beim Menschen eine Beeinflussung der IGF (*insulin like growth factor*) -1-Achse durch eine Erhöhung der Konzentrationen an IGF-1 und IGF-2 infolge einer gesteigerten oralen

Carnitinaufnahme (Di Marzio *et al.*, 1999; Heo *et al.*, 2001; Kita *et al.*, 2002; Doberenz *et al.*, 2006).

Carnitin wurde in den letzten Jahren zunehmend in der Fütterung von landwirtschaftlichen Nutztieren eingesetzt. Die Basis für den Einsatz lieferten die Befunde zahlreicher Studien an Sportpferden, Milchkühen, Legehennen, Ferkeln und Sauen, bei denen der Einfluss von Carnitin auf die Leistung der Tiere untersucht wurde. Bei der trächtigen und laktierenden Sau konnten durch unsere Arbeitsgruppe eine Reihe von positiven Wirkungen durch den Einsatz von Carnitin in der Fütterung nachgewiesen werden. Es existieren Befunde, dass eine Supplementierung von Carnitin während der Trächtigkeitsphase der Sau, die Lebendmassezunahmen der Sau, sowie der Ferkel in der Säugeperiode erhöht (Musser *et al.*, 1999; Eder *et al.*, 2001; Ramanau *et al.*, 2002; 2004). Weiterhin wurde gezeigt, dass die Fütterung hoher Mengen Carnitins zu einer erhöhten Anzahl lebendgeborener Ferkel und einer Verringerung totgeborener Ferkel bei der Sau führt (Musser *et al.*, 1999; Ramanau *et al.*, 2004). Einen weiteren interessanten Aspekt stellt die Beobachtung dar, dass die Supplementierung von Schweinefutter mit Carnitin zu einem gesteigerten Proteinbestand auf Kosten des Fettansatzes bei den Tieren führt (Owen *et al.*, 1996; 2001a; 2001b; Penn *et al.*, 1997; Rincker *et al.*, 2003; Birkenfeld *et al.*, 2005). Der Proteinturnover setzt sich aus einem ständigen Auf- und Abbau von Proteinen im Organismus zusammen und unterliegt strikten Regulationsmechanismen. Bei einem normalgewichtigen erwachsenen Menschen werden täglich etwa 280 g Protein synthetisiert und abgebaut, wobei den Hauptanteil intrazelluläre Proteine ausmachen (Mitch und Goldberg, 1996). Viele regulatorische Proteine besitzen eine sehr geringe Halbwertszeit und werden relativ schnell zu einzelnen Aminosäuren hydrolysiert, um zur erneuten Proteinsynthese genutzt werden zu können. Der Zelle stehen mehrere Mechanismen zum Abbau von Proteinen zur Verfügung. Neben dem Abbau durch lysosomale Proteasen, durch welche vor allem extrazelluläre Proteine und Membranproteine abgebaut werden können, werden intrazelluläre, kurzlebige Proteine hauptsächlich dem so genannten Ubiquitin-Proteasom-System (UPS) zugeführt (Goldberg und Rock, 1992; Ciechanover, 1994; Rock *et al.*, 1994; Lecker *et al.*, 2006). Das UPS ist Bestandteil aller eukaryotischen Zellen und besteht aus einer komplexen Kaskade von enzymatischen Reaktionen mit einer hohen Substratspezifität. Der proteasomale Abbau eines Proteins beginnt mit der Markierung des abzubauenden Proteins mit Ubiquitin, welches über eine Isopeptidbindung an die ε-Aminogruppen mehrerer Lysinreste mit dem zum Abbau vorgesehenen Protein verknüpft wird. Zunächst wird das Ubiquitinmolekül an seiner terminalen Carboxylgruppe mit der Sulfhydrylgruppe des ubiquitinaktivierenden Enzyms

(E1) verknüpft und somit aktiviert. Anschließend wird es auf die Sulfhydrylgruppe des ubiquitinkonjugierenden Enzyms (E2) übertragen (Jentsch, 1992). Zuletzt katalysieren spezifische Ubiquitin-Protein-Ligasen (E3s) den Transfer des Ubiquitins von E2 auf eine ε-Aminogruppe des Zielproteins, welches dann durch das 26S Proteasom, einem multikatalytischen Proteasekomplex, erkannt und unter Energieverbrauch zu kleineren Peptiden abgebaut wird (Thrower *et al.*, 2000; Cao *et al.*, 2005). Das 26S Proteasom besteht aus insgesamt 60 Untereinheiten (UE), die sich in zwei Komplexe gliedern, dem katalytischen 20S Proteasom und der regulatorischen 19S UE (Voges *et al.*, 1999). Die UE sind so angeordnet, dass eine fassartige Struktur entsteht, in welche die Proteine aufgenommen und durch verschiedene Peptidasen abgebaut werden können. Durch die Arbeit von deubiquitinierenden Enzymen (Isopeptidasen), die in der 19S UE lokalisiert sind, können die Polyubiqutinketten abgespalten und wiederverwendet werden (Glickman und Ciechanover, 2002). Die freigelassenen kleineren Peptide werden anschließend durch zelluläre Proteasen zu einzelnen Aminosäuren abgebaut. Der Organismus ist in der Lage Proteine mithilfe des Proteasoms innerhalb kürzester Zeit abzubauen, um sich neuen physiologischen Bedingungen kurzfristig anpassen zu können. Dies betrifft unter anderem die Familie der nukleären Transkriptionsfaktoren. Die Ubiquitinierung und anschließende Proteolyse der verbrauchten Aktivatoren führt zu einer Stimulation der transkriptionellen Aktivität, da die Promotorregion des Gens für die erneute Bindung weiterer Aktivatoren zur Verfügung steht und somit die Transkription verstärkt wird (Lipford *et al.*, 2005). Weiterhin werden durch den proteasomalen Abbau fehlgefaltete oder geschädigte Proteine aus dem Stoffwechsel eliminiert. Diese entstehen durch Mutationen oder durch die Einwirkung von reaktiven Sauerstoffradikalen bzw. Denaturierung durch Hitze. Somit ist das UPS in vielerlei Regulationsmechanismen des Körpers involviert, unterliegt jedoch selbst zahlreichen Einflussfaktoren. So konnte gezeigt werden, dass erhöhte IGF-1-Konzentrationen eine Verringerung der Expression der beiden E3-Ligasen MuRF1 (*muscle RING finger-1*) und Atrogin-1 (MAFbx, *muscle atrophy F-box*) im Skelettmuskel zur Folge hat (Schiaffino und Mammucari, 2011). Die Expression dieser beiden Ligasen ist in katabolen Stoffwechsellagen bis zu 20-fach erhöht, weswegen sie als spezifische Marker für proteolytische Prozesse und Muskelschwund herangezogen werden können. Eine *Knockout*-Studie an Mäusen bekräftigt die essenzielle Bedeutung der E3-Ligasen bei der Vermittlung von zum Abbau vorgesehenen Proteinen durch das UPS (Bodine *et al.*, 2001). Es konnte gezeigt werden, dass der proteolytische Abbau von Muskelmasse, welcher durch Denervation des entsprechenden Muskels induziert wurde, bei einem Defekt des

MuRF1- bzw. Atrogin-1-Gens drastisch verringert wird. Die transkriptionelle Regulation von MuRF1 und Atrogin-1 wird durch den Insulin/IGF-1-Signalweg realisiert. Die Bindung von Insulin und/oder IGF-1 an spezifische Tyrosinkinaserezeptoren führt über eine komplexe, intrazelluläre Signalkaskade zur Hemmung der Transkription von MuRF1 und Atrogin-1 und somit zu einem verminderten proteasomalen Proteinabbau. Inwiefern Carnitin dieses komplexe System und somit auch den Proteinabbau beeinflussen kann, wurde bislang nicht untersucht.

Die Bedeutung von Carnitin für den Energiestoffwechsel der eukaryotischen Zelle aufgrund seiner zentralen Rolle als Cofaktor des mitochondrialen Fettsäuretransportes ist in der Vergangenheit Gegenstand zahlreicher Untersuchungen gewesen. Carnitin nimmt daher eine fundamentale Stellung im Fettsäurekatabolismus ein. Gleichzeitig liefern neuere Studien Hinweise auf weitere interessante Effekte von Carnitin und bilden die Grundlage weiterer Untersuchungen zum Einfluss einer Carnitinsupplementierung auf verschiedene Stoffwechselwege im Organismus. Molekularbiologische Untersuchungen zur Klärung der zugrundeliegenden Mechanismen der in vorangegangenen Human- und Tierstudien beobachteten Wirkungen von Carnitin auf die Glukosetoleranz und Insulinsensitivität in der Leber und Skelettmuskulatur fehlen bislang. Weiterhin fehlen in der Literatur Studien zur Aufklärung der molekularen Ursachen der in zahlreichen Fütterungsversuchen bei verschiedenen Nutztierarten festgestellten Beeinflussung der Leistungsphysiologie durch Carnitin. Das Schwein stellt aufgrund seiner genetischen und physiologischen Ähnlichkeit mit dem Menschen ein optimales Modelltier zur Untersuchung molekularer Wirkmechanismen von Nahrungsmittelzusatzstoffen auf den Stoffwechsel des Organismus dar. Daher liegt es nahe, Mechanismen der Carnitinwirkung anhand der Beeinflussung des Genexpressionsprofils beim Schwein zu untersuchen. Diese Erkenntnisse lassen sich auf den Menschen übertragen und liefern ferner neue Erklärungsansätze für die phänotypischen Veränderungen, die bereits durch eine Carnitinsupplementierung in verschiedenen Fütterungsstudien beobachtet werden konnten.

2. Zielstellung

Aufgrund der viel diskutierten günstigen Wirkungen von Carnitin auf die Leistungsparameter verschiedener landwirtschaftlicher Nutztierarten sollten im Rahmen dieser Arbeit zunächst die Gewebe vom Schwein, die über eine aktive Form der BBD verfügen und somit zur vollständigen Carnitinsynthese befähigt sind, identifiziert werden. Darüber hinaus sollten zugrundeliegende molekulare Mechanismen, über welche Carnitin seine Wirkungen beim Schwein auf den Stoffwechsel der Leber und Skelettmuskulatur vermittelt, aufgeklärt werden. Weiterhin war das Ziel dieser Arbeit den Einfluss einer trainingsinduzierten Aktivierung des nukleären Kernrezeptors PPARα auf die Regulation der Carnitinhomöostase am Modelltier Maus zu untersuchen.

2.1 Untersuchungen zur Carnitinsynthese beim Schwein

In vorangegangenen Studien konnten bereits die Orte der Carnitinsynthese bei verschiedenen Säugetieren identifiziert werden (Vaz und Wanders, 2002). So hat sich gezeigt, dass die Aktivität der BBD gewebe- und speziesspezifisch reguliert wird. In einer Reihe von Untersuchungen konnte nachgewiesen werden, dass Ratte, Maus, Schaf, Hund, Meerschwein und Cebusaffe einzig in der Leber nachweisliche Aktivitäten der BBD aufweisen (Vaz und Wanders, 2002). Beim Menschen konnten sowohl in der Leber und in der Niere hohe Aktivitäten, als auch geringe Aktivitäten der BBD im Gehirn nachgewiesen werden (Rebouche und Engel, 1980; Vaz und Wanders, 2002; Rigault *et al.*, 2006). Weitere Spezies, wie Hamster, Kaninchen, Rhesusaffe, Rind und Katze sind, ähnlich wie der Mensch, einzig in der Niere und Leber zur vollständigen Carnitinsynthese befähigt (Englard und Carnicero, 1978). Des Weiteren wird in der Literatur diskutiert, dass die Aktivität der BBD bei Säugetieren in verschiedenen Altersstufen variiert (Hahn, 1981; Olson und Rebouche, 1987; Galland *et al.*, 1999). Das Schwein, als bedeutendes landwirtschaftliches Nutztier, ist hinsichtlich der Aufklärung des Carnitinmetabolismus noch nicht hinreichend untersucht worden. Daher war das Ziel der ersten Studie, Aktivität und Vorkommen der BBD beim Schwein zu identifizieren. Dazu wurde bei Schweinen nach *ad libitum* Verfütterung einer Standarddiät mit einem geringen nativen Gehalt an Carnitin die Konzentration von Carnitin in verschiedenen Geweben bestimmt. Darüber hinaus wurden mRNA- und Proteinkonzentrationen und die Aktivität der BBD in verschiedenen Geweben der Schweine ermittelt. Um zu untersuchen, inwieweit der Carnitinstatus beim jungen Schwein beeinträchtigt ist, wurden die Konzentrationen von Carnitin in den Geweben und die Aktivität der BBD in Leber und Niere der Ferkel vom Tag der Geburt bis zur 7. Lebenswoche

bestimmt. Weitere Details zur Versuchsdurchführung, Material und Methodik, sowie die ausführliche Diskussion der Ergebnisse dieser Studie sind ersichtlich in:

Studie 1:
Fischer M, Keller J, Hirche F, Kluge H, Ringseis R, Eder K (2009) Activities of gamma-butyrobetaine dioxygenase and concentrations of carnitine in tissues of pigs. ***Comp Biochem Physiol A Mol Integr Physiol*** 153:324-331; ***reproduced with permission of Elsevier***

2.2 Untersuchungen zu molekularen Wirkmechanismen von Carnitin beim Schwein

Carnitin hat aufgrund seiner günstigen Wirkungen auf die Leistungsmerkmale von landwirtschaftlichen Nutztieren in den letzten Jahrzehnten großes Interesse in der Tierernährung geweckt. So konnten verbesserte Wachstumsraten, eine höhere Futterverwertung und ein gesteigerter Proteinansatz direkt mit einer Carnitinsupplementierung in Verbindung gebracht werden. Auch in der menschlichen Ernährung gewinnt Carnitin zunehmend an Bedeutung. Neuere Untersuchungen haben gezeigt, dass sich der Einsatz von Carnitin positiv auf die Behandlung von degenerativen Stoffwechselerkrankungen auswirkt. In vorangegangenen Studien am Menschen konnte gezeigt werden, dass Carnitin protektive Eigenschaften im Zusammenhang mit der Entwicklung neurodegenerativer Erkrankungen, altersbedingter Beeinträchtigung der Mitochondrienfunktion und oxidativem Stress besitzt (Rani und Panneerselvam, 2001; Hagen *et al.*, 2002; Calabrese *et al.*, 2005). Des Weiteren liefert die Literatur viele Hinweise auf weitere günstige Wirkungen des Carnitins auf den Glukose- und Lipidstoffwechsel sowohl bei Stoffwechselgesunden als auch bei Kranken. Die zugrundeliegenden Mechanismen der günstigen Wirkungen von Carnitin sind bislang weitgehend unbekannt. Ziel dieser zweiten Studie war es daher, Einblicke in die Mechanismen und Stoffwechselwege, in welche Carnitin eingreift, aufzuklären. Da Schweine im Hinblick auf genetische und physiologische Eigenschaften dem Menschen sehr ähnlich sind, werden sie vielfach in der Wissenschaft als Modelltier genutzt. Daher wurden in dieser Arbeit Ferkel entweder mit einer carnitinangereicherten Standarddiät (500 mg Carnitin/kg Diät) oder einer Standarddiät ohne Zusatz von Carnitin über einen Zeitraum von drei Wochen gefüttert, um anschließend mithilfe der *microarray*-Technologie Einblicke in das Transkriptprofil der Leber zu bekommen. Ausführliche Details zur Versuchsdurchführung, Material und Methodik, sowie die Diskussion der gewonnenen Daten dieser Studie sind ersichtlich in:

Studie 2:

Keller J, Ringseis R, Priebe S, Guthke R, Kluge H, Eder K (2011) Effect of L-carnitine on the hepatic transcript profile in piglets as animal model. ***Nutr Metab (Lond)*** 8:76

In einer weiteren Studie wurde der Einfluss einer Carnitinsupplementierung auf das Transkriptprofil im Skelettmuskel untersucht. Dazu wurden von den Tieren aus der vorangegangenen Studie Gewebeproben des *M. longissimus dorsi* gewonnen und hinsichtlich des Transkriptprofils untersucht, um weitere Aussagen über die durch Carnitin beeinflussten Stoffwechselwege in der Muskulatur treffen zu können. Besonders interessant ist die Beobachtung, dass steigende Carnitindosierungen beim Schwein mit einem erhöhten Proteinansatz auf Kosten des Fettansatzes einhergehen (Rincker *et al.*, 2003). Owen *et al.* (1996; 2001a; 2001b) konnten ähnliche Effekte eines verringerten Fettansatzes mit steigenden Carnitinkonzentrationen im Futter bei Absetzferkeln beobachten. Des Weiteren konnte mit steigenden Carnitindosen beim Ferkel eine Verbesserung der täglichen Stickstoffretention, ein erhöhter Proteinansatz und ein verminderter Fettanteil im Schlachtkörper beobachtet werden (Heo *et al.*, 2000). Es wird vermutet, dass die erhöhten muskulären Carnitinkonzentrationen eine verbesserte Energieversorgung der Ferkel aufgrund einer gesteigerten Fettsäureoxidation im Mitochondrium zur Folge haben (Rincker *et al.*, 2003). Um die zugrundeliegenden Mechanismen der Carnitinwirkung auf die Muskulatur beim Ferkel aufzuklären, wurde mithilfe der *microarray*-Technologie das Transkriptom im Muskel untersucht. Weitere Einzelheiten der Untersuchung im Hinblick auf Material und Methodik, Ergebnisdarstellung und Diskussion sind folgender Studie zu entnehmen:

Studie 3:

Keller J, Ringseis R, Priebe S, Guthke R, Kluge H, Eder K (2011) Dietary L-carnitine alters gene expression in skeletal muscle of piglets. ***Mol Nutr Food Res*** 55:419-429; ***reproduced with permission of John Wiley and Sons***

Die Datenlage der wissenschaftlichen Literatur zeigt eindeutig einen positiven Zusammenhang zwischen einem Carnitin-Einsatz und einer Verbesserung der Leistungsmerkmale von landwirtschaftlichen Nutztieren und Sporttieren (Foster *et al.*, 1989; LaCount *et al.*, 1995; Musser *et al.*, 1999; Heo *et al.*, 2000; Owen *et al.*, 2001a; 2001b;

Rincker *et al.*, 2003; Geng *et al.*, 2007). Die molekularen Mechanismen, die diesem Phänomen zugrunde liegen, konnten bislang nicht endgültig geklärt werden. Basierend auf den Daten der *microarray*-Technologie in Leber und Muskel von Ferkeln der beiden vorangegangenen Studien, lässt sich vermuten, dass Carnitin die Gene des UPS beeinflusst. Das UPS ist bei der Regulation des Proteinturnover in den Geweben, vor allem in der Muskulatur, von entscheidender Bedeutung und stellt somit einen Ansatzpunkt für den in zahlreichen Voruntersuchungen beobachteten erhöhten Proteinstatus beim Nutztier dar. Um diese Hypothese zu prüfen, wurden in einer weiteren Studie die bereits gewonnenen Leber- und Muskelproben von Ferkeln hinsichtlich der relativen mRNA-Konzentrationen klassischer Gene des UPS untersucht. Um feststellen zu können, ob Carnitin seine Wirkungen auf die Expression von Genen des UPS auf direktem oder indirektem Weg vermittelt, wurden Inkubationsversuche an humanen Leberzellen (HepG2-Zellen) und murinen Muskelzellen (C2C12-Zellen) durchgeführt. Die detaillierte Beschreibung der Versuchsdurchführung, sowie Darstellung und Diskussion der Ergebnisse sind in folgender Studie ersichtlich:

Studie 4:

Keller J, Ringseis R, Koc A, Lukas I, Kluge H, Eder K (2011) Supplementation with L-carnitine downregulates genes of the ubiquitin proteasome system in the skeletal muscle and liver of piglets. ***Animal*** 6:70-78; ***reproduced with permission of Cambridge University Press***

2.3 Untersuchungen zur Wirkung einer PPARα-Aktivierung auf die Carnitinhomöostase am Modelltier Maus

Zahlreiche Studien, sowohl *in vitro*, als auch *in vivo* haben eindeutig gezeigt, dass die Expression verschiedener Gene der Carnitinhomöostase, wie OCTN2, TMABA-DH und BBD der transkriptionellen Kontrolle des PPARα unterliegen. PPARα zählt zu den Liganden-aktivierbaren Transkriptionsfaktoren und nimmt eine zentrale Stellung bei der Regulation des Fettsäurekatabolismus ein (Desvergne und Wahli, 1999; Kersten *et al.*, 1999). Die Aktivierung von PPARα in der Leber führt nachweislich zu einer gesteigerten Carnitinaufnahme in die Leber und einer erhöhten hepatischen Carnitinsyntheserate (van Vlies *et al.*, 2007; Koch *et al.*, 2008; Ringseis *et al.*, 2009; Wen *et al.*, 2010). Die essenzielle Bedeutung des PPARα für die Aufrechterhaltung der Carnitinhomöostase wurde durch Untersuchungen unserer Arbeitsgruppe bestätigt. Hier konnte gezeigt werden, dass eine *Down*-Regulierung des PPARα während der Laktation zu einem verminderten Carnitinstatus

in der Leber führt (Gutgesell *et al.*, 2009). Weiterhin wiesen PPARα-defiziente Mäuse sowohl erniedrigte Carnitinkonzentrationen in den Geweben, als auch verminderte relative mRNA- Konzentrationen des OCTN2, TMABA-DH und BBD in der Leber auf (van Vlies *et al.*, 2007; Koch *et al.*, 2008; Makowski *et al.*, 2009). Darüber hinaus konnte in einigen Studien gezeigt werden, dass eine *Down*-Regulierung des *PPARγ-co-activator α* (PGC1α), einem Transkriptions-Co-Aktivator von PPARα, hervorgerufen durch eine Hochfettdiät, zu einer Beeinträchtigung der transkriptionellen Aktivität des PPARα führt (Li *et al.*, 2007; Koves *et al.*, 2008). Interessanterweise konnte gezeigt werden, dass Ausdauertraining zu einer Stimulierung des PPARα über die Aktivierung des PGC1α und somit zu einer gesteigerten Transkription von PPARα-Zielgenen führt (Koves *et al.*, 2005). Um die Hypothese zu prüfen, dass regelmäßiges Ausdauertraining die hochfettinduzierte Beeinträchtigung des PPARα aufheben kann und somit in einer gesteigerten hepatischen Carnitinsynthese und -aufnahme resultiert, erhielten Mäuse *ad libitum* über einen Zeitraum von zehn Wochen entweder eine Standarddiät mit einem moderaten Fettgehalt (10%) oder eine Diät mit stark erhöhtem Fettgehalt (45%). Anschließend wurde ein Teil der Tiere einem regelmäßigen Ausdauertraining auf einem Laufband unterzogen und nach Versuchsende die Genexpressionen und die Proteinkonzentrationen von Enzymen der Carnitinsynthese sowie des OCTN2 ermittelt. Weiterhin wurde geprüft, inwieweit regelmäßiges Ausdauertraining den Carnitinstatus in Leber und Skelettmuskulatur sowie die hepatischen Konzentrationen der Carnitinvorstufen TML und BB der Tiere beeinflusst. Ferner sollte untersucht werden, inwiefern sich die langfristige Fütterung einer Hochfettdiät und kompensatorisches Ausdauertraining auf die Glukosetoleranz der Mäuse auswirkt. Ausführliche Details zur Versuchsdurchführung, Material und Methodik, sowie die Diskussion der Ergebnisse dieser Studie sind ersichtlich in:

Studie 5:

Ringseis R, Mooren FC, Keller J, Couturier A, Wen G, Hirche F, Stangl GI, Eder K, Krüger K (2011) Regular endurance exercise improves the diminished hepatic carnitine status in mice fed a high-fat diet. ***Mol Nutr Food Res*** 55:S193-202; ***reproduced with permission of John Wiley and Sons***

3. Originalarbeiten

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Activities of γ-butyrobetaine dioxygenase and concentrations of carnitine in tissues of pigs

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ABSTRACT

In contrast to other species, less is known about carnitine homeostasis in the pig. This study was performed to yield information about the site of carnitine synthesis and carnitine concentrations in various tissues of pigs (*Sus scrofa*). We found that among several pig tissues, a considerable activity of γ-butyrobetaine dioxygenase (BBD), the last enzyme of carnitine synthesis, exists, like in humans and several other species, only in liver and kidney. Activity of that enzyme in liver and kidney was lower at birth than in the subsequent weeks of life. Highest carnitine concentrations were found in skeletal muscle and heart. Carnitine concentrations in plasma, liver and kidney at birth were higher than in the subsequent weeks of life in spite of the low BBD activity at birth. In conclusion, this study shows that liver and kidney are the major sites of carnitine synthesis and that neonatal pigs do not have an insufficient carnitine status.

1. Introduction

Carnitine (L-3-hydroxy-4-N-N-N-trimethylaminobutyrate) is an essential metabolite, which has a number of indispensable functions in intermediary metabolism (Steiber et al., 2004). All tissues that use fatty acids as a fuel source require carnitine for normal function. Carnitine is derived from dietary sources and endogenous biosynthesis (Hoppel and Davis, 1986; Rebouche and Seim, 1998). Carnitine biosynthesis involves a complex series of reactions. Lysine in protein peptide linkages provides the carbon backbone of carnitine. It undergoes methylation of the ε-amino group to yield trimethyllysine, which is released upon protein degradation. The released trimethyllysine is further oxidised to γ-butyrobetaine which is then hydroxylated by γ-butyrobetaine dioxygenase (BBD) to form carnitine (Vaz and Wanders, 2002). Carnitine produced in tissues expressing an active BBD is secreted into the blood and taken up into tissues by novel organic cation transporters (OCTN), particularly OCTN2 which is the most important carnitine transporter (Tamai et al., 2000; Lahjouji et al., 2001).

Tissue distribution of BBD is different between various mammalian species. In all mammals studied so far, BBD activity has been found in the liver (Vaz and Wanders, 2002). In some species such as in humans, cats, cows, hamsters, rabbits or Rhesus monkeys, BBD activity has been detected also in the kidney; in these species, activity of BBD in the kidney is even higher than in the liver (Vaz and Wanders, 2002). In contrast, in several other species such as Cebus monkeys, sheep, dogs, guinea pigs, mice and rats, BBD is not, or only at very low activity, present in the kidney (Vaz and Wanders, 2002). In humans, BBD activity has been also found in the brain which is in contrast to other species (Rebouche and Engel, 1980). In rats, one study detected BBD activity in testis and epididymis (Carter et al., 1987), which could, however, not be confirmed by another study (Galland et al., 1999). In sheep, BBD activity was also observed in muscle (Erfle, 1975). There does not appear to be any evolutionary pattern with respect to the activity of BBD in tissues, since even very closely related species, like the Rhesus and Cebus monkeys, exhibit a different pattern.

In contrast to various other animal species, less is known about carnitine metabolism in the pig. For instance, the site of carnitine biosynthesis in pigs has not yet identified. Moreover, there is also less information about carnitine concentrations in tissues of pigs. In humans carnitine concentrations are highest in skeletal muscle which is regarded as a carnitine storage. Concentration of carnitine in muscle (2000–4000 nmol/g wet weight) is as much as 100times higher than that in plasma (Bertoli et al., 1981; Moorthy et al., 1983; Angelini et al., 1992). Organs such as kidney, liver and brain contain intermediate levels of 300–1000 nmol/g wet weight (Moorthy et al., 1983; Angelini et al., 1992). It is actually unclear whether a similar pattern of tissue carnitine concentrations exists also in pigs.

In humans, activity of BBD in liver is particularly low at birth (Rebouche and Engel, 1980). Moreover, it has been shown that infants fed a formula without supplemented carnitine have low plasma carnitine concentration (Olson et al., 1989). Therefore, it has been suggested that carnitine is an essential nutrient in the newborn (Borum, 1981; Penn et al., 1981). Some studies suggested that pigs have also an insufficient carnitine status at birth (van Kempen and Odle, 1995; Penn et al., 1997; Heo et al., 2000). Therefore, the neonatal

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pig has been suggested as model to study human neonatal carnitine metabolism (Baltzell et al., 1978; Penn et al., 1997). We are however not aware of any study which investigated the carnitine status of neonatal pigs relative to older pigs.

The aim of this study was to gain more insight into carnitine homeostasis in the pig. First, we intended to identify the sites of carnitine biosynthesis in pigs. Therefore, we determined mRNA and protein concentration and activities of BBD in various tissues of pigs. Second, to characterize the tissue carnitine concentration pattern in pigs, we determined carnitine concentrations in various tissues. Third, to find out whether newborn pigs indeed have a low carnitine status at birth, we determined concentrations of carnitine in various tissues of pigs at birth and in the subsequent weeks of life. In order to investigate the hypothesis that carnitine biosynthesis rate is particularly low at birth, we also determined the activity of BBD and concentrations of γ-butyrobetaine, the precursor of carnitine, in pig tissues of pigs at birth and in the subsequent weeks. These studies should also be helpful to characterize the carnitine homeostasis in the pig compared to humans and other mammalian species.

2. Materials and methods

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

2.1. Animals and diets

In order to identify tissues containing BBD, we used four pigs (*Sus scrofa*) (one female, two castrates, one uncastrated male of a crossbred race [(German Landrace × Large White) × Pietrain] with a body mass between 60 and 70 kg. These pigs were fed ad-libitum a nutritionally adequate standard pig diet containing 13.4 MJ metabolizable energy and 170 g crude protein/kg. The diet had a low native carnitine concentration (<5 mg/kg).

In order to investigate the effect of age on activity of BBD and concentrations of carnitine and precursors in plasma and tissues, we used litters of five crossbred sows [Large White × German Landrace × Hermitage) × Pietrain]. During pregnancy, these sows received a standard lactation diet for sows containing 13.4 MJ metabolizable energy and 175 g crude protein/kg. The carnitine concentration of this diet was below 5 mg/kg. Immediately after birth, the litters of those sows were standardized to eight (four male, four female) pigs/litter. From day 11 until weaning, the piglets were offered a creep feed for ad-libitum consumption which contained 15.6 MJ metabolizable energy and 200 g crude protein/kg diet; the carnitine concentration was 35 mg/kg. The piglets were weaned at day 28 and were offered the creep feed for ad libitum consumption until day 35. Thereafter, they were switched to a nutritionally adequate piglet diet which contained 13.7 MJ metabolizable energy and 185 g crude protein/kg. The native carnitine concentration of this diet was below 5 mg/kg. Immediately after birth and at the end of each following week, one piglet/litter was removed from each of the five sows and used for the collection of samples.

Concentrations of crude protein in the diets were analysed according to the official German VDLUFA methodology (Bassler and Buchholz, 1993). The metabolisable energy of the diet was calculated as recommended by the German Nutrition Society (Gesellschaft für Ernährungsphysiologie, 2006).

2.2. Sample collection

The animals were anaesthesised and exsanguinated. Blood samples were collected into heparinised polyethylene tubes. In the first experiment, liver, kidneys, heart, proximal segments of small intestine (duodenum) and colon and samples from m. *longissimus dorsi*, brain, lung and spleen were excised. In the uncastrated male animal, additionally one testis and epididymis were prepared. In the second experiment, liver, kidneys, heart and samples from m. *longissimus dorsi* and m. *semimembranosus* were excised from each animal. Plasma was obtained in each experiment by centrifugation of the blood samples (1100 *g*, 10 min, 4 °C). Plasma and tissue samples were stored at −20 °C.

2.3. RNA isolation and RT-PCR analysis

Total RNA was isolated from tissue samples using Trizol™ reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocol. Total RNA concentration and purity were estimated from the optical density at 260 and 280 nm, respectively. Synthesis of cDNA and determination of mRNA abundance by RT-PCR with real-time detection (Rotorgene 6000, Corbett Research, Australia) using Sybr Green I was performed as recently described in detail (Ringseis et al., 2007). For absolute quantification of mRNA abundance of BBD and GAPDH standard curves were generated with purified PCR products of BBD and GAPDH which were obtained by extraction of cut ethidium bromide-stained bands following 2% agarose gel electrophoresis by MinElute Gel Extraction Kit (Qiagen, Hilden, Germany). Ct values for each amplification curve were obtained using Rotorgene Series Software 1.7 (Corbett Research, Australia). Quantification of double-stranded DNA concentration of purified PCR products was performed using the PicoGreen DNA Quantitation Kit (Molecular Probes) and a spectrofluorometer (excitation: 480 nm, emission: 520 nm). For normalization purposes, the copy number of the housekeeping gene GAPDH served as an independent internal control. Sequences of gene-specific primers obtained from Operon (Köln, Germany) were as follows (forward, reverse; NCBI Genbank): BBD (5′-GTG CCG AAA GCT CAA GGA AAA A-3′, 5′-CTC TGC CGG CCG TGA AGT AAC-3′; partial sequence according to Ruan et al. (2007) and GAPDH (5′-AGG GGC TCT CCA GAA CAT CAT CC-3′, 5′-TCG CGT GCT CTT GCT GGG GTT GG-3′; AF017079).

2.4. Immunoblot analysis of BBD

For immunoblotting, homogenates of all tissues were prepared by homogenising tissue aliquots in 10 mM 3-morpholinepropansulfonic acid buffer (pH 7.4) containing 0.9% (w/v) sodium chloride, 10% (w/v) glycerol, and 5 mM dithiothreitol, and protein concentrations were determined by the Bradford method (Bradford, 1976). The brain was not considered for immunoblot analysis, because this tissue could not be completely homogenised using the above mentioned buffer. Equal amounts of protein (50 µg) were electrophoresed by 12.5% SDS-PAGE, and transferred to a nitrocellulose membrane (Pall, Pensacola, USA). The membranes were blocked overnight at 4 °C in 5% skim milk in Tris-buffered saline containing 0.2% Tween (TBS-T), and then incubated with a 1:500 dilution of a mouse monoclonal anti-BBD primary antibody (ab56350; Abcam, Cambridge, UK) for 2 h at room temperature. Membranes were washed with TBS-T, and incubated with a HRP conjugated secondary antibody anti-mouse IgG (Sigma-Aldrich, Taufkirchen, Germany) for 1.5 h at room temperature. Afterwards, blots were washed again, and developed using ECL Plus (Western Blotting Detection Reagents, GE Healthcare Europe GmbH, Freiburg, Germany). For normalisation purposes, membranes were also incubated with a mouse monoclonal anti-GAPDH primary antibody (ab8245; Abcam, Cambridge, UK). The signal intensities of specific bands (BBD and GAPDH) were detected with a Bio-Imaging system (Bio-Imaging Systems, F-ChemiBIS 3.2 M, biostep GmbH, Jahnsdorf, Germany) and quantified using TotalLab TL100-Quick Start analysis software (nonlinear dynamics). The normalised protein concentration was calculated as the ratio of the band intensities of BBD and GAPDH.

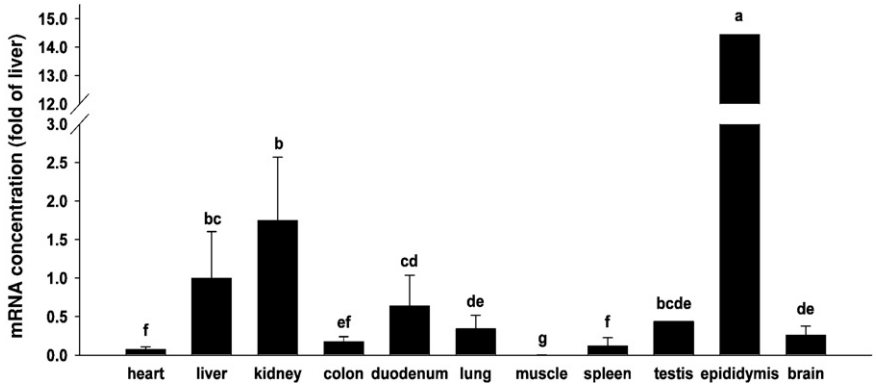

Fig. 1. Relative mRNA concentrations of BBD in various tissues of pigs. Total RNA was extracted from the samples and relative mRNA levels of γ-BBD were determined by real-time detection RT-PCR analysis using glyceraldehyde-3-phosphate dehydrogenase for normalization. Bars represent means ± standard deviations ($n = 4$ for all tissues with the exception of testis and epididymis for which $n = 1$), expressed relative to liver (= 1.00). Bars marked without a common superscript letter differ ($p < 0.05$).

2.5. Analysis of carnitine and γ-butyrobetaine

Concentrations of free carnitine, acetyl carnitine, propionyl carnitine and γ-butyrobetaine in plasma and tissues as well as concentration of carnitine in the diet were determined by tandem mass spectrometry using deuterated analoga as internal standard (Ringseis et al., 2007). Carnitine-d_3 (N-methyl-d3) was supplied by Cambridge Isotope Laboratories (Andover, MA, USA), acetyl carnitine-d_3 and propionyl carnitine-d_3 were products of Larodan Fine Chemicals (Malmö, Sweden). Propionyl carnitine and γ-butyrobetaine-d_3 were synthesized as described in literature (Vaz et al., 2002; Konishi and Hashimoto, 1992). Concentration of carnitine represents the sum of free carnitine, acetyl carnitine and propionyl carnitine.

2.6. Determination of the activity of BBD

Activity of BBD in tissues was determined as described previously in detail by van Vlies et al. (2006). Homogenates from tissues were prepared by homogenising tissue in 10 mM 3-morpholinepropansulfonic acid buffer (pH 7.4) containing 0.9% (w/v) sodium chloride, 10% (w/v) glycerol, and 5 mM dithiothreitol.

2.7. Statistical analysis

In both experiments, data were treated by one factorial analysis of variance (ANOVA) using the Minitab statistical software (Release 13, Minitab Inc., State College, PA, USA). In the first experiment, tissue was used as factor of ANOVA; in the second experiment age was used as factor of ANOVA. For statistically significant F values ($p < 0.05$) means were compared by Fisher's multiple range test. In the case of large differences in the variances, data were transformed to their logarithms prior to analysis. Means were considered significantly different for $p < 0.05$. Results in the text are given as means ± SD.

3. Results

3.1. mRNA concentrations, protein concentrations and activity of BBD in various tissues of pigs

In almost all tissues examined, we detected measurable amounts of BBD mRNA, with the only exception of skeletal muscle in which BBD mRNA was almost completely absent. The highest mRNA concentration of BBD was observed in epididymis obtained from one male pig which was 14.4-fold higher than in liver (Fig. 1). Relative mRNA concentration of BBD in kidney was also higher than that in liver (1.74-fold, Fig. 1). mRNA concentrations of BBD in heart, colon, small intestine, lung, spleen, testes and brain were lower than those in the liver (Fig. 1).

