Reperfusion injury in skeletal muscle is reduced in inducible nitric oxide synthase knockout mice

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Reperfusion injury in skeletal muscle is reduced in inducible nitric oxide synthase knockout mice. J Appl Physiol 97: 1323–1328, 2004. First published June 4, 2004; 10.1152/japplphysiol.00380.2004.—Inducible nitric oxide synthase (iNOS) participates in many pathological events, and selective inhibition of iNOS has been shown to reduce ischemia-reperfusion (I/R) injury in different tissues. To further confirm its role in this injury process, I/R injury was observed in denervated cremaster muscles of iNOS-deficient (iNOS−/−) and wild-type mice. After 3-h ischemia and 90-min reperfusion, blood flow in reperfused muscle was 80 ± 8.5% (mean ± SE) of baseline at 10-min reperfusion and completely returned to the preischemia baseline after 20 min in iNOS−/− mice. In contrast, blood flow was 32 ± 7.4% at 10 min and increased to 60 ± 20% of the baseline level at 90 min in wild-type mice (P < 0.001 vs. iNOS−/− mice at all time points). The increased muscle blood flow in iNOS−/− mice was associated with significantly less vasospasm in all three sizes of arterial vessel size categories. The weight ratio to the contralateral muscle not subjected to I/R was greater in wild-type mice (173 ± 11%) than in iNOS−/− mice (117 ± 3%; P < 0.01). Inflammation and neutrophil extravasation were also more severe in wild-type mice. Western blot analysis demonstrated an absence of iNOS protein band in iNOS−/− mice and upregulation of iNOS protein expression in wild-type mice. Our results confirm the importance of iNOS in I/R injury. Upregulated iNOS exacerbates I/R injury and appears to be a therapeutic target in protection of tissues against this type of injury.

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ILE. The cremaster muscle was prepared according to our laboratory’s previously described technique (27, 34, 48). The muscle was exposed, opened, and then separated from the testis. The pudic-epigastric artery and vein and the genitofemoral nerve were isolated and separated from each other. The cremaster muscle sac was cut circumferentially, rendering the muscle totally isolated but still attached to the body by its neurovascular pedicle. A 3-mm segment of the genitofemoral nerve was removed proximally to the muscle. The isolated muscle was then spread flat onto the surface of a transparent acrylic microscope stage and secured with five peripheral sutures. The exposed surface of the muscle was moistened with warm (34 ± 0.5°C) lactated Ringer solution (Abbott Laboratories), covered with a thin layer of oxygen-impermeable plastic polymer (Saran Wrap) to prevent diffusion of oxygen and other gases from the environment to the muscle, and warmed by using a heat lamp. The muscle preparation was left undisturbed for 30 min before a baseline measurement was made to allow any effect of the muscle isolation procedure to dissipate. Three hours of complete warm ischemia were achieved by clamping the main vascular pedicle of the isolated muscle by using a microvascular clamp (ST-B-1 VB, ASSI, Westbury, NY). After completion of the ischemic period, the clamp was removed, and the muscle underwent 90 min of reperfusion.

**Measurement of blood flow.** See Refs. 34 and 48. Overall blood flow of the cremaster muscle was measured with laser Doppler flowmetry (MBF3, Moor Instruments, Devon, UK). A 1-mm-diameter double-fiber probe was positioned by a manipulator (MM 33, Stoeltling, Wood Dale, IL) throughout the consecutive measurements in eight animals from each group. Via a small window on the covering plastic polymer, the tip of the probe was placed as close as possible to the surface of the vascular pedicle of the isolated muscle without causing a compressive effect on the vasculature. The position of the laser probe tip was kept constant throughout the experiment. During reperfusion, blood flow was recorded every 10 min for 90 min. Background flux detected on the vascular pedicle during ischemia was subtracted from measurements.

**Measurement of vessel diameter.** See Refs. 34 and 48. Using intravital microscopy (Zeiss binocular microscope, model 473028) equipped with a video monitor (Sony monitor, model PVM-1343MD, a Sony Beta ED video cassette recorder, model EDW-30F), we measured vessel diameters from 10 to 70 μm in a selected arteriolar tree of each muscle (n = 8 in each group) during the reperfusion phase of the experiment. Ten to twenty vessel measurement sites within the vascular tree were selected in each muscle. Sequential measurements were taken at the same sites throughout the experiment. The internal luminal diameter of this selected vessel site was measured from a recorded image by using a FOR/A IV-560 video-measuring gauge. The exposed surface of the muscle was divided into three categories: small arterioles (10–20 μm), large arterioles (21–40 μm), and small arteries (41–70 μm). During reperfusion, the vessel diameters were measured every 10 min for 90 min.

