Reperfusion injury is reduced in skeletal muscle by inhibition of inducible nitric oxide synthase

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Zhang, Li, Colin G. Looney, Wen-Ning Qi, Long-En Chen, Anthony V. Seaber, Jonathan S. Stamler, and James R. Urbaniak. Reperfusion injury is reduced in skeletal muscle by inhibition of inducible nitric oxide synthase. J Appl Physiol 94: 1473–1478, 2003. First published December 27, 2002; 10.1152/japplphysiol.00789.2002.—This study evaluated the effects of the selective inducible nitric oxide synthase (iNOS) inhibitor N-[3-(aminomethyl)benzyl]acetamide (1400W) on the microcirculation in reperfused skeletal muscle. The cremaster muscles from 32 rats underwent 5 h of ischemia followed by 90 min of reperfusion. Rats received either 3 mg/kg 1400W or PBS subcutaneously before reperfusion. We found that blood flow in reperfused muscles was <45% of baseline in controls but sharply recovered to near baseline levels in 1400W-treated animals. There was a significant (P < 0.01 to P < 0.001) difference between the two groups at each time point throughout the 90 min of reperfusion. Vessel diameters remained <80% of baseline in controls during reperfusion, but recovered to the baseline level in the 1400W group by 20 min, and reached a maximum of 121 ± 14% (mean ± SD) of baseline in 10- to 20-μm arterioles, 121 ± 6% in 21- to 40-μm arterioles, and 115 ± 8% in 41- to 70-μm arteries (P < 0.01 to P < 0.001). The muscle weight normalized to muscle wet weight at the baseline level was 80% of controls in 1400W group (P < 0.001). Histology showed that neutrophil extravasation and edema were markedly reduced in 1400W-treated muscles compared with controls. We conclude that ischemia-reperfusion leads to increased generation of NO from iNOS in skeletal muscle and that the selective iNOS inhibitor 1400W reduces the negative effects of ischemia-reperfusion on vessel diameter and muscle blood flow. Thus 1400W may have therapeutic potential in treatment of ischemia-reperfusion injury.

ischemia; microcirculation; selective inhibitor; rat

The discovery that mammalian cells generate nitric oxide (NO) has led to important information about many biological processes, including ischemia-reperfusion (I/R) injury. I/R injury compromises the clinical outcome of patients undergoing re plantation, free muscular flap, free myocutaneous flap, compartment syndrome, or any revascularization procedures, even with technically successful procedures (38).

I/R injury is a complex process, and its severity is affected by many factors. During recent years, it has been recognized that NO may play a prominent role in I/R injury, both in microcirculation and muscle function. Reduced NO production has been found in rabbit skeletal muscle (18) and pig myocutaneous flap (11) during reperfusion. Our laboratory’s previous work has demonstrated that an exogenous NO donor improved microcirculation (10, 26) and preserved contractile function in reperfused skeletal muscle (7), whereas use of a nonselective NO synthase (NOS) inhibitor proved detrimental (21). As a prominent mediator of local response, NO is likely to have both pathogenic and protective effects in I/R injury.

NO is synthesized from L-arginine by NOS and decays within seconds in biological systems (5). NO is produced by at least three NOS isoforms: by the calcium/calmodulin-dependent neuronal (nNOS) and endothelial (eNOS) isoforms and by the inducible calcium-independent isoform (iNOS) (27). All three NOS isoforms are present in normal and I/R skeletal muscle (31, 35, 41). Among them, iNOS is induced mainly by endotoxins and cytokines in most cells. iNOS releases large amounts of NO to exert a defense function that may be important in host defense, and this increased iNOS activity can lead to cytotoxicity when the levels of NO released are excessive (12, 27). Despite our incomplete understanding of the contributions of iNOS-derived NO (23), mice deficient in iNOS have been shown to be protected from the detrimental effects of I/R injury in skeletal muscle (4). Our laboratory’s previous work has also shown significant upregulation of iNOS expression in both skeletal muscle (31) and peripheral nerve (32) during early reperfusion. Inhibition of iNOS by dexamethasone reduces the loss of contractile function in reperfused muscle (9). These observations lead us to hypothesize that selective inhibition of the iNOS isoenzyme will reduce the incidence of reperfusion failure. To investigate this hypothesis, we observed the
effects of a highly selective iNOS inhibitor, N-[3-(aminomethyl)benzyl]acetamidine (1400W), on microcirculation in rat skeletal muscle during early reperfusion.

