Human skeletal muscle carnitine palmitoyltransferase I activity determined in isolated intact mitochondria

PHANÉLIE M. BERTHON,1 RICHARD A. HOWLETT,1 GEORGE J. F. HEIGENHAUSER,2 AND LAWRENCE L. SPRIET

1Department of Human Biology and Nutritional Sciences, University of Guelph, Guelph, Ontario N1G 2W1; and 2Department of Medicine, McMaster University, Hamilton, Ontario, Canada L8N 3S5

Berthon, Phanely M., Richard A. Howlett, George J. F. Heigenhauser, and Lawrence L. Spriet. Human skeletal muscle carnitine palmitoyltransferase I (CPT I) in trained and inactive men (n = 14) and women (n = 12). CPT I activity was measured in intact mitochondria, isolated from needle biopsy vastus lateralis muscle samples (60 mg). The variability of CPT I activity determined on two biopsy samples from the same leg on the same day was 4.4, whereas it was 7.8% on two biopsy samples from the same leg on different days. The method was sensitive to the CPT I inhibitor malonyl-CoA (88% inhibition) and therefore specific for CPT I activity. The mean CPT I activity for all 26 subjects was 141.1 ± 10.6 µmol · min⁻¹ · kg wet muscle (wm)⁻¹ and was not different when all men vs. all women (140.5 ± 15.7 and 142.2 ± 14.5 µmol · min⁻¹ · kg w⁻¹, respectively) were compared. However, CPT I activity was significantly higher in trained vs. inactive subjects for both men (176.2 ± 21.1 vs. 104.1 ± 13.6 µmol · min⁻¹ · kg w⁻¹) and women (167.6 ± 14.1 vs. 91.2 ± 9.5 µmol · min⁻¹ · kg w⁻¹). CPT I activity was also significantly correlated with citrate synthase activity (all subjects, r = 0.76) and maximal oxygen consumption expressed in milliliters per kilogram per minute (all subjects, r = 0.69). The results of this study suggest that CPT I activity can be accurately and reliably measured in intact mitochondria isolated from human muscle biopsy samples. CPT I activity was not affected by gender, and higher activities in aerobically trained subjects appeared to be the result of increased mitochondrial content in both men and women.

Long-chain fatty acid transport; vastus lateralis muscle; aerobic training; malonyl-CoA; citrate synthase activity

An important step in beta-oxidation of long-chain fatty acids (FAs) is believed to be transport across the mitochondrial membranes (3, 12-14, 28). This transport is L-carnitine dependent and is catalyzed by the carnitine palmitoyltransferase (CPT) enzyme system, which consists of CPT I, carnitine-acylcarnitine translocase, and CPT II. CPT I is located in the outer mitochondrial membrane and catalyzes the conversion of acyl-CoA to acylcarnitine, which is then transported across the inner mitochondrial membrane via carnitine-acylcarnitine translocase. The translocase acts mainly as an antiport, allowing the simultaneous export of L-carnitine from the mitochondrial matrix. The acylcarnitine is reconverted into acyl-CoA via CPT II on the matrix side of the inner mitochondrial membrane.

It has been well established, by using in vitro assays and tissues of different species, that the activity of the rate-limiting enzyme CPT I can be reversibly inhibited by malonyl-CoA (13, 14, 18), whereas CPT II is unaffected. This inhibition could be an important in vivo regulatory mechanism because skeletal muscle malonyl-CoA levels may be high enough to limit FA transport into the mitochondria at rest. During exercise, decreased malonyl-CoA contents have been observed in rat skeletal muscle, suggesting that the decrease contributes to increased FA transport and oxidation (29, 30). However, the importance of malonyl-CoA in the regulation of CPT I activity has been recently questioned because no consistent decreases in malonyl-CoA contents were found during aerobic exercise in human skeletal muscle when FA oxidation was high (16, 17).

Most investigations studying the CPT enzyme complex have examined rat tissues, whereas those utilizing human skeletal muscle often involved CPT measurements on homogenized skeletal muscle (1, 4, 21, 33, 34) or freeze-thawed mitochondrial membranes (10, 27). Both methods of preparation do not allow for the specific measurement of CPT I activity because mitochondria are broken, leaving both CPT I and II exposed to substrates and products (20). Distinction between CPT I and II activities was based on the assumption that only CPT I was malonyl-CoA and Triton X-100 sensitive (31), which does not allow for a clear discrimination between enzyme activities. Disruption of the mitochondrial membranes by freeze-thawing and homogenization or freeze-thawing alone may also alter the kinetic properties of CPT I. McGarry et al. (14) appear to be the only investigators who measured the activity of CPT I in isolated and intact mitochondria but report data from the semitendinosus muscle of only one person.

The purpose of this study was to characterize skeletal muscle CPT I activity in inactive and aerobically trained men and women. To achieve this, CPT I activity was determined with a radioisotope assay in intact mitochondria, isolated from 50-70 mg of fresh muscle, obtained with the needle-biopsy technique.

**METHODS**

**Subjects.** Fourteen men and twelve women volunteered to participate in the study. Their mean weight, height, and age were 72.0 ± 2.5 kg, 174 ± 1.5 cm and 24.1 ± 0.9 yr, respectively. Subjects were accepted for the study on the basis of their training status and classified as inactive (essentially...
0 aerobic workouts/wk) or trained (4 or more aerobic workouts/ wk). The trained subjects were involved in many aerobic activities including running, cycling, swimming, rowing, aerobics, and team sports involving large aerobic involvement. The experimental procedure and possible risks were explained to each subject before written consent was obtained. The experiment was approved by the University of Guéph Ethics Committee.

Experimental protocol. Before a muscle biopsy was obtained, subjects performed an incremental maximal O2 uptake (V̇O2max) test on a cycle ergometer. On the day of the experiment, one leg was prepared for muscle biopsy. The skin and underlying fascia covering the vastus lateralis muscle were anesthetized with lidocaine (without epinephrine), and an incision was made with a scalpel. A resting biopsy was obtained as described by Bergström (2). Visible fat and blood were removed from the biopsy sample, one piece of the wet muscle was used for the mitochondrial isolation procedure, and the remainder was frozen in liquid N2 for the whole muscle homogenate preparation. Four volunteers had two biopsies taken from the same leg on the same day, and four others had one biopsy taken from the same leg on two different days.

Isolation of intact mitochondria. The mitochondrial isolation methods were adapted from Mäkinen and Lee (11) as previously described (8), and the entire procedure was performed at 0–4°C. The buffer solutions used were the following (in mM): solution I (100 KCl, 40 Tris-methane (Tris·HCl), 10 Tris base, 5 MgCl2, 1 EDTA, and 1 ATP, pH 7.4); solution II (100 KCl, 40 Tris·HCl, 10 Tris base, 1 MgSO4, 0.1 EDTA, and 0.2 ATP, as well as 1.5% fatty acid-free BSA, pH 7.4); and solution III (same as solution II without BSA).

Muscle samples (~50–70 mg wet muscle [wm]) were rapidly weighed, minced, homogenized by using a hand-driven all-glass homogenizer in 20 vol/wt of solution I, and centrifuged at 700 g for 10 min. Supernatants were centrifuged at 14,000 g for 10 min, and the pellet was resuspended in 10 vol/original wt of solution II and centrifuged at 7,000 g for 10 min. The mitochondrial pellet was resuspended in 10 vol/original wt of solution III and centrifuged at 3,500 g for 10 min. Finally, the pellet was resuspended in 1 vol/wt of a mannitol-sucrose buffer that contained (in mM) 220 sucrose, 70 mannitol, 10 Tris·HCl, and 1 EDTA, pH 7.4, and was used for enzymatic analysis.

CPT I activity. CPT I (EC 2.3.1.21) activity measurements were carried out with the sensitive forward radioisotope assay (13, 14, 18) as modified below. This assay measured the amount of labeled palmitoyl-l-carnitine formed when both palmitoyl-CoA and labeled l-carnitine were added to the medium surrounding the intact mitochondria. The standard incubation mixture contained the following (in mM) in a volume of 90 µl: 117 Tris·HCl (pH 7.4), 0.28 reduced glutathione, 4.4 ATP, 4.4 MgCl2, 16.7 KCl, and 2.2 KCN as well as 40 mg/ml rotenone, 0.5% BSA, 100 µM palmitoyl-CoA, and 400 µM l-carnitine with 1 µCi of l-[3H]carnitine. The reaction was initiated by the addition of 10 µl of mitochondrial suspension (1:3 dilution) and stopped 6 min later with 60 µl of cold 1 M HCl. The reaction was linear for at least 8 min, as also reported by McGarry et al. (14). To extract the palmitoyl-[3H]carnitine formed during the reaction, 200 µl of water-saturated butanol was added. After mixing and brief centrifugation, the butanol layer was retrieved and mixed with 200 µl of water-saturated butanol and 100 µl of distilled water before brief centrifugation. The butanol layer was then washed twice with an equal volume of distilled water. Finally, 100 µl of the butanol layer together with 5 ml of liquid scintillation cocktail were assayed for radioactivity in a liquid scintillation counter. Blanks were measured and subtracted. All assays were performed in duplicate at 30°C. To establish that CPT I activity was specifically measured, the suppression of the activity in the presence of a high malonyl-CoA concentration (2.1 µM) was evaluated because CPT I, but not CPT II, is inhibited by malonyl-CoA (13, 14, 18). The average malonyl-CoA inhibition of CPT I activity was 88%, which is comparable to other studies with isolated mitochondria from varying tissues (9, 14, 19) and confirms the specificity of the method.

Muscle homogenate preparation. Muscle samples (~20–30 mg wm) were mechanically homogenized in 80 vol/wt of a 100 mM phosphate buffer solution containing 0.5 g/l BSA, pH 7.3. The homogenates were frozen in liquid N2 and thawed three times to disrupt the mitochondrial membranes.

Citrate synthase activity. Citrate synthase (CS; EC 4.1.3.7) activity was measured spectrophotometrically as previously described (24). Three measurements of CS activity were made: the first in the whole muscle homogenate samples, the second in the intact mitochondrial suspension, and the third in the mitochondrial suspension after disruption of the mitochondrial membranes by three freeze-thaw cycles. The first and third measurements also included 1% Triton X-100 in the assay medium to ensure mitochondrial membrane disruption.

Expression of CPT I activity. CPT I activities were measured in the mitochondrial suspensions in nanomoles of substrate per minute per milliliter and were normalized to the muscle wet weight in the following manner. The difference between the CS activities in the mitochondrial suspension before and after disruption of the membranes was used to determine the percentage of the extracted mitochondria that were intact (88.1 ± 1.6%). The whole muscle homogenate CS activity was used to normalize the intact mitochondrial CPT I activities to the muscle wet mass (µmol·min−1·kg wt−1). The yield of intact mitochondria averaged 12.6 ± 1.3% of the total mitochondrial volume.

Statistical analysis. All data are reported as means ± SE. The reliability of the measurement was assessed with the coefficient of variation (CV) of CPT I activities measured from two muscle samples obtained from two biopsies on the same day or two biopsies obtained on separate days. The formula used to determine CV was

$$CV = \sqrt{\frac{D - D}{2(n - 1)}} \left(\frac{X_1 + X_2}{2}\right)$$

where n is the number of subjects who had two biopsies (n = 4), D is the difference between duplicate measurements in the same subject, D̄ is the mean D for the four subjects, and X1 and X2 are the mean CPT I activities for the first and second determinations, respectively. Differences between data for men and women were analyzed with unpaired t-tests. The effects of gender and training status on all variables were analyzed with a two-way analysis of variance (gender × training). When a significant F-ratio was obtained, Tukey’s post hoc test was used to locate significant differences. Linear regression analysis was performed by the least squares method. Results were significant at P < 0.05.

RESULTS

The men in this study were heavier, taller, and had a higher absolute V̇O2max than did the women (Table 1).
However, age, relative $\dot{V}O_{2\text{max}}$, and the activities of CS and CPT I were not different between men and women. The average CPT I activity of all 26 subjects (14 men, 12 women) was 141.1 ± 10.6 µmol·min⁻¹·kg⁻¹·wm⁻¹.

The data were then analyzed on the basis of gender and aerobic training status: inactive (0 aerobic workouts/wk) vs. trained (4 or more aerobic workouts/wk). Aerobic workouts included cycling, running, aerobics, rowing, playing soccer or basketball, and so on. In both men and women, the trained individuals had higher absolute and relative $\dot{V}O_{2\text{max}}$ values and higher activities of CS and CPT I than did untrained subjects. In addition, trained men had higher absolute and relative $\dot{V}O_{2\text{max}}$ values than did trained women, whereas enzyme activities were similar between the trained men and women.

The CV for CPT I determinations between two biopsies from the same leg on the same day was 4.4%. When two biopsies were obtained from the same leg on separate days, the CV was 7.0%.

A significant positive correlation was found when the CPT I activity of all subjects was plotted against the activity of CS, a tricarboxylic acid cycle enzyme and mitochondrial marker (Fig. 1). This correlation remained significant when women and men were examined separately (Fig. 2). Significant positive correlations were also observed when CS or CPT I activities were plotted against $\dot{V}O_{2\text{max}}$ expressed in milliliters per kilogram per minute (Fig. 3). The correlation between $\dot{V}O_{2\text{max}}$ and CPT I remained significant when women and men were examined separately (Fig. 4).

