Muscle Carnitine Deficiency in Patients With Severe Peripheral Vascular Disease

Gregorio Brevetti, MD; Corrado Angelini, MD; Maurizio Rosa, MS; Rosalba Carrozzo, MS; Sergio Perna, MD; Marco Corsi, MD; Angelo Matarazzo, MD; and Alberto Marcialis, MD

Background. This study was designed to evaluate the effect of severe peripheral arterial insufficiency on carnitine concentrations and carnitine acetyltransferase and palmitoyltransferase activities in the ischemic skeletal muscles of patients with severe peripheral vascular disease.

Methods and Results. Nine biopsy specimens of ischemic muscles were obtained from five patients undergoing reconstructive vascular surgery. Biopsies from 35 normal subjects served as controls. Ischemic muscles showed a significant reduction in total carnitine from the control value of 20.9±5.2 to 11.6±6.2 nmol/mg noncollagen protein (p<0.01). A significantly lower free carnitine and acylcarnitine content contributed to this reduction. Similarly, carnitine acetyltransferase activity was reduced in the ischemic muscles from the control value of 102.1±41.2 to 52.9±22.1 nmol/min/mg noncollagen protein (p<0.01). On the contrary, carnitine palmitoyltransferase activity did not show any change (0.29±0.05 nmol/min/mg noncollagen protein in the ischemic muscles and 0.28±0.07 nmol/min/mg noncollagen protein in controls). Carnitine, acylcarnitines, and enzyme activities were also measured in the ischemic muscles in four additional patients 2 days after intravenous administration of L-propionylcarnitine (1.5 g as a single bolus followed by an infusion of 1 mg/kg/min for 30 minutes). Treatment restored normal levels of carnitine and its esters in the ischemic muscles but did not affect enzyme activities.

Conclusions. Demonstration of carnitine deficiency in severe peripheral vascular disease substantiates previous findings showing the efficacy of carnitine supplementation to ischemic muscles. Furthermore, the feasibility of restoring carnitine homeostasis with L-propionylcarnitine provides the basis for clinical trials aimed at assessing the efficacy of this carnitine ester in the treatment of peripheral vascular disease. (Circulation 1991;84:1490–1495)

Carnitine is a quaternary amine that plays an important role in regulating substrate flux and energy balance across cell membranes. It is a cofactor for the shuttle mechanism whereby long-chain fatty acids are transformed into acylcarnitine derivatives (acylcarnitines whose acyl moiety consists of 10 or more carbon atoms) and may be transported into mitochondria for energy liberation via β-oxidation.1 Carnitine palmitoyltransferase (CPT) is the mandatory enzyme for this shuttle mechanism.1 In addition, carnitine and the enzyme carnitine acetyltransferase (CAT)1 are involved in the following reaction:

\[
\text{Acetylcoenzyme A + carnitine} \rightarrow \text{acylcarnitine + coenzyme A}
\]

This reaction modulates the intracellular concentration of coenzyme A (CoA) and acetylCoA. Through this reaction, carnitine produces free CoA for other metabolic reactions and reduces the ratio of acetyl-CoA to CoA. This reduction stimulates the activity of pyruvate dehydrogenase and thus enhances the oxidative use of glucose.2–4

As a consequence of these mechanisms of action, an adequate carnitine availability and the efficiency of the carnitine-linked system are important factors for muscle metabolism, especially in providing efficient regulation of the energy flow from the different oxidative sources. Patients with primary carnitine deficiency are characterized by cardiomyopathy and progressive muscle weakness, which can be partially reversed by carnitine supplementation.5–6 One of the possible consequences of ischemia is secondary carnitine deficiency. A decrease in myocardial carnitine content has been observed in experimental animals as a result of acute and chronic coronary ischemia7,8 and in humans after acute myocardial infarction.9 Replacement of carnitine to the ischemic myocard-
medium has been found to improve energy metabolism and mechanical function.\textsuperscript{10,11} Furthermore, carnitine enhances the stress tolerance of the heart in coronary patients.\textsuperscript{12-14} Carnitine supplementation has also been reported to increase muscle carnitine levels and improve walking ability\textsuperscript{15} in patients with peripheral arterial insufficiency of stage II of Fontaine’s classification (i.e., patients with intermittent claudication without pain at rest and/or trophic lesions in the affected leg). Such patients have normal levels of carnitine in the ischemic muscles;\textsuperscript{15} however, they show a negative correlation between plasma short-chain acylcarnitines at rest and subsequent exercise performance.\textsuperscript{16} Because short-chain acylcarnitines (acylcarnitines with acyl moiety of fewer than 10 carbon atoms) are formed from and in equilibrium with short-chain acylCoA esters, this negative relation suggests that the more severe the ischemic disease, the larger the amount of carnitine that is required to remove the excess acylCoA esters produced by ischemia. In the more advanced stages of peripheral vascular disease, this process could lead to a depletion of carnitine in the ischemic muscles. To test this hypothesis, we measured the carnitine and acylcarnitines as well as CAT and CPT activities in the ischemic skeletal muscles of patients with peripheral arterial insufficiency of stage III or IV of Fontaine’s classification (i.e., patients with rest pain and/or trophic lesions in the affected leg).

