Contractile function and energy metabolism of skeletal muscle in rats with secondary carnitine deficiency

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Contractile function and energy metabolism of skeletal muscle in rats with secondary carnitine deficiency. Am J Physiol Endocrinol Metab 309: E265–E274, 2015.—The consequences of carnitine depletion upon metabolic and contractile characteristics of skeletal muscle remain largely unexplored. Therefore, we investigated the effect of N-trimethyl-hydrazone-3-propionate (THP) administration, a carnitine analog inhibiting carnitine biosynthesis and renal reabsorption of carnitine, on skeletal muscle function and energy metabolism. Male Sprague-Dawley rats were fed a standard rat chow in the absence (CON; n = 8) or presence of THP (n = 8) for 3 wk. Following treatment, rats were fasted for 24 h prior to excision of their soleus and EDL muscles for biochemical characterization at rest and following 5 min of contraction in vitro. THP treatment reduced the carnitine pool by ~80% in both soleus and EDL muscles compared with CON. Carnitine depletion was associated with a 30% decrease soleus muscle weight, whereas contractile function (expressed per gram of muscle), free coenzyme A, and water content remained unaltered from CON. Muscle fiber distribution and fiber area remained unaffected, whereas markers of apoptosis were increased in soleus muscle of THP-treated rats. In EDL muscle, carnitine depletion was associated with reduced free coenzyme A availability (~2%, P < 0.05), impaired peak tension development (~44%, P < 0.05), and increased glycogen hydrolysis (52%, P < 0.05) during muscle contraction, whereas PDC activation, muscle weight, and water content remained unaltered from CON. In conclusion, myopathy associated with carnitine deficiency can have different causes. Although muscle atrophy, most likely due to increased apoptosis, is predominant in muscle composed predominantly of type I fibers (soleus), disturbance of energy metabolism appears to be the major cause in muscle composed of type II fibers (EDL).

N-trimethyl-hydrazone-3-propionate; secondary carnitine deficiency; carbohydrate metabolism; muscle atrophy; apoptosis

CARNITINE IS A NATURALLY OCCURRING COMPOUND that is found in all mammalian tissues. L-Carnitine, the biologically effective isomer, plays a key role within several cellular energy producing pathways (9). For instance, carnitine is essential for the transport of long-chain fatty acids across the inner mitochondrial membrane toward their oxidative fate inside the mitochondrial matrix (15), is important for the removal of potentially toxic acyl-CoAs from the mitochondria by forming acylcarnitines (3, 6), and serves as a temporal acetyl group buffer in the oxidation of carbohydrates during periods of augmented pathway flux (14, 31).

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Fundamental to our understanding of the role of carnitine within the body is the ability to manipulate the size of the tissue carnitine pool and to investigate the consequences of such upon cellular, tissue, and whole body functions at rest and in response to external stresses such as muscular contraction. Although recent studies have shown that the skeletal muscle carnitine content can be increased in the presence of high circulating insulin concentrations by ~20% in humans (39, 40, 46), it appears to be more difficult to increase the skeletal muscle carnitine content in rodents (26). On the other hand, well-characterized models of carnitine deficiency are available in rodents (21, 37). Therefore, in rodents it is easier to assess the effect of carnitine deficiency on skeletal muscle function and energy metabolism.

In humans and in animals, carnitine deficiency can be divided into two forms, primary and secondary. Primary carnitine deficiency is associated with mutations in OCTN2, a sodium-dependent, high-affinity carnitine carrier with a high expression in the renal proximal tubules (29, 42). Lack of OCTN2 activity is associated with systemic carnitine deficiency both in humans (29) and in mice (21). Secondary carnitine deficiency is associated with diseases such as organic acidurias (35), hemodialysis (13, 19, 44), drugs such as valproate or pivmecillinam (7), or reduced dietary intake (24). Patients with primary carnitine deficiency typically present in early childhood with recurrent episodes of hypoketotic hypoglycemia and possibly coma (43). Patients with secondary carnitine deficiency typically present later in life and myopathy as well as cardiomyopathy are leading symptoms (19, 35, 43).