**DISCUSSION**

The mitochondrial oxidation of long-chain FAs is an important source of energy in resting and contracting skeletal muscle. For beta-oxidation to proceed, long-chain FAs must be transferred across the mitochondrial membranes, utilizing the CPT system (3, 12, 28). CPT I is localized on the outer surface of the outer mitochondrial membrane and catalyzes the conversion of palmitoyl-CoA and L-carnitine to palmitoyl-L-carnitine and free CoA in the so-called “forward reaction.” CPT II is localized on the inner surface of the inner mitochondrial membrane and catalyzes the reverse or “backward reaction.” CPT I is described as the rate-limiting step of this process (3, 12, 28) and is reversibly inhibited by malonyl-CoA (9, 14, 19). However, it is

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**Table 1. Comparison of physical characteristics and skeletal muscle enzyme activities between men and women**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Men (n = 14)</th>
<th>Women (n = 12)</th>
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</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>24.0 ± 1.0</td>
<td>24.2 ± 1.5</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>79.7 ± 2.8</td>
<td>63.0 ± 2.4*</td>
</tr>
<tr>
<td>Height, cm</td>
<td>178.0 ± 1.6</td>
<td>169.5 ± 1.8*</td>
</tr>
<tr>
<td>$\dot{V}O_{2\text{max}}, l/min$</td>
<td>4.16 ± 0.20</td>
<td>2.93 ± 0.18†</td>
</tr>
<tr>
<td>$\dot{V}O_{2\text{max}}, ml·min⁻¹·kg⁻¹·wm⁻¹$</td>
<td>52.6 ± 3.1</td>
<td>46.9 ± 3.3</td>
</tr>
<tr>
<td>CS, mmol·min⁻¹·kg⁻¹·wm⁻¹</td>
<td>18.5 ± 1.3</td>
<td>18.86 ± 1.6</td>
</tr>
<tr>
<td>CPT I, µmol·min⁻¹·kg⁻¹·wm⁻¹</td>
<td>140.5 ± 15.7</td>
<td>142.15 ± 14.5</td>
</tr>
</tbody>
</table>

Values are means ± SE. n, No. of subjects; $\dot{V}O_{2\text{max}}$, maximal O₂ uptake; wm, wet muscle; CS, citrate synthase activity; CPT I, carnitine palmitoyltransferase I activity. Significantly different from men: *P < 0.01; †P < 0.05.

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Fig. 1. Correlation between vastus lateralis muscle citrate synthase (CS) activity and carnitine palmitoyltransferase I (CPT I) in human subjects ($y = 8.03x - 8.64, r = 0.76, P = 0.0001$), wm, Wet muscle.

Fig. 2. Correlations between vastus lateralis CS and CPT I activity in men (A; $y = 9.37x - 32.66, r = 0.79, P = 0.0008$) and women (B; $y = 6.76x + 14.74, r = 0.73, P = 0.007$).
not clear whether malonyl-CoA is the sole regulator of CPT I activity because conflicting reports regarding exercise-induced changes in malonyl-CoA exist (16, 17, 29, 30).

Most of the investigations studying the human CPT enzyme system in healthy subjects and patients with lipid metabolism disorders have used skeletal muscle homogenates (1, 4, 10, 21, 33, 34) or freeze-thawed mitochondrial membranes (10, 27). In skeletal muscle homogenate and freeze-thawed preparations, where the integrity of the mitochondrial membranes has not been maintained, both CPT I and II are exposed to the substrates and products of the reaction. Both enzymes are capable of catalyzing the forward and reverse reactions, although CPT I has the greatest capacity for the forward reaction and CPT II has the greatest capacity for the backward reaction (15). Disruption of the mitochondrial membranes may also alter the kinetic properties of CPT I because the malonyl-CoA inhibition of CPT I activity is reduced (27). When muscle homogenates and freeze-thawed mitochondrial membranes have been used, some form of total CPT activity is measured, but the information is difficult to interpret. Thus isolated mitochondria with intact membranes are required to accurately measure CPT I activity (14, 32).