**Histology.** After weighing was completed, the left cremaster from each animal was immersed in 10% formaldehyde and then histologically examined by using a hematoxylin and eosin stain. Each animal was immersed in 10% formaldehyde and then histologically examined by using a hematoxylin and eosin stain. After weighing was completed, the right cremaster was homogenized in boiling lysis buffer and insoluble material was then discarded. Protein content was determined by using a BCA Protein Assay Reagent (Pierce, Rockford, IL). Proteins (30 μg) were loaded onto NuPAGE 4–12% bis–(2-hydroxyethyl)amino-tris(hydroxymethyl)methane gel (Invitrogen, Carlsbad, CA). After proteins were transferred onto polyvinylidene difluoride membrane, the membrane was blocked by 5% milk Tris-buffered saline-Tween 20 for 1 h, incubated at 4°C overnight with primary antibody (Ab) [monoclonal Ab for nNOS (1:1,500; BD Transduction, San Diego, CA) and eNOS (1:1,000; BD Transduction) and polyclonal Ab for iNOS (1:750, Upstate, Lake Placid, NY)] and then incubated with second Ab [goat anti-mouse IgG + IgM-horse-radish peroxidase (1:5,000, Pierce) for nNOS and eNOS and goat anti-rabbit IgG-horseradish peroxidase (1:12,000, Santa Cruz Biotechnology, Santa Cruz, CA) for iNOS] for 1 h at room temperature. Immunoreactivity was detected with a SuperSignal West Pico Chemiluminescent Substrate detection kit (Pierce). Kodak film was used to visualize chemiluminescent signals. A mouse anti-actin monoclonal Ab (1:1,000, Chemicon, Temecula, CA) was used to detect actin as an internal control protein to demonstrate that all wells were loaded with equal amounts of cytoplasmic protein. The density was measured by Scion Image program.

**Statistical analysis.** Vessel diameter for each vessel site and blood flow changes for each muscle at each time point were expressed as percentages of the baseline value. All values are expressed as means ± SE. Statistical analysis was performed by a repeated-measures two-way ANOVA. The post hoc analysis of specific values at each time point was performed by one-way ANOVA. A P value < 0.05 was considered statistically significant. For the measurement of muscle wet weight ratio and NOS protein expressions, statistical analysis was performed by a one-way ANOVA. A P value of < 0.05 was considered statistically significant.

**RESULTS**

**Measurement of blood flow.** At 10 min of reperfusion, the cremaster muscle blood flow was 32 ± 7.4% (mean ± SE) of baseline in the wild-type mice group and 80 ± 8.5% in the iNOS−/− group. Whereas the blood flow in the wild-type group gradually increased to a maximum of 60 ± 20% at 90 min, blood flow in the iNOS−/− group reached baseline level at 20 min and remained at that level throughout the experiment. Blood flow was significantly greater in the iNOS−/− group than in wild-type animals at all time points (P < 0.01 to <0.001) (Fig. 1).

**Measurement of vessel diameter.** The average baseline vessel diameters are summarized in Table 1, with no significant difference in each vessel size category between the iNOS−/− and wild-type groups. As shown in Fig. 2, the average vessel diameters in the wild-type group were between 39 and 59% of baseline at 10 min of reperfusion and increased to a maximum of 72 ± 4% in 10- to 20-μm, 71 ± 5% in 21- to 40-μm, and 58 ± 4% in 41- to 70-μm vessels in the iNOS−/− group. Whereas the blood flow in the wild-type group gradually increased to a maximum of 60 ± 20% at 90 min, blood flow in the iNOS−/− group reached baseline level at 20 min and remained at that level throughout the experiment. Blood flow was significantly greater in the iNOS−/− group than in wild-type animals at all time points (P < 0.01 to <0.001) (Fig. 1).

