Postconditioning for salvage of ischemic skeletal muscle from reperfusion injury: efficacy and mechanism

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McAllister SE, Ashrafpour H, Cahoon N, Huang N, