Skeletal muscle reperfusion injury is enhanced in extracellular superoxide dismutase knockout mouse

Jong Woong Park,¹ Wen-Ning Qi,³ Yongting Cai,³ Igor Zelko,²
John Q. Liu,² Long-En Chen,³ James R. Urbaniak,³ and Rodney J. Folz²

¹Department of Orthopaedic Surgery, College of Medicine, Korea University, Seoul, Korea; and ²Division of Pulmonary, Allergy, and Critical Care, Departments of Medicine and Cell Biology, and ³Orthopaedic Microsurgery Laboratory, Department of Surgery, Duke University Medical Center, Durham, North Carolina

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PArk, Jong Woong, Wen-Ning Qi, Yongting Cai, Igor Zelko, John Q. Liu, Long-En Chen, James R. Urbaniak, and Rodney J. Folz. Skeletal muscle reperfusion injury is enhanced in extracellular superoxide dismutase knockout mouse. Am J Physiol Heart Circ Physiol 289: H181–H187, 2005. First published March 18, 2005; doi:10.1152/ajpheart.00458.2004.—This study investigates the role of extracellular SOD (EC-SOD), the major extracellular antioxidant enzyme, in skeletal muscle ischemia and reperfusion (I/R) injury. Pedicled cremaster muscle flaps from homozygous EC-SOD knockout (EC-SOD⁻/⁻) and wild-type (WT) mice were subjected to 4.5-h ischemia and 90-min reperfusion followed by functional and molecular analyses. Our results revealed that EC-SOD⁻/⁻ mice showed significantly profound I/R injury compared with WT littermates. In particular, there was a delayed and incomplete recovery of arterial spasm and blood flow during reperfusion, and more severe acute inflammatory reaction and muscle damage were noted in EC-SOD⁻/⁻ mice. After 90-min reperfusion, intracellular SOD [copper- and zinc-containing SOD (CuZn-SOD) and manganese-containing (Mn-SOD)] mRNA levels decreased similarly in both groups. EC-SOD mRNA levels increased in WT mice, whereas EC-SOD mRNA was undetectable, as expected, in EC-SOD⁻/⁻ mice. In both groups of animals, CuZn-SOD protein levels decreased and Mn-SOD protein levels remained unchanged. EC-SOD protein levels decreased in WT mice. Histological analysis showed diffuse edema and inflammation around muscle fibers, which was more pronounced in EC-SOD⁻/⁻ mice. In conclusion, our data suggest that EC-SOD plays an important role in the protection from skeletal muscle I/R injury caused by excessive generation of reactive oxygen species.

reactive oxygen species; vessel diameter; blood flow

ISCHEMIA-REPERFUSION (I/R) injury is deleterious to the clinical outcome of replantation or free flap surgery, even after a technically successful procedure. Although the exact mechanism of this potentially catastrophic phenomenon is still unclear, reactive oxygen species (ROS) have been shown to be important contributors because of their toxicity to living tissue (19, 55).

I/R-mediated ROS formation can be generated from both intracellular and extracellular sources. The intracellular pathway is likely mediated via the xanthine dehydrogenase/xanthine oxidase system (20). In this pathway, ATP is utilized during ischemia and broken down to hypoxanthine. Concurrently, some of the xanthine dehydrogenase is converted to xanthine oxidase that then catalyzes the oxidation of hypoxanthine and xanthine to uric acid while reducing molecular oxygen to superoxide (O₂⁻) and hydrogen peroxide (H₂O₂) (19). One of the major sources of extracellular ROS formation is leukocyte-derived reduced NADP (NADPH) oxidase and the myeloperoxidase (MPO) system (6). It has been estimated that up to 70% of O₂⁻ production by activated leukocytes occurs via membrane-associated NADPH oxidase (3, 42). Whether or not O₂⁻ is generated from intra- or extracellular sources, SOD functions as an enzymatic scavenger of O₂⁻, leading to its dismutation to H₂O₂ (35). If SOD activation is insufficient to clear the enhanced levels of O₂⁻ formed during I/R, tissue damage will likely ensue (12, 34).