We have reported previously on a rat model with secondary carnitine deficiency induced by treatment with the carnitine analog N-trimethyl-hydrazone-3-propionate (THP) (37). Two to three weeks of THP treatment has been shown to reduce the carnitine content of heart, liver, plasma, and skeletal muscle (quadriceps femoris) by 72–83% (37) to cause liver steatosis and peroxisomal proliferation (38) and impaired myocardial function (47). To date, the characterization of this model of carnitine deficiency has focused predominantly upon THP’s effect upon liver, heart, and kidney, whereas skeletal muscle metabolism and function have received little attention. Taking into account that myopathy is a leading symptom of secondary carnitine deficiency, rats treated with THP appear to be a good animal model to learn more about this disease. For instance, it is currently not known whether the observed ~80% reduction in the skeletal muscle carnitine pool in THP-treated rats is sufficient to induce alterations in energy metabolism or a decrease in muscle function.

The present study wishes to address this point by examining the consequences of carnitine deficiency induced by THP
treatment upon the composition of coenzyme A and carnitine pools, tissue water content, and substrates of energy metabolism at rest and in response to short-term contraction in rat skeletal muscle. To further our understanding, we examined the above in two functionally and biochemically distinct muscle groups, namely soleus (SOL; 84% slow-twitch fibers) (1) and extensor digitorum longus (EDL; 98% fast-twitch fibers) (2), where the role of the carnitine pool varies from predominantly facilitating fat translocation (SOL) to preserving the free coenzyme A content for sustained carbohydrate oxidation (EDL).

METHODS

Animal handling. All in vivo procedures were performed in full accordance with the Ethical Principles and Guidelines for Scientific Experiments on Animals of the Swiss Academy of Medical Sciences and following Cantonal approval from the Veterinary Department of Basel, Switzerland. Twenty-six male Sprague-Dawley rats weighing ~200 g were obtained from the Süddeutsche Versuchstierfarm (Tübingen, Germany) and were acclimatized to the laboratory 1 wk prior to the start of the study. The animals were housed in a temperature-(20–22°C) and light-controlled (12:12-h light-dark cycle) animal facility.

Study design. Two different types of experiments were performed. In the first experiment (experimental group 1), THP-treated (n = 8) and control rats (n = 8) were used to study the strength of SOL and EDL muscle as well as for biochemical characterization. In the second experiment (experimental group 2), THP-treated (n = 5) and control rats (n = 5) were used for studying muscle histology, mechanisms of cell death, and mRNA expression.

Following 1 wk of habitation, animals were randomly assigned to one of the treatments groups and received a standard powdered rat chow (no. 3433; Kliba Futter, Basel, Switzerland) ad libitum. Treatment with THP was performed as described earlier (20 mg THP·100 g body wt⁻¹·day⁻¹ for 21 days) (37). THP was added to the rat chow, which was obtained ground. Based upon the findings of our previous study, rats were fed a standard rat chow (carnitine content 16.9 nmol/g; Kliba Futter), as the effect of carmine-deficient food upon carmine homeostasis is minor in rats treated with THP. Water was provided ad libitum throughout the study. The THP was commercially prepared by ReseChem (Burgdorf, Switzerland), as described previously (37). In the first experimental group, food consumption and changes in body weight were examined daily.

Following 21 days of treatment, animals were starved for 24 h prior to the induction of anesthesia with 160 mg/kg ip ketamine (Ketalar; Park-Davis, Zurich, Switzerland). Once adequate anesthesia was established, the animal’s SOL and EDL muscles were carefully isolated (total time <5 min), tendon to tendon, from both the left and right hindlimbs and processed as described below.

Muscle strength and biochemistry (experimental group 1). For experimental group 1, one SOL and one EDL muscle were snap-frozen in liquid nitrogen for future biochemical analysis of basal metabolite values. The distal and proximal tendons of the remaining soleus and EDL muscles were tied with nylon suture (Surgilon, grade 3-0) prior to their removal from the animal and immediately placed in continually gassed (95% oxygen-5% carbon dioxide) Krebs buffer (135 mM NaCl, 5 mM KCl, 1 mM NaH₂PO₄, 15 mM NaHCO₃, 11 mM glucose, 1 mM MgSO₄, 2.5 mM CaCl₂, pH 7.4, 25°C). In an attempt to maintain the in vivo appearance (fiber alignment and 3D architecture), the muscles were loaded with ~1.2 g upon the distal tendon while in Krebs suspension prior to the assessment of their in vitro contractile properties.