**Fig. 1.** Blood flow in denervated cremaster muscle of inducible nitric oxide synthase-deficient mice (iNOS−/−) and wild-type mice (n = 8 for each) during 90-min reperfusion following 3 h of ischemia. Values are means ± SE. **P < 0.01 and ***P < 0.001 compared with wild-type mice.
maximum diameters of 92% of baseline in each vessel category at 10 min and reached diameters in the iNOS

Fig. 2. Vascular diameter changes in denervated cremaster muscles of
64 ± 4% in 41- to 70-μm vessels at 90 min. In contrast, the diameters in the iNOS−/− group sharply increased to >70% of baseline in each vessel category at 10 min and reached maximum diameters of 92 ± 2.8, 88 ± 2.7, and 86 ± 2.3% at

Table 1. Baseline values of vessel diameters in the iNOS−/− and wild-type groups

<table>
<thead>
<tr>
<th>Group</th>
<th>10–20 μm</th>
<th>21–40 μm</th>
<th>41–70 μm</th>
</tr>
</thead>
<tbody>
<tr>
<td>iNOS−/−</td>
<td>15.5 ± 0.7</td>
<td>26.5 ± 1.3</td>
<td>49.0 ± 2.6</td>
</tr>
<tr>
<td>Wild-type</td>
<td>14.9 ± 0.7</td>
<td>28.2 ± 1.2</td>
<td>47.1 ± 2.5</td>
</tr>
</tbody>
</table>

Values are means ± SE. iNOS, inducible nitric oxide synthase.

90 min, with a significant (P < 0.05 to <0.001) difference at each time point.

Wet weight ratio evaluation. The weight ratio (percentage of normal) of the muscles was 173 ± 11% in the wild-type group and 117 ± 3% in the iNOS−/− group, with a significant difference (P < 0.01) between the two groups (Fig. 3).

Histological evaluation. There was normal skeletal muscle cell shape and no thrombosis in the lumen of vessels in both iNOS−/− and wild-type animals. However, there were reduced edema, muscle necrosis, and neutrophil extravasation in the iNOS−/− group compared with the wild-type group (Fig. 4).

NOS protein expression. The band of iNOS protein was not detected in normal and reperfused cremaster muscle in iNOS−/− mice, whereas iNOS protein expression was upregulated from normal in the reperfused muscle in wild mice (Fig. 5). For nNOS and eNOS proteins, the expression was downregulated to ~50–60% of normal in the reperfused cremaster muscle in both iNOS−/− and wild mice. There was no significant difference between the two groups (Fig. 5).

DISCUSSION

In a cremaster muscle model of I/R injury, iNOS−/− mice demonstrated significantly less vasospasm, complete restoration of blood flow, reduced muscle fiber necrosis on histological examination, and decreased edema, as indicated by wet weight ratio, compared with wild-type mice. Those alternations were accompanied by an undetectable band of iNOS protein in the muscle in iNOS−/− mice. Therefore, I/R injury is clearly reduced in iNOS−/− mice in which the iNOS gene has been selectively eliminated.

Our results have confirmed that iNOS is an important factor in the amplification of I/R injury in skeletal muscle, which was previously suggested by studies using the relatively selective iNOS inhibitor dexamethasone (7) or highly selective iNOS inhibitor 1400W, which significantly increased blood flow, reduced vasoconstriction and tissue edema, and preserved muscle contractile function (32, 34, 48). Although 1400W is a highly selective inhibitor of iNOS, there is an unavoidable redundancy in functional actions to nNOS and eNOS isoforms. In fact, 1400W has been shown to have weak inhibition for nNOS and eNOS (12), specifically under a condition of dramatic upregulation of nNOS and eNOS (32). Thus the exact role of iNOS in I/R injury requires further direct confirmation. In this regard, the present study has shown a reduced I/R injury in iNOS−/− mice, in which the iNOS gene is eliminated by state-of-the-art gene knockout technology. Because the control

Fig. 3. The wet weight ratio (means ± SE) of denervated cremaster muscles of iNOS−/− and wild-type mice (n = 16 for each) at the end of 90-min reperfusion. ** P < 0.01 compared with wild-type mice.
mice and iNOS−/− mice were of the same genetic background and have no known genotypic differences other than the absence of the iNOS gene, the deficiency of this enzyme appears responsible for the reduction of I/R injury in the cremaster muscle.