BBD protein was detected in all tissues examined. However, the pattern of BBD protein concentrations in tissues reflected that of BBD mRNA concentrations only partially. Highest BBD protein concentrations were observed in epididymis, followed by kidney and testes (Fig. 2). Protein concentration of BBD in testes was also higher than in liver (Fig. 2). BBD protein concentrations in lung and spleen were similar to

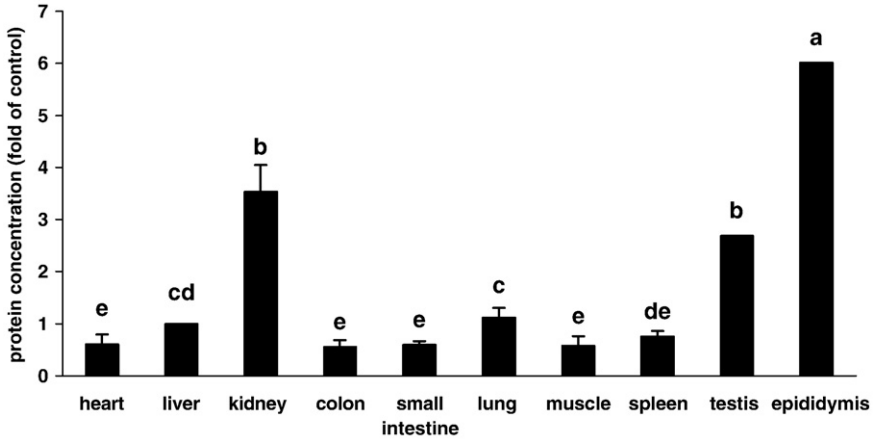

Fig. 2. Protein concentration of BBD in various tissues of pigs. Bars represent means ± standard deviations ($n = 4$ for all tissues with the exception of testis and epididymis for which $n = 1$), expressed relative to liver (= 1.00). Bars marked without a common superscript letter differ ($p < 0.05$).

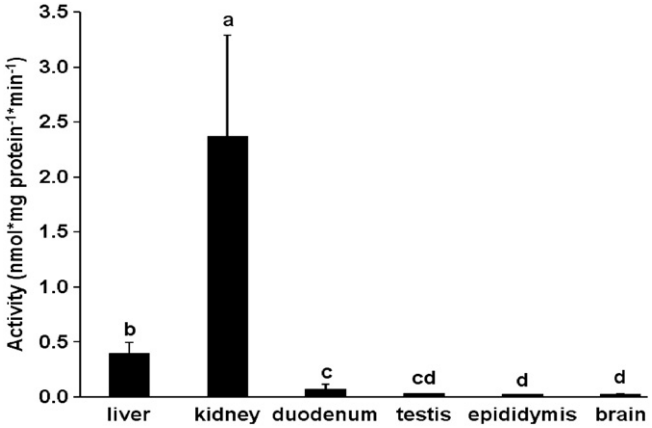

Fig. 3. Activities of BBD in various tissues of pigs. Bars represent means ± standard deviations ($n = 4$ for all tissues with the exception of testis and epididymis for which $n = 1$). Bars marked without a common superscript letter differ ($p < 0.05$).

that in the liver; BBD protein concentrations in heart, muscle, colon, small intestine and skeletal muscle were lower than in the liver (Fig. 2).

Activities of BBD in tissues were in strong contrast to mRNA and protein concentrations of BBD. Among the tissues examined, a considerable activity of that enzyme was found only in kidney and liver (Fig. 3). A small but detectable activity was also found in small intestine, epididymis, testes and brain (Fig. 3). In contrast, activities of this enzyme in all other tissues (skeletal muscle, heart, colon, lung, spleen) were below the detection limit (<0.01 nmol mg protein^{-1} min^{-1}).

3.2. Concentrations of carnitine in various tissues of pigs

The highest concentrations of free carnitine, acetyl carnitine and total carnitine were observed in heart and skeletal muscle (Fig. 4). Kidney and small intestine had the next highest concentrations of free and total carnitine which were 3- to 6-fold lower than those in heart and skeletal muscle (Fig. 4). Concentrations of total carnitine in colon, spleen and epididymis were similar to those in liver while carnitine

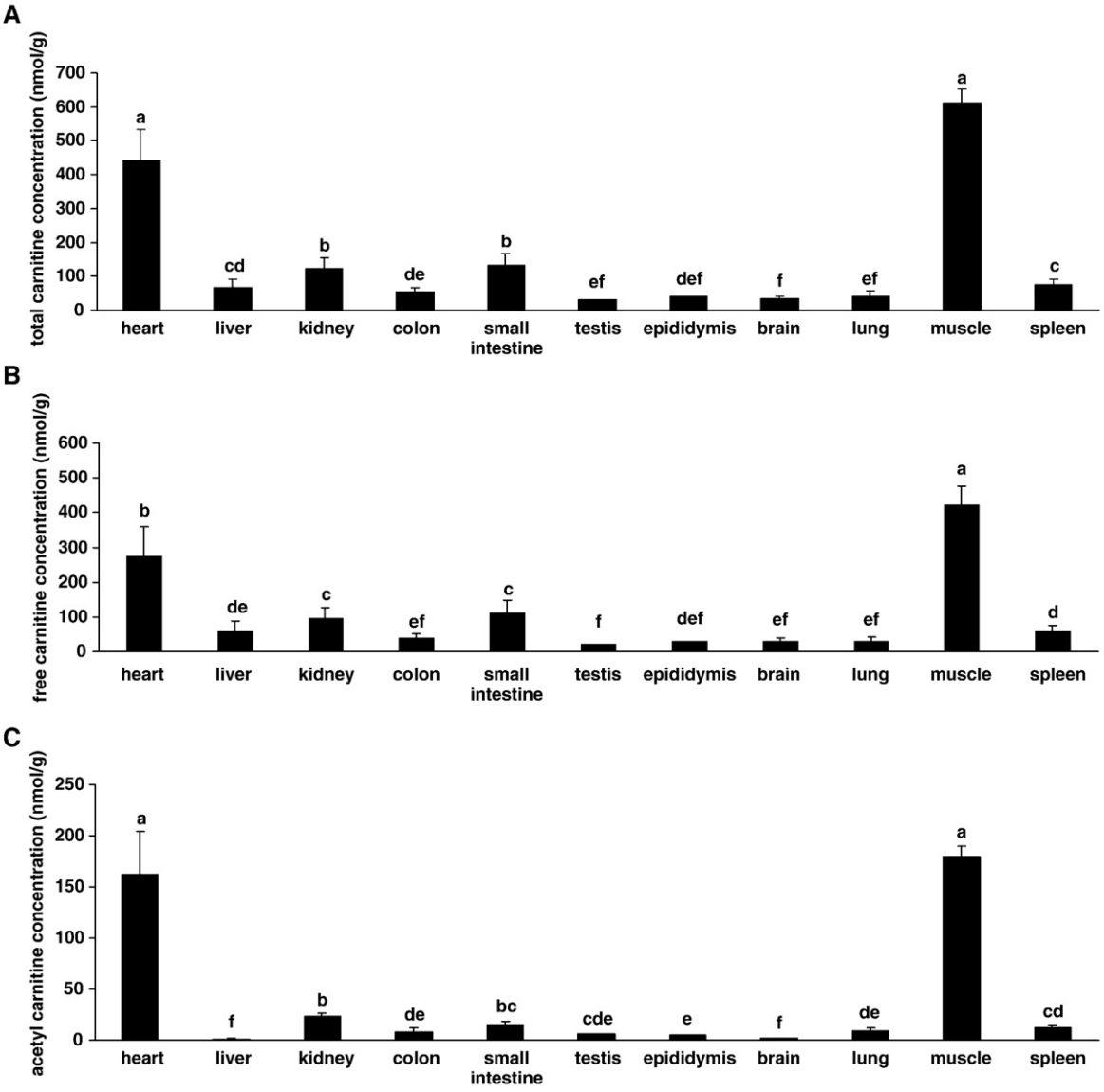

Fig. 4. Concentration of total carnitine (A), free carnitine (B) and acetyl carnitine (C) in various tissues of pigs. Bars represent means ± standard deviations ($n = 4$ for all tissues with the exception of testis and epididymis for which $n = 1$). Bars marked without a common superscript letter differ ($p < 0.05$).

concentrations in testes, brain and lung were lower than in liver ($p<0.05$, Fig. 4). Concentrations of free carnitine in colon, epididymis, brain, lung and spleen did not differ from that of liver while that in testes was lower ($p<0.05$, Fig. 4). Liver and brain had among all tissues examined the lowest concentrations of acetyl carnitine (Fig. 4). Accordingly, these tissues had lower ratios between acetyl carnitine and free carnitine than all the other tissues (ratio of acetyl carnitine to free carnitine: heart, 0.62 ± 0.26; liver, 0.02 ± 0.01; kidney, 0.21 ± 0.01; colon, 0.20 ± 0.05; small intestine, 0.13 ± 0.02; testis, 0.27; epididymis, 0.15; brain, 0.05 ± 0.02; lung, 0.28 ± 0.06; muscle, 0.43 ± 0.08; spleen, 0.21 ± 0.04; $n = 4$ for all tissues with exception of testis and epididymis for which $n = 1$).

3.3. Concentrations of carnitine in plasma and tissues of pigs from birth to 7 weeks of age

Concentrations of total carnitine in plasma, liver and kidney were highest at birth and thereafter declined until an age of 4 weeks (Table 1). Total carnitine concentrations in liver and kidney thereafter remained constant until an age of 7 weeks, whereas plasma carnitine concentration further declined until an age of 7 weeks (Table 1). In contrast, carnitine concentrations in heart and skeletal muscle rose from birth until an age of 3–4 weeks and thereafter declined (Table 1). The ratio of acetyl carnitine to free carnitine in plasma was falling from birth until week 4 and was thereafter, in weeks 5–7, increasing again. The ratio of acetyl carnitine to free carnitine in the liver was generally very low and remained unchanged during the first weeks of life (Table 1). In contrast, the ratios of acetyl carnitine to free carnitine in kidney and heart decreased from birth to week 7 of life (Table 1). The ratio of acetyl carnitine to free carnitine in skeletal muscle was generally higher than that of the other tissues investigated (Table 1). This ratio in muscle was falling during the first 2–3 weeks of life and was then increasing to values in excess of those at birth (Table 1).

3.4. Activity of BBD and concentrations of BB in tissues of pigs from birth to 7 weeks of age

Activity of BBD in both, liver and kidney was lowest at birth (Fig. 5). It thereafter increased, reached its maximum already at an age of 2 weeks and remained on this level until 7 weeks of age (Fig. 5). Among the tissues considered, γ-butyrobetaine concentrations were generally highest in skeletal muscle (m. *longissimus dorsi*, m. *semimembranosus*), followed by heart (Table 2). γ-Butyrobetaine concentrations in liver and kidney were around and 20- and 15-fold lower, respectively, than those in skeletal muscle (Table 2). γ-Butyrobetaine concentrations in liver, kidney and m. *longissimus dorsi* were highest at birth and declined during the first weeks of life (Table 2). Between an age of 1 and 7 weeks, γ-butyrobetaine concentrations in those tissues remained at a constant level (Table 2). In plasma, γ-butyrobetaine concentration was also highest at birth. Between 1 and 6 weeks of age, plasma BB concentration remained at a constant level (Table 2). At 7 weeks of age, a significant decline of plasma BB concentration was observed. γ-Butyrobetaine concentrations in heart and m. *semimembranosus* were not significantly changing from birth until an age of 7 weeks (Table 2).

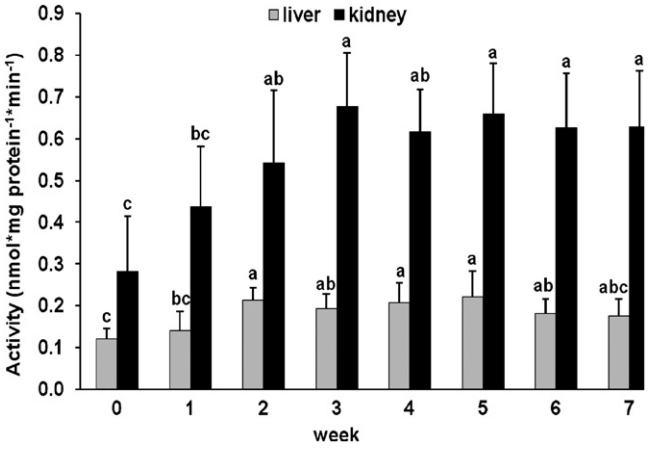

Fig. 5. Activity of BBD in liver and kidney from birth to 7 weeks of age. Bars represent means ± standard deviations ($n = 5$). Bars marked without a common superscript letter differ ($p<0.05$).

4. Discussion

One aim of this study was to detect the site of carnitine synthesis in the pig. Therefore, we determined mRNA concentrations, protein concentrations and activities of BBD in various tissues. The study shows for the first time that mRNA and protein of BBD is expressed in several tissues, while a considerable activity of BBD occurs only in kidney and liver. With respect to the pattern of BBD activity in tissues, the pig obviously behaves like humans, cats, cows, hamsters, rabbits or Rhesus monkeys, in which liver and kidney are also the primary sites of carnitine synthesis (Vaz and Wanders, 2002; Englard and Carnicero, 1978). In all those species, activity of BBD in the kidney exceeds that in

Table 1
Concentrations of total carnitine and rations of acetyl carnitine:free carnitine in plasma and tissues of pigs differing in life age[†].

Age [weeks]	Plasma [μmol/L]	Liver [nmol/g]	Kidney [nmol/g]	Heart [nmol/g]	M. *longissimus dorsi* [nmol/g]	M. *semimembranosus* [nmol/g]
Total carnitine						
Birth	21.8 ± 3.6^{a}	200 ± 22^{a}	123 ± 27^{a}	316 ± 53^{d}	284 ± 56^{d}	333 ± 74^{f}
1	17.6 ± 4.3^{b}	165 ± 58^{ab}	119 ± 35^{ab}	380 ± 58^{cd}	475 ± 58^{c}	575 ± 87^{e}
2	17.4 ± 4.0^{bc}	118 ± 37^{b}	118 ± 30^{ab}	475 ± 69^{ab}	706 ± 75^{a}	761 ± 99^{bc}
3	13.6 ± 2.0^{c}	79.1 ± 4.9^{c}	95.4 ± 11.3^{b}	571 ± 78^{a}	811 ± 32^{a}	897 ± 35^{a}
4	14.0 ± 3.1^{bc}	47.7 ± 6.1^{d}	73.6 ± 10.0^{cd}	550 ± 142^{a}	747 ± 91^{a}	818 ± 101^{ab}
5	15.8 ± 1.9^{bc}	49.3 ± 15.3^{d}	77.9 ± 5.9^{bc}	513 ± 55^{a}	596 ± 36^{b}	684 ± 44^{cd}
6	16.6 ± 1.2^{bc}	45.7 ± 9.1^{d}	72.8 ± 8.5^{cd}	408 ± 38^{bc}	586 ± 24^{b}	668 ± 95^{cde}
7	7.62 ± 0.81^{d}	41.2 ± 8.6^{d}	61.0 ± 5.1^{d}	339 ± 27^{cd}	538 ± 79^{bc}	580 ± 64^{de}
Acetyl carnitine:free carnitine						
Birth	0.17 ± 0.02^{b}	0.010 ± 0.003	0.031 ± 0.005^{a}	0.060 ± 0.022^{a}	0.34 ± 0.11^{c}	0.22 ± 0.05^{b}
1	0.13 ± 0.03^{cd}	0.008 ± 0.002	0.027 ± 0.006^{ab}	0.050 ± 0.021^{ab}	0.36 ± 0.02^{bc}	0.20 ± 0.11^{bc}
2	0.10 ± 0.01^{de}	0.011 ± 0.003	0.025 ± 0.010^{ab}	0.036 ± 0.008^{bc}	0.24 ± 0.04^{d}	0.18 ± 0.03^{bc}
3	0.08 ± 0.01^{e}	0.012 ± 0.002	0.022 ± 0.003^{b}	0.025 ± 0.007^{cd}	0.20 ± 0.01^{d}	0.15 ± 0.01^{c}
4	0.09 ± 0.01^{e}	0.012 ± 0.003	0.026 ± 0.003^{ab}	0.017 ± 0.003^{de}	0.33 ± 0.04^{c}	0.26 ± 0.09^{b}
5	0.13 ± 0.02^{c}	0.011 ± 0.005	0.020 ± 0.005^{bc}	0.015 ± 0.003^{e}	0.44 ± 0.05^{ab}	0.55 ± 0.06^{a}
6	0.27 ± 0.02^{a}	0.012 ± 0.003	0.016 ± 0.006^{c}	0.012 ± 0.006^{e}	0.44 ± 0.08^{ab}	0.43 ± 0.06^{a}
7	0.19 ± 0.03^{b}	0.011 ± 0.003	0.014 ± 0.003^{c}	0.012 ± 0.002^{e}	0.52 ± 0.10^{a}	0.45 ± 0.08^{a}

[†]Means and standard deviations ($n = 5$). Means without the same superscript letters (a, b, c, d) are significantly different ($p<0.05$).

Table 2
Concentration of γ—butyrobetaine in plasma and tissues of pigs differing in life age[†].

Age [week]	Plasma [μmol/L]	Liver [nmol/g]	Kidney [nmol/g]	Heart [nmol/g]	M. *longissimus dorsi* [nmol/g]	M. *semimembranosus* [nmol/g]
Birth	1.56 ± 0.64^{a}	7.75 ± 1.81^{a}	11.5 ± 1.4^{a}	98.1 ± 26.1	187 ± 60^{a}	144 ± 64
1	0.79 ± 0.15^{cd}	5.51 ± 1.26^{b}	7.44 ± 1.57^{c}	82.1 ± 29.9	104 ± 7^{b}	96.3 ± 21.9
2	0.84 ± 0.19^{bcd}	4.60 ± 0.52^{bc}	7.35 ± 1.74^{c}	83.0 ± 18.5	92.8 ± 11.7^{b}	93.5 ± 21.0
3	0.64 ± 0.07^{d}	3.92 ± 0.55^{c}	7.00 ± 1.21^{c}	91.8 ± 26.2	106 ± 15^{b}	129 ± 33
4	0.91 ± 0.13^{bcd}	3.80 ± 0.92^{c}	6.67 ± 0.82^{c}	70.3 ± 13.7	95.1 ± 21.0^{b}	113 ± 27
5	1.31 ± 0.27^{a}	4.25 ± 0.78^{bc}	9.38 ± 2.01^{b}	74.5 ± 14.4	102 ± 12^{b}	126 ± 19
6	1.15 ± 0.30^{bc}	4.78 ± 0.58^{bc}	8.26 ± 1.13^{bc}	71.8 ± 11.4	97.1 ± 12.8^{b}	109 ± 18
7	1.05 ± 0.19^{bc}	4.28 ± 0.29^{c}	7.73 ± 1.07^{bc}	72.7 ± 16.8	113 ± 29^{b}	129 ± 33

[†]Means and standard deviations ($n = 5$). Means without the same superscript letters (a, b, c, d) are significantly different ($p < 0.05$).

the liver as also observed for pigs in this study. In opposite, there are several other species such as rats, mice, sheep, dogs or guinea pigs which do not exhibit any measurable activity of BBD in kidney (Lindstedt et al., 1982; Erfle, 1975; Englard and Carnicero, 1978; Englard, 1979). The activity of BBD in pig liver determined in this study was two- to three fold higher than BBD activities in liver rats and mice determined with the same assay (van Vlies et al., 2006). Although enzyme activities determined in different laboratories cannot be directly compared, this finding suggests that pig liver has a relatively high capacity to convert γ-butyrobetaine into carnitine.

Interestingly, we detected mRNA and protein concentrations of BBD in nearly all tissues, also in those which did not show any activity of that enzyme such as testis, colon, lung, and spleen. From the data of this study, we cannot provide an explanation for the quite different pattern of mRNA, protein and activity of BBD in various tissues. However, investigations in rats also revealed that BBD mRNA is present in tissues such as testis and epididymis, although no significant activity of BBD has been found in these tissues (Galland et al., 1999). Noteworthy, Galland et al. (1999) observed that the size of BBD mRNA in testis (3.5 kb) and epididymis (4.5 kb) was markedly different from that in the liver (1.9 kb). This has been suggested to be the result of a tissue-specific alternative splicing of the BBD mRNA (Vaz and Wanders, 2002), which could result in either no translation or translation of the BBD mRNA into a protein with similar size as the hepatic or renal BBD but without significant BBD activity. In addition, tissue-specific posttranslational inactivation of the BBD protein, possibly mediated by phosphorylation or dephosphorylation of serine or threonine residues of the protein, could also explain the differences in BBD activities between tissues. Whether the BBD protein belongs to such interconvertible enzymes, however, is currently unknown, and, therefore, should be investigated in future studies. Furthermore, it might be also conceivable that putative inhibitors of BBD are present in specific tissues leading to a tissue-specific inhibition, either competitive, non-competitive or allosteric, of the BBD protein. This, however, is highly speculative and, thus, remains to be established. Moreover, it is known that the BBD activity is also dependent on several cofactors including molecular oxygen, Fe^{2+} and ascorbate, and that BBD activity was stimulated considerably by 2-oxoglutarate (Lindstedt, 1967; Lindstedt et al., 1968; Lindstedt and Lindstedt, 1970). Hence, differences in the availability of these cofactors between tissues might also explain tissue-specific variations in the activity of BBD.

In the present study, we determined also the concentrations of carnitine in various tissues of the pig. We found that carnitine concentrations in tissues of pigs are generally markedly lower than those reported for humans. As in humans, highest carnitine concentrations were found in skeletal muscle. This suggests that muscle serves as a carnitine storage in pigs like in humans. In humans, approximately 95% of the carnitine is localized in skeletal muscle (Evans and Fornasini, 2003). Carnitine concentrations in skeletal muscle of pigs, being around 600 nmol/g, are comparable with those of rat muscle but they are markedly lower than those of human muscle which are in the range between 2000 and 4000 nmol/g (Bertoli et al., 1981; Moorthy et al., 1983; Angelini et al., 1992). Concentrations of carnitine in other tissues such as liver, kidney or brain of pigs are also three- to five-fold lower than those of the respective human tissues suggesting that pigs have generally a lower carnitine status than humans (Moorthy et al., 1983; Angelini et al., 1992). Total carnitine concentrations in liver, kidney and heart of pigs are moreover also much lower than in rodents (van Vlies et al., 2006; Koch et al., 2008; Ringseis et al., 2007; Luci et al., 2008).

It has been suggested that newborn infants or animals have a low carnitine status at birth which could even lead to carnitine deficiency (Borum, 1981; Penn et al., 1981). To investigate the carnitine status of piglets at birth and in the subsequent weeks of life, we considered piglets of litters adjusted to a size of eight animals each. As we removed one piglet from each litter at each of the next three subsequent weeks for analysis of tissue carnitine concentrations, litter sizes were even reduced to four piglets/litter until week 4. It is known that piglets of smaller litters receive more milk than those in larger litters (Auldist et al., 1998). Accordingly, it is likely that the piglets in this study received more carnitine through the milk than piglets of larger litters such as under practical farming conditions. The present study shows that, nevertheless, concentrations of carnitine in plasma, liver and kidney of pigs are even higher at birth than in the subsequent weeks of suckling. It is moreover shown that concentrations of carnitine in muscle are lowest at birth and are increasing thereafter. In order to achieve an indication of carnitine synthesis, we determined activities of BBD in liver and kidney and the concentrations of γ-butyrobetaine in various tissues. In humans, not the activity of BBD but the availability of γ-butyrobetaine as precursor is rate-limiting for carnitine synthesis (Olson and Rebouche, 1987; Rebouche et al., 1989). It is likely that the availability of γ-butyrobetaine in liver and kidney is the limiting factor for carnitine synthesis in pigs, too (Fischer et al., 2009a). In the present study, we found that the activity of BBD was lowest at birth. This finding agrees with reports in human neonates which have also a low activity of BBD in the liver (Rebouche and Engel, 1980). In contrast, concentrations of γ-butyrobetaine in liver and kidney were even higher at birth than in the subsequent weeks. Under the assumption that the concentration of γ-butyrobetaine is the rate-limiting factor for carnitine synthesis (Olson and Rebouche, 1987; Rebouche et al., 1989), the high γ-butyrobetaine concentrations in liver and kidney provide an explanation for the elevated carnitine concentrations at birth. It is known that γ-butyrobetaine is produced in all tissues from trimethyllysine (Vaz and Wanders, 2002). The finding that γ-butyrobetaine concentrations are highest in skeletal muscle suggests that muscle is the most important supplier of γ-butyrobetaine for carnitine synthesis in liver and kidney in the pig. Interestingly, γ-butyrobetaine concentrations in muscle of pigs are 10–20 fold higher than those reported for rat or mouse muscle (Noël et al., 1984; van Vlies et al., 2006; Ringseis et al., 2008; Koch et al., 2008). This suggests that pigs have a higher capacity to produce γ-butyrobetaine from trimethyllysine in muscle than rodents. Concentrations of γ-butyrobetaine in plasma, liver and kidney of pigs are in the same order as in rats or mice (Noël et al., 1984; van Vlies et al., 2006; Ringseis et al., 2008; Koch et al., 2008) but only one half to one

fifth of those of humans (Sandor et al., 1988). This finding suggests that pigs have a lower rate of carnitine synthesis in these tissues than humans which in turn could be an explanation for the markedly lower tissues carnitine concentrations in pigs compared to humans.

The observation that γ-butyrobetaine concentrations in muscle were even higher at birth than in the subsequent weeks indicates that even the fetus has a high capacity in muscle to form γ-butyrobetaine from trimethyllysine. In humans, carnitine can be delivered in the placenta from maternal to fetal blood by OCTN2 (Lahjouji et al., 2004; Grube et al., 2007). Although such a mechanism has not yet been reported in pigs, it is likely that carnitine can cross the placenta in pregnant sows, too. Accordingly, it is possible that the high carnitine concentrations at birth could also be due to the transfer of carnitine from maternal to fetal blood during late gestation.

In humans and rats, milk intake during the early suckling period is important for the development of plasma and tissue carnitine concentrations. In the rat, carnitine concentrations in tissues are rising several-fold within a few days after birth due to carnitine intake via milk (Davis, 1989; Flores et al., 1996). The role of carnitine from milk for human infants is evident by the finding that infants of an age of 3 months fed a soy protein formula without added carnitine have markedly lower plasma carnitine concentrations than infants fed a formula supplemented with carnitine or breast-fed infants (Novak et al., 1983; Olson and Rebouche, 1987; Olson et al., 1989). Interestingly, a different picture was observed in pigs of this study. Although sow milk has even a higher carnitine concentration than human milk (120–180 vs. 30–95 μmol/L; Ramanau et al., 2005; Birkenfeld et al., 2006a; Rebouche and Paulson, 1986), concentrations of carnitine in plasma, liver and kidney in pigs were even decreasing during the suckling period. A possible explanation for this finding could be that carnitine absorbed from milk was deposited in the muscle as carnitine concentrations in skeletal muscle indeed increased during the suckling period. Normally, the movement of carnitine from blood into muscle is a slow process (Evans and Fornasini, 2003). Nevertheless, an uptake of carnitine from blood into muscle could also contribute to the decline of plasma carnitine concentration during the first weeks of life. After 4 weeks of life the pigs were switched from sows' milk to a diet with relatively low carnitine concentrations. It is likely that the decline of carnitine concentrations in all tissues after 5 weeks of life could be due, at least in part, to the lower carnitine intake by the diet. Plasma and tissue carnitine concentrations of the 7 week old pigs of the present study were close to those of pigs in a body weight range between 20 and 30 kg used in two recent studies (Fischer et al., 2009a,b).

Determination of free carnitine and carnitine esters shows that the ratio of acetyl carnitine to free carnitine in plasma, kidney and heart is highest at birth and decreases thereafter. This ratio reflects the intramitochondrial relationship between acetyl-CoA and free CoA and is very sensitive to metabolic changes in the mitochondria (Böhles et al., 1994). The high ratio at birth, which is also noted in plasma of human neonates, is thought to reflect the increased production of acetyl-CoA produced by the enhanced fatty acid oxidation in the newborn period (Warshaw and Curry, 1980; Girard et al., 1992). The reduction of this ratio in liver and kidney during the first weeks of life suggests that there is a shift from fatty acid oxidation to utilization of glucose for energy production in these tissues during this time period. Interestingly, this ratio in plasma was unexpectedly increasing at an age of 4 weeks. We have no explanation for this observation, but it is possible that the increase of this ratio was due to the switch from sow's milk to a vegetable diet with a relatively low native carnitine content.

Plasma and tissue carnitine concentrations are regulated by an interplay of absorption of carnitine from the diet, endogenous biosynthesis, uptake from blood into tissues, storage in muscle and excretion via the urine (Evans and Fornasini, 2003). Although we were not able to consider all these aspects of carnitine metabolism in detail, consideration of plasma and tissue carnitine concentrations shows that neonatal pigs in our study did not have an insufficient carnitine status. Indeed, concentrations of carnitine in plasma, liver and kidney at birth were higher than in the subsequent weeks of life which is in contrast to rats or humans (Davis, 1989; Flores et al., 1996; Rebouche, 1992). Plasma carnitine concentrations in neonatal and suckling pigs (being in the range between 14 and 21 μmol/L) were clearly below those of infants receiving carnitine-containing formulas (being in the range between 30 and 60 μmol/L, Olson et al., 1989) but they were in excess of those of growing–finishing pigs (Owen et al., 2001a) or pregnant or lactating sows (Doberenz et al., 2006; Birkenfeld et al., 2006a,b). Moreover, carnitine concentration in the liver was two-fold higher in neonatal pig compared to growing–finishing pigs with a body weight in excess of 100 kg (Owen et al., 2001a,b). The comparison of carnitine concentrations between young and older pigs clearly indicates that young pigs do not have an insufficient carnitine status.

In conclusion, the present study shows for the first time that liver and kidney are the only tissues in pigs with a considerable activity of BBD. In pigs, like in humans, these tissues might be therefore regarded as the major sites of carnitine synthesis. It is moreover shown that the activity of BBD in liver and kidney of pigs is lowest at birth. Nevertheless, carnitine concentrations in plasma, liver and kidney at birth are even higher than at the subsequent weeks. This study therefore does not confirm the view that neonatal pigs have a low carnitine status compared to older pigs.

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Effect of L-carnitine on the hepatic transcript profile in piglets as animal model

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Abstract

Background: Carnitine has attracted scientific interest due to several health-related effects, like protection against neurodegeneration, mitochondrial decay, and oxidative stress as well as improvement of glucose tolerance and insulin sensitivity. The mechanisms underlying most of the health-related effects of carnitine are largely unknown.

Methods: To gain insight into mechanisms through which carnitine exerts its beneficial metabolic effects, we fed piglets either a control or a carnitine supplemented diet, and analysed the transcriptome in the liver.

Results: Transcript profiling revealed 563 genes to be differentially expressed in liver by carnitine supplementation. Clustering analysis of the identified genes revealed that most of the top-ranked annotation term clusters were dealing with metabolic processes. Representative genes of these clusters which were significantly up-regulated by carnitine were involved in cellular fatty acid uptake, fatty acid activation, fatty acid β-oxidation, glucose uptake, and glycolysis. In contrast, genes involved in gluconeogenesis were down-regulated by carnitine. Moreover, clustering analysis identified genes involved in the insulin signaling cascade to be significantly associated with carnitine supplementation. Furthermore, clustering analysis revealed that biological processes dealing with posttranscriptional RNA processing were significantly associated with carnitine supplementation.

Conclusion: The data suggest that carnitine supplementation has beneficial effects on lipid and glucose homeostasis by inducing genes involved in fatty acid catabolism and glycolysis and repressing genes involved in gluconeogenesis.

Keywords: carnitine, pig, microarray, gene expression, liver

Background

Carnitine (3-hydroxy-4-*N, N, N*-trimethylaminobutyric acid) belongs to the class of conditionally essential nutrients and has a number of indispensable functions in intermediary metabolism. Carnitine is necessary for fatty acid metabolism due to its role in the transfer of long-chain fatty acids (acyl groups) from the cytosol into the mitochondrial matrix for subsequent β-oxidation [1]. Moreover, carnitine facilitates the transport of peroxisomal β-oxidation products to the mitochondria, the export accumulating acyl-groups and acts as a CoA buffer in mammalian cells [2]. Carnitine in the body originates from intestinal absorption from dietary sources, especially meat, fish and dairy products [3] and enzyme catalyzed endogenous synthesis [4], which involves five enzymatic steps. Given that the last enzyme required for carnitine synthesis, γ-butyrobetaine hydroxylase, is only active in liver and kidney, other tissues than liver and kidney are dependent on the active uptake of carnitine from blood into tissues which is catalyzed by novel organic cation transporters (OCTN), particularly OCTN2 which is the physiologically most important carnitine transporter [5].

In livestock animal nutrition, supplementation with L-carnitine has attracted great interest due to its ability to improve performance characteristics, such as growth rate, feed conversion ratio, protein:fat accretion [6-8]. However, L-carnitine is also of interest for human nutrition because recent studies indicated that L-carnitine exerts several other effects which may be useful for the treatment of degenerative and metabolic disorders. For instance, supplementation of L-carnitine or acyl-carnitines has been

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associated with protecting against neurodegeneration [9], age-dependent mitochondrial decay [10], and oxidative stress [11]. In addition, L-carnitine was shown to improve glucose tolerance and insulin sensitivity in animals as well as in healthy and diabetic patients [12,13]. Moreover, L-carnitine supplementation was shown to be useful for the treatment of hepatic steatosis induced by total parenteral nutrition in rodents [14] and nonalcoholic steatohepatitis in humans [15,16]. Furthermore, L-carnitine was reported to reduce hepatic inflammation and plasma levels of cytokines and acute phase proteins in patients with chronic hepatitis C [16]. The mechanisms underlying many of these beneficial effects of L-carnitine are largely unknown. Therefore, the present study was designed to gain insight into mechanisms and pathways influenced by L-carnitine. For this end, we used piglets, indicating many genetical and physiological similarities with humans, making it an optimal species to study the effect of L-carnitine on transcript profile.

We considered the liver as the target organ because it plays a central role in whole body metabolism by regulating glucose and lipid homeostasis as well as protein synthesis. In this sense, we used liver samples taken from a previous experiment with piglets [17] which were fed a either a control diet with a low native carnitine content or a diet supplemented with 500 mg/kg diet L-carnitine and performed genome-wide transcript profiling in the liver of these piglets.

Methods

Animal experiment

The animal experiment was approved by the local Animal Care and Use Committee. As described recently in more detail [17], the experiment was performed with sixteen male crossbred pigs [(German Landrace × Large White) × Pietrain] with an average body weight of 10 ± 1 (mean ± SD) kg. The pigs were assigned to two groups (control group and carnitine group) and fed experimental diets for a period of 21 days. The control group received a basal diet with a low native carnitine concentration (< 5 mg/kg) which was nutritionally adequate for growing pigs in a body weight range between 10 and 20 kg, according to the recommendations of the German Society for Nutrition Physiology (Gesellschaft für Ernährungsphysiologie, 2006). The carnitine group received the same diet supplemented with 500 mg L-carnitine (obtained from Lohmann Animal Health, Cuxhaven, Germany) per kg. Blood was collected and plasma obtained by centrifugation of the blood, and liver was excised. Plasma and liver samples were immediately stored at -80°C until analysis. A full description of diet composition, feeding regime, sample collection and carnitine analysis of diets and tissues can be found in our recent publication [17].

Carnitine analysis

Concentrations of free carnitine, acetyl carnitine and propionyl carnitine in liver of pigs fed either a control diet or a diet supplemented with 500 mg/kg carnitine for 20 days were determined by tandem mass spectrometry using deuterated carnitine-d_3 (Larodane Fine Chemicals, Malmö, Sweden) as internal standard as described recently in detail [17]. Concentration of total carnitine represents the sum of free carnitine, acetyl carnitine and propionyl carnitine.

RNA isolation and quality control

Total RNA was prepared from 20-30 mg of frozen liver tissue using the RNeasy Minikit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Afterwards, the RNA concentration and purity were estimated from the optical density at 260 and 280 nm, respectively. The A260/280 ratio of all individual samples was 1.98 ± 0.02 (mean ± SD). The integrity of the total RNA was checked by 1% agarose gel electrophoresis. RNA was judged as suitable for array hybridization only if the samples exhibited intact bands corresponding to the 18S and 28S ribosomal RNA subunits.

Microarray analysis

For microarray analyses, two RNA pools for each group (control, n = 2; carnitine group, n = 2) were prepared, with RNA from 4 animals contributing to each RNA pool. Hybridization to the Affymetrix GeneChip porcine genome arrays (Affymetrix) containing 23,937 probe sets that represent approximately 23,256 porcine transcripts and quality assessment of the hybridization process were performed at the Center of Excellence for Fluorescent Bioanalytics (KFB) at the University of Regensburg for hybridization to the Affymetrix GeneChip porcine genome arrays (Affymetrix, UK). The microarray data related to all samples have been deposited in NCBI's Gene Expression Omnibus (GEO) public repository (GEO accession number GSE22931) [18]. Data analyses and functional interpretation of microarray data using the bioinformatic tools from the Database for Annotation, Visualization and Integrated Discovery (DAVID) bioinformatic resource [19] were performed as described recently in detail [17].

Quantitative real-time RT-PCR (qPCR)

Differential expression data of selected genes, FbxL3 (F-box/LRR-repeat protein 3), FbxL20 (F-box/LRR-repeat protein 20), FbxO32 (F-box protein 32), ESRRG (estrogen-related receptor gamma isoform 2), HECTD2 (HECT domain containing 2 isoform b), DRE1 (DRE1 protein), GPD1 (Glycerol-3-phosphate dehydrogenase), MTTP (Microsomal triglyceride transfer protein), ACSL3 (Long-chain-fatty-acid-CoA ligase 3), ACADSB

(Acyl-CoA dehydrogenase, short/branched chain specific), GLUT8 (Glucose transporter type 8), GCK (Glucokinase), GPAT (Glycerol-3-phosphate acyltransferase) and USP10 (ubiquitin specific peptidase 10) obtained from Affymetrix GeneChip analysis were validated by using qPCR carried out on a Rotorgene 2000 system (Corbett Research, Mortlake, Australia). For qPCR all individual samples (n = 8/group) contributing to the RNA pools for microarray analysis were used. cDNA synthesis and qPCR analysis was performed as described recently in detail [17]. Gene-specific primers (Eurofins MWG Operon, Ebersberg, Germany) were designed using Primer3 and BLAST. Characteristics of the porcine primer pairs are listed in Table 1. Expression values of selected genes were normalized using the GeNorm normalization factor. Procedure of normalization, characteristics of gene-specific primers and the average expression stability ranking of the six potential reference genes in liver of piglets were previously described in detail [20]. Relative expression ratios are expressed as fold changes of mRNA abundance in the carnitine group compared to the control group.

Statistical analysis

Values presented in the text are means ± SD. Data were analyzed by one factorial analysis of variance with dietary carnitine concentration as factor using the Minitab statistical software (Release 13, Minitab Inc., State College, PA, USA). For statistical significant *F*-values ($P < 0.05$), means of both groups were compared by Fisher's multiple range test.

