The role of ascorbic acid and exercise in chronic ischemia of skeletal muscle in rats

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1Laboratory of Physiology and 2Laboratory of Histology-Embryology, Faculty of Medicine; 3Laboratory of Physiology, Department of Physical Education and Sports Science; and 4Laboratory of Analytical Chemistry, Chemistry Department, Aristotle University of Thessaloniki, Thessaloniki, Greece

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Loizidis T, Sioga A, Economou L, Frosinis A, Kyparos A, Zotou A, Albani M. The role of ascorbic acid and exercise in chronic ischemia of skeletal muscle in rats. J Appl Physiol 102: 321–330, 2007. First published August 31, 2006; doi:10.1152/japphysiol.00251.2005.—This study was designed to investigate the effects of peripheral arterial insufficiency, exercise, and vitamin C administration on muscle performance, cross-sectional area, and ultrastructural morphology in extensor digitorum longus (EDL) and soleus (Sol) muscles in rats. Adult Wistar rats were assigned to ischemia alone (isch), ischemia-exercised (exe), ischemia-vitamin C (vit C), and ischemia-exercise-vitamin C (vit C + exe) groups. Ischemia was achieved via unilateral ligation of the right common iliac artery. Contractual muscles within the same animal served as controls. Exercise protocol consisted of 50-min intermittent level running performed every other day for 5 days. Vitamin C (100 mg/kg body wt) was administered intraperitoneally on a daily basis throughout the 14 days of the experiment. With regard to the EDL muscle, ischemia alone reduced muscle strength, which was not recovered after vitamin C administration. Exercise alone following ischemia induced the most severe structural damage and cross-sectional area decrease in the muscle, yet the reduction in tetanic tension was not significant. Exercise in conjunction with vitamin C administration preserved ischemia-induced EDL muscle tetanic tension. In the Sol muscle, a significant reduction in single twitch tension after vitamin C administration was found, whereas the tetanic force of the ischemic Sol was not significantly decreased compared with the contractual muscles in any group. Ischemic Sol muscle cross-sectional area was reduced in all but the exe groups. In Sol, muscle strength was reduced in the vit C group, and mean cross-sectional area of ischemic Sol muscles was reduced in all groups except the exe group. These results illustrate that mild exercise, combined with a low dose of vitamin C supplementation, may have beneficial effects on ischemic EDL muscle with a smaller effect on the Sol muscle.

vitamin C; soleus; extensor digitorum longus; intermittent claudication

Peripheral arterial disease (PAD) constitutes the major cause of morbidity and mortality in older populations (40). The most common symptom of this disease is intermittent claudication, which is expressed as ischemic pain occurring in skeletal muscle during walking (16, 42). PAD-associated intermittent claudication is related to chronic changes in morphology and function of the affected muscle, such as denervation as well as a reduction in the cross-sectional area of type II fibers, compared with healthy, age-matched controls (41).

The hypothesis that the claudication-associated mild transient ischemia is capable of inducing systemic vascular injury is also supported by evidence from animal models (31). In rats subjected to claudication, systemic vascular injury (endothelial cell swelling) was detected using electron microscopy after stimulating ischemic hindlimb muscles (27).

Previous studies have shown that after unilateral ligation of iliac artery in rats, the chronic ischemic muscles exhibited increased white blood cell adhesion in postcapillary vessels (13), while capillary endothelial cell swelling was present after 7 days (43). This pathogenesis could be related to functional hyperemia in chronically ischemic muscles, when natural movements are increasing their activity (1).

Although the effect of exercise on muscle function is well established, it is also known that exhaustive exercise is related to muscle fiber degeneration and regeneration, such as has been described in marathon runners (28) and in elite sprinters (18). However, even limited exercise can be damaging to muscles with limited blood supply. Intermittent claudication patients engaged in prolonged walks encountered muscle fiber pathological alterations more frequently than their healthy counterparts (44).

Data from animal studies suggest that exercise does not increase total blood flow in the chronically ischemic muscle; instead, there is a redistribution of blood flow at the microvascular level within the muscles (46). The application of a mild regimen of electrical stimulation to chronic ischemic muscles, which combines short periods of stimulation with adequate recovery intervals, and mimics severe, yet not exhaustive, exercise, has been shown to restore arteriolar dilatation, improve blood flow during muscle activity, increase perfusion pressure, and enhance capillary supply to the muscles (33).

Free radicals have been implicated in the pathophysiology of intermittent claudication. More specifically, patients (suffering from) with intermittent claudication show a decrease in antioxidant levels and a rise in microalbuminuria after exercise, the latter being an indication of an endothelial dysfunction (36). It has been reported that supplementation with an antioxidant (vitamin C) for 2 wk appears to offer some benefits to the recovery process following unaccustomed demanding exercise (47). Administration of extra antioxidants to claudicants reduced the oxidative stress in these patients (48).

In various animal models, it has been also demonstrated that free radical production is a key element during reperfusion phase and that vitamin C has an antioxidant effect. Furthermore, systemic exercise has been proposed as a potential therapeutic approach to chronic ischemic muscles, although it causes histological damage to normal and ischemic muscles.

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To our knowledge, little emphasis has been placed on assessing the combined effect of exercise and antioxidant treatment on chronic ischemic muscles. In the present study, we investigate the effect of mild exercise on ischemia-induced functional impairment and tissue damage in an animal model of chronic ischemia of skeletal muscles. Moreover, the possibility that the minimal dose of an antioxidant, such as vitamin C, would limit injury in mild exercised chronic ischemic skeletal muscles is examined.

