Effects of S-nitroso-N-acetylcysteine on contractile function of reperfused skeletal muscle

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The Orthopaedic Research Laboratories, Department of Surgery; and 2Howard Hughes Medical Institute, The Pulmonary and Cardiovascular Divisions, Departments of Medicine and Cell Biology, Duke University Medical Center, Durham, North Carolina 27710

Chen, Long-En, Anthony V. Seaber, Rima M. Nasser, Jonathan S. Stamler, and James R. Urbaniaik. Effects of S-nitroso-N-acetylcysteine on contractile function of reperfused skeletal muscle. Am. J. Physiol. 274 (Regulatory Integrative Comp. Physiol. 43): R822–R829, 1998.—The ultimate goal of replantation and microsurgical reconstructive operations is to regain or improve impaired function of the tissue. However, the data related to the influence of NO on tissue function are limited. This study evaluated the effects of the NO donor S-nitroso-N-acetylcysteine (SNAC) on contractile function of skeletal muscle during reperfusion. Forty-nine rats were divided into six groups. The extensor digitorum longus (EDL) muscles in groups I and II were not subjected to ischemia-reperfusion but were treated with a low (100 nmol/min) or high (1 µmol/min) dose of SNAC. In groups III–V, the EDL underwent 3 h of ischemia and 3 h of reperfusion and was also treated with low (100 nmol/min) or high doses (1 or 5 µmol/min) of SNAC. Group VI was a phosphate-buffered saline (PBS)-treated control group. Twenty additional animals were used to document systemic effects of SNAC and PBS only. SNAC or PBS was infused for 6.5 h, beginning 30 min before ischemia and continuing throughout the duration of reperfusion. Contractile testing compared the maximal twitch force, isometric tetanic contractile forces, fatigue, and fatigue half time of the experimental EDL and the contralateral nontreated EDL. The findings indicate that 1) SNAC does not influence contractile function of EDL muscle not subjected to ischemia-reperfusion, 2) SNAC significantly protects the contractile function of ischemic skeletal muscle against reperfusion injury in the early reperfusion period, and 3) the protective role of SNAC is critically dosage dependent; protection is lost at higher doses. The conclusion from this study is that supplementation with exogenous NO exerts a protective effect on the tissue against reperfusion injury.

nitric oxide; muscle force; ischemia; rat

IT IS NOW WELL ACCEPTED that NO, a signaling molecule, is involved in many important physiological and pathological functions, including vessel relaxation, neurotransmission, and pathogen suppression (5).

NO is synthesized from L-arginine by NO synthase (NOS). Three major NOS isoforms exist. NOS 1 is a constitutive isoform found in the central and peripheral nervous systems, epithelial cells, and skeletal muscle (11). Neurotransmission, bronchodilation, glucose transport, and regulation of force development are among its functions. NOS 2 (iNOS) is induced by endotoxins and cytokines in most cells (20). This enzyme is believed to release larger amounts of NO (nitrogen oxides), which may be important in host defense (20). NOS 3 is a Ca2+-calmodulin-dependent constitutive isoform found in vascular endothelial cells, some neuronal populations, platelets, cardiac muscle, and skeletal muscle. Its major activities include relaxation of vascular smooth muscle, inhibition of platelet activation and adhesion, and control of cell respiration (16, 24).

Although many studies have examined the role of NO in ischemia-reperfusion injury of cerebral, myocardial, mesenteric, gastric, and lung tissues (2, 17, 19), the results are paradoxical and no consensus has been reached. In skeletal muscle, the one existing report suggested that NO production during reperfusion injury may be deleterious to survival of muscle tissue (26). However, it has become apparent that the success of replantation and microsurgical reconstructive operations should not be measured by survival alone but also by quantification of subsequent function. The ultimate goal is to regain normal function or, at least, to improve impaired function. To our knowledge, there are no data in the literature pertaining to functional regulation by NO in the reperfused skeletal muscle. The present study was designed to test the hypothesis that NO influences the contractile function of reperfused skeletal muscle.