MATERIALS AND METHODS

Thirty-two male Sprague-Dawley rats weighing 90–110 g received a standard diet and water ad libitum. The animals were purchased from Charles River Laboratories (Raleigh, NC). The rats were randomly divided into two groups, which received a standard diet and water ad libitum. The animals

The rats were anesthetized with an intraperitoneal injection of Nembutal (50 mg/kg, Abbott Laboratories, North Chicago, IL). The left cremaster muscle was exposed, and the cremaster muscle was prepared according to our previously described technique (8, 26). Briefly, the cremaster muscle was exposed, opened, and then separated from the testis. The pudic-epigastric artery and vein and the genitofemoral nerve were isolated from the posterior peritoneum along the inguinal canal and were 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 isolated muscle was then spread flat onto the surface of a transparent acrylic microscope stage by five peripheral sutures. The exposed surface of the muscle was moistened with warm (34–35°C) lactated Ringer solution and covered with a thin layer of oxygen-impermeable plastic polymer to prevent diffusion of oxygen and other gases from the environment to the muscle. The temperature of the muscle was maintained at 34 ± 0.5°C throughout the duration of the experiment by using a heat lamp. The cremaster preparation was left undisturbed for 30 min before baseline measurements were obtained, to allow any transient effects of the muscle isolation procedure to dissipate. After baseline vessel diameters were obtained, 5 h of ischemia were achieved by clamping the main vascular pedicle of the muscle by using an atraumatic microvascular clamp (ST-B-1 VB, ASSI, Westbury, NY). At the end of the ischemia period and after agent administration, the microclamp was released to restore circulation, and evaluation of microcirculation was started and continued at 10-min intervals for 90 min.

**Measurement of vessel diameters.** With the use of intravital videomicroscopy (Super-Lux 40, Carl Zeiss, Oberkochen, Germany) to view the vasculature of the prepared cremaster muscle, observation areas were selected in an arterial tree for monitoring during the duration of the experiment. Only arteries that were between 10- and 70-μm diameter were evaluated. The internal luminal diameters of each selected vessel was measured from the recorded image by using a FOR/A IV-560 video-measuring gauge. According to the number of branches of the arterial tree, 8–12 sites were selected for measurement in each muscle. Sequential measurements were taken at the same sites throughout the experiment. Vessels were divided into three categories according to their baseline diameters: small arterioles (10–20 μm), large arterioles (21–40 μm), and small arteries (41–70 μm).

**Measurement of blood flow (10).** Overall blood flow in the cremaster muscle was measured with laser-Doppler flowmetry (Moor Instruments, Devon, UK). A 1-mm-diameter double-fiber probe was positioned by a manipulator (MM 33, Stoelting, Wood Dale, IL) throughout the consecutive measurements. The tip of the probe was placed 1 mm above the surface of the vascular pedicle of the cremaster muscle so that there was no compressive effect of the probe on the vasculature. The position of the laser probe tip relative to the selected measured site on the vascular pedicle was kept constant through the course of the procedure by using a suture mark as a guide. Background flux recorded from the vascular pedicle during ischemia was subtracted from every measurement before data comparison.

**Surgical procedure.** The rats were anesthetized with an intraperitoneal injection of Nembutal (50 mg/kg body wt, Abbott Laboratories, North Chicago, IL). The left cremaster muscle was cannulated with polyethylene tubing (PE-10, Clay Adams), and a patient monitoring system (MR-1300, Mennen Medical, New York, NY) was used to monitor MAP, HR, and RR in response to the administration of 1400W (3 mg/kg) or PBS at 10-min intervals for 120 min. These 10 rats were not subjected to other surgical procedures or to I/R.

