Ischemic tolerance in skeletal muscle: role of nitric oxide

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Pudupakkam, S., K. A. Harris, W. G. Jamieson, G. DeRose, J. A. Scott, M. W. Carson, M. G. Schlag, P. R. Kviets, and R. F. Potter. Ischemic tolerance in skeletal muscle: role of nitric oxide. Am. J. Physiol. 275 (Heart Circ. Physiol. 44): H94–H99, 1998.—We tested the hypothesis that ischemic preconditioning (PC) of skeletal muscle provided tolerance to a subsequent ischemic event 24 h later, and that such protection was due to nitric oxide (NO). Male Wistar rats, anesthetized with halothane, were randomly assigned to groups: ischemic (no PC; n = 11), PC (n = 11), PC + N-nitro-L-arginine methyl ester (L-NAME; 100 µmol/l; n = 5), PC + N-nitro-o-arginine methyl ester (100 µmol/l; n = 4), PC + aminoguanidine (AMG; 100 µmol/l; n = 4), ischemic + L-NAME (n = 4), or ischemic + AMG (n = 4). PC consisted of 5× 10 min of ischemia and reperfusion, and, 24 h later, 2 h of ischemia were induced by a tourniquet applied to the limb. With the use of intravital microscopy, the number of perfused capillaries (Npc) in the extensor digitorum longus (EDL) muscle was measured over a 90-min reperfusion period. The ratio of ethidium bromide- to bisbenzimide-labeled nuclei was used to estimate tissue injury. PC preserved Npc (23.6 ± 2.5) following 2 h of ischemia compared with sham muscles (11.5 ± 5.1), significantly elevating inducible NO synthase (iNOS) activity (81% increase), but did not affect protection to the parenchyma. L-NAME and AMG prevented ischemia-reperfusion-induced reduction in Npc in muscles without PC. However, after 90 min of reperfusion, L-NAME (Npc = 15.0 ± 1.7), but not AMG (Npc = 22.8 ± 3.1), significantly reduced the microvascular protection afforded by PC. We conclude that PC of the EDL muscle resulted, 24 h later, in protection to microvascular perfusion only, and that such protection was due to NO from sources other than iNOS.

Ischemic preconditioning; microvascular perfusion; tissue injury

Over the last decade a phenomenon called ischemic preconditioning (PC) has shown promise as a means of preventing ischemia-reperfusion (I/R)-induced injury to the myocardium (13, 14, 18, 28), brain (2), and skeletal muscle (1, 21). However, these studies were limited to a description of the acute benefits of PC that lasted only 1–2 h. Recent studies have shown that a “second window of protection” or “ischemic tolerance” occurs 24 h after application of the PC stimuli in the myocardium (3, 16), intestine (19), kidney (31), and brain (11). Although very little is known regarding the mechanisms responsible for the delayed benefits of PC, the increased production of cytoprotective proteins such as antioxidants is thought to play an important role (29).

We recently showed (7) that the reduction in microvascular perfusion (no reflow) associated with I/R in skel-etal muscle was prevented by induction of neutropenia and that such treatment significantly reduced parenchymal injury. We believed that if ischemic tolerance could be demonstrated in skeletal muscle, the use of PC might provide significant clinical advantage in situations that induce periods of limb ischemia such as during reconstruction of abdominal aortas, free flap transfer, or orthopedic surgery. Thus we hypothesized that rat skeletal muscle would demonstrate increased tolerance to a 2-h period of ischemia 24 h after PC and that such ischemic tolerance would be characterized by preserved microvascular perfusion and reduced parenchymal cell injury following the second insult.

A potential mechanism by which ischemic tolerance can develop was suggested from studies in which an in vitro model of I/R was used (30). Exposing endothelial cells to anoxia-reoxygenation (A/R), their in vitro counterpart to I/R, resulted in an increase in neutrophil adhesion to endothelial cells. Such neutrophil adhesion was completely prevented by pretreating the endothelial cells 24 h earlier with an A/R challenge (i.e., A/R tolerance). Recent studies using this approach indicated that inhibition of nitric oxide (NO) synthesis prevented the development of A/R tolerance (15). This result would be consistent with the in vivo observation that endogenous NO, at least partially, controls the degree of leukocyte sticking within the microvasculature (12, 23, 26). In addition, recent in vivo studies have suggested that the delayed benefits of PC in the myocardium result as a consequence of the induction of NO (4, 32). Thus the second major aim of the present study was to test the hypothesis that induction of NO synthesis in skeletal muscle is involved in ischemic tolerance in our in vivo model of I/R.