MATERIALS AND METHODS

Sixty-nine male Sprague-Dawley rats with body weights ranging from 90 to 120 g were used. The systemic influence of infusion was studied in 20 rats. The remaining 49 animals were randomly divided into six groups in which the extensor digitorum longus (EDL) muscle was used for evaluating contractile function. The animal grouping and experimental protocol are summarized in Table 1.

Anatomy of the EDL. The EDL is a fusiform muscle located in the deep layer between the peroneus longus muscle and the tibialis anterior muscle. It arises from the lateral epicondyle of the femur and inserts on the base of the third phalanx of digits two to five. In animals of the size used in this study, the EDL has an average length of 23.5 ± 1.2 mm and an average weight of 48 ± 2 mg (4). Ordinarily, there are three nutritional branches entering the EDL arising from the anterior tibial artery at the proximal one-third of the muscle. Branches of the deep peroneal nerve accompany the vessel branches to the muscle.

Surgical procedure. Procedures were based on those of Bolognesi et al. (4). Each animal was anesthetized with intraperitoneal Nembutal (Abbott Laboratories, North Chicago, IL) at a dose of 50 mg/kg body wt. The left femoral artery was cannulated in 20 rats for monitoring systemic influence. Blood pressure, heart rate (HR), and respiratory rate (RR) in response to the infused drugs were monitored by a patient monitor (MR-1300, Mennen Medical, New York, NY) at 10-min intervals for 6.5 h. These 20 rats were not subjected to other surgical procedures or to ischemia-reperfusion.
S-nitroso-N-acetylcysteine (SNAC; 100, 1, and 5 μmol/min) and phosphate-buffered saline (PBS) were administered through the distal end of the muscle connected to an amplifier. The distal end was attached to the isometric force transducer (Radnoti Monrovia, CA). The proximal end of the muscle was attached by the suture tail to an isometric force transducer (Radnoti Glass Technology, Monrovia, CA). The tissue bath was filled with Krebs solution (pH 7.4, concentration in mM: 122.0 NaCl, 4.7 KCl, 1.2 MgCl2, 2.5 CaCl2, 15.4 NaHCO3, 1.2 KH2PO4, and 5.5 glucose). The solution was aerated with a 95% O2-5% CO2 gaseous mixture, maintained at 37°C with the help of a Cole-Parmer polystat circulator, and changed at 15-min intervals (4).

The muscle contractile test was performed with the EDL muscle at its optimal contractile length, which was adjusted by rotating the micrometer head to lengthen and shorten the muscle belly length. Electrical stimulation provided by a neurostimulator (model S44B, Grass Instruments, Quincy, MA), with a duration of 0.2 ms and a delay of 2 ms, was delivered to the muscle through platinum electrodes using a gradually increasing stimulus voltage until the maximal twitch force was achieved and recorded. Isometric tetanic contractile forces were then measured using the optimal muscle length and the rate of force rise required to create the previously obtained maximal twitch force, with a duration of 2 ms and a delay of 2 ms. The tetanic contractile testing was tested at frequencies of 40, 70, 100, and 120 Hz, with 1.5 s for each stimulation; a 3-min rest period followed each stimulation. After the final 1.5-s train, the muscle was allowed to rest for 10 min before a fatigue test was begun. Fatigue was induced by stimulation for 30 s at 70 Hz. The experimental protocol was carried out in <40 min to limit tissue damage.

All force measurements were recorded on a chart recorder (MFE model 8000, Beverly, MA). The average of the maximal twitch force, isometric tetanic forces, and the fatigue testing peak value of the treated EDL were compared with those of the contralateral nontreated EDL, and the results were expressed as a percentage of the contractile force achieved by the contralateral nontreated EDL. One-way analysis of variance (ANOVA) and then repeated-measures analysis of variance were performed. Bonferroni correction was applied where appropriate. A P < 0.05 was taken as significant. Furthermore, a fatigue half time (FT1/2) was calculated from the recorded fatigue curve.

Histological examination. Two EDLs each from groups III and VI were harvested for histological examination. A piece of the muscle measuring 1.0 × 0.3 × 0.3 cm was excised and fixed to a wooden bar to prevent contraction of the muscle. The preparation was immediately immersed in 4% glutaraldehyde buffered with 0.1 M sodium cacodylate for transmission electron microscopy (TEM) examination. The remaining muscle was fixed in 10% formaldehyde and histologically examined using phosphotungstic acid hematoxylin (PTAH) and hematoxylin and eosin stain.