**MATERIALS AND METHODS**

Experiments were performed on 30 Wistar rats of both sexes (body wt 179.38 ± 26.45 g) and were approved by the local ethical committee in accordance with the EEC Council Directive 86/609. Animals were housed in a temperature- and light-controlled environment. Standard rat chow and tap water were provided ad libitum. Rats were divided into four groups as follows: 1) ischemia (isch) (n = 5); 2) ischemia-exercised (exe) (n = 6); 3) ischemia-vitamin C (vit C) (n = 8); and 4) ischemia-exercised-vitamin C (vit C + exe) (n = 11). Vitamin C (100 mg/kg body wt ip) was administered intraperitoneally on a daily basis throughout the 14 days of the experiment. Contralateral muscles in all groups served as controls.

**Vitamin C Determination-Chromatographic Conditions**

Vitamin C (ascorbic acid, AA) was determined by a novel method using high-performance liquid chromatography (HPLC). The analysis was performed under isocratic conditions at ambient temperatures with a mobile phase consisting of a mixture of 0.05 M acetate buffer (pH 3.8) and methanol (95:5 vol/vol) and at a flow rate of 0.8 ml/min. The detection was performed at 265 nm with a sensitivity setting of 0.002 AUFS.

**Plasma samples.** To 100-μl aliquots of plasma from rats to which no AA had been administered, a 250-μl volume of AA standard solutions in buffer containing 1% wt/vol metaphosphoric acid (MPA) as stabilizer and a 50-μl volume of buffer were added. Denaturation of plasma proteins was achieved by addition of 100 μl of 10% vol/vol perchloric acid (PCA) containing 1% wt/vol MPA followed by the addition of a 250 μl of AA standard solutions in buffer, containing 1% wt/vol MPA (as stabilizer) and 50 μl of buffer. The mixtures were then vortex-mixed, allowed to stand in the refrigerator for 20 min to ensure complete deproteinization, and centrifuged at 3,500 rpm for 15 min. Blank plasma was also prepared. The standard solutions used for spiking were 1.07, 2.14, 6.42, 10.70, 16.04, and 21.40 ng/ml in AA. The supernatants were filtered through 0.2-μm syringe filters and injected into the HPLC system. During analysis the samples were kept on ice to deter oxidation of AA (39). Five replicate injections were made for each sample, and the peak areas were plotted vs. AA concentration.

The study of AA metabolism in plasma of ischemic rats was performed as follows: intraperitoneal injections of 200-μl volumes of a 5 g/l, freshly prepared AA solution, were administered simultaneously to five rats (200 g body wt). At 10 min, 30 min, 1 h, 4 h, and 24 h after AA administration, blood was drawn from the animals and centrifuged, and plasma samples were stored at −20°C. The analysis was carried out the next day, immediately after the deproteinization step, according to the procedure described above.

**Exercise Protocol-Surgical Procedure**

Animals were anesthetized by intraperitoneal injections of chloral hydrate (4.5%, 1 ml/100 g body wt), and ligation of the right common iliac artery was performed via an abdominal incision. The peritoneal cavity was opened by a midline incision, and the intestines were wrapped in gauze soaked in warm saline and shifted sideways. A silk ligature was then passed under the right common iliac artery ~1 mm below the bifurcation of the aorta and firmly tied. All animals were able to walk within 24 h of the operation.

The exercise protocol consisted of 50-min intermittent running on a motor-driven treadmill. The rats became accustomed to the procedure by an initial 2-min running period. Afterward, the exercised groups performed 10 × 5 min running bouts at a speed of 16 m/min, separated by 2-min rest periods. The animals were exercised every other day for a total of 5 times within a period of 2 wk (days 2 and 12 were defined as the first and the last exercise trial, respectively).

**Tension Recordings**

Muscle tension recording was performed 14 days after ligation under chloral hydrate anesthesia (4.5%, 1 ml/100 g body wt ip) as previously described (23). Briefly, longitudinal incisions were made over the distal tendons of both extensor digitorum longus (EDL) and soleus (Sol) muscles, and tendons were detached distally. The sciatic nerve was carefully dissected from its bed through a posterior thigh incision ensuring that the nerve was left intact. Both lower limbs were immobilized on the operating board, and the distal ends of the muscles were attached to an isometric force transducer via a 3-0 silk suture. First EDL and then Sol muscles were recorded. Each time the muscle tendon was attached to a strain-gauge transducer (Dynamometer UFI, Devices) by a short silk suture, and bipolar silver electrodes were placed under the sciatic nerve, which was held in relaxed position.

Isometric contractions were evoked by stimulating the sciatic nerve (Digitimer D59A stimulator), using supramaximal (3-8 V) square pulses of duration of 0.5 ms. The signal from the transducer was amplified by a DC transducer amplifier (Neurolog NL 107), displayed on an oscilloscope screen (Fluke PM 3380A), stored in a computer, and calculated with data-acquisition software (Fluke View combscope software). The muscle was adjusted to optimal length (L_o) as previously described (23). All devices during the tension-recording procedure were controlled by a pulse programmer (Digitimer D4030).