**Histological examination.** After being weighed, both 1400W-treated and PBS-treated cremaster muscles were immersed in 10% formalin. The muscle samples were sectioned and stained with hematoxylin and eosin.

**Data and statistical analysis.** MAP, HR, RR, vessel diameter, and blood flow changes observed during reperfusion were expressed as percentages of the baseline values. All values in the figures and the text are expressed as means ± SD. The data were analyzed 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 of <0.05 was considered to be statistically significant.

### RESULTS

**Systemic effects of 1400W administration.** The baseline values of MAP, HR, and RR are summarized in Table 1. The baseline measures did not differ significantly between the PBS (control) and 1400W-treated groups. After agent administration, MAP was reduced in both groups during the 120-min observation period, with a range from 80 ± 14 to 96 ± 4% of baseline in the PBS group and from 90 ± 17 to 100 ± 12% in the 1400W group. Compared with the PBS group, 1400W-treated animals showed a significant overall increase in MAP but no significant difference at any specific time point during the experiment (Fig. 1A).

After agent administration, HR and RR in the PBS group remained between 82 ± 15 and 109 ± 15% of baseline and between 84 ± 10 and 98 ± 4%, respec-

<table>
<thead>
<tr>
<th>Group</th>
<th>MAP, mmHg</th>
<th>Heart Rate, beats/min</th>
<th>Respiratory Rate, breaths/min</th>
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<tr>
<td>PBS</td>
<td>87.4 ± 9.1</td>
<td>262.6 ± 20</td>
<td>56.2 ± 5.7</td>
</tr>
<tr>
<td>1400W</td>
<td>96.6 ± 11.5</td>
<td>230.6 ± 24.3</td>
<td>68.4 ± 7.3</td>
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Values are means ± SE for 5 rats per group. MAP, mean arterial pressure; 1400W, N-[3-(aminomethyl)benzyl]acetamidine.
respectively, during the 120 min of observation. The ranges in the 1400W group were between 84 ± 10 and 125 ± 26% and between 84 ± 11 and 117 ± 38%, respectively (Fig. 1B). Compared with the PBS group, 1400W administration resulted in a decrease in HR and an increase in RR between 50 and 100 min of observation, but those differences were not statistically significant at any given time point. However, a statistically significant overall difference in HR and RR was present between the two groups when analyzed by using a repeated-measures two-way ANOVA.

Gross observation of microcirculation in the reperfused cremaster muscle. Gross hemorrhage was observed in all rats in the PBS group during reperfusion. Red blood cell accumulation in the extravascular space was more severe in the PBS group than in the 1400W group. During the first 20 min of reperfusion, the reperfused muscles were noted to be darkly erythematous with areas of blood splotching from capillary hemorrhage. This erythema and blood splotching were more severe in the control group. Four of the 16 cremaster muscles in the PBS group had cessation of blood flow during the first 30 min of reperfusion, whereas 14 of the 16 muscles in the 1400W group reperfused successfully on removal of the microvascular clamp.

Measurement of blood flow in the reperfused cremaster muscle. At 10 min of reperfusion, the mean blood flow as measured by laser-Doppler flowmetry was 28 ± 11% of baseline in the PBS group and 64 ± 28% in the 1400W-treated group (P < 0.001). The blood flow gradually increased to 45 ± 28% in the PBS group at 40 min and remained at this level throughout the remainder of the 90-min reperfusion period. In the 1400W-treated group, the mean blood flow reached its maximum of 98 ± 22% at 60 min reperfusion. When compared with the controls, the 1400W-treated cremaster muscles had a significantly (P < 0.01 to P < 0.001) greater flow at all time points (Fig. 2).

Measurement of vessel diameter in the reperfused cremaster muscle. The mean vessel diameter of 10- to 20-μm arterioles in the PBS group was 61 ± 6% (mean ± SD) of baseline at 10 min of reperfusion and gradually increased to a maximum level of 72 ± 14% at 90 min. The mean diameter in the 1400W-treated group was 92 ± 11% at 10 min, rising to 118 ± 30% at 30 min, and increasing to a maximum value of 121 ± 14% at 70 min (P < 0.01 to P < 0.001).