Results

Feed intake, final body weight and feed conversion ratio

Feed intake, final body weights and feed conversion ratio of piglets were not different between both groups [17].

Concentration of carnitine in the liver

Pigs supplemented with L-carnitine had approximately 10-fold higher concentrations of free and total carnitine in the liver ($P < 0.05$; Figure 1) than control pigs.

Table 1 Characteristics of porcine primer pairs used for validation of microarray analysis using RT-PCR

Gene symbol	Primer sequence (5´-3´)	GenBank accession no.	Product size (bp)
ACADSB	For:TCGTGATACCGAGGGCCTCCG	XM_001926297.2	196
	Rev:TCCCAGCATCTGTGCCGCAA		
ACSL3	For:TCGCTGCACAGGCCTGCTTC	NM_001143698.1	174
	Rev:GCAGGCGCGGCACTAGAGAG		
DRE1	For:CAACAACCTCCGATACTACC	NP_060114.2	158
	Rev:GGTCCTCCACCAATCACAAA		
ESRRG	For:GGATCAGATGAGTCTTCTGC	XM_003357621.1	127
	Rev:GGACTGGTCTTCATCCATTAT		
FbxL3	For:CATAGGAGACACACCGTCTA	Q9UKT7	637
	Rev:GTGGGCATCATGTCTGGAAA		
FbxL20	For:GTGAGGGATGTCCACTGTTG	XM_003131523.2	128
	Rev:CTGTGTGCAGCCTTTTAAGAA		
FbxO32	For:TCACAGCTCACATCCCTGAG	NM_001044588	167
	Rev:GACTTGCCGACTCTCTGGAC		
GCK	For:GAGCGAGAGAGCAGAGCCTCAGA	XM_003356680.1	221
	Rev:CTGGAGCCAGCCTCCGAACG		
GLUT8	For:GTGGAGCCCACCGATGCCAG	EU012361.2	145
	Rev:CCACGCCCTTGACGTGCAGA		
GPAT	For:GAATTGATCTCTCCACGTTG	XM_001927875.1	257
	Rev:CCTCCATGATAAAGTCGTGG		
GPD1	For:GGCCGGCTGGCACACTTTGA	NM_001190240.1	354
	Rev:CATGGGGATGCCAAGGCGCT		
HECTD2	For:GGTTTGGACAGAGGATCCAAA	XM_003361201.1	130
	Rev:CATTCTTGATGTTAGGGAAAAC		
MTTP	For:TCCCGCTGCACCAAGAGAACT	NM_214185.1	151
	Rev:TACCTCGGCACGGTGCATCGT		
USP10	For:GTGGTGTACCAGCAGAGCT	XM_003126825.2	157
	Rev:GCTTGGTTTTGGTGGTGTAG		

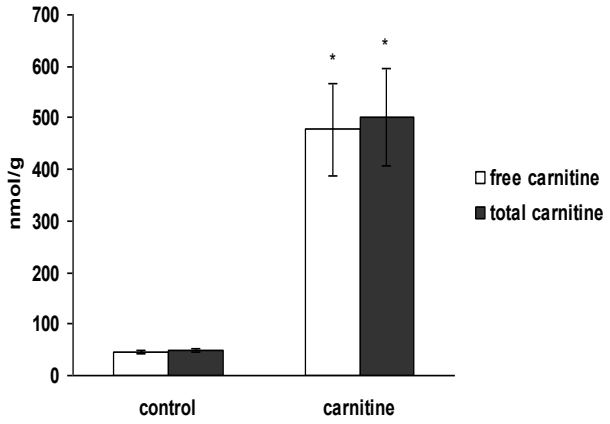

Figure 1 Concentrations of free and total carnitine in the liver of growing piglets fed either a control diet or a diet supplemented with 500 mg L-carnitine per kg diet for 21 days. Bars are mean ± SD (n = 8/group), * indicates significant differences (P < 0.05) between groups (Fisher's multiple range test).

Identification of differentially expressed genes

A total of 638 probe sets were differentially expressed (fold change ≥ 2.0 and ≤ -2.0) between the L-carnitine and the control group. Of these probe sets, 372 were up-regulated by L-carnitine and 266 probe sets were down-regulated by L-carnitine. The 15 most strongly up-regulated and down-regulated probe sets are shown in Table 2. Because the Affymetrix GeneChip porcine genome array is poorly annotated, the differentially expressed probe sets were largely annotated by the annotation list created by Tsai et al. [21]. 563 porcine Affymetrix probe sets were matched to human RefSeq entries and converted into human Affymetrix probeset IDs. Conversion of porcine Affymetrix probe set IDs into the human probe set IDs was necessary for subsequent analysis by the DAVID bioinformatic resource because this platform cannot use porcine gene information. The distribution of signal intensities of the differentially expressed probe sets of the two control and carnitine arrays are shown in Figure 2.

Real-time RT-PCR verification of microarray data

A total of 14 genes were selected to validate the microarray data by the use of real-time RT-PCR. As shown in Table 3, all tested genes were differentially expressed with microarray analysis, however 3 genes were not significant at an FDR P value < 0.05. The magnitude of differential expression tended to be higher by qPCR than those obtained from microarray analysis.

Identification of overrepresented annotation terms

563 genes identified to be differentially expressed were used for gene-term enrichment analysis using the DAVID Functional Annotation Chart tool. The analyses were based on the GO category biological process. The GO analysis assigned the 563 differentially expressed genes to 21 biological processes (p-value < 0.01). Most genes were allocated to the annotation terms cellular process (357 genes), metabolic process (262 genes), and cellular metabolic process (239 genes). The most significantly enriched annotation terms were (top-ranked: lowest p-values): cellular process (P = 2.0E-04), ribosome biogenesis (P = 3.8E-04), developmental process (P = 4.0E-04), ribonucleoprotein complex biogenesis (P = 4.6E-04), cellular metabolic process (P = 6.5E-04), multicellular organismal development (P = 1.5E-03), cellular protein metabolic process (P = 1.9E-03), rRNA processing (P = 2.1E-03), and rRNA metabolic process (P = 2.8E-03). The annotation terms with the highest fold enrichment were triglyceride metabolic process (4.5-fold), rRNA processing (3.5-fold), ribosome biogenesis (3.4-fold), and rRNA metabolic process (3.4-fold).

Identification of clusters of functionally related annotation terms

To identify clusters of functionally related biological processes we used the DAVID functional annotation clustering tool. Clusters were ranked according to the enrichment score for each cluster reflecting the geometric mean of all the enrichment p-values (EASE scores) of each annotation term in the cluster. The 10 top-ranked clusters showing the highest enrichment scores are shown in Table 4. The top-ranked clusters allocated annotation terms dealing with RNA splicing (cluster 1), ribosomal RNA processing and non-coding RNA processing (cluster 2), triglyceride, acylglycerol, neutral lipid and glycerol ether metabolism (clusters 3 and 4) and regulation of glucose import and glucose transport (cluster 5). Further clusters summarized annotation terms dealing with chromosome localization (cluster 6), posttranslational protein folding (cluster 7), carbohydrate biosynthetic processes (cluster 8) and organic acid metabolic processes (cluster 9), and modification-dependent protein catabolic process and proteolysis involved in cellular protein catabolic process (cluster 10).

Discussion

In the present study we aimed to get insight into potential mechanisms of L-carnitine by applying genome-wide transcript profiling in the liver of piglets. After feeding carnitine supplemented diets for 3 weeks, concentrations of free and total carnitine in the liver of the piglets were markedly increased (approximately 10-fold) compared to piglets fed diets without supplemental L-carnitine, indicating that the supplemental L-carnitine significantly improved the carnitine status of the piglets. As a main result we observed that 563 genes were differentially

Table 2 The 15 most strongly up- and down-regulated genes in the liver of growing piglets fed with or without L-carnitine

Probe set ID	Gene name (Gene symbol)	FC*	Probe set ID	Gene name (Gene symbol)	FC*
Up-regulated genes			*Down-regulated genes*		
Ssc.16377.2.A1_at	Glutathione S-transferase A3-3 (GSTA3)	129.6	Ssc.27111.1.A1_at	Kelch-like protein 8 (KLHL8)	-7.9
Ssc.18484.1.S1_at	Hexokinase D (GCK)	26.5	Ssc.30350.1.A1_at	Homeobox protein Meis1 (MEIS1)	-6.6
Ssc.14503.1.S1_at	Apolipoprotein A-IV precursor (ApoA4)	16.6	Ssc.15845.1.S1_at	Mannose-binding protein C precursor (MBP-C)	-5.8
Ssc.13302.1.A1_at	Sentrin-specific protease 6 (SENP6)	13.0	Ssc.451.1.A1_at	Insulin-like growth factor binding protein 1 precursor (IGFBP-1)	-5.8
Ssc.12965.1.A1_at	Sprouty homolog 3 (SPRY3)	11.8	Ssc.22959.1.S1_at	Phosphoenolpyruvate carboxykinase, cytosolic (PCK1)	-5.7
Ssc.30459.1.A1_at	R3H domain protein 1 (R3HDM)	11.3	Ssc.24758.1.A1_at	estrogen-related receptor gamma isoform 2 (ESRRG)	-5.3
Ssc.25850.1.A1_at	Telomerase-binding protein p23 (TEPB)	9.2	Ssc.21169.1.S1_at	Synaptic vesicular amine transporter (SLC18A2)	-5.1
Ssc.5327.2.A1_at	Cytochrome P450 2J2 (CYP2J2)	9.2	Ssc.20502.1.S1_at	Serine/threonine-protein kinase (ULK1)	-5.0
Ssc.9177.1.A1_at	SPARC related modular calcium-binding protein 1 precursor (SMOC1)	8.5	Ssc.29392.1.A1_at	DRE1 protein (DRE1)	-4.7
Ssc.30207.1.A1_at	Ubiquitin carboxyl-terminal hydrolase 1 (USP1)	8.3	Ssc.14386.1.A1_at	Cyclin G2 (CCNG2)	-4.7
Ssc.8700.1.A1_at	Heterogeneous nuclear ribonucleoprotein M (hnRNP M)	8.1	Ssc.29946.1.A1_at	T-cell lymphoma breakpoint-associated target 1 (TCBA1)	-4.2
Ssc.8308.1.A1_at	cell adhesion molecule with homology to L1CAM precursor (CHL1)	7.6	Ssc.28087.1.A1_at	oxidation resistance 1 (OXR1)	-4.2
Ssc.18681.1.A1_at	Metabotropic glutamate receptor 5 precursor GRM5)	7.5	Ssc.2274.1.A1_at	Ephrin-A1 precursor (EFNA1)	-4.1
Ssc.29205.1.A1_at	Serine/threonine-protein kinase (Nek7)	7.3	Ssc.13343.1.A1_at	CD109 (CD109)	-4.1
Ssc.18831.1.A1_at	Glutaminase, kidney isoform, mitochondrial precursor (GLS)	6.8	Ssc.30210.1.A1_at	Testican-1 precursor (SPOCK)	-4.0

* FC = fold change

expressed by L-carnitine. This shows that supplemental L-carnitine influences gene expression in the liver of piglets and indicates that at least some of the biological effects of L-carnitine are mediated by altering gene transcription. To extract biological meaning from the observed alterations in gene expression we performed gene term enrichment analysis and functional clustering analysis with the 563 differentially expressed genes. Gene term enrichment analysis revealed that the most frequent biological processes associated with L-carnitine supplementation were dealing with metabolic processes. This was not surprising considering that the main function of L-carnitine is to stimulate energy metabolism by acting as shuttling molecule for long-chain fatty acids which also enhances the metabolic flux of glucose through the glycolytic chain. This was also confirmed by clustering analysis showing that 6 out of the 10 top-ranked clusters were dealing with metabolic processes. Representative genes from one of these clusters dealing with metabolic processes (carboxylic acid metabolic process, oxoacid metabolic process, organic acid metabolic process) encoded proteins or enzymes involved in cellular fatty acid uptake (SLC27A6, solute carrier family 27/fatty acid transporter, member 6), fatty acid activation (ACSL3, Long-chain-fatty-acid-CoA ligase 3) and fatty acid β-oxidation (ACADSB, Acyl-CoA dehydrogenase, short/branched chain specific), and most of these genes including SLC27A6, ACSL3 and ACADSB were found to be significantly up-regulated by L-carnitine supplementation by microarray analysis and confirmed by qPCR, a more sensitive method for gene expression analysis. Thus, our data indicate that the well-known stimulatory effect of carnitine on fatty acid β-oxidation [22,23] is at least partially mediated by stimulating the transcription of genes involved in cellular fatty acid uptake, fatty acid activation and β-oxidation.

Clustering analysis further revealed that L-carnitine supplementation was significantly associated with biological processes involved in glucose metabolism, like glucose transport, conversion of glucose into glucose 6-

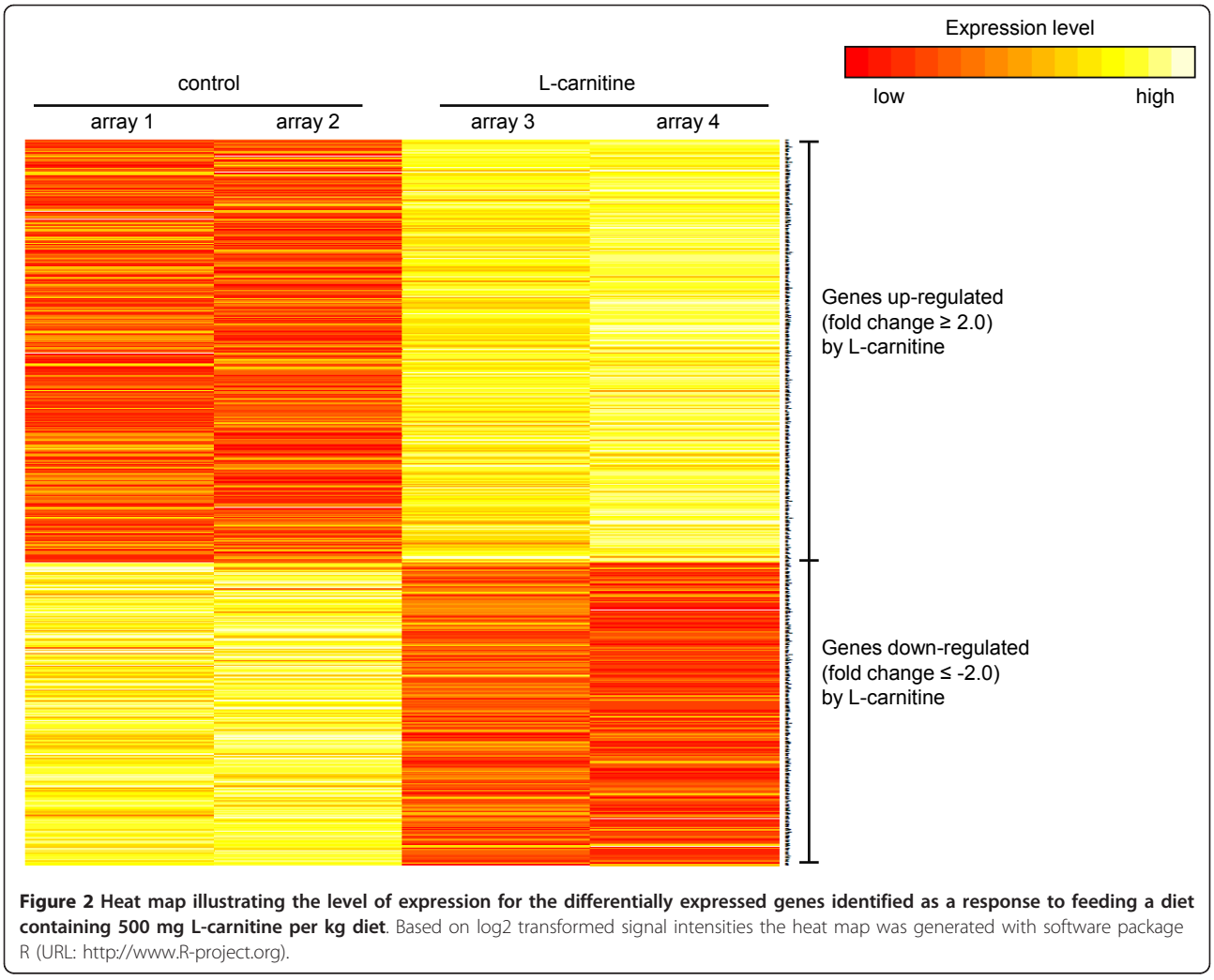

Figure 2 Heat map illustrating the level of expression for the differentially expressed genes identified as a response to feeding a diet containing 500 mg L-carnitine per kg diet. Based on log2 transformed signal intensities the heat map was generated with software package R (URL: http://www.R-project.org).

Table 3 qPCR and microarray gene expression analyses of liver tissue

Gene symbol	Mean fold changes	P-value	
	Microarray	qPCR	qPCR
ACADSB	2.24	4.61	0.057
ACSL3	2.15	3.48	0.001
DRE1	-4.8	-3.3	0.018
ESRRG	-5.6	-12.5	0.003
FbxL20	-2.9	-14.3	0.002
FbxL3	-2.4	-4.4	0.026
FbxO32	-3.6	-3.4	0.009
GCK	26.53	1.97	0.107
GLUT8	3.55	4.55	0.018
GPAT	-2.84	-1.67	0.173
GPD1	2.87	3.11	0.031
HECTD2	-2.9	-4.6	0.004
MTTP	2.14	1.75	0.031
USP10	-2.2	-3.2	0.026

phosphate, and glycolysis, and hexose biosynthetic processes, like gluconeogenesis. Representative genes included GLUT8 (glucose transporter type 8), GCK (Hexokinase D), GPD1 (Glycerol-3-phosphate dehydrogenase), PCK1 (Phosphoenolpyruvate carboxykinase), and FBP2 (Fructose-1,6-bisphosphatase isozyme 2). Moreover, the tandem enzyme PFKFB3 (6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3) which is responsible for maintaining the cellular levels of fructose-2,6-biphosphate, the most potent allosteric activator of one of the key regulatory enzymes of glycolysis, 6-phosphofructo-1-kinase, was also identified to be differentially expressed by L-carnitine. All the genes dealing with glucose metabolism like GLUT8, GCK and GPD1 were markedly up-regulated, at least 4-fold, by the supplemental L-carnitine. These strongly up-regulation could also observed by qPCR, supporting microarray analysis. GCK which is the predominant hexokinase isoenzyme in the liver phosphorylating glucose for

Table 4 Identification of functionally related annotation groups (GO category biological process)

Cluster	GO terms	P-value
1	RNA splicing, via transesterification reactions	7.8E-03
	RNA splicing, via transesterification reactions with bulged adenosine as nucleophile	7.8E-03
	nuclear mRNA splicing, via spliceosome	7.8E-03
2	ribosomal RNA processing	2.1E-03
	ribosomal RNA metabolic process	2.8E-03
	non-coding RNA processing	1.2E-01
3	triglyceride metabolic process	9.9E-03
	acylglycerol metabolic process	1.7E-02
	neutral lipid metabolic process	1.8E-02
	glycerol ether metabolic process	2.0E-02
	organic ether metabolic process	2.3E-02
4	triglyceride biosynthetic process	3.0E-02
	neutral lipid biosynthetic process	5.1E-02
	acylglycerol biosynthetic process	5.1E-02
	glycerol ether biosynthetic process	6.0E-02
5	positive regulation of glucose import	2.9E-02
	positive regulation of glucose transport	2.9E-02
	regulation of glucose import	8.0E-02
	regulation of glucose transport	8.6E-02
6	mitotic metaphase plate congression	2.9E-02
	metaphase plate congression	4.3E-02
	chromosome localization	7.6E-02
	Establishment of chromosome localization	7.6E-02
7	chaperone mediated protein folding requiring cofactor	5.3E-02
	'de novo' posttranslational protein folding	5.3E-02
	'de novo' protein folding	5.3E-02
8	hexose biosynthetic process	7.1E-01
	monosaccharide biosynthetic process	7.1E-01
	alcohol biosynthetic process	7.1E-01
	cellular carbohydrate biosynthetic process	7.1E-01
	carbohydrate biosynthetic process	7.1E-01
9	carboxylic acid metabolic process	1.9E-01
	oxoacid metabolic process	1.9E-01
	organic acid metabolic process	2.0E-01
10	modification-dependent protein catabolic process	2.4E-01
	modification-dependent macromolecule catabolic process	2.4E-01
	proteolysis involved in cellular protein catabolic process	3.0E-01
	cellular protein catabolic process	3.1E-01
	protein catabolic process	3.7E-01

subsequent metabolism by either glycolysis, pentose phosphate shunt or glycogen synthesis was even induced 27-fold by L-carnitine supplementation - indicating that L-carnitine has a dramatic effect on glucose metabolism. Several studies have already shown that carnitine supplementation increases glucose disposal and glucose oxidation in animals as well as in healthy and diabetic patients [12,24,25] due to activation of the pyruvate dehydrogenase complex [26]. Besides allosteric regulation of the activity of enzymes of the glycolytic pathway, it is also well known that the flux through the glycolytic pathway can be increased by induction of the rate-limiting enzymes of this pathway, GCK and PFK1 (6-phosphofructo-1-kinase). Thus, our observations suggest that up-regulation of genes involved in glucose uptake, glucose phosphorylation and glycolysis also contributes to increased glucose oxidation by supplemental L-carnitine. In contrast to the genes involved in glucose metabolism, genes involved in gluconeogenesis, like PCK1 and FBP2 were significantly down-regulated in the liver of piglets by L-carnitine supplementation. This indicates that the positive effect of carnitine on glucose utilization is explained not only by stimulation of glycolysis but also suppression of gluconeogenesis in the liver. It has been recently shown in rats that dietary L-carnitine is capable of restoring an increase in the activity of the gluconeogenic enzymes PCK1, FBP2 and glucose 6-phosphatase caused by feeding fructose [27], which increases the availability of the gluconeogenic substrates pyruvate, lactate and glycerol. The present findings suggest that inhibition of transcription of gluconeogenic genes by L-carnitine also contributes to the suppression of gluconeogenesis by carnitine, which has not been demonstrated yet. The exact reason for this effect of L-carnitine remains to be established. However, it has been suggested that the inhibitory effect of L-carnitine on gluconeogenic enzyme activities is the consequence of an improvement in the action of insulin [12,28], which is a known repressor of expression of gluconeogenic genes. Supportive of this assumption is also the identification of another annotation term cluster dealing with positive regulation of glucose import. Considering that insulin is the most important regulator of glucose import, it was not surprising that genes belonging to this cluster were involved in insulin signaling, like IRS2 (Insulin receptor substrate-2), PIK3R1 (Phosphatidylinositol 3-kinase regulatory alpha subunit), and ERBB3 (Receptor protein-tyrosine kinase erbB-3 precursor). Besides regulation by insulin action, key gluconeogenic enzymes such as FBP2 are also known to be subject to complex allosteric regulation. Noteworthy, one of the allosteric inhibitors of FBP2 is fructose-2,6-biphosphate whose cellular levels are controlled by the abovementioned tandem enzyme PFKFB3. Thus, the activity of gluconeogenic enzymes may be influenced by L-carnitine through regulating the cellular availability of allosteric enzyme regulators as well.

Two other identified annotation term clusters were dealing with triglyceride metabolic and triglyceride biosynthetic processes. Representative genes were GPAT, which esterifies acyl-groups from acyl-ACP to the sn-1 position of glycerol-3-phosphate, an essential step in glycerolipid biosynthesis, and MTTP, which catalyses the transport of triglyceride, cholesteryl ester, and phospholipid between phospholipid surfaces, and is required for the secretion of apolipoprotein B containing lipoproteins from the liver. In rodents, L-carnitine administration was demonstrated to decrease liver lipids and hepatic steatosis after administration of a high fat diet, after parenteral nutrition, or after alcohol intoxication [14,29,30], with the mechanisms of action being largely unknown. Our observations that GPAT was down-regulated whereas MTTP and genes involved in fatty acid catabolism (SLC27A6, ACSL3, ACADSB) were up-regulated by L-carnitine supplementation in the liver of the piglets, confirmed by qPCR. Thus, our data indicates that inhibition of glycerolipid biosynthesis and stimulation of lipoprotein secretion and fatty acid catabolism may contribute to the decreased liver lipids in rodents fed L-carnitine.

Besides metabolic processes, clustering analysis revealed that biological processes dealing with posttranscriptional RNA processing (mRNA splicing, ribosomal RNA processing, non-coding RNA processing) were significantly associated with L-carnitine supplementation. Noteworthy, almost all of these genes were significantly up-regulated in the liver of the piglets by the supplemental L-carnitine indicating that the biological functions exerted by the encoded proteins are stimulated by L-carnitine. Posttranscriptional RNA processing, which includes precursor-mRNA splicing, is one of the main regulatory mechanisms of gene expression which results in a repertoire of mRNAs, and consequently of proteins, much larger than expected from the number of genes. This process contributes substantially to cell-specific and tissue-specific gene expression, and it is estimated that over 60% of human genes are alternatively spliced [31]. Although the exact biological meaning of the up-regulation of genes dealing with posttranscriptional RNA processing by L-carnitine is unclear, it is obvious that L-carnitine supplementation has a stimulatory effect on this important regulatory mechanism of gene expression. It is therefore possible that the alterations in gene expression observed with L-carnitine supplementation are mediated, at least partially, by modulating posttranscriptional RNA processing.

As far as the physiological relevance of the carnitine dose (500 mg/kg) used in this study is concerned, it has

to be noted that omnivorous humans are reported to generally ingest 0.3-1.9 mg of carnitine per day and kg of body weight [32]. Based on a daily feed consumption of approximately 500 g per day and a final body weight of the piglets of about 17 kg this relates to 15 mg of carnitine/kg body weight indicating that the carnitine dose applied to the piglets was at least 8-fold higher than achieved in humans by a normal diet. However, when compared to several clinical trials in which free carnitine was supplemented up to 3 g per day and subject [33-35], which relates to 40 mg carnitine/kg body weight for a human weighing 70 kg, the dose applied in our study can be regarded as low. Therefore, the beneficial effects of carnitine supplementation on several metabolic parameters observed in the abovementioned clinical trials may be at least partially explained by the alterations in gene expression found in our pig model. In several clinical trials, carnitine supplementation was shown to improve glucose homeostasis and insulin sensitivity in obese, insulin resistant and diabetic subjects [36,37]. Due to theses effects carnitine supplementation has gained significant attention as a tool for the treatment or prevention of insulin resistance and type 2 diabetes mellitus [38]. The improvement of glucose tolerance has been explained by a normalization of mitochondrial fuel metabolism, which is perturbed during insulin resistance due to an intracellular accumulation of acyl-CoA derivatives. Recent evidence indicates that carnitine supplementation corrects these mitochondrial perturbations through an increased efflux of acylcarnitine out of mitochondria and cells into the blood [39]. Based on our transcriptomic data it cannot be evaluated whether or not acyl-carnitine efflux and mitochondrial function was stimulated by carnitine in the piglets of this study. However, since convincing evidence from both human and animal studies suggests that carnitine lowers "mitochondrial stress" and improves mitochondrial function, in particular in the face of energy surplus, we suggest that carnitine also improved mitochondrial function in our pig model.

In conclusion, results of the present study show that supplemental L-carnitine influences gene expression in the liver of growing piglets. Our data suggest that L-carnitine supplementation has beneficial effects on lipid and glucose homeostasis by inducing genes involved in fatty acid catabolism and glycolysis and repressing genes involved in gluconeogenesis. In addition, the data indicate that the effects of L-carnitine on transcription of glycolytic and gluconeogenic genes are mediated by potentiating the action of insulin and that at least some of the alterations in gene expression observed with L-carnitine supplementation are mediated by modulating posttranscriptional RNA processing. Regarding the strong similarities between pigs and humans with regard to metabolism, our data obtained in piglets can be considered as relevant for humans. Future studies employing both, transcriptomics and metabolomics are required to confirm a correlation between alterations in gene expression and modulation of carbon fluxes through metabolic pathways.

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Authors' contributions
RR and KE designed research and coordinated the study; JK carried out the feeding experiment and the molecular biological analyses; HK supervised the feeding experiment and performed the chemical analysis of experimental diets; JK and RR performed bioinformatical data analyses; SP and RG were involved in microchip data visualization; JK, RR and KE wrote the paper. KE had primary responsibility for final content. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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RESEARCH ARTICLE

Dietary L-carnitine alters gene expression in skeletal muscle of piglets

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Scope: Carnitine improves protein accretion, muscle mass, and protein:fat accretion in piglets. The underlying mechanisms, however, are largely unknown.
Methods and results: To gain insight into mechanisms through which carnitine exerts these effects, we fed piglets either a control or a carnitine-supplemented diet, and analyzed the transcriptome in skeletal muscle. Carnitine concentrations in plasma and muscle were about four-fold higher in the carnitine group when compared to the control group. Transcript profiling revealed 211 genes to be differentially expressed in muscle by carnitine supplementation. The identified genes were mainly involved in molecular processes such as cytoskeletal protein binding, insulin-like growth factor (IGF) binding, transcription factor activity, and insulin receptor binding. Identified genes with the molecular function transcription factor activity encoded primarily transcription factors, most of which were down-regulated by carnitine, including pro-apoptotic transcription factors such as proto-oncogene c-fos, proto-oncogene c-jun and activating transcription factor 3. Furthermore, atrophy-related genes such as atrogin-1, MuRF1, and DRE1 were significantly down-regulated by carnitine. IGF signalling and insulin signalling were identified as significantly up-regulated regulatory pathways in the carnitine group.
Conclusion: Carnitine may have beneficial effects on skeletal muscle mass through stimulating the anabolic IGF-1 pathway and suppressing pro-apoptotic and atrophy-related genes, which are involved in apoptosis of muscle fibers and proteolysis of muscle proteins, respectively.

Keywords:
Carnitine / Gene array / Gene expression / Muscle / Pig

1 Introduction

Carnitine is a water soluble quaternary amine (3-hydroxy-4-*N,N,N*-trimethylaminobutyric acid), which is essential for normal function of all tissues. The most documented function is its role in energy homeostasis by acting as a shuttling molecule for the translocation of long-chain fatty acids (acyl groups) from the cytosol into the mitochondrial matrix, which is referred to as the carnitine shuttle, for subsequent β-oxidation [1, 2]. Moreover, carnitine regulates the intramitochondrial acyl-CoA/CoA ratio and acts as a CoA buffer in mammalian cells [3]. Carnitine is derived from dietary sources and endogenous biosynthesis [4, 5]. It is taken up from blood into tissues by novel organic cation transporters (OCTN), particularly OCTN2 which is the physiologically most important carnitine transporter [6, 7]. The fact that inborn or

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Abbreviations: DAVID, database for annotation, visualization, and integrated discovery; **FOS,** proto-oncogene c-fos; **GO,** gene ontology; **IGF,** insulin-like growth factor; **JUN,** proto-oncogene c-jun; **MuRF1,** muscle RING finger-1 protein; **OCTN2,** novel organic cation transporters

acquired defects of OCTN2 lead to primary or secondary systemic carnitine deficiency demonstrates the essential role of these transporters for reabsorption of carnitine in the kidney and delivery of carnitine from blood into body cells [6].

Supplementation of L-carnitine was reported to cause several pleiotropic and often beneficial effects. For instance, supplementation of L-carnitine or various acyl-carnitines has been associated with protecting against neurodegeneration and mitochondrial decay resulting from aging [8, 9]. Feeding trials showed that L-carnitine supplementation is capable of improving performance characteristics of several livestock or sport animals such as horses [10, 11], dairy cows or steers [12, 13], laying hens and broilers [14, 15]. In addition, in sows L-carnitine supplementation was shown to improve their reproductive performance, *i.e.* L-carnitine increases birth weights of piglets born to sows fed carnitine [16–18]. Moreover, beneficial effects of L-carnitine supplementation on performance characteristics were also reported for growing–finishing pigs [19–21] and, in particular, in suckling and weanling piglets [19, 20, 22–24]. In piglets, an improvement in the rate of protein accretion and an increase in the rate of protein:fat accretion was reported with increasing dietary carnitine concentrations [23]. Similar effects were observed by Owen *et al.* [19, 24], which reported a decrease in daily fat accretion rates when pigs were fed with increasing dietary carnitine concentrations during the nursery phase. Moreover, Heo *et al.* [20] observed an improvement in daily nitrogen retention, an increase in daily protein accretion and a decrease in carcass fat concentration when piglets were fed carnitine. In parts, the effects of L-carnitine on growth performance in piglets have been attributed to an increase in available energy to the growing piglet through an improvement in fatty acid oxidation which likely enhances the energy availability for protein accretion and/or growth [23]. It is however unclear whether L-carnitine causes additional effects in skeletal muscle, beyond its stimulatory role on energy metabolism, which might also contribute to its beneficial effect on protein:fat accretion. The aim of the present study, therefore, was to gain insight into mechanisms and pathways through which L-carnitine exerts potential effects in skeletal muscle. For this end we performed a feeding experiment with growing piglets that were fed either a control diet with a low native L-carnitine concentration or the control diet with the addition of supplemental L-carnitine and performed genome-wide transcription profiling in skeletal muscle of these pigs.

2 Materials and methods

2.1 Animals

Sixteen male crossbred pigs [(German Landrace × Large White) × Pietrain] with an average body weight of 10 (±1) kg at four weeks of age were used. The animals were used for an experiment which lasted 21 days. They were kept in an environmentally controlled facility with a temperature of 23°C, a relative humidity between 55 and 60%, and light from 06:00 to 19:00 h.

2.2 Experimental design

One day before the start of the experimental feeding period the pigs were weighed and randomly assigned to two groups of eight animals each. One group received a basal diet which was nutritionally adequate for growing pigs in a body weight range between 10 and 20 kg [25] containing (in g/kg diet) wheat (490), barley (201.4), soy bean meal, 44% CP (240), soy oil (30), calcium carbonate (1.5), sodium chloride (0.3), mineral feed (30), lysine-HCL (0.5), DL-methionine (0.8), L-threonine (0.3), L-tryptophan (0.2), and titanium dioxide (5). This diet contained 13.6 MJ metabolisable energy and 179 g crude protein *per* kg. Concentration of crude protein in the diets was analysed according to the official German VDLUFA methodology [26]. The metabolisable energy of the diet was calculated as recommended by the German Nutrition Society [27]. The native carnitine concentration of the diet was low (below 5 mg/kg as determined by liquid chromatography-tandem mass spectrometry [28]). The treatment group received the same diet supplemented with 500 mg L-carnitine (obtained from Lohmann Animal Health, Cuxhaven, Germany) *per* kg. All pigs were housed in pairs in flat-deck pens. During this time, the animals were given free access to the diets. To control the feed intake, unconsumed feed was weighed weekly. At the evening of day 20 the feed was removed and at the morning of day 21, body weights of all pigs were recorded. They received 300 g of feed and were killed 2.5 h thereafter. Water was available ad libitum from a nipple drinker system during the whole experiment. All experimental procedures were approved by the local Animal Care and Use Committee.

2.3 Sample collection

The animals were anaesthesised and exsanguinated 2.5 h after their last meal. Blood samples were collected into heparinised polyethylene tubes. Muscle samples (M. longissimus dorsi) were excised. Plasma was obtained by centrifugation of the blood samples (1100 g, 10 min, 4°C). Plasma and muscle samples were stored at −80°C pending further analysis.

2.4 Carnitine analysis

To investigate the carnitine status of the pigs after the 20-day feeding period, concentrations of free and total carnitine in plasma and muscle were determined by liquid chromatography-tandem mass spectrometry using deuterated carnitine-d3 (Larodane Fine Chemicals, Malmö, Sweden) as internal standard as described recently [28].

2.5 RNA isolation and quality control

Total RNA was isolated from muscle samples using the RNeasy Fibrous Tissue Minikit (Qiagen, Hilden, Germany). After RNA isolation, concentration and purity were estimated from the optical density at 260 and 280 nm, respectively. The integrity of the total RNA was checked by 1% agarose gel electrophoresis. RNA was judged as suitable for array hybridization only if the samples exhibited intact bands corresponding to the 18S and 28S ribosomal RNA subunits.

2.6 Microarray analysis

For microarray analyses, two RNA pools, for the control group and the carnitine group each, were used. Each RNA pool comprised RNA from four animals. The RNA pools were sent to the Center of Excellence for Fluorescent Bioanalytics (KFB) at the University of Regensburg for hybridization to the Affymetrix GeneChip porcine genome arrays (Affymetrix, UK). The Affymetrix GeneChip porcine genome array contains 23 937 probe sets that interrogate approximately 23 256 transcripts from 20 201 *S. scrofa* genes. In brief, total RNA was checked for quality and quantity using an Agilent Bioanalyzer 2100 machine, and total RNA was transcribed to first- and second-strand cDNAs. After purification and testing on an Agilent Bioanalyzer 2100 machine, the double-stranded cDNA served as a template for the *in vitro*-transcription reaction for cRNA synthesis. The cRNA was labeled with biotin using the Affymetrix GeneChip labeling kit. After checking the quality and quantity of the labeled cRNA, cRNA was fractionated and hybridized with the Affymetrix GeneChips. GeneChips were washed and stained with the Affymetrix GeneChip Fluidics Station 450. The GeneChips were then scanned with an Affymetrix GeneChip scanner 3000. All procedures were performed according to Affymetrix protocols (GeneChip expression analysis, Technical manual from Affymetrix). The quality of hybridization was assessed in all samples following the manufacturer's recommendations. The microarray data related to all samples have been deposited in NCBI's Gene Expression Omnibus public repository [29].

2.7 Data analyses and functional interpretation of microarray data

After scanning the arrays, cell intensity files containing a single intensity value for each probe cell were computed from the image data with the Affymetrix GeneChip Operating Software. Probe cell intensity data were further analyzed in the Affymetrix Expression Console 1.1 software using the Affymetrix Microarray Suite Version 5.0 (MAS 5.0) algorithm to create chip files. In the MAS 5.0 algorithm a global scaling strategy is applied where the average signal intensity of all probe sets is scaled to a default target signal and a detection *p*-value and detection call (present, marginal, absent) for each probe set is calculated. Probe sets with absent detection calls in all four arrays were eliminated from further analysis. In addition, probe sets with disparate detection calls (one absent call and one present call) within the same treatment group were also not considered for data analysis. Of the remaining probe sets, those that had a signal log ratio of ≥ 1.0 and ≤ -1.0 corresponding to a fold change of ≥ 2.0 and ≤ -2.0 between the carnitine group and the control group were designated as differentially expressed. Identified probe sets were annotated by a published annotation list [30].