### Methods

During reconstructive vascular surgery, nine biopsy specimens of ischemic skeletal muscles were obtained from five patients with severe peripheral vascular disease. No patient was diabetic or affected by other metabolic or neuromuscular diseases. All were on a low-fat, low-cholesterol diet. All had been drug free for at least 1 week; the only exceptions were analgesics and nitrates when required for concomitant coronary heart disease. Patient characteristics and muscles that were biopsied are given in Table 1. Muscle specimens were immediately frozen in liquid nitrogen–cooled isopentane and stored at \(-70^\circ C\). Each biopsy was initially divided in half; one half was prepared for morphological and histochemical analyses, and the other half was prepared for measurements of carnitine, acylcarnitines, and enzyme activities. Free carnitine was assayed by the radiochemical method of Cederblad and Linstedt\textsuperscript{17} in the presence of 0.5 mM N-ethylmaleimide. Short- and long-chain acylcarnitines were measured during alkaline hydrolysis, as described by Pearson and Tubbs.\textsuperscript{18} Noncollagen proteins were determined according to the method of Lilienthal et al.\textsuperscript{19} CAT activity was measured by the method of Di Donato et al\textsuperscript{20} and modified as described: 50–100 mg of tissue was homogenized in Chappel-Perry medium and centrifuged at 8,500g for 5 minutes. Next, 10, 20, or 30 \(\mu l\) of suramin was incubated for 10 minutes at 30°C in 200 \(\mu l\) of a mixture containing (mM) phosphate buffer 250 (pH 7.5), \(\ell\)-carnitine 10, N-ethylmaleimide 0.5, (1-C\textsuperscript{14})acetyl-CoA (5 nCi/mM) 1, and eserine 0.12. At the end of incubation, 300 \(\mu l\) of anion exchange resin Dowex 2-X8 200–400 mesh was added, and the mixture was vortexed and chilled in an ice bath for 10 minutes. Then, the mixture was centrifuged, and the final suramin containing labeled acetylcarnitine was counted in a \(\beta\)-scintillation counter. CPT activity was determined by the isotope exchange method described by Solberg\textsuperscript{21} as modified by Trevisan et al.\textsuperscript{22} Citrate synthase was measured according to the method of Srere.\textsuperscript{23} NADH dehydrogenase was assayed as described by Singer.\textsuperscript{24} Creatine phosphokinase (CPK) was determined according to the method of Hess et al.\textsuperscript{25}

In four additional patients, carnitine, acylcarnitines, and enzyme activities were measured in the ischemic muscles 2 days after intravenous administration of \(\ell\)-propionylcarnitine (1.5 g as a single bolus followed by an infusion of 1 mg/kg/min for 30 minutes. The characteristics of these patients (Table 2) were similar to those of the untreated patients.

Biopsy specimens were obtained under local anesthesia from the normoperfused muscles of four healthy male subjects (mean age, 61.4±4.0 years) who received \(\ell\)-propionylcarnitine at the dosage indicated above.

Informed consent was obtained from all patients and control subjects before the study began.

The data of the present study were compared with those obtained from the muscles of 35 normal subjects previously studied in our laboratory. Group

### Table 1. Characteristics and Biopsied Muscles of Untreated Patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (years)</th>
<th>Arterial pressure (mm Hg)</th>
<th>Fontaine disease stage</th>
<th>Disease duration (years)</th>
<th>Concomitant treatment</th>
<th>Biopsied muscles</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>70</td>
<td>140/90</td>
<td>III</td>
<td>9</td>
<td>...</td>
<td>Gastrocnemius, sartorius</td>
</tr>
<tr>
<td>2</td>
<td>60</td>
<td>160/80</td>
<td>III</td>
<td>7</td>
<td>Nitrates</td>
<td>Gastrocnemius, sartorius</td>
</tr>
<tr>
<td>3</td>
<td>61</td>
<td>150/90</td>
<td>IV</td>
<td>3</td>
<td>...</td>
<td>Sartorius, adductor magnus</td>
</tr>
<tr>
<td>4</td>
<td>61</td>
<td>160/100</td>
<td>III</td>
<td>8</td>
<td>...</td>
<td>Gastrocnemius, sartorius</td>
</tr>
<tr>
<td>5</td>
<td>68</td>
<td>170/105</td>
<td>IV</td>
<td>9</td>
<td>Nitrates</td>
<td>Gastrocnemius</td>
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</table>
values are expressed as mean±SD. Statistical analysis was performed by the t test for unpaired data.

**Results**

**Histochromal Findings**

Biopsy specimens from patients with peripheral vascular disease showed typical ischemic alterations. Trichrome stain showed several atrophic, angulated, and vacuolated fibers. Increased subsarcolemmal mitochondrial rims were observed with oxidative enzyme stains, such as cytochrome oxidase or NADH-TR-reductase. Oil-red-O stain showed increased lipid droplets.

**Figure 1.** Bar graphs of total carnitine levels in skeletal muscles of control subjects and patients with peripheral vascular disease (PVD). Open bars indicate values in untreated subjects; hatched bars indicate subjects treated with L-propionylcarnitine. *Significantly lower than untreated controls (p<0.01). †Significantly higher than untreated PVD patients (p<0.05).

**Table 2. Characteristics and Biopsied Muscles of Patients Treated With L-Propionyl Carnitine**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (years)</th>
<th>Arterial pressure (mm Hg)</th>
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<th>Disease duration (years)</th>
<th>Concomitant treatment</th>
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<td>IV</td>
<td>8</td>
<td>...</td>
<td>Gastrocnemius</td>
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</tbody>
</table>

**Table 3. Muscle Levels of Carnitine and Its Fractions**

Muscular levels of total carnitine observed in patients with peripheral vascular disease and in normal subjects are shown in Figure 1. Compared with the normoperfused muscles of control subjects, the ischemic muscles of untreated patients showed a significant reduction in total carnitine, from 20.9±5.2 to 11.6±6.2 nmol/mg noncollagen protein (p<0.01). This reduction can be attributed to a significantly lower content in free carnitine and acylcarnitines (Table 3). On the contrary, the content of carnitine and its fractions in the ischemic muscles of patients treated with L-propionylcarnitine did not differ from those of controls. Furthermore, total and free carnitine levels were significantly higher than those in untreated patients (Table 3).

As shown in Table 3, L-propionylcarnitine did not modify the muscle levels of carnitine and its fractions in the four normal subjects treated before biopsy. This demonstrates that at the dose used in the present study, L-propionylcarnitine is taken up only by carnitine-deficient muscles.

**Enzyme Activities**

The activities of CAT and CPT in the skeletal muscles are shown in Figure 2. A significant reduction in CAT activity from the control value of 102.1±41.2 to 52.9±22.1 nmol/min/mg noncollagen protein (p<0.01) was observed in the ischemic muscles of untreated patients. On the contrary, CPT activity did not show any change (0.29±0.05 nmol/min/mg noncollagen protein in the ischemic muscles and 0.28±0.07 nmol/min/mg noncollagen protein in controls).

**Table 3. Muscle Levels of Carnitine and Its Fractions in Normal Subjects and Patients With Peripheral Vascular Disease**

<table>
<thead>
<tr>
<th></th>
<th>Normal subjects</th>
<th>PVD patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated</td>
<td>Treated</td>
</tr>
<tr>
<td>Total carnitine</td>
<td>20.9±5.2</td>
<td>18.0±5.0</td>
</tr>
<tr>
<td>Free carnitine</td>
<td>16.3±4.8</td>
<td>13.3±2.0</td>
</tr>
<tr>
<td>Short-chain</td>
<td></td>
<td></td>
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<tr>
<td>acylcarnitines</td>
<td>3.4±1.6</td>
<td>4.1±2.0</td>
</tr>
<tr>
<td>Long-chain</td>
<td>0.9±0.6</td>
<td>0.6±0.4</td>
</tr>
</tbody>
</table>

PVD, peripheral vascular disease.

*Significantly lower than untreated normal subjects (p<0.01).
†Significantly lower than untreated normal subjects (p<0.05).
‡Significantly higher than untreated PVD patients (p<0.05). Values are expressed in nmol/mg noncollagen protein.
Treatment with L-propionylcarnitine did not modify the activities of these enzymes in the ischemic muscles. After treatment, CAT activity was still lower than that in controls (44.9±23.8 nmol/min/mg noncollagen protein, p<0.01), and CPT remained within the normal range (0.29±0.06 nmol/min/mg noncollagen protein).

Similar to findings obtained in ischemic muscles, in normal muscles L-propionylcarnitine did not affect CAT and CPT activities, which were 109.2±54.7 and 0.29±0.05 nmol/min/mg noncollagen protein, respectively.

Citrate synthase activity was slightly decreased in patients compared with controls (184.8±43.6 versus 221.3±23.7 nmol/min/mg noncollagen protein, a decrease of 16%). However, after treatment with L-propionylcarnitine, it increased in patients to 362.4±201.4 nmol/min/mg noncollagen protein, demonstrating a 96% increase compared with the untreated group. Furthermore, a linear correlation (r=0.98, p<0.01) between total carnitine levels and citrate synthase activity was observed in patients after treatment. NADH dehydrogenase was 446.1±56.9 nmol/min/mg noncollagen protein in patients and 518.7±82.1 nmol/min/mg noncollagen protein in controls (−14%), and it increased in treated patients to 592.8±368.0 nmol/min/mg noncollagen protein, demonstrating a 32% increase compared with the untreated group. No significant correlation was found after treatment between the activity of NADH dehydrogenase and total carnitine levels. Both citrate synthase and NADH dehydrogenase activities measured in normal subjects after treatment with L-propionylcarnitine were similar to those observed in untreated controls. In patients with peripheral vascular disease, CPK was 26.5±5.3 μmol/min/mg noncollagen protein, a value similar to that observed in controls (22.6±2.6 μmol/min/mg noncollagen protein). Treatment with L-propionylcarnitine did not modify these values.

No side effects were observed during L-propionylcarnitine infusion.

**Discussion**

The importance of carnitine for muscle function was recognized in 1973 when Engel and Angelini described the first case of human myopathy caused by carnitine deficiency. Carnitine deficiency has since been reported in a number of disorders associated with alterations of muscle function, including postdiabetic syndrome, cachexia, and myocardial ischemia. The present study demonstrates that ischemic skeletal muscles of patients with severe peripheral vascular disease exhibit a marked decrease in the content of carnitine and its esters and a significant reduction in the activity of the enzyme CAT. In a previous study, we found normal levels of carnitine and acylcarnitines in the ischemic muscles of patients with peripheral vascular disease of stage II of Fontaine’s classification. Patients included in the present study were in a more advanced stage of the disease (stage III or IV); it is thus reasonable to assume that muscle carnitine is progressively lost as the ischemic process advances. This hypothesis is supported by findings from patients who died from myocardial infarction. In such patients, the necrotic myocardial areas had lower carnitine levels, whereas the border zone tissue showed values intermediate between those of necrotic and healthy surrounding tissue.