The diameter of 21- to 40-μm arterioles in the PBS group was 58 ± 17% of baseline at 10 min and 78 ± 11% at 90 min. These values were 90 ± 9 and 121 ± 6% in the 1400W group (P < 0.001), respectively (Fig. 3).
For the 41- to 70-μm arteries, the diameter in the PBS group was 63 ± 11% at 10 min, increased to 72 ± 8% at 30 min, and remained at this level for the remainder of the observation period. The diameter in the 1400W group reached 98 ± 15% at 10 min and remained above the baseline level throughout the experiment, with a significant difference (P < 0.001) between the two groups at each time point.

Muscle wet weight ratio. The weight ratio (percentage of normal) of the muscles was 124 ± 12% in the 1400W group and 193 ± 42% in the PBS group, with a significant difference (P < 0.001) between the two groups.

Histological examination. Overall, the inflammatory changes were significantly reduced in the 1400W group. Venous congestion, neutrophil presence in the tissues, muscular fiber edema, and eosinophilic changes in the muscle were greatly diminished (Fig. 4).

DISCUSSION

This study was undertaken to determine the role of iNOS in I/R injury in skeletal muscle by using a highly selective iNOS inhibitor 1400W. Compared with controls, the 1400W group demonstrated increased vessel diameter as observed by intravital microscopy, improved blood flow determined by laser-Doppler flowmetry, and reduced tissue necrosis on histological examination. Furthermore, there was decreased vessel congestion in the muscle cells of the 1400W group compared with the control group as observed by in vivo microscopy. Inflammatory changes such as edema and white blood cell extravasation in posts ischemic cremaster muscle were less severe in the 1400W group than in controls, as observed by muscle weight ratio and histology. All of the above outcome measures indicate that injury to the microcirculation and microvasculature in rat reperfused cremaster muscle was reduced as a result of 1400W administration; therefore, I/R injury is clearly reduced via 1400W inhibition of iNOS.

Our results confirm the hypothesis that selective inhibition of iNOS reduces the incidence of reperfusion failure in this model. Our data are in agreement with findings in other tissues. After focal cerebral ischemia in the rat, an increase in iNOS mRNA expression and enzymatic activity is present (20, 33) and ischemia-induced brain injury is reduced in both iNOS knockout mice (19) and rats after treatment with a partially selective iNOS inhibitor (42). Similarly, iNOS has been shown to contribute to posts ischemic heart dysfunction (1), renal I/R injury (6), and pancreatitis after pancreatic I/R injury (3). That iNOS appears to play a beneficial role against I/R injury in the myocardium (43) and liver (17) may be due to the different experimental conditions or organ-specific effects. Regardless, iNOS can adversely affect many biological activities through mechanisms such as ion currents (13), respiration (39), enzyme activities (40), and DNA integrity (24), thereby leading to cellular necrosis or apoptosis (36). Taken together, the published data support the concept that NO produced from iNOS may play a deleterious role in I/R injury and that its inhibition by a pharmacological agent could attenuate injury.

In concurrence with the earlier finding of upregulation of iNOS expression in reperfused skeletal muscle (31) and peripheral nerve (32), our results also support the premise that I/R leads to nitrosative stress in skeletal muscle. Although free radicals have been shown to play an important role in complex mechanisms of damage in skeletal muscle after reperfusion (30), it is uncertain what effect 1400W has on superoxide or hydroxyl radical generation by iNOS. In cytokine-activated macrophages, inhibition of reactive ox-
xygen production prevents oxidation but not cell death (12). It is now clear that NO can impair cellular function through mechanisms that are nonoxidative in nature. We have termed such impairment of cellular function “nitrosative stress” (16). Nitrosative stress is characterized by accumulation of nitrosylated proteins to hazardous levels. In cells, for example, specific enzymes protect against NO and nitrosothiols but confer no protection against reactive oxygen species (34). Conversely, superoxide dismutase and catalase protect against reactive oxygen species. In macrophages stimulated with cytokines, nitrosative stress is responsible against reactive oxygen species. In macrophages stimulated with cytokines, nitrosative stress is responsible for apoptotic cell death (12). Collectively, these findings support the use of the term nitrosative stress. That said, nitrosylation and, more generally, nitrosative chemistry can lead to secondary oxidative modifications, and very high levels of reactive nitrogen species in combination with reactive oxygen species can produce synergistic toxic effects by oxidizing cellular constituent. Thus peroxynitrite mediates both oxidative and nitrosative chemistry.

