Chronically ischemic mouse skeletal muscle exhibits myopathy in association with mitochondrial dysfunction and oxidative damage

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Peripheral arterial disease (PAD) is a manifestation of systemic atherosclerosis, producing stenoses and occlusions in the arterial tree supplying the lower extremities. PAD affects ~10 million people in the United States, most of them elderly (10). In the early stages of PAD, patients present with a significant impairment of their ability to walk (claudication), whereas, in more advanced stages, claudication worsens, and patients also may develop nonhealing foot ulcers and gangrene. Procedures to treat these patients constitute some of the most common operations performed by vascular specialists nationwide (18). With the expected increases in the elderly population, PAD is fast becoming one of the most significant causes of morbidity and mortality in the United States (18).

Claudication is a consequence of compromised blood supply to the ischemic limb and a metabolic myopathy affecting the ischemic muscles (4, 5, 22, 23). Defective mitochondria are central to this myopathy through compromised performance as primary energy producers and regulators of oxygen radical species, contributing to a progressive deterioration in muscle function and microanatomy (4, 5, 17, 22, 23). PAD myopathy is characterized by an increased content of dysfunctional mitochondria having significant defects in electron transport chain (ETC) complexes I, III, and IV (17, 24). These defects are associated with a bioenergetic decline, characterized by inadequate oxidative phosphorylation, decreased ATP energy production, and increased oxidative stress (4, 5, 17, 23–25). All start with inflow arterial occlusions, which produce low blood supply and myopathy (due to ischemic injury), and the two, in conjunction, produce the manifestations of PAD claudication (5, 14, 22, 23).

The long-term effects of arterial occlusive disease on the physiology of skeletal muscle, particularly in relation to its microanatomy and mitochondrial function, have been evaluated only in atherosclerotic PAD patients (5, 14, 22, 23). Patients with PAD differ in disease severity, mode of presentation, and presence of comorbid conditions, and such differences may complicate the study of skeletal muscle physiology in these patients. Rodents subjected to inflow arterial ligation exhibit characteristics resembling moderate-severe PAD in humans and have been used to evaluate the effects of occlusive arterial disease on angiogenesis and muscle physiology and bioenergetics (8, 10). In the present study, we evaluated skeletal muscle microanatomy, mitochondrial respiration, and oxidative stress in an established mouse model of chronic inflow arterial occlusion. The hypothesis driving the present study is that inflow arterial occlusion alone, in the absence of comorbidities, causes myopathy with mitochondrial dysfunction, increased mitochondrial content, and increased oxidative stress.
**METHODS**

**Animals**

Female C57Bl/6 mice (N = 48) (Charles River Laboratories), weighing 30–35 g, were placed on study at 6 mo of age, when ligation of the left femoral artery (N = 24) and sham operation (N = 24) were implemented. The experimental protocol was approved by the Institutional Animal Care and Use Committee of the University of Nebraska Medical Center.

**Hindlimb Ischemia Model**

Arterial ligation and division and tissue collection were carried out on mice anesthetized with an intraperitoneal injection of tribromethanol (Avertin; 0.25 mg/g body wt). Arterial occlusions were induced in two stages. First, the left femoral artery was ligated proximal to the superficial epigastric artery. Two weeks later, the common iliac artery was ligated distal to the aortic bifurcation. These animals exhibited no gross changes in hindlimb appearance. Control mice received similarly staged incisions and anatomic dissections without arterial ligation. Twelve weeks after the second operation, both ligated and control mice were anesthetized. Soleus muscle was harvested and used fresh for measurement of mitochondrial respiration. Tibialis anterior muscle was harvested and fixed in methacarn (methanol-chloroform-acetic acid 60:30:10) for microscopy. The remainder of the (below knee) hindlimb muscles (including gastrocnemius, plantaris, tibialis posterior, flexor hallucis longus, flexor digitorum longus, extensor digitorum longus, and peroneus muscles) was removed and immediately frozen for later biochemical measurements, including analysis of manganese superoxide dismutase (MnSOD) and citrate synthase (CS) activities and quantification of MnSOD, 4-hydroxy-2-nonenal (HNE) adducts, and carbonyl groups by reverse-phase protein array analysis (RPPA).

**Bright-Field and Fluorescence Microscopy**

Tibialis anterior muscles were taken from chronically ligated and control mice. Gastrocnemius muscle samples from a PAD patient and a control subject were harvested, in accordance with a protocol approved by the Internal Review Board of the University of Nebraska Medical Center. The samples were obtained from the anteromedial aspect of the muscle belly 10 cm distal to the tibial tuberosity. Muscle specimens were fixed in methacarn, embedded in paraffin, sectioned at 4 µm, and mounted to glass slides. For both bright-field and fluorescence microscopy, slide specimens were deparaffinized in xylene and rehydrated to water. Images of specimens stained with hematoxylin and eosin were captured under bright-field illumination with a Leica DMRXA2 microscope (North Central Instruments), the coverslips were sealed with clear lacquer, and the specimens were mounted with ProLong Gold antifade reagent (Molecular Probes), the coverslips were sealed with clear lacquer, and the slides were stored at 5°C. Fluorescence images were captured with the Leica DMRXA2 microscope configured with a charge-coupled device B/W camera (Hamamatsu) and ImagePro Plus software.

**Evaluation of Mitochondrial ETC Complexes**

Respiration of skinned (saponin-permeabilized) soleus muscle fibers (26) was measured with a Clark electrode (YSI), in four separate assays corresponding to the following sets of conditions: 1) 5 mM glutamate, 5 mM malate, and 1 mM ADP (complex I-dependent respiration); 2) 3 µM rotenone (to inhibit complex I), 1 mM ADP, and 10 mM succinate (complex II-dependent respiration); 3) 3 µM rotenone, 1 mM ADP, and 1 mM duroquinol (complex III-dependent respiration); 4) 3 µM rotenone, 1 mM ADP, 10 mM ascorbate, and 0.2 mM N,N,N',N'-tetramethyl-p-phenylenediamine (complex IV-dependent respiration). Respiratory rates (nanomolar of oxygen per minute) were normalized to CS activity and total protein, immediately after completion of the respiratory measurements.

CS activity was measured as the increase in absorbance (412 nm) from the reduction of 5,5′-dithiobis-2-nitrobenzoic acid by newly formed CoA-SH (30). Total protein concentration of each sample was determined with the BCA Protein Assay Kit (Pierce).

**Analysis of MnSOD Activities in Calf Muscle Homogenates**

MnSOD activity was quantified by a modification of the nitrite method (3, 11, 20). Briefly, a SOD activity reference set (0, 6.25, 12.5, 25, 50, 100, 200, and 400 ng MnSOD/20 µl) was prepared with standard MnSOD (Sigma-Aldrich, St. Louis, MO; catalogue no. S-5639), and each sample set was prepared by adding muscle homogenate at a dilution of 1/20 to each solution of a duplicate reference set. For reference and sample sets, signal reduction was plotted as a function of the quantity of MnSOD in the activity reference set. The shift of the X-intercept at the 50% end point of the sample curve relative to the 50% end point of the reference curve was a linear function \( R^2 = 0.997 \) of MnSOD added to the reference set. Standard SOD activity units in each sample were computed as nanogram equivalents of standard MnSOD and normalized to homogenate protein.

**Quantification of MnSOD, HNE Adducts, and Carbonyl Groups in Whole Muscle Homogenates by RPPA**

Preparation of muscle homogenates for RPPA. Calf muscle homogenates were analyzed by the methods of Calvert et al. (7). Homogenates were diluted in extraction buffer to 100 µg protein/ml (final volume 250–500 µl) and then heated at 70°C for 2 h (Eppendorf Thermomixer 5436). Samples were sonicated (Branson 2110 Branson Ultrasonics, Danbury, CT) for 10 min, heated at 95°C for 10 min, and then centrifuged (Fisher Micro-Centrifuge model 235V, Fisher Scientific, Pittsburgh, PA) at 5,000 g and room temperature for 10 min.

General procedures for RPPA. Samples were spotted (50 nl/slot) in triplicate on duplicate nitrocellulose slides (LI-COR Biosciences Laboratories), with an eight-pin arrayer (VP478; V&P Scientific), according to manufacturer instructions. Slides were treated with primary Ab or isotype control for 2 h at room temperature and then with IRDye 800-conjugated secondary Ab (1:2,500 dilution; LI-COR Biosciences Laboratories). Washed slides were air dried and then analyzed with the Odyssey IR imaging system (LI-COR Biosciences Laboratories). Integrated fluorescence intensities were determined for each spot.

