S-NITROSO-N-ACETYLCYSTEINE PROTECTS SKELETAL MUSCLE AGAINST REPERFUSION INJURY

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The effects of a nitric oxide (NO) donor on microcirculation and contractile function of reperfused skeletal muscle were studied. Rat cremaster muscles underwent 5 hours of ischemia and 90 minutes of reperfusion and were divided into two groups systemically infused with S-nitroso-N-acetylcysteine (SNAC, 100 nmol/min) and phosphate-buffered saline (PBS), respectively. The results showed that the vessels in the SNAC group had more rapid and complete recovery than that in controls. A significant difference was found from 10 to 40 minutes and at 90 minutes in 10–20-µm arterioles, from 10 to 90 minutes in 20–40-µm arterioles, and at 10 and 90 minutes in 40–70-µm arteries. When compared to controls, SNAC-treated muscles showed larger fluorescein filling areas at 15, 30, 60, and 90 minutes and greater isometric tetanic contractile forces in response to stimulation frequencies of 40, 70, 100, and 120 Hz. The data indicate that supplementation of exogenous NO could effectively improve microcirculation and contractile function of skeletal muscle during early reperfusion.

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Nitric oxide (NO) has been considered a unique messenger molecule that can exert multiple biological functions, including vessel relaxation, neurotransmission, and pathogen suppression. In blood vessels, NO is released from the vascular endothelium in response to vasoactive agents and maintains the basal vascular tone through elicitation of vasodilation. NO release is dependent on normal function of vascular endothelium, but is independent of tissue innervation. Regulation by NO is reflected in microvessels as well as in larger vessels. The observation of microvascular response to NO showed that exogenous NO can elicit a dose-dependent dilatory response of both arterial and venous microvessels in skeletal muscle and reverse the endothelial dysfunction-induced NO depression. Conversely, inhibition of NO generation results in the constriction of arterioles, increase of peripheral resistance, and even increase of systemic blood pressure. Studies have also verified that both exogenous and endogenous NO result in a significant increase in developed force of normal fast-twitch and slow-twitch skeletal muscle.

Since NO is involved in mediating normal microcirculation and function of skeletal muscle, the question arises as to whether it exerts an influence on the muscle under pathological states, such as ischemia/reperfusion (I/R) injury, a serious complication affecting the success of tissue replan-
tation. Both protective and deleterious roles of NO production during reperfusion on the survival of skeletal muscle have been reported. While it has been concluded that NO is associated with events leading to injury, there is also evidence showing this mediator’s protection against I/R injury. This apparent paradox may be related with its redox state. The expression of destructive effects may derive from reaction of superoxide generated locally during reperfusion with NO, forming toxic peroxynitrite (ONOO−) and subsequently resulting in cytotoxicity and loss of skeletal muscle function. In contrast, compounds containing the NO group in an alternative redox state could prevent excessive influx of Ca2+ during I/R and its associated cytotoxic effects. Based on the notion that I/R is associated with diminished production of NO, which is supported by observations from our previous study, the present study was designed to determine whether supplementation of NO attenuates the microcirculation damage observed in rat cremaster muscle following I/R injury, and to assess the contribution of NO donor to preserving contractile function of reperfused cremaster muscle.

MATERIALS AND METHODS

Sixty-eight male Sprague-Dawley rats weighing 120 to 150 g were studied. The experiment was performed in the following protocols.

One, observation of systemic blood pressure in normal rats during 120-minute infusion of S-nitroso-N-acetylcysteine (SNAC, n = 5) and phosphate-buffered saline (PBS, n = 5).

Two, observation of microcirculation in normal rat cre-
master muscles during 120-minute infusion of SNAC (n = 5) and PBS (n = 5).

Three, observation of microcirculation, fluorescein filling area, and contractile function in ischemia/reperfused (I/R) cremaster areas during 120-minute infusion of SNAC (n = 24) and PBS (n = 24). The muscles underwent 5-hour ischemia and 90-minute reperfusion. SNAC and PBS were systemically infused at 30 minutes before and during reperfusion.

**Surgical Procedure**

The rats were anesthetized with an intraperitoneal injection of Nembutal (50 mg/kg body weight; Abbott Laboratories, North Chicago, IL). The right jugular veins of all 68 rats were catheterized with polyethylene tubing (PE-10, Clay Adams) for agent administration. The tubing was connected to a 3-ml syringe and fixed to a Sage syringe pump (model M362; Analytical Technology, Inc., Boston, MA), SNAC with a dosage of 100 nmol/min in the 34 experimental animals and PBS with the same volume in the 34 control animals were infused through the pump for a duration of 120 minutes. In protocol 1, the left femoral artery was cannulated and blood pressure was monitored by a patient monitor (MR-1300, Mennen Medical Inc., New York, NY) at 10-minute intervals for 2 hours. These 10 rats were not subjected to other surgical procedures or I/R.