To extract biological meaning from the identified differentially expressed genes, we used the bioinformatic tools from the Database for Annotation, Visualization, and Integrated Discovery (DAVID) bioinformatic resource [31]. The DAVID Functional Annotation Chart tool was used for gene-term enrichment analysis with a modified Fisher's exact test (EASE score) in order to identify enriched (overrepresented) Gene Ontology (GO) terms with the differentially expressed genes. GO terms were ranked according to their *p*-values (EASE scores) describing the significance of gene-term enrichment. Only GO terms with an EASE score <0.1 were considered. GO terms constitute a controlled vocabulary of biological processes, molecular functions and cellular components for gene products [32]. GO has been widely used as a tool for the interpretation of microarray differential gene expression by grouping genes according to mapped GO terms. The DAVID Functional Annotation Chart tool was also used to identify enriched regulatory pathways with the differentially expressed genes by the integrated analysis of molecular interaction network databases such as Biocarta. In addition, the DAVID Functional Annotation Clustering tool was used for functionally clustering similar terms associated with the differentially expressed genes by integrated analysis of the GO database. The grouping algorithm is based on the hypothesis that similar annotations should have similar gene members. The DAVID Functional Annotation Clustering tool integrates κ-statistics, which is a chance corrected measure of agreement between two sets of categorized data, to measure the degree of the common genes between two annotations, and fuzzy heuristic clustering to classify the groups of similar annotations according κ-values. The enrichment score for each annotation group, which is the geometric mean of all enrichment *p*-values (EASE scores) of each annotation term in the group, was used to rank the importance of the identified clusters.

2.8 Quantitative real-time RT-PCR (qPCR)

Differential expression data of selected genes obtained from Affymetrix GeneChip analysis were validated by using real-time RT-PCR carried out on a Rotorgene 2000 system (Corbett Research, Mortlake, Australia). For this end, 1.2 μg of total RNA from all individual samples ($n = 8$/*per* group) contributing to the RNA pools for microarray analysis was subjected to cDNA synthesis. Real-time RT-PCR analysis of selected genes

was performed by the use of gene-specific primers (Table 1) and the cDNA as a template as described recently in detail [33]. Expression values of selected genes were normalized to the individual expression of the housekeeping gene β-actin. Relative expression ratios are expressed as fold changes of mRNA abundance in the carnitine group compared with the control group.

2.9 Statistical analysis

Values presented in the text are means ± SD. Treatment effects were analyzed using one-way analysis of variance. For significant *F*-values, means were compared by Fisher's multiple range test. Differences with $p < 0.05$ were considered significant.

3 Results

3.1 Feed intake, final body weight development and feed conversion ratio

Feed intake, final body weights after three weeks and feed conversion ratio were not different between both groups. Food intake was 489 ± 121 g/day and 576 ± 90 g/day, final weights were 17.2 ± 3.3 kg and 16.9 ± 3.9 kg, and feed conversion ratio were 1.35 ± 0.13 g feed/g body weight gain and 1.41 ± 0.29 g feed/g body weight gain in the control group and the carnitine group, respectively (means ± SD, $n = 8$).

3.2 Concentrations of carnitine in plasma and skeletal muscle

Pigs supplemented with L-carnitine had higher concentrations of free and total carnitine in muscle and plasma than control pigs (Fig. 1).

3.3 Identification of differentially expressed genes

A total of 231 probe sets were differentially expressed (fold change ≥2.0 and ≤−2.0) between the L-carnitine and the control group. Of these probe sets, 83 were up-regulated by L-carnitine. Only nine of these, equivalent to 3.9 % of the 231 identified probe sets, which are shown in Table 2, showed a fold change of ≥4.0. Hundred and forty-eight probe sets were down-regulated by carnitine. Eighteen of these probe sets, equivalent to 7.8% of the 231 identified probe sets, showed a fold change ≤ −4.0 (Table 2). Because the Affymetrix GeneChip porcine genome array is poorly annotated, the differentially expressed probe sets were largely annotated by the annotation list created by Tsai *et al.* [30]. This annotation of the porcine probe sets is based on BLAST comparison of EnsEMBL human cDNA and genomic sequences and the Affymetrix porcine target sequence, which were extended with porcine sequence information of the Pig Gene Index (Institute for Genome Research, TIGR). Two hundred and twenty-seven porcine Affymetrix probe sets were matched to human RefSeq entries, from which 211 could be converted into human Affymetrix probeset IDs. Conversion of porcine Affymetrix probe set IDs into the human probe set IDs was necessary for subsequent analysis by the DAVID bioinformatic resource because this platform cannot use porcine gene information. The distribution of signal intensities of the differentially expressed probe sets of the two control and carnitine arrays are shown in Fig. 2.

3.4 Real-time RT-PCR verification of microarray data

Five genes (SERTAD1, RXRγ, atrogin-1, FOS, DRE1) were randomly selected to validate the microarray data by the use of real-time RT-PCR. As shown in Table 3, the expression patterns of these genes observed by microarray analysis could be confirmed by real-time RT-PCR analysis. However, the fold changes obtained from real-time RT-PCR analysis for atrogin-1 were higher and those for FOS and DRE1 were slighty lower than those obtained from microarray analysis.

3.5 Identification of overrepresented annotation terms

Two hundred and eleven genes identified to be differentially expressed were used for gene-term enrichment analysis

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

Gene	Forward primer (from 5′ to 3′)	Reverse primer (5′ to 3′)	PCR product size (bp)	NCBI GenBank
β-Actin	GACATCCGCAAGGACCTCTA	ACATCTGCTGGAAGGTGGAC	205	NM_001167795
atrogin-1	TCACAGCTCACATCCCTGAG	GACTTGCCGACTCTCTGGAC	167	NM_001044588
MuRF1	ATGGAGAACCTGGAGAAGCA	ACGGTCCATGATCACCTCAT	201	XM_001926657
DRE1	CAACAACCTCCGATACTACC	GGTCCTCCACCAATCACAAA	158	NP_060114.2
SERTAD1	GCCGCCACCGCTTCCTGATT	AGCCACCAGGCGTCAACTGC	154	Q9UHV2
RXRγ	CTTGTCCACAGGGAAGCCAA	CAGATTGATTCCTGGAGGGG	181	NM_001130213
FOS	GTCTCCAGTGCCAACTTCAT	CATGGTCTTCACGACTCCAG	183	NM_001123113

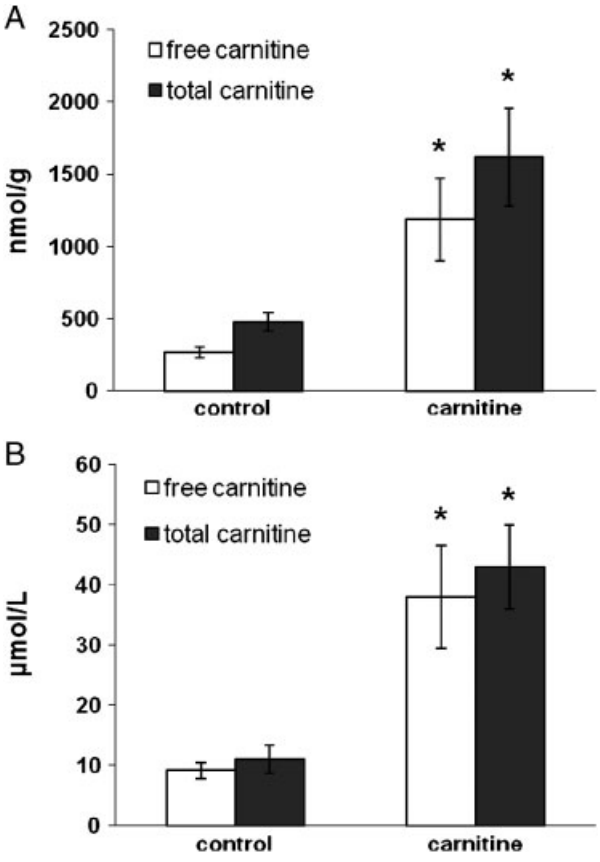

Figure 1. Concentrations of free and total carnitine in skeletal muscle (A) and plasma (B) of growing piglets fed either a control diet or a diet supplemented with 500 mg L-carnitine *per* kg diet.

using the DAVID Functional Annotation Chart tool. The analyses were based on the GO category molecular function. The GO analysis assigned the 211 differentially expressed genes to 23 molecular functions (p value <0.1). According to their ranking (first: lowest p value, last: highest p value) these molecular functions were: cytoskeletal protein binding ($p = 3.2E{-}03$), protein complex binding ($p = 5.3E{-}03$), protein binding ($p = 6.2E{-}03$), actin binding ($p = 8.8E{-}03$), binding ($p = 1.0E{-}02$), double-stranded DNA binding ($p = 2.1E{-}02$), peptide binding ($p = 2.3E{-}02$), heparin binding ($p = 2.5E{-}02$), insulin-like growth factor (IGF) binding ($p = 2.9E{-}02$), steroid binding ($p = 3.2E{-}02$), transcription factor activity ($p = 3.4E{-}02$), insulin receptor binding ($p = 3.6E{-}02$), transcription regulator activity ($p = 5.6E{-}02$), substrate-specific transmembrane transporter activity ($p = 6.2E{-}02$), sequence-specific DNA binding ($p = 6.4E{-}02$), glycosaminoglycan binding ($p = 6.5E{-}02$), structure-specific DNA binding ($p = 7.2E{-}02$), Ras GTPase activator activity ($p = 7.5E{-}02$), transcription activator activity ($p = 7.6E{-}02$), oxygen binding ($p = 7.8E{-}02$), pattern binding ($p = 8.5E{-}02$), polysaccharide binding ($p = 8.5E{-}02$), and steroid hormone receptor activity ($p = 9.8E{-}02$). Most genes were allocated to the GO terms binding (147 genes), protein binding (105 genes), transcription regulator activity (24 genes), transcription factor activity (18 genes), substrate-specific transmembrane transporter activity (15 genes), and cytoskeletal protein binding (14 genes). The GO terms with the highest fold enrichment were IGF factor binding (11.1-fold) and insulin receptor binding (9.9-fold).

3.6 Identification of clusters of functionally related annotation terms

To identify clusters of functionally related molecular functions we used the DAVID functional annotation clustering tool. Clusters were ranked according to the enrichment score for each cluster reflecting the geometric mean of all the enrichment p-values (EASE scores) of each annotation term in the cluster. The eight top-ranked clusters showing the highest enrichment scores are shown in Table 4. Two of the significantly enriched clusters, cluster 2 and cluster 5, allocated GO terms dealing with gene transcription such as transcription factor activity, transcription regulator activity, sequence-specific DNA binding, DNA binding, nucleic acid binding, transcription factor binding, transcription cofactor activity, transcription corepressor activity, transcription repressor activity, and transcription coactivator activity. Three clusters, cluster 1, cluster 3, and cluster 4, allocated molecular functions dealing with binding (cluster 1: *e.g.* polysaccharide binding, carbohydrate binding; cluster 3: *e.g.* insulin receptor binding, kinase binding; cluster 4: *e.g.* peptide binding, peptide receptor activity). The clusters with the lowest enrichment scores of the eight top-ranked clusters allocated molecular functions dealing with transmembrane transporter and channel activity (cluster 6), peptidase activity (cluster 7), and transferase activity (cluster 8).

3.7 Identification of overrepresented regulatory pathways

To identify regulatory pathways associated with L-carnitine supplementation, DAVID gene-term enrichment analysis was also performed on the 211 differentially expressed genes using the Biocarta database, which provides knowledge on molecular interaction networks such as pathways. Identified pathways (EASE score <0.05) included the IGF-1 signalling pathway ($p = 1.9E{-}03$), the insulin signalling pathway ($p = 2.7E{-}02$), the TPO signalling pathway ($p = 3.0E{-}02$), the PDGF signalling pathway ($p = 4.6E{-}02$), and the EGF signalling pathway ($p = 4.9E{-}2$).

4 Discussion

In the present study, transcript profiling was applied to identify genes regulated by supplemental L-carnitine in

Table 2. The most strongly up-regulated (fold change ≥ 4.0) and down-regulated (fold change ≤ -4.0) genes in skeletal muscle of growing piglets fed with or without L-carnitine

Probe set ID	Gene name (Gene symbol)	FC[a)]
Up-regulated genes (fold change ≥ 4.0)		
Ssc.13228.1.A1_at	Protein kinase C, nu type (PRKCN)	26.4
Ssc.24519.2.S1_a_at	Exocyst complex component Sec8 (SEC8L1)	11.2
Ssc.16363.1.S1_at	Ubiquitous tropomodulin (TMOD3)	6.3
Ssc.19009.1.S1_at	Insulin-like growth factor I receptor precursor (IGFR1)	4.9
Ssc.14511.1.A1_at	cytidine monophosphate-N-acetylneuraminic acid hydroxylase (Q5TD44)	4.3
Ssc.12965.1.A1_at	Sprouty homolog 3 (SPRY3)	4.3
Ssc.8410.1.A1_at	dipeptidylpeptidase 10 isoform 1 (DPP10)	4.1
Ssc.8394.1.A1_at	Protein C10orf11 (C10orf11)	4.0
Ssc.28906.1.S1_at	Leucine-rich alpha-2-glycoprotein precursor (LRG1)	4.0
Down-regulated genes (fold change ≤ -4.0)		
Ssc.20728.1.S1_at	Fibrinogen beta chain precursor (FGB)	−13.7
Ssc.13954.1.A1_at	PREDICTED: similar to DKFZp761A052 (Q5VV17)	−9.5
Ssc.10439.1.S1_at	Serum albumin precursor (ALB)	−7.4
Ssc.37.1.S1_at	Haptoglobin-related protein precursor (HRP)	−7.2
Ssc.29156.1.A1_at	BAG-family molecular chaperone regulator-3 (BAG3)	−6.6
Ssc.2875.1.S1_at	Thyrotropin-releasing hormone degrading ectoenzyme (THDE)	−6.5
Ssc.15142.1.A1_at	Nedd-4-like E3 ubiquitin-protein ligase WWP2 (WWP2)	−6.2
Ssc.22266.1.A1_at	Protocadherin beta 16 precursor (PCDHB16)	−5.9
Ssc.6189.1.A1_at	Cystine/glutamate transporter (SLC7A11)	−5.0
Ssc.12502.2.S1_at	Zinc finger protein 38 homolog (ZNF38)	−5.0
Ssc.11563.1.S1_at	T-cell surface glycoprotein CD1e precursor (CD1E)	−4.4
Ssc.7724.1.A1_at	Myosin heavy chain, fast skeletal muscle, embryonic (MYH3)	−4.3
Ssc.22241.1.A1_at	Zinc finger protein 212 (ZNF212)	−4.2
Ssc.30498.1.A1_at	zinc finger, CSL domain containing 3 (ZCSL3)	−4.2
Ssc.1121.1.S1_at	Pyruvate dehydrogenase [lipoamide] kinase isozyme 4, mitochondrial precursor (PDK4)	−4.1
Ssc.9962.1.A1_at	Low-density lipoprotein receptor-related protein 1B precursor (LRP1B)	−4.1
Ssc.22521.1.A1_a_at	Protein- tyrosine kinase 2 (PTK2)	−4.0
Ssc.22974.1.A1_at	Metabotropic glutamate receptor 1 precursor (GRM1)	−4.0

a) FC = fold change.

skeletal muscle of piglets. Using this approach, we successfully identified 211 differentially expressed genes. Considering that the carnitine status of the piglets was significantly improved (about 3-fold increase in muscle carnitine content) by the supplemental L-carnitine, this finding indicates that supplemental L-carnitine, by enhancing carnitine concentration in skeletal muscle, influences gene expression in skeletal muscle of piglets.

To gain insight into molecular functions/processes influenced by L-carnitine we performed annotation term enrichment analysis. This analysis revealed several important molecular processes to be significantly associated with L-carnitine supplementation. Amongst them, those dealing with gene transcription, like double-stranded DNA binding and transcription factor activity, were dominating. This could also be confirmed by functional clustering analysis identifying two annotation term groups (cluster 2 and cluster 5) dealing with gene transcription. Noteworthy, most of the differentially expressed genes assigned to these groups encoded transcription factors, and most of them were down-regulated by L-carnitine. These transcription factors included RXRγ (retinoic acid receptor RXR-gamma), ZNF38 (Zinc finger protein 38 homolog), CITED2 (Cbp/p300-interacting transactivator 2), KLF5 (krueppel-like factor 5), HIF1A (hypoxia-inducible factor 1 alpha), ESRRG (estrogen-related receptor gamma), ARID3A (AT-rich interactive domain-containing protein 3A), and members of the activator protein (AP)-1 family such as activating transcription factor 3, JUN (proto-oncogene c-jun), and FOS (proto-oncogene c-fos). The observed down-regulation of genes encoding members of the AP-1 family by L-carnitine and the association of L-carnitine supplementation with decreased DNA binding is in line with recent observations demonstrating that carnitine injection reduces AP-1 DNA-binding activity [34], and carnitine deficiency up-regulates JUN and FOS [35]. There is compelling evidence that elevated AP-1 binding activity and expression of JUN, FOS and activating transcription factor 3 induce apoptosis in various cell types [36], whereas inhibition of AP-1 signalling has the opposite effect [37]. Our findings therefore suggest that L-carnitine might have anti-apoptotic effects in skeletal muscle of piglets. Indeed, this suggestion is supported by

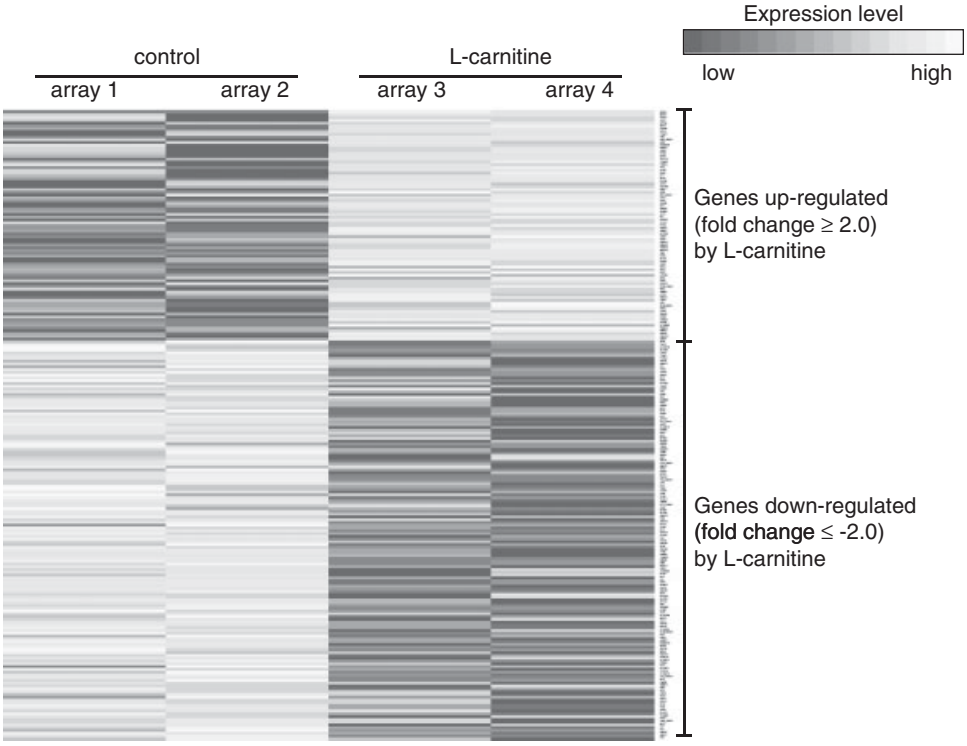

Figure 2. Heat map representing the expression levels of the 211 identified differentially expressed genes between piglets fed either the control diet or the diet supplemented with 500 mg L-carnitine *per* kg diet. The heat map was generated with the freely available software package R (URL: http://www.R-project.org) using the log2 transformed signal intensities.

Table 3. Validation of microarray results using real-time RT-PCR

Gene symbol	GenBank accession no.	Mean fold changes		*p*-value	
		qPCR	Microarray	qPCR	Microarray
SERTAD1	Q9UHV2	2.1	2.2	0.008	n.d.[a)]
RXRγ	NM_001130213	−2.2	−2.0	0.019	n.d.
atrogin-1	NM_001044588	−7.1	−2.9	0.001	n.d.
FOS	NM_001123113	−1.5	−2.5	0.001	n.d.
DRE1	NP_060114.2	−1.7	−3.3	0.038	n.d.

a) n.d.: not determined.

recent reports showing that L-carnitine has anti-apoptotic effects on lymphocytes of HIV-infected subjects [38–40]. Although the anti-apoptotic effect of L-carnitine has been explained to be related to recovery from mitochondrial dysfunction [41], our findings indicate that the inhibition of pro-apoptotic signalling pathways might also contribute to this effect. From a medical perspective, a putative anti-apoptotic effect of L-carnitine on skeletal muscle might be beneficial considering that accelerated apoptosis of muscle fibers has been attributed to increased muscle loss (sarcopenia) as observed with advancing age and different pathological conditions [42, 43]. Anti-apoptotic effects of L-carnitine on muscle fibers might also explain, at least partially, the increased muscle mass and the increased protein:fat accretion in piglets fed L-carnitine [19, 20, 22–24].

The observation that genes associated with IGF binding and insulin binding were enriched by L-carnitine supplementation is also of biological relevance. This was also evidenced by functional clustering analysis (cluster 3). Genes with the annotation term IGF receptor binding identified to be differentially expressed by L-carnitine were IGF-1 receptor, PDPK1 (3-phosphoinositide-dependent protein kinase-1), and SORBS1 (sorbin and SH3 domain containing 1). All these genes were significantly up-regulated by the supplemental L-carnitine – IGF-1 receptor was one of the most strongly up-regulated genes of all identified genes – indicating that carnitine influences IGF binding and insulin binding. This indication could be further strengthened by gene-annotation term enrichment analysis using the molecular interaction network database Biocarta, identifying IGF binding and insulin binding as one of the regulatory pathways significantly associated with L-carnitine supplementation. Recent studies in sows, broiler chicks, rats, and humans revealed that L-carnitine influences the

Table 4. Identification of functionally related annotation groups (GO category molecular function)

Cluster	GO terms	Count	*p*-Value	Genes
1	Heparin binding	5	2.5E−02	ADAMTS3, CTGF, CYR61, KLRG1, PTPRC, VTN
	Glycosaminoglycan binding	5	6.5E−02	
	Pattern binding	5	8.5E−02	
	Polysaccharide binding	5	8.5E−02	
	Carbohydrate binding	6	3.3E−01	
2	Transcription factor activity	18	3.4E−02	ABRA, ABTB2, ALB, ANKRD1, ARID3A, ASCC1,
	Transcription regulator activity	24	5.6E−02	ATF3, CETN1, CITED2, CPEB4, DZIP3, EGR1,
	Sequence-specific DNA binding	12	6.4E−02	ESRRG, FOS, FUSIP1, HIF1A, HNRPDL, HRB2,
	DNA binding	28	3.7E−01	ILF2, JUN, KLF5, MYF6, NR2F1, POU2F1, PRRX1,
	Nucleic acid binding	35	6.2E−01	RBPSUH, RMP, RXRG, SATB1, SERTAD1, ZFPL1, ZNF38, ZNF212, ZNF282
3	Insulin receptor binding	3	3.6E−02	HIF1A, IGF1R, PDPK1, PTPRC, RASA1, S100A1,
	Kinase binding	4	3.0E−01	SL2B,
	Enzyme binding	8	3.3E−01	SORBS1
	Protein kinase binding	3	4.8E−01	
4	Peptide binding	7	2.3E−02	AGTR2, EDG7, GPR126, GRM1, HCRTR2, HTR4,
	Peptide receptor activity	3	3.5E−01	IGF1R, KCNIP2, PCSK5, SORCS1, TCA
	Peptide receptor activity, G-protein coupled	3	3.5E−01	
	G-protein coupled receptor activity	7	9.1E−01	
5	Transcription factor binding	9	1.9E−01	ABRA, ANKRD1, ATF3, CITED2, HIF1A, JUN,
	Transcription cofactor activity	7	2.0E−01	NR2F1, POU2F1, PRRX1, RMP, ZNF282
	Transcription corepressor activity	4	2.1E−01	
	Transcription repressor activity	6	2.5E−01	
	Transcription coactivator activity	4	4.1E−01	
6	Substrate-specific transmembrane transporter activity	15	6.2E−02	AQP5, ATP13A4, ATP10D, CATSPER2, COX11,
	Transmembrane transporter activity	15	1.1E−01	GLRB, GRID2, KCNE1, KCNIP2, MTMR6,
	gated channel activity	7	1.2E−01	SCN10A, SF21, SLC7A11, SLC15A3, TDE2
	Substrate-specific channel activity	8	1.4E−01	
	Channel activity	8	1.6E−01	
	Substrate-specific transporter activity	15	1.6E−01	
	Passive transmembrane transporter activity	8	1.6E−01	
	Ion transmembrane transporter activity	12	1.6E−01	
	Ion channel activity	7	2.4E−01	
	Cation channel activity	5	3.4E−01	
	Voltage-gated ion channel activity	4	3.5E−01	
	Voltage-gated channel activity	4	3.5E−01	
	Transporter activity	15	3.9E−01	
	Cation transmembrane transporter activity	8	3.9E−01	
	Potassium channel activity	3	4.2E−01	
	Metal ion transmembrane transporter activity	5	4.7E−01	
	Voltage-gated cation channel activity	3	4.8E−01	
	Alkali metal ion binding	3	7.1E−01	
7	Peptidase activity, acting on L-amino acid peptides	10	1.4E−01	ADAM12, ADAMTS3, CASP8, CPB1, DPP10,
	Peptidase activity	10	1.7E−01	HPR, PCSK5, THDE,USP32, ZRANB1
	Metallopeptidase activity	4	3.1E−01	
	Endopeptidase activity	5	5.8E−01	
8	Transferase activity, transferring glycosyl groups	5	3.0E−01	ART3, CHSY2, DSCR5, PPAT, UGT2B11
	UDP-glycosyltransferase activity	3	3.4E−01	
	Transferase activity, transferring hexosyl groups	3	5.6E−01	

IGF axis by increasing plasma concentrations of IGF-1 and IGF-2 [16, 44–47]. The increase in plasma levels of especially IGF-1 is considered to be responsible for the phenomenon that carnitine increases birth weights of piglets born to sows fed carnitine [16–18], because IGF-1 is a key hormone favoring placenta development and intra-uterine nutrition [48, 49]. These findings therefore strongly suggest that carnitine activates the IGF-1 signalling pathway. Activation of this pathway is also of relevance in the context of the abovementioned inhibition of sarcopenia because the IGF-1 signalling pathway was shown to be responsible for regulating protein synthesis pathways [50], and overexpression of IGF-1 in muscle was demonstrated to protect against age-related sarcopenia [51]. Moreover, it has been shown that IGF-1 can also block the transcriptional up-regulation of the key mediators of skeletal muscle atrophy, the ubiquitin-ligases muscle RING finger-1 protein and atrogin-1 [52]. Atrogin-1 could also be identified as one of the significantly down-regulated genes in skeletal muscle of the L-carnitine group by the microarray analysis and by confirmatory real-time RT-PCR measurement. In addition, muscle RING finger-1 protein, which was identified to be slightly down-regulated by L-carnitine in the microarray analysis (−1.0-fold), was shown to be significantly down-regulated in the muscle of the L-carnitine group (−2.0-fold) as determined by real-time RT-PCR analysis (data not shown). Moreover, the F box protein DRE-1, which is also involved in the ubiquitin-proteasome pathway regulating muscle atrophy [53], was also identified as a significantly down-regulated gene in skeletal muscle of the L-carnitine group by both, microarray (−3.3-fold) and real-time RT-PCR analysis (−1.7-fold).

Three other clusters of annotation terms dealing with transmembrane transporter and channel activity (cluster 6), peptidase activity (cluster 7), and transferase activity (cluster 8) were also identified to be associated with L-carnitine supplementation. The biological significance of over-representation of these clusters with L-carnitine supplementation, however, is less clear because genes belonging to these clusters were inconsistently regulated by L-carnitine, *e.g.* the number of genes up- and down-regulated within each cluster was similar. Considering this as well as the fact that the degree of differential expression (fold change) of genes belonging to these clusters and the enrichment score of these three clusters was rather low we suggest that the observed association of L-carnitine supplementation with these annotation clusters is of minor biological importance.

Some of the most enriched annotation terms like cytoskeletal protein binding, protein complex binding, protein binding, and actin binding were not clustered, probably because related annotation terms were not co-enriched. Nevertheless, this shows that L-carnitine supplementation is also associated with the interaction of components of the cytoskeleton, in particular the actin filaments. Differentially expressed genes assigned to these molecular functions included the myogenesis-related genes MYH3 (myosin, heavy chain 3), MYH8 (myosin, heavy chain 8), and MYL4 (myosin, light chain 4). Interestingly, Lösel *et al.* [54] have previously shown that L-carnitine supplementation during suckling intensifies the early postnatal skeletal myofiber formation in piglets of low birth weight through stimulating myogenic proliferation indicating that piglets, particularly those of low birth weight, could profit from an early postnatal L-carnitine supplementation by attenuating the negative consequences of low birth weight on body composition. Our findings suggest that the effect of L-carnitine on myogenic proliferation is mediated by modulating the expression of myogenesis-related genes. In addition, the actin-binding Rho activating protein ABRA was also one of the differentially expressed genes assigned to cytoskeletal protein and actin binding. Interestingly, recent research has demonstrated that actin and some of the actin-binding proteins play important roles in processes such as chromatin remodelling, transcriptional regulation, RNA processing, and nuclear export [55]. Moreover, it was shown that ABRA links changes in actin dynamics to gene transcription in striated muscle cells [56]. Hence, our microarray data also indicate that L-carnitine may influence gene transcription *via* actin-binding proteins. It is noteworthy in this context that vitamin E deficiency was shown to induce a similar response in skeletal muscle like L-carnitine, such as up-regulation of myogenesis-related genes and actin [57]. This has been suggested to reflect an adaptive regenerative process in myofibres aiming to maintain muscle structure during vitamin E deficiency.

In conclusion, the present study shows that supplemental L-carnitine influences gene expression in skeletal muscle of growing piglets. Our data suggest that L-carnitine supplementation may have beneficial effects on maintaining skeletal muscle mass through stimulating IGF-1 signalling and inhibiting the expression of pro-apoptotic and atrophy-related genes. These effects might explain, at least partially, the increased muscle mass and the increased protein:fat accretion in piglets fed dietary L-carnitine [19, 20, 22–24].

The authors have declared no conflict of interest.

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Supplementation with L-carnitine downregulates genes of the ubiquitin proteasome system in the skeletal muscle and liver of piglets

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Supplementation of carnitine has been shown to improve performance characteristics such as protein accretion in growing pigs. The molecular mechanisms underlying this phenomenon are largely unknown. Based on recent results from DNA microchip analysis, we hypothesized that carnitine supplementation leads to a downregulation of genes of the ubiquitin proteasome system (UPS). The UPS is the most important system for protein breakdown in tissues, which in turn could be an explanation for increased protein accretion. To test this hypothesis, we fed sixteen male, four-week-old piglets either a control diet or the same diet supplemented with carnitine and determined the expression of several genes involved in the UPS in the liver and skeletal muscle. To further determine whether the effects of carnitine on the expression of genes of the UPS are mediated directly or indirectly, we also investigated the effect of carnitine on the expression of genes of the UPS in cultured C2C12 myotubes and HepG2 liver cells. In the liver of piglets fed the carnitine-supplemented diet, the relative mRNA levels of atrogin-1, $E_2$14k and Psma1 were lower than in those of the control piglets ($P < 0.05$). In skeletal muscle, the relative mRNA levels of atrogin-1, MuRF1, $E_2$14k, Psma1 and ubiquitin were lower in piglets fed the carnitine-supplemented diet than that in control piglets ($P < 0.05$). Incubating C2C12 myotubes and HepG2 liver cells with increasing concentrations of carnitine had no effect on basal and/or hydrocortisone-stimulated mRNA levels of genes of the UPS. In conclusion, this study shows that dietary carnitine decreases the transcript levels of several genes involved in the UPS in skeletal muscle and liver of piglets, whereas carnitine has no effect on the transcript levels of these genes in cultivated HepG2 liver cells and C2C12 myotubes. These data suggest that the inhibitory effect of carnitine on the expression of genes of the UPS is mediated indirectly, probably via modulating the release of inhibitors of the UPS such as IGF-1. The inhibitory effect of carnitine on the expression of genes of the UPS might explain, at least partially, the increased protein accretion in piglets supplemented with carnitine.

Keywords: carnitine, ubiquitin proteasome system, skeletal muscle, liver, pig

Implications

Our findings show for the first time that carnitine inhibits the expression of genes of the ubiquitin proteasome system (UPS), which is the most important system for protein breakdown in tissues. This finding may provide an explanation for the previous observation that carnitine stimulates protein accretion in growing pigs, although a direct relationship between the downregulation of UPS and an increased protein has to be shown in future studies. Nevertheless, this study, overall, confirms recent studies indicating that carnitine has a beneficial effect on protein metabolism in pigs.

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Introduction

L-Carnitine (3-hydroxy-4-*N,N,N*-trimethylaminobutyric acid) is an essential compound that serves a number of indispensable functions in intermediary metabolism. The most important function is in its role in the transport of activated long-chain fatty acids from the cytosol to the mitochondrial matrix in which β-oxidation takes place. Thus, all tissues that use fatty acids as an energy source require carnitine for normal function (McGarry and Brown, 1997; Kerner and Hoppel, 2000).

Evidence suggests that the supplementation of carnitine improves the performance characteristics of livestock or sport animals, such as horses (Foster *et al.*, 1989; Rivero *et al.*, 2002) dairy cows or steers (LaCount *et al.*, 1995; Greenwood

et al., 2001), laying hens and broilers (Geng *et al.*, 2007; Zhai *et al.*, 2008). In addition, an improvement of performance characteristics in response to carnitine supplementation was reported for sows (Musser *et al.*, 1999; Ramanau *et al.*, 2002 and 2004), growing–finishing pigs (Heo *et al.*, 2000; Owen *et al.*, 2001a; Birkenfeld *et al.*, 2005) and, in particular, suckling and weanling piglets (Wolfe *et al.*, 1978; Heo *et al.*, 2000; Rincker *et al.*, 2003). In piglets, dietary carnitine supplementation increased whole-body protein accretion and simultaneously decreased fat deposition (Owen *et al.*, 1996; Heo *et al.*, 2000). Regarding the underlying mechanisms of action, it has been postulated that the beneficial effect of carnitine on growth performance in piglets is due to an increase in available energy to the growing piglet. It has been suggested that the increase in energy availability is due to an improvement in fatty acid oxidation, which in turn enhances energy availability for protein accretion and/or growth (Owen *et al.*, 2001b; Rincker *et al.*, 2003). In an attempt to explore further effects of carnitine that might contribute to its beneficial effect on protein accretion, we have recently performed genome-wide transcript profiling in skeletal muscle of piglets fed supplemental carnitine (Keller *et al.*, 2011). Using this approach, we observed that several genes belonging to the UPS were downregulated by carnitine, indicating that carnitine may inhibit the UPS. The UPS is the most important system for intracellular protein degradation in mammalian cells. It consists of several components, namely the 26S proteasome and three enzymatic components, designated as E1, E2 and E3 classes. The 26S proteasome is a large multicatalytic protease complex that degrades ubiquitinated proteins to small peptides. The β subunits of this complex are of particular significance functionally as they are responsible for its proteolytic activities (Baumeister *et al.*, 1998). Substrates for proteasomal proteolysis are marked by the addition of ubiquitin molecules. E1 (ubiquitin-activating enzymes) and E2 (ubiquitin carrier or conjugating proteins) prepare ubiquitin for conjugation, whereas E3, which is the key enzyme class in this process, recognizes a specific protein substrate and catalyses the transfer of the activated ubiquitin to it. Successful ubiquitination of the target protein by E3 is followed by their unfolding and importing into the proteasome by an ATP-dependent process, where it is degraded (Attaix *et al.*, 2005). The significance of the UPS for whole-body protein status is shown by the fact that the stimulation of this system under certain pathological (e.g. metabolic acidosis, kidney failure, muscle denervation, diabetes mellitus, thermal injury, glucocorticoid treatment, hyperthyroidism) as well as physiological conditions (e.g. fasting) leads to muscle atrophy (Mitch and Goldberg, 1996; Costelli and Baccino, 2003; Murton *et al.*, 2008). As stimulators of the UPS in response to pathological conditions, pro-inflammatory cytokines and glucocorticoids have been identified (Nury *et al.*, 2007), whereas insulin and IGF-1 were shown to suppress the UPS (Mitch and Goldberg, 1996; Tisdale, 2005).

In light of the above-mentioned findings, we hypothesized that carnitine supplementation inhibits protein breakdown by downregulating genes of the UPS. To test this hypothesis, we used tissue samples taken from a previous experiment with piglets (Keller *et al.*, 2011) that were fed a diet supplemented with carnitine and determined the transcript levels of important genes involved in the UPS in skeletal muscle, namely ubiquitin, the subunits *Psma1* and *Psmb1* of the 20S proteasome, E_2*14k* as a member of the E2 enzyme class and *atrogin-1* and *MuRF1* as members of the E3 enzyme class. In this recent study, carnitine concentrations in tissues and plasma were markedly increased (approximately fourfold) by carnitine, indicating that the carnitine status of these piglets was significantly improved by the supplement. As the UPS is also important for protein degradation in visceral tissues, we also investigated changes in the liver as a tissue with a high protein turnover. To explore whether carnitine has a direct effect on the expression of components of the UPS, we also investigated the effect of carnitine on the expression of genes of the UPS in C2C12 myotubes and HepG2 cells, which are established *in vitro* model systems for skeletal muscle cells and hepatocytes, respectively.

Material and methods

Animal experiment

The animal experiment was approved by the local Animal Care and Use Committee. As described recently in more detail (Keller *et al.*, 2011), the experiment was performed with sixteen male crossbred pigs ((German Landrace × Large White) × Pietrain) with an average body weight of 10 ± 1 (mean ± s.d.) kg. The pigs were assigned to two groups (control and carnitine) and fed experimental diets for a period of 21 days. The control group received a basal diet with a low native carnitine concentration (<5 mg/kg), which was nutritionally adequate for growing pigs in a body weight range between 10 and 20 kg, according to the recommendations of the German Society for Nutrition Physiology (Gesellschaft für Ernährungsphysiologie, 2006). The carnitine group received the same diet supplemented with 500 mg L-carnitine/kg (obtained from Lohmann Animal Health, Cuxhaven, Germany). At the end of the feeding experiment, pigs with a mean body weight of 17.0 ± 3.2 kg were sacrificed. The feed intake, final body weights and feed conversion ratio were not different between both the groups (Keller *et al.*, 2011). Blood sample was collected and plasma was obtained by centrifugation of the blood, and the skeletal muscle and liver were excised. Plasma and tissue samples were immediately stored at −80°C until analysis. A full description of the diet composition, feeding regime, sample collection and carnitine analysis of the diets and tissues can be found in our recent publication (Keller *et al.*, 2011).