The use of intravital video microscopy applied to our model of skeletal muscle I/R (6) afforded the unique opportunity to directly test our hypotheses. The results are unique, because they suggest that, 24-h after ischemic PC, the EDL muscle showed evidence of preserved microvascular perfusion following prolonged ischemia, without concurrent parenchymal protection.

In addition, we showed that although the protection afforded microvascular perfusion was due to NO, such benefits could not be linked solely to the increased inducible NO synthase (iNOS) activity following PC.

METHODS

Animal Description and Care

Male Wistar rats used in this study were maintained according to the Guide for the Care and Use of Laboratory
Animals of the Institute of Laboratory Animal Resources, National Research Council (Washington, DC). All rats were housed in cages and had food and water available ad libitum. The experimental protocol was approved by the Council on Animal Care of the University of Western Ontario.

Experimental Groups

Intravital microscopy. To investigate the development of ischemic tolerance in skeletal muscle, rats (250–400 g) were randomly assigned to either ischemic (ischemia without PC; n = 11) or PC (n = 11) groups. To test the role of NO as a possible mechanism leading to microvascular protection, additional rats were assigned to either PC + N-nitro-l-arginine methyl ester (L-NAME; n = 5), PC + N-nitro-o-arginine methyl ester (o-NAME; n = 4), or PC + aminoguanidine (AMG; n = 4) groups. As controls for L-NAME and AMG, additional rats were assigned to either ischemic (no PC) + L-NAME (n = 4), naive (no ischemia or PC) + L-NAME (n = 3), or ischemic + AMG (n = 3) groups. All PC muscles received 2 h of ischemia 24 h after application of the PC stimuli.

Measurement of NOS activity. To determine NOS activity within the EDL muscle, rats were assigned to one of two groups: a group that did not receive PC (n = 6), or a group that did receive PC (n = 6). After 24 h, rats from both groups were reanesthetized with 1% inhalational halothane, and the right EDL muscle in the hindlimb was harvested for the measurement of NOS activity.

Experimental Protocol

Rats in all groups were anesthetized by inhalation of halothane (1–2%), and the internal jugular and carotid arteries were cannulated for infusion of fluids and measurement of blood pressure, respectively. The cannulas were fed subcutaneously to the back of the neck, where they were inserted into a swivel device. During anesthesia, the PC stimulus consisted of 5 cycles of 10 min of hindlimb ischemia, each separated by 10 min of reperfusion. Ischemia was produced by the application of a tourniquet to the hindlimb above the greater trochanter. The rats were allowed to recover from the anesthetic and received continuous saline infusion (300–400 ml kg⁻¹ day⁻¹) containing fentanyl as an analgesic (400 µg kg⁻¹ day⁻¹) via the internal jugular line.

The preparation of the EDL muscle for video microscopy has been described elsewhere in detail (6). Briefly, the skin overlying a portion of the hindlimb was removed to expose the biceps femoris muscle, which was incised and reflected to expose the underlying tibialis anterior and gastrocnemius muscles. Separation of the latter muscles permitted exposure of the EDL muscle, which was dissected free to the level of its distal tendon. The tendon was tied with a suture and cut from its bony insertion, allowing the EDL muscle to be gently reflected into a bath of Krebs solution on the stage of an inverted intravital microscope (Nikon Diaphot model 300; Nikon Canada).

After animals were prepared for intravital microscopy, the level of anesthetized was reduced to 0.8–1.0% for the duration of the experiment. The EDL muscle was covered with a glass coverslip, and all other exposed tissue was covered with plastic film (Saran Wrap, Dow Chemical) to isolate the preparation from the atmosphere and prevent drying. Muscle temperature was continuously monitored via a thermocouple probe placed within the bath and positioned so that it was in contact with the muscle. Body temperature was continuously monitored via a rectal probe. Muscle and body temperatures were maintained constant at 32 and 37°C, respectively, using heat lamps.

The muscle preparation was allowed to recover for 30 min to allow the surgically induced hyperemic blood flow to return to normal (27). During this recovery period, two fields of view were randomly chosen, each containing at least one complete microvascular unit (i.e., an arteriole, capillary bed, and postcapillary venule). After the recovery period, these fields of view were recorded on videotape ( × 20 objective) with a final magnification at the video monitor of × 700. These images were used to determine microvascular perfusion.

