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Overexpression of carnitine palmitoyltransferase I in skeletal muscle in vivo increases fatty acid oxidation and reduces triacylglycerol esterification

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Bruce CR, Brolin C, Turner N, Cleasby ME, Leij FR, Cooney GJ, Kraegen EW. Overexpression of carnitine palmitoyltransferase I in skeletal muscle in vivo increases fatty acid oxidation and reduces triacylglycerol esterification. *Am J Physiol Endocrinol Metab* 292: E1231–E1237, 2007. First published December 19, 2006; doi:10.1152/ajpendo.00561.2006.—A key regulatory point in the control of fatty acid (FA) oxidation is thought to be transport of FAs across the mitochondrial membrane by carnitine palmitoyltransferase I (CPT I). To investigate the role of CPT I in FA metabolism, we used in vivo electrotransfer (IVE) to locally overexpress CPT I in muscle of rodents. A vector expressing the human muscle isoform of CPT I was electrotransferred into the right lateral muscles of the distal hindlimb [tibialis cranialis (TC) and extensor digitorum longus (EDL)] of rats, and a control vector expressing GFP was electrotransferred into the left muscles. Initial studies showed that CPT I protein expression peaked 7 days after IVE (+104%, $P < 0.01$). This was associated with an increase in maximal CPT I activity (+30%, $P < 0.001$) and a similar increase in palmitoyl-CoA oxidation (+24%; $P < 0.001$) in isolated mitochondria from the TC. Importantly, oxidation of the medium-chain FA octanoyl-CoA and CPT I sensitivity to inhibition by malonyl-CoA were not altered by CPT I overexpression. FA oxidation in isolated EDL muscle strips was increased with CPT I overexpression (+28%, $P < 0.01$), whereas FA incorporation into the muscle triacylglycerol (TAG) pool was reduced (−17%, $P < 0.01$). As a result, intramyocellular TAG content was decreased with CPT I overexpression in both the TC (−25%, $P < 0.05$) and the EDL (−45%, $P < 0.05$). These studies demonstrate that acute overexpression of CPT I in muscle leads to a repartitioning of FAs away from esterification and toward oxidation and highlight the importance of CPT I in regulating muscle FA metabolism.

mitochondria; muscle lipids; substrate metabolism

LIPIDS REPRESENT THE LARGEST STORE of nutrient energy in the human body. Defects in lipid metabolism are associated with the development of cardiovascular disease, obesity, insulin resistance, and type 2 diabetes. Therefore, the normal control of fatty acid (FA) metabolism is not only important in energy production, but it is also essential to maintain good health. Skeletal muscle is the primary tissue contributing to basal metabolic rate and is also the major site of FA oxidation (25); however, the control of FA oxidation in skeletal muscle is not completely understood. A number of factors, such as free fatty acid availability and the content of FA transport proteins, may affect the rate of FA oxidation in muscle. However, the principal regulatory point appears to be the transport of long-chain fatty acyl-CoA across the mitochondrial membrane by carnitine palmitoyltransferase I [CPT I (15, 19)].

CPT I (EC 2.3.1.21) is a transmembrane enzyme of the mitochondrial outer membrane which catalyzes the transfer of an acyl-CoA moiety from a long-chain acyl-CoA ester to carnitine to form acylcarnitine. The generated acylcarnitine can then enter the mitochondria and undergo β-oxidation (14). CPT I is considered the rate-limiting step in the oxidation of long-chain fatty acids and is an important site in the regulation of flux through β-oxidation (15, 19). The activity of CPT I is largely controlled by cytosolic levels of its biological inhibitor malonyl-CoA (15, 19). As a result, malonyl-CoA has been suggested to be a key regulator of fatty acid partitioning in skeletal muscle by virtue of its ability to inhibit CPT I.

As CPT I is an important control point in the regulation of skeletal muscle FA oxidation, it would appear to be a viable target to manipulate FA metabolism. A number of interventions that could potentially do this, such as exercise training or pharmacological activation of AMP-activated protein kinase, cause many metabolic and biochemical changes that can increase FA oxidation, which cannot be attributed solely to an increase in CPT I activity. Therefore, a more direct means of enhancing CPT I activity is required to gain a further understanding of its role in regulating FA metabolism in skeletal muscle. One approach to achieve this would be to genetically manipulate animals, resulting in the "knocking in" or knocking out" of the CPT I gene. However, we are not aware of any studies that have utilized such an approach to manipulate CPT I expression in muscle, possibly due to technical difficulties encountered when attempts have been made to transgenically overexpress CPT I in muscle (Brown NF and O'Doherty RM, personal communication). In addition, by use of such an approach there is the possibility of developmental adaptations to the genetic manipulation that may hamper the interpretation of the results. Therefore, it may be more informative to use a technique to acutely manipulate gene expression of a single muscle in mature animals. A promising approach is to alter local gene expression in skeletal muscle through a direct intramuscular DNA injection in combination with in vivo electrotransfer [IVE (5)]. This novel technique allows for the specific overexpression of proteins of interest in a single muscle in vivo, with the contralateral muscle acting as a

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control. Such an approach represents a powerful comparative tool for metabolic studies because of the limited time for the development of confounding counterregulatory changes in metabolism and the fact that both muscles are exposed to identical in vivo factors, such as the circulating substrate-endocrine milieu.

Although CPT I is recognized as an important regulatory point in the control of FA oxidation, it is not known whether an increase in CPT I content alone is sufficient to upregulate FA metabolism in skeletal muscle. Given the relationship between CPT I and malonyl-CoA, it would be predicted that simply overexpressing CPT I would not activate FA oxidation in muscle, as malonyl-CoA levels would be sufficient to inhibit all of the CPT I activity. However, to the best of our knowledge, no studies have been performed to examine the effect of CPT I overexpression on FA metabolism in skeletal muscle in vivo. Therefore, the purpose of this study was to use the novel in vivo electroporation technique to examine the functional effects of localized CPT I overexpression in skeletal muscle on parameters of FA metabolism. Specifically, we examined the effects of CPT I overexpression on *1*) aspects of FA metabolism in isolated mitochondria and *2*) the rates of palmitate metabolism in intact skeletal muscle. We hypothesized that CPT I overexpression would upregulate FA oxidation in isolated mitochondria. However, we predicted that, in intact skeletal muscle, overexpressing CPT I would have little effect on palmitate metabolism due to inhibition of CPT I activity by constitutively high levels of malonyl-CoA.

RESEARCH DESIGN AND METHODS

Male Wistar rats (~200 g) were used for all experiments. Animals were obtained from the Animal Resources Centre (Perth, Australia) and were acclimatized to their new surroundings for 1 wk. Animals were maintained at 22 ± 0.5°C under a 12:12-h light-dark cycle. Rat chow (6% calories from fat, 21% calories from protein, 71% calories from carbohydrate; Gordon's Specialty Stock Feeds, Yanderra, NSW, Australia) and water were available ad libitum. The *CPT I* gene was overexpressed in mature rat tibialis cranialis (TC) muscle (subsequently described). Studies were performed to examine the time course of CPT I overexpression (3, 7, and 10 days) and the functional effects of overexpressing CPT I in vivo. In addition, we explored the possibility of developing a system to study the effects of overexpressing CPT I on FA metabolism in an intact isolated muscle preparation. Therefore, the expression of CPT I following the electroporation procedure was determined in the extensor digitorum longus (EDL) muscle. As the EDL lies in close proximity to the TC muscle, it is possible that, along with the TC, the EDL may also overexpress the gene of interest. All experimental procedures were approved by the Garvan Institute/St. Vincent's Hospital Animal Experimentation Ethics Committee and were in accordance with the National Health and Medical Research Council of Australia Guidelines on Animal Experimentation.

Vector construction. The human *CPT IB* cDNA in pCE200 (23) is a translational fusion with green fluorescent protein (EGFP). The 5′ end of the *CPT IB* cDNA was excised from pCE200 with the restriction enzymes *Not*I and *Bln*I. This fragment was fused to a *Bln*I-*Eco*RI fragment representing the 3′ end of a human *CPT IB* cDNA from clone pCP205, which contains the natural *CPT IB* stop codon (24) in a *Not*I-*Eco*RI digest of pIRES-EGFP (Clontech). The resulting plasmid encodes for muscle-type *CPT I*, cotranscribed with EGFP and translationally separated by the internal ribosome entry site (IRES), yielding two independent proteins when expressed in mammalian cells. EH114-EGFP was constructed as previously described (5).

In vivo electrotransfer. The electrotransfer procedures used have been previously described (5). Briefly, vectors were propagated in selective media and DNA extracted, purified using Endotoxin-free Maxi- or Mega-Prep kits (Qiagen, Melbourne, Victoria, Australia), and resuspended in sterile 0.9% saline. Anesthesia was induced with 5%, and maintained with 1–2%, halothane in oxygen. Thereafter, animals' hindlimbs were shaved and prepared with a chlorhexidine-ethanol solution. The TC muscles were injected in an oblique fashion transcutaneously along their length with 0.5 μg/μl total DNA in saline with an insulin syringe. Rats received six spaced 50-μl injections along the TC muscle. This was followed by the application of a pair of plate electrodes across the distal limb connected to an ECM-830 electroporator device (BTX, Holliston, MA). The electroporation protocol consisted of one 800 V/cm, 100-ms pulse followed by four 80 V/cm, 100-ms pulses at 1 Hz. This protocol has been shown to result in a transfection rate of ~50% of fibers (5). The TC muscle was sampled either 3, 7, or 10 days after the tissue was transfected. In a subset of animals, the EDL muscle was sampled 7 days after the electroporation procedure. Unless otherwise stated, the muscles were rapidly freeze-clamped using precooled tongs and stored at −80°C.

Western blot anaylsis. Muscle tissue (~20 mg) was homogenized (Polytron, Kinematica, Switzerland) in ice-cold buffer containing 50 mM HEPES, 150 mM NaCl, 10 mM NaF, 1 mM Na_3VO_4, 5 mM EDTA, 0.5% Triton X-100, 10% glycerol (vol/vol), 2 mg/ml leupeptin, 100 mg/ml phenylmethylsulfonyl fluoride, and 2 mg/ml aprotinin. Homogenates were spun at 16,000 *g* for 60 min at 4°C, and the supernatant was removed and protein content determined. Muscle lysates were solubilized in Laemmli buffer and boiled for 5 min, resolved by SDS-PAGE on 10% polyacrylamide gels, transferred to a nitrocellulose membrane, and blocked with 5% nonfat milk powder. Membranes were immunoblotted overnight with antibodies specific for the muscle isoform of CPT I (1:2,000; Alpha Diagnostic International, San Antonio, TX) and uncoupling protein-3 (UCP3, 1:1,000; Affinity Bioreagents, Golden, CO). After incubation with horseradish peroxidase-conjugated secondary antibody (1:5,000; Jackson ImmunoResearch Laboratories, West Grove, PA), the immunoreactive proteins were detected with enhanced chemiluminescence and quantified by densitometry.

Isolation of mitochondria from skeletal muscle. The TC muscle was cleaned of any visible nonmuscle tissue and immediately placed in ice-cold *medium I* for isolation of mitochondria. To obtain a pure and intact mitochondrial fraction, differential centrifugation was used (3, 6). All procedures were performed at 0–4°C. Media used were as follows: *medium 1*: 100 mM KCl, 5 mM $MgSO_4 \cdot 7H_2O$, 5 mM EDTA, and 50 mM Tris·HCl, pH 7.4; *medium II*: solution I plus 1 mM ATP, pH 7.4; *medium III*: 220 mM sucrose, 70 mM mannitol, 10 mM Tris·HCl, and 1 mM EDTA, pH 7.4. Fresh TC muscle was immediately placed in ice-cold *medium I* and then blotted and weighed. Muscle was minced with scissors in 1 ml of *medium II*. Minced muscle was homogenized in 20 vol of ice-cold *medium II* using a polytron at the precise low setting of 3 for 2 × 15 s. The homogenate was spun at 800 *g* for 10 min at 4°C. Subsarcolemmal (SS) mitochondria remained in the supernatant, which was removed and kept on ice. The intermyofibrillar (IMF) mitochondria were pulled down in the pellet, which was resuspended in 5 vol of *medium II* and treated with a protease (Sigma P5380, 0.025 ml/g) for 5 min to digest the myofibrils. Addition of 15 ml of ice-cold *medium II* was used to diminish the action of the protease. Samples were spun at 5,000 *g* for 5 min, and the supernatant was removed. The pellet was resuspended in 10 vol of *medium II* and spun at 800 *g* for 10 min. The IMF mitochondria found in the supernatant were combined with the SS supernatant from the first 800-*g* spin to increase the mitochondrial yield and were spun at 10,000 *g* for 10 min. The pellet was washed twice in *medium II* and spun at 10,000 *g* for 10 min at 4°C. The pellet was resuspended in 1 μl *medium III*/mg tissue and used for CPT I and mitochondrial respiratory activity measurements.

CPT I activity. The forward radioisotope assay for the determination of CPT I activity was used as described by McGarry et al. (15), with minor modifications (1). Briefly, the assay was conducted at 37°C and was initiated by the addition of 10 μl of mitochondrial suspension (1:3 dilution) to 90 μl of the following standard reaction medium: 117 mM Tris·HCl (pH 7.4), 0.28 mM reduced glutathione, 4.4 mM ATP, 4.4 mM $MgCl_2$, 16.7 mM KCl, 2.2 mM KCN, 40 mg/l rotenone, 0.5% BSA, 300 μM palmitoyl-CoA, and 5 mM L-carnitine with 1 μCi of L-[^{3}H]carnitine and a final pH of 7.1. The sensitivity of CPT I to malonyl-CoA was also determined with the addition of malonyl-CoA in concentrations of 0.2, 0.7, 2.0, 5.0, and 10.0 μM. The reaction was stopped after 6 min with the addition of ice-cold 1 N HCl. Palmitoyl-[^{3}H]carnitine was extracted in water-saturated butanol in a process involving three washes with distilled water and subsequent recentrifugation steps to separate the butanol phase, in which the radioactivity was counted. CPT I activity was normalized to milligrams of protein.

Mitochondrial respiratory activity. Mitochondrial oxygen consumption was measured polarographically using a Clark-type electrode (Strathkelvin Instruments, Glasgow, UK) at 25°C. Respiration was measured in a medium containing 225 mM mannitol, 75 mM sucrose, 10 mM Tris, 10 mM K_2HPO_4, 0.1 mM EDTA, and 0.3% BSA, pH 7.4. For the measurement of palmitoyl-CoA oxidation, the following substrate combinations were used: 2 mM carnitine + 2.5 mM malate + 0.3 mM palmitoyl-CoA. To determine rates of octanoyl-CoA oxidation, the same combination of substrates was used; however, octanoyl-CoA (0.3 mM) was substituted for palmitoyl-CoA. Oxidative phosphorylation (state III respiration) was initiated by adding ADP to a final concentration of 0.6 mM.

Palmitate metabolism in isolated EDL muscle. To examine palmitate metabolism in control and transfected muscles, the EDL muscle was carefully dissected into longitudinal strips from tendon to tendon by use of a 27-gauge needle. Strips were removed and placed in a 20-ml glass reaction vial containing 2 ml of warmed (30°C), pregassed (95% O_2-5% CO_2, pH 7.4), modified Krebs-Henseleit buffer containing 4% FA-free BSA (Sigma Chemical, St. Louis, MO), 5 mM glucose, and 0.5 mM palmitate, giving a palmitate-to-BSA molar ratio of 1:1. Following a 30-min preincubation period, muscle strips were transferred to vials containing 0.5 μCi/ml [1-^{14}C]palmitate (GE Healthcare Life Sciences, Buckinghamshire, UK) for 60 min. During this phase, exogenous palmitate oxidation and esterification were monitored by the production of $^{14}CO_2$ and incorporation of [1-^{14}C]palmitate into endogenous lipids.