Analysis of MnSOD. One slide was treated with rabbit anti-MnSOD Ab (Stressgen) diluted to 2 µg/ml blocking buffer, and the duplicate with rabbit IgG (2 µg/ml) (Vector Laboratories). Monospecificity of the Ab was determined by Western analysis of muscle homogenate (data not shown). MnSOD concentration (µg/ml) of each homogenate specimens were mounted with ProLong Gold antifade reagent (Molecular Probes), the coverslips were sealed with clear lacquer, and the slides were stored at 5°C. Fluorescence images were captured with the Leica DMRXA2 microscope configured with a charge-coupled device B/W camera (Hamamatsu) and ImagePro Plus software.
was determined with a standard curve expressing MnSOD concentration (μg/ml) as a linear function \((R^2 = 0.999)\) of integrated fluorescence intensity. Expression was normalized to total protein in each homogenate.

**Analysis of HNE adducts.** Adducts were labeled with monoclonal Ab (2 μg/ml) (kind gift of Dr. Koji Uchida, Institute for Advanced Research, Nagoya University, Japan), specific for the tetrahydrofuran (ring) form of the Michael adduct (32), and the isotype control slide was treated with mouse IgG (2 μg/ml) (eBiosciences Laboratories, San Diego, CA; 2 μg/ml; 2 mouse monoclonal anti-dinitrophenol Ab (Sigma-Aldrich, St. Louis, MO; 2 μg/ml), and standards were arrayed on duplicate slides. Reduced samples served as background controls. Protein carboxyls were labeled with oxidizing BSA (OxBSA) with ferrous ammonium sulfate (1), and a negative control was prepared by reducing OxBSA with sodium borohydride (1). Borohydride-reduced and nonreduced homogenates and standards were arrayed on duplicate slides. Reduced samples served as background controls. Protein carbonyls were labeled with mouse monoclonal anti-dinitrophenol Ab (Sigma-Aldrich, St. Louis, MO; 2 μg/ml), and the isotype control slide was treated with mouse IgG (eBiosciences Laboratories, San Diego, CA; 2 μg/ml). Protein carbonyls were determined from a standard curve relating integrated intensity of the HNE-BSA standards (Alpha Diagnostics) was a linear function of concentration of the standards \((R^2 = 0.996)\), and HNE adducts in sample homogenates were expressed as HNE-BSA standard units per microgram protein.

**Analysis of carbonyl groups.** A carbonyl standard was prepared by oxidizing BSA (OxBSA) with ferrous ammonium sulfate (1), and a negative control was prepared by reducing OxBSA with sodium borohydride (1). Borohydride-reduced and nonreduced homogenates and standards were arrayed on duplicate slides. Reduced samples served as background controls. Protein carbonyls were labeled with mouse monoclonal anti-dinitrophenol Ab (Sigma-Aldrich, St. Louis, MO; 2 μg/ml), and the isotype control slide was treated with mouse IgG (eBiosciences Laboratories, San Diego, CA; 2 μg/ml). Protein carbonyls were determined from a standard curve relating integrated intensity of OxBSA standards as a linear function \((R^2 = 0.999)\) of OxBSA concentration (μg/ml) and expressed as micrograms of OxBSA per milligram homogenate protein.

**Data Analysis**

Results were expressed as means ± SE. Sigmastat (Jandel) and PlotIT (Scientific Programming Enterprises) software packages were used for computation of means and SE and for statistical analysis. The statistical significance of differences between measurements of ischemic and control muscle was evaluated by a two-tailed Student’s t-test for unequal variance. \(P\) values of ≤0.05 were considered indicative of significance. Curves were fitted to linear and nonlinear data by regression analyses with Sigmastat and PlotIT software.
Mitochondrial Respiration Is Impaired in Muscle Fibers From Ischemic Mice

Compared with control, the respiratory activities of ETC complexes I, III, and IV were significantly reduced in fibers of ischemic muscle, when normalized to CS activity (Table 1). The activities of complexes I, III, and IV were reduced 34, 45, and 42%, respectively. Normalized to myofiber protein, respiratory activity of complex III was significantly reduced, whereas the activities of complexes I and IV exhibited a downward trend (Table 1). These results are consistent with the higher SEs of complex activities normalized to total protein (Table 1). In agreement with our findings from whole calf muscle homogenates, CS in the soleus muscles used for respirometry was significantly (×670) with a ×20 objective (A and B) revealed the classic tubular microanatomy of the mitochondrial system and intensely fluorescent nodules associated with mitochondrial labeling. At a lower magnification (×260) with a ×10 objective (C and D), labeling exhibits subsarcolemmal aggregates and a sarcoplasmic dotlike pattern typical of mitochondrial labeling of skeletal myofibers. ATP synthase expression is variable among myofibers of ischemic and control muscle, reflecting differences in mitochondrial content. Overall, ischemic muscle exhibits increased ATP synthase expression compared with control muscle. The same pattern of labeling was seen in adjacent serial sections of control (E) and ischemic (F) muscle labeled with a monoclonal antibody specific for MnSOD. Myofibers treated with isotype control exhibited no fluorescence labeling (not shown). Calibration bars at the bottom right of B and F represent 25 μm. Magnification was calculated by dividing the length of each bar in the image by 25 μm.