Contractile function of isolated muscles (experimental group 1). Muscles were mounted within the electrical stimulatory apparatus following their excision from the animal (<10 min). The contractile properties of the isolated muscles were assessed individually, with the EDL muscle always the first to be studied. Muscles were mounted vertically in the water-tight chamber of the electrical stimulatory apparatus (manufactured in-house) by the anchoring of the distal tendon to a fixed hook at the base of the bath and the attachment of the proximal tendon, by thread, to the isometric force transducer (Grass FT03; Quincy, Medfield, MA). The force transducer was mounted upon the micropositioner and was calibrated over the range of 0.25–1.00 g prior to each electrical stimulation protocol. Following muscle connection, the chamber of the apparatus was filled with 20 ml of Krebs buffer and continually gassed with a mixture of 96% O₂ and 4% CO₂ and maintained at 37.5°C (Julabo 5B; JD Instruments, Houston, TX) for the duration of the muscle stimulation protocol. Optimal muscle length (length producing maximal twitch tension) was determined by varying the muscle length in increments of 1 mm with the micropositioner and by performing a single “twitch” stimulus. Once optimal muscle length was determined, all subsequent measurements were made at this setting, and the muscle was allowed to equilibrate for 5 min prior to the commencement of the contractile electrical stimulation protocol. Muscular contraction was electrically induced by two platinum plate electrodes located at the proximal and distal origins of the muscle. Muscles were stimulated for 5 min (1-s train interval, 333-ms train duration, 25-ms pulse interval, 1-ms pulse duration, 130 V), in accord with the work of Brass et al. (8). Contractile function was recorded using a one-channel chart recorder (LKB Bromma 2210; Bromma).

Biochemical analysis of skeletal muscle (experimental group 1). All muscle samples, basal and postcontraction, were divided into two equal portions under liquid nitrogen. Subsequently, one portion was freeze-dried, dissected free from visible blood and connective tissue, and powdered. Total muscle water content of samples was determined by weighing the samples before and after freeze-drying. Freeze-dried samples were then extracted in 0.5 M perchloric acid containing 1 mM E64, with the resulting supernatant neutralized with 2.2 M KHCO₃ and used for the spectrophotometric determination of ATP, phosphocreatine, creatine, and muscle lactate (18). The extract was also used for the radioenzymatic determination of free carnitine, acetylcarnitine, and total acid soluble carnitine (5, 10) as well as for free CoA (CoASH) and acetyl-CoA (10). Finally, the extract was analyzed fluorimetrically for the determination of the tricarboxylic acid cycle intermediate citrate (25). Freeze-dried muscle powder was also used for the spectrophotometric determination of muscle glycogen (18). The remaining portion of frozen, wet muscle was used to assess the activation status of the pyruvate dehydrogenase complex (11).

Muscle histology, TUNEL assay and mRNA and protein expression (experimental group 2). For experimental group 2, both EDL and soleus muscles were isolated, snap-frozen, and stored at −80°C. Histology was performed using cryosections, as described previously (26). Frozen sections were stained with hematoxylin and eosin and with NADH-tetrazolium to differentiate type I from type II muscle fibers, and photographs were captured using an Olympus BX61 microscope (Olympus, Hamburg, Germany). The fiber area was determined by randomly selecting muscle fibers with a distinct cell membrane and excluding elongated fibers indicating an oblique section. We employed ImageJ (version 1.41) software to measure muscle fibers within at least four muscle cross-sections of each muscle and each rat.

Mechanisms of cell death were studied by quantifying the expression of cleaved caspase 3, the Bax/Bcl2 mRNA ratio, and a TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP nick-end label-
ing) assay. Cleaved caspase 3 was quantified by Western blotting according to Bonifacio et al. (4). Isolation of mRNA from frozen skeletal muscle, synthesis of cDNA, and real-time quantitative PCR for Bax and Bcl2 and for glucose transporter 4 (GLUT4) and hexokinase were performed as described previously (4, 41).

TUNEL staining of myonuclei positive for DNA strand breaks was performed using a commercially available fluorescence detection kit (Life Technologies, Zug, Switzerland). Cross-sections (10 μm) of muscles cut with a cryostat microtome were fixed with 4% paraformaldehyde for 15 min and permeabilized with 2 mg/ml proteinase K. The TUNEL reaction mixture containing terminal deoxynucleotidyltransferase (TdT) and fluorescein-labeled dUTP was added to the sections in portions of 50 μl and then incubated for 60 min at 37°C in a humidified chamber in the dark. After incubation, the sections were rinsed three times in PBS for 1 min each. Following embedding with ProLong diamond antifade mountant with DAPI (Life Technologies), the sections were investigated with a fluorescence microscope (×40 objective; Olympus BX61).