To determine the radiolabeled palmitate incorporation into intramuscular lipid pools, muscles were placed in 13-ml plastic centrifuge tubes containing 5 ml of ice-cold 2:1 chloroform-methanol (vol/vol) and were homogenized using a polytron. After homogenization, samples were centrifuged at 2,000 *g* (4°C) for 10 min. The supernatant was removed with a glass Pasteur pipette and transferred to a clean centrifuge tube. Distilled water (2 ml) was added, and samples were shaken for 10 min and centrifuged as before to separate the aqueous and lipophilic phases. Five hundred microliters of the aqueous phase were quantified by liquid scintillation counting to determine the amount of ^{14}C-labeled oxidative intermediates resulting from isotopic fixation. This represented a twofold correction factor for exogenous [^{14}C]palmitate oxidation, as previously described (8). The chloroform phase, which contains the total lipids extracted from muscle, was gently evaporated under a stream of N_2 and redissolved in 100 μl of 2:1 chloroform-methanol. Small amounts of dipalmitin and tripalmitin (Sigma Chemical) were added to the 2:1 chloroform-methanol to facilitate the identification of lipid bands on the silica gel plates. Fifty microliters of each sample were spotted onto an oven-dried silica gel plate (Sigma Chemical). Silica gel plates were placed in a sealed tank containing solvent (60:40:3 heptane-isopropyl ether-acetic acid) for 40 min. Plates were then dried, sprayed with dichlorofluorescein dye (0.02% wt/vol in ethanol), and visualized under long-wave ultraviolet light. The individual lipid bands were marked on the plate with a scalpel and scraped into vials for liquid scintillation counting.

Gaseous $^{14}CO_2$ produced from the exogenous oxidation of [1-^{14}C]palmitate during the incubation was measured by transferring 1 ml of the chase incubation medium to a 20-ml glass scintillation vial containing 1 ml of 1 M H_2SO_4 and to a 0.5-ml microcentrifuge tube containing 1 M benzethonium hydroxide. Liberated $^{14}CO_2$ was trapped in the benzethonium hydroxide over 60 min, the microcentrifuge tube containing trapped $^{14}CO_2$ was placed in a scintillation vial, and radioactivity was counted.

Intramuscular triacylglycerol content. Intramuscular triacylglycerol (TAG) content was determined in the TC and EDL muscles by means of an enzymatic colorimetric technique (Triglycerides GPO-PAP; Roche Diagnostics, Indianapolis, IN). Total lipids were extracted from muscle (~30 mg) in 4 ml of chloroform-methanol (2:1) by the method of Folch et al. (9). Phases were separated by addition of 2 ml of 0.6% (wt/vol) NaCl, and the organic phase was evaporated under nitrogen gas. The dried lipid extract was dissolved in ethanol and used for determination of TAG content.

Citrate synthase activity. Muscle was homogenized in 1:100 (wt/vol) dilution of a 175 mM potassium buffer solution, and citrate synthase (CS) activity was assayed spectrophotometrically at 37°C according to the method of Srere et al. (20). Mitochondrial recovery and quality were calculated as follows:

$$\text{recovery} = \frac{CS_{TS} - CS_{EM}}{CS_{MH}} \times 100$$

$$\text{quality} = \frac{CS_{TS} - CS_{EM}}{CS_{TS}} \times 100$$

where CS_{TS} is CS activity in the total mitochondrial suspension after lysing of the mitochondria with 0.04% Triton X-100 and repeated freeze-thaw cycles; CS_{EM} is CS activity in the extramitochondrial suspension in intact mitochondria; and CS_{MH} is CS activity in the muscle homogenate.

Statistics. All data are reported as means ± SE. Differences between control and transfected muscles were analyzed with paired *t*-tests. The sensitivity of CPT I to malonyl-CoA was examined using a two-way ANOVA. Statistical significance was accepted at $P < 0.05$.

RESULTS

CPT I expression in electrotransfected TC muscle. CPT I protein expression in the TC muscle was unchanged 3 days following electroporation (+17%, $P = 0.26$; Fig. 1). Peak CPT I overexpression occurred 7 days after IVE (+104%, $P < 0.001$; Fig. 1). Ten days after electroporation, CPT I protein remained elevated compared with control muscles (+36%, $P = 0.01$; Fig. 1). Subsequent experiments were therefore performed 7 days following IVE.

CPT I activity. Maximal CPT I activity in isolated mitochondria was increased by 31% ($P < 0.001$; Fig. 2*A*). The sensitivity of CPT I to inhibition by malonyl-CoA was not altered in the TC muscles transfected with the *CPT I* DNA (Fig. 2*B*). The mitochondrial yield was 22 ± 1%, whereas the quality was 85 ± 1% and was not different in the control and test TC muscles transfected with the *CPT I* DNA.

Mitochondrial respiratory activity. To further evaluate the functional effects of CPT I overexpression, palmitoyl-CoA oxidation rates were determined in isolated mitochondria. In muscles overexpressing CPT I, the rate of palmitoyl-CoA oxidation was increased by 24% ($P < 0.001$; Fig. 3*A*). The oxidation of the medium-chain fatty acyl-CoA octanoyl-CoA, which enters the mitochondria independently of the CPT I pathway, was unaffected by CPT I overexpression (Fig. 3*B*).