### RESULTS

Systemic effects of SNAC infusion. During infusion, mean arterial pressure (MBP), HR, and RR gradually decreased in both the low-dose SNAC (100 nmol/min) and PBS-treated animals, with no significant difference between the two treatments. Average BP fell 12% in SNAC-treated rats and 11% in PBS-treated rats (Fig. 1). HR fell 8 and 5%, respectively, and RR fell 17 and 12%, respectively. Compared with the same parameters at initiation of infusion and after SNAC infusion, there was no significant difference in MBP, HR, or RR.

In 1 μmol/min-SNAC-treated animals, MBP fell beginning 20 min after the commencement of SNAC infusion.
and reached the lowest level (17% decrease) at 60 min. From 160 min onward, MBP increased and gradually recovered to a nearly normal level (Fig. 1). There was no significant difference in MBP at any time compared with low-dose SNAC or PBS-treated animals. RR and HR in these rats were also slightly lower than those in low-dose SNAC- or PBS-treated rats from 80 min, but the difference did not reach significance.

At a SNAC dose of 5 µmol/min, MBP exhibited a 22% decrease at 20 min of infusion and reached the lowest level (31% decrease) at 80 min. MBP remained at this low level until 220 min and subsequently gradually increased (Fig. 1). A significant (P < 0.05) decrease was present between 20 and 120 min compared with low-dose SNAC- and PBS-treated animals. RR and HR in these rats were also slightly lower than those in low-dose SNAC- or PBS-treated rats from 80 min, but the difference did not reach significance.

Influence of SNAC dosage on contractile function in the EDL not subjected to ischemia-reperfusion (groups I and II). After 6.5 h of 100 nmol/min SNAC infusion (group I), the average maximal twitch force in the infused right EDL was 91 ± 9% (mean ± SE) of the contralateral non-treated muscle force. The average isometric tetanic contraction was 114 ± 8% of the contralateral non-treated EDL force at 40-Hz stimulation and near 100% at the remaining three stimulations. When SNAC concentration was increased to 1 µmol/min (group II), the maximal twitch force was 110 ± 6% and the isometric tetanic force remained near 100% at all four stimulation frequencies. There were no statistically significant differences between groups I and II. There was no statistically significant difference in the absolute contractile tension between the right EDL with SNAC infusion and the left EDL without SNAC infusion in either group I or group II. After fatigue induction, peak tension and FT₁/₂ were 92 ± 2% and 10.7 s in group I and 88 ± 6% and 7.8 s in group II, respectively (average contralateral FT₁/₂ was 9.3 s; P = NS).

Influence of SNAC on contractile function in EDL after ischemia-reperfusion (groups III and VI). The average maximal twitch force was 46 ± 3% of the contralateral nontreated EDL in the 100 nmol/min-SNAC-infused EDL (group III) and 15 ± 3% in the PBS-infused EDL (group VI). This difference was highly significant (Fig. 2). The average isometric tetanic force was 43 ± 4% in group III at 40-Hz stimulation and 59 ± 5, 80 ± 4, and 91 ± 1% at 70-, 100-, and 120-Hz stimulation, respectively. The relative values were 15 ± 4, 25 ± 7, 36 ± 6, and 42 ± 8% in group VI, respectively. When these two groups were compared, the contractile force in SNAC-treated group III muscles was found to be significantly greater than that in the PBS-treated group VI muscles at all four stimulation rates (Fig. 3). After fatigue induction, test peak tension in group III was 86.6 ± 0.96%, whereas it was only 36.3 ± 5.9% in the PBS group. This difference was highly significantly different (Table 2). The average FT₁/₂ was 7.3 s in group III and only 4 s in group VI, with a significant difference.