Michael Adducts of HNE and Carbonyl Groups, Indexes of Oxidative Stress, Are Quantitatively Increased in the Ischemic Muscle of Chronically Ligated Mice

Myofibers of ischemic muscle (Fig. 3B) exhibited brighter fluorescence labeling for HNE-protein adducts compared with control muscle (Fig. 3A), indicating greater oxidative stress (28). Isotype controls did not exhibit fluorescence signal. In addition, labeling specificity was demonstrated by 90% inhibition of fluorescence signal (data not shown) when primary monoclonal Ab specific for MnSOD. In the isotype control slides, myofibers exhibited no fluorescence labeling (data not shown).

Analysis of CS activity in muscle homogenates. Total CS activity was measured in homogenates of whole calf muscle. CS activity was significantly increased (P < 0.05) in ischemic (N = 10; 752.42 ± 35.03 μmol·ml⁻¹·min⁻¹·mg protein⁻¹) compared with control muscle (N = 12; 466.97 ± 9.38 μmol·ml⁻¹·min⁻¹·mg protein⁻¹). This increased specific activity was not due to the small but significant (P < 0.05) reduction of total protein in ischemic (11.45 ± 0.44 mg) compared with control (12.63 ± 0.35 mg) calf muscle. Total wet weights of ischemic and control muscles were 0.218 ± 0.015 and 0.243 ± 0.007 g, respectively, and were not significantly different.

Mitochondrial Respiration Is Impaired in Muscle Fibers From Ischemic Mice

Compared with control, the respiratory activities of ETC complexes I, III, and IV were significantly reduced in fibers of ischemic muscle, when normalized to CS activity (Table 1).
Table 1. Analysis of mitochondrial complex I- through IV-dependent respiration in ischemic and control soleus muscle of chronically ligated and sham-operated mice

<table>
<thead>
<tr>
<th>Complex-dependent respiration normalized to CS activity, nanoatom O₂·min⁻¹·mg⁻¹ protein⁻¹</th>
<th>Control Muscle</th>
<th>Ischemic Muscle</th>
<th>P Value</th>
</tr>
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<tbody>
<tr>
<td>Complex I</td>
<td>148.0±16.3</td>
<td>98.1±5.5</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Complex II</td>
<td>124.4±7.8</td>
<td>100.4±9.6</td>
<td>NS</td>
</tr>
<tr>
<td>Complex III</td>
<td>364.9±43.3</td>
<td>202.2±16.2</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Complex IV</td>
<td>337.8±23.7</td>
<td>196.4±15.0</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Values are means ± SE; N = 12 mice. Respiration determined by polarography as nanoatom O₂/min was normalized to both citrate synthase (CS) activity (determined as μmol·ml⁻¹·min⁻¹) and total protein (mg). NS, not significant.

Ab was pretreated with 2 × 10⁻⁵ M HNE-histidine conjugate (kind gift of Dr. K. Uchida).

HNE adducts in homogenates of whole calf muscle from control (N = 9) and ischemic (N = 9) mice were measured by RPPA (7). HNE adducts expressed as HNE-BSA units/μg protein were two times more abundant in homogenates of ischemic muscle (5.66 ± 0.43) compared with control muscle (2.77 ± 0.33). This is in agreement with findings from a previous study evaluating muscle from PAD patients (24). Results obtained with homogenates from three ligated and three control mice are presented in Fig. 3C. Arrays treated with nonimmune IgG did not exhibit fluorescence signal. In addition, fluorescence labeling was reduced by 90% (data not shown) when primary Ab was pretreated with the 2 × 10⁻⁵ M HNE-histidine conjugate (kind gift of Dr. K. Uchida).

To confirm the increase in oxidative stress, we quantified carbonyl groups in the same muscle as derivatives of dinitrophenyl hydrazine, by RPPA. In agreement with our measurements of HNE adducts and similar to findings with muscle from PAD patients (24), carbonyls were significantly increased (P < 0.05) in ischemic muscle (14.7 ± 1.10 μg OxBSA/mg protein) compared with control (9.70 ± 0.44 μg OxBSA/mg protein). Arrays treated with nonimmune IgG did not exhibit fluorescence signal. In addition, reduction of homogenates and OxBSA standard with NaBH₄ eliminated the carbonyl signal.