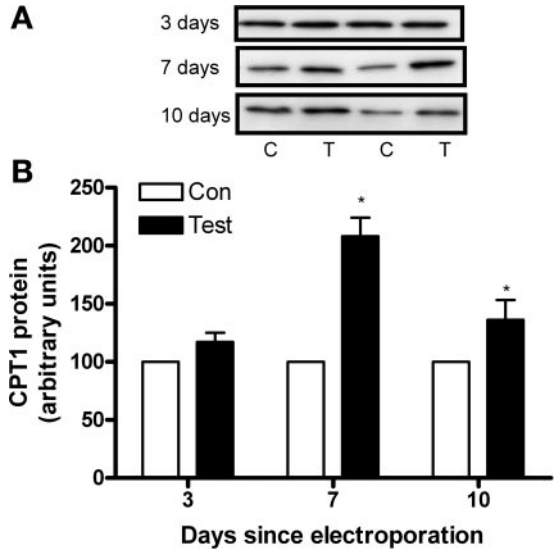

Fig. 1. Time course of carnitine palmitoyltransferase I (CPT I) protein expression in nontransfected (Con, C) and transfected (Test, T) tibialis cranialis (TC) muscles. *A*: representative immunoblots of CPT I protein 3, 7, and 10 days after electroporation. *B*: densitometric quantification of CPT I protein. Values are relative to corresponding control. Data are means ± SE; $n = 4$–12. *Significantly different from control ($P < 0.01$).

Effect of CPT I overexpression on markers of muscle lipid metabolism. No changes were observed in the protein content of UCP3 (12.3 ± 0.6 vs. 11.6 ± 0.8 arbitrary units for control and test leg, respectively) or the activity of CS (26.2 ± 2.1 vs. 23.5 ± 2.0 μmol·min^{-1}·g wet wt^{-1} for control and test leg, respectively). Muscle TAG content was significantly reduced in the TC muscles overexpressing CPT I (−25%, $P < 0.05$; Table 1).

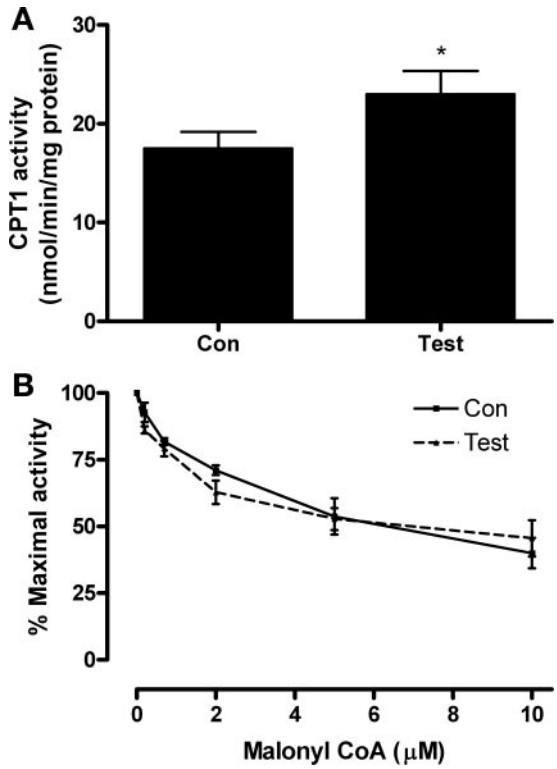

Fig. 2. Effect of CPT I overexpression on CPT I activity 7 days after electroporation. *A*: maximal CPT I activity in isolated mitochondria from TC muscle. *B*: CPT I sensitivity to inhibition by malonyl-CoA. Data are means ± SE; $n = 12$. *Significantly different from control ($P < 0.001$).

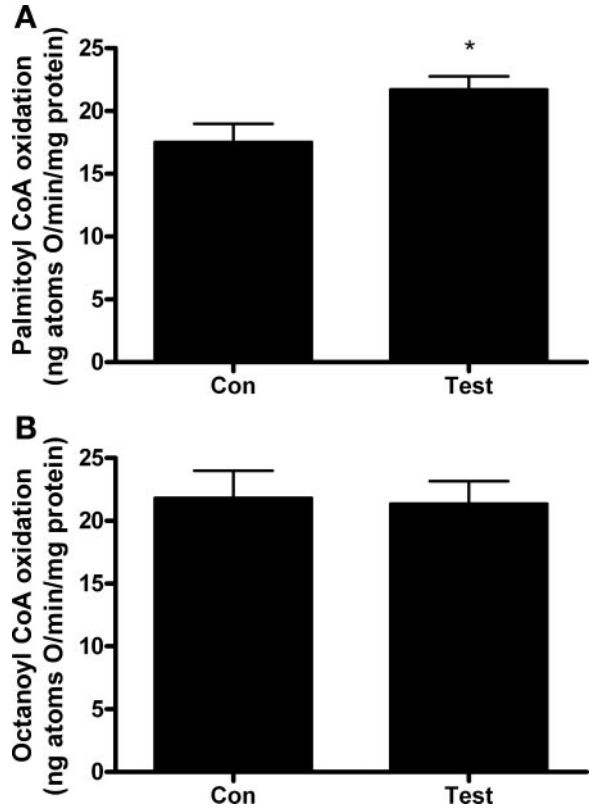

Fig. 3. Effect of CPT I overexpression on mitochondrial respiratory activity 7 days after electroporation. *A*: palmitoyl-CoA oxidation rates. *B*: octanoyl-CoA oxidation rates in isolated mitochondria from TC muscle. Data are means ± SE; $n = 5$–12. *Significantly different from control ($P < 0.001$).