After preschismic microvascular blood flow was recorded on video, all animals underwent 2 h of normothermic no-flow ischemia induced by tightening a tourniquet around the hindlimb proximal to the EDL muscle. A condition of no-flow ischemia was confirmed using intravital microscopy. Monitoring throughout the ischemic period ensured that a state of no-flow persisted. Immediately before the onset of reperfusion, the nuclear fluorescent dyes bisbenzimide (Hoechst 33342; 5 µg/ml), which permeates all cells, and ethidium bromide (5 µg/ml), which stains the nuclei of injured cells only, were added to the muscle baths of all groups. Tissue viability was assessed by epifluorescence illumination with the appropriate filters for bisbenzimide (excitation = 343 nm, emission = 483 nm) and ethidium bromide (excitation = 482 nm, emission = 610 nm) (5, 6, 22). To inhibit NOS activity, the NOS inhibitor L-NAME (100 µmol/l), its inactive enantiomer D-NAME (100 µmol/l), or the iNOS-specific inhibitor AMG (100 µmol/l) was added to the baths of separate groups of rats. L-NAME, D-NAME, or AMG was applied to the muscle bath immediately before reperfusion.

Analytical Procedures

Video analysis. Video recordings of each field of view were obtained before ischemia was induced and every 15 min during the 90-min reperfusion period. Microvascular perfusion and tissue injury were measured from the playback of the video recordings.

An index of microvascular perfusion was obtained by counting the number of perfused capillaries crossing three parallel lines drawn on the video monitor perpendicular to the capillary axis. This index was expressed as the number of perfused capillaries per millimeter of line length (Npc) (6, 7, 22).

An index of cellular injury was obtained from the ratio of the number of nuclei stained with ethidium bromide (injured cells) to the number stained with bisbenzimide (total number of cells) and was expressed as a percentage. The number of nuclei stained with bisbenzimide was determined 15 min after the topical application of the dyes. This population of cells was then followed throughout the 90-min reperfusion period and observed for evidence of initial or subsequent staining with ethidium bromide. It is well known that ethidium bromide is the most permeable of the impermeant vital dyes and thus would provide an index of the total number of injured cells (i.e., minor through severe injury).

Measurement of NOS activity. NOS activity was estimated from the conversion of ³H-labeled l-arginine to ³H-labeled l-citrulline (l-[³H]citrulline), as previously described (25). Briefly, EDL muscles harvested from PC (n = 6) and control (i.e., no PC) rats (n = 6) were homogenized and assayed for calcium-dependent (constitutive) NOS activity (cNOS) by 10.220.33.3 on March 31, 2017 http://ajpheart.physiology.org/ Downloaded from by 10.220.33.3 on March 31, 2017 http://ajpheart.physiology.org/ Downloaded from by
as the blank. cNOS activity was calculated as the difference between the calcium/calmodulin sample (cNOS + iNOS activities) and the EDTA/EGTA sample (iNOS activity only). Nonspecific radioactivity and metabolism of l-arginine were accounted for by incubating tissue homogenate with L-NAME (10 µM) in the incubation buffer containing EDTA/EGTA. iNOS activity was calculated as the L-NAME-inhibitable portion of the activity in the samples with EDTA and EGTA. Resultant enzyme activities were expressed as picomoles of L-[3H]citrulline evolved per minute per milligram of protein.

**Results**

**Effect of Preconditioning**

The mean N_{pc} in all groups was not significantly different during the preischemic period (ischemic: 31.7 ± 3.9; PC: 26.7 ± 0.8; PC + L-NAME: 27.4 ± 2.6; PC + d-NAME: 27.8 ± 0.3; and PC + AMG: 29.7 ± 0.5). After the second insult (2 h of ischemia), the EDL muscles demonstrated a progressive reduction in N_{pc} that became significant at 75 min of reperfusion (11.5 ± 5.1) and was significantly lower than the N_{pc} in PC muscles at 90 min of reperfusion. In contrast, the PC group showed no decrease in N_{pc} throughout the 90-min reperfusion period (N_{pc}: 23.6 ± 2.5) (Fig. 1A).