SOD is an endogenous enzymatic ROS scavenger that protects many organs against I/R injury (13, 43). Among three SOD isoforms, a copper- and zinc-containing form of SOD (CuZn-SOD or SOD1) is widely distributed in the cell cytosol and nucleus (11). Manganese SOD (Mn-SOD or SOD2) is a manganese-containing isoenzyme localized to the mitochondria (56). According to their specific localizations, CuZn-SOD and Mn-SOD are classified as intracellular SODs.

The third isoform is extracellular SOD (EC-SOD or SOD3). EC-SOD was first detected in extracellular fluids (33) and subsequently recognized to be a major extracellular form of SOD (31). It is secreted from smooth muscle cells and macrophages and is heavily targeted to smooth muscle-containing vasculature and airway (17, 32, 54). EC-SOD constitutes the predominant form of SOD, accounting for up to 45–70% of total SOD activity in the human or mouse aortic wall (54). EC-SOD protects cells from oxidative stress by binding to cell surface membranes through its heparin-binding carboxy terminal tail (36) and by preventing fragmentation of collagen type I in the extracellular matrix (45). Although EC-SOD has been shown to have a potent anti-inflammatory and scavenging effect against toxic ROS (15, 16, 49), there are no in vivo data concerning the specific role of EC-SOD in skeletal muscle I/R injury.

With the development of gene cloning technology, mice with a deficiency in or an overexpression of a specific SOD gene have been created and used to examine the role of individual SODs in I/R injury (1, 9, 25, 27, 28, 52, 53). In this study, we used EC-SOD knockout (EC-SOD⁻/⁻) mice (7) to examine the functional role that EC-SOD plays in skeletal muscle I/R injury.

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MATERIALS AND METHODS

The generation of EC-SOD−/− mice as described previously (7) except that the EC-SOD−/− mice used in the present study were bred and maintained in a C57BL/6 genotype. The EC-SOD−/− genotype was confirmed by PCR analysis of tail DNA (58). Wild-type (WT) C57BL/6 mice, purchased from Taconic (Germantown, NY), were used as controls. This investigation conformed to the NIH Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 85–23, Revised 1996). The animal protocol was approved by the Institutional Animal Care and Use Committee of Duke University Medical Center.

Surgical procedure. Anesthesia was induced with an intraperitoneal injection of Nembutal (50 mg/kg; Abbott Laboratories, North Chicago, IL) and maintained with a continuous intraperitoneal infusion of Nembutal at a rate of 17 mg·kg−1·h−1 by syringe pump (Stoelting, Wood Dale, IL), starting 40 min after the initial dose. The left cremaster muscle of the mice was prepared according to our previously described technique (10, 30). Briefly, the cremaster muscle was exposed, opened, and then separated from the testis. The pudendal epigastric artery and vein and the genitofemoral nerve were isolated and subsequently separated from each other. The cremaster muscle sac was cut circumferentially, rendering the muscle totally isolated but still attached to the body through its main neurovascular pedicle. The genitofemoral nerve was cut to make a denervated pedicled muscle flap. The prepared cremaster muscle was spread over a transparent acrylic microscope stage and maintained in the expanded position to the muscle. The temperature of the muscle was maintained at 34°C and covered with a thin layer of O2-impermeable plastic polymer (Saran Wrap; Dow Chemical, Indianapolis, IN) to prevent diffusion of O2 and other gases from the environment to the muscle. The temperature of the muscle was maintained at 34 ± 0.5°C throughout the experiment with a heat lamp. The muscle preparation was left undisturbed for 30 min to allow any transient effects of the muscle isolation procedure to dissipate. After baseline vessel diameter and blood flow were obtained, muscle ischemia was induced by clamping the vascular pedicle with an atrumatic microvascular clamp (ST-B-1 VB; ASSI, Westbury, NY). The vascular clamp was changed every 2 h during ischemia to prevent intimal injury due to the prolonged pressure of the clamp.