Cell culture experiments

HepG2 cells. As a model for hepatocytes, the hepatoma cell line HepG2, obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany), was used. HepG2 cells were grown in RPMI1640 medium (GIBCO/Invitrogen, Karlsruhe, Germany) supplemented with 10% foetal calf serum (GIBCO/Invitrogen) and 0.5% gentamycin

(GIBCO/Invitrogen) at 37°C in 5% CO_2 (Eder *et al.*, 2002). Cells were seeded in 24-well culture plates (Catalogue no. 662960; Greiner Bio-One, Frickenhausen, Germany) at a density of 2.2×10^5 cells/well and, before reaching confluence (usually 3 days after seeding), incubated with different concentrations (0, 50, 100, 500 and 1000 μM) of L-carnitine (≥98% pure; Sigma-Aldrich, Taufkirchen, Germany) for 24 h. Incubation media containing carnitine were prepared by diluting the carnitine stock solution (100 mM in bi-destilled water) with RPMI1640 medium without supplements to the concentrations indicated. At the end of incubation, the media were discarded, the cell layer was washed once with phosphate-buffered saline (PBS) and plates including the attached cells were immediately placed in a refrigerator at −80°C.

C2C12 myoblasts. As a model for skeletal muscle cells, the muscle-derived C2C12 myoblast cell line, obtained from Cell Lines Service (Eppelheim, Germany), was used. Undifferentiated myoblasts were grown in Dulbecco's modified Eagle's medium (DMEM; GIBCO/Invitrogen, Karlsruhe, Germany) supplemented with 10% foetal calf serum and 0.5% gentamycin at 37°C in 5% CO_2 (Mordier *et al.*, 2000). Cells were seeded in 24-well culture plates (Catalogue no. 662960; Greiner Bio-One) at a density of 1.5×10^4 cells/well. After reaching 60% to 70% confluence (after 3 days), the medium was replaced by DMEM supplemented with 2% horse serum to induce myogenic differentiation into myotubes. In preliminary experiments, successful differentiation into myotubes was confirmed by determining the mRNA levels of *myogenin* – a myogenesis-stimulating factor that is upregulated during differentiation – and AT-motif-binding factor 1 (*ATBF1-A*) – a myogenesis-inhibiting factor that is downregulated during differentiation (Figure 1). For this, cells were seeded as mentioned above and cultured with differentiation medium for different durations (0, 24, 48 and 72 h). Subsequently, RNA was isolated as described below and analysed for *myogenin* and *ATBF1-A* mRNA levels. The medium was changed every 48 h, and differentiation was allowed to continue for 96 h. Subsequently, myotubes were incubated with different concentrations (0, 50, 100, 500 and 1000 μM) of carnitine for 24 h. Incubation media containing carnitine were prepared as described in the section 'HepG2 cells', except that DMEM was used as a medium.

To study the effect of carnitine on stimulus-induced expression of genes of the UPS, cells were treated in parallel with different carnitine concentrations and 10 μM hydrocortisone. Cells treated with 10 μM hydrocortisone without carnitine were used as controls (stimulated control). Cells treated without hydrocortisone were used as a negative control (unstimulated control). At the end of incubation, the media were discarded, the cell layer was washed once with PBS and plates including the attached cells were immediately placed in a refrigerator at −80°C.

RNA isolation and quantitative real-time PCR (qPCR). For the determination of mRNA expression levels, total RNA was isolated from the liver, skeletal muscle and cells using

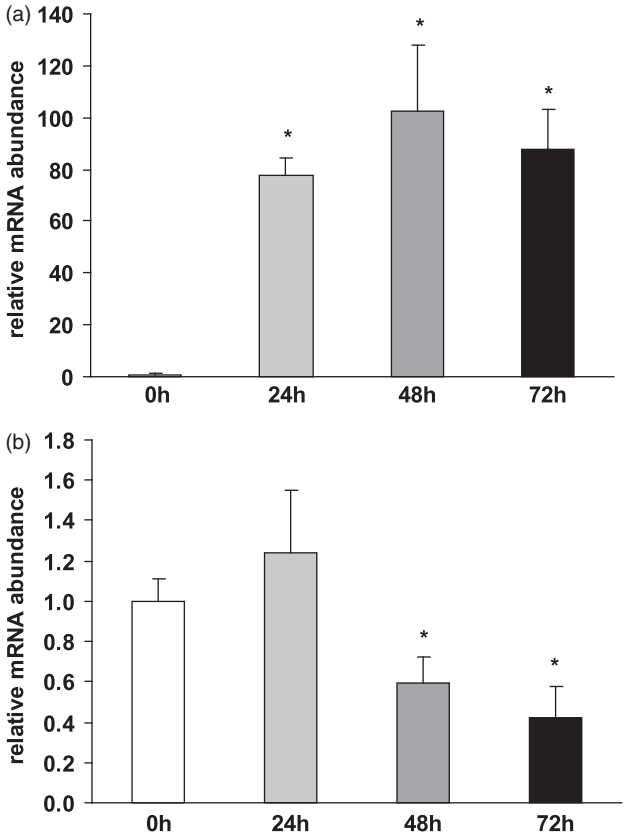

Figure 1 Relative mRNA abundance of *myogenin* (a) and AT-motif-binding factor 1 (*ATBF1-A*) (b) in C2C12 myotubes treated with differentiation medium for different durations (0, 24, 48 and 72 h). Data are expressed relative to the mRNA concentrations of time point 0 h (= 1.0). Bars are mean ± s.d. from three independent experiments performed in quadruplicate. Bars marked without a common superscript letter differ significantly at $P < 0.05$.

Trizol™ reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocol. RNA from 15 mg of each tissue was isolated within 1 week after completion of the trial. RNA from cells (24 well at 90 (HepG2 cells) and 80% (C2C12 myotubes) confluence) was isolated within 1 week following cell incubation by adding Trizol directly to the wells, and pipetting the lysed cells up and down 2 to 3 times. Genomic DNA was removed from total RNA isolated with on-column DNase I digestion using RNeasy Mini Kit columns (Qiagen, Germany). Isolated RNA was preserved at −80°C until use. The RNA concentration and purity were estimated from the optical density at 260 and 280 nm, respectively. The A260/A280 ratios were 1.98 ± 0.02 (Mean ± SD) (liver), 2.00 ± 0.02 (muscle), 1.89 ± 0.04 (HepG2 cells) and 1.92 ± 0.04 (C2C12 myotubes). cDNA was synthesized in less than a week after RNA extraction from 1.2 μg of total RNA using 100 pmol dT18 primer (Eurofins MWG Operon, Ebersberg, Germany), 1.25 μl 10 mmol/l dNTP mix (GeneCraft, Lüdinghausen, Germany), 5 μl buffer (MBI Fermentas, St. Leon-Rot, Germany) and 60 units M-MuLV reverse transcriptase (MBI Fermentas) at 42°C for 60 min, and a final inactivating step at 70°C for 10 min in a Biometra Thermal Cycler

(Whatman Biometra®, Göttingen, Germany). Subsequently, cDNA was stored in aliquots at −20°C. For the standard curve, a cDNA pool each from the liver, muscle, HepG2 cells and C2C12 myotubes was prepared. qPCR was performed using 2 μl cDNA combined with 18 μl of a mixture composed of 10 μl KAPA SYBR FAST qPCR Universal Mastermix (Peqlab, Erlangen, Germany), 0.4 μl each of 10 μM forward and reverse primers and 7.2 μl DNase/RNase free water in 0.1 ml tubes (Ltf Labortechnik, Wasserburg, Germany). Gene-specific primer pairs obtained from Eurofins MWG Operon were designed using Primer3 and BLAST. The features of the primer pairs are listed in Table 1. All primer pairs were designed to have melting temperatures of about 60°C and, if possible, both primers of a primer pair were designed to be located in different exons. qPCR runs were performed using a Rotorgene 2000 system (Corbett Research, Mortlake, Australia) and included all samples and a 5-point relative standard curve plus the non-template control. The qPCR protocol was as follows: 3 min at 95°C, followed by 40 cycles of a two-step PCR consisting of 5 s at 95°C (denaturation) and 20 s at 60°C (annealing and extension). Subsequently, melting curve analysis was performed from 50°C to 95°C to verify the presence of a single PCR product. In addition, the amplification of a single product of the expected size was confirmed using 2% agarose gel electrophoresis stained with GelRed™ nucleic acid gel stain (Biotium, Hayward, CA, USA). C_t values of target genes and reference genes were obtained using Rotorgene Software 5.0 (Corbett Research). For the determination of the relative expression levels, relative quantities were calculated using

Table 1 *Characteristics of the primers used for quantitative real-time PCR analysis*

Gene	F-primer[1]	R-primer[2]	Product size (bp)	Accession no.
Homo sapiens				
ATP5B	TCGCGTGCCATTGCTGAGCT; 1261	CGTGCACGGGACACGGTCAA; 1478	218	NM_001686.3
Atrogin-1	TCCCTGAGTGGCATCGCCCA; 280	CTGAGCACGTGCAGGTCGGG; 653	374	NM_148177.1
CYC1	TTCGCTTCGCGGGGTAGTGTTGG; 61	GACAAGGCCACTGCCTGAGGT; 186	126	NM_991916.3
$E2_{14}k$	TGTGGGTGTCAGTGGCGCAC; 480	CTTCAAAAGGTGTCCCTTCTGGTCC; 565	86	NM_003337.2
EIF4A2	GCGCAAGGTGGACTGGCTGA; 888	GCACATCAATCCCGCGAGCC; 1057	170	NM_001967.3
Psma1	CAGTTTTGCTGGAGGCCGCAACCAG; 107	ATGGTCTCCGGCCATATCGTTGTGT; 528	422	NM_002786.3
Psmb1	GGCAGCCATCTCGCCGTGAG; 33	GCGGCTCTGTGCGGTTCCAT; 155	123	NM_002793.3
SDHA	CCAAGCCCATCCAGGGGCAAC; 1935	TCCAGAGTGACCTTCCCAGTGCCAA; 2034	100	NM_004168.2
Ubc	AGCGCTGCCACGTCAGACGAA; 111	CGGCTGCGACGGAACTAGCTG; 407	297	NM_021009
Ubiquitin	GGATAAAGAAGGCATCCCTCCC; 459	GCTCCACCTCCAGAGTGATGG; 649	191	NM_018955.2
YWHAZ	TGGGGACTACGACGTCCCTCAA; 82	CATATCGCTCAGCCTGCTCGG; 196	115	NM_003406.3
Mus musculus				
ATP5B	GCCAGAGACTATGCGGCGCA; 147	CCCCCAAATGCTGGGCCACC; 333	187	NM_016774.3
Atrogin-1	ATGCACACTGGTGCAGAGAG; 858	TGTAAGCACACAGGCAGGTC; 1025	168	AF441120
CANX	GTCCCCGGGAGGCTCGAGATAGAT; 164	ACCTCCCCTGTTGGAACTGGAGC; 397	234	NM_007597.3
CYC1	GCTTCGCCGGACGGTACTGG; 62	CGCAATGGAAGCTGCCGGGA; 160	99	NM_025567.2
$E2_{14}k$	CAGAAGGGACACCCTTTGAA; 171	GTTGGCTGGACTGTTTGGAT; 412	242	U57690
EIF4A2	ACATGGCGGCCCAGAGGGAA; 346	TGGTGGGGGCCAATACTAGTGCT; 644	299	NM_013506.2
MuRF1	GACAGTCGCATTTCAAAGCA; 83	AACGACCTCCAGACATGGAC; 321	239	NM_001039048
Psma1	TGGAGTGCAATTTGGATGAA; 652	CATTGGTTCATCGGCTTTTT; 896	245	NM_011965
Psmb1	TTGACCCAGTGGGCTCTTAC; 466	CTCTTTGGTCACGATGCAGA; 698	233	NM_011185
RPL13A	GTGGTCGTACGCTGTGAAGGCATC; 121	GGCCTCGGGAGGGGTTGGTATT; 229	109	NM_009438.4
SDHA	GCCCATCCCAGTCCTCCCCA; 1226	TTGCTCCAAGCCGGTTGGCA; 1395	170	NM_023281.1
Ubiquitin	CGCACCCTGTCAGACTACAA	CTAAGACACCTCCCCCATCA	239	BC021837
Sus scrofa				
ATP5G1	CAGTCACCTTGAGCCGGGCGA; 24	TAGCGCCCCGGTGGTTTGC; 117	94	NM_001025218.1
Atrogin-1	TCACAGCTCACATCCCTGAG; 430	GACTTGCCGACTCTCTGGAC; 596	167	NM_001044588
$E2_{14}k$	CCAAATAAACCGCCAACTGT; 229	GTTCAACAATGGCCGAAACT; 478	250	AK240614
GAPDH	AGGGGCTCTCCAGAACATCATCC; 935	TCGCGTGCTCTTGCTGGGGTTGG; 1380	446	AF017079.1
GPI	CACGAGCACCGCTCTGACCT; 87	CCACTCCGGACACGCTTGCA; 451	365	NM_214330.1
GPX1	GGCACAACGGTGCGGGACTA; 163	AGGCGAAGAGCGGGTGAGCA; 397	235	NM_214201.1
MuRF1	ATGGAGAACCTGGAGAAGCA; 138	ACGGTCCATGATCACCTCAT; 356	219	NM_001184756
RPS9	GTCGCAAGACTTATGTGACC; 20	AGCTTAAAGACCTGGGTCTG; 344	327	CAA23101
SDHA	CTACGCCCCCGTCGCAAAGG; 813	AGTTTGCCCCCAGGCGGTTG; 1192	380	DQ402993
Ubiquitin	GGTGGCTGCTAATTCTCCAG; 767	TTTTGGACAGGTTCAGCTATTAC; 893	127	EF688558
Psma1	CCGGAGGCCGTGACTAGGCT; 42	GCATCGGCAGTAAGTCCCGCA; 315	274	AY609452
Psmb1	CTGTGGGGTCCTACCAGAGA; 612	CCCAGTGTACACGTCCCTCT; 805	194	AK345051.1

[1] Forward primer (from 5′ to 3′) and hybridization position.
[2] Reverse primer (from 5′ to 3′) and hybridization position.

Table 2 *Average expression stability ranking of six candidate reference genes used in pig liver and skeletal muscle, HepG2 cells and C2C12 myotubes**

	Liver		Skeletal muscle		HepG2 cells		C2C12 myotubes	
Ranking	Gene	*M*-value	Gene	*M*-value	Gene	*M*-value	Gene	*M*-value
Most stable	*GAPDH*	0.667	*RPS9*	0.470	*YWAHZ*	0.190	*SDHA*	0.185
	ATP5G1	0.690	*GAPDH*	0.480	*EIF4A2*	0.191	*RPL13A*	0.196
	GPI	0.730	*ATP5G1*	0.494	*ATP5B*	0.198	*EIF4A2*	0.209
	GPX1	0.774	*GPI*	0.570	*SDHA*	0.204	*ATP5B*	0.211
	SDHA	0.809	*SDHA*	0.608	*CYC1*	0.234	*CANX*	0.221
Least stable	*RPS9*	0.888	*β-Actin*	0.816	*Ubc*	0.247	*CYC1*	0.230

*Ranking of the candidate reference genes according to their stability score *M* as calculated by the Microsoft Excel-based application GeNorm.

the GeNorm normalization factor. To calculate the normalization factor, all C_t values were transformed into relative quantification data using the $2^{-\Delta Ct}$ equation (Livak and Schmittgen, 2001), and the highest relative quantities for each gene were set to 1. From these values, the normalization factor was calculated as the geometric mean of expression data of the three most stable out of six tested potential reference genes (Table 2). Reference gene stability across samples from each tissue and each cell line was determined by performing GeNorm analysis (Vandesompele *et al.*, 2002). After normalization of gene expression data using the calculated GeNorm normalization factor, the mean and s.d. were calculated from normalized expression data for samples of the same treatment group. The mean of the control group was set to 1 and the mean and s.d. of the other treatment groups were scaled proportionally. Data on qPCR performance for each gene measured in the tissues and cell lines are shown in Table 3.

Statistical analysis

Data of all experiments were analysed using the Minitab Statistical Software Rel 13.0 (Minitab, State college, PA, USA). Treatment effects were analysed using one-way ANOVA. For significant *F*-values, means were compared using Fisher's multiple-range test. Means were considered significantly different at $P < 0.05$. Data shown from the animal experiment are mean ± s.d. for $n = 8$/group. Data shown from cell culture experiments are mean ± s.d. for $n = 3$ independent experiments.

Table 3 *Quantitative real-time PCR performance data*

Gene	Slope	R^2*	Efficiency#
Homo sapiens			
ATP5B	−0.29	0.999	1.96
Atrogin-1	−0.26	0.981	1.84
EIF4A2	−0.29	0.998	1.97
E2$_{14}$k	−0.29	0.999	1.96
Psma	−0.26	0.998	1.83
Psmb	−0.28	0.996	1.92
ubiquitin	−0.27	0.998	1.92
YWHAZ	−0.29	0.998	1.93
Mus musculus			
Atrogin-1	−0.24	0.997	1.75
EIF4A2	−0.29	0.998	1.95
E2$_{14}$k	−0.28	0.999	1.90
MuRF1	−0.27	0.991	1.86
Psma	−0.28	0.997	1.89
Psmb	−0.26	0.997	1.83
RPL13	−0.29	0.999	1.95
SDHA	−0.30	0.998	1.99
ubiquitin	−0.28	0.998	1.93
Sus scrofa			
ATP5B	−0.29	0.998	1.93
Atrogin-1	−0.27	0.998	1.87
GAPDH	−0.28	0.998	1.88
GPI	−0.22	0.999	1.67
E2$_{14}$k	−0.29	0.995	1.95
MuRF1	−0.29	0.997	1.94
Psma	−0.32	0.990	2.08
Psmb	−0.32	0.995	2.08
ubiquitin	−0.29	0.999	1.97

*Coefficient of determination of the standard curve.
#The efficiency is determined by [10^{-slope}].

Results

Effect of carnitine on mRNA abundance of selected genes of the UPS in skeletal muscle and liver of piglets

Carnitine supplementation decreased the relative mRNA concentrations of *atrogin-1*, *MuRF1*, *E$_2$14k*, *Psma1* and *ubiquitin* in the skeletal muscle of piglets by 40% to 80% ($P < 0.05$; Figure 2a). The relative mRNA level of Psmb1 in the skeletal muscle did not differ between both the groups (Figure 2a).

The relative mRNA levels of *atrogin-1*, *E$_2$14k* and *Psma1* in the liver were 40% to 80% lower ($P < 0.05$) in the carnitine group than in piglets fed the control diet (Figure 2b), whereas the mRNA levels of *Psmb1* and *ubiquitin* in the liver were not affected by carnitine supplementation (Figure 2b).

Effect of carnitine on mRNA abundance of selected genes of the UPS in C2C12 myotubes and HepG2 liver cells

Incubating C2C12 myotubes and HepG2 liver cells with increasing concentrations of carnitine had no effect on the relative mRNA levels of genes of the UPS (Figure 3a and b),

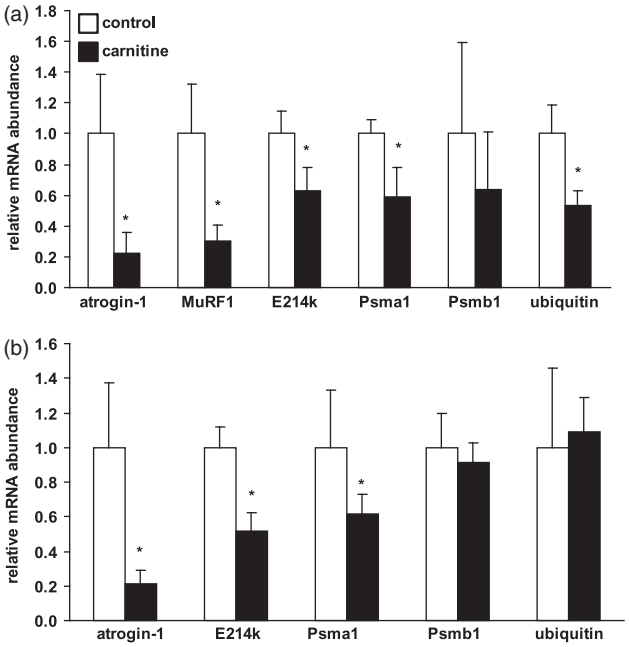

Figure 2 Relative mRNA abundance of selected genes of the ubiquitin proteasome system in the skeletal muscle (a) and liver (b) of growing pigs fed either a control diet or a diet supplemented with 500 mg carnitine/kg diet. Bars are mean ± s.d. ($n = 8$/group). The normalized expression ratio in the control group is set to 1.0. *Significantly different from the control group (0 mg carnitine/kg diet) at $P < 0.05$.

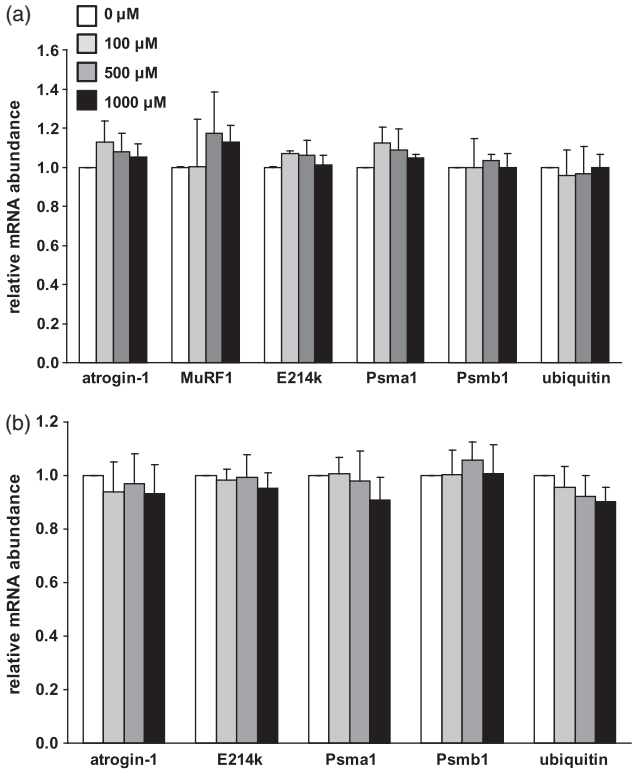

Figure 3 Relative mRNA abundance of selected genes of the ubiquitin proteasome system in C2C12 myotubes (a) and HepG2 liver cells (b) treated with increasing concentrations of carnitine (0, 100, 500 and 1000 µM). Data are expressed relative to the mRNA concentrations of the control group ($n = 1.0$). Bars are mean ± s.d. from three independent experiments performed in quadruplicate. Bars marked without a common superscript letter differ significantly at $P < 0.05$.

indicating that the effect of carnitine on the expression of genes of the UPS in skeletal muscle and liver of piglets is not a direct effect.

In Figure 4, the effect of carnitine on the relative mRNA levels of genes of the UPS in C2C12 myotubes under stimulated conditions (hydrocortisone) is shown. The relative mRNA levels of *atrogin-1*, *MuRF1* and *$E_2$14k*, but not *ubiquitin*, *Psma1* and *Psmb1*, were increased in cells stimulated with hydrocortisone relative to unstimulated cells ($P < 0.05$). However, incubating hydrocortisone-stimulated C2C12 myotubes with increasing concentrations of carnitine also had no effect on the relative mRNA levels of genes of the UPS (Figure 4).

Discussion

As a main finding, we observed that the transcript levels of genes belonging to the UPS were markedly reduced in both the skeletal muscle and the liver of piglets fed supplemental carnitine. These findings confirm indications from a recently performed DNA microchip analysis in which the expression levels of genes of the UPS pathway were reduced in pigs with carnitine supplementation (Keller *et al.*, 2011). The most drastic downregulation by the supplemental carnitine of genes belonging to the UPS was observed for genes encoding the E3 ligases (*atrogin-1* and *MuRF1*). Both *atrogin-1* and *MuRF1* are considered key mediators and established markers of protein degradation via the UPS because they are responsible for connecting multiple ubiquitin monomers to the target protein, which is an essential step in the proteolytic breakdown of intracellular proteins (Thrower *et al.*, 2000; Cao *et al.*, 2005). The importance of these E3 ligases is underlined by the fact that under pathological conditions (e.g. metabolic acidosis, kidney failure or muscle denervation) of increased protein loss, these two E3 ligases are consistently upregulated, whereas E1 and E2 ligases and *ubiquitin* are not or only marginally upregulated under such conditions (Wray *et al.*, 2003; Lecker *et al.*, 2004). The role of the E3 ligases in muscle atrophy has also been clearly demonstrated in knockout studies with mice in which the deletion of *MuRF1* and *atrogin-1* resulted in 36% and 56%, respectively, sparing of muscle mass loss after denervation of the right hindlimb muscle compared with the controls (Bodine *et al.*, 2001). Consistent with these findings, it was suggested that *atrogin-1* and *MuRF1* are, at least in part, responsible for the muscle protein degradation observed under muscle atrophy conditions and thus represent highly reliable markers of skeletal muscle atrophy by the UPS (Latres *et al.*, 2005). Thus, it is likely that the observed increases in protein mass in growing pigs supplemented with carnitine (Owen *et al.*, 2001a; Rincker *et al.*, 2003) might be, at least in part, due to an inhibition of protein degradation via the UPS in skeletal muscle, even though we observed no effect of carnitine supplementation on protein accretion in this experiment. One might speculate that the duration of our feeding trial was sufficient to induce changes in the gene expression level but

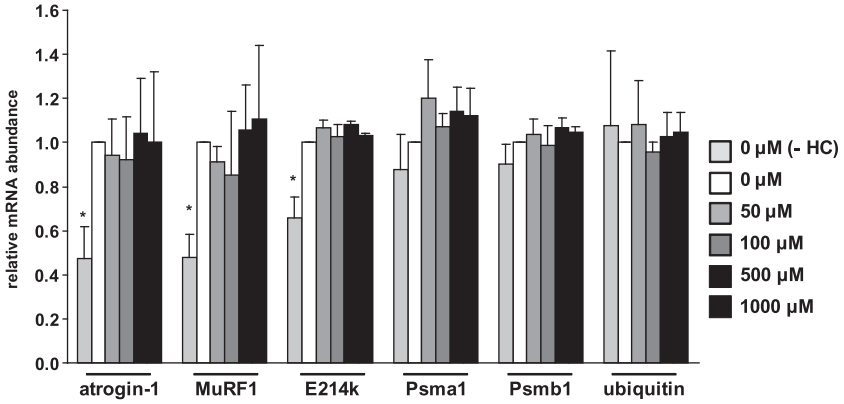

Figure 4 Relative mRNA abundance of selected genes of the ubiquitin proteasome system in C2C12 myotubes treated with increasing concentrations of carnitine (0, 50, 100, 500 and 1000 μM) and 10 μM hydrocortisone (+HC). C2C12 myotubes treated with 10 μmol/l hydrocortisone served only as controls. C2C12 myotubes treated without hydrocortisone served as unstimulated controls. Data are expressed relative to the mRNA concentrations of the control group ($n = 1.0$). Bars are mean ± s.d. from three independent experiments performed in quadruplicate. Bars marked without a common superscript letter differ significantly at $P < 0.05$.

too short to induce phenotypic changes. A further reason for the lack of effect on protein accretion might be that the food intake and daily body weight gain of the piglets were on a relatively low level under the experimental conditions applied. Inhibition of the UPS by carnitine in the liver is probably of less importance for the recent observations showing that carnitine increases protein accretion in growing pigs (Owen *et al.*, 1996 and 2001a; Rincker *et al.*, 2003) because the contribution of hepatic protein pool to the total body protein pool is comparatively small. In contrast to skeletal muscle, the main function of the UPS in the liver is to degrade cytosolic proteins that are either short-lived or folded abnormally (Hamel *et al.*, 2004), and not to provide other tissues with amino acids. This largely explains why, under, the above-mentioned pathological conditions, but also during fasting, in which the UPS is stimulated, protein loss occurs preferentially in skeletal muscle, whereas protein loss in visceral organs like the liver and kidney is kept to a minimum to facilitate biological functions essential for survival (Mitch and Goldberg, 1996).

To investigate whether the inhibitory effect of carnitine on the expression of genes of the UPS is mediated directly by carnitine, we treated muscle and liver cells with different carnitine concentrations, ranging from physiological (<100 μM) to pharmacological (>500 μM). We observed that incubating the cells with carnitine even at pharmacological concentrations had no effect on the basal expression of genes involved in the UPS pathway (e.g. *atrogin-1*, *MuRF1*, $E2_{14k}$, *Psma1*, *Psmb1*, *ubiquitin*). To further study whether carnitine might exert an effect on the expression of genes of the UPS under stimulated conditions, we treated muscle cells with different concentrations of carnitine in the presence of hydrocortisone, which is a known stimulator of the UPS (Price *et al.*, 1996; Combaret *et al.*, 2004; Nury *et al.*, 2007). As expected, hydrocortisone treatment markedly upregulated genes of the UPS pathway. However, carnitine failed to exert an inhibitory effect on hydrocortisone-stimulated expression of genes of the UPS pathway. Thus, the findings from our cell culture experiments indicate that the inhibitory effect of carnitine on the expression of genes of the UPS observed in the animal experiment is not a direct one but likely mediated by modulating the release of inhibitors of the UPS pathway. Indeed, convincing evidence exists showing that carnitine supplementation influences the IGF axis as shown by increased plasma concentrations of IGF-1 and IGF-2 observed in pigs (Doberenz *et al.*, 2006; Woodworth *et al.*, 2007; Brown *et al.*, 2008). Similar observations were found in broiler chicks, rats and humans (Di Marzio *et al.*, 1999; Heo *et al.*, 2001; Kita *et al.*, 2002). Recently, it was identified that the IGF-1 signalling pathway is one regulatory pathway associated with carnitine supplementation in skeletal muscle of piglets, indicating that carnitine activates the IGF-1 pathway (Keller *et al.*, 2011). IGF-1 is well known to inhibit the UPS by blocking the transcriptional upregulation of the key mediators of this pathway, *MuRF1* and *atrogin-1* (Sacheck *et al.*, 2004; Sandri *et al.*, 2004; Stitt *et al.*, 2004; Tong *et al.*, 2009), thereby reducing protein degradation and muscle wasting (Sacheck, 2003). The beneficial effect of IGF-1 in preventing protein loss is also shown by the fact that overexpression of IGF-1 in muscle protects against age-related sarcopenia (Li *et al.*, 2003). From these findings, we suggest that the marked inhibitory effect of carnitine on genes of the UPS in skeletal muscle and liver could be due to the known stimulatory effect of carnitine on IGF-1 secretion and signalling.

In conclusion, this study shows that dietary carnitine decreases the transcript levels of several genes involved in the UPS in skeletal muscle and liver of piglets, whereas carnitine has no effect on the transcript levels of these genes in cultivated HepG2 liver cells and C2C12 myotubes. These data suggest that the inhibitory effect of carnitine on the expression of genes of the UPS is mediated indirectly, probably via modulating the release of inhibitors of the UPS such as IGF-1. The inhibitory effect of carnitine on the expression of genes of the UPS might explain, at least partially, the increased protein accretion in piglets supplemented with carnitine.

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RESEARCH ARTICLE

Regular endurance exercise improves the diminished hepatic carnitine status in mice fed a high-fat diet

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Scope: Metabolic stress induced by chronic high-fat (HF) diet feeding or genetically induced diabetes impairs carnitine status. Herein, we tested the hypothesis that regular endurance exercise (EE) improves the HF diet-induced impairment of carnitine status through stimulating the expression of hepatic genes involved in carnitine synthesis and uptake.
Methods and results: Eighteen male C57BL/6 mice were assigned to three groups: group S received a standard diet, group HF received a HF diet, and group HF+EE received an HF diet and was regularly exercised on a treadmill. After 10 wk, mice of the HF and the HF+EE groups were highly obese and insulin resistant compared with mice of the S group ($p<0.05$), but mice of the HF+EE group were less insulin resistant than those of the HF group ($p<0.05$). The HF group had lower carnitine concentrations and mRNA and protein levels of genes involved in carnitine synthesis and uptake in the liver than the S group ($p<0.05$), whereas these parameters did not differ between the S group and the HF+EE group.
Conclusion: These findings indicate that regular EE reverses an HF diet-induced impairment of hepatic carnitine content by stimulating hepatic carnitine synthesis and uptake.

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1 Introduction

Carnitine is an essential metabolite that has a number of indispensable functions in intermediary metabolism. The most documented function is the translocation of long-chain fatty acids (acyl groups) from the cytosol into the mitochondrial matrix for subsequent β-oxidation [1]. Besides its role in permitting mitochondrial oxidation of long-chain fatty acids, more recent studies showed that carnitine also stimulates whole body glucose oxidation and improves insulin sensitivity in distinct models of glucose intolerance including high-fat (HF) feeding and genetic diabetes [2]. Carnitine is derived from dietary sources and synthesized endogenously from trimethyllysine (TML), which originates from protein degradation [3, 4]. The released TML is further oxidized to γ-butyrobetaine (BB) by different enzymatic reactions involving TML dioxygenase, 3-hydroxy-*N*-TML aldolase and 4-*N*-trimethylaminobutyraldehyde dehydrogenase (TMABA-DH). In the final biosynthetic step, BB is hydroxylated by γ-butyrobetaine dioxygenase (BBD) to form carnitine [5]. In mice, the principal site of carnitine synthesis is the liver because it is the only tissue with a considerable activity of BBD [6]. From extrahepatic tissues, BB is excreted and transported via the circulation to the liver, where it is converted into carnitine [5].

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Abbreviations: ACO, acyl-CoA oxidase; **BB,** γ-butyrobetaine; **BBD,** γ-butyrobetaine dioxygenase; **CPT I,** carnitine-palmitoyltransferase I; **EE,** endurance exercise; **HF,** high fat; **OCTN2,** novel organic cation transporter 2; **PGC,** PPARγ co-activator; **PPARα,** peroxisome proliferator-activated receptor α; **TMABA-DH,** 4-*N*-trimethylaminobutyraldehyde dehydrogenase; **TML,** trimethyllysine

All tissues which are incapable of producing carnitine are highly dependent on active carnitine uptake from blood. Delivery of carnitine from plasma into cells is catalyzed by novel organic cation transporters (OCTN) from which the OCTN2 isoform has the highest binding affinity for carnitine and is therefore the physiologically most important carnitine transporter [7, 8].

Evidence from both in vitro and in vivo studies clearly showed that genes involved in carnitine homeostasis such as OCTN2, TMABA-DH, and BBD are transcriptionally regulated by peroxisome proliferator-activated receptor α (PPARα), which is a ligand-activated transcription factor that acts as an important regulator of fatty acid catabolism [9, 10], and that activation of hepatic PPARα increases carnitine concentrations due to an increased carnitine uptake and elevated carnitine biosynthesis [11–14]. The essential role for PPARα in regulating carnitine homeostasis is confirmed by the observation that downregulation of PPARα as observed during lactation causes a reduction of hepatic carnitine concentrations [15], and that PPARα-deficient mice have markedly reduced carnitine levels in tissues along with reduced hepatic expression of OCTN2 and genes involved in carnitine biosynthesis including BBD and TMABA-DH [11, 13, 16].

Interestingly, recent studies showed that whole body carnitine status is significantly compromised in rodent models of genetic and diet-induced obesity and diabetes [17]. This effect has been attributed to a decreased hepatic expression of genes involved in carnitine biosynthesis and uptake [17]. Inhibition of expression of these genes by chronic HF feeding is supposed to be mediated by downregulation of PPARγ coactivator (PGC)-1α [18, 19], a transcriptional coactivator of PPARα which stimulates mitochondrial biogenesis and regulates genes involved in fatty acid catabolism through coactivation of PPARα, leading to a disturbed PPARα function. In contrast to chronic HF feeding, endurance exercise (EE) is known to stimulate PPARα function through upregulating PGC-1α, thereby, activating PPARα-dependent gene transcription [20]. We therefore hypothesized that regular EE may improve a HF diet-induced impairment of carnitine status through normalizing the disturbed PPARα function, thereby, leading to an increased expression of hepatic genes involved in carnitine synthesis and uptake. To test this hypothesis, we investigated the effect of regular EE on liver and skeletal muscle carnitine status and on the expression of genes regulating carnitine homeostasis in a mouse model of HF diet-induced obesity and insulin resistance.

2 Materials and methods

2.1 Animals and diets

The study was performed with a total of 18 male C57BL/6 mice (Charles River, Sulzfeld, Germany) aged 8–12 wk and weighing 19.5 ± 1.4 g. Mice were housed in groups of four to six animals per cage at $21 \pm 1^{\circ}$C in standard cages and had free access to food and water. After a 1 wk acclimation period, mice were randomly assigned to three groups. The first group (''standard diet,'' S) received a standard low-fat diet (Ref. C1090-10; Altromin, Lage, Germany) which provided 14.6 MJ metabolizable energy/kg diet and consisted of 24% of total energy as protein, 66% as carbohydrate, and 10% as fat. The second group (''HF'' diet) received a HF diet (Ref. C1090-45; Altromin) which provided 18.4 MJ metabolizable energy/kg diet and consisted of 20% of total energy as protein, 35% as carbohydrate, and 45% as fat. The third group received the same diet as the second group but had to perform regular EE (''HF diet+endurance exercise,'' HF+EE). The experimental diets were fed ad libitum for 10 wk. All experimental procedures described followed established guidelines for the care and handling of laboratory animals and were approved by the local Animal Care and Use Committee (Regierungspräsidium Giessen; permission no: GI20/24 No. 94/2010).

2.2 Exercise protocol

All animals were housed on a reverse light–dark cycle (lighting from 21:00 to 09:00 h). Mice of the HF+EE group were exercise trained during their dark cycle (i.e. during their active period) between 09:00 and 12:00 h on a motorized treadmill (customer made) for 35 min/day, 12% grade, five times per wk, for 10 wk. Running speed was 12.0 ± 2.5 m/min corresponding to 80% maximum oxygen consumption (VO_{2max}).

2.3 Glucose tolerance test

Insulin resistance was estimated by using an intraperitoneal glucose tolerance test performed at the end of the experimental period. Following a 12-h fasting period, tail vein blood was taken before, 30 min after, 60 min after, and 2 h after intraperitoneal application of 2 g glucose (dissolved in phosphate-buffered saline) per kilogram body weight. Blood glucose concentration was measured using a Glucometer (Roche Diagnostics, Mannheim, Germany). Total area under the curve (AUC) was calculated by using the trapezoidal rule [21].

2.4 Sample collection

The mice were killed by cervical dislocation under anaesthesia with CO_2. Whole blood was collected into ethylenediaminetetraacetic acid-containing tubes, and plasma was obtained by centrifugation ($1100 \times g$, 10 min, 4°C). Samples of liver and skeletal muscle (*M. gastrocnemius*) destined for RNA isolation and plasma were immediately snap-frozen in liquid nitrogen and stored at −80°C.