Among the available iNOS inhibitors, 1400W is a particularly potent and highly selective inhibitor (2). 1400W is an irreversible or an extremely slowly reversible inhibitor of iNOS, whereas its inhibition of nNOS and eNOS is weak and rapidly reversible. 1400W has >5,000-fold and ≥2 greater selectivity against purified iNOS than eNOS and nNOS, respectively, and it has similar selectivity in organ chamber bioassays (14). In isolated blood-perfused rabbit hearts, 1400W administration increases coronary flow during early reperfusion, accompanied by decreased neutrophil accumulation and a trend toward increased contractility (29). 1400W has also been shown to significantly reduce ischemic lesion volume by 31% and to attenuate weight loss and neurological dysfunction in rat ischemic brain (28). Recent data from our laboratory indicate that 1400W reduces contractile dysfunction and downregulates iNOS protein expression from 27 times the normal value in controls to only 4 times in reperfused rat extensor digitorum longus muscle (P. Patel, W. N. Qi, L. E. Chen, A. V. Seaber, J. S. Stamler, and J. R. Urbaniak, unpublished observations). These findings, in combination with the results from this study, suggest that 1400W may thus have therapeutic potential in treatment of I/R injury.

Dose effects of 1400W were not investigated in this study. Application of 3 mg/kg 1400W was based on our laboratory’s previous studies in which dose-dependent effects on muscle contractile function were not demonstrated between 3 and 10 mg/kg (P. Patel, W. N. Qi, L. E. Chen, A. V. Seaber, J. S. Stamler, and J. R. Urbaniak, unpublished observations). Recently, similar results were also found in motor functional recovery in 1400W-treated reperfused peripheral nerve (unpublished data). Thus a dosage of 3 mg/kg is evidently sufficient to effectively inhibit iNOS and reduce the loss of contractile function subsequent to I/R; dose-dependent effects of 1400W are mainly found at lower dosages (0.1–1 mg/kg) (14, 22, 37).

The present study has shown that 1400W administration at a dosage of 3 mg/kg has systemic effects. Compared with controls, a significant overall increase in MAP and RR and decrease in HR was found in nonischemic cremaster muscle in the 1400W-treated group over 120 min of observation. Our results differ from those of other investigators who reported that MAP remained unchanged in rats throughout a 3- or 1-h investigation period after 1400W administration at a dosage up to 20 mg/kg (28) or 5 mg/kg (25). However, the difference (1–19%) in MAP, HR, and RR between the 1400W-treated group and controls compared with the difference (~30–55%) in muscle blood flow and arterial diameters between the two groups (Fig. 3) is very modest and therefore unlikely to explain the protective effects by 1400W.

Cremaster muscle is an extensively used model to study microcirculation in experimental investigations. It is an extension of the abdominal muscles and mainly consists of type II muscle fibers. The blood supply of the cremaster muscle is primarily from the pudic-epigastic vessel. Innervation is from the genitofemoral nerve, ilioepigastric nerve, ilioinguinal nerve, and lateral cutaneous nerve, but most of the nerve fibers emanate from the genitofemoral nerve (15). Compared with skeletal muscle of the limbs, such as extensor digitorum longus, the cremaster muscle contains a greater proportion of type I muscle fibers and is an involuntary muscle. Although numerous studies of metabolism, free radicals, and NO have been performed in the cremaster muscle, the absolute differences in the responses of the cremaster muscle and extensor digitorum longus muscle remain to be investigated.

In conclusion, our results have demonstrated that 1400W administration can significantly improve microcirculation in reperfused skeletal muscle. Thus these findings confirm the hypothesis that I/R leads to increased generation of NO from iNOS and that selective inhibition of iNOS reduces the incidence of reperfusion failure in skeletal muscle.

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