Contractile properties measured included maximal tension (g) of single isometric twitch tension (Pt), tetanic isometric tension at frequencies of 40, 80, and 100 Hz, and time course of single twitch contraction, namely time to peak tension (TTP) (ms) and half-relaxation time (½ RT) (ms). Tetanic isometric tension was recorded at different frequencies to evaluate the ability of the muscles to contract in various energy demands. During the recording procedure, the rat was kept warm with a heating pad. The muscles and sciatic nerves were periodically moistened with nonoxygenated Krebs solution contained no glucose (30–35°C) (38). At the end of the recordings, calibration of the setting was performed, and the measurements were converted into grams. Finally, data were normalized, and the generated force was expressed in grams per gram muscle weight.

**Immunohistochemistry**

Frozen sections obtained from the middle third of EDL and Sol muscles were used for immunohistochemistry. Antigen unmasking was performed by adding citric buffer and heating in a microwave oven. Sections were incubated at 4°C overnight with primary antibody (1:100, polyclonal anti-Actin, Biogenex). After incubation with primary antibody, the avidin-streptavidin-peroxidase complex method was used for detection of immunopositive muscle fibers (Universal Streptavidin/Biotin Detection System, Immunon Shandon). Immunoreactivity was visualized using the Sigmafast diaminobenzidine tablet set (Sigma). In negative controls, incubation with the primary antibody was omitted from the procedure. In each muscle, the number and mean cross-sectional area of muscle fibers was counted in 20 muscle bundles (300–400 muscle fibers). Quantitative analysis was accomplished using a color interactive quantitative image analysis system (ImagePro Plus, Media Cybernetics). Serial adjacent sections were stained with hematoxylin-eosin.
Electron Microscopy

Sol and EDL muscle samples for ultrastructural analysis were obtained from random locations of the middle third of the muscles of both legs. Samples were cut in small pieces (3–5 mm), fixed with 3% glutaraldehyde in sodium phosphate buffer at pH 7.3 (temperature 4°C) for 2 h, postfixed with 2% OsO4 for 1.5 h, and stained with 1% aqueous uranyl acetate for 14–18 h. The tissue samples were dehydrated in a series of aqueous alcohol solutions and finally 100% alcohol and then embedded in EPON 812 (Serve, Heidelberg, Germany). Semithin sections (2–3 μm) were stained with aqueous solution of 1% toluidine blue and 1% borax. Ultrathin sections cut in a Reichert ultramicrotome were stained with lead citrate (Reynolds) and were examined in a Jeol TEM 2000 FxII at 80 keV.

Statistics

Results were expressed in the text as means ± SD. Group means were compared by Student’s paired or unpaired t-test where appropriate and by single-factor ANOVA. P < 0.05 was accepted as significant.

RESULTS

No significant differences in rat body weight in any experimental group before and after the ligation of common iliac artery was found at the time of recording (Table 1).

The preserved rat plasma samples were analyzed directly after deproteinization and filtration through 0.2-μm syringe filters. A small peak at the retention time of AA in blank plasma was due to the presence of AA traces from food intake, which were subtracted from the treated rat plasma using the methodology of standard additions. The determination of AA in spiked rat plasma samples gave precise and accurate readings. The relative standard deviations (RSD) varied from 1.1 to 6.3% and the recoveries from 85.1 to 104.1% of the true value. The data on the AA metabolism in the plasma presented on Table 2 show a maximum plasma level at 30 min after administration and provide information for establishing the necessary amount of AA after 24 h.

Tension Recordings

EDL muscle. The EDL muscle tension recording data are presented in Table 3. Ischemic EDL muscle weight in all groups but the exe group was decreased. However, this reduction was significant only in the vit C group. In all groups, ischemic EDL muscle single twitch tension was lower compared with the contralateral muscle. However, significant differences (P < 0.05) in twitch tension between ischemic (58.06 ± 24.29 g) and contralateral (84.42 ± 25.50 g) EDL muscle were found only in the vit C group.

Ischemic muscles exhibited almost equal time of contraction (TTP) compared with the contralateral muscles of the isch and exe groups. In contrast, a slight prolongation of TTP was observed in the ischemic muscles of the vit C and vit C + exe groups, although statistical analysis did not reveal any significant difference compared with the contralateral muscles.

The 1/2 RT of ischemic EDL was significantly longer compared with the contralateral in the vit C + exe group. Tetanic tensions (40, 80, and 100 Hz) recorded by the ischemic muscles were significantly decreased compared with the contralaterals in isch and vit C groups (P < 0.05). The force of the ischemic muscle was also decreased in exe and vit C + exe groups, although this was not statistically significant (Figs. 1–3). No statistical difference was observed among the contralateral muscles of all the examined groups with regard to tension recordings. In contrast, among the ischemic muscles there was a significant difference in single twitch tension (P = 0.015). Post hoc Bonferroni analysis revealed a statistically significant difference between the vit C group and the exe group (P = 0.013) (Table 3).

Expression of the tensions as grams per gram muscle weight revealed that single twitch tension (ischemic 605.5 ± 262.5 vs. contralateral 766.1 ± 283.1) and tetanic tension at 100 Hz (ischemic 1,296.5 ± 761.3 vs. contralateral 1,576.7 ± 579.1) recorded by the ischemic muscle remained significantly decreased compared with the contralateral muscle in the vit C group (P < 0.05) (Table 4).