2.5 RNA isolation and RT-PCR analysis

Total RNA was isolated from liver and skeletal muscle using TrizolTM reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocol. RNA concentration and purity were estimated from the optical density at 260 and 280 nm, respectively. An aliquot of 1.2 μg RNA was subjected to cDNA synthesis using M-MuLV Reverse Transcriptase (MBI Fermentas, St. Leon-Rot, Germany). For the determination of mRNA expression levels, real-time detection RT-PCR using the Rotorgene 2000 system (Corbett Research, Mortlake, Australia) was applied. In all, 2 μL cDNA templates were amplified using the KAPA SYBR FAST qPCR Universal Mastermix (Peqlab, Erlangen, Germany) and 26.7 pmol of each primer pair. The PCR protocol comprised an initial denaturation at 95°C for 3 min and 35 cycles of amplification comprising denaturation at 95°C for 5 s and annealing and elongation at 60°C for 20 s. Subsequently, melting curve analysis was performed from 50 to 99°C with continuous fluorescence measurement. The amplification of a single product of the expected size was confirmed using 1.5% agarose gel electrophoresis. Relative quantification was performed using the $2^{-\Delta\Delta CT}$ method [22]. *Ct*-values of target genes and the reference gene were obtained using the Rotorgene Software 5.0. Relative expression ratios are expressed as fold changes of mRNA abundance in the HF and HF+EE groups compared with the S group. Characteristics of gene-specific primers obtained from Eurofins MWG Operon (Ebersberg, Germany) are listed in Table 1.

2.6 Immunoblot analysis

Homogenates were prepared from frozen liver aliquots using RIPA buffer (50 mM Tris, 150 mM NaCl, 10% glycerol, 0.1% SDS, 1% Triton X-100, 1 mM EDTA, 0.5% deoxycholate, 1% protease inhibitor mix; pH 7.5). Protein concentrations in the homogenates were determined by the bicinchoninic acid protein assay kit (Interchim, Montluçon, France) with BSA as standard. From each homogenate, 30 μg protein was separated on 12.5% SDS-PAGE and electrotransferred to a nitrocellulose membrane (Pall, Pensacola, FL, USA). Loading of equal amounts of protein in each line was verified by Ponceau S (Carl Roth, Karlsruhe, Germany) staining. After incubation, the membranes overnight at 4°C in blocking solution, membranes were incubated with primary antibodies against BBOX1 (BBD) (monoclonal anti-BBOX1 antibody; Abcam, Cambridge, UK), OCTN2 (polyclonal anti-OCTN2 antibody; LifeSpan Biosciences, Seattle, WA, USA), ALDH9A1 (TMABA-DH) (polyclonal anti-ALDH9A1 antibody, Abnova, Heidelberg, Germany), and β-actin (monoclonal anti-β-actin antibody, Abcam) as a reference protein to control for adequate normalization at room temperature. The membranes were washed, and then incubated with a horseradish peroxidase-conjugated secondary monoclonal anti-mouse-IgG antibody (Sigma-Aldrich, Steinheim, Germany) for BBD and β-actin and polyclonal anti-mouse-IgG antibody (DakoCytomation, Glostrup, Denmark) for OCTN2 and TMABA-DH at room temperature. Afterward, blots were developed using ECL Plus (GE Healthcare, München, Germany). The signal intensities of specific bands were detected with a Bio-Imaging system (Syngene, Cambridge, UK) and quantified using Syngene GeneTools software (nonlinear dynamics).

2.7 Carnitine analysis

Concentrations of free carnitine, acetylcarnitine, propionylcarnitine, palmitoylcarnitine, stearoylcarnitine, TML, and γ-BB in plasma and tissues were determined by tandem mass spectrometry according to Hirche et al. [23]. In brief, freeze-dried tissue samples were extracted with methanol:water (2:1 v/v) by homogenization (Tissue Lyser, Qiagen, Hilden, Germany), followed by sonification for 20 min and incuba-

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

Gene	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)	PCR product size (bp)	NCBI GenBank
ACO	CAGGAAGAGCAAGGAAGTGG	CCTTTCTGGCTGATCCCATA	189	NM_015729
BBD	CGAAGCTAACTGGCTGAAGA	CCACATTGTTGGCATCAATCT	200	BC019406
GAPDH	AACGACCCCTTCATTGAC	TCCACGACATACTCAGCAC	191	XM_001476707
L-CPT I	CCAGGCTACAGTGGGACATT	GAACTTGCCCATGTCCTTGT	209	NM_013495
M-CPT I	GTCGCTTCTTCAAGGTCTGG	AAGAAAGCAGCACGTTCGAT	232	NM_009948
OCTN2	CCTGTGCCTCACACCGTGAA	CCTAGCTCAGAGAAGTTGGC	213	AF110417
PGC-1α	AAACTTGCTAGCGGTCCTCA	TGTTGACAAATGCTCTTC	342	NM_008904
PGC-1β	AACCCAACCAGTCTCACAGG	TGCTGCTGTCCTCAAATACG	371	NM_133249
PPARα	CGGGAAAGACCAGCAACAAC	TGGCAGTGGAAGAATCG	137	NM_011144
TMABA-DH	AGCTGAAGACGGTGTGTGTG	CTAATGACCCAAAGCCTGGA	154	NM_019993

ACO, acyl-CoA oxidase; BBD, γ-butyrobetaine dioxygenase; GAPDH, glyceraldehyde-3 phosphate dehydrogenase; L-CPT I, liver-type carnitine-palmitoyltransferase I; M-CPT I, muscle-type carnitine-palmitoyltransferase; OCTN2, novel organic cation transporter 2; PGC-1α, PPARγ co-activator-1α; PGC-1β, PPARγ co-activator-1β; PPARα, peroxisome proliferator-activated receptor α; TMABA-DH, 4-N-trimethylaminobutyraldehyde dehydrogenase.

tion at 50°C for 30 min in a shaker. After centrifugation (13 000 × *g*, 10 min) 20 μL of the supernatant were added with 100 μL methanol containing the internal standards, mixed, incubated for 10 min, and centrifuged (13 000 × *g*, 10 min). Plasma samples were handled at 4°C in the same manner as the supernatant after tissue extraction. The final supernatants were used for quantification of the compounds by a 1100 series 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 LC-MS/MS-System (Applied Biosystems, Darmstadt, Germany). As eluents, methanol and a methanol:water:ACN:acetic acid mixture (100:90:9:1 v/v/v/v) were used.

2.8 Statistical analysis

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

3 Results

3.1 Body weight development and development of glucose intolerance

Body weights of the mice were recorded at the beginning and at the end of the 10 wk experimental period, to evaluate body weight development. Mice fed the HF diets had higher final body weights and body weight gains than mice fed the standard diet ($p < 0.05$; Table 2). Final body weights and total body weight gains did not differ between exercising and nonexercising mice fed the HF diets. To evaluate glucose intolerance of the mice, an intraperitoneal glucose tolerance test was performed at the last experimental day. This test revealed that mice fed the HF diets had an impaired glucose tolerance as shown by the high AUC for glucose compared with mice fed the standard diet ($p < 0.05$, Table 2). However, the AUC for glucose was lower in exercising than in nonexercising mice fed the HF diet ($p < 0.05$, Table 2).

Table 2. Body weight development and glucose tolerance of non-exercising mice fed either a standard diet (S) or a high-fat diet (HF) and of exercising mice fed a high-fat diet (HF+EE)

	S	HF	HF+EE
Body weight development			
Initial body weight (g)	20.1 ± 1.1	19.5 ± 1.3	18.8 ± 1.3
Final body weight (g)	29.4 ± 1.7^b	45.3 ± 2.3^a	42.9 ± 4.2^a
Total body weight gain (g)	9.3 ± 0.9^b	25.4 ± 1.9^a	23.8 ± 3.6^a
Glucose tolerance			
Glucose (AUC)	16.4 ± 2.5^c	26.1 ± 4.2^a	22.0 ± 3.0^b

Values are mean ± SD ($n = 6$ per group). Means with different superscript letters differ ($p < 0.05$).

3.2 Concentrations of free and acetylcarnitine in plasma of mice

To evaluate the carnitine status of the mice, we determined the concentrations of free carnitine and acylcarnitines in plasma. Concentrations of free carnitine and acetylcarnitine in plasma were lower in the HF diet groups than in the standard diet group ($p < 0.05$, Table 3). Other acylcarnitines were below the limit of detection (0.01, 0.01, and 0.027 nmol/g wet weight for propionylcarnitine, palmitoylcarnitine and stearoylcarnitine, respectively). In addition, the concentration of total carnitine (sum of free carnitine and acetylcarnitine) in plasma was lower in the HF diet groups than in the standard diet group ($p < 0.05$, Table 3). In mice fed the HF diets, the concentrations of free carnitine, acetylcarnitine, and total carnitine in plasma did not differ between exercising and nonexercising mice (Table 3).

3.3 Concentrations of free carnitine, acylcarnitines, and total carnitine in liver and skeletal muscle of mice

To evaluate tissue carnitine status of the mice, we determined the concentrations of free carnitine and acylcarnitines and calculated the concentration of total carnitine in liver and skeletal muscle. In the liver, concentration of free and total carnitine was lower in nonexercising mice fed the HF diet than in those fed the standard diet ($p < 0.05$, Table 4). In exercising mice fed the HF diet, the concentration of free and total carnitine in the liver did not differ from mice fed the standard diet but was higher than in nonexercising mice fed the HF diet (Table 4). The concentrations of acylcarnitines in the liver did not differ between the three groups (Table 4). In skeletal muscle, concentrations of free carnitine, acetylcarnitine, palmitoylcarnitine, stearoylcarnitine, and total carnitine did not differ between the three groups (Table 4). The concentration of propionylcarnitine in skeletal muscle was lower in the exercising mice fed the HF diet than in the two other groups ($p < 0.05$, Table 4).

3.4 Concentrations of carnitine precursors, TML, and BB, in plasma, liver, and skeletal muscle of mice

To study whether carnitine biosynthesis was influenced by the treatment protocol, we determined the concentrations of the carnitine precursors TML and BB in tissues of the mice. The concentration of BB in plasma, liver, and skeletal

Table 3. Concentrations of free carnitine, acylcarnitines and total carnitine in plasma of non-exercising mice fed either a standard diet (S) or a high-fat diet (HF) and of exercising mice fed a high fat diet (HF+EE)

	S	HF	HF+EE
Free carnitine (μmol/L)	13.4±0.8[a]	10.2±1.3[b]	8.5±2.8[b]
Acetylcarnitine (μmol/L)	6.13±0.67[a]	2.66±0.86[b]	3.04±1.19[b]
Propionylcarnitine (μmol/L)	n.d.*	n.d.*	n.d.*
Palmitoylcarnitine (μmol/L)	n.d.*	n.d.*	n.d.*
Stearoylcarnitine (μmol/L)	n.d.*	n.d.*	n.d.*
Total carnitine # (μmol/L)	19.5±1.4[a]	12.9±2.2[b]	11.6±3.8[b]

Values are mean±SD (n = 5–6 per group). Means with different superscript letters differ significantly ($p<0.05$). #Total carnitine: sum of free carnitine, acetylcarnitine and propionylcarnitine. *n.d., not detectable; Limit of detection: 0.01, 0.01 and 0.027 nmol/g wet weight for propionylcarnitine, palmitoylcarnitine and stearoylcarnitine, respectively.

Table 4. Concentrations of free carnitine, acylcarnitines and total carnitine in liver and skeletal muscle of non-exercising mice fed either a standard diet (S) or a high-fat diet (HF) and of exercising mice fed a high-fat diet (HF+EE)

	S	HF	HF+EE
Liver			
Free carnitine (nmol/g wet weight)	149±25[a]	121±18[b]	146±13[a]
Acetylcarnitine (nmol/g wet weight)	0.45±0.09	0.55±0.43	0.53±0.21
Propionylcarnitine (nmol/g wet weight)	0.17±0.09	0.20±0.08	0.19±0.04
Palmitoylcarnitine (nmol/g wet weight)	0.03±0.02	0.04±0.04	0.04±0.03
Stearoylcarnitine (nmol/g wet weight)	n.d.*	n.d.*	n.d.*
Total carnitine (nmol/g wet weight)	150±25[a]	122±81[b]	147±13[a]
Skeletal muscle			
Free carnitine (nmol/g wet weight)	51.7±8.7	56.7±11.4	64.0±12.8
Acetylcarnitine (nmol/g wet weight)	54.1±14.6	41.4±14.3	43.1±6.5
Propionylcarnitine (nmol/g wet weight)	1.62±0.44[a]	1.48±0.63[a]	0.59±0.31[b]
Palmitoylcarnitine (nmol/g wet weight)	2.46±1.66	2.14±2.34	2.26±1.08
Stearoylcarnitine (nmol/g wet weight)	0.50±0.31	0.64±0.51	0.68±0.21
Total carnitine# (nmol/g wet weight)	110±20	102±20	111±16

Values are means±SD (n = 5-6 per group). Means with different superscript letters differ ($p<0.05$). #Total carnitine: sum of free carnitine, acetylcarnitine, propionylcarnitine, palmitoylcarnitine and stearoylcarnitine. *n.d., not detectable. Limit of detection: 0.01 nmol/g wet weight and 0.027 nmol/g wet weight for palmitoylcarnitine and stearoylcarnitine, respectively.

muscle was lower in mice fed the HF diets than in mice fed the standard diet ($p<0.05$, Table 5). In mice fed the HF diets, the concentration of BB in these tissues did not differ between exercising and nonexercising mice. The concentration of TML in liver was lower in mice fed the HF diets than in mice fed the standard diet ($p<0.05$, Table 5). In mice fed the HF diets, the concentration of TML in the liver did not differ between exercising and nonexercising mice. The concentration of TML in plasma and skeletal muscle did not differ between mice of all groups.

3.5 Relative mRNA and protein concentrations of genes involved in carnitine biosynthesis and uptake in the liver of mice

To further study whether the altered concentration of carnitine in the liver might be explained by alterations in the

Table 5. Concentrations of the carnitine precursors trimethyllysine (TML) and γ-butyrobetaine (BB) in plasma, liver and skeletal muscle of non-exercising mice fed either a standard diet (S) or a high-fat diet (HF) and of exercising mice fed a high-fat diet (HF+EE)

	S	HF	HF+EE
Plasma			
TML (μmol/L)	0.55±0.06	0.82±0.24	0.58±0.16
BB (μmol/L)	0.71±0.19[a]	0.45±0.09[b]	0.33±0.11[b]
Liver			
TML (nmol/g)	33.2±4.8[a]	24.1±3.8[b]	23.6±3.1[b]
BB (nmol/g)	6.20±0.75[a]	1.96±0.58[b]	1.73±0.38[b]
Skeletal muscle			
TML (nmol/g)	8.53±2.92	6.89±2.10	5.99±0.64
BB (nmol/g)	5.40±1.31[a]	4.04±0.59[b]	3.47±0.48[b]

Values are mean±SD (n = 5–6 per group). Means with different superscript letters differ significantly ($p<0.05$).

expression of genes involved in carnitine biosynthesis and uptake, we determined mRNA and protein levels of respective genes. Relative mRNA and protein levels of OCTN2, BBD, and TMABA-DH in the liver were markedly lower in nonexercising mice fed the HF diet than in mice fed the standard diet ($p<0.05$, Fig. 1A and B). In mice fed the HF diets, the mRNA and protein levels of BBD in the liver were higher in exercising than in nonexercising ones ($p<0.05$, Fig. 1A and B). The relative mRNA level of OCTN2 in the liver tended to be higher in exercising mice fed the HF diet than in nonexercising mice fed the HF diet ($p<0.15$, Fig. 1A). The protein level of OCTN2 was higher in exercising mice fed the HF diet than in nonexercising mice fed the HF diet ($p<0.05$), but lower in exercising mice fed the HF than in mice fed the standard diet ($p<0.05$, Fig. 1B). The relative mRNA and protein levels of TMABA-DH in the liver tended to be higher in exercising mice fed the HF diet than in nonexercising mice fed the HF diet ($p<0.15$, Fig. 1A and B).

3.6 Relative mRNA concentrations of PPARα, PPARα coactivators, and genes involved in fatty acid oxidation in liver and skeletal muscle of mice

To finally investigate whether changes in the expression of genes involved in carnitine synthesis and uptake were due to alterations in the transcriptional activity of PPARα, we determined mRNA levels of PPARα, PPARα coactivators, and classical downstream targets of PPARα. Relative mRNA levels of acyl-CoA oxidase (ACO), PGC-1α, and PGC-1β in the liver were markedly lower in nonexercising mice fed the HF diet than in mice fed the standard diet ($p<0.05$, Fig. 2). Exercising mice fed the HF diet had a higher relative mRNA level of PGC-1β in the liver than nonexercising mice fed the HF diet but a lower relative mRNA level of PGC-1β in the liver than mice fed the standard diet ($p<0.05$, Fig. 2). The relative mRNA levels of ACO and PGC-1α in the liver did not differ between exercising and nonexercising mice fed the HF diet (Fig. 2). The mRNA levels of PPARα and

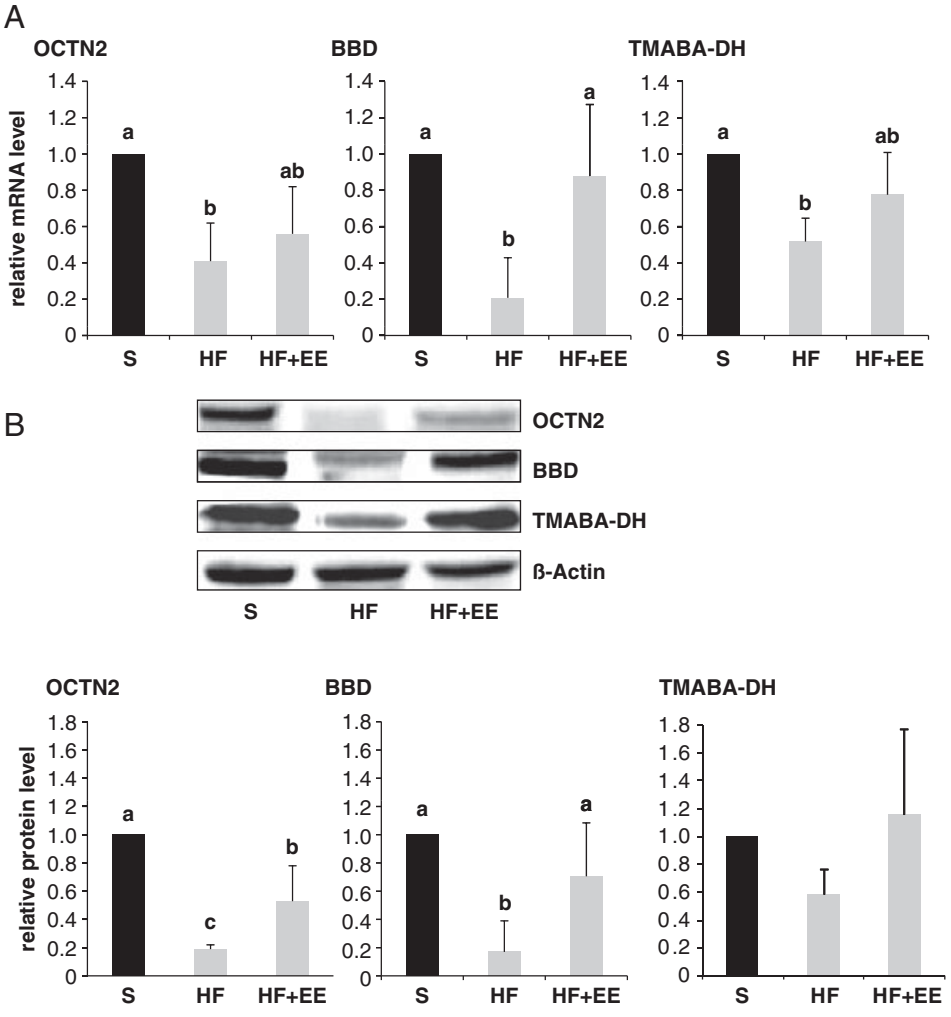
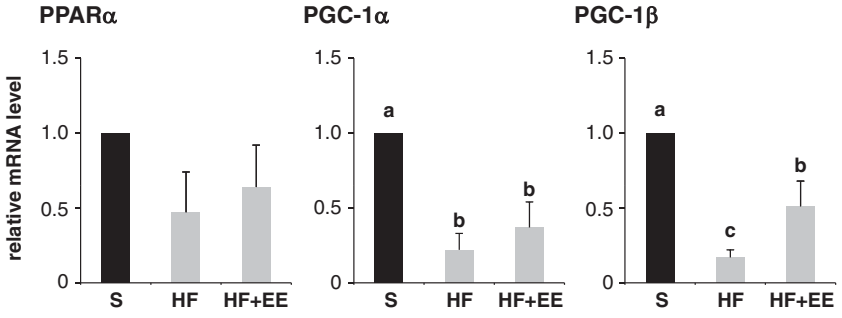


Figure 1. Relative mRNA and protein concentrations of genes involved in carnitine uptake (OCTN2) and carnitine synthesis (BBD, TMABA-DH) in the liver of nonexercising mice fed either a standard diet (S) or a HF diet and of exercising mice fed a HF diet (HF+EE). (A) Relative mRNA concentrations of OCTN2, BBD, and TMABA-DH; bars represent mean ± SD ($n=6$ animals/group) and are expressed relative to the mRNA level of group S (= 1.00). (B) Representative immunoblots specific to OCTN2, BBD, TMABA-DH, and β-actin as internal control are shown for one animal per group; immunoblots for the other animals revealed similar results; bars represent data from densitometric analysis and represent mean ± SD ($n=6$ animals/group); bars are expressed relative to the protein level of group S (= 1.00). [a,b,c]Bars with different superscript letters differ significantly, $p<0.05$.



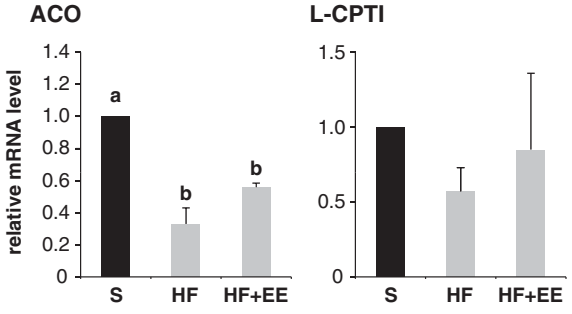
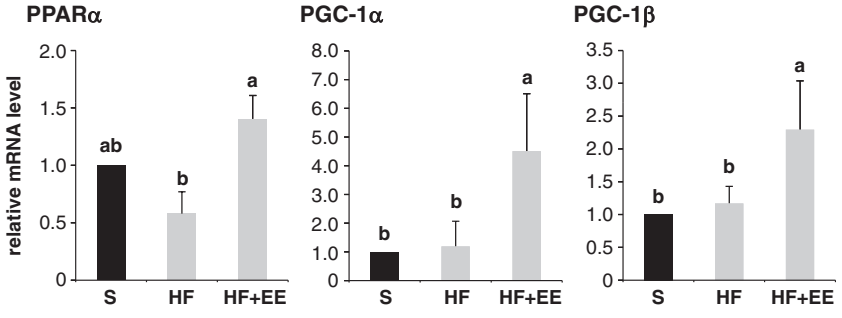


Figure 2. Relative mRNA concentrations of PPARα, PPAR coactivators (PGC-1α, PGC-1β), and PPARα target genes (ACO, L-CPT I) in the liver of nonexercising mice fed either a standard diet (S) or a HF diet and of exercising mice fed a HF diet (HF+EE). Bars represent mean ± SD ($n = 6$ animals/group) and are expressed relative to the mRNA level of group S (= 1.00). [a,b,c]Bars with different superscript letters differ significantly, $p < 0.05$.



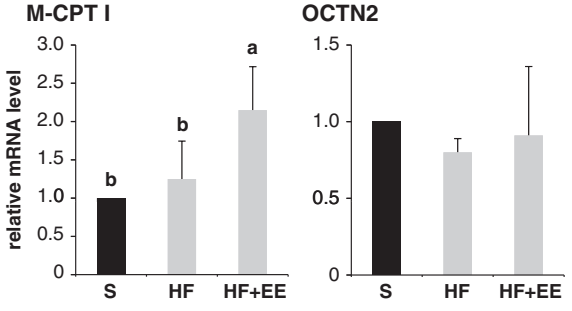


Figure 3. Relative mRNA concentrations of PPARα, PPAR coactivators (PGC-1α, PGC-1β), and PPARα target genes (ACO, L-CPT I, and OCTN2) in the gastrocnemius muscle of nonexercising mice fed either a standard diet (S) or a HF diet and of exercising mice fed a HF diet (HF+EE). Bars represent mean ± SD ($n = 6$ animals/group) and are expressed relative to the mRNA level of group S (= 1.00). [a,b,c]Bars with different superscript letters differ significantly, $p < 0.05$.

L-carnitine-palmitoyltransferase I (CPT I) in the liver did not differ between the three groups (Fig. 2). Relative mRNA levels of PPARα, PGC-1α, and PGC-1β and M-CPT I in skeletal muscle did not differ between mice fed the standard diet and nonexercising mice fed the HF diet (Fig. 3). Exercising mice fed the HF diet had higher mRNA levels of PPARα, PGC-1α, PGC-1β, and M-CPT I in skeletal muscle than nonexercising mice fed the HF diet ($p < 0.05$, Fig. 3). Relative mRNA level of OCTN2 in skeletal muscle did not differ between the three groups (Fig. 3).

4 Discussion

It has been shown that metabolic stress induced by chronic HF diet feeding or genetically induced diabetes is accompanied by an impaired carnitine status, and, as a consequence, a diminished mitochondrial fuel metabolism and glucose disposal [17]. In the present study, we tested the hypothesis that regular EE improves the HF diet-induced impairment of carnitine status through stimulating the expression of hepatic genes involved in carnitine synthesis

and uptake. As a model, we used mice that were fed either a high-caloric HF diet or a normo-caloric standard diet. After 10 wk of feeding, the HF diet-fed mice were severely obese when compared with mice fed the standard diet. Interestingly, final body weights of the HF diet-fed mice did not significantly differ between exercising and nonexercising mice. However, glucose tolerance was significantly improved in exercising compared with nonexercising mice fed the HF diet indicating that EE lowered metabolic stress. The main finding of the present study is that nonexercising mice fed the HF diet had approximately 20% reduced free and total carnitine levels in the liver, whereas exercising mice fed the HF diet had similar hepatic carnitine levels as nonobese mice fed a standard diet. Noteworthy, the concentrations of acylcarnitines in the liver were not altered by feeding the HF diet demonstrating that the decrease in hepatic-free carnitine content was not compensated by a parallel increase in the hepatic content of acylcarnitine species. Thus, our findings indicate that regular EE in mice fed a HF diet lowers metabolic stress, thereby, preventing the HF diet-induced impairment of carnitine status in the liver.

To gain insight into the mechanisms underlying the beneficial effect of EE on hepatic carnitine content, we determined hepatic mRNA and protein levels of genes involved in carnitine uptake and carnitine biosynthesis and concentrations of carnitine precursors in the liver. In agreement with the recent study of Noland et al. [17], our study shows that feeding a HF diet to nonexercising mice resulted in decreased mRNA and protein levels of the plasmalemmal carnitine transporter OCTN2 and enzymes involved in carnitine biosynthesis, TMABA-DH, and BBD and reduced concentrations of BB in plasma, liver, and skeletal muscle, indicating that the enzymatic conversion of BB into carnitine and the uptake of carnitine into cells was lowered in these mice. Our study, moreover, shows that obese mice fed the HF diet had reduced concentrations of BB, the direct precursor of carnitine, in plasma, liver, and skeletal muscle. In rats and humans, it has been shown that the availability of BB can be rate-limiting for the synthesis of carnitine rather than the activity of BBD [24, 25]. Therefore, it is likely that a reduced availability of BB as a precursor of carnitine synthesis contributed to the diminished carnitine concentrations in the mice fed the HF diet.

A key finding of the present study is that concomitant regular EE (five times a wk) in mice fed the HF diet caused a significant increase in the hepatic mRNA and protein levels of BBD, the last enzyme of carnitine biosynthesis, and protein level of OCTN2. In addition, the mRNA and protein level of TMABA-DH in the liver tended to be increased when compared with nonexercising mice fed the HF diet. These observations suggest that EE stimulates hepatic carnitine biosynthesis by an increased expression of enzymes involved in the carnitine synthesis pathway and, to a lesser extent, carnitine uptake into the liver. As plasma and tissue concentrations of BB were not different between exercising and nonexercising mice, it is unlikely that carnitine synthesis differed between the two groups of mice due to a different availability of BB. Collectively, our findings suggest that regular EE is capable of completely reversing the HF diet-induced impairment of hepatic carnitine status by stimulating carnitine synthesis and uptake.

Regarding that PPARα plays an important role for carnitine homeostasis by transcriptionally regulating genes involved in carnitine synthesis and uptake [11–14], we also considered transcript levels of classical PPARα target genes as well as PPAR coactivators in the liver. In agreement with recent evidence that chronic HF feeding disrupts hepatic PPARα function [18, 19], we observed that, besides OCTN2, TMABA-DH, and BBD, the classical PPARα target gene ACO and the PPAR coactivators PGC-1α und PGC-1β, which cooperate with PPARα in the transcriptional control of PPARα target genes [26, 27], were markedly downregulated by feeding the HF diet. In addition, the mRNA level of L-CPT I was at least numerically decreased by the HF diet although this effect was not significant. Regardless, this strongly suggests that PPARα-dependent gene transcription in the liver of nonexercising mice was impaired by feeding the HF diet. Moreover, this finding indicates that hepatic carnitine status was diminished by the HF diet through inhibiting PPARα-dependent gene transcription. Noteworthy, considering that concomitant application of EE to mice fed the HF diet significantly elevated hepatic expression of BBD and PGC-1β and tended to increase that of TMABA-DH and OCTN2, our data suggest that EE was able to restore, at least partially, the HF diet-induced perturbation of PPARα function.

In skeletal muscle, concentration of free carnitine was markedly lower than in the liver. This finding is in agreement with the recent reports [6, 28], although the concentration of free carnitine in skeletal muscle of mice in the present study (wet weight, 52–64 nmol/g) was lower than reported from others. However, the values for free carnitine concentration in skeletal muscle of mice reported in the literature vary greatly (wet weight, 120–200 nmol/g; [6, 28, 29]), indicating that variations from this range, which may be caused by differences in strain, age or muscle type, may be not unexpected. Regardless, we exclude the possibility that the low skeletal muscle carnitine content is due to an inappropriate tissue extraction protocol because we have found a complete recovery for carnitine using this method [23]. In contrast to the liver, both carnitine content and expression of PPARα and PPARα target genes in the gastrocnemius muscle of the mice were not decreased by the HF diet. This finding contrasts that of a recent study reporting that carnitine levels in gastrocnemius muscle were diminished in multiple rat models of insulin resistance, including ZDF rats and diet-induced obesity [17]. The authors of this study [17], however, also observed that a high endurance capacity rat strain could maintain gastrocnemius muscle levels of carnitine when fed a HF diet, indicating

that the susceptibility to deplete muscle carnitine levels in response to a HF diet is dependent on the model used. Moreover, it is conceivable that the depletion of muscle carnitine is dependent on the duration of HF diet feeding which was shorter in our study when compared with that in the study from Noland et al. [17]. Regardless of this, we found that PPARα in skeletal muscle was strongly activated in exercising compared with nonexercising mice fed the HF diet as evidenced by markedly increased mRNA levels of M-CPT 1, PPARα, PGC-1α, and PGC-1β. This effect is in-line with the previous studies demonstrating an upregulation of PPARα target genes by EE in skeletal muscle of laboratory animals and normal weight, overweight, and obese humans [30–34] and has been interpreted to be indicative of an enhanced mitochondrial function and fatty acid β-oxidation in the exercising muscle [34]. In contrast to the other PPARα target genes investigated, the expression of OCTN2 in skeletal muscle was not upregulated in exercising compared with nonexercising mice. Although we have currently no explanation for this, the unaltered expression of OCTN2 concurs well with the unchanged carnitine concentrations in skeletal muscle between groups. Since the skeletal muscle acts as the body's carnitine storage site, it is likely that the enhanced carnitine demand for mitochondrial fatty acid oxidation in skeletal muscle of exercising mice was adequately fulfilled by the skeletal muscle carnitine, thus, making an increased carnitine uptake from plasma into the skeletal muscle unnecessary. One might speculate that a stimulatory effect of EE on OCTN2 expression would have been observed in a skeletal muscle with a higher type I (oxidative/slow) fiber content than in gastrocnemius muscle, which is a representative muscle composed of a mixture of type I, IIa, and IIb fibers. Type I fibers are mitochondria-rich and mainly use fatty acids for energy production and are therefore more dependent on carnitine than type II (glycolytic/fast) fibers which have a low content of mitochondria and oxidative enzymes.

In conclusion, our study shows for the first time that regular EE in mice fed a HF diet prevents the HF diet-induced impairment of carnitine status in the liver. Although the present study has the limitation that the sample size was relatively small, we observed increased transcript and protein levels of BBD, TMABA-DH, and OCTN2 in the liver of exercising mice fed the HF diet, indicating that EE is capable of reversing the HF diet-induced impairment of hepatic carnitine content by stimulating hepatic carnitine synthesis and uptake. It is well known that EE induces several adaptations in skeletal muscle leading to enhancements of insulin-mediated glucose metabolism and overall oxidative enzyme activities in skeletal muscle [35, 36]. Since skeletal muscle is a major organ contributing to the development of peripheral insulin resistance [37], the abovementioned adaptations in fuel utilization have to be considered as key events responsible for the prevention from HF diet-induced insulin resistance. Nevertheless, future studies have to clarify whether the improvement of carnitine status observed in exercising mice on a HF diet also contributes to the improved glucose tolerance in these mice.

The authors have declared no conflict of interest.

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4. Diskussion

4.1 Carnitinsynthese beim Schwein

Der Mensch nimmt mit der normalen Mischkost täglich 0,3-1,9 mg Carnitin pro kg Körpergewicht und Tag auf (Rebouche *et al.*, 1989). Vor allem tierische Produkte wie Fleisch, Fisch und Milchprodukte, weisen hohe Konzentrationen an Carnitin auf und stellen somit die wesentlichen Quellen in der menschlichen Ernährung dar (Rebouche *et al.*, 1989; Rebouche, 1992). Der Körper ist in der Lage, sich einer geringen Carnitinzufuhr über die Nahrung, wie es beispielsweise bei vegetarischer Ernährung der Fall ist, anzupassen. So konnte gezeigt werden, dass im Gegensatz zu Omnivoren, welche etwa 75% des im Körper verfügbaren Carnitins aus der Nahrung beziehen und lediglich 25% des Carnitinbestandes über *de novo* Synthese decken, strikte Veganer 90% des verfügbaren Carnitins über die endogene Synthese bereitstellen können (Rebouche, 1992; Vaz und Wanders, 2002). Bekannt ist, dass die Synthese von Carnitin nicht in allen Geweben bei allen Spezies gleichermaßen erfolgt. Darüber hinaus existieren in der Literatur Hinweise auf eine Altersabhängigkeit der der BBD-Aktivität. So konnte beim Menschen gezeigt werden, dass die Aktivität der BBD in der Leber von Säuglingen geringer ist im Vergleich zur vollständigen Aktivität des Enzyms im Erwachsenenalter (Rebouche und Engel, 1980; Olson und Rebouche, 1987). Darüber hinaus konnten Galland *et al.* (1999) mithilfe der Methode des *Northern Blottings* bei der Ratte zeigen, dass die Expression der BBD-mRNA in der Leber erst nach dem Absetzen der Jungtiere erfolgt und diese mit zunehmendem Alter der Tiere ansteigt. In ***Studie 1*** sollte daraufhin zunächst geklärt werden, welche Gewebe vom Schwein zur vollständigen Carnitinsynthese befähigt sind. Weiterhin sollten in dieser Studie altersabhängige Unterschiede hinsichtlich der Aktivität der BBD, dem Schlüsselenzym der Carnitinsynthese, ermittelt werden. So konnte in Leber und Niere der Schweine eine messbare Aktivität der BBD nachgewiesen werden, wobei die renale Aktivität der BBD um das 6-fache höher war im Vergleich zur BBD-Aktivität in der Leber. Des Weiteren konnte gezeigt werden, dass die Aktivität der BBD in Niere und Leber der Ferkel vom Tag der Geburt bis zur 3. Lebenswoche ansteigt und somit altersabhängig reguliert wird (für einen vertiefenden Einblick in die Ergebnisse sei auf die Originalarbeit ***Studie 1*** verwiesen). Infolgedessen ist anzunehmen, dass sowohl die Lokalisation, als auch die Aktivitätsunterschiede der BBD in den Geweben beim Schwein ähnlich der des Menschen sind, wobei lediglich die hepatische und nicht die renale BBD beim Menschen altersabhängig reguliert wird (Rebouche und Engel, 1980; Olson und Rebouche, 1987; Vaz und Wanders, 2002). Interessanterweise konnten beim Schwein messbare mRNA- und Proteinkonzentrationen der BBD in nahezu allen untersuchten

Geweben detektiert werden, auch in den Geweben, in denen keine aktive Form der BBD nachgewiesen werden konnte. Galland *et al.* (1998; 1999) konnten in Leber, Hoden und Nebenhoden von Ratten BBD-mRNA, das aktive BBD-Protein jedoch lediglich in der Leber nachweisen. Die Enzyme der Carnitinsynthese unterliegen während allen Prozessierungsschritten einer Reihe von Einflussfaktoren. Galland *et al.* (1999) begründen die gewebespezifischen Unterschiede mit dem Vorhandensein verschiedener spezifischer Spleißvarianten der BBD-mRNA, welche sich in verschiedenen Größen des Transkriptes widerspiegeln. Auch in der menschlichen Leber und Niere sowie in der Leber von Ratten und Kälbern konnten drei Isoformen der BBD nachgewiesen werden, welche die gleichen Cofaktoren benötigen und auch die gleiche spezifische Aktivität aufweisen (Lindstedt und Nordin, 1984). Isoenzyme sind unterschiedliche molekulare Formen eines Enzymproteins, die ein und dieselbe Reaktion katalysieren, welche einerseits speziesspezifisch auftreten, andererseits aber auch innerhalb desselben Organismus in unterschiedlichen Geweben und Zellkompartimenten vorkommen können. Einzelne Isoformen können unterschiedliche Affinitäten zu ihrem Substrat aufweisen und sich auch bezüglich ihres kinetischen Verhaltens und hinsichtlich der bevorzugten Coenzyme und der regulatorischen Eigenschaften unterscheiden. Fragwürdig ist, ob auch beim Schwein durch die Prozessierung des Transkriptes verschiedene Isoformen organspezifisch auftreten. Die Aktivität eines fertig translatierten Proteins wird durch gewebespezifische posttranslationale Prozesse reguliert. Unsere Untersuchungen zeigen, dass lediglich in Leber und Niere vom Schwein ein aktives BBD-Protein synthetisiert wird, obwohl in nahezu allen untersuchten Geweben die Transkription des BBD-Gens stattgefunden hat. Daher ist anzunehmen, dass die BBD posttranslational, beispielsweise durch Interkonversion, reguliert wird. Interkonversion ist eine reversible enzymkatalysierte chemische Modifikation von Enzymen, wodurch ein vollständiges An- bzw. Abschalten der katalytischen Wirksamkeit eines Enzyms erfolgt. Je nach Enzym kann eine Phosphorylierung ein Anschalten der Enzymaktivität oder ein Abschalten hervorrufen. Unklar ist allerdings inwiefern Phosphorylierungs- bzw. Dephosphorylierungsreaktionen in unterschiedlichen Geweben im Körper unterschiedliche Effekte hinsichtlich der Aktivität ein und desselben Enzyms erzielen können, so dass in einigen Geweben (wie beispielsweise Leber und Niere) dieses Enzym aktiviert, in einem Anderen jedoch inaktiviert wird. Die Klärung der Frage inwieweit posttranslationale und/oder posttranskriptionale Regulationsmechanismen ursächlich für die gewebespezifischen Aktivitätsunterschiede der BBD sind, sollte Gegenstand zukünftiger Untersuchungen sein. Die Verfügbarkeit von Cofaktoren der Carnitinsynthese und Carnitin-Precursoren im

Organismus stellen weitere Einflussfaktoren dar. Es wird vermutet, dass der Precursor BB kompetitiv den Transport von Carnitin in den Muskel hemmt, indem er mit Carnitin um die Aufnahme in dieses Gewebe konkurriert. Die Folge davon könnte ein Anstieg der Plasmakonzentration an Carnitin und somit auch ein Anstieg der renalen Exkretion sein, was sich wiederum in einer gesteigerten Carnitinsynthese widerspiegeln könnte (Olson und Rebouche, 1987). Kinder und Erwachsene reagieren auf die Aufnahme einer BB angereicherten Nahrung mit einer stark erhöhten renalen Carnitinausscheidung (Olson und Rebouche, 1987; Rebouche *et al.*, 1989). Bereits 1983 wurde bei Ratten entdeckt, dass eine 1%ige BB-Diät zu einer Hemmung des Carnitintransportes führte, im Gegensatz zu einer Diät mit 0,1% BB (Rebouche, 1983). Ferner zeigten Rebouche *et al.* (1986), dass die Fütterung von BB an Ratten die Rate der Carnitinbiosynthese stark erhöht. Dies lässt vermuten, dass auch bei Schweinen die Verfügbarkeit des Precursors BB und nicht die Aktivität der BBD den limitierenden Faktor der Carnitinsynthese darstellt.