Influence of SNAC dosage on contractile function in EDL after ischemia-reperfusion (groups III-VI). Although the maximal twitch force was significantly greater in all three SNAC-treated groups than in the PBS-treated group, the higher doses of SNAC (1 and 5 µmol/min) were less effective than the lower dose (100 nmol/min). Groups IV (1 µmol/min) and V (5 µmol/min) did not differ significantly from one another in the degree of protection conferred (Fig. 2). Measurement of the isometric tetanic contractile forces showed similar
and that NOS activity varies among several respiratory and limb muscles (15, 16, 22). Immunohistochemistry has shown that NOS activity in rat muscles correlates with type II fiber density (15). Because the EDL is a fast muscle composed mainly of type II fibers, it was hypothesized that NO may play a role in the pathophysiology of skeletal muscle injury.

The effect of ischemia-reperfusion on NO production is controversial. Although some investigators have reported that ischemia-reperfusion decreases NOx production (15), others observed increases in NOx in both humans (33) and animals (21). A study of rat hindlimb ischemia suggested that endothelium-derived NO plays an important role in the maintenance of vascular tone, and a reduction of NO release during reperfusion may predispose vessels to vasoconstriction (31). Although NO generation was not measured in the present study, our results suggest that ischemia-reperfusion results in a relative deficiency of NO in skeletal muscle, inasmuch as SNAC significantly protects the contractile function of reperfused skeletal muscle. Our findings are in agreement with those of others (10) who showed that constitutive NO release is impaired in rat ischemia-reperfused heart muscle because of endothelial dysfunction and can be ameliorated with L-arginine pretreatment.

We also found that SNAC exerts a complex systemic response. Although the lower dose had no significant effects, higher-dose (5 µmol/min) SNAC resulted in a reduction of blood pressure and RR of ~1/3 and an increase in HR of ~50%. However, the changes lasted only 3–4 h. Subsequently, they appeared to be diminished or reversed despite continuous SNAC infusion. The phenomenon may reflect a negative feedback regulation that protects and maintains the body in a normal physiological condition. Such tolerances and tachyphylaxis to the systemic effects of S-nitrosothiols have not been previously reported. The fact that systemic effects of SNAC at 100 nmol/min occurred mainly during the ischemic period rather than during reperfusion suggests that the changes in muscle function resulted predominantly from NO supplementation.

In the present study, a 6.5-h infusion of SNAC showed significant functional protection of both the maximal twitch and tetanic contractile forces in the EDL after 3-h ischemia and 3-h reperfusion, with no statistically significant systemic side action at <1 µmol/min. Several mechanisms may be related to the NO protective function. First, NO can reverse the vessel spasm that is known to occur during reperfusion injury (32). Our earlier study (12) showed that acetyl-

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**DISCUSSION**

Recent studies have shown that rat skeletal muscle expresses neuronal and endothelial constitutive NOSs and that NOS activity varies among several respiratory and limb muscles (15, 16, 22). Immunohistochemistry has shown that NOS activity in rat muscles correlates with type II fiber density (15). Because the EDL is a fast muscle composed mainly of type II fibers, it was hypothesized that NO may play a role in the pathophysiology of skeletal muscle injury.

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**Table 2. Effects of SNAC infusion on EDL fatigue testing**

<table>
<thead>
<tr>
<th>SNAC</th>
<th>EDL Without Ischemia (SNAC)</th>
<th>EDL With I-R</th>
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<tbody>
<tr>
<td></td>
<td>Contralateral EDL 100 nmol 1 µmol</td>
<td></td>
</tr>
<tr>
<td>Peak tension, %</td>
<td>100 91.9 ± 2.4 87.9 ± 5.5</td>
<td>86.6 ± 1.0† 66.8 ± 8.1*† 60.4 ± 4.1† 36.3 ± 5.9</td>
</tr>
<tr>
<td>FT½, s</td>
<td>9.3 ± 0.4 10.8 ± 0.9 7.8 ± 0.4</td>
<td>7.1 ± 1.5* 5.9 ± 0.7 6.4 ± 1.0 4.0 ± 0.4</td>
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Values are means ± SE. FT½, fatigue half time. *P < 0.05, †P < 0.01, and ‡P < 0.001 compared with group VI (PBS group).
choline-induced NO release significantly relieved nor-
epinephrine-induced vasoconstriction in 40- to 80-µm
arteries of rat cremaster muscle. More recently, we
found that SNAC infusion significantly relieved vaso-
spasm and improved microcirculation in rat cremaster
muscle after 5-h ischemia and 90-min reperfusion
(unpublished observations). Therefore, the protective
function by SNAC on contraction in the present study
may be related to NO-mediated vasodilation, thereby
improving the microcirculation and increasing nutri-
tion input to the muscle. A feline hindlimb study (9) also
suggested that basal NO production is required to
maintain resting vasomotor status of vascular smooth
muscle. The inhibition of such an effect, as might occur
with ischemia followed by reperfusion, would be ex-
pected to increase vascular resistance and thereby
impair blood flow and tissue nutrition.