The left cremaster muscle in the remaining 58 rats was prepared as described previously to produce a neurovascular pedicle muscle flap. Ten muscles (5 SNAC, 5 PBS) without I/R were studied for protocol 2. In the 48 rats of protocol 3, muscle ischemia was induced by clamping the vascular pedicle (the pudic epigastric artery and vein) with an atraumatic vascular clamp (ASSI Model ST-B-1 VB).

**Measurement of Vessel Diameter**

The prepared cremaster muscles in protocol 2 and 8 I/R muscles from each group of protocol 3 were spread over a transparent acrylic microscope stage, moistened with Ringer’s solution (34 ± 0.5°C) and covered with a thin layer of oxygen impermeable plastic polymer (Saran Wrap, Dow Chemical Company, Indianapolis, IN). The muscle preparation was left undisturbed for 30 minutes before clamping to allow any effects of the muscle isolation procedure to dissipate. By using an operating microscope (Zeiss, Inc., Germany) connected to a video camera (3CCD, Sony, Japan) and a recording system (EDW-30F, Sony), 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, 8 to 15 sites were selected for measurement in each rat. Sequential measurements were taken at the same sites throughout the experiment. The data obtained from each muscle at each time point were divided in three categories: small arterioles (10–20 μm), large arterioles (21–40 μm), and small arteries (41–70 μm), depending on the baseline diameters of the measured vessels. Diameter changes at each time point for each animal were expressed as percentages of the baseline value. Statistical analysis was performed by one-way analysis (ANOVA). Bonferroni correction was applied where appropriate. A P < 0.05 was considered significant.

**Fluorescein Filling Study**

Eight rats from each group of protocol 3 were received 0.1-ml 10% sodium fluorescein (Smith, Miller & Patch, New Brunswick, NJ) through the jugular catheter at the start of reperfusion. In a darkened room under a long-wave ultraviolet light (365 nm, Spectroline Model EN-160L), photographs were taken through the operating microscope following 15, 30, 60, and 90 minutes of reperfusion, with a Minolta X-700 camera using 160 ASA tungsten color slide film (Kodak, Ektachrome, Rochester, NY), to determine the time course and pattern of fluorescent staining. The percentage of fluorescent filling area was scored as 25%, 25–50%, 50–70%, and >75% filling.

**Muscle Contractile Testing**

Both cremaster muscles of the remaining eight rats from each group of protocol 3 were isolated as described above, except that the muscles were neither divided down to the ventral side nor affixed to the microscope stage. Contractile function in the right cremaster muscle was assessed immediately. Ischemia and reperfusion were then induced only in the left one. At the end of reperfusion, contractile testing of the left muscle was performed as described in an earlier study. Briefly, the muscle was placed in an organ bath system. Isometric tetanic contractile forces were measured using the voltage required to elicit the maximal twitch force and consisted of four 1.5-second stimulation trains at frequencies of 40, 70, 100, and 120 Hz. A three-minute rest period followed each stimulation. All force measurements were recorded on a chart recorder. The response of the reperfused muscle was expressed as a percentage of the right cremaster muscle force (normal). Statistical analysis was performed by one-way analysis of variance (ANOVA) for the tetanic contraction. A P < 0.05 was considered significant.

**RESULTS**

**Effect of SNAC Infusion on Blood Pressure**

Mean arterial blood pressure in the control group gradually decreased from 98 ± 1% of baseline (mean ± SEM) at 20 minutes to 91 ± 2% at 120 minutes of infusion. A similar tendency was observed in the SNAC group, with corresponding values of 97 ± 2% and 88 ± 3%, respectively. Although mean blood pressure was generally lower in the SNAC group than in the controls, there was no significant
difference between the two groups throughout the experiment (Fig. 1).

**Influence of SNAC on Vessel Diameter of Normal Cremaster Muscle**

The mean vessel diameter in the controls remained at the baseline level during the 120-minute infusion. In the SNAC group, the vessel diameter increased in all three vessel categories, beginning at 20 minutes of reperfusion and continuing for the duration of the experiment, with a 1–5% increase in the 10–20-µm vessels, an 11–20% increase in the 20–40-µm vessels, and an 8–13% increase in the 40–70-µm vessels when compared to baseline level. When compared to the controls, the vessel diameter in the SNAC group had no statistical increase in the 10–20-µm vessels at any time point during infusion, but the diameter was significantly greater in the 21–40-µm vessels from 20 to 120 minutes and in the 41–70-µm vessels from 30 to 120 minutes.

