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


Surgical preparation of the cremaster muscle. The mice were anesthetized with an intraperitoneal injection of pentobarbital sodium (Nembutal, 50 mg/kg body wt; Abbott Laboratories, North Chicago, IL). The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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arterioles (21–divided into three categories: small arterioles (10–lnuminal diameter of each selected vessel site was measured from a
were taken at the same sites throughout the experiment. The internal
vascular tree were selected in each muscle. Sequential measurements
of each muscle (n = 8 in each group) during the reperfusion phase of
ischemia was 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
period, the clamp was removed, and the muscle underwent
reperfusion, blood
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 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 consid-
ered 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 ves-
sel 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

Histology. After weighing was completed, the left cremaster from
each animal was immersed in 10% formaldehyde and then histolog-
ically examined by using a hematoxylin and eosin stain.

Determination of expressions of NOS protein. See Ref. 34. After
we completed measurement of the vessel diameters and blood flow,
four muscle samples were harvested and frozen at −80°C immedi-
ately. The muscle samples were homogenized in boiling lysis buffer
(1% Triton X-100, 10 mM sodium orthovanadate, 10 mM Tris, pH 7.4) and
microwaved for 10–15 s. The homogenate was centrifuged, and
insoluble material was then discarded. Protein content was determined
by using Coomassie Plus 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
Fluorescent Tissue Bonding (Abbott Laborato-
ries), covered with a thin layer of oxygen-
permeable plastic (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

Measurement of blood flow. See Refs. 34 and 48. Overall blood
flow of the cremaster muscle was measured with laser Doppler
metry (MBF3, Moor Instruments, Devon, UK). A 1-mm-diameter
double-fiber probe was positioned by a manipulator (MM 33, Stoel-
ing, 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 arterial
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 each selected vessel site was measured from a
recorded image by using a FORA IV-560 video-measuring gauge.
According to the baseline diameters, the observed vessels were
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.

Wet weight ratio evaluation. To determine the extent of IR-
induced tissue edema, the cremaster muscles from both sides were
removed at the end of each experiment (n = 16 in each group), and
their weights were measured. The differences between these two
groups were expressed as a percentage of the weight of the opposite
nontreated cremaster muscle.

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 92/11006 of baseline in each vessel category at 10 min and reached diameters in the iNOS/11002/iNOS/11002/11002 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 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

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

<table>
<thead>
<tr>
<th>Group</th>
<th>Vessel Diameter</th>
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<tr>
<td></td>
<td>10–20 μm</td>
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<tr>
<td>iNOS−/−</td>
<td>15.5 ± 0.7</td>
</tr>
<tr>
<td>Wild-type</td>
<td>14.9 ± 0.7</td>
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Values are means ± SE. iNOS, inducible nitric oxide synthase.

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
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 cel-
lular 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 nitrative 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 acti-
vated iNOS leads to excess NO production, subsequently
potentiating I/R injury and the resulting dysfunction and ne-
crosis 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 con-
tactile function and reduced apoptosis were demonstrated in
iNOS−/− mice after myocardial infarction (35). Similarly, the
infarct and the motor de


cicits 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 intesti-
nal I/R-induced bacterial translocation (39), increased micro-
vascular reactivity and improved survival in septic shock (17),
diminished myocardial dysfunction in endotoxin-induced mu-
rine sepsis (41), and improved apoptosis in ischemic prolifer-
active retinopathy (36) have also been reported in iNOS−/−
mice compared with wild-type mice. Furthermore, the protec-
tive 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 isoforms 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 systematically 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 generic manipulation of the knockout animals.

GRANTS

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REFERENCES