Die Ergebnisse der ersten Studie deuten insgesamt darauf hin, dass das Schwein einzig in der Leber und Niere zur vollständigen Carnitinsynthese befähigt ist. Darüber hinaus konnte erstmalig gezeigt werden, dass die Aktivität der BBD in diesen beiden Geweben beim Ferkel altersabhängig reguliert wird. In zukünftigen Studien gilt es zu klären, inwiefern das BBD-Protein gewebespezifisch prozessiert und damit die Aktivität dieses Enzyms reguliert wird.

4.2 Molekulare Wirkmechanismen von Carnitin beim Schwein

Carnitin hat in den letzten Jahren großes wissenschaftliches Interesse aufgrund seiner günstigen Wirkungen auf die Leistungsphysiologie beim Nutztier auf sich gezogen. Trotz der Fähigkeit des Organismus, Carnitin in einigen Geweben eigenständig zu synthetisieren, konnten durch Carnitinzulagen bei einer Reihe von Nutztierarten phänotypische Veränderungen hinsichtlich der Lebendmasseentwicklung und Futterverwertung aber auch in Bezug auf die Reproduktionsleistung nachgewiesen werden. Auch beim Menschen deuten einige Befunde aus wissenschaftlichen Untersuchungen auf einen günstigen Einfluss von Carnitin in der Therapie zahlreicher Erkrankungen wie *Diabetes mellitus* Typ II, Aids und Krebskachexie sowie altersbedingten mitochondrialen und neurodegenerativen Dysfunktionen hin. Die zugrundeliegenden molekularen und biochemischen Mechanismen, über welche Carnitin seine vielfältigen Wirkungen im Organismus vermittelt, sind bislang unbekannt. Zur Aufklärung dieser Mechanismen von Carnitin stellt das Schwein aufgrund seiner Ähnlichkeit mit dem Menschen bezüglich der Stoffwechselphysiologie sowie der Sequenz- und

Strukturhomologie des Genoms einen optimalen Modellorganismus dar (Lunney, 2007). Im Rahmen dieser Arbeit konnte mithilfe der *microarray*-Technologie erstmalig gezeigt werden, dass eine Carnitinsupplementierung mit dem Futter zahlreiche Veränderungen der Genexpression in Leber und Muskel von Ferkeln bewirkt. Die *microarray*-Technologie ermöglicht den Vergleich von Genexpressionsstärken und -profilen in einer Behandlungsgruppe gegenüber unbehandelten Kontrollgeweben und kann somit unter anderem der Aufklärung komplexer molekularer Wirkmechanismen von Futtermittelzusatzstoffen auf das Transkriptom beim Nutztier dienen. Bei dieser Art von Expressionsanalysen wird dabei der momentane Expressionsstatus aller Gene eines Gewebes zu einem bestimmten Zeitpunkt erfasst. Die Anwendung dieser Technologie ermöglicht Aussagen über den Einfluss bestimmter Nährstoffe (in diesem Fall Carnitin) auf die Expression zahlreicher Zielmoleküle. Allerdings werden Prozesse, die auf Proteinebene reguliert werden, wie beispielsweise über die Phosphorylierung von Proteinen oder deren Abbau, nicht erfasst. Mithilfe der sogenannten Clusteranalyse ist, auf der Basis von statistischen Methoden, die Interpretation der Fülle an Daten möglich mit deren Hilfe Zusammenhänge und Analogien in der Expression einzelner Gene hergestellt werden können. Ähnlich exprimierte Gene werden dabei in verschiedenen Clustern zusammengefasst. Die Analyse des Transkriptprofils der Leber und des Muskels hat ergeben, dass die dreiwöchige Carnitinsupplementierung der Ferkel zu einer differenziell regulierten Expression von insgesamt 563 Genen in der Leber und 211 Genen im Muskel führt. Die zahlreichen Befunde der ***Studien 2-4*** sollen im Folgenden getrennt nach den Geweben Leber und Muskel diskutiert werden.

4.2.1 Transkriptionelle Veränderungen in der Leber

Stimulierung des Fettsäureabbaus

Die Supplementierung der Ferkel mit Carnitin führte neben einer gesteigerten hepatischen Expression von Genen, die in die zelluläre Aufnahme und Aktivierung von Fettsäuren involviert sind, auch zu einer erhöhten Expression von Genen des mitochondrialen Fettsäureabbaus in der Leber. Fettsäuren sind aufgrund ihrer geringen Löslichkeit im Plasma extrazellulär an Serumalbumin gebunden. Für die Aufnahme in die Zelle müssen Fettsäuren zunächst aus ihrer Proteinbindung gelöst werden, um anschließend mithilfe verschiedener Transportsysteme die Plasmamembran passieren zu können. Neben dem Transport durch Fettsäuretranslokasen und Plasmamembran-assoziierten Fettsäurebindungsproteinen fungieren *fatty acid transport proteins* (FATP) 1-6 als Carriermoleküle für den Übertritt von

Fettsäuren in die Zelle. Da Fettsäuren relativ reaktionsträge Verbindungen sind, müssen sie, um unter Energiegewinn abgebaut werden zu können, aktiviert werden. Dies geschieht durch die Bildung eines energiereichen Thioesters mit CoA. Diese Reaktion wird unter anderem durch die Funktion spezifischer Ligasen, wie beispielsweise der *acyl-CoA synthetase, long-chain family member 3* (ACSL3), realisiert. Bei allen drei Stoffwechselwegen Aufnahme-, Aktivierung- und Abbau von Fettsäuren konnte durch Carnitin eine trankriptionelle Stimulierung der Gene FATP6, ACSL3 und ACADSB (*acyl-CoA dehydrogenase, short/branched chain*) in der Leber der Ferkel nachgewiesen werden. Diese Befunde bekräftigen den hinreichend bekannten Einfluss des Carnitins auf den Fettsäurestoffwechsel aufgrund seiner Rolle als Cofaktor beim mitochondrialen Fettsäuretransport.

Hemmung der Triglyzeridsynthese

Weiterhin ergab die Analyse der *microarray*-Daten eine hemmende Wirkung des Carnitins auf die Transkription von Genen der Triglyzeridsynthese (*glycerol-3-phosphate acyltransferase*, GPAT) sowie eine stimulierende Wirkung auf die Transkription von Genen die für den Transport und Einbau von Triglyzeriden, Cholesterinestern und Phospholipiden in Lipoproteine (*microsomal triglyceride transfer protein*, MTTP) von Bedeutung sind. Dies hat zur Folge, dass die Synthese von Leberlipiden gesenkt wird, die Lipoproteinsekretion und der Fettsäureabbau in der Leber jedoch gesteigert wird. Bereits in früheren Studien an Nagern konnte gezeigt werden, dass sich die Gabe von Carnitin bei der Behandlung von Lebersteatosen, induziert durch eine fettreiche Ernährung oder auch durch alkoholische Intoxikation, aufgrund seiner triglyzeridsenkenden Eigenschaften in der Leber als nützlich erweist (Liang *et al.*, 1999; Diraison *et al.*, 2003; Fabbrini *et al.*, 2008). Des Weiteren liefert die Literatur Hinweise darauf, dass die Gabe von Carnitin, aufgrund seiner plasma- und gewebslipidsenkenden Wirkung bei vielerlei Erkrankungen, wie kardiovaskulären Störungen und chronischem Alkoholkonsum, den Krankheitsverlauf positiv beeinflussen kann (Maebashi *et al.*, 1978; Khan und Bamji, 1979). Richter *et al.* (1987) berichteten, dass die Gabe von Carnitin die Sukrose-induzierte Hypertriglyzeridämie bei weiblichen Ratten und damit verbundenen erhöhten Konzentrationen an freien Fettsäuren im Plasma verringerte. Die Autoren begründeten diesen Effekt wiederum mit einer Steigerung der Fettsäureoxidation in den Mitochondrien und nicht mit einer gesteigerten Aktivität von lipogenen Enzymen in der Leber. In einer weiteren Studie führte die Carnitingabe bei Ratten mit Energiemangel zu einer gesteigerten Aktivität der Plasma-Lipoproteinlipase und zu einer Normalisierung der

Sekretionsrate von Triglyzeriden in das Plasma (Feng *et al.*, 2001). So könnten die Befunde der ***Studie 2*** hinsichtlich der Beeinflussung der Transkription von Genen des Fettstoffwechsels eine Erklärung für die in vorangegangenen Studien beobachtete Senkung des Leberfettgehaltes und den günstigen Effekt von Carnitin bei der Entwicklung von Lebersteatosen liefern.

Stimulierung der Aufnahme und des Abbaus von Glukose

Die Carnitinsupplementierung der Ferkel bewirkte eine drastisch gesteigerte Expression von Genen, welche für die zelluläre Aufnahme von Glukose und die anschließende Metabolisierung von Glukose im Rahmen der Glykolyse in der Leber verantwortlich sind. So ist davon auszugehen, dass vermehrt Glukose über die gesteigerte Expression der Glukosetransporter in die Hepatozyten gelangt, wo sie anschließend über die verstärkt exprimierten Schlüsselenzyme der Glykolyse umgesetzt wird. In einer Untersuchung unserer Arbeitsgruppe konnte bereits gezeigt werden, dass die Fütterung von Sauen mit Carnitin während der Trächtigkeit eine deutliche Steigerung der Proteinkonzentration des membranständigen Glukosetransporter 1 (GLUT1) im Chorion bewirkt und somit mehr Glukose über die Plazenta zum Fötus gelangt, was eine verbesserte Versorgung des Fötus mit Nährstoffen zur Folge hat (Doberenz *et al.*, 2006). Nach der Einschleusung der Glukose in die Zelle können zwei Wege der Glukoseverwertung eingeschlagen werden. In anabolen Zuständen kann Glukose-6-Phosphat in der Leber zu Glykogen umgewandelt und die Energie somit gespeichert werden. Andererseits kann es in der Glykolyse unter Energiegewinn verbraucht werden. Dieser gesteigerte Verbrauch bzw. die vermehrte Speicherung von Glukose führt zu einer verbesserten Glukosetoleranz des Körpers und könnte letztendlich in einer verbesserten Insulinsensitivität resultieren. Die Analyse der *microarray*-Daten in der Leber der Ferkel ergab ferner einen Einfluss von Carnitin auf die Expression des bifunktionellen Enzyms Fruktose-6-Phosphat-2-Kinase/Fruktose-2,6-Bisphosphatase. Ein Teil des Enzyms stellt die Phosphofruktokinase-2 dar, die die Herstellung von Fruktose-2,6-bisphosphat aus Fruktose-6-phosphat katalysiert. Fruktose-2,6-bisphosphat kommt ausschließlich in der Leber und der Muskulatur vor. Dort erfüllt es die Signalfunktion als starker allosterischer Stimulator der Phosphofruktokinase-1 und fungiert dadurch als Beschleuniger der Glykolyse. In zahlreichen Tier- und Humanstudien konnte bereits bewiesen werden, dass eine erhöhte Carnitinaufnahme zu einer verbesserten Glukosetoleranz und Glukoseoxidation führt. Es konnte gezeigt werden, dass Carnitin das intramitochondriale Acetyl-CoA/CoA-Verhältnis moduliert und somit zu einer Steigerung der Aktivität des

Pyruvatdehydrogenasekomplexes (PDHC), einem Schlüsselmediator der Glukoseoxidation, führt. Intramitochondriales Acetyl-CoA wird mithilfe der CAT zu Acetylcarnitin umgesetzt und wird anschließend in das Zytosol transportiert. Somit sinkt der Gehalt an Acetyl-CoA im Mitochondrium und führt zu einer Hemmung der Pyruvatdehydrogenasekinase, wodurch es über eine verminderte Phosphorylierung des PDHCs zu einer gesteigerten Aktivierung des PDHC kommt (Lysiak *et al.*, 1988; Uziel *et al.*, 1988; Ramsay und Zammit, 2004). Viele Autoren erklären über diese biochemische Wirkung des Carnitins dessen günstigen Effekt auf die Glukoseverwertung sowohl bei gesunden Menschen als auch Menschen mit *Diabetes mellitus* Typ II (De Gaetano *et al.*, 1999; Mingrone *et al.*, 1999; Derosa *et al.*, 2003).

Hemmung der Glukoneogenese

Interessanterweise konnte in der Leber der Ferkel, welche eine Carnitinsupplementierung mit dem Futter erhalten haben, eine Hemmung von glukoneogenetischen Enzymen auf Transkriptionsebene beobachtet werden. Dazu zählen die PCK1 und die Fructose-1,6-Bisphosphatase (FBP2). Beide katalysieren eine geschwindigkeitsbestimmende Teilreaktion der Glukoneogenese. Es ist davon auszugehen, dass eine Hemmung der Transkription dieser beiden Gene zu verringerten Proteinkonzentrationen der PCK1 und FBP2 in der Leber der carnitinsupplementierten Ferkel führt und somit eine verringerte Neusyntheserate von Glukose zu erwarten ist. Untersuchungen an Ratten bekräftigen diese Vermutung dahingehend, dass eine Erhöhung der Fruktoseaufnahme über die Nahrung eine gesteigerte Aktivität glukoneogenetischer Enzyme zur Folge hat und dies durch Carnitingaben wieder aufgehoben werden konnte (Rajasekar und Anuradha, 2007).

Stimulierung des Insulin/IGF-1-Signalweges

In der Literatur gibt es bereits einige Hinweise auf eine direkte insulinstimulierende Wirkung von Carnitin, welche für die Stimulation der Glukoseverwertung und -oxidation bei gesunden Probanden nach Carnitingabe verantwortlich ist. Die Autoren vermuten, dass Carnitin direkt auf der Ebene des Insulinrezeptors wirkt, so dass es zu einer gesteigerten Aufnahme der Glukose über insulinabhängige Glukosetransporter oder zu einer Modulation der Insulinsignalkaskade kommt (De Gaetano *et al.*, 1999; Galloway *et al.*, 2011). Auch in den eigenen Untersuchungen konnte ein positiver Zusammenhang zwischen Carnitin und der Insulin/IGF-1-Signalkaskade festgestellt werden. Insulin ist bekanntermaßen das wichtigste anabole Hormon im Organismus und nimmt bei der Regulation der Glukosehomöostase eine zentrale Rolle ein. Dabei vermittelt Insulin, aber auch andere Wachstumsfaktoren wie

IGF-1 und -2, seine biologische Wirkung über die Bindung an spezifische Rezeptoren, die der Familie der Rezeptor-Tyrosin-Kinasen angehören. Diese Rezeptoren stellen Transmembranproteine dar, die aus jeweils zwei durch Disulfid-Brücken verbundenen α- und β-UE bestehen (Cheatham und Kahn, 1995). Nach Bindung des Substrates an seinen Rezeptor kommt es zur Aktivierung der Tyrosin-Kinasen in den β-UE. Dies bewirkt eine schnelle Autophosphorylierung des Rezeptors und somit seine Aktivierung. Der aktivierte Rezeptor rekrutiert mehrere Proteine, die SH2 (*Src-Homology-2*) -Domänen aufweisen. Dazu gehören neben drei SHC (*Src-Homology-Collagen*) -Proteinen und Gab-1 (*Grb-2 associated binder-1*) vier IRS (Insulinrezeptorsubstrat) -Proteine. IRS-Proteine sind cytoplasmatische Adapter-Moleküle, die andere Proteine zu den Zellmembranrezeptoren rekrutieren und somit die Signal-Komplexe organisieren. Die rekrutierten IRS-Proteine werden an mehreren Tyrosin-Resten phosphoryliert und dienen dadurch als Bindungsstelle verschiedener intrazellulärer Proteine, wodurch die Signaltransduktion des Insulins intrazellulär weitergeleitet wird. Eines der wichtigsten Proteine ist die Phosphoinositid 3-Kinase (PI3K). Wird diese aktiviert, phosphoryliert sie beispielsweise PI-4,5-P_2 (*phosphatinositol-4,5-bisphosphate*; PIP_2) und generiert PI-3,4,5-P_3 (*phosphatinositol-3,4,5-triphosphate*; PIP_3). Die durch PI3K phosphorylierten Lipide beeinflussen den weiteren Informationsfluss in der Zelle. Zahlreiche Proteine, die an der Signaltransduktion beteiligt sind, besitzen Domänen die spezifisch phosphorylierte Phosphoinositide binden. Diese akkumulieren nach Stimulation der Zellen an der Membran, wo sie durch PIP_3 aktiviert werden. Die Serin/Threonin-Kinase Akt1 (Proteinkinase B, PKB) und die 3´-Phosphoinositid-abhängige Proteinkinase (PDK, PDPK1) sind bei der intrazellulären Signaltransduktion von besonderer Bedeutung. Die Lipide PIP_3 und PIP_2 können an die im Amino-Terminus enthaltene PH (Pleckstrin-Homologie)-Domäne von Akt1 mit relativ hoher Affinität und Spezifität binden, wodurch es zu einer Konformationsänderung des Proteins kommt. Diese ist Voraussetzung für die Aktivierung von Akt1 durch Phosphorylierung an Threonin-308 durch die ebenfalls über eine PH-Domäne an PIP_3 bindende PDPK1. Zusätzlich erfordert die Aktivierung von Akt1 die Phosphorylierung an Serin-473 durch die PDPK1 (Hofler *et al.*, 2011). Aktiviertes Akt1 kann mit seiner Kinase-Domäne selbst Zielsubstrate an Serin- oder Threoninresten phosphorylieren und dadurch aktivieren oder inhibieren. In den letzten Jahren konnte eine Vielzahl von Akt-Substraten, welche alle eine Konsensussequenz aufweisen, identifiziert werden (Alessi *et al.*, 1996). Eines der ersten bekannten Akt1-Substrate ist die Glykogensynthasekinase 3β (GSK3β) (Cross *et al.*, 1995). Ein Substrat der GSK3β ist die Glykogensynthase, welche von Akt1 über die Inhibierung der GSK3β aktiviert wird. So kann

in den eigenen Untersuchungen davon ausgegangen werden, dass über eine Aktivierung des PDPK1/Akt1-Signalweges die Glykogensynthese in der Leber der Ferkel, welche Carnitin erhalten haben, gesteigert wurde und dies einen weiteren Regulationsmechanismus der Glukosehomöostase durch Carnitin darstellen könnte. Die Aktivierbarkeit des Insulin/IGF-1-Signalweges kann durch verschiedene Einflussfaktoren moduliert werden. So hat sich gezeigt, dass IGF-bindende Proteine (IGFBPs), vor allem IGFBP5, in der Lage sind die Bindung von IGF-1 an den IGF-1-Rezeptor (IGF1R) zu inhibieren (Schiaffino und Mammucari, 2011). Interessanterweise hat die Analyse der *microarray*-Daten in der Leber der Ferkel mit Carnitinzulage ergeben, dass es zu einer Verringerung der Expression von IGFBP5 kommt. Daher ist anzunehmen, dass eine bindungshemmende Wirkung von IGFBP5 durch Carnitin vermindert werden kann und letztendlich zu einer gesteigerten Aktivität des Insulin/IGF-1-Signalweges führt.

Mechanismen von Carnitin in der Modulation der Genexpression

Die Clusteranalyse der *microarray*-Daten in der Leber der Ferkel hat ergeben, dass Carnitin Einfluss auf die posttranskriptionelle RNA-Prozessierung hat. Der Anteil der RNA macht in der eukaryotischen Zelle 3-4% des organischen Materials aus und kann in kodierende RNA (z.B. mRNA) und nicht kodierende RNA (z.B. tRNA, rRNA, snRNA, snoRNA, scRNA) untergliedert werden. Nichtkodierende RNA wird nicht translatiert, erfüllt als RNA jedoch viele verschiedene Aufgaben. So dient sie unter anderem der Prozessierung anderer RNA-Moleküle oder der Modifikation ribosomaler RNA. Dazu zählt der posttranslative Prozess des Spleißens. Der Prozess des Spleißens dient nicht nur dem Entfernen der Introns, den nichtcodierenden Sequenzen für die Reifung der mRNA aus der prä-mRNA, sondern auch der Entstehung unterschiedlicher mRNA-Transkripte. Somit können durch alternatives Spleißen von einem Gen verschiedene verwandte Proteine mit veränderten Eigenschaften erzeugt werden. Dies resultiert in einer großen zell- und gewebespezifischen Expression der Gene im Organismus. So ist es nicht überraschend, dass über 60% der menschlichen Gene alternativ gespleißt werden (Wang *et al.*, 2008). Die biologische Relevanz der Beeinflussung posttranskriptioneller RNA-Prozessierungen durch Carnitin in dieser Untersuchung kann nicht eindeutig geklärt werden, dennoch erscheint es plausibel, dass die beobachteten Veränderungen der Expression einer ganzen Vielfalt von Genen in der Leber der Ferkel durch eine Modulation dieser Prozesse vermittelt werden.

Zusammenfassend zeigen die Ergebnisse dieser Untersuchung, dass die Supplementierung von Carnitin zu umfassenden Veränderungen der Genexpression in der Leber des Ferkels

führt. Neben einer hemmenden Wirkung von Carnitin auf die Expression von Genen der Triglyzeridsynthese und Glukoneogenese, konnte eine günstige Beeinflussung der Genexpression bezüglich des Fettsäureabbaus und der Glykolyse nachgewiesen werden. Diese Effekte können unter anderem über eine Steigerung der Insulinwirkung und eine Modulation der posttranskriptionellen RNA-Prozessierung erklärt werden.

4.2.2 Transkriptionelle Veränderungen in der Muskulatur

Stimulierung des Insulin/IGF-1-Signalweges

Neben den interessanten Befunden in der Leber, führte die Carnitinsupplementierung auch im Muskel zu einer Beeinflussung der Transkription von Genen, die mit der Bindung von IGF-1 und Insulin assoziiert sind. IGF-1 ist für das Wachstum und die Differenzierung unerlässlich und stellt einen Hauptregulator der Muskelmasseentwicklung, v.a. der Myogenese dar (Florini *et al.*, 1996; Adams und McCue, 1998; Chakravarthy *et al.*, 2000). Ferner führt eine Anreicherung von IGF-1 in der Muskulatur zu einer Verlangsamung des Proteinkatabolismus. Die Literatur liefert bereits einige Hinweise auf eine durch Carnitin vermittelte Beeinflussung der IGF-1 Sekretion. Heo *et al.* (2001) konnten eine verminderte Expression der IGF-1-mRNA in der Leber von Ratten mit Streptozotozin-induziertem Diabetes beobachten. Diese Verminderung konnte durch Carnitinsupplementierung der Tiere wieder aufgehoben werden. Im der eigenen Untersuchung (***Studie 3***), konnte im Skelettmuskel der Ferkel mit Carnitinsupplementierung ebenfalls eine gesteigerte mRNA-Expression des IGF1R und der PDPK1 nachgewiesen werden, wobei der IGF1R zu den am stärksten regulierten Genen (*fold change*: 4,9) zählte. Die umfangreiche Anzahl an wissenschaftlichen Studien bei Mensch, Schwein, Ratte und Huhn belegen den positiven Zusammenhang zwischen Carnitingaben und einer Steigerung der Plasmaspiegel an IGF-1 und IGF-2 (Di Marzio *et al.*, 1999; Heo *et al.*, 2001; Kita *et al.*, 2002; Doberenz *et al.*, 2006; Woodworth *et al.*, 2007; Brown *et al.*, 2008). Durch eine gesteigerte Bindung von IGF-1 an seinen Rezeptor kommt es, ähnlich wie in der Leber bei der Vermittlung des intrazellulären Insulinsignals, zu einer Aktivierung des PDPK1/Akt1-Signalweges. Wie bereits bei den Befunden in der Leber eingehend beschrieben wurde, kann das phosphorylierte und damit aktive Akt1-Protein eine Reihe von Substraten aktivieren oder inhibieren und somit viele Abläufe in der Zelle beeinflussen. So könnten die zahlreichen Befunde, bei denen eine Carnitinsupplementierung in einem gesteigerten Proteinansatz beim Nutztier resultierte, durch die Stimulierung des muskulären PDPK/Akt1-Signalweges durch Carnitin erklärt werden. Studien an IGF1R-*Knockout*-Mäusen, nicht aber beim *Knockout* des IGF-1-Gens, haben

gezeigt, dass es durch das Fehlen des Rezeptors zu einer verringerten Anzahl und Größe der Myofibrillen und einer verringerten Anzahl an Muskelfasern des Typ 1 im *M. gastrocnemius* der Tiere kam (Mavalli *et al.*, 2010). Daher ist anzunehmen, dass Carnitin eine gesteigerte Expression des IGF1R in der Muskulatur und somit eine Steigerung der Anzahl-, Größe- und Zusammensetzung der Muskelfasern bewirken könnte. Die Bestätigung für diese Vermutung sollten zukünftige Untersuchungen beim Schwein erbringen.

Stimulierung der Glukoseaufnahme

Neben den beobachteten Effekten von Carnitin auf die Aufnahme von Glukose in die Leberzellen der Ferkel, konnte auch im Muskel eine stimulierende Wirkung von Carnitin auf die Expression von Genen, welche an der Glukoseaufnahme beteiligt sind, beobachtet werden. So konnte bei den Tieren mit Carnitinsupplementierung eine gesteigerte Expression der SORBS1-mRNA (*Sorbin and SH3 containing-1*, CAP, SH3P12) im Muskel nachgewiesen werden (***Studie 3***). Das humane SORBS1 Gen kodiert für ein Insulin-Signal-Molekül, welches vor allem in insulinsensitiven Geweben eine Rolle spielt, wobei die Expression in Leber und Skelettmuskel besonders hoch ist (Lin *et al.*, 2001). Durch die Stimulation des Insulinrezeptors kommt es zur Phosphorylierung des Cbl-CAP-Komplexes, der daraufhin an die Zellmembran rekrutiert wird. Anschließend interagiert dieser mit weiteren Proteinen, wodurch es zur Translokation des GLUT4 aus dem Zytoplasma in die Zellmembran kommt (Saltiel und Kahn, 2001; Liu *et al.*, 2002). Ribon *et al.* (1998) konnten zeigen, dass es durch eine Thiazolidindion-vermittelte PPARγ-Aktivierung zu einer höheren SORBS1-Expression in den Adipozyten kommt und dies vermutlich über eine gesteigerte Glukoseaufnahme zu einer verbesserten Insulinsensitivität führt. Im Umkehrschluss dazu könnte davon ausgegangen werden, dass eine pathologisch bedingte verringerte Expression von SORBS1 zu einer verringerten Aufnahme von Glukose aus dem Plasma in die insulinsensitiven Gewebe führt und dies zur Entwicklung von Übergewicht und Insulinresistenz beiträgt. Dieser molekulare Mechanismus stellt eine weitere Möglichkeit dar, inwiefern Carnitin seine positiven Wirkungen auf die Glukosetoleranz im Muskel beim Ferkel auf Ebene der Transkription vermittelt. Einen Einsatz von Carnitin in der Humanmedizin bei der Therapie von Übergewicht und damit verbundenen Stoffwechselerkrankungen wie *Diabetes mellitus* Typ II könnte auf Grundlage der gewonnenen Daten beim Ferkel als sinnvoll erachtet werden, bedarf jedoch weiterer klinischer Studien am Menschen.

Hemmung des proteasomalen Proteinabbaus

Die Muskelmasse wird als Antwort auf Belastung, Aktivität oder pathophysiologische Vorgänge reguliert und stellt eine Balance zwischen anabolen und katabolen Prozessen dar. Muskelhypertrophy ist assoziiert mit einer gesteigerten Proteinsynthese und einer gesteigerten muskulären Expression von IGF-1, während atrophische Zustände im Muskel mit einer gesteigerten Proteindegradation einhergehen (Mitch und Goldberg, 1996; Jagoe und Goldberg, 2001; Rommel *et al.*, 2001; Li *et al.*, 2003; Fang *et al.*, 2005). In den eigenen Untersuchungen am Schwein konnte eindrucksvoll gezeigt werden, dass eine Carnitinsupplementierung der Ferkel über das Futter in einer massiven Verringerung der Transkriptlevel von Genen des UPS im Muskel resultiert (***Studie 3***, ***Studie 4***). Ähnliche Befunde konnten auch in der Leber der Ferkel aus der Carnitingruppe beobachtet werden, verglichen mit den Kontrolltieren (***Studie 4***). Prolongiertes Fasten sowie zahlreiche Erkrankungen wie Krebskachexie, Niereninsuffizienz, Sepsis, *Diabetes mellitus* und Aids aber auch die Therapie mit Glukokortikoiden gehen mit einem erhöhten Verlust an Muskelmasse einher (Mitch und Goldberg, 1996). Während der Muskelatrophy wird eine Reihe an biochemischen und transkriptionellen Veränderungen im Skelettmuskel, das sogenannte „Atrophy-Programm“, angeschalten, wobei die Proteindegradation im atrophierenden Muskel hauptsächlich auf die Aktivität des UPS zurückzuführen ist (Jagoe *et al.*, 2002; Lecker *et al.*, 2004). In den eigenen Untersuchungen kam es sowohl im Muskel als auch in der Leber durch die Carnitinsupplementierung zu einer signifikanten Hemmung der mRNA-Expression von Atrogin-1 (in Leber und Muskel) und MuRF1 (im Muskel). Beide Proteine erfüllen die Funktion spezifischer E3-Ligasen und fungieren als Schlüsselmediatoren bei der Markierung von Proteinen, die für den Abbau durch das 26S Proteasom vorgesehen sind. Studien an Tier und Mensch haben gezeigt, dass in pathologischen Zuständen eines verstärkten Proteinverlustes die E3-Ligasen MuRF1 und Atrogin-1 verstärkt exprimiert werden (Mansoor *et al.*, 1996; Tiao *et al.*, 1997; Bodine *et al.*, 2001; Gomes *et al.*, 2001; Pickering *et al.*, 2002; Wray *et al.*, 2003; Lecker *et al.*, 2004). Die Befunde aus den ***Studien 3*** und ***4*** haben hingegen gezeigt, dass die Expression dieser beiden E3-Ligasen durch Carnitin vermindert wird und lassen somit den Schluss zu, dass dies in einer verringerten Verknüpfung von Proteinen mit Ubiquitin und letztendlich in einem verminderten Eintritt der abzubauenden Proteine in das 26S Proteasom resultiert. Diese Beobachtungen liefern eine mögliche Erklärung für die in einigen Untersuchungen beobachtete Erhöhung des Proteinansatzes bei Ferkeln, welche mit Carnitin angereichertes Futter erhielten (Owen *et al.*, 2001b; Rincker *et al.*, 2003). In pathologischen

Zuständen obliegt die Versorgung anderer Gewebe mit Aminosäuren vor allem der Muskulatur, um viszerale Organe vor größeren Proteinverlusten zu schützen und deren physiologische Funktion aufrecht zu erhalten (Mitch und Goldberg, 1996). Im Fastenzustand dient der Proteinabbau in der Muskulatur der Bereitstellung glukoplastischer Aminosäuren für die Leber, um lebenswichtige Organe wie das Gehirn mit dem Energiesubstrat Glukose zu versorgen oder neue Proteine zu synthetisieren (Mitch und Goldberg, 1996). Die Bedeutung des proteasomalen Proteinabbaus in der Leber hingegen ist im Vergleich zur Skelettmuskulatur von geringerer Bedeutung, da die Leber lediglich einen geringen Anteil am Gesamtproteinbestand ausmacht. In der Leber werden vor allem kurzlebige und fehlerhaft gefaltete Proteine dem proteasomalen Abbau unterzogen.

Weiterhin sollte im Rahmen dieser Arbeit geklärt werden, ob Carnitin seine Effekte auf die Leber und Muskulatur der Ferkel auf direktem oder indirektem Wege vermittelt. Dazu wurden Zellkulturstudien mit HepG2- und C2C12-Zellen, welche über 24 h mit verschiedenen Carnitinkonzentrationen inkubiert wurden, durchgeführt (***Studie 4***). Zellkulturen stellen ein isoliertes, in sich abgeschlossenes System dar und ermöglichen somit Aussagen darüber, ob der Effekt eines Wirkstoffes direkt durch das betreffende Gewebe oder die Gesamtheit des Intermediärstoffwechsels hervorgerufen wird. Es hat sich gezeigt, dass die Inkubation der Zellen mit Carnitin keinen Effekt auf die Expression der Gene des UPS hatte und somit eine direkte Wirkung des Carnitin sowohl in der Leber als auch in der Muskulatur ausgeschlossen werden kann. Eine Behandlung der murinen C2C12-Zellen mit Hydrocortison, einem potenten Stimulator des UPS, führte wie erwartet zu einer gesteigerten Expression der proteasomalen Gene. In der Literatur ist die stimulierende Wirkung von Glukokortikoiden auf den proteasomalen Proteinabbau im atrophierenden Muskel hinreichend beschrieben (Wing und Goldberg, 1993; Tiao *et al.*, 1996; Mitch *et al.*, 1999). Sacheck *et al.* (2004) konnten ebenfalls durch die Behandlung von kultivierten *Myotubes* mit dem synthetischen Glukokortikoid Dexamethason eine gesteigerte Expression von Atrogin-1 und MuRF1 sowie eine 22%ige Steigerung der Proteolyse feststellen. Interessanterweise kam es durch die Behandlung der *Myotubes* mit IGF-1 zu einer Absenkung des basalen Levels des Proteinabbaus und einem erhöhten Gehalt an Gesamt-RNA. Die Autoren konnten zeigen, dass IGF-1 den durch Dexamethason induzierten Abbau myofibrillärer Proteine verminderte und zu einem gesteigerten Proteingehalt führte. Weiterhin kam es zu einer Absenkung der mRNA-Konzentrationen von Atrogin-1 und MuRF1 durch die Inkubation der Zellen mit IGF-1. Weitere Untersuchungen an *Myotubes* haben gezeigt, dass die mRNA-Konzentration von Atrogin-1 nach IGF-1-Inkubation sehr viel schneller sinkt als die von MuRF1

(Sacheck *et al.*, 2004). Experimente mit Actinomycin D haben gezeigt, dass die mRNA von Atrogin-1 eine sehr kurze Halbwertszeit (ca. 1 h) im Vergleich zur mRNA von MuRF1 besitzt. Der Zerfall der Atrogin-1-mRNA unterschied sich nicht in An- oder Abwesenheit von IGF-1, so dass die Autoren davon ausgehen, dass IGF-1 seine Effekte eher über eine Repression der Transkription von Atrogin-1 über die FoxO-Transkriptionsfaktoren (*Forkhead transcription factors of the O class*) vermittelt als über die Beeinflussung der Abbaurate der mRNA (Sandri *et al.*, 2004). Es konnte gezeigt werden, dass Veränderungen der MuRF1-mRNA nach Hormongabe (IGF-1 und/oder Insulin) viel langsamer und geringer ausfallen als bei Atrogin-1, was die hohe Stabilität der mRNA von MuRF1 widerspiegelt (Sandri *et al.*, 2004). Diese Befunde unterstützen die eigenen Ergebnisse aus ***Studie 4***, die gezeigt haben, dass die Carnitinsupplementierung zu einem stärkeren Abfall der mRNA-Konzentration von Atrogin-1 führt, als der von MuRF1.

Die Beobachtungen in Leber und Muskulatur sowie in den Zellkulturstudien lassen den Schluss zu, dass Carnitin seine Wirkungen auf den proteasomalen Proteinabbau indirekt vermittelt. Denkbar ist hierbei eine Beeinflussung des Insulin/IGF-1-Signalweges. Im Folgenden sollen mögliche molekulare Mechanismen, über welche Carnitin seine Wirkungen beim Ferkel vermittelt, diskutiert werden.