Calculations and statistics. All data are reported as means ± SE. Comparisons between treatments were carried out using two-way analysis of variance (ANOVA) with repeated measures. When a significant F value was obtained (P < 0.05), a least significant difference post hoc test was used to locate any differences (SPSS Base 12.0).

RESULTS

Animal data. There were no significant differences in body mass between the treatment groups during the 21 days of chow feeding in the presence and absence of THP, with each group linearly gaining ~90 g during the course of the study. Daily food consumption was not different between treatment groups during the 21 days of treatment (CON = 38.7 ± 0.2 vs. THP = 38.3 ± 0.3 g/day). The activity of creatine kinase in plasma was numerically higher in THP-treated rats without reaching statistical significance (CON = 79 ± 7 vs. THP = 105 ± 12 U/l, P = 0.098).

Muscle mass, water content, histology, and apoptosis. No differences in muscle water content existed between the CON and THP groups following 21 days of treatment in either the EDL (CON = 76.9 ± 0.3 vs. THP = 76.5 ± 0.2% water) or soleus muscles (CON = 75.4 ± 0.2 vs. THP = 75.3 ± 0.1% water).

The absolute muscle weight of tendon-to-tendon excised SOL muscle was reduced by 30% in THP-treated animals compared with CON (CON = 187 ± 19 vs. THP = 132 ± 15 mg wet muscle, P < 0.05). This muscle atrophy was maintained when SOL weight was expressed relative to the animals’ body weight (CON = 0.47 ± 0.05 vs. THP = 0.34 ± 0.03 mg wet muscle/g body mass, P < 0.05). In contrast, no significant reduction in absolute weight (CON = 141 ± 4 vs. THP = 124 ± 15 mg wet muscle) or muscle weight relative to body weight (CON = 0.35 ± 0.01 vs. THP = 0.32 ± 0.03 mg wet muscle/g body mass) was observed for EDL muscle of THP-treated compared with CON rats.

There was no significant change in muscle fiber type or fiber area in SOL muscle (Fig. 1, A and B). In contrast, in EDL

Fig. 1. Muscle fiber composition and fiber area. Muscle fibers were stained with NADH-tetrazolium, using cryosections as described in METHODS. Type I fibers are dark; type II fibers are bright. Fiber area was quantified using an Olympus BX61 microscope equipped with ImageJ (version 1.41) software. A: soleus muscle sections. B: quantification of type I fiber area in soleus muscle. C: extensor digitorum longus (EDL) muscle sections. D: quantification of type I and type II fiber area in EDL muscle. CON, control rats (n = 5); THP, N-trimethyl-hydrazine-3-propionate-treated rats (n = 5). Results are expressed as means ± SE. There were no statistically significant differences in fiber area between control and THP-treated animals.
muscle, the fiber area was 25% larger for THP-treated compared with control rats regarding type I fibers and 27% larger regarding type II fibers (Fig. 1, C and D). Because of a large variability mainly in THP-treated rats, these differences did not reach statistical significance ($P = 0.199$ for type I and $0.123$ for type II fibers). On sections stained with hematoxylin and eosin, no necrotic areas could be detected in either SOL or EDL muscle (data not shown).

To find out the mechanism for SOL muscle atrophy in THP-treated rats, we assessed markers of apoptosis. As shown in Fig. 2, A and B, expression of cleaved caspase 3 was significantly higher in soleus muscle from THP-treated to CON rats, suggesting increased myocyte apoptosis in the SOL of THP-treated rats. This was not the case for EDL muscle. Similarly, TUNEL-positive nuclei were detected only in SOL muscle from THP-treated rats but not in SOL from control rats or in EDL muscle (Fig. 2C). In addition, the ratio of the mRNA expression of Bax/Bcl2 was increased in SOL from THP-treated compared with control rats but not in EDL muscle (Fig. 2D).

Skeletal muscle contractile properties. Peak isometric force generated was reduced by 44% in the EDL muscle following THP treatment compared with CON, and the force remained significantly lower throughout contraction (Fig. 3 and Table 1). Despite the difference in peak force generation, no difference in the rate of fatigue development existed between the two treatment groups, as reflected by similar slopes of the decline in the force development curve (Fig. 3 and Table 1). Similarly to peak isometric force generation, the entire isometric force generated, as reflected by the area under the force development curve (AUC$_{0-300}$), was reduced by 48% in EDL muscles from THP-treated compared with control rats (Table 1).

