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

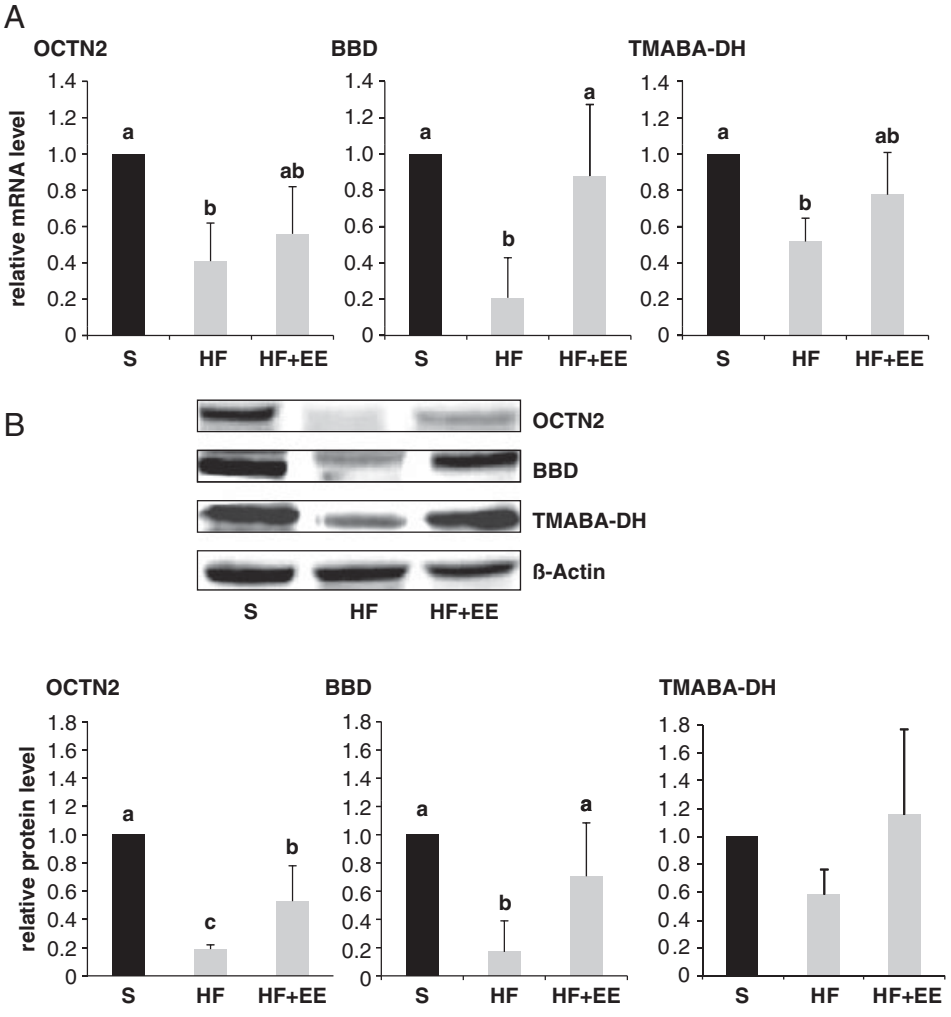
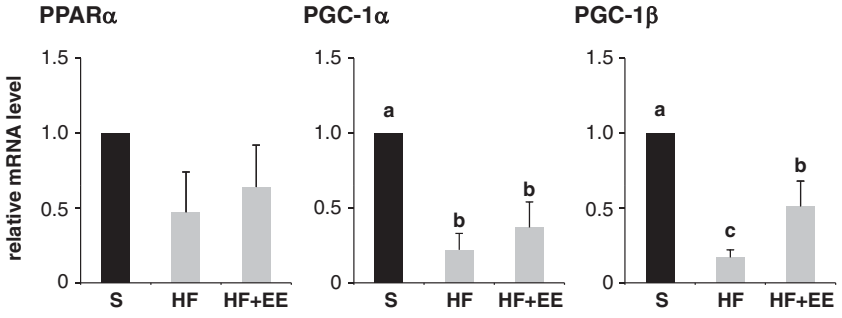


Figure 1. Relative mRNA and protein concentrations of genes involved in carnitine uptake (OCTN2) and carnitine synthesis (BBD, TMABA-DH) in the liver of nonexercising mice fed either a standard diet (S) or a HF diet and of exercising mice fed a HF diet (HF+EE). (A) Relative mRNA concentrations of OCTN2, 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, $p<0.05$.



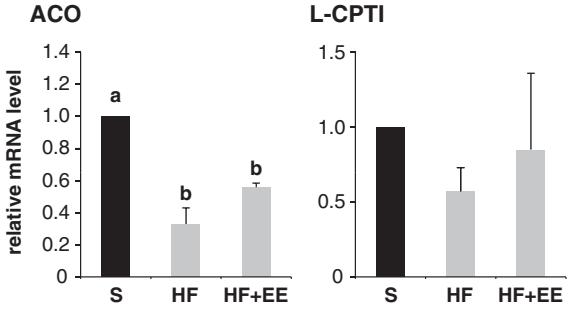
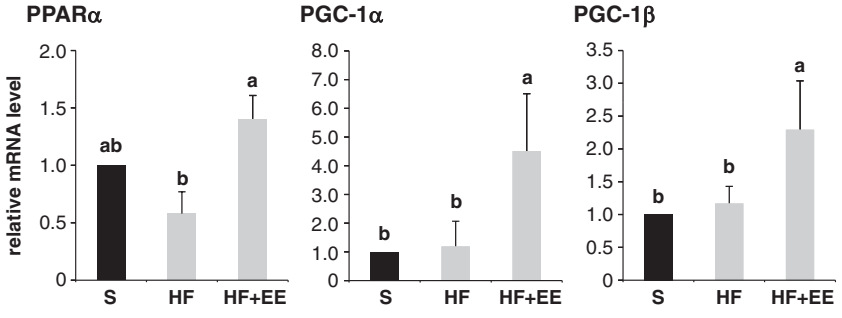


Figure 2. Relative mRNA concentrations of PPARα, PPAR coactivators (PGC-1α, PGC-1β), and PPARα target genes (ACO, L-CPT I) in the liver of nonexercising mice fed either a standard diet (S) or a HF diet and of exercising mice fed a HF diet (HF+EE). Bars represent mean ± SD ($n = 6$ animals/group) and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



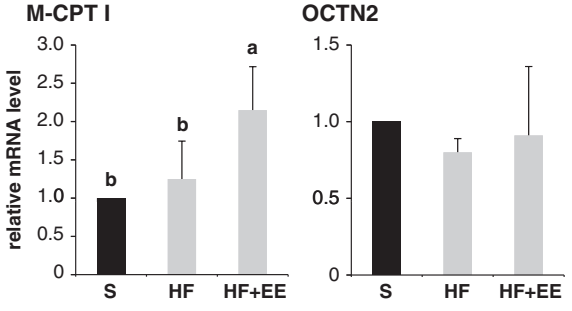


Figure 3. Relative mRNA concentrations of PPARα, PPAR coactivators (PGC-1α, PGC-1β), and PPARα target genes (ACO, L-CPT I, and OCTN2) in the gastrocnemius muscle of nonexercising mice fed either a standard diet (S) or a HF diet and of exercising mice fed a HF diet (HF+EE). Bars represent mean ± SD ($n = 6$ 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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