No significant difference in parenchymal injury was measured between the ischemic and PC groups. Both groups demonstrated a significant increase in tissue injury compared with naive muscle as early as 15 min after reperfusion (naive: 27.4 ± 3.4%; ischemic: 64.5 ± 6.6%; and PC: 64.9 ± 12.6%; Fig. 1B). The extent of tissue injury reached a maximum after 60–75 min of reperfusion (ischemic: 83.4 ± 11.2%; PC: 82.3 ± 5.4%).

**NOS Activity**

iNOS activity in PC muscles (1.94 ± 0.24 pmol·min⁻¹·mg protein⁻¹) was significantly higher (81%) compared with that in non-PC muscles (1.07 ± 0.12 pmol·min⁻¹·mg protein⁻¹) (Fig. 2). However, cNOS activity in the PC muscles (1.14 ± 0.25 pmol·min⁻¹·mg protein⁻¹) was significantly lower by 53% compared with that in non-PC muscles (2.44 ± 0.19 pmol·min⁻¹·mg protein⁻¹).

**Inhibition**

After 90 min of reperfusion, the average N_{pc} in PC + L-NAME-treated muscles (15.0 ± 1.7) was significantly lower than the average N_{pc} in PC (23.6 ± 2.5) or PC + d-NAME-treated muscles (26.5 ± 1.7) (Fig. 3). In fact, treating PC muscles with L-NAME resulted in a reduction in the mean N_{pc} that was not significantly different from that measured in ischemic (non-PC) muscles (11.5 ± 2.2). Interestingly, treating ischemic muscles (without PC) with L-NAME resulted in a significant increase in N_{pc} compared with the ischemic group (without L-NAME), whereas L-NAME treatment in naive rats (i.e., no ischemia or PC) had no effect on the average N_{pc} (Fig. 4). Unlike PC + L-NAME-treated muscles, the N_{pc} in PC + AMG-treated muscles (22.8 ± 3.1) was not significantly different from the mean N_{pc} in untreated PC muscles (23.6 ± 2.5) (Fig. 3). However, the dose of AMG used in these experiments was sufficient to significantly improve N_{pc} in ischemic muscles not exposed to prior PC (Fig. 4).

The change in N_{pc} in PC + L-NAME-treated muscles was gradual, requiring 60 min of reperfusion before becoming significantly lower than the preischemic level. In contrast, PC + d-NAME- or PC + AMG-treated muscles did not demonstrate a statistically significant reduction in N_{pc} throughout the reperfusion period (Fig. 5).

**Discussion**

The use of intravital video microscopy provided the unique opportunity to directly test the development of ischemic tolerance by simultaneously measuring the temporal progression of parenchymal injury and micro-

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**Fig. 1.** A: average number of perfused capillaries (N_{pc}; mm⁻¹) at surface of rat extensor digitorum longus (EDL) muscles 15 min before ischemia (−15 min) was not different between muscles with preconditioning (PC; ■) and ischemic muscles without PC (○). A progressive decline in N_{pc} during reperfusion occurred following ischemia in muscles without PC, whereas such a perfusion deficit did not occur in muscles with PC. *P < 0.05 compared with preischemia; #P < 0.05 compared with PC group. B: an estimate of tissue injury in EDL muscle was obtained from ratio of ethidium bromide-to-bisbenzimide-labeled nuclei (E/B) and expressed as a percentage. Both PC (■) and ischemic muscles without PC (○) had significantly higher estimates of tissue injury compared with naive muscles (▲) 15 min after 2 h of ischemia. *P < 0.05 compared with naive group.
vascular perfusion following a prolonged ischemic insult. Using this technique, we have shown highly significant microvascular protection in rat skeletal muscle 24 h after ischemic PC. Although delayed or late protection had been shown to follow PC in various organs (3, 11, 16, 19, 31), our results are unique because they suggest that preservation of microvascular perfusion and parenchymal protection may not occur concurrently within skeletal muscle.

Fluorescent vital dyes have been used previously to great effect in the study of I/R-induced injury in skeletal muscle (6, 7). Our application of this technique used the ratio of the number of nuclei stained with ethidium bromide (an impermeant fluorescent vital dye) to the number stained with bisbenzimide (a permeant fluorescent dye) as an index of tissue injury (6, 7, 22). Compared with other vital dyes such as propidium
iodide, ethidium bromide is the most permeable, and thus we believe it more closely estimates total cell injury (minor to severe). The degree of tissue injury measured in the naive rats used in the present study was comparable to that reported previously (6, 7) using the same experimental protocol. We previously showed (6, 7) that such baseline tissue injury did not increase over time in our EDL preparation, and thus we established the stability of the muscle preparation.