Although peak isometric force and entire force generation (AUC$_{0-300}$) of SOL muscle were reduced by 17 and 20%, respectively, in THP-treated compared with CON rats, this reduction did not reach statistical significance (Fig. 3 and Table 1). Similarly to EDL muscle, the development of muscle fatigue (as reflected by the slope of the force development decline) was not different for SOL muscle obtained from THP-treated or CON rats.

Skeletal muscle carnitine pool. Treatment with THP reduced all carnitine fractions determined (free carnitine, acetylcarnitine, total acid soluble carnitine) by 70 – 85% compared with CON in both EDL and SOL muscles both at rest and following muscle contraction (Table 2). The individual carnitine fractions determined (free carnitine, acetylcarnitine, total acid soluble

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Fig. 2. Markers of apoptosis in EDL and soleus muscle. Apoptosis was investigated by quantifying cleaved caspase 3, TUNEL staining, and determination of the Bax/Bcl2 mRNA ratio. A: Western blot for cleaved caspase 3. GAPDH protein expression was used to correct for different protein loading. B: quantification of the Western blots ($n = 3$ independent experiments). C: TUNEL staining of EDL and soleus muscle sections. Arrows show TUNEL-positive nuclei. D: ratio of the Bax/Bcl2 mRNA expression in EDL and soleus muscle. Results are expressed as means ± SE; $n = 5$. *$P < 0.05$ vs. control rats.
carnitine) did not change with muscle contraction in either SOL or EDL muscle for either treatment group (Table 2).

The acetyl-carnitine-to-free carnitine ratio was increased significantly by ~95% in EDL muscle and by ~125% in SOL muscle following THP treatment compared with CON rats at rest and also following contraction (Table 2). Muscle contraction did not change this ratio in either the CON or the THP group in either muscle type investigated.

**Skeletal muscle CoA pool.** The free CoA (CoASH) content was decreased by ~12% in SOL and by ~25% in EDL muscle in THP-treated compared with CON rats, reaching statistical significance only for EDL muscle, both at rest and after muscle contraction (Table 3). Regarding acetyl-CoA, there were no significant differences between THP-treated and CON rats for either SOL or EDL muscle. Similar to carnitine, muscle contraction was not associated with significant changes in the CoASH or acetyl-CoASH pools in either muscle studied or in THP-treated or CON rats.

The acetyl-CoA/CoASH ratio was increased in THP-treated compared with CON rats, reaching statistical significance for EDL muscle both at rest (46% increase) and after muscle contraction (53% increase). Muscle contraction was associated with slight increases in the acetyl-CoA to CoASH ratio in EDL and soleus muscle in both THP-treated and CON rats, reaching statistical significance only for EDL muscle of THP-treated rats.

**Muscle metabolites at rest and during contraction.** No differences in the ATP content existed between THP-treated and CON rats at rest or during contraction in either EDL or SOL muscle (Table 4). During contraction, the ATP content had a tendency to decrease compared with resting conditions, reaching statistical significance in EDL muscle from THP-treated rats (40% decrease).

Similar to the ATP content, the skeletal muscle creatine, phosphocreatine, and total creatine contents were not different between THP-treated and CON rats for either EDL or SOL muscle under resting conditions or during contraction. Skeletal muscle metabolism was associated with a significant decrease in the phosphocreatine content in both muscle types and both treatment groups studied (decrease by ~70% irrespective of treatment and muscle type), whereas the creatine content was not affected. The total creatine content decreased by 10–20% with muscle contraction; this decrease reached statistical significance for SOL muscle in both treatment groups.

The resting skeletal muscle lactate content was not affected by treatment with THP in either muscle type studied. During

