Xanthine oxidase and activated neutrophils cause oxidative damage to skeletal muscle after contractile claudication

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Several studies have shown that ischemia-reperfusion (I/R) caused by exercise claudication elevates markers of oxidative damage in the plasma. Hickman et al. (16) exercised claudicant patients for 10 min and found an increase in plasma malondialdehyde levels after exercise, whereas Silvestro et al. (32) showed an increase in thiobarbituric acid-reactive substances, as a marker of lipid peroxidation, after maximal, but not submaximal, exercise in claudicants.

Likewise, markers of oxidative damage are also elevated in skeletal muscle after claudication. We recently found (21) an increase in protein oxidation and lipid peroxidation and a reduction in total glutathione levels 1 h after exercise with an animal model of claudication. This was associated with an increase in xanthine oxidase activity and neutrophils—both sources of oxidizing species.

To further explore these findings and determine the relative contributions of each pathway, we chose to 1) inhibit xanthine oxidase activity and 2) deplete neutrophils and then measure markers of oxidative damage. Thus the purpose of this study was to determine whether inhibition of xanthine oxidase activity or induction of neutropenia attenuates markers of oxidative stress and edema within skeletal muscle after an acute bout of contractile claudication.

We refer to our model as contractile claudication simply because we use electrical stimulation to cause muscle contraction-induced claudication. Claudication is caused by the combination of this muscle contraction and blood flow restriction, caused by femoral artery ligation. Because of the number of collateral vessels, and the low metabolic demand of a resting skeletal muscle, the ligation does not cause ischemia at rest, only during muscle contraction when the blood flow demands are increased greatly (8, 31). This model has been suggested to closely resemble the circulatory conditions seen in human claudicants (15) and has been used to examine the effects of exercise claudication on such parameters as angiogenesis (26), bioenergetics (4, 8), and leukocyte adherence and vascular permeability (14).

EXPERIMENTAL PROCEDURES

Animals. Male Sprague-Dawley rats (120 days old) were provided food and water ad libitum and maintained on a 12:12-h light-dark photoperiod for 7 days before the beginning of these experiments. During this 7-day period, animals were handled daily to prevent a stress hormone-induced reduction in body weight at the beginning of the experiments. These experiments were approved by the institutional animal care and use committee and conformed to the “Guiding Principles in the Care and Use of Animals” of the American Physiological Society.

Experimental designs. Animals were assigned to one of three experimental groups: control (n = 6), allopurinol (n = 6), and cyclophosphamide (n = 6). Control animals received intraperitoneal saline injections twice a day for 2 days and 30 min before claudication. Allopurinol-treated animals received allopurinol (50 mg/kg body wt ip) twice a day for 2 days and 30 min before claudication. This dose of allopurinol has been shown to cause 80% inhibition of xanthine oxidase activity without significant oxidant scavenging (36). Cyclophosphamide-treated animals received cyclophosphamide (20 mg/100 mg body wt ip) 4 days before claudication. Cyclophosphamide, a nitrogen mustard derivative, is an immunosuppressant often used experimentally to induce neutropenia (22, 30). The dose used in this study was used previously to protect against an increase in microvascular permeability associated with short-term I/R (6).

The limbs of each rat were randomly assigned to a ligated-stimulated (LS; claudicant) or a sham ligated-stimulated (SS; control) group. After 24-h recovery, both hindlimbs were stimulated in vivo for 30 min and force production was measured. One hour after stimulation, gastrocnemius muscles were removed for determination of lipid hydroperoxides, 4-hydroxy-2-nonenal (4-HNE), protein car-
bonyls, xanthine oxidase activity, MPO activity, lactate dehydrogenase (LDH) activity, and wet-to-dry muscle weight ratio.

Surgical procedure. After isoflurane anesthesia (5% for induction, 1.5–2.5% for maintenance), a small incision was made directly above the inguinal fold and the femoral artery was exposed and isolated by blunt dissection. Two ligatures were placed tightly around the vessel, with the vessel cut between the ties. This ensured cessation of blood supply via the femoral artery. The same procedure was performed on the contralateral limbs, with the exception being that the femoral artery was left intact.