This study measured CPT I activity by using intact mitochondria, isolated from small human vastus lateralis muscle biopsy samples. The mitochondrial isolation procedure employed in this study resulted in 88% of the extracted mitochondria (~13% of total mitochondria) being intact, assessed by measuring CS activity in the mitochondrial pellet before and after mitochondrial disruption (see METHODS). CPT I activity measurements were then performed with the sensitive forward isotope assay (13, 14), which measures the amount of labeled palmitoyl-L-carnitine formed when both palmitoyl-CoA and labeled L-carnitine were added to the medium surrounding the intact mitochondria. This permits a specific measurement of CPT I activity because the substrates (palmitoyl-CoA and L-carnitine) were not available for CPT II. Normally, these substrates are moved across the inner mitochondrial membrane via acylcarnitine-carnitine translocase. However, the CPT I assay medium contains respiratory inhibitors that prevent the movement of palmitoyl-CoA into the mitochondria (and carnitine out) and ultimately limit CPT II activity. This is confirmed by the specificity of malonyl-CoA inhibition of CPT I activity. The malonyl-CoA...
CoA inhibition of CPT I averaged 88% in this study, which is in accordance with the inhibition reported in previous studies using mitochondria extracted from a variety of tissues (9, 14, 19) and confirms that this method leads to the specific determination of CPT I activity. The reproducibility of the CPT I determinations by using mitochondria isolated from small human skeletal muscle samples was also good, with low CV values for CPT I activity between duplicate biopsies obtained on the same day and on separate days.

There has been recent interest in the potential for gender differences in skeletal muscle metabolism. A series of studies by Tarnopolsky and colleagues (25, 26) has demonstrated lower whole body respiratory exchange ratios during exercise at ~62–75% of V̇O₂max in women vs. men, when subjects were matched on the basis of V̇O₂max expressed as milliliters per minute per kilogram lean body mass (25, 26; for review, see Ref. 20). On the basis of the respiratory exchange ratio measurements, it appears that women were oxidizing fat at approximately twice the rate of men during exercise. Assuming that the musculature required to perform cycling is proportionally the same in men and women, the working muscles in women should also be oxidizing fat at twice the rate of those in men. Consequently, researchers have attempted to identify the reasons for this higher fat utilization in women. The results of the present study indicate that there is no difference in the maximal activity of CPT I under the in vitro conditions of the assay. This suggests that the difference in the ability to use fat during submaximal exercise is not due to gender differences in total CPT I activity. However, it must be remembered that measurements of maximal activities of rate-limiting enzymes may not predict the in vivo regulation of the enzyme during submaximal aerobic exercise. Further kinetic studies are required to determine whether the enzyme is regulated differently in men and women.

The activity of CPT I in this study was related to the muscle mitochondrial content, both in women and men, as demonstrated by the strong relationships between CPT I and CS activities (Figs. 1 and 2). A comparison of CS and CPT I activities between untrained men and women and trained men and women revealed no significant differences (Table 2), which agrees with previous CS activity measurements (5, 22). However, Costill et al. (4) reported higher total CPT activity in skeletal muscle homogenates of men vs. women. This discrepancy could be because of the fact that Costill et al. (4) measured total CPT activity and not CPT I activity and/or that the trained subjects in the present study were only recreationally trained, but the subjects in the study by Costill et al. (4) were well trained. The lack of gender effect with CS and CPT I activities in the present study is consistent with previous data showing no gender difference in the activity of the beta-oxidation enzyme, beta-hydroxyacyl-CoA dehydrogenase, in inactive (23) and active (6) subjects. Thus, taken together, the mitochondrial enzyme data suggest that men and women have similar muscle mitochondrial oxidative capacities when matched on the basis of relative whole body V̇O₂max (ml·kg⁻¹·min⁻¹ or ml·kg lean mass⁻¹·min⁻¹).

The higher CS and CPT I activities, and their strong relationships with V̇O₂max in trained men and women suggest higher mitochondrial oxidative capacities than in inactive men and women (Table 2, Fig. 3). These data are in accordance with the higher skeletal muscle capacity for FA oxidation reported after endurance training in previous studies, as reviewed by Holloszy and Coyle (7). This parallel increase in CPT I activity with increasing V̇O₂max remained significant when men and women were examined separately (Fig. 4). Moreover, the significant correlation found between CPT I and CS activities (Figs. 1 and 2) and the increase in mitochondrial volume that occurs with training (7) suggest that the increase in CPT I activity was primarily because of an elevated mitochondrial content.

In the present study, CPT I activity, a key regulatory enzyme in long-chain FA metabolism, was measured with a radioisotopic assay by using intact mitochondria isolated from human skeletal muscle biopsy samples. The method was sensitive to the CPT I inhibitor malonyl-CoA and therefore specific to CPT I. It was also reliable because between-biopsy variability was low. The mean CPT I activity in women was not significantly different from that in men and was higher in aerobically trained individuals in both genders.
REFERENCES