### Table 2. **Skeletal muscle Cn content**

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<th>Extensor digitorum longus</th>
<th>Soleus</th>
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<tr>
<td></td>
<td>CON</td>
<td>THP</td>
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<tr>
<td>Force&lt;sub&gt;max&lt;/sub&gt;, N/g wet wt</td>
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<tr>
<td>9.65 ± 0.81</td>
<td>5.36 ± 1.00&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.80 ± 0.33</td>
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<td>40 ± 13</td>
<td>55 ± 17</td>
<td>35 ± 4</td>
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<td>AUC&lt;sub&gt;CoASH&lt;/sub&gt;, N/g wet wt&lt;sup&gt;-1·s&lt;/sup&gt;-1</td>
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<tr>
<td>2.520 ± 170</td>
<td>1.320 ± 270&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>960 ± 110</td>
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<td>Slope, N/g wet wt&lt;sup&gt;-1·s&lt;/sup&gt;-1&lt;sup&gt;-1&lt;/sup&gt;</td>
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<td>−11.1 ± 1.8</td>
<td>−8.98 ± 2.36</td>
<td>−5.91 ± 0.73</td>
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Results are expressed as means ± SE; n = 8. CON, control; THP, N-trimethyl-hydrazine-3-propionate; T<sub>max</sub>, point in time of maximal force; AUC<sub>CoASH</sub>, area under the force development curve. Isometric tension production and fatigue development were determined in extensor digitorum longus and soleus muscles excised tendon to tendon from CON and THP-treated rats, as described in METHODS. Muscles were incubated at 37°C, and electrical stimulation was performed for 5 min. *Different from respective CON (P < 0.05).
muscle contraction, the lactate content showed a tendency to increase in both treatment groups and both muscles studied; this increase reached statistical significance for EDL muscle in CON rats (54% increase).

The glycogen content was not significantly different between treatment groups in EDL or SOL muscles under resting conditions or after muscle contraction. The only exception was a 52% decrease in EDL muscle of THP-treated compared with control rats after muscle contraction.

The muscle citrate content at resting conditions was not statistically different between the treatment groups in SOL but was increased by 108% in THP-treated compared with control rats after muscle contraction.

The muscle citrate content at resting conditions was not statistically different between the treatment groups in SOL but was increased by 108% in THP-treated compared with control rats after muscle contraction.

Active form of the pyruvate dehydrogenase complex. At rest, the active form of pyruvate dehydrogenase complex activity was increased in both muscle types investigated in THP-treated compared with control rats by 50–100% in muscles from THP-treated rats and by ~200% in muscles from control rats. This increase was significantly greater for all conditions studied, except for SOL muscle of THP-treated rats. After electrical stimulation, there were no significant differences in PDCa activity between muscles from THP-treated or control rats.

mRNA expression of GLUT4 and hexokinase. mRNA expression of GLUT4 was decreased by 25% in SOL and increased by 54% in EDL muscle in THP-treated compared with CON rats. These differences did not reach statistical significance. mRNA expression of hexokinase was increased by 24% in soleus and by 67% in EDL muscle of THP-treated compared with control rats, reaching statistical significance (P < 0.05) for EDL.

**DISCUSSION**

This is the first study to examine the consequences of carnitine depletion upon the metabolic and contractile characteristics of predominantly slow- and fast-twitch skeletal muscles from otherwise healthy rats. Twenty-one days of treatment with THP, a competitive inhibitor of carnitine biosynthesis and renal reabsorption (37), reduced the total carnitine pool to the same extent in both SOL and EDL muscles from that measured in control animals by ~80% to a level comparable with that reported in JVS mice (20) and in human sufferers of primary carnitine deficiency (36). The THP-induced decrease of the muscle carnitine pool was associated with a marked atrophy in soleus muscles, whereas contractile function, total-carnitine A, and water contents were not significantly altered compared with control muscle. In comparison, carnitine depletion was associated with impaired peak tension development, ATP homeostasis, a reduction in free coenzyme A availability, and increased glycogen hydrolysis during contraction in EDL muscle, whereas muscle weight and water content were not significantly altered from control muscle.

Skeletal muscle atrophy. Taking into account that the body weight increased by 21% during the observation period but that SOL muscle in THP-treated rats was 30% lower than in control rats, it can be assumed that the decrease in SOL muscle weight in THP-treated rats cannot be explained only by impaired muscle growth but that muscle atrophy has to at least contribute.

Muscle atrophy refers to the wasting or loss of muscular tissue that occurs as a result of disuse or damage to the nerves that supply the muscle from a disease of the muscle itself or...
from toxic effects on the muscle (22). In the present study, we observed a significant 30% reduction in the mass of the SOL muscle in response to THP treatment, whereas the effect on EDL did not reach statistical significance (minus 14% in THP-treated rats, P = 0.292). Because the SOL muscle is composed of more than 80% slow-twitch fibers and the fiber composition remained unchanged in THP-treated compared with control rats (Fig. 1), our findings imply a predominant type I fiber loss in response to carnitine depletion. Given that THP treatment results in no alteration in rodent overnight activity (Kaufmann P, unpublished observation), it would appear that the atrophy reported herein was not the result of disuse but from an abnormality within the muscle itself. It is noteworthy that a type I atrophy has been reported in an 18-yr-old female with a secondary carnitine deficiency (16).