Measurement of vessel diameter. Intravitral videomicroscopy (Super-Lux 40; Carl Zeiss, Oberkochen, Germany) was used to view the vasculature of the prepared cremaster muscle. Observation areas were selected in an arterial tree for monitoring during the whole period of the experiment. The internal luminal diameters of the vessels in selected areas containing arteries of 10–70 μm in diameter were measured from the recorded image by means of a video-measuring gauge (FOR/A IV-560, Sony). Depending on the number of branches in the arterial tree, 12–15 sites were selected for the measurement in each muscle. Sequential measurements were taken at the same sites throughout the experiment, using the map of the vascular tree drawn at the time of baseline measurement. The data obtained from each muscle at each time point were divided into three categories, small arteries (10–20 μm), large arteries (21–40 μm), and small arteries (41–70 μm), depending on the baseline diameters of the measured vessels.

Measurement of blood flow. Overall blood flow in the cremaster muscle was measured with laser-Doppler flowmetry (Moor Instrument, Axminster, UK). A 1-mm-diameter double-fiber probe was positioned with a manipulator (MM 33, Stoelting). The tip of the probe was placed 1 mm above the surface of the cremaster vascular pedicle, so that there was no compressive effect of the probe on the vasculature. The position of the laser-Doppler tip was kept constant throughout the course of the procedure by monitoring with an operating microscope.

Histological examination. After ischemia followed by 90 min of reperfusion, two cremaster muscles from each group were harvested and immersed in 10% formalin. Overall blood flow in the cremaster muscle was also harvested and was identical immersed in formalin. The muscle samples were then embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin. To quantitatively evaluate tissue inflammation, polymorphonuclear neutrophil (PMN) infiltration in both groups was measured by cell density counts per high-power field under ×400 magnification (n = 10 for each group).

Skeletal muscle levels of SOD mRNA and protein. After measurements of vessel diameters and blood flow, muscle samples (n = 7 for each group) were immediately harvested and frozen at −80°C. Total RNA was extracted from half of each muscle sample with an RNeasy Mini kit plus DNase I digestion (Qiagen, Valencia, CA) and quantified with RiboGreen (Molecular Probes, Eugene, OR). Reverse transcription was performed in a total volume of 20 μl containing 0.4 μg of total RNA. First-strand cDNA was synthesized with a SuperScript II reverse transcriptase kit (Invitrogen, Carlsbad, CA) and quantitated with PicoGreen (Molecular Probes). Quantitative real-time PCR was performed with an iCycler Thermal Cycler (Bio-Rad, Hercules, CA) and an iQ SYBRgreen Supermix real-time PCR kit (Bio-Rad). The primer sequences and product sizes for each SOD and 18S rRNA (as the endogenous reference) are summarized in Table 1. The relative amounts of the target genes were calculated by the comparative threshold cycle method (47).

The remaining half of each muscle (n = 6 for each group) was homogenized in boiling lysis buffer (1% SDS, 1.0 mM sodium orthovanadate, and 10 mM Tris, pH 7.4) and microwaved for 10–15 s. The homogenate was centrifuged, and the pellet was then discarded. Protein concentrations were determined with Coomassie Plus protein assay reagent (Pierce, Rockford, IL). Equivalent amounts of cleared homogenate (total protein 30 μg) were loaded onto a NuPAGE 4–12% Bis-Tris gel (Invitrogen). After electrophoresis, the proteins were transferred onto polyvinylidene difluoride (PVDF) membranes. The PVDF membrane was blocked with 5% milk-Tris-buffered saline-Tween 20 for 1 h and incubated at 4°C overnight with a polyclonal primary antibody for each of the SOD isoforms, followed by incubation with a goat anti-rabbit IgG-horseradish peroxidase (sec-

Table 1. Oligonucleotides of primers for quantitative real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense (5–3’)</th>
<th>Antisense (5–3’)</th>
<th>Product Size, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>CuZn-SOD</td>
<td>TGGACCCAGTGTTGTTGTCAGG</td>
<td>TCACATGACTGTTGTCAGTCCG</td>
<td>123</td>
</tr>
<tr>
<td>Mn-SOD</td>
<td>TGGCTTGGCCCTAATAAGGA</td>
<td>AAGGTAGTAAAGGTGCTCACCACA</td>
<td>129</td>
</tr>
<tr>
<td>EC-SOD</td>
<td>AGGTGAGATCCGGAGAT</td>
<td>TCACAGCCATGAAATAGGCTCACA</td>
<td>142</td>
</tr>
<tr>
<td>18S rRNA</td>
<td>GTACACCGGTGAACCCGATT</td>
<td>CCATCCGATGGAACCCGAGG</td>
<td>151</td>
</tr>
</tbody>
</table>

CuZn-SOD, copper- and zinc-containing SOD; Mn-SOD, manganese-containing SOD; EC-SOD, extracellular SOD.