**Effect of SNAC on Cremaster Muscle With I/R**

**Measurement of vessel diameter.** In the 10–20-µm vessels, the mean diameter in the controls was only 28 ± 5% of baseline at 10 minutes of reperfusion and gradually increased to a maximum level of 74 ± 5% at 70 minutes. In contrast, the diameter in the SNAC group rapidly returned to 95 ± 4% at 10 minutes of reperfusion, and subsequently slowly decreased, with a diameter of 88 ± 4% at 90 minutes. There was a significant difference between the two groups during the first 40 minutes and at 90 minutes of reperfusion (Fig. 2).

The mean diameter of 21–40-µm vessels in the controls was 63 ± 5% of baseline at 10 minutes and increased to a maximum recovery of 88 ± 2% at 40 minutes. The diameter in the SNAC group reached baseline level at 10 minutes of reperfusion and then remained at that level throughout the experiment, with a significant difference at each time point of reperfusion when compared to the controls (Fig. 3).

For the 41–70-µm vessels, more rapid and complete recovery was again seen in the SNAC group than in the controls, with a significant difference at 10 (100 ± 2% vs. 82 ± 3%) and 90 minutes (100 ± 4% vs. 88 ± 2%) (Fig. 4).

**Fluorescein filling study.** At 15 minutes after fluorescein administration, the cremaster muscles in five of eight SNAC-treated rats fluoresced in 25–50% of the total muscle area. Only two of the controls reached this level. In the SNAC group, half of the cremasters at 30 minutes and all at 60 minutes showed more than 50% fluorescence filling, while only one and four control muscles, respectively, fluoresced to this degree. At 90 minutes, all muscles were perfused completely by fluorescein in the SNAC group, but only five of eight in the controls were in this range and one control muscle failed to fluoresce at all.

**Muscle contractile testing.** Mean isometric tetanic contractile force of the muscles at 90 minutes of reperfusion was significantly greater in the SNAC group than in the controls in response to the stimulation frequency of 40 Hz (27 ± 3% vs. 12 ± 2% of normal contralateral muscle force), 70 Hz (33 ± 4% vs. 17 ± 3%), 100 Hz (35 ± 4% vs. 20 ± 3%), and 120 Hz (37 ± 4% vs. 19 ± 3%), with a significant difference between the two groups at all four stimulation frequencies (Fig. 5).

**DISCUSSION**

It is well accepted that the constitutive NO continually released from endothelial cells regulates vascular tone by
relaxing vascular smooth muscle. Previous studies verified that NO can modulate arteriolar response to vasoactive agents in skeletal muscle microcirculation.3,19,20 In the present study, NO generated from the infused SNAC caused vasodilation in normal and reperfused cremaster, indicating that exogenous NO affects the vascular tone of microvessels under both physiological and pathological conditions. The fact that SNAC significantly increases the diameters of larger vessels (20–70 µm), but not those of small arterioles (10–20 µm), may relate to the presence of fewer smooth muscle cells in the vascular walls of small arterioles than in the walls of larger vessels. These data are consistent with the findings that the main site of NO action is located in the large-bore arterial resistance vessels,20,21 and that NOS inhibitors are unable to change the resting tone of small arterioles.7 Because SNAC-produced NO is independent of the endothelium, investigation of the pharmacological characterization of NO donors may facilitate development of a substitution therapy for the diseases associated with endothelial dysfunction.

I/R injury results from multiple causes and leads to complex pathophysiological and mechanical changes that compromise tissue function and lead to irreversible lack of tissue perfusion. The exact mechanisms of this catastrophic phenomenon remain unknown, but it has been shown to be influenced by many systemic and local factors,22 such as the severity and type of tissue damage, duration of ischemia, ambient and tissue temperatures, and pharmacologic treatment. Local generation of free radicals during reperfusion could be exaggerated by acidosis during ischemia because free radicals can be catalyzed by ions released from protein bindings at a lowering PH.23 Excess hydrogen peroxide (H₂O₂) combines with superoxide anion (O²⁻) to produce highly toxic hydroxyl radical (OH•), and superoxides combine with NO to form anion peroxynitrite (ONOO⁻), which could be further converted to peroxyxynitric acid (HOONO) under acidic conditions.24 These products cause a series of damages, including phospholipid peroxidation and loss of membrane integrity, intracellular calcium overload, increase in capillary permeability, impaired NO metabolism, and so on.

