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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

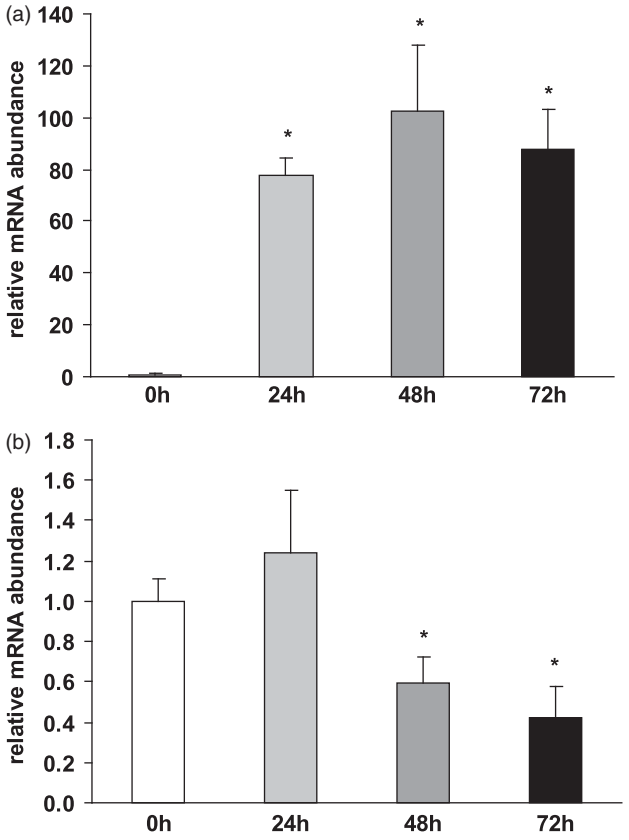

Figure 1 Relative mRNA abundance of *myogenin* (a) and AT-motif-binding factor 1 (*ATBF1-A*) (b) in C2C12 myotubes treated with differentiation medium for different durations (0, 24, 48 and 72 h). Data are expressed relative to the mRNA concentrations of time point 0 h (= 1.0). Bars are mean ± s.d. from 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),

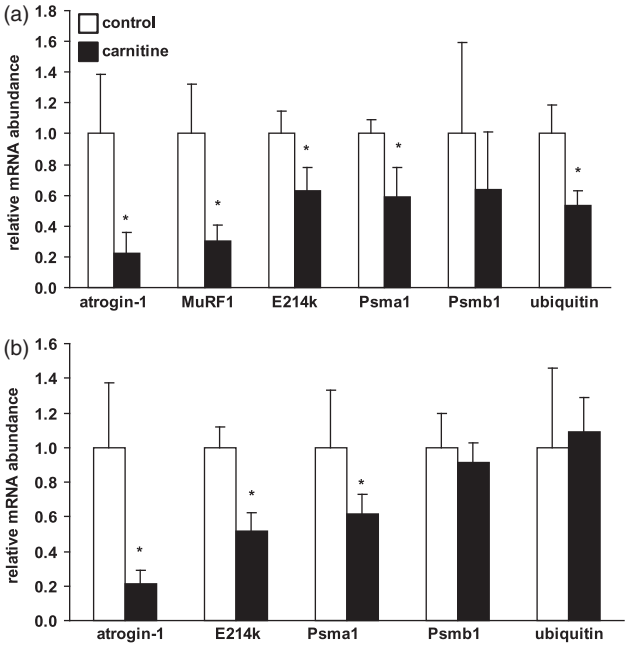

Figure 2 Relative mRNA abundance of selected genes of the ubiquitin proteasome system in the skeletal muscle (a) and liver (b) of growing pigs fed either a control diet or a diet supplemented with 500 mg carnitine/kg diet. Bars are mean ± s.d. ($n = 8$/group). The normalized expression ratio in the control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

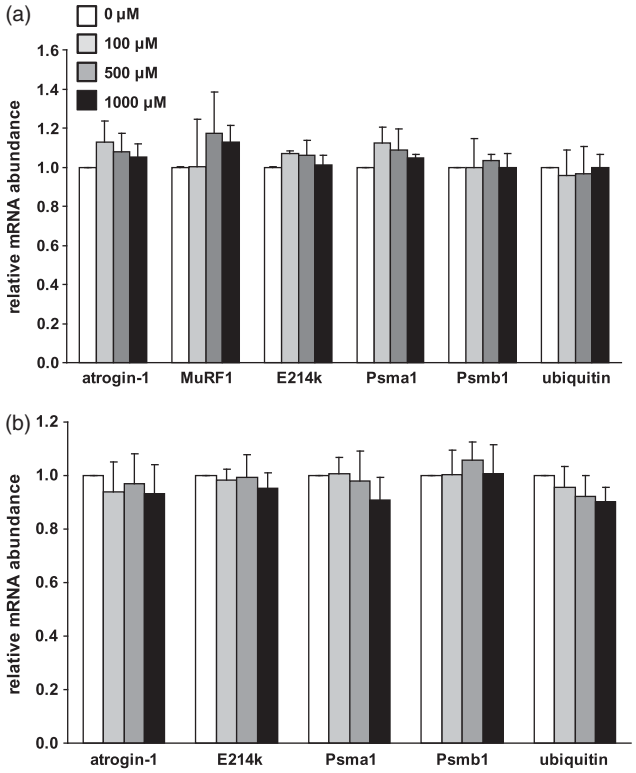

Figure 3 Relative mRNA abundance of selected genes of the ubiquitin proteasome system in C2C12 myotubes (a) and HepG2 liver cells (b) treated with increasing concentrations of carnitine (0, 100, 500 and 1000 µM). Data are expressed relative to the mRNA concentrations of the control group ($n = 1.0$). 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

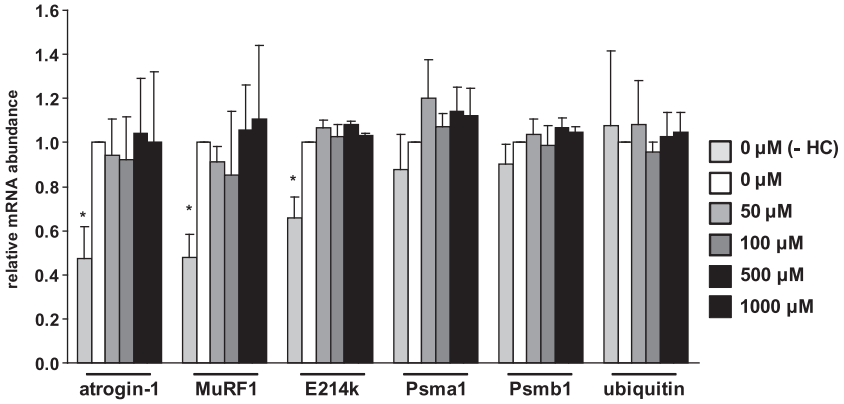

Figure 4 Relative mRNA abundance of selected genes of the ubiquitin proteasome system in C2C12 myotubes treated with increasing concentrations of carnitine (0, 50, 100, 500 and 1000 μM) and 10 μM hydrocortisone (+HC). C2C12 myotubes treated with 10 μmol/l hydrocortisone served only as controls. C2C12 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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