Hemmung der FoxO-Transkriptionsfaktoraktivität

In der Literatur gibt es einige Hinweise darauf, dass eine Störung der Insulin/IGF-1-Signalkaskade eine verminderte Aktivität der beiden Kinasen PI3K und Akt1 zur Folge hat und somit eine Aktivierung von spezifischen Transkriptionsfaktoren bewirkt. Diese translozieren anschließend in den Zellkern und veranlassen eine verstärkte Transkription der beiden E3-Ligasen Atrogin-1 und MuRF1 (Lee *et al.*, 2004; Sandri *et al.*, 2004). Im Gegenzug ist eine Aktivierung der Hypertrophy im Skelettmuskel begleitet von einer gesteigerten Expression von IGF-1 (DeVol *et al.*, 1990; Musaro *et al.*, 2001; Philippou *et al.*, 2007). So konnte in Untersuchungen an transgenen Mäusen mit erhöhten IGF-1-Spiegeln eine gesteigerte Muskelmasse beobachtet werden (Coleman *et al.*, 1995; Musaro *et al.*, 2001). Wie bereits erwähnt worden ist, führt die Bindung von IGF-1 an seine entsprechenden Rezeptoren zur Aktivierung des PDPK1/Akt1-Signalweges. Aktiviertes Akt1 wird nicht mehr an der Zellmembran gebunden und gelangt in den Zellkern (Meier und Hemmings, 1999), wo es die Aktivität spezifischer Transkriptionsfaktoren beeinflusst. So werden unter anderem die FoxO-Transkriptionsfaktoren durch die Aktivität des Akt1 reguliert. Sie gehören zur Gruppe der *Forkhead*-Proteine, welche eine koservierte

DNA-Bindungsdomäne, die *Forkhead-Box* (Fox), aufweisen. Die Mitglieder der FoxO-Familie sind beteiligt an der Regulation von Targetgenen, die in die Kontrolle der Apoptose und des Zellzyklus involviert sind (Carlsson und Mahlapuu, 2002; Tran *et al.*, 2003). Die Zielgene der FoxOs besitzen alle eine spezifische DNA-Bindesequenz, welche nach den FoxO-Homologen aus dem Fadenwurm *Caenorhabditis elegans (C.elegans)* daf-16-Bindeelement (DBE) benannt wurden (Biggs *et al.*, 2001). In Säugerzellen konnten bislang die drei Mitglieder Foxo1 (FKHR), Foxo3a (FKHRL1) und Foxo4 (AFX) der FoxO-Familie identifiziert werden (Burgering und Kops, 2002; Tran *et al.*, 2003; Greer und Brunet, 2005). In der atrophierenden Skelettmuskulatur nehmen Foxo1 und Foxo3 eine zentrale Stellung bei der Regulation der Transkription spezieller Gene des „Atrophy-Programmes“ ein (Kamei *et al.*, 2003; Lecker *et al.*, 2004; Giresi *et al.*, 2005). Aktiviertes Akt1 phosphoryliert im Zellkern die an der nukleären DNA der Zielgene bindenden FoxO-Transkriptionsfaktoren an den drei spezifischen Aminosäureresten Threonin-24, Serin-256 und Serin-319 (Biggs *et al.*, 1999; Brunet *et al.*, 1999; Kops *et al.*, 1999; Rena *et al.*, 1999). Daraufhin dissoziieren diese von der DNA ab und werden an spezifische Proteine (14-3-3 Proteine) gebunden aus dem Zellkern in das Zytosol exportiert. Dies hat eine Senkung der Transkription von FoxO-Zielgenen zur Folge (Biggs *et al.*, 1999; Brunet *et al.*, 1999; Kops *et al.*, 1999; Rena *et al.*, 1999). Im Gegensatz dazu führt die Dephosphorylierung der FoxOs, infolge einer verminderten Aktivität der Insulin/IGF-1-Signalkaskade, zu deren Aktivierung und Translokation in den Zellkern, worauf die Bindung an responsive Elemente in der Promotorregion spezifischer Targetgene erfolgt. Interessanterweise deuten genetische Studien darauf hin, dass eine verminderte Aktivität des PDPK1/Akt1-Signalweges eine Verlängerung der Lebensspanne bei Würmern, Fliegen und Säugetieren bewirkt (Bartke, 2008; Kenyon, 2010). Mutationen des Insulin/IGF-1-Rezeptor-Homologes (*daf 2*), der katalytischen Untereinheit der PI3K (*age 1*) oder des Akt-Homologes (*akt1* und *akt2*) in *C.elegans* resultierten in einer deutlich erhöhten Lebensdauer der Fadenwürmer (Dorman *et al.*, 1995; Kimura *et al.*, 1997; Paradis und Ruvkun, 1998). Diese Verlängerung konnte durch die Mutation von *daf-16*, dem Homolog des FoxO-Transkriptionsfaktors in *C.elegans*, wieder aufgehoben werden (Lin *et al.*, 1997; Ogg *et al.*, 1997; Paradis und Ruvkun, 1998). Somit scheint eine Verringerung der FoxO-Transkriptionsfaktoren nach Aktivierung des Insulin/IGF-1-Signalweges paradoxerweise mit einer verminderten Lebensdauer einher zu gehen. Inwiefern dieser Zusammenhang, auch im Hinblick auf die Effekte einer Carnitinsupplementierung, beim Schwein eine Rolle spielen könnte, sollte Gegenstand zukünftiger Studien sein.

Stimulierung der Proteinsynthese

Der erhöhte Anteil an Protein im Vergleich zu Fett im Schlachtkörper, wie es nach Carnitingaben beim Schwein gezeigt werden konnte, könnte nicht nur auf die Repression der FoxO-Transkriptionsfaktoren und der daraus resultierenden verminderten Expression der E3-Ligasen MuRF1 und Atrogin-1 zurückzuführen sein. Weiterhin ist es denkbar, dass die gesteigerte Aktivität des PDPK1/Akt1-Signalweges durch Carnitin zu einer gesteigerten Proteinsynthese im Skelettmuskel über eine indirekte Aktivierung des mTOR (*mammalian target of rapamycin*) und einer Inhibierung der GSK3β führt (Manning und Cantley, 2007). Durch die Stimulierung des Insulin/IGF-1-Signalweges aktivierte Kinasen vermitteln die Phosphorylierung von mTORC1, welches wiederum den nachgeschalteten Effektor S6-Kinase aktiviert. Dieser phosphoryliert das ribosomale Protein S6 und weitere Faktoren, die in die Translationsinitiation- und elongation involviert sind, was in einer gesteigerten Proteinsynthese resultiert (Schiaffino und Mammucari, 2011). Weiterhin kommt es durch die Akt1-vermittelte Repression der GSK3β zu einer Aufhebung der Inhibierung des eIF2B (*eukariotic translation initiation factor 2B*) und somit zu einer Steigerung der Proteinsynthese (Welsh *et al.*, 1998; Schiaffino und Mammucari, 2011). Dies ist ein weiterer Mechanismus über den Insulin aber auch IGF-1 die Proteinsynthese in verschiedenen Geweben wie Leber und Skelettmuskel stimuliert. Die Befunde, dass Carnitin diesen Signalweg stimuliert, stellen somit einen weiteren Ansatzpunkt dar, um die zahlreichen phänotypischen Veränderungen der Körperzusammensetzung beim Nutztier erklären zu können.

Hemmung der Apoptose

Die Analyse der *microarray*-Daten im Muskel der Ferkel mit Carnitinsupplementierung ergab interessanterweise eine verminderte Expression von Genen die für proapoptotisch wirkende Transkritpionsfaktoren kodieren (***Studie 3***). Eine Hemmung der Expression dieser Faktoren durch Carnitin könnte demnach in einer Hemmung der Apoptose münden und somit eine Erklärung für die in zahlreichen Studien beobachtete protektive Wirkung von Carnitin bei neurodegenerativen Erkrankungen, mitochondrialer Dysfunktion und Sarkopenie liefern. In einigen Humanstudien konnte dieser Effekt ebenfalls beobachtet werden. So hat sich gezeigt, dass Carnitin antiapoptotisch auf Lymphozyten bei HIV-infizierten Patienten wirkt. Die Aktivierung des Insulin/IGF-1-Signalweges durch Carnitin und somit die Repression der transkriptionellen Aktivität der FoxO-Transkriptionsfaktoren schützt Zellen ebenfalls vor dem programmierten Zelltod (Dijkers *et al.*, 2000; Zheng *et al.*, 2000). Durch die Hemmung der

FoxOs bleibt die Aktivierung verschiedener proapoptotisch wirkender Proteine, wie beispielsweise der Mitglieder der Bcl-2 Familie sowie von Bcl-6, einem Repressor des antiapoptotischen Bcl-X_L, aus (Dijkers *et al.*, 2000; 2002; Tang *et al.*, 2002). Ferner wird die FoxO-vermittelte Induktion der Synthese von Cytokinen, wie *FAS-Ligand* und *TRAIL*, welche für die Apoptose von entscheidender Bedeutung sind, gehemmt (Brunet *et al.*, 1999; Modur *et al.*, 2002). Diese Beobachtungen belegen die antiapoptotischen Wirkungen von Akt1 und somit vermutlich auch von Carnitin und könnten ursächlich für die günstigen Wirkungen von Carnitin bei verschiedenen degenerativen Erkrankungen sein.

Abschließend sollte erwähnt werden, dass die Gabe von Carnitin (***Studie 2-4***) keine Auswirkungen auf den Phänotyp der Ferkel hatte. Die Lebendmasseentwicklung, Futteraufnahme und Futterverwertung der Tiere aus der Behandlungsgruppe unterschieden sich nach Versuchsende nicht von denen der Kontrollgruppe. Die Carnitindosierung betrug in der Behandlungsgruppe 500 mg/kg Diät. Bei einer LM der Ferkel von 17 kg entspricht das einer Carnitinaufnahme von 15 mg/kg LM/d. In einigen Humanstudien wurde eine Tagesdosis von 3 g Carnitin eingesetzt, dies würde bei einem normalgewichtigen 70 kg schweren Menschen einer Carnitindosis von 43 mg/kg KG/d entsprechen und liegt somit über den Carnitindosierungen in den eigenen Untersuchungen. Dies könnte das Ausbleiben phänotypischer Veränderungen der Ferkel erklären, ist jedoch eher unwahrscheinlich. Wahrscheinlicher hingegen ist, dass die Dauer des Fütterungsexperimentes Einfluss auf die Ausprägung phänotypischer Veränderungen hat. Zu vermuten wäre, dass die dreiwöchige Versuchsdauer zwar zu einer Induktion der Transkritption führt, jedoch nicht für eine Ausprägung im Phänotyp ausreichend ist.

Zusammenfassend zeigen die Befunde erstmalig, dass Carnitin Einfluss auf die Transkription zahlreicher Gene der Muskulatur des Ferkels ausübt. Eine Stimulierung des Insulin/IGF-1-Signalweges und die vermutlich damit verbundene Hemmung der Aktivität der FoxO-Transkriptionsfaktoren resultieren in einer verringerten Expression einiger Gene des UPS. Diese Hemmung des Proteinabbaus in der Muskulatur sowie die Hemmung von apoptotischen Prozessen und die Stimulierung der Proteinsynthese durch Carnitin sind mögliche Erklärungen für den gesteigerten Proteinansatz, welcher in vielen Studien bei verschiedenen Nutztierarten beobachtet wurde.

Die nachfolgende Abbildung stellt ein hypothetisches Modell dar, in welchem die im Rahmen dieser Arbeit ermittelten transkriptionellen Veränderungen, welche durch eine Carnitinsupplementierung beim Ferkel induziert wurden, mit den bereits aus der Literatur

bekannten zahlreichen phänotypischen Veränderungen beim Schwein in Zusammenhang gebracht werden sollen.

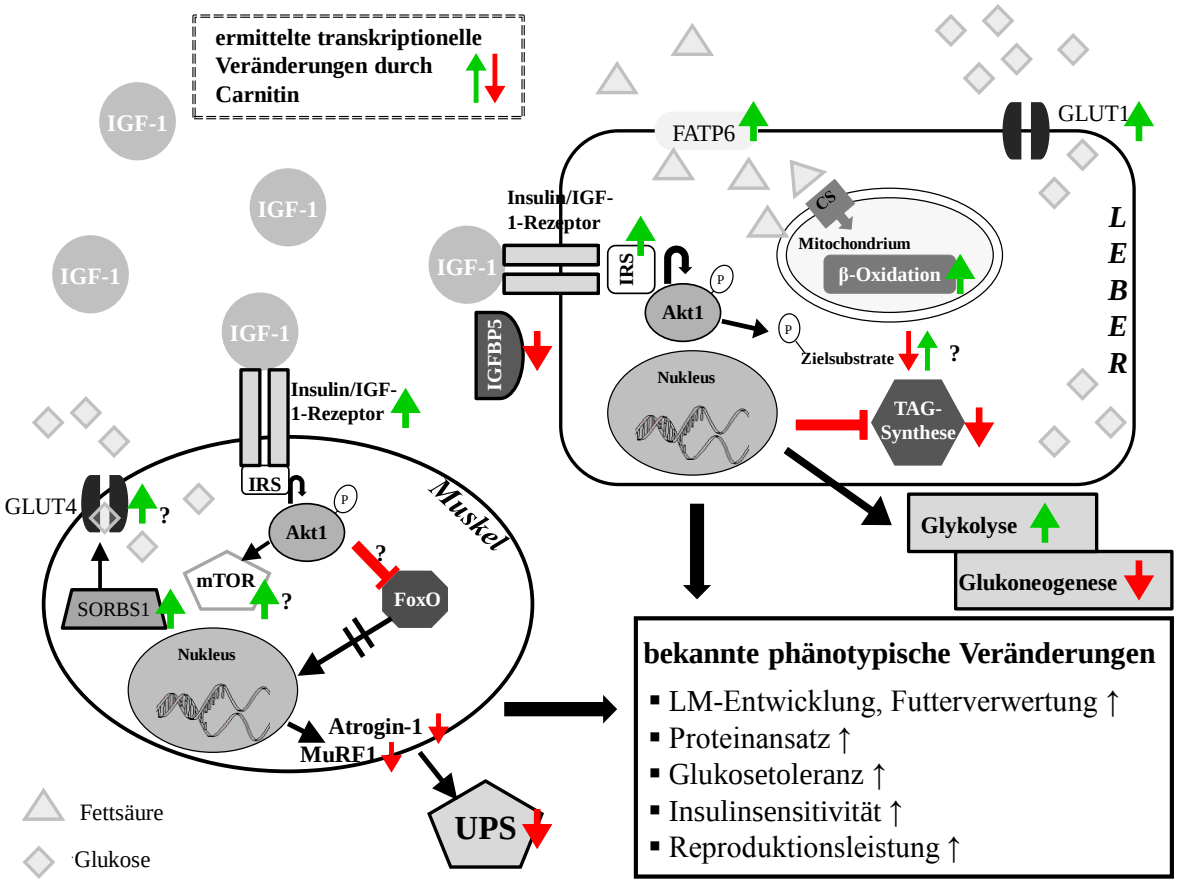

Abb. 1: Hypothetisches Modell der molekularen Wirkmechanismen von Carnitin beim Schwein
Vorangegangene Studien konnten bereits belegen, dass eine Carnitinsupplementierung einen Anstieg der Plasma-IGF-1-Konzentrationen beim Schwein zur Folge hat. Im Rahmen dieser Arbeit konnte nach Carnitingabe eine gesteigerte mRNA-Expression des Insulin/IGF-1-Rezeptors im Muskel und des IRS in der Leber der Ferkel mittels *microarray*-Technologie beobachtet werden. Die Hemmung der Transkription von IGFBP5 durch Carnitin in der Leber führt zu einer verstärkten Bindung des IGF-1 an den Rezeptor. Dies könnte eine vermehrte Aktivierung der PDPK1/Akt1-Signalkaskade und damit eine Modulation der Gentranskription und der Aktivität verschiedener Enzyme in der Leber- und Muskelzelle bewirken. In der Muskulatur könnte die Aktivierung von Akt1 eine Hemmung der FoxO-Transkriptionsfaktoren und somit eine Repression des UPS über eine verminderte Expression der beiden E3-Ligasen Atrogin-1 und MuRF1 bewirken. Weiterhin könnte die Proteinsynthese über eine Akt1-induzierte Steigerung von mTOR erhöht werden. Weiterhin konnte in der Muskulatur eine gesteigerte Expression von SORBS1 festgestellt werden, was in einer gesteigerten Expression von GLUT4 resultieren müßte. Auch in der Leber kam es durch die Carnitinsupplementierung zu einer gesteigerten Expression von GLUT1 sowie der Enzyme der Glykolyse. Ferner konnte eine verminderte Expression von Enzymen der TAG-Synthese und Glukoneogenese festgestellt werden. Ebenfalls kam es zu einer gesteigerten Transkription von Genen der Fettsäureaufnahme in die Leberzelle (FATP6) und der Fettsäureaktivierung sowie des mitochondrialen Fettsäureabbaus. Diese ermittelten transkriptionellen Veränderungen in der Leber- und Muskelzelle stellen molekulare Wirkmechanismen dar, die eine Vielzahl an bereits publizierten phänotypischen Veränderungen, welche nach Carnitinsupplementierung beim Schwein beobachtet wurden, erklären.
Abkürzungen: IGF-1, *insulin like growth factor*; IRS, Insulinrezeptorsubstrat; IGFBP5, IGF-bindendes Protein 5; GLUT1,4, Glukosetransporter 1,4; CS, Carnitin-*Shuttle*; UPS, Ubiquitin-Proteasom-System; FATP6, *fatty acid transport protein 6*; SORBS1, *Sorbin and SH3 containing-1*; TAG, Triazylglyzerin; LM, Lebendmasse

4.3 Wirkungen einer PPARα-Aktivierung auf die Carnitinhomöostase am Modelltier Maus

In der wissenschaftlichen Literatur wurde hinreichend belegt, dass metabolischer Stress ausgelöst durch eine langfristige hyperkalorische Ernährung und daraus resultierendem Übergewicht aber auch eine genetische Prädisposition für metabolische Störungen wie *Diabetes mellitus* Typ II den Carnitinstatus im Organismus beeinträchtigt (An *et al.*, 2004; Koves *et al.*, 2008; Noland *et al.*, 2009). Die Folge einer verminderten Versorgung des Organismus mit Carnitin ist eine Beeinträchtigung des mitochondrialen Energiestoffwechsels und eine gestörte Glukoseverwertung der Zellen (Noland *et al.*, 2009). Ziel der ***Studie 5*** war es daher, zu klären inwieweit regelmäßiges Ausdauertraining den durch eine langfristig zu hohe Aufnahme von Nahrungsenergie beeinträchtigten Carnitinstatus beeinflussen kann. Erwartungsgemäß führte die zehnwöchige Aufnahme der fettreichen Diät bei den Tieren zu einer beeinträchtigten Glukosetoleranz und einem signifikanten Abfall der hepatischen Carnitinkonzentrationen. Erstaunlich war jedoch, dass diese fettinduzierte Beeinträchtigung des Carnitinstatus in den Lebern der trainierten, fettreich ernährten Tiere ausblieb. In Übereinstimmung mit den Befunden von Noland *et al.* (2009) hat sich gezeigt, dass die langfristige Verabreichung einer Hochfettdiät an Mäuse sowohl zu verringerten mRNA- und Proteinkonzentrationen von Enzymen der hepatischen Carnitinsynthese und des Carnitintransporters OCTN2 führt als auch zu einer Verminderung der Konzentration der Carnitinvorstufen in den Geweben. Daher liegt die Schlussfolgerung nahe, dass eine langfristige hyperkalorische, fettreiche Ernährung mit einer Abnahme der hepatischen Carnitinsynthese und einer parallelen Verringerung der Carnitinaufnahme in die Leber einhergeht. Im Gegensatz dazu konnte in dieser Untersuchung eindrucksvoll bewiesen werden, dass die Beeinträchtigung des Carnitinstatus durch fettreiche Ernährung in Verbindung mit regelmäßigem Ausdauertraining vermieden werden kann. So zeigten die trainierten Mäuse eine gesteigerte hepatische mRNA-Konzentration der BBD, dem Schlüsselenzym der Carnitinsynthese, sowie des OCTN2. Diese Beobachtungen legen den Schluss nahe, dass diese transkriptionellen Anpassungen des Organismus an die erhöhte körperliche Leistung über eine Aktivierung des nukleären Transkriptionsfaktors PPARα vermittelt werden. Durch körperliche Belastung wie Ausdauersport werden nachweislich endogen vermehrt Fettsäuren aus den Speichergeweben freigesetzt und führen anschließend zu einer PPARα-Aktivierung in den Geweben, welche vornehmlich Fettsäuren als Energiesubstrat nutzen. Dies stellt einen Anpassungsmechanismus des Organismus an die erhöhten Leistungsansprüche dar. Die Befunde der letzten Untersuchung zeigen eindeutig,

dass eine langfristig zu hohe Aufnahme von Nahrungsenergie die PPARα-abhängige transkriptionelle Regulation seiner Zielgene in hohem Maße beeinträchtigt (für einen vertiefenden Einblick in die Ergebnisse sei auf die Originalarbeit ***Studie 5*** verwiesen). In der wissenschaftlichen Literatur wurde jüngst bewiesen, dass einige Gene der Carnitinhomöostase funktionelle PPREs aufweisen und somit der transkriptionellen Kontrolle des PPARα unterliegen (Wen *et al.*, 2010; 2011). Dies lässt vermuten, dass regelmäßiges Ausdauertraining die übergewichtinduzierte Beeinträchtigung des hepatischen Carnitinstatus über eine trainingsinduzierte Aktivierung des PPARα aufzuheben vermag. Im Gegensatz zu den Befunden in der Leber hatte die fettreiche Ernährung der Tiere keinen Einfluss auf die muskuläre Expression PPARα-abhängiger Zielgene und den Carnitinstatus. Die Skelettmuskulatur dient als körpereigener Speicher von Carnitin. So konnten beim Menschen Konzentrationen von 2000-4000 nmol Carnitin/g Skelettmuskelmasse nachgewiesen werden, welche um den Faktor 100 höher sind als in Plasma, Niere, Leber und Gehirn des Menschen (Bertoli *et al.*, 1981; Moorthy *et al.*, 1983; Angelini *et al.*, 1992). Es ist daher anzunehmen, dass der gesteigerte Bedarf an Carnitin für die mitochondriale Oxidation von Fettsäuren in der Skelettmuskulatur der trainierten Mäuse ausreichend über die Entleerung der muskulären Carnitinspeicher gedeckt werden kann, so dass eine erhöhte Carnitinaufnahme aus dem Plasma in die extrahepatischen Gewebe überflüssig ist. Die Beobachtung des fehlenden Einflusses einer fettreichen Ernährung auf den Carnitinstatus der Mäuse in dieser Untersuchung stehen im Kontrast zu den Befunden von Noland *et al.* (2009), welche anhand von verschiedenen Rattenmodellen einen carnitinsenkenden Effekt im Muskel durch genetisch oder nahrungsinduzierte Fettleibigkeit und Insulinresistenz postulieren. Es wäre denkbar, dass der fettinduzierte Abbau von Carnitin in der Muskulatur von der Fütterungsdauer der fettreichen Diät abhängt. So war die zehnwöchige Versuchsdauer dieser Studie wesentlich kürzer verglichen mit der dreimonatigen Fütterungsdauer in der Studie von Noland *et al.* (2009). Unabhängig davon hat sich gezeigt, dass Ausdauertraining in einer starken muskulären PPARα-Aktivierung resultiert. Dieser Effekt wird durch zahlreiche Befunde aus vorangegangenen Studien an Labortieren, normalgewichtigen, übergewichtigen sowie adipösen Menschen bestätigt, in welchen Training zu einer gesteigerten Expression von PPARα-Zielgenen in der Muskulatur führte (Horowitz *et al.*, 2000; Lee *et al.*, 2007; Smith *et al.*, 2009; Leick *et al.*, 2010).

Zusammenfassend konnte durch die Untersuchung erstmalig gezeigt werden, dass regelmäßiges körperliches Training die fettinduzierte Beeinträchtigung des Carnitinstatus über eine trainingsinduzierte Aktivierung des nukleären Transkriptionsfaktors PPARα

verbessern kann. Durch die verbesserte Versorgung des Organismus mit Carnitin kommt es aufgrund seiner elementaren Rolle im intrazellulären Fettsäuretransport zu einer günstigen Beeinflussung des Lipid- und Energiehaushaltes. Gegenstand weiterer Untersuchungen könnte sein, ob und in welchem Maße Carnitin auch die Glukosetoleranz und Insulinsensitivität beeinflussen kann.

5. Zusammenfassung

Carnitin ist ein essenzieller Bestandteil der Zelle und nimmt eine Schlüsselrolle beim Transport aktivierter langkettiger Fettsäuren in die Mitochondrien ein, wo sie unter Energiegewinn abgebaut werden können. Zahlreiche Studien an Tier und Mensch haben gezeigt, dass der Körper in der Lage ist sich einer geringen exogenen Zufuhr von Carnitin über die Nahrung anzupassen, wobei sich die Rate der *de novo* Synthese spezies-, gewebe- und altersabhängig unterscheidet. Studien am Menschen haben gezeigt, dass die γ-Butyrobetaindioxygenase (BBD), das Schlüsselenzym der Carnitinsynthese, hauptsächlich in Leber und Niere aktiv ist. Darüber hinaus wird die Aktivität der BBD in der menschlichen Leber, nicht aber in der Niere altersabhängig reguliert. Grundlegende Untersuchungen zur Carnitinsynthese beim Schwein fehlen bislang jedoch. In ***Studie 1*** sollte daher untersucht werden, welche Gewebe vom Schwein zur vollständigen Carnitinsynthese befähigt sind. Ferner sollten altersabhängige Unterschiede hinsichtlich der Aktivität der BBD beim Schwein ermittelt werden. In der vorliegenden Arbeit konnte erstmalig gezeigt werden, dass das Schwein, ähnlich wie der Mensch, vorrangig in Leber und Niere zur vollständigen Carnitinsynthese befähigt ist. Ebenso konnte gezeigt werden, dass die Aktivität der porzinen BBD in Leber und Niere altersabhängig reguliert wird. Erstaunlicherweise konnten die ermittelten Proteinkonzentrationen der BBD in den verschiedenen Geweben nicht in Übereinstimmung mit den mRNA-Expressionen und der letztendlichen Enzymaktivität gebracht werden. Gründe hierfür stellen unter anderem posttrankriptionale und posttranslationale Regulationsmechanismen dar. Daraus können einerseits verschiedene Isoformen der BBD in den Geweben resultieren, andererseits können chemische Veränderungen von Enzymen zu einer Modifikation der Wirksamkeit führen. Weiterer Grund für die trotz nachweislich ausgeprägter Protein- und mRNA-Konzentration drastisch unterschiedlichen BBD-Aktivitäten kann die Verfügbarkeit von Cofaktoren der Carnitinsynthese sein.

Die Befunde verschiedenster Studien an Mensch und Nutztier belegen die Bedeutung von Carnitin bezüglich der günstigen Beeinflussung von Körperzusammensetzung, Lipid- und Insulinstoffwechsel sowie der Entwicklung degenerativer Erkrankungen. Die zugrundeliegenden molekularen Mechanismen sind bislang unbekannt und wurden in dieser Arbeit untersucht. Mithilfe der *microarray*-Technologie konnte der Einfluss von Carnitin auf das Transkriptprofil in Leber und Muskel von Ferkeln ermittelt werden. Dazu wurden die Ferkel in zwei Gruppen aufgeteilt (n=8) und erhielten über einen Zeitraum von drei Wochen

eine Standarddiät mit bzw. ohne 500 mg Carnitin/kg Futter. Nach Versuchsende wurden Muskel-, Leber- und Plasmaproben gewonnen, welche anschließend in ***Studie 2-4*** hinsichtlich des Expressionsprofils sowie der Carnitinkonzentrationen analysiert wurden. Die Befunde aller drei Studien deuten stark darauf hin, dass Carnitin Einfluss auf die Transkription zahlreicher Gene aus dem Protein-, Lipid- und Glukosestoffwechsel hat. Die erhöhte Zufuhr von Carnitin führte in der Leber der Ferkel zu einer verstärkten Transkription von Genen des Fettsäurekatabolismus. Die Hemmung der Transkription von Genen der Triglyzeridsynthese durch Carnitin und Steigerung der Transkription von Genen, die für den Transport und Einbau von Triglyzeriden, Cholesterinestern und Phospholipiden in Lipoproteine von Bedeutung sind, hat zur Folge, dass die Synthese von Leberlipiden gesenkt wird, die Lipoproteinsekretion und der Fettsäureabbau in der Leber jedoch gesteigert werden. Die hepatische Expression von Genen der zellulären Glukoseaufnahme und Metabolisierung war bei den Ferkeln mit Carnitinsupplementierung erhöht, wohingegen Gene, die für Enzyme der Glukoneogenese kodieren, in ihrer Expression durch Carnitin vermindert waren. Weiterhin konnte in der Muskulatur ein steigernder Effekt der Carnitinsupplementierung auf die Transkription des SORBS1-Gens (*Sorbin and SH3 containing-1*), welches für ein Insulin-Signal-Molekül kodiert, ermittelt werden. Dies resultiert letztendlich in einer vermehrten Translokation von GLUT4 (Glukosetransporter 4) an die Zellmembran wodurch mehr Glukose in die Muskelzelle aufgenommen werden kann. Sowohl in der Leber als auch in der Muskulatur zeigte sich bei den Ferkeln durch die erhöhte Carnitinzufuhr eine stimulierende Wirkung auf die Expression von Genen des Insulin/IGF-1-Signalweges, was wiederum weitere Veränderungen auf die Transkription vieler Gene bedingt. So konnte in ***Studie 4*** mithilfe der *real-time* PCR eine signifikante Verringerung der relativen mRNA-Konzentration von Genen des proteasomalen Proteinabbaus [Muskel: Atrogin-1, MuRF1 (*muscle RING-finger protein-1*), $E2_{14}k$, Psma1, Ubiquitin; Leber: Atrogin-1, MuRF1, $E2_{14}k$] bei den Ferkeln der Carnitingruppe nachgewiesen werden. Anhand von Zellkulturstudien mit HepG2- und C2C12-Zellen, welche mit steigenden Carnitinkonzentrationen (0, 100, 500 und 1000 µM) inkubiert wurden, konnte festgestellt werden, dass Carnitin keinen direkten Einfluss auf das Ubiquitin-Proteasom-System (UPS) hat. Eher ist anzunehmen, dass Carnitin über die Stimulierung des Insulin/IGF-1-Signalweges die Transkription proteasomaler Gene über die Repression der sogenannten FoxO-Transkriptionsfaktoren (*Forkhead transcription factors of the O class*) hemmt. Neben der Hemmung des Proteinabbaus auf Transkriptionsebene kann vermutet werden, dass Carnitin über eine Aktivierung des Insulin/IGF-1-Signalweges und somit einer Aktivierung von Akt1 die Proteinsynthese über

die Beeinflussung verschiedener Transkriptionsfaktoren stimuliert. Darüber hinaus führte die Fütterung der Ferkel mit Carnitin im Muskel zu einer Hemmung der Expression von Genen, die für proapoptotisch wirksame Transkriptionsfaktoren kodieren. Die Ergebnisse dieser drei Versuche liefern insgesamt neue Einblicke bezüglich der indirekten Regulation der Transkription zahlreicher Gene durch Carnitin und könnten die Erklärung für die in zahlreichen Fütterungsstudien beobachtete Verbesserung der Leistungsphysiologie bei verschiedenen Nutztieren sein.

Ziel der ***Studie 5*** war es zu klären, inwieweit regelmäßiges Ausdauertraining den durch eine zu hohe Aufnahme von Nahrungsenergie beeinträchtigten Carnitinstatus beeinflussen kann. Dazu wurden 18 C57BL/6 Mäuse in drei Gruppen aufgeteilt. Die Kontrollgruppe erhielt eine Standarddiät (10% Fett), die beiden Behandlungsgruppen eine fettreiche Diät (45% Fett) mit bzw. ohne Ausdauertraining. Die Aufnahme der fettreichen Diät führte zu einer beeinträchtigten Glukosetoleranz und einem signifikanten Abfall der hepatischen Carnitinkonzentrationen. Ebenso konnten bei diesen Tieren verringerte mRNA- und Proteinkonzentrationen von Enzymen der hepatischen Carnitinsynthese und des Carnitintransporters OCTN2 (*novel organic cation transporter 2*) sowie verminderte Konzentrationen der Carnitinvorstufen in den Geweben beobachtet werden. So resultiert eine fettreiche Ernährung vermutlich in einer Abnahme der hepatischen Carnitinsynthese und einer parallelen Verringerung der hepatischen Carnitinaufnahme. Im Gegensatz dazu konnte in dieser Untersuchung erstmalig bewiesen werden, dass die Beeinträchtigung des Carnitinstatus durch fettreiche Ernährung in Verbindung mit regelmäßigem Ausdauertraining vermieden werden kann. So zeigten die trainierten Mäuse eine gesteigerte hepatische mRNA-Konzentration der BBD und des OCTN2. Da aus der Literatur bereits bekannt ist, dass sowohl Gene der Carnitinsynthese als auch des Carnitintransportes zu den Zielgenen des nukleären Transkriptionsfaktors PPARα (*peroxisome proliferator-activated receptor α*) gehören, legen die Befunde aus der letzten Studie den Schluss nahe, dass die transkriptionellen Anpassungen des Organismus an die erhöhte körperliche Leistung über eine trainingsinduzierte Aktivierung des PPARα vermittelt werden.

6. Summary

Carnitin is an essential metabolite and plays a key role in the transport of activated long-chain fatty acids into the mitochondrion, where fatty acids are oxidized. Numerous studies in animals and humans have shown that the body is able to adapt to a low exogenous carnitine intake from the diet because of its ability to stimulate carnitine synthesis, whereat the rate of de novo synthesis differ species-, tissue- and age- dependent. Human studies have shown that γ-butyrobetaine dioxygenase (BBD), the key enzyme of carnitine synthesis, is active mainly in liver and kidney. In addition, it was shown that the activity of BBD in the human liver, but not in the kidney, depends on the age. However, basic studies on carnitine synthesis in pigs have not been conducted yet. ***Study 1*** aimed to explore which tissues from pigs are capable of complete carnitine synthesis. Furthermore, this study aimed to age-dependent differences in the activity of the BBD should be determined in pigs. The present work shows for the first time that BBD-activity takes place mainly in liver and kidney of piglets and is regulated age-dependently. Interestingly, the protein concentrations of BBD determined in different tissues did not correlate with the mRNA expression and the final enzyme activity. Reasons for this might be different regulatory mechanisms on posttranscriptional or posttranslational level, resulting in different isoforms of BBD or chemical modifications leading a modification of the activity. Moreover, the availability of cofactors of carnitine synthesis could be another reason for the dramatic differences between protein- and mRNA-concentrations and the activity of the BBD.

Studies in humans and livestock animals have shown the importance of carnitine regarding the favorable influence of body composition, lipid and insulin metabolism and the development of degenerative diseases. The underlying molecular mechanisms are still unknown and were investigated in this work. Using microarray technology, the influence of carnitine on the transcript profile was determined in liver and muscle of piglets. For this end two groups of piglets (n=8) aged four weeks were given a standard diet with or without 500 mg carnitine/kg diet over a period of three weeks. At the end of the feeding period muscle, liver and plasma samples were collected and used in ***study 2-4*** for subsequent analysis. The findings of all three studies strongly suggest that carnitine influences the transcription of numerous genes from protein, lipid and glucose metabolism. The intake of carnitine led to an increased transcription of genes involved in fatty acid catabolism and the transport and incorporation of triglycerides, cholesterol esters and phospholipids in lipoproteins in the liver of piglets. Furthermore it has been shown that carnitine causes a reduced transcription of genes encoding for enzymes of triglyceride synthesis. Thus leads to

reduced synthesis of lipids and lipoprotein secretion but increases fatty acid degradation in the liver. The hepatic expression of genes involved in cellular glucose uptake and metabolism was elevated in piglets due to supplementation of carnitine whereas genes encoding for enzymes of gluconeogenesis were decreased in their expression. Furthermore, in muscle of piglets fed carnitine the transcription of the gene SORBS1 (Sorbin and SH3 containing-1), which encodes an insulin-signaling molecule, was increased. This ultimately results in an increased translocation of GLUT4 (glucose transporter 4) to the cell membrane leading to an increased glucose absorption into the muscle cell. Supplementation of carnitine causes a stimulation of genes involved in insulin/IGF-1 signaling pathway in both, liver and muscle of piglets, which in turn explains further changes in transcription of many genes. Using real-time PCR, a significant decrease in the relative mRNA levels of genes involved in proteasomal protein degradation [muscle: atrogin-1, MuRF1 (muscle RING-finger protein-1), E214k, Psma1, ubiquitin; liver: Atrogin-1, MuRF1, E214k] was observed in the fourth study in piglets fed increased carnitine doses. Based on cell culture studies with HepG2 and C2C12 cells, which were incubated with increasing carnitine concentrations (0, 100, 500 and 1000 µM), it could be shown that carnitine has no direct effect on the ubiquitin-proteasome-system (UPS). It is more likely that carnitine inhibits transcription of genes involved in the UPS through stimulation of the insulin/IGF-1 signaling pathway via repression of FoxO-transcription factors. In addition, the findings of this study support that carnitine activates, through stimulation of insulin/IGF-1 signaling pathway, the Akt1 protein and thereby the activity of several transcription factors resulting in elevated rates of protein synthesis. Moreover, the feeding of carnitine led to an inhibition of the expression of genes encoding for proapoptotic transcription factors in muscle of piglets. The results of these experiments provides insights into the indirect regulation of transcription of numerous genes by carnitine and could explain the improvement of physiological performance characteristics observed in many feeding studies with various livestock animals.

The aim of ***study 5*** was to clarify whether regular endurance exercise influences the high-fat-feeding induced impairment of carnitine status. Therefore 18 male C57BL/6 mice were divided into three groups which received either a standard diet (10% fat) or a high-fat diet (45% fat) with or without endurance training. The intake of the high-fat diet led to an impaired glucose tolerance and a significant decrease in hepatic carnitine concentrations. Furthermore, decreased mRNA and protein levels of hepatic enzymes of carnitine synthesis and carnitine transporter OCTN2 (novel organic cation transporter 2) as well as reduced concentrations of carnitine and precursors were observed in tissues of these animals. Thus, the

high-fat diet probably resulted in a decrease in hepatic carnitine synthesis and a parallel reduction of hepatic carnitine intake. In contrast, it could be demonstrated for the first time that regular endurance exercise combined with high-fat diet improves carnitine status. Exercise trained mice had increased hepatic mRNA concentrations of BBD and OCTN2. Since BBD and OCTN2 are PPARα (peroxisome proliferator-activated receptor α) target genes the findings from the last study suggest that the transcriptional changes of the organism to excessive workload are due to a training-induced activation of nuclear transcription factor PPARα.

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Erklärung

„Ich erkläre: Ich habe die vorgelegte Dissertation „**Molekulare Wirkmechanismen von Carnitin und Regulation der Carnitinhomöostase beim Modell- und Nutztier**“ selbstständig und ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Dissertation angegeben habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der „Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis“ niedergelegt sind, eingehalten.“

Gießen, den …….

Janine Keller

Der Lebenslauf wurde aus der elektronischen Version der Arbeit entfernt.

The curriculum vitae was removed from the electronic version of the paper.

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