Alteration of Activity and Expression of MnSOD in Ischemic Muscle of the Mouse Hindlimb

Expression of MnSOD increased significantly (P < 0.05) in ischemic compared with control muscle, but activity displayed no significant change (Table 2). The ratio of MnSOD activity to expression was reduced in ischemic muscle to ~80% of the ratio in control muscle. The divergence of MnSOD activity and expression supports inactivation of the enzyme.

DISCUSSION

In this study, we have evaluated mice with hindlimb ischemia secondary to inflow arterial occlusion. The skeletal muscle of these animals exhibited typical histological and biochemical features found in human patients with arterial occlusive disease. Development in ischemic mice, of a myopathy associated with proliferation of dysfunctional mitochondria, oxidative stress, and alterations in MnSOD expression and activity provides the first direct demonstration that arterial occlusive disease produces muscle and mitochondrial pathology.
Table 2. Quantitative analysis of MnSOD in homogenates of ischemic and control calf muscle of chronically ligated and sham-operated mice

<table>
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<tr>
<th></th>
<th>Control Muscle</th>
<th>Ischemic Muscle</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MnSOD activity (standard SOD activity) normalized per mg total protein</td>
<td>1.75±0.14</td>
<td>1.90±0.05</td>
<td>NS</td>
</tr>
<tr>
<td>MnSOD expression (ng) normalized per mg total protein</td>
<td>310.3±14.8</td>
<td>412.1±16.0</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Values are means ± SE; N = 9 mice. Manganese superoxide dismutase (MnSOD) concentration (µg/ml) and MnSOD activity (SOD activity units/ml) were normalized to total homogenate protein (mg).

Myopathic Histological Features in Chronically Ischemic Muscle of the Mouse Hindlimb

Our work demonstrates that mouse skeletal muscle responds to chronic inflow arterial occlusion with the development of appreciable myopathic histological changes, a drop in total protein content, and a trend toward decreased wet weight. These findings are similar to the significant myopathic and atrophic changes that have been well described in the ischemic muscle of PAD patients (22, 23). These changes in human PAD muscle are thought to be related to and responsible for the sarcopenia, loss of strength, and functional impairment that is so characteristic of this patient population (5, 22, 23).

Increased Mitochondrial Content

Our work further demonstrates that arterial occlusive disease generates a significant increase in the mitochondrial content of skeletal muscle (documented both by elevated CS activity and increased ATP synthase expression). Increased mitochondrial expression reflected by CS activity and mitochondrial DNA levels has been documented previously for claudicating PAD muscle (22, 23). These changes in human PAD muscle are thought to be related to and responsible for the sarcopenia, loss of strength, and functional impairment that is so characteristic of this patient population (5, 22, 23).

Inflow Arterial Occlusion in the Mouse Model

Exercise-induced ischemia-reperfusion is the central problem in PAD patients and in animals with inflow arterial occlusion (2, 6, 29). The ischemia-reperfusion of every bout of claudication increases oxidative stress, triggers inflammation and oxidative damage to the tissues (4, 5, 12, 13, 22, 23), and initiates mitochondrial injury and dysfunction (4, 5, 22, 23). Mitochondrial dysfunction can then be perpetuated by repeated destructive cycles of ischemia-reperfusion, causing amplification of respiratory chain defects, compromised bioenergetics, increased ROS production, diminished MnSOD antioxidant activity, and oxidative stress (5, 17). In this process, mitochondrial biogenesis may be induced as the ischemic muscle attempts to compensate for its compromised bioenergetic state. The combination of compromised bioenergetics and worsening oxidative stress may then lead to progressive oxidative damage
of structures in the myocytes, eventually producing the myopathic histological changes of chronically ischemic muscle.

**Perspectives and Significance**

Our data have significant implications for modeling mitochondrial dysfunction and oxidative stress in the pathophysiology of PAD. We have evaluated mice with hindlimb ischemia secondary to inflow arterial occlusion. These mice reproduce important histopathological and biochemical features found in patients with arterial occlusive disease. These features include myopathic histological alterations, increased mitochondrial content, compromised mitochondrial respiration with dysfunctional ETC complexes I, III, and IV, increased oxidative stress, and alterations in MnSOD expression and activity. Our findings indicate that chronic arterial occlusion is the underlying mechanism for the myopathy described in patients with PAD and that the key factors in this myopathy are mitochondrial dysfunction and oxidative stress. Future animal model work will allow further delineation of the mechanisms responsible for the production of this myopathy. Such research is of central importance in delineating the pathology of claudication and will support the development of novel therapies for patients with arterial disease.

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