CPT I expression in electrotransfected EDL muscle. Along with overexpression of CPT I in the TC muscle, we also observed significant overexpression of CPT I in the EDL 7 days after IVE (+82%, $P < 0.001$; Fig. 4).

Effects of CPT I overexpression on palmitate metabolism in isolated EDL muscle. CPT I overexpression increased the rate of palmitate oxidation by 28% ($P = 0.001$; Fig. 5*A*). Palmitate incorporation into diacylglycerol was unchanged in muscles that overexpressed CPT I (10.3 ± 0.6 vs. 11.4 ± 0.7 nmol/g for control and test leg, respectively). However, CPT I overexpression reduced the rate of palmitate incorporation into the muscle TAG pool (−17%, $P < 0.01$; Fig. 5*B*). As a result of the increase in palmitate oxidation and reduction in TAG incorporation, the esterification-to-oxidation ratio was reduced with

Table 1. *Effect of CPT I overexpression on muscle TAG content*

	Control	Test
TC TAG	4.9±0.9	3.7±0.4*
EDL TAG	5.3±1.0	2.9±0.2*

Data are means ± SE in μmol/g wet wt; $n = 8$–12. CPT I, carnitine palmitoyltransferase I; TAG, triacylglycerol; TC, tibialis cranialis; EDL, extensor digitorum longus. *$P < 0.05$ for control vs. test.

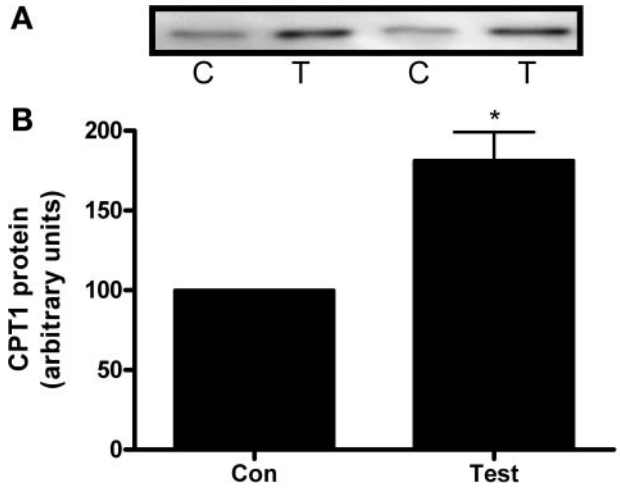

Fig. 4. Effect of electroporation on CPT I protein expression in extensor digitorum longus (EDL) muscle 7 days after electroporation. *A*: representative immunoblots of CPT I protein. *B*: densitometric quantification of CPT I protein 7 days after electroporation. Values are relative to corresponding control. Data are means ± SE; $n = 15$. *Significantly different from control ($P < 0.001$).

CPT I overexpression (−27%, $P < 0.001$; Fig. 5*C*). Total palmitate uptake (esterification + oxidation) was not different between the control and transfected muscles (Fig. 5*D*). Intramuscular TAG content was decreased in the EDL muscles overexpressing CPT I (−45%, $P < 0.05$; Table 1).

DISCUSSION

The regulation of FA oxidation in skeletal muscle is not completely understood. However, CPT I is recognized as an important protein in modulating FA oxidation in muscle. CPT I is the rate-limiting step in the transport of long-chain FAs into the mitochondria and is considered to be the most critical step in controlling FA flux through β-oxidation. Consequently, CPT I would appear to be a viable target to manipulate FA metabolism in muscle. However, to the best of our knowledge, no studies have examined the effect of genetically manipulating CPT I expression on FA metabolism in mature skeletal muscle. Therefore, the purpose of this study was to examine the effect of local CPT I overexpression on skeletal muscle FA metabolism. The results from this study provide direct evidence that overexpressing CPT I protein content alone is adequate to increase CPT I function and FA oxidation not only in isolated mitochondria but also in intact skeletal muscle. Importantly, CPT I overexpression and the subsequent upregulation of FA oxidation lead to a reduction in muscle TAG storage, findings that may have implications for the treatment of insulin resistance, which is strongly associated with the accumulation of muscle lipids.

Characterization of the model. In vivo electrotransfer is emerging a powerful tool to examine the function of specific genes on metabolic pathways in skeletal muscle (4, 5, 10). The advantage of transfecting a single muscle with the gene of interest is that whole body metabolism is not altered and the functional consequences of altered gene expression can be examined in isolated, mature skeletal muscle while the contralateral muscle acts as a control (4). We initially used the IVE technique to examine the feasibility of overexpressing CPT I in skeletal muscle. In agreement with previous results from our laboratory, we found that, in the initial days (3 days) after IVE, the expression of the electroporated DNA was relatively low. Peak protein expression of CPT I occurred 7 days after electroporation, which is consistent with previous reports examining the time course of protein expression in the days following IVE (5, 7, 16). As CPT I is a mitochondrial protein, we next performed functional assessments of CPT I overexpression on aspects of FA metabolism in isolated mitochondria. The twofold increase in CPT I protein expression resulted in an increase in maximal CPT I activity. Importantly, malonyl-CoA sensitivity was retained in the muscles overexpressing CPT I. In association with the increase in CPT I activity, we found a concomitant increase in palmitoyl-CoA oxidation, the substrate for CPT I. The oxidation of octanoyl-CoA, a medium-chain

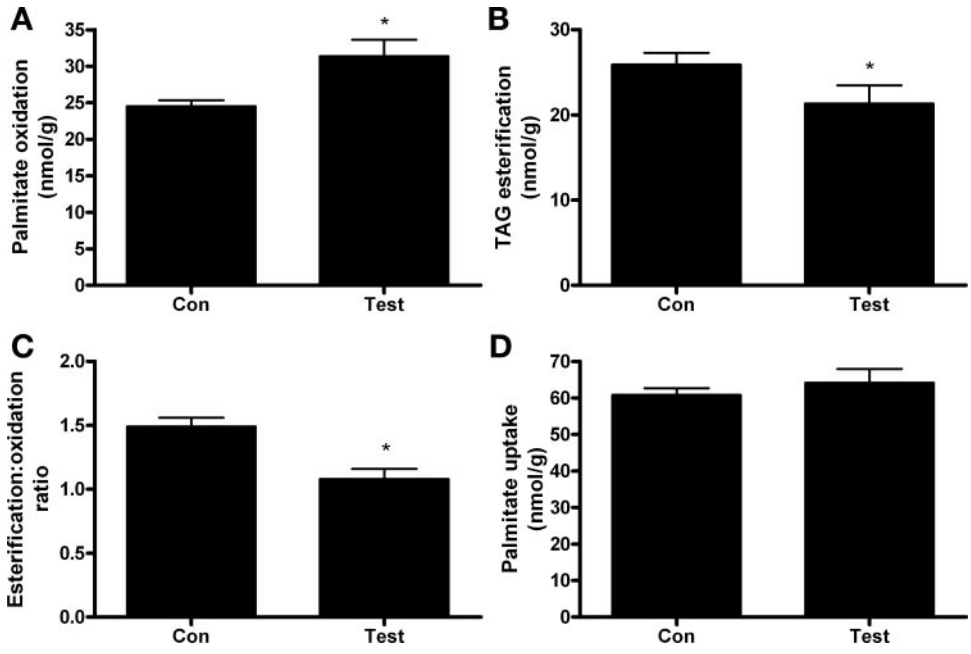

Fig. 5. Effect of CPT I overexpression on palmitate metabolism in isolated EDL muscle strips 7 days after electroporation. *A*: rates of exogenous palmitate oxidation. *B*: palmitate esterification to triacylglycerol (TAG). *C*: esterification/oxidation ratio. *E*: rates of palmitate uptake. Data are means ± SE; $n = 15$. *Significantly different from control ($P < 0.01$).

fatty acyl-CoA, which enters the mitochondria in a CPT I-independent manner, was not affected by CPT I overexpression. Furthermore, other markers of mitochondrial function, such as UCP3 protein content and citrate synthase activity, were not altered. Therefore, CPT I overexpression resulted in a specific increase in the capacity for mitochondrial oxidation of long-chain FAs that can be attributed to an increase in long-chain FA transport across the mitochondrial membrane leading to increased flux through β-oxidation rather than a consequence of an alteration in mitochondrial function.

In addition to examining the feasibility of using IVE to overexpress CPT I in the TC muscle, we also explored the possibility of developing a system to study the effects of CPT I overexpression in an intact muscle preparation. During the IVE procedure, the DNA is injected into the TC muscle and is followed by electroporation of the distal hind limb. Since the EDL lies in close proximity to the TC muscle, we hypothesized that, along with the TC, the EDL might also be transfected with the gene of interest. Indeed, we found that, 7 days after the IVE procedure, CPT I expression was increased approximately twofold in the EDL. The EDL has well-defined tendons so that it can be isolated without damage and used for incubation in vitro. Therefore, on the basis of our observation that CPT I was also overexpressed in the EDL, we examined FA metabolism in isolated EDL muscle strips.

Effect of CPT I overexpression on palmitate metabolism in isolated EDL muscle. A major finding of the present study was that CPT I overexpression increased FA oxidation and reduced esterification of exogenous palmitate into the muscle TAG pool. Thus, the ratio of FA esterification to oxidation was reduced, indicating a repartitioning of FAs away from esterification and toward oxidation. This repartitioning of FAs ultimately caused a reduction in muscle TAG content in both the EDL and TC muscles. To the best of our knowledge, this is the first study to investigate the effect of overexpressing CPT I on FA metabolism in mature skeletal muscle. It has recently been reported that overexpression of CPT I in L6 muscle cells increased FA oxidation without any effect on intracellular lipid accumulation (17). However, in that study, FA oxidation was increased only in cells overexpressing the liver-type isoform of CPT I, the endogenous CPT I isoform expressed in this system, and not in cells where the muscle isoform was overexpressed. It was suggested that the malonyl-CoA levels in the L6 cells were appropriate for the regulation of the liver isoform, which is less sensitive to inhibition by malonyl-CoA compared with the muscle isoform (15). On the other hand, the malonyl-CoA concentrations may have been too high to allow significant flux through the muscle CPT I isoform, thus providing a mechanism as to why no increase in FA oxidation was observed despite a substantial overexpression of muscle CPT I (17).

Therefore, given the relationship between muscle CPT I and malonyl-CoA, our finding that CPT I overexpression increased FA oxidation may be considered somewhat surprising. CPT I is reversibly inhibited by malonyl-CoA, thereby reducing FA oxidation (15, 21). The malonyl-CoA concentration required to inhibit CPT I by 50% (IC_{50}) is 0.03 μM in skeletal muscle (15), which is lower than the malonyl-CoA concentrations reported in muscle. Thus, irrespective of whether or not CPT I is overexpressed, it would be expected that CPT I activity would be continually inhibited. In our system, in which both muscles were exposed to identical in vivo factors and the contralateral muscle served as the control, it would be expected that malonyl-CoA levels would be similar in both EDL muscles obtained from the same animal. Thus, our finding that CPT I overexpression enhanced FA oxidation under basal conditions, where malonyl-CoA concentrations would be expected to be high enough to inhibit CPT I, suggests that factors other than malonyl-CoA are important in regulating FA oxidation in muscle.

One potential explanation for this is that CPT I is regulated independently of malonyl-CoA, as Kim et al. (12) have demonstrated the presence of a malonyl-CoA-resistant pool of CPT I in rodent muscle. Indeed, our findings in relation to inhibition of CPT I by malonyl-CoA support this concept. We found that 10 μM malonyl-CoA inhibited CPT I activity by ~60%, which is in contrast to the findings of Bezaire et al. (2), who reported a similar inhibition of CPT I in mitochondria isolated from human skeletal muscle in the presence of only 2 μM malonyl-CoA. However, in support of our results, a recent report demonstrated a similar inhibition of CPT I (~60%) when mitochondria were incubated in the presence of 10 μM malonyl-CoA (11). In addition, Kim et al. (12) have shown that 10 μM malonyl-CoA was unable to fully inhibit palmitiate oxidation or CPT I activity in mitochondria isolated from a number of different rodent muscles. Thus, the results from the current study and those of others (11, 12) have demonstrated that a significant portion of CPT I activity that is not inhibited by malonyl-CoA. This could be attributed to the action of CPT II, which could contribute to the measured CPT activity if the mitochondria have been ruptured. However, given that our mitochondria were of high quality, we think that this is unlikely. It has also been suggested that malonyl-CoA sensitivity may vary according to muscle fiber composition. Indeed, it has been postulated that the presence of a malonyl-CoA-resistant pool of CPT I may be closely linked to the amount of type IIA muscle fibers (12). Given that the TC muscle is composed of ~50% type IIA fibers (22), this may explain why a significant portion of CPT I activity was not inhibited by malonyl-CoA. Furthermore, it has been suggested that malonyl-CoA may be compartmentalized, such that malonyl-CoA in the direct vicinity of CPT I may be the true regulator of CPT I activity (14). Nonetheless, our in vitro findings demonstrate that an increase in CPT I protein can directly enhance FA oxidation in skeletal muscle.

In addition to stimulating rates of FA oxidation, CPT I overexpression also resulted in a reduction in FA esterification into the TAG pool, subsequently leading to a decrease in muscle TAG content. As accumulation of muscle TAG is strongly associated with the development of insulin resistance (13), it has been proposed that interventions that increase FA oxidation may lead to a reduction in muscle lipids, thereby exerting an insulin-sensitizing effect on skeletal muscle (3, 17). Thus, on the basis of the results of the present study, activation of CPT I could be a potential means to reduce the lipid content of skeletal muscle, potentially resulting in enhanced insulin action.

Taken together, the results obtained from experiments performed in both the isolated mitochondria and intact muscle suggest that simply increasing CPT I protein expression can activate FA oxidation in skeletal muscle. Other approaches have been used to increase CPT I activity in muscle, such as endurance exercise training (3) and high-fat diets (18), but

these interventions cause many other metabolic adaptations favorable to increasing the capacity to oxidize FAs, such as increased mitochondrial content, increased expression of FA transport proteins, and increased activity of enzymes involved in β-oxidation. Here, for the first time, we have clearly shown that FA oxidation can be activated by increasing CPT I independently of any of other confounding effects on muscle FA metabolism. These observations highlight that CPT I is a critical enzyme in regulating skeletal muscle FA oxidation and provide some evidence that factors other than malonyl-CoA (i.e., CPT I protein content) may be important in regulating FA metabolism.

In conclusion, the results from this study demonstrate that in vivo electrotransfer can be used to successfully overexpress CPT I in skeletal muscle. The overexpression of CPT I leads to increased CPT I function in isolated mitochondria. Furthermore, in an intact isolated muscle preparation, CPT I overexpression repartitions FAs away from esterification and toward oxidation, leading to a reduction in muscle TAG content. These findings demonstrate that CPT I is an important protein in regulating FA partitioning in skeletal muscle and may have implications for identifying potential targets for the treatment of muscle insulin resistance. Importantly, we have now developed a novel experimental system to study the importance of CPT I and malonyl-CoA in regulating skeletal muscle metabolism and insulin sensitivity in vivo.

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