Sol muscle. The Sol muscle tension recording data are presented in Table 5. Single tension recordings expressed either as grams of force or as grams per gram muscle weight showed that single twitch tension of ischemic muscles in the vit C group was significantly decreased compared with the contralateral muscles (P < 0.05), whereas 1/2 RT was significantly prolonged in the vit C + exe group (P < 0.05). All ischemic muscles showed decreased tension peak values (single and tetanic) and prolonged TTP and 1/2 RT compared with the contralateral muscles. However, statistical analysis did not reveal any significant difference. One-way ANOVA performed among the contralateral as well as the ischemic muscles of all groups did not result in any significant difference.

Immunohistochemistry

EDL muscle. Mean muscle fiber area of the ischemic EDL muscle was significantly decreased in vit C (P < 0.05) and in exe (P < 0.001) groups. The mean muscle fiber area reduction

<table>
<thead>
<tr>
<th>Groups</th>
<th>Weight on Ligation, g</th>
<th>Weight on Recording, g</th>
<th>Weight Difference, g</th>
</tr>
</thead>
<tbody>
<tr>
<td>isch</td>
<td>186.48 ± 32.09</td>
<td>209.48 ± 37.76</td>
<td>17.60 ± 43.4</td>
</tr>
<tr>
<td>vit C</td>
<td>179.75 ± 25.84</td>
<td>190 ± 19</td>
<td>10.25 ± 14.2</td>
</tr>
<tr>
<td>exe</td>
<td>167.67 ± 7.51</td>
<td>191.17 ± 9.83</td>
<td>23.50 ± 5.6</td>
</tr>
<tr>
<td>vit C + exe</td>
<td>178.43 ± 28.69</td>
<td>192.64 ± 22.83</td>
<td>14.20 ± 11.1</td>
</tr>
</tbody>
</table>

Values are means ± SD. Groups: isch, ischemia; vit C, ischemia-vitamin C; exe, exercise-ischemia; vit C + exe, ischemia-exercise-vitamin C.

Table 2. Within-day and between-day precision and accuracy of the assay in standard solutions in the presence of internal standard

<table>
<thead>
<tr>
<th>Precision and Accuracy</th>
<th>Injected Quantity, ng</th>
<th>Mean Measured Quantity ± SD, ng</th>
<th>RSD, %</th>
<th>Recovery, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Within day</td>
<td>32.1</td>
<td>32.2 ± 0.72</td>
<td>2.2</td>
<td>100.3</td>
</tr>
<tr>
<td></td>
<td>53.5</td>
<td>51.9 ± 0.81</td>
<td>1.6</td>
<td>97.0</td>
</tr>
<tr>
<td></td>
<td>74.9</td>
<td>74.1 ± 1.15</td>
<td>1.6</td>
<td>98.9</td>
</tr>
<tr>
<td>Between day</td>
<td>32.1</td>
<td>32.7 ± 0.48</td>
<td>1.5</td>
<td>101.9</td>
</tr>
<tr>
<td></td>
<td>53.5</td>
<td>53.7 ± 1.73</td>
<td>3.2</td>
<td>100.4</td>
</tr>
<tr>
<td></td>
<td>74.9</td>
<td>73.0 ± 3.22</td>
<td>4.4</td>
<td>97.5</td>
</tr>
</tbody>
</table>

*Mean values obtained from the linear regression equations (of n = 8 determinations within a day; n = 3 determinations/day over a period of 6 days). RSD, relative SD.
was not significant in the ischemia group, whereas the ischemic and contralateral muscle fiber areas were almost equal in the vit C + exe group (Fig. 4, Table 3).

Sol muscle. Mean muscle fiber area of the ischemic Sol muscle showed a significant decrease compared with the contralateral muscle in all groups but the exe group (Fig. 5, Table 5).

Electron Microscopy

EDL muscle. In the isch group, a small dilation of the sarcoplasmic reticulum in the ischemic EDL muscle fibers was observed. This was present in all ischemic muscles in all groups examined. A reduction in the number of mitochondria with few crests was also observed. Additionally, a decrease in muscle fiber diameter and an increase in the intrafiber connective tissue in all ischemic EDL muscles were also noticed. An extended dilation of the sarcoplasmic reticulum in the exe group was observed. In addition, a reduction in mitochondria number as well as glycogen granules was noted. A disorganization of sarcomeres as indicated by the disappearance of Z-bands and A and I regions and Z-banding disappeared, and glycogen granules were depleted. The effect of exercise on the unoperated muscle was evident considering that there was a small and localized reduction in glycogen granules, less frequent changes in the orientation of the microfilaments and local disorganization, and overall less extensive damage (Figs. 9 and 10).

The morphological appearance of the ischemic Sol muscle was improved in the vit C group compared with this in the isch group. More normal fibers were clearly seen and fewer sarcomeres with A and I regions and Z-band disturbances were observed. Reduction in glycogen granules and disorganization of microfilaments were locally seen (Fig. 10). In the exe + vit C group, the disorganization of microfilaments was evident, and the presence of large vacuoles between microfilaments, as well as around nuclei, was observed (Figs. 9 and 10).

Table 3. All variables of EDL muscle

<table>
<thead>
<tr>
<th></th>
<th>Isch</th>
<th>Contra</th>
<th>Ischem</th>
<th>Vit C</th>
<th>Exe</th>
<th>Exe</th>
<th>Vit C + Exe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muscle weight, g</td>
<td>0.126±0.003</td>
<td>0.119±0.002</td>
<td>0.113±0.002</td>
<td>0.098±0.001*</td>
<td>0.106±0.009</td>
<td>0.106±0.003</td>
<td>0.108±0.015</td>
</tr>
<tr>
<td>Single tension, g</td>
<td>91.36±31.15</td>
<td>82.59±29.57</td>
<td>84.42±25.5</td>
<td>58.06±24.29**</td>
<td>128.64±31.77</td>
<td>113.6±22.01</td>
<td>93.02±13.93</td>
</tr>
<tr>
<td>TTP, ms</td>
<td>33.67±7.63</td>
<td>34.67±9.0</td>
<td>36.28±8.97</td>
<td>38.75±3.54</td>
<td>41.33±3.06</td>
<td>41.33±4.16</td>
<td>36.55±8.58</td>
</tr>
<tr>
<td>1/2 RT, ms</td>
<td>25.0±7.64</td>
<td>22.17±11.32</td>
<td>22.5±3.51</td>
<td>25.13±11.7</td>
<td>21.33±2.31</td>
<td>29.67±3.79</td>
<td>22.91±5.49</td>
</tr>
<tr>
<td>40-Hz tension, g</td>
<td>185.66±46.75</td>
<td>142.37±50.99*</td>
<td>155.87±44.0</td>
<td>123.96±59.54*</td>
<td>224.96±79.11</td>
<td>194.32±59.19</td>
<td>167.05±30.25</td>
</tr>
<tr>
<td>80-Hz tension, g</td>
<td>220.89±26.45</td>
<td>159.18±57.36*</td>
<td>171.97±48.63</td>
<td>124.11±66.90*</td>
<td>232.66±80.02</td>
<td>197.29±52.38</td>
<td>183.97±28.19</td>
</tr>
<tr>
<td>100-Hz tension, g</td>
<td>238.86±24.64</td>
<td>157.08±49.73*</td>
<td>174.50±55.63</td>
<td>123.12±68.92*</td>
<td>224.07±91.88</td>
<td>197.93±60.33</td>
<td>190.17±29.74</td>
</tr>
<tr>
<td>Muscle fiber area, μm²</td>
<td>944.07±606.81</td>
<td>921.91±422.91</td>
<td>1,883.5±1,042.6</td>
<td>1,729.2±1,041.8*</td>
<td>2,552.7±951.91</td>
<td>2,161.6±866.2*</td>
<td>1,608.0±989.53</td>
</tr>
</tbody>
</table>

Values are means ± SD. EDL, extensor digitorum longus; Contra, contralateral leg; Ischem, ischemic leg; TTP, time to peak tension; ½ RT, half-relaxation time. *P < 0.05, Ischemic vs. contralateral leg; †P < 0.05, vit C vs. exe group.

Fig. 1. Mean values ± SD of extensor digitorum longus (EDL) tetanic twitch tension at 40 Hz in all groups. *P < 0.05, ischemic vs. contralateral muscle.

Fig. 2. Mean values ± SD of EDL tetanic twitch tension at 80 Hz in all groups. *P < 0.05, ischemic vs. contralateral muscle.
In the Sol muscle of the nonoperated limb, most of the fibers were normal. In some fibers of the exe group, a reduction of glycogen granules, small dilatation of sarcoplasmic reticulum, local disorganization of the microfilaments, and few small vacuoles under sarcolemma were observed. In the vit C and vit C + exe groups, the morphological alterations were less pronounced and less frequently encountered.

DISCUSSION

In the present study the effect of exercise and vitamin C administration on skeletal muscles in a rat model of chronic ischemia was examined. The ligation of the iliac artery, the exercise protocol, and/or vitamin C administration had no effect on the food and water intake of the rats or their body weight gain. The complete unilateral ligation of iliac artery did not visibly restrict rats from their habitual activities, other than avoiding standing on the operated leg during the first 24 h following the operation.

Ligation of iliac artery is a well-established experimental model of chronic ischemia resembling intermittent claudication more than critical limb ischemia (19) and resulting in functional and structural disturbances in skeletal muscles either under sedentary or demanding exercise conditions (1, 9, 10, 30). In our study, ligation affected functional performance of the EDL muscle by decreasing tetanic tensions at all frequencies examined. It is known that ligation of iliac artery decreases resting blood flow to ~70% and working blood flow to as little as 25% of the contralateral control leg values (34). This might result in a decrease in high-energy tensions of the ischemic muscles, which in turn might contribute to ischemia-induced functional disturbances of EDL. This is in accordance with previous studies (29) showing that after ligation the resting blood flow had minimal effect on structural integrity of EDL muscle.

In the present study a different effect of ischemia on fast EDL muscle was evident. A recent study suggests that ATP concentration, at least during contractile-induced activity, may not be the fundamental factor in protecting the mechanical integrity of the muscle (37). According to current thinking, metabolic by-product accumulation resulting in changes in the intracellular milieu as a result of high-energy transfer reactions and glycolysis might be the decisive consideration (17). If this is the case, fast-twitch muscles might demonstrate a greater contractile dysfunction than slow-twitch muscles given the greater absolute increase that would be expected in a range of selected metabolites such as inorganic phosphate and hydrogen ions following ischemia.

In contrast to the fast-twitch muscle EDL, Sol muscle, a representative slow-twitch muscle, exhibited more extensive structural damages, whereas functional disturbances were less pronounced. This is in agreement with a previous study demonstrated extensive structural damage in the rat Sol muscle (9).

Vitamin C administration in rats subjected to iliac artery ligation appears to have reduced the functional performance of the ischemic EDL muscle and also resulted in a significant decrease in muscle weight. In addition, a significant decrease in muscle fiber area in ischemic compared with contralateral EDL muscle, corroborated by electron microscopy observations, leads to the hypothesis that vitamin C administration can limit the damage in the contralateral EDL.

It is known that under ischemic conditions, even a mild type of activity results in some swelling of the capillary endothelium, not only in the directly active muscles but also in muscles remote from the site to injury (15, 33). This swelling has been associated with the increased vascular permeability to macromolecules and increased adhesion of leukocytes (26, 27).

Vitamin C administration does not seem to affect the ischemia-induced damages in the Sol muscle other than a significant reduction in the mean fiber area of the ischemic Sol. A variety of exogenously applied intracellular and extracellular antioxidants (superoxide dismutase, catalase) might cause alterations of exogenously applied intracellular and extracellular antioxidants. Reduced intracellular free radical formation might be the reason that in our study single twitch tension is reduced in the vit C group both in EDL and Sol muscles (9, 30).

The ischemic EDL muscle of the exe group presented the highest single twitch tension compared with the ischemic EDL muscles of the other three groups. The tension recorded was almost equal to that of the contralateral muscle. The mean muscle area was significantly decreased, and severe damage of

Table 4. All variables of EDL muscle express as gram of force per gram of muscle weight

<table>
<thead>
<tr>
<th></th>
<th>Contra</th>
<th>Ischem</th>
<th>Contra</th>
<th>Ischem</th>
<th>Contra</th>
<th>Ischem</th>
<th>Contra</th>
<th>Ischem</th>
<th>Contra</th>
<th>Ischem</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single tension, g/g</td>
<td>724.57±174.9</td>
<td>713.58±261.8</td>
<td>766.16±283.2</td>
<td>605.5±262.5*</td>
<td>1,284.1±315.1</td>
<td>1,124.2±173.2</td>
<td>1,223.75±699.9</td>
<td>1,917.6±505.7</td>
<td>2,304.35±718.02</td>
<td>1,948.7±438.2</td>
</tr>
<tr>
<td>40-Hz tension, g/g</td>
<td>1,511.6±319.8</td>
<td>1,237.2±466.6</td>
<td>1,408.7±467.5</td>
<td>1,298.8±467.46</td>
<td>1,307.45±739.8</td>
<td>1,307.45±739.8</td>
<td>2,213.8±829.9</td>
<td>1,953.3±515.5</td>
<td>1,622.1±568.9</td>
<td>1,518.3±570.4</td>
</tr>
<tr>
<td>80-Hz tension, g/g</td>
<td>1,852.5±455.3</td>
<td>1,375.3±502.6</td>
<td>1,555.6±532.5</td>
<td>1,307.45±739.8</td>
<td>1,307.45±739.8</td>
<td>1,307.45±739.8</td>
<td>2,213.8±829.9</td>
<td>1,953.3±515.5</td>
<td>1,622.1±568.9</td>
<td>1,518.3±570.4</td>
</tr>
<tr>
<td>100-Hz tension, g/g</td>
<td>2,043.1±653.3</td>
<td>1,359.4±457.9</td>
<td>1,576.7±579.1</td>
<td>1,296.57±761.3*</td>
<td>1,284.1±315.1</td>
<td>1,124.2±173.2</td>
<td>1,223.75±699.9</td>
<td>1,917.6±505.7</td>
<td>2,304.35±718.02</td>
<td>1,948.7±438.2</td>
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Values are means ± SD. *P < 0.05, ischemic vs. contralateral leg.
muscle fibers was observed in electron microscopy. These findings were not observed in ischemic Sol muscles where ligation of iliac artery did not affect either the performance or the structure. These data are in accordance with findings of previous studies in rats subjected to chronic ischemia (30) or in patients with intermittent claudication (3) where mild exercise improves the performance, even though it worsens the histological appearance of damage.

It is necessary to examine the possible causes of the detrimental effect of excessive activity in muscles with limited blood supply. The combination of repeated muscle activity and restricted blood supply leads to progressive muscle glycogen loss and degenerative changes possibly through the additive effects of substrate deficiency and high metabolic load (32). Limitation of blood supply, even on an acute basis, results in a greater glycogen loss than in intact muscle (2). In our experimental model the restriction of oxygen supply was not substantial during resting periods, yet it was sufficient to induce damage to ischemic muscles in exercised groups. The exercise protocol used in this study involved short periods of running (5 min) that mimic ischemia by increasing oxygen demands, followed by periods of rest (2 min), which is equivalent to reperfusion injury. This type of activity might induce formation of reactive oxygen species (ROS). It is likely that in extreme conditions, the delicate balance between oxygen supply and demand can be compromised regionally. In such conditions, tissue oxygen supply can fall below a critical level in some portions of the cell, resulting in a build up of reducing equivalents and enhanced ROS formation. Data from previous studies suggest that antioxidants protect critical metabolic pathways during hypoxia, as well as pathways that are responsible for preservation of creatine phosphate, the major energy shuttle system of contracting muscle (7).

Claudicants suffer from chronic ischemia-reperfusion injury. This presents as a repetitive low-grade ischemia with calf or buttock pain that subsides when exercise stops and reperfusion starts (48). This not only damages the muscles involved, as well as other tissues (endothelium, kidney) subjected to these periods of ischemia-reperfusion, but also causes a systemic response and remote organ impairment (36).

There are a number of studies on fast and slow fibers following ischemia and reperfusion, but the conclusions are conflicting. Studies regarding fiber type and reperfusion injury indicate that fast fibers are more prone to injury (4, 49). These investigations were focused on the function of the remaining fibers rather than the degree of injury. Our findings are in agreement with data of previous studies indicating a significant disturbance in structural integrity of the EDL muscle, yet a preservation of its functional status.

Fast-twitch (type 2) myosin fibers are glycolysis dependent and predominant in muscles of locomotion. In contrast, slow-twitch myosin fibers are capable of sustained contraction and are the fiber type mostly found in muscles of posture (5). After reperfusion, fast-twitch muscles exhibit a greater degree of injury, yet are still functional, most likely because of the low metabolic demand of the surviving fibers. One possible explanation for this finding is that fast-twitch muscle has a higher metabolic rate than slow twitch and is therefore intrinsically more susceptible to ischemia-induced loss of substrate. It has been reported that the predominantly fast-twitch muscles lost force at a faster rate than the predominantly slow-twitch muscles (21). These observations indicate that ische-

### Table 5. All variables of Sol muscle

<table>
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<tr>
<th></th>
<th>Contra</th>
<th>Ischem</th>
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<th>Contra</th>
<th>Ischem</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muscle weight, g</td>
<td>0.118 ± 0.004</td>
<td>0.109 ± 0.002</td>
<td>0.105 ± 0.002</td>
<td>0.094 ± 0.002</td>
<td>0.084 ± 0.005</td>
<td>0.084 ± 0.004</td>
<td>0.110 ± 0.016</td>
<td>0.16 ± 0.21</td>
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<tr>
<td>Single tension, g</td>
<td>64.11 ± 47.15</td>
<td>33.21 ± 10.02</td>
<td>52.60 ± 23.62</td>
<td>41.46 ± 22.61*</td>
<td>39.77 ± 19.73</td>
<td>30.01 ± 6.79</td>
<td>52.23 ± 44.5</td>
<td>46.43 ± 38.2</td>
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<td></td>
</tr>
<tr>
<td>TTP, ms</td>
<td>60.0 ± 33.9</td>
<td>76.0 ± 27.2</td>
<td>70.0 ± 28.43</td>
<td>74.0 ± 24.17</td>
<td>87.32 ± 22.12</td>
<td>72.0 ± 13.86</td>
<td>69.45 ± 26.76</td>
<td>80.18 ± 20.09</td>
<td></td>
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</tr>
<tr>
<td>1/2 RT, ms</td>
<td>83.60 ± 36.56</td>
<td>117.4 ± 41.33</td>
<td>90.86 ± 20.33</td>
<td>125.14 ± 46.51</td>
<td>89.67 ± 10.9</td>
<td>137.33 ± 31.75</td>
<td>79.91 ± 32.39</td>
<td>140.18 ± 81.84*</td>
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<tr>
<td>40-Hz tension, g</td>
<td>144.0 ± 74.06</td>
<td>105.41 ± 12.57</td>
<td>120.47 ± 35.04</td>
<td>111.74 ± 46.23</td>
<td>117.09 ± 31.28</td>
<td>87.89 ± 26.11</td>
<td>114.57 ± 52.04</td>
<td>124.58 ± 59.99</td>
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<tr>
<td>80-Hz tension, g</td>
<td>167.27 ± 87.89</td>
<td>128.86 ± 10.64</td>
<td>131.29 ± 37.46</td>
<td>116.36 ± 51.33</td>
<td>131.52 ± 33.58</td>
<td>99.12 ± 26.67</td>
<td>122.78 ± 52.52</td>
<td>139.25 ± 62.29</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100-Hz tension, g</td>
<td>164.67 ± 92.47</td>
<td>131.14 ± 13.98</td>
<td>131.94 ± 37.18</td>
<td>114.45 ± 55.21</td>
<td>123.18 ± 52.95</td>
<td>99.36 ± 26.25</td>
<td>125.32 ± 55.9</td>
<td>129.2 ± 49.07</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Muscle fiber area, μm²</td>
<td>1,261.6 ± 558.14</td>
<td>1,213.6 ± 552.5*</td>
<td>3,297.7 ± 1,819.7</td>
<td>2,809.7 ± 1,794.4*</td>
<td>2,918.1 ± 2,809.7</td>
<td>2,805.1 ± 1,559.2</td>
<td>3,007.8 ± 1,260.2</td>
<td>2,562.03 ± 1,107.7*</td>
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Values are means ± SD. Sol, soleus. *P < 0.05, ischemic vs. contralateral leg.

**Fig. 4. Mean values ± SD of EDL muscle fiber cross-sectional area in all groups. *P < 0.05, **P < 0.001, ischemic vs. contralateral muscle.**

**Fig. 5. Mean values ± SD of soleus (Sol) muscle fiber cross-sectional area in all groups. *P < 0.05, ischemic vs. contralateral muscle.**
mia-reperfusion injury greatly affects intracellular organelles of fast-twitch fibers (49).