No agreement has been achieved regarding increase or decrease of NO production during I/R. Paradoxical observations of protective effects of both addition and removal of
NO in I/R exist in different tissues. Some studies showed that destruction of NO exacerbates I/R injury in the heart and skeletal muscle, and agents that release NO have a protective effect against I/R injury in the gastric mucous, heart, and splanchnic circulation. A recent study suggested that temporary endothelial cell dysfunction during early reperfusion resulted in lack of NO production, thereby contributing to vasospasm of reperfused skeletal muscle. Similarly, the present study demonstrated more rapid and complete recovery of vessel diameter, larger fluorescein filling area, and stronger muscle contractile force in SNAC-infused rat cremaster muscle during early reperfusion following 5-hour ischemia than that in the controls. NO-induced vasodilation may be responsible for its protection of reperfused skeletal muscle because vessel spasm is a common phenomenon in I/R skeletal muscle. Except the differences in the experimental protocol, animal species, dosing, harvest time, and a multitude of related factors that might influence the contribution of NO to muscle microcirculation and function, sufficient precedent and rationale exist for dual actions and paradoxical effects of NO. Thus, the protective role of NO in I/R injury of skeletal muscle in our studies cannot be generalized to all clinical ischemic conditions of the muscle or other organs. Its devise characterization, however, helps us to understand a wide variety of NO effects under normal physiological and pathological condition, such as I/R injury. Coupled with the data derived from another study showing that NO-induced vasodilation was impaired following I/R and reduction of NO may predispose to vasoconstriction, our findings suggest that NO plays an important role in the maintenance of vascular tone of skeletal muscle microcirculation.

The enhancement of adhesion and emigration of leukocytes (PMN) is thought to play a key role in I/R injury. It was reported that inhibiting NO can elicit significant leukocyte adhesion to microvessels and mimic the inflammatory response observed in reperfused tissues. In contrast, NO donors can effectively attenuate PMN adhesion and emigration and reduce albumin leakage and mast cell degranulation elicited by I/R. Thus, NO donors appear to exert considerable role in maintaining the normal barrier function of microvasculature.

The decrease of NO production during I/R may result from injury of endothelium (a major source of NO) and/or destruction of endogenous NO by superoxide produced during reperfusion. Reduced NO production leads to excessive accumulation of superoxide, thereby resulting in formation of inflammatory mediators, increase of expression of adhesion molecules, and enhancement of protein leakage. Evidence that NO donor blunts leukocyte–endothelial cell adhesion response in mesenteric venules that were exposed to the oxygen radical-generating system suggests that NO is an effective scavenger of superoxide.

The data from the present study have shown that SNAC effectively preserves contractile function of reperfused rat cremaster muscle. The results concur with our earlier work in which SNAC assumed a protective role and NOS inhibitor L-NMMA (unpublished data) assumed a deteriorative role in contractile function of reperfused rat extensor digitorum longus muscle. Although exact mechanisms remain undefined, improvement of microcirculation produced by SNAC is definitely helpful for the delivery of nutrient molecules into and removal of cytotoxic metabolic substances from reperfused muscle. Second, SNAC as a superoxide scavenger may reduce adverse effects produced by superoxide on the balance between intra- and extracellular calcium and facilitate the recovery of depressed muscle function by I/R. Free-radical-induced cell membrane damage accelerates this abnormal calcium entry, thereby impairing the muscle contractility. Third, NO may modulate mitochondrial function and oxygen consumption. Constitutive NO is expressed in skeletal muscle cells and there is a potential functional correlation between NOS and mitochondria. NO appears to be a regulator to modulate cellular respiration and act as a “brake” on respiration through binding cytochrome oxidase or inhibiting electron transport. NO donor has been reported to improve the maintenance of force in normal muscle during repetitive tetanic contractions and fatigue. I/R injury may inhibit the expression of both NOS isoforms (nc- and ec-) and supplementation of NO into the reperfused muscle may serve a protective role for cell respiration. Further studies are needed to clarify the exact mechanism of NO in regulating contraction of skeletal muscle under I/R condition.

In conclusion, the findings from the present study suggest the protective role of NO in reperfused skeletal muscle and its mechanism of action, which might influence the contribution of NO to muscle microcirculation and function.
gest that, one, infusion of exogenous NO alters microcirculation of cremaster muscle in normal rats by significantly increasing vessel diameters of large arterioles and small arteries. Two, treatment of reperfused skeletal muscle with SNAC provides effective protection for microcirculation and contractile function against reperfusion injury in this model. NO donor may be beneficial in clinical reconstructive microsurgery. Three, the protective effect of NO donors may also be related to their ability to attenuate I/R-induced inflammatory responses, vasodilation of microvessels, and scavenging of superoxide.

REFERENCES