Contractile claudication. To cause contractile claudication, animals were placed in a prone position in a specially fabricated Flexiglas apparatus that allowed the animal to be secured in a reproducible position with limited mobility of the lower leg except at the tibiotarsal joint. The animals were kept warm by heating pads and an incandescent light. The core temperature was measured with a thermistor placed in the rectum and maintained from 35 to 38°C. A force-displacement ergometer was calibrated and secured to the forefoot between the first and second footpads by a lightweight chain such that the tibiotarsal angle was 90°. The voltage signal from the force transducer was processed via a computerized data acquisition system (LabView, National Instruments, Austin, TX).

A stainless steel stimulating electrode (cathode) was placed transcutaneously near the sciatic nerve midway between the posterior ischial spine and the greater femoral trochanter. Another stainless steel stimulating electrode (anode) was inserted 3 mm subdermally in the midline of the lower back. The sciatic nerve was then stimulated proximally with a supramaximal voltage (90–125 V), 0.5 pulses/s, and a stimulus time of 0.05 ms (model S48, Grass Instruments, West Warwick, RI) to cause muscular contraction of the hindlimb muscles, as previously described (9).

Model. This model was used previously (11, 14) to mimic the condition of claudication. Because of the number of collateral and reentrant vessels, ischemia is not apparent at rest (31). However, during stimulation, blood flow only increases to ~40% of the expected increase in controls (2). Thus only during the increased demand for blood flow during stimulation is the muscle ischemic and, therefore, only the limbs undergoing ligation and stimulation experienced acute contractile claudication.

Muscle removal. One hour after stimulation the gastrocnemius muscles were removed, placed in cold antioxidant buffer (100 μm EDTA, 50 mM Na3HPO4, and 1 mM butylated hydroxytoluene), blotted dry, weighed, rapidly frozen in liquid nitrogen, and stored at −80°C until being assayed.

Biochemical assays. Protein carbonyls were measured spectrophotometrically as described by Reznick and Packer (29). Lipid hydroperoxides were measured with the ferrous oxidation-xenolol orange technique reported by Hermes-Lima et al. (13). Total 4-HNE binding was measured with standard Western blot analysis (primary antibody, Alpha Diagnostics, San Antonio, TX). Blots were developed with enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech) and imaged with an Image Station (model 440cF, Eastman Kodak).

MPO is an enzyme specific to granulocyte lysosomes and, therefore, directly correlates with the number of neutrophils. MPO activity was assayed according to methods by Allan et al. (1). Change in absorbance at 460 nm was monitored with a spectrophotometer, with 1 unit of MPO defined as that yielding a change in absorbance of 1.0 and expressed as units per gram of wet weight.

Xanthine oxidase activity was measured with a modified version of the Amplex Red Xanthine/Xanthine Oxidase Assay Kit from Molecular Probes (Eugene, OR).

LDH activity was determined according to the methods of Bergmeyer et al. (5). This cytoplasmic protein of 140,000 kDa is highly impermeable to the muscle cell membrane, and therefore a loss in LDH activity may reflect the enzyme’s escape from the cell due to membrane disruption (10).

Wet-to-dry weight ratio was determined by placing a precise wet weight of muscle in a freeze-dry unit (Virtis Sentry Benchtop 3L). The dry weighing was terminated when the same weight was recorded three times in succession. The wet-to-dry ratio provides an index of edema formation (18).

Statistical analysis. All data are expressed as means ± SE. Statistical analyses were performed with a two-way ANOVA (GraphPad Software, San Diego, CA). Significance was established at the P < 0.05 level.

RESULTS

Claudication. To ensure that each treatment group endured the same degree of contractile claudication, we measured force generation from the triceps surae muscle group throughout the 30-min stimulation period (Table 1). During the final minute of stimulation, force production from the LS limbs had been reduced to 31.4%, 31.2%, and 32.7% of initial force in the control, allopurinol, and cyclophosphamide groups, respectively. This clearly demonstrates the effects of blood flow restriction during muscle contraction and validates that each treatment group reached the same degree of claudication. In contrast, force production from the SS limbs was reduced to only 87.2%, 87.6%, and 86.0% of initial force in the control, allopurinol, and cyclophosphamide groups, respectively, demonstrating the mild intensity of stimulation.

Lipid peroxidation. We chose to analyze two markers of lipid peroxidation, lipid hydroperoxides and 4-HNE. Lipid hydroperoxides were significantly elevated (P < 0.001) in the LS limbs of both control and allopurinol-treated animals compared with SS limbs. However, the levels in LS limbs of allopurinol animals were significantly lower (P < 0.05) than in the LS limbs of control animals. There were no differences in lipid hydroperoxide levels between the LS and SS limbs of the cyclophosphamide-treated animals (Fig. 1A).