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Table 2. Baseline MAP and HR in mice before ischemia-reperfusion

<table>
<thead>
<tr>
<th>Group</th>
<th>EC-SOD (^{-/-})</th>
<th>WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAP, mmHg</td>
<td>82.0±3.7</td>
<td>78.8±6.9</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>352.0±12.9</td>
<td>364.0±14.1</td>
</tr>
</tbody>
</table>

Values are means ± SE; \(n = 4\) mice/group. EC-SOD \(^{-/-}\), EC-SOD knockout mice; WT, wild-type mice; MAP, mean arterial blood pressure; HR, heart rate.

Statistical analysis. Changes in vessel diameter and blood flow are expressed as a percentage of baseline measurements. Changes in mRNA and protein levels of I/R-exposed muscles are expressed as percentages of normal muscle tissue levels. All values are represented as means ± SE. The vessel diameter and blood flow data were analyzed by two-way repeated-measures ANOVA. One-way ANOVA was used for the post hoc analysis of specific values at each time point of vessel diameter and blood flow changes and for the comparison of RT-PCR and Western blot results. A \(P\) value of <0.05 was considered to be statistically significant.

RESULTS

Systemic effects of EC-SOD deficiency. The MAP and HR values for EC-SOD \(^{-/-}\) and WT littermates are summarized in Table 2. There were no significant differences in basal hemodynamics from either group.

Measurement of vessel diameter. After 4.5 h of cremaster muscle ischemia, reperfusion-induced vasoconstriction reached a maximum at 10 min and gradually relaxed thereafter. These changes were more dramatic in EC-SOD \(^{-/-}\) mice, where 10- to 20-μm arterioles constricted to 53.0 ± 4.5% of baseline at 10 min and gradually recovered to a maximum level of 85.7 ± 1.0% at 90 min. WT mice had a more blunted response to reperfusion, with vessel diameters only decreasing to 76.6 ± 1.1% of baseline at 10 min and completely recovering back to 100.7 ± 0.5% at 90 min (Fig. 1A). Similar results were found in 21- to 40-μm arterioles (Fig. 1B) and 41- to 70-μm arteries (Fig. 1C). Significant differences \((P < 0.001)\) in the measurements of vessel diameter changes were also found between EC-SOD \(^{-/-}\) and WT mice at each time point of measurement.

Measurement of blood flow. The mean blood flow at 10 min of reperfusion was 16.4 ± 3.4% of baseline in EC-SOD \(^{-/-}\) mice vs. 56.2 ± 2.1% in WT mice. The blood flow gradually increased and reached 71.5 ± 3.3% of baseline in EC-SOD \(^{-/-}\) mice and 93.1 ± 1.2% in WT mice. Compared with WT mice, the muscle blood flow in EC-SOD \(^{-/-}\) mice was significantly reduced \((P < 0.001)\) at all time points of measurement (Fig. 2).

Histological examination. After I/R, we examined the muscle for pathological effects. We found venous congestion, platelet aggregation, extravascular hemorrhage, and PMN interstitial infiltration in both groups, but these findings were more prominent in EC-SOD \(^{-/-}\) mice (Fig. 3). In particular,
interstitium and muscle fiber edema associated with a disorganized myofibril arrangement were more obvious in the EC-SOD+/−/− mice compared with WT littermates. PMN counts per high-power field were 63.5 ± 5.1 in EC-SOD+/−/− mice vs. 42.2 ± 5.4 in WT mice, a finding that demonstrates the more prominent acute inflammatory reaction in EC-SOD+/−/− mice (P < 0.05).