Second, NO may modulate the function of blood
elements that influence the microcirculation. NO re-
duces clotting by inhibiting platelet aggregation and
adhesion (8) as well as by inhibiting the adhesion of
neutrophils and monocytes (18).

Third, NO may modulate muscle contractile function
directly or through effects on reactive oxygen species
(ROS). Muscle contraction increases intracellular oxi-
dant levels, and an excessive level of ROS promotes
muscle fatigue (27). It is believed that the protective
function of NO may relate to the scavenging of superox-
ide in such settings (23). Our results appear to support
one of these possibilities, because fatigue peak tension
and FT ¼ were greater in the SNAC-treated EDL than
in the control. Thus NO donor administration in-
creased the resistance of skeletal muscle to fatigue
after ischemia-reperfusion. That constitutive ecNOS

Fig. 4. Photomicrographs of rat EDLs after 3-h ische-
ia and 3-h reperfusion. A: PBS-infused muscle. B:
SNAC-infused muscle. There was no apparent differ-
ence between muscles, and inflammation was present
in both (hematoxylin and eosin stain). ×170 Magnifica-
tion.
(NOS 3) associates closely with mitochondria in skeletal muscle and influences muscle respiration is consistent with a role in scavenging ROS (16). In the present study, the mitochondrial edema seen after 3 h of reperfusion is highly suggestive of functional impairments that might adversely influence mitochondrial permeability, NO production, and reactive oxygen generation (28).

Our findings indicate that the function-sparing effect of the NO donor SNAC is critically dependent on the dosage. As an adjunct to the present study, we have examined the effects of lower (10 nmol/min) and higher (10 µmol/min) SNAC dosages in two additional small groups of rats (n = 2/group). After 3-h ischemia and 3-h reperfusion, the EDL tetanic contractile force in the animals with a 10 nmol/min SNAC infusion was <30% (23–29%) of normal at each of four stimulation rates, much lower than that observed in rats receiving a 100-nmol/min dose (group III), but not statistically different from controls (group VI). When a high dose (10 µmol/min) of SNAC was administered, the contractile forces were 30–55% of normal at the four different stimulation rates, a level less than that observed after 5-µmol/min SNAC administration. The findings suggest that there is a threshold between 10 and 100 nmol/min at which SNAC becomes dramatically more effective. As the SNAC dose was increased from 100 nmol/min to 10 µmol/min, however, SNAC progressively lost effectiveness. At the higher doses of SNAC, there was no significant difference in tetanic forces generated between the SNAC-infused and PBS-infused EDLs. This may reflect a direct inhibitory effect of SNAC on muscle under these conditions (15) or the deleterious effects of excessive NO-related activity. Indeed, S-nitrosothiols such as SNAC have been reported to exert an inhibitory effect on isolated normal diaphragm contractile function (15). The inhibitory effect of NO-related activity may be manifest in EDL after muscle function is compromised by ischemia.

The muscle function that we have documented after SNAC administration does not correlate well with the results of other investigators who reported that NOS inhibitors improved the survival of skeletal muscle (26) and skin flaps (14), suggesting that NO production was...
biological regulation. The protective role of NO in ischemia-reperfusion injury of skeletal muscle in our studies cannot be generalized to all clinical ischemic conditions of the muscle or to its effects on other organs. Specifically, studies concerning NO/NOS influence on cerebral reperfusion have shown inconclusive and conflicting results (7). Studies of reperfusion injury in skeletal muscle preserved cellular viability after extended periods of warm ischemia. J. Cardiovasc. Surg. (Torino) 32: 664–676, 1991.