Histological analysis of ischemic muscle of PAD patients demonstrated denervation and a reduction in the cross-sectional area of type II fibers compared with healthy, age-matched controls (41). Patients with severe PAD developed structural alterations in skeletal muscles such as denervation, fiber atrophy, and a selective loss of type II fibers relative to type I fibers that may contribute to the muscle dysfunction (24). Studies on chronic ischemic muscles showed an improvement in slow-twitch muscle performance, namely fatigue resistance and peak tension. In contrast, increased activity had a dramatic impact on muscle, causing structural damage (9), such as satellite cell activation, centrally located nuclei, and signs of muscle repair (myoblast).

Comparable results are obtained from studies performed in patients suffering from chronic ischemia. Although increased activity along with restriction of blood supply damages muscle fibers, exercise training in patients with chronic ischemia increases peak exercise performance in all treated subjects,

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**Fig. 6.** Ischemic EDL muscle of all groups (cross sections) [electron microscopy (EM)]. A: ischemic. B: vit C. C: exe. D: vit C + exe. ↑, dilatation of sarcoplasmic reticulum; *, and dm, disorganization of myofilaments; m, mitochondria.

**Fig. 7.** Ischemic EDL muscle of all groups (longitudinal section) (EM). A: ischemic. B: vit C. C: exe. D: vit C + exe. ↑, dilatation of sarcoplasmic reticulum; dm, disorganization of myofilaments; v, vacuoles; nm, normal muscle.
delays onset and progression of claudication pain during exercise, and improves community-based ability (25). A supervised exercise program is mandatory for patients suffering from intermittent claudication, and it is found to increase claudication-free distance walking, improve the ability to perform activities of higher intensity, and improve quality of life (20, 42, 45).

Sjostrom (44) observed a more frequent occurrence of pathologically changed muscle fibers in intermittent claudication-diagnosed patients who were capable of longer distance walking than those undertaking less exertion and concluded that “an extremely delicate adaptation in musculature at light as well as electron microscopic level occurs in relation to the ischemia and functional demand.” It was also shown that exercise rehabilitation improved ambulatory function, endothelium-dependent dilation, and calf blood flow in older PAD patients with intermittent claudication symptoms, which might be responsible for the overall improvement in performance (3, 22).

Additional proposed mechanisms that enhance exercise tolerance in arterial insufficiency include increase in blood flow, favorable redistribution of blood flow, improved hemorheological and fibrinolytic properties of blood, greater reliance on aerobic metabolism due to a higher concentration of oxidative...
enzymes, and less reliance on anaerobic metabolism (14). In addition, studies in humans subjected to hypoxia for a limited period of time followed by exercise demonstrate enhanced fiber size, capillarity, myoglobin concentration, and muscle oxidative capacity (29).

Studies in rats have shown that after ligation of a major artery such as iliac or femoral, muscle blood flow and functional hyperemia during activity are impaired for days or even weeks (12, 33). Further mechanisms of muscle adaptation to ischemic conditions are the muscle is swift to use anaerobic metabolism as well as collateral circulation (10).

Ligation of the iliac artery itself for 2 wk impairs blood flow and prevents arterial dilatation during muscular contractions in rat skeletal muscle while the intermittency of individual capillary flow is increased (11).

Since our study was conducted in a time frame of 2 wk, the above-mentioned mechanisms were not examined. One possible mechanism of improvement for the muscle function improvement found in our study is the redistribution of blood flow at the microvascular level within the muscles (1). Furthermore, exercise might improve the impaired responses to endothelium-dependent dilators in precapillary arterioles of ischemic muscles. It is proposed that chronic ischemia curtails production of nitric oxide (NO), vasodilator agent, in small arteries, and increased muscle activity or electrical stimulation of muscles were able to counteract this effect, either by normalizing availability of NO or by induction of compensatory mechanisms even in 1 wk after ligation of iliac artery (35).

Vitamin C administration in exercised rats improved the ischemia-induced structural damage in EDL muscle. Neither mean muscle fiber cross-sectional area nor muscle function was different compared with these in the contralateral EDL muscle. The ultrastructure of the filaments showed small areas of disorganization of the fibers and vacuoles in sarcoplasmic reticulum although histological appearance was improved compared with this in the exe group. A decrease in muscle fiber cross-sectional area was evident in the ischemic Sol muscle in the vit C + exe group, but this was not the case in the exe group. This finding might be due to the fact that administration of vitamin C improved the contralateral Sol muscle.

Prolongation of ½ RT was found in both muscles (EDL and Sol) in the vit C + exe group compared with the contralateral muscles. Although the effect of exercise under ischemic conditions was different on the two types of muscles, it appears that the exercise combined with an antioxidant agent resulted in prolongation of ½ RT. Contraction times are dependent in part on the sarcoplasmic reticulum and its ability to sequester calcium (8). Ischemia resulted in TTP and ½ RT prolongation of single twitch tension. Although these alterations were present in both muscles in all groups, there was a significant ½ RT delay in ischemic EDL and Sol muscles of vit C + exe group. This prolongation might be due to Ca2+ reabsorption delay by the sarcoplasmic reticulum (8).

In our study, vitamin C at the dose administered could not protect the ischemic muscles in rats with normal activity. Mild exercise improved the performance of the ischemic muscles but damaged the muscle fibers. In exercised rats, an increased damage of muscle fibers is observed, but vitamin C administration reduced the structural damage and preserved function. An exercise program combined with low dose of vitamin C showed protection mainly in type II muscle fibers and reduced the structural damage in both types of muscle fibers.

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REFERENCES