Effect of I/R on SOD mRNA and protein expression. After I/R, CuZn-SOD mRNA levels decreased by 15% in EC-SOD−/− mice and by 10% in WT mice (Fig. 4). More prominently, Mn-SOD mRNA levels decreased by 50% in both groups. These differences were not significant within groups. In contrast, EC-SOD mRNA levels were elevated to 115.5 ± 12.5% of normal in WT mice. As expected, EC-SOD mRNA levels were undetectable in EC-SOD−/−/− mice (Fig. 4).

CuZn-SOD protein levels in the reperfused cremaster muscles decreased similarly in both EC-SOD−/− and WT mice (61.8 ± 5.7% and 59.3 ± 4.5%, respectively) (Fig. 5). Mn-SOD protein levels were unchanged in WT mice (100.0 ± 11.81%) and slightly increased to 117.1 ± 17.4% of normal in EC-SOD−/−/− mice, without a significant difference between the groups. Whereas EC-SOD protein was absent in EC-SOD−/−/− mice, EC-SOD protein levels decreased to 60.5 ± 9.0% in WT mice (Fig. 5).

DISCUSSION

Our results show that a deficiency of EC-SOD significantly increases cremaster muscle susceptibility to I/R injury in a mouse model of ischemia (4.5 h) and reperfusion (90 min). Despite similar basal hemodynamics, the cremaster muscle from EC-SOD−/− mice showed an increased susceptibility to I/R injury compared with that from WT mice. This was best demonstrated by a slower, incomplete recovery from vascular spasm and reduced blood flow and a more severe acute inflammatory reaction in the muscle of EC-SOD−/− mice. Molecular studies showed that after I/R EC-SOD mRNA levels increased in WT mice but intracellular SOD (CuZn-SOD and Mn-SOD) mRNA levels decreased in the cremaster muscles exposed to I/R event. Protein levels of these antioxidant enzymes after I/R were decreased for CuZn-SOD and EC-SOD but remained unchanged for Mn-SOD.

To the best of our knowledge, this is the first in vivo study to show a protective role of EC-SOD in skeletal muscle I/R injury. Our results are consistent with studies that have reported that EC-SOD−/− mice subjected to transient focal cerebral ischemia had a 81% greater infarct size compared with WT mice (52). Other evidence that transgenic EC-SOD overexpression in mice preserved myocardial function and attenuated infarct size after cardiac I/R injury (1, 9, 27, 28) and ameliorated infarct volume and cell death after cerebral I/R injury (53) also suggests that EC-SOD plays a key role in the pathogenesis of I/R injury.
Several potential mechanisms may explain our results. First, a complete absence of EC-SOD may amplify an imbalance in the ratio of O$_2^-$ produced vs. the ability to dismutate O$_2^-$. Most studies suggest that increased levels of ROS enhance vasoconstriction (29, 37), although exceptions do occur (40). The generation of ROS after I/R is strongly implicated in microvascular damage and dysfunction, leading to vasoconstriction and leukocyte adhesion on the endothelial surface (5). SOD and allopurinol have been shown to protect contractile function of skeletal muscle against I/R injury (39). In the present study, the protective effect of EC-SOD, which attenuates cellular toxicity of O$_2^-$ in WT mice (7), is absent in EC-SOD$^{-/-}$ mice. Second, EC-SOD null mice may have reduced bioactivity of NO, because the resulting increased levels of O$_2^-$ may react with NO to form peroxynitrite. This nearly diffusion-limited reaction between NO and O$_2^-$ (4, 46) limits NO-dependent vascular homeostasis and vessel relaxation during I/R insult. The high concentration of EC-SOD localized to the vascular interstitial space between the endothelium and smooth muscle likely further augments this function (41). This is the same intercellular space that endothelium-derived NO must pass through to stimulate smooth muscle relaxation. By reducing levels of vascular O$_2^-$ (22), EC-SOD is thought to preserve NO-mediated vessel relaxation (14, 24). The absence of EC-SOD, however, results in the loss of this function. Finally, EC-SOD-null mice may exaggerate inducible nitric oxide synthase (iNOS)-mediated tissue damage. We previously showed that I/R leads to increased expression of iNOS in skeletal muscle, whose enzymatic activity was attenuated by the selective use of an iNOS inhibitor (44, 48). This iNOS inhibition significantly reduced muscle damage and preserved muscle contractile function. It is known that infiltrating leukocytes and macrophages are major cellular sources of iNOS. Similar to endothelial nitric oxide synthase in the vessel wall, excessive NO produced by iNOS reacts with O$_2^-$ to form the toxic peroxynitrite species that could lead to muscle fiber damage. EC-SOD-null mice would be predicted to increase peroxynitrite formation, thereby exaggerating iNOS toxicity to tissues.