When oxidative metabolism is altered, the metabolic flux into the Krebs’ cycle decreases. As a consequence, acylCoAs and acetylCoA esters in particular accumulate within the mitochondria, increasing the acetylCoA-to-CoA ratio and thus inhibiting the oxidative use of glucose. Under these conditions, carnitine serves as a buffer of the metabolically critical acetylCoA pool. Through the action of CAT, which is very active in the skeletal muscle, carnitine may relieve the excess acylCoA esters by forming short-chain acylcarnitines that may be transported out of the mitochondria and the cell. This peculiar detoxifying action requires an adequate pool of carnitine and an efficient CAT system.
In normal subjects, exercise of sufficient intensity to qualitatively alter muscle substrate metabolism produces a redistribution from free to short-chain acylcarnitines, as indicated by the decrease in free carnitine in skeletal muscle and the concomitant increase in short-chain acylcarnitines in both plasma and skeletal muscle.31,32 Similarly, patients with obstructive vascular disease of stage II of Fontaine’s classification show an increase in plasma short-chain acylcarnitines when claudication pain develops in the ischemic leg.16 Such patients also demonstrate a statistically significant negative correlation between short-chain acylcarnitine concentration at rest and subsequent exercise performance,16 suggesting that short-chain acylcarnitines are formed in proportion to the severity of the ischemic disease. These findings demonstrate that in patients with peripheral vascular disease of stage II of Fontaine’s classification, the efficiency of the carnitine-linked system is preserved and able to buffer the excess acylCoA esters produced by ischemia.

In other disease states, however, the continued generation and excretion of acylcarnitines have metabolic consequences. For example, inherited disorders of organic acid metabolism are associated with an export of carnitine from the tissue in the form of acylcarnitines, yet an increased amount of carnitine is required to buffer the acylCoA pool and a secondary carnitine deficiency takes place under the impaired metabolic conditions.33 Accordingly, it is possible that in patients with peripheral vascular disease, the increasing severity of the ischemic process parallels a progressive increase in the production of acylcarnitines, leading to a depletion of carnitine from the ischemic muscle. The present study demonstrates that patients of stage III or IV of Fontaine’s classification show a marked reduction in the muscle content of carnitine. Loss of carnitine through functionally impaired cell membranes may contribute to the extent of this phenomenon.

Our patients also exhibited a decreased activity of CAT. This, in addition to a decreased availability of carnitine, is responsible for the reduced formation of short-chain acylcarnitines and a consequent noxious accumulation of acylCoA esters in the ischemic muscles. The possibility that CAT activity is decreased as a consequence of cell necrosis appears to be ruled out by the finding that the activities of the other enzymes measured in the ischemic muscles were within the normal range.

Administration of L-carnitine to patients with peripheral vascular disease of stage II of Fontaine’s classification significantly increases muscle total carnitine levels and improves maximal walking ability.15 In the present study, we used L-propionylcarnitine, a naturally occurring derivative of carnitine, because it has been postulated that the propionyl structure of this compound accelerates carnitine entry into myocytes.34 Our results demonstrate that L-propionylcarnitine administration to patients with severe peripheral vascular disease restores normal levels of carnitine and its esters in the ischemic muscles. Treatment significantly increased total and free carnitine compared with values observed in the untreated group. Short- and long-chain acylcarnitines also increased twofold, although this increase was not significant. These findings indicate that part of the administered L-propionylcarnitine (a short-chain acyl derivative) was taken up by the ischemic muscles and that a consistent portion was transformed into free carnitine. In effect, L-propionylcarnitine exhibits a very high affinity for CAT35 and thus is readily converted into propionylCoA and free carnitine. PropionylCoA does not affect β-oxidation, but it may be converted into succinylCoA36 and thus may contribute to the beneficial action of carnitine by increasing the flux in the Kreb’s cycle with an anaplerotic mechanism. Furthermore, the linear correlation observed between total carnitine levels and citrate synthase activity suggests that L-propionylcarnitine increases the functional activity of this matrix enzyme, probably through acylCoA removal.37

It is interesting that administration of L-propionylcarnitine to normal subjects did not modify the muscle content of carnitine and its esters. This indicates that at the dosage used in the present study, L-propionylcarnitine is taken up only by muscles showing a condition of carnitine insufficiency.

Conclusions

Carnitine is a crucial factor for energy production in the ischemic skeletal muscle.15 Therefore, a decrease in both carnitine levels and the activity of CAT may play an important role in the pathophysiology of peripheral vascular disease. The feasibility of carnitine replacement in the ischemic muscles by L-propionylcarnitine provides the basis for clinical trials aimed at assessing the efficacy of this carnitine ester in the treatment of peripheral vascular disease.

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**KEY WORDS**: carnitine acyltransferase • L-propionylcarnitine • muscle, skeletal • ischemia
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