Our mechanistic studies are compatible with increased apoptosis of type I muscle fibers as the mechanism for SOL muscle atrophy (Fig. 2). Apoptosis is indeed one of the major determinants of skeletal muscle atrophy (23, 45). In vitro studies have demonstrated that L-carnitine can inhibit apoptosis at several levels (12, 28). For example, L-carnitine can interfere with Fas ligand-mediated apoptosis, thereby reducing acid sphingomyelinase activity and the ability of ceramide to stimulate the proapoptotic Bax/Bad complexes (12, 27). L-Carnitine can also impair opening of the mitochondrial membrane permeability transition pore, which normally leads to apoptosis (28, 30). Clearly, low skeletal muscle carnitine stores are likely to promote rather than suppress apoptosis. Further studies are required, however, to understand the predominant effect of the decreased carnitine muscle stores on type I vs. type II muscle fibers. This could be attributable to the higher mitochondrial content and higher carnitine palmitoyl transferase I levels in type I compared with type II muscle fibers. In this context, it is worth noting that 24 wk of L-carnitine administration has been shown to have a selective trophic effect upon type I muscle fibers in man (17).

Compared with soleus, EDL muscle of THP-treated rats was not atrophic but showed a nonsignificant ~25% increase in the area of type I and II fibers, which was compatible with fiber hypertrophy. A possible explanation for these findings could be that low skeletal muscle carnitine content is associated with muscle fiber loss. Certain muscles (such as EDL) can compensate for this loss with fiber hypertrophy, but this is not possible for other muscles (such as SOL). The reason for the assumed difference in the adaptation to fiber loss is currently not known and deserves further investigation. In total, THP-treated rats can preserve their muscle mass, as suggested by maintained body weight and urinary excretion of creatinine compared with control rats (37).

**Contractile function.** At present, very little is known regarding carnitine’s effect upon the contractile properties of skeletal muscle, especially with regard to peak tension development and toward the maintenance of contractile function. Work by Brass et al. (8) examining the contractile properties of SOL and EDL muscle strips in vitro documented an improved maintenance of contractile function for 5 min of electrical stimulation, when soleus was incubated in a medium rich in carnitine. However, no carnitine-mediated improvements in contractile function were observed in EDL muscle in this study.

Since we wanted to be comparable with existing data, we used the protocol of Brass et al. (8) for skeletal muscle stimulation. Using this protocol, an intense muscle contraction is induced, for which carbohydrates are the main energy source (32). Based on these considerations and the previous findings of Brass et al. (8), we expected carnitine depletion to impair peak tension and increase fatigability of the musculature mainly by impairing the ability to buffer excess acetyl-CoA generated through carbohydrate oxidation.

However, peak tension and the decline in contractile function during 5 min of electrical stimulation of SOL muscle in vitro were not significantly impaired in THP-treated compared with control rats. The absence of THP-induced impairment in function of the SOL muscle could possibly be explained by the intensity of the stimulation. Although we observed the expected decrease in the phosphocreatine content in THP-treated and control muscle in response to contraction, there was no increase in the acetyl/carnitine content or in the acetylcarnitine/carnitine ratio. Therefore, it appears that acetyl-CoA delivery through the carbohydrate pathways did not exceed the demands of the TCA cycle for oxidative substrates. Therefore, carnitine’s ability to buffer excess acetyl-CoA may not have been sufficiently tested using the current intensity of contraction.

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**Fig. 4. Pyruvate dehydrogenase complex activation (PDCa) in EDL and soleus muscles.** Muscles were studied at rest and following 5 min of electrically induced contraction from CON (n = 8) and THP-treated (n = 8) rats. The reaction was carried out at 37°C, and the results are expressed as means ± SE. *P < 0.05 vs. resting conditions within the same group. There were no significant differences between THP-treated and control rats.
In contrast to soleus, peak isometric tension production was reduced in EDL muscle in response to THP pretreatment, although the maintenance of peak tension was not different from control muscle. As will be discussed below, a likely explanation for this finding is the reduced availability of free CoASH to stimulate oxidative ATP synthesis in response to treatment with THP.