The generation of ROS occurs rapidly during reperfusion of ischemic tissue, peaking in <20s (2). Thus, because EC-SOD$^{-/-}$ mice have reduced blood flow and increased arterial tone during reperfusion, it might be predicted that EC-SOD$^{-/-}$ mice have reduced generation of O$_2^-$, thereby providing protection to the tissue. Our results, however, do not appear to support this possibility, as histological analysis of the muscle revealed more severe tissue damage and inflammation in the muscle of EC-SOD$^{-/-}$ mice compared with WT mice. Specifically, worse reperfusion injury accompanied with upregulated creatine phosphokinase (a marker of tissue damage) (39) and MPO activity (a marker of leukocyte adhesion) (26) has been reported, supporting a parallel relationship between tissue damage and microcirculation during I/R.

Under physiological conditions, endogenous CuZn-SOD and Mn-SOD are sufficient to handle intracellularly generated O$_2^-$ (59). During sustained ischemia, however, an anoxic environment damages tissue and appears to disturb intracellular SOD activities (8, 57). Our findings of decreased expression of CuZn-SOD and Mn-SOD mRNA in both groups appear to reasonably support this concept. During reperfusion, activated PMNs produce large amount of O$_2^-$. Under such conditions, decreased levels of intracellular SODs may magnify the imbalance of oxidants/antioxidants in favor of oxidative injury. Thus the more severe I/R injury is in the EC-SOD$^{-/-}$ mice rather than the WT mice. Furthermore, EC-SOD mRNA is upregulated in the WT mice, which supports the idea that there is an increased need for EC-SOD to scavenge excessive O$_2^-$ during reperfusion. Although EC-SOD mRNA levels were increased in WT mice, this was not translated into elevated levels of EC-SOD protein expression. We speculate that there might be a time lag between transcriptional and translational regulation of EC-SOD gene expression, and the 90 min of reperfusion in this study may not be sufficient to complete this process.

It is not completely understood how EC-SOD attenuates I/R injury and how exogenous administration of SOD protects against intracellular events (18, 23, 51). The three isoforms of SOD are highly compartmentalized and cannot readily change their positions across the cell membrane, even in the cell (13, 21), although O$_2^-$ has been reported to cross cell membranes via an anion exchange channel (38, 50). Although this may not routinely occur under physiological conditions because of a relative excess of SOD over O$_2^-$, I/R-induced cell damage might allow this movement more easily, resulting in diffusion...
of O$_2^-$ between intra- and extracellular compartments. Therefore, under pathological conditions such as I/R, extracellularly positioned EC-SOD may play an important role in scavenging excessive O$_2^-$ diffusing from an intracellular source, whereas the activity of intracellular SODs is restricted because of sustained ischemia. Specifically, the extracellular position of EC-SOD may be more effective in guarding cells against extracellularly generated O$_2^-$.

In summary, the present study demonstrates that EC-SOD plays a very important role in preserving skeletal muscle function after I/R injury. Upregulation of EC-SOD mRNA in the reperfused muscle of WT mice suggests that there is an increased need for EC-SOD in the defense mechanism against I/R injury, whereas the expression of intracellular SODs (CuZn-SOD and Mn-SOD) are depressed as a consequence of I/R. Thus enhancing endogenous EC-SOD activity may provide a potential therapeutic strategy for the treatment and prevention of skeletal muscle injury after I/R.

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