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

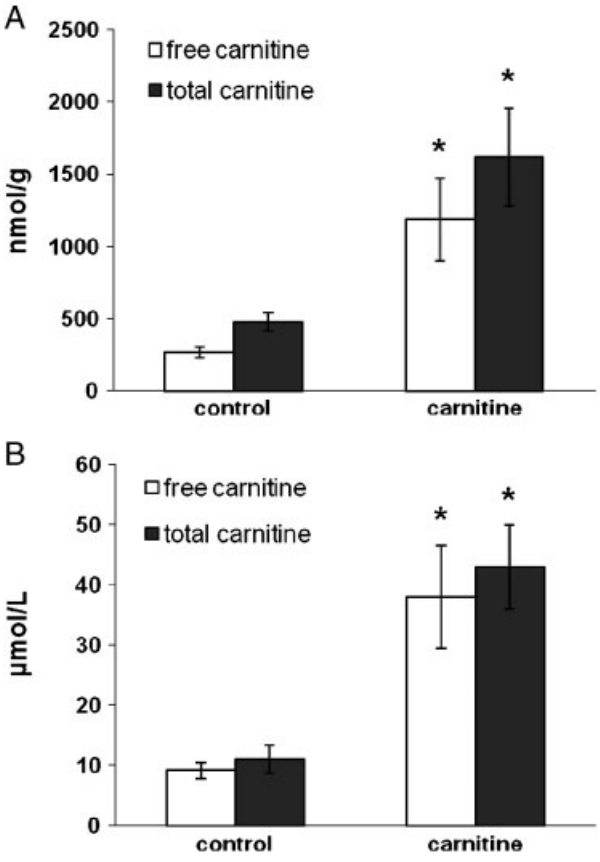

Figure 1. Concentrations of free and total carnitine in skeletal muscle (A) and plasma (B) of growing piglets fed either a control diet or a diet supplemented with 500 mg L-carnitine *per* kg diet.

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

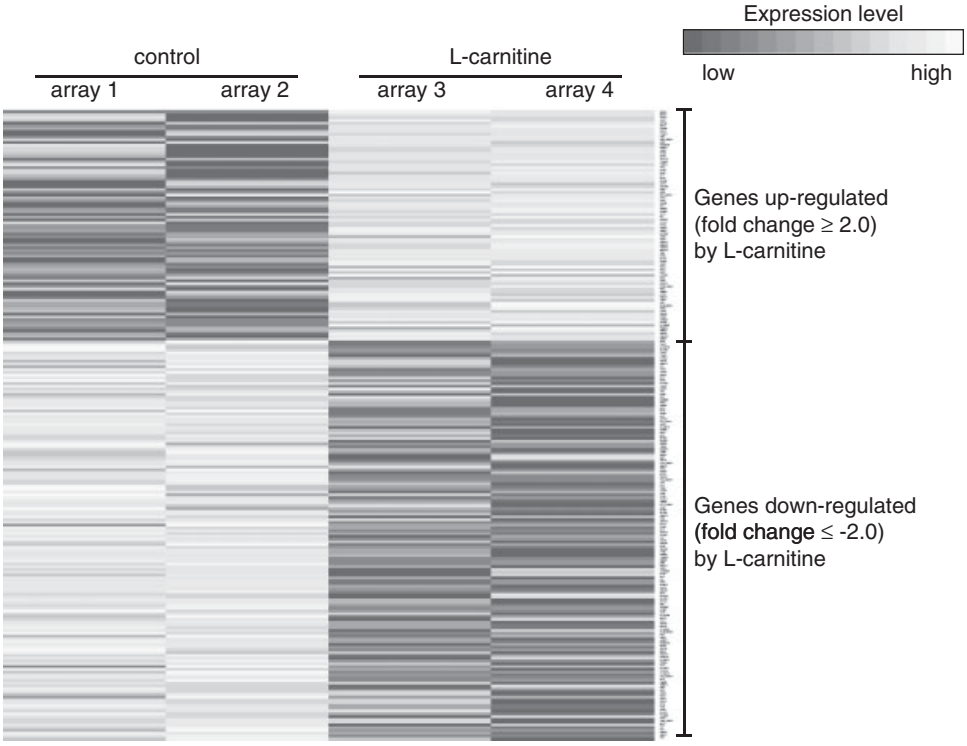

Figure 2. Heat map representing the expression levels of the 211 identified differentially expressed genes between piglets fed either the control diet or the diet supplemented with 500 mg L-carnitine *per* kg diet. The heat map was generated with the freely available software package R (URL: http://www.R-project.org)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

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