The lack of parenchymal protection within skeletal muscle was surprising in view of the reported benefit afforded the myocardium following PC (3, 16). Such results suggest that the mechanisms underlying parenchymal protection may be different from those that preserve microvascular perfusion in skeletal muscle. Although the mechanisms responsible for the late benefits of PC remain an active area of study, it is believed that protection may be the result of the increased synthesis of cytoprotective proteins (e.g., heat stress proteins, endogenous antioxidants) (9, 29). It is generally argued that the time required for the induction of protein synthesis is the time-dependent mechanism leading to ischemic tolerance. Presumably, different tissues may require different times for the production of such protective proteins. Thus the 24 h that followed PC may not have been sufficient time for the parenchyma of the EDL muscle to produce the proteins necessary for parenchymal protection. Further studies are required regarding the time-dependent nature of parenchymal protection within skeletal muscle.

Measurements of the activity of cNOS and iNOS within the EDL muscle (without PC) showed the presence of both NOS isoforms and showed that the ratio of cNOS to iNOS activity was reversed after PC. L-NAME treatment after ischemia (without PC) resulted in improved microvascular perfusion. Interestingly, application of AMG to ischemic muscles (without PC) resulted in the same increase in the microvascular perfusion as that measured after L-NAME treatment. Such results suggest not only that NO may be injurious during reperfusion of skeletal muscle, an observation recently suggested by others (25), but also that the source of such NO may be from iNOS.

To test the role of NO during ischemic tolerance in skeletal muscle, we inhibited the activity of the NOS enzymes. For this purpose we made use of L-NAME, a nonselective inhibitor of NOS activity, as well as AMG, a selective NOS inhibitor. Treating PC muscles with L-NAME significantly reduced the benefit afforded by ischemic PC. However, treating PC muscles with AMG had no effect. Such observations suggest that although NO was essential for the late benefits afforded PC, the source of NO was not via iNOS activity, despite elevated iNOS activity within these tissues.

Recent evidence has suggested that preconditioning the myocardium with the use of either brief periods of ischemia (4) or monophosphoryl lipid A (32) resulted, 24 h later, in ischemic tolerance. These studies provided evidence suggesting that NO was important for the expression of such tolerance. The results of the present study concur with these results and, taken together, suggest that NO may be a common final pathway for the expression of tolerance in different tissues. However, conclusions regarding the source of NO appear to differ. For example, the use of either intravenously (32) or subcutaneously (4) administered AMG has suggested that iNOS was important for the expression of ischemic tolerance, whereas in the present study administration of AMG to the organ bath was ineffective in altering the PC-induced protection to microvascular perfusion. Although such disparate results may be due to differences between the models or organs being studied, it is also possible that differences in the concentration of AMG used in these studies could account for such disparities. The use of 100 µmol/l of AMG in the present study was chosen because this dosage, when used in organ bath preparations, has been shown to have high selectivity for iNOS (8, 17, 24). The preparation of the EDL muscle is equivalent to that used in organ bath studies (i.e., AMG applied to solution bathing the tissue). The selectivity of AMG for iNOS is dose dependent such that, at higher doses, AMG may act on other NOS isoforms such as cNOS and thus mimic the results of L-NAME treatment. Because it is difficult to establish the exact concentration of AMG that would be present after whole body applications, it is difficult to determine whether, in such studies, the actions of AMG remained selective for iNOS. The fact that the same concentration of AMG was effective after ischemia without PC but was after ischemia in PC muscles suggests that the dose of AMG used in the present study was likely selective for iNOS. The observations that iNOS activity was significantly elevated 24 h after PC but apparently had no influence on the outcome of PC suggest that the muscle may have developed tolerance to the increased NO via the PC-induced increase in iNOS activity. Such an intriguing hypothesis awaits further study.

In conclusion, ischemic tolerance has been shown to occur in skeletal muscle 24 h after ischemic PC. Such tolerance was reflected in preserved capillary perfusion within the EDL muscle after a 2-h ischemic period. Although microvascular perfusion was protected, no coexisting reduction in parenchymal injury was measured 24 h after the PC stimuli. The delayed benefits afforded by PC appeared to be due to NO; however, the source of NO does not appear to be via the increased iNOS activity per se.

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