Effect of carnitine deficiency on skeletal muscle energy metabolism. Similarly to SOL muscle, treatment with THP reduced the carnitine content in EDL muscle by ~80%. However, in contrast to soleus, the acetyl-CoA/free CoASH ratio was significantly increased and the concentration of free CoASH, a key substrate for the PDC reaction, was significantly lowered compared with CON muscle. Despite these differences in free CoASH availability in EDL muscle, no difference in PDC activation existed between THP-treated and control rats. These findings indicate that, despite a similar degree of PDC activation, acetyl-CoA and NADH delivery via this enzyme complex (i.e., flux) was reduced in EDL muscle from THP-treated vs. CON rats.

The decline in free-CoASH concentration in EDL vs. SOL muscle following 21 days of THP is a surprising observation that warrants further investigation. One explanation is the increased acetyl-CoA/free-CoASH ratio in response to the decline in tissue carnitine content and a concomitant reduced ability of carnitine acyltransferase to buffer excess acetyl groups to maintain free CoASH. An additional complication of an elevated acetyl-CoA/free-CoASH ratio may be its effect upon pantothenate kinase, the master regulator of coenzyme A biosynthesis in mammalian cells (33). Evidence is emerging that acetyl-CoA and free-CoASH differentially regulate pantothenate kinase, with acetyl-CoA potently inhibiting activity, leading to a reduction in total CoASH content and with free CoASH stimulating coenzyme A biosynthesis (34).

Interestingly, the citrate content in EDL muscle was increased in THP-treated compared with control rats under resting conditions. Increased formation of citrate by the Krebs cycle is unlikely to be taking into account the impaired flux across the PDC. Impaired activity of the Krebs cycle distal to the formation of citrate is also unlikely regarding the maintained ATP and phosphocreatine content without a significant increase in the lactate content. A more likely possibility is a decreased flux across the cytosolic citrate lyase reaction, which uses free CoASH as a substrate to produce acetyl-CoA and oxaloacetate from citrate.

Another important observation in EDL muscle is the decrease in the glycogen content in response to muscle contraction in THP-treated compared with control rats. This could result from impaired transport and/or phosphorylation of glucose as well as impaired glycogen synthesis. Taking into account the increased mRNA expression of GLUT4 and hexokinase and the maintained cellular ATP concentrations in resting EDL of THP-treated rats, impaired transport and phosphorylation of glucose are both unlikely. Glycogen synthesis was not assessed directly, but maintained glycogen stores under resting conditions in EDL of THP-treated rats suggest a normal activity of glycogen synthesis. Therefore, a more likely explanation for the reduced glycogen content in postcontraction EDL muscle of THP-treated rats is increased glycogen consumption. As mentioned above, the flux across the PDC reaction, which is at the same time an important source of substrates for the Krebs cycle, was decreased in EDL of THP-treated rats. Under resting conditions, this decrease was not large enough to impair oxidative metabolism of glucose. During muscle contraction, however, Krebs cycle activity may have been too limited in EDL from THP-treated rats to maintain the cellular ATP stores, and glycogenolysis/glycolysis may have been necessary for ATP generation. The lactate content after contraction was numerically increased in EDL muscle from THP-treated compared with control rats (70 ± 7 vs. 49 ± 15 mmol/kg dry muscle, P = 0.225), supporting this explanation.

Impaired flux across PDC and citrate lyase and, as a consequence, increased cellular citrate and reduced glycogen stores may explain the observed decrease in maximal contraction in the EDL of THP-treated rats. In comparison, the findings in the SOL muscle concerning the effect of the loss of carnitine on the CoA pool go into the same direction as that observed in the EDL muscle but are less accentuated. Accordingly, citrate did not accumulate, glycogen stores were maintained during contraction in SOL from THP-treated rats, and SOL muscle contraction was not significantly impaired.

It is also possible that reduced contractile function of EDL is not a consequence of changes in energy metabolism but may be related to the observed morphological changes that may affect muscle contraction directly or via impairing excitation-contraction coupling.

In conclusion, we present novel data demonstrating that a THP-induced carnitine deficiency in otherwise healthy skeletal muscle results in a predominant loss of SOL (type I) muscle fibers, whereas the biochemical and functional properties of the soleus muscle remained largely normal. Conversely, despite the preservation of EDL muscle mass, the biochemical and functional properties of this predominantly type II fibered muscle were impaired, with an increased reliance upon energy production from non-oxygen-dependent routes compared with control muscle. Our findings suggest that myopathy associated with carnitine deficiency can have different reasons, namely muscle atrophy in muscles composed predominantly of type I fibers and impaired mitochondrial energy production in muscles composed predominantly of type II fibers.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

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