iNOS has been identified as a major source of NO generation in tissues. It has been reported that ~80% of the basal NO in the respiratory tract originates from iNOS and that 99% of NOS activity is attributable to iNOS after ovalbumin challenge (8). In skeletal muscle, most of the NO production during I/R injury may be generated by iNOS (30). Although the exact role of iNOS in I/R injury is controversial (23), high concentrations of NO produced by iNOS can affect many cellular functions simultaneously by changing ion currents (11), inhibiting cellular respiration (44) and various enzyme activities (45), and mediating DNA damage (24). iNOS appears to be responsible for nitrotyrosine formation, which is a nitration product of tyrosine and is a possible biomarker for cytotoxicity of NO in I/R injury (15). iNOS-mediated apoptosis appears to be through nitrosative mechanisms (10). Other reactive nitrogen species generated from iNOS, including peroxynitrite, may also contribute to I/R injury (43). Furthermore, iNOS may cause alterations in the expression or function of proteins involved in the regulation of tissue function (41). Thus activated iNOS leads to excess NO production, subsequently potentiating I/R injury and the resulting dysfunction and necrosis of reperfused tissues.

Our findings are in agreement with those of others who demonstrated that targeted disruption of the iNOS gene could protect tissues against ischemic and I/R injury. After 70-min tourniquet ischemia and 24-h reperfusion, 40% of muscle fiber necrosis was found in the gatrocnemius muscle in wild-type mice, but this severity of necrosis was seen in iNOS−/− mice only after 90-min ischemia (2). Improved ventricular contractile function and reduced apoptosis were demonstrated in iNOS−/− mice after myocardial infarction (35). Similarly, the infarct and the motor deficits produced by occlusion of the middle cerebral artery were smaller in iNOS−/− mice compared with those in wild-type mice (19). Attenuated renal I/R injury (26), increased resistance to mucosal injury and intestinal I/R-induced bacterial translocation (39), increased microvascular reactivity and improved survival in septic shock (17), diminished myocardial dysfunction in endotoxin-induced murine sepsis (41), and improved apoptosis in ischemic proliferative retinopathy (36) have also been reported in iNOS−/− mice compared with wild-type mice. Furthermore, the protective role in iNOS−/− mice exhibits a gene-dose effect in ischemic brain injury (49). Taken together, the data strongly support the conclusion that upregulated iNOS has detrimental effects on I/R injury to tissues.
Our results, however, are different from those of other studies in which flow, function, and infarct size were not significantly different in isolated, reperfused heart between wild-type and iNOS−/− mice (46). Moreover, evidence showed that NO generated from iNOS might contribute to protection of heart function in isolated mouse heart (13). Extensive myocardial injury associated with a higher mortality rate and neutrophil infiltration were found in the iNOS−/− mouse heart after I/R compared with wild-type mice (50). A similar conclusion was reported in liver I/R injury (14). It appears that different experimental conditions can affect the molecular reactivity of NO. These variables include differences in animal species, rate and timing of NO release, type of tissue, surgical skill, extent of tissue damage, and, consequently, the cellular environment of target molecules. It is noted that the denervated cremaster muscle underwent 3 h of ischemia in the present study and 20 or 60 min of ischemia in those myocardial studies.

Knockout mice are useful tools for studying the contribution of individual NOS isoform to specific pathophysiological processes and can provide many insights into the complex roles of NO (18, 31). Targeted gene disruption or transgenic overexpression will likely augment pharmacological approaches to define the roles of particular gene products in normal tissue function and in pathophysiology of disease processes (18). However, disruption of a single gene may have systemically significant effects, such as increased susceptibility to pathogens in iNOS−/− mice, in addition to those being studied (31). In addition, a potential adaptive mechanism between NOS genes should be considered. Although this adaptation was not found in the present study (Fig. 5), recent data from an ex vivo heart study showed a remarkable iNOS induction in I/R myocardium in eNOS−/− mice (21). Furthermore, the role of NO production generated from NOS-independent pathways (25) should also be considered when evaluating I/R injury in NOS−/− mice. Thus future work needs to include direct measurement of NO to determine the exact mechanism by which iNOS exerts its effect in I/R injury.

In summary, the present study shows that, compared with wild-type mice, I/R injury is reduced in iNOS−/− mice during early reperfusion. Combined with earlier results from inhibiting the iNOS isoform, our results conclusively demonstrate that iNOS is deleterious to I/R injury in skeletal muscle and that selective inhibition of iNOS protects tissues against I/R injury. Whereas our results have demonstrated that knockout mice are valuable tools for the study of the mechanisms of pathophysiological processes, one must be cognizant of unknown systemic effects and adaptive mechanisms related to the genetic manipulation of the knockout animals.

GRANTS

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