For analysis of 4-HNE, the levels in LS limbs of each treatment group were selected and expressed as a percentage of the levels in control SS limbs. HNE levels were significantly attenuated only in the cyclophosphamide animals (Fig. 1B).

Protein oxidation. Both the control and allopurinol groups showed a significant increase (P < 0.05) in protein carbonyl content in the LS limbs compared with SS. However, there were no differences between the LS and SS limbs in cyclophosphamide animals, and the carbonyl content in the LS limbs of cyclophosphamide animals was significantly lower than that in the LS limbs of control animals (Fig. 1C).

Table 1. Force production from triceps surae muscle group during last minute of 30-min stimulation period

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Force Production, % of initial force</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
</tr>
<tr>
<td>SS</td>
<td>87.17±2.310</td>
</tr>
<tr>
<td>LS</td>
<td>31.41±2.044*</td>
</tr>
<tr>
<td>Allopurinol</td>
<td></td>
</tr>
<tr>
<td>SS</td>
<td>87.57±3.647</td>
</tr>
<tr>
<td>LS</td>
<td>31.22±2.228*</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td></td>
</tr>
<tr>
<td>SS</td>
<td>85.98±4.772</td>
</tr>
<tr>
<td>LS</td>
<td>32.68±1.473*</td>
</tr>
</tbody>
</table>

Values are means ± SE. SS, sham ligated stimulated; LS, ligated stimulated.

*Significantly different (P < 0.001) from the SS limb undergoing the same treatment.

CAUSES OF OXIDATIVE STRESS AFTER CLAUDICATION  
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Xanthine oxidase activity. In control and cyclophosphamide animals, xanthine oxidase activity was significantly increased \((P < 0.001)\) in the LS limb compared with the SS limb. This increase was attenuated in the allopurinol group, confirming that allopurinol served its purpose in inhibiting xanthine oxidase (Table 2).

MPO activity. Control and allopurinol animals had significantly elevated MPO activity in the LS limb compared with the SS limb. However, the MPO activity in the LS limb of the allopurinol group was significantly lower \((P < 0.01)\) than in the LS limb of control animals. There was no increase in MPO activity in cyclophosphamide-supplemented animals, confirming that cyclophosphamide served its purpose in reducing neutrophil infiltration (Table 2).

LDH activity. The LS limbs of control animals showed a significant decrease \((P < 0.05)\) in LDH activity. This reflects damage to the muscle cell membrane because the enzyme may escape from its cytosolic residence into the vasculature, thereby decreasing enzyme activity within the muscle (10). The decrease in LDH activity was attenuated in the allopurinol and cyclophosphamide groups (Table 3).

Wet-to-dry ratio. The control \((P < 0.01)\) and allopurinol \((P < 0.05)\) groups showed a significant increase in muscle wet-to-dry ratio after contractile claudication in the LS limb compared with the SS limb. This increase was attenuated in the LS limbs of cyclophosphamide animals (Table 3).

DISCUSSION

Using an animal model to mimic the effects of exercise claudication, we (21) previously showed that oxidative damage occurs within skeletal muscle after acute claudication and that this is associated with an increase in xanthine oxidase activity and MPO activity. Therefore, the purpose of this study was to further explore these potential sources of oxidizing species.

The role of xanthine oxidase in I/R injury has been described extensively (3, 24, 27), and the discovery that the activity of this enzyme is elevated after contractile claudication is in agreement with our previous findings (21). However, the novel finding is that by administering allopurinol to attenuate the increase in xanthine oxidase activity, we were able to show attenuation of lipid peroxidation, muscle damage, and neutrophil infiltration. That inhibition of xanthine oxidase activity significantly reduced lipid peroxidation confirms the enzyme as a source of oxidizing species after contractile claudication.

In addition, attenuation of xanthine oxidase activity protected against the loss in LDH activity seen in control animals. Because a decrease in LDH activity is reflective of muscle cell membrane damage, these data suggest that xanthine oxidase-derived oxidants may target the sarcolemma. A possible explanation for this could be the enzyme’s location. Although immunolocalization techniques demonstrate that xanthine oxidase is highly concentrated in capillary endothelial cells (20), histochemical localization studies indicate that the enzyme is also localized in the sarcolemma (19). This latter fact might explain why xanthine oxidase-derived oxidants cause muscle cell membrane damage. In addition, the lipid composition of the sarcolemma could provide an easy target for oxidative modification and help explain the lipid peroxidation caused by xanthine oxidase-derived oxidants.

It should be pointed out that a loss of LDH activity could conceivably be caused by oxidation of the enzyme’s sulfhydryl groups. However, a recent study showed that LDH activity was unchanged under oxidative conditions (33). Likewise, although glutathione was significantly reduced, LDH activity was unaffected by treatment with diamide, a thiol-specific oxidant (7). Therefore, we believe the loss of LDH activity is more likely caused by oxidative damage.
due to increased membrane permeability, as previously suggested (10).

Furthermore, although MPO activity was significantly elevated in the claudicant limb of the allopurinol group compared with the control limb, it was significantly lower than the claudicant limb of control animals. Therefore, it can be concluded that xanthine oxidase-derived oxidants are important in the accumulation of neutrophils after contractile claudication. This chemotactic potential of oxidants from xanthine oxidase is in agreement with the findings of Seekamp et al. (30), who used allopurinol to inhibit xanthine oxidase activity and observed a significant reduction in MPO content after prolonged I/R. The chemotactic potential of xanthine oxidase-derived oxidants may be due to its high concentration in endothelial cells, because isolated endothelial cells or isolated vessels exposed to hydrogen peroxide show increased sensitivity to neutrophils (12, 23). There are several potential mechanisms to explain this. One is that oxidants stimulate endothelial cells to synthesize and/or release chemoattractants, such as platelet-activating factor and leukotriene B4 (34). Another potential mechanism is that oxidants may directly induce the expression of endothelial cell adhesion molecules. Indeed, endothelial cells exposed to hydrogen peroxide have been shown to induce P-selectin expression (25), and neutrophils incubated in hydrogen peroxide increase their expression of CD11 and CD18 (28).

Circulating neutrophil levels have been shown to increase after exercise claudication (17, 35) and we previously showed (21) significant neutrophil infiltration into skeletal muscle after contractile claudication. In this study, we used cyclophosphamide to reduce neutrophil infiltration. Cyclophosphamide-treated animals showed only a 17% increase in MPO activity after contractile claudication compared with a 94% increase in control animals, indicating that the treatment was successful in reducing neutrophil infiltration. This was associated with diminished lipid peroxidation, protein oxidation, muscle cell membrane damage, and edema, clearly demonstrating the ability of neutrophils to cause oxidative damage and edema. Although no previous studies have measured tissue neutrophil levels after claudication or depleted neutrophils before claudication, our findings are in agreement with others showing attenuation of lipid peroxidation and edema (18) and reduction of muscle permeability (30) with neutrophil depletion before prolonged I/R.

The attenuation of edema with neutropenia observed here was shown previously after prolonged I/R (6). It appears that as neutrophils migrate through the vascular endothelium into the muscle they may release oxidizing species and/or lysosomal enzymes. These molecules can damage the endothelium and alter membrane permeability, thereby resulting in edema. A second possibility is that the diapedesis process itself may widen endothelial gap junctions, thereby contributing to edema. Although neither of these potential mechanisms was addressed in this study, it is clear that neutrophils cause significant edema after contractile claudication.

The finding that neither allopurinol nor cyclophosphamide treatment reduced xanthine oxidase activity or MPO activity, respectively, in the SS muscles is somewhat confusing. In fact, the SS muscles are not different on any measure, across all treatments. However, because SS muscles do not experience contractile claudication, they do not generate significant amounts of oxidants. Therefore, the xanthine oxidase and MPO values obtained in all SS muscles should be baseline values and it is possible they may not be reduced further.

In summary, an acute bout of contractile claudication causes oxidative stress, muscle damage, and edema in skeletal muscle. Inhibition of xanthine oxidase activity attenuated lipid peroxidation, neutrophil infiltration, and muscle damage; neutropenia attenuated lipid peroxidation, protein oxidation, muscle cell membrane damage, and edema. Whether inhibition of xanthine oxidase activity directly protected against oxidative stress or was protective because of a reduction in neutrophil infiltration is unknown and should be addressed in future studies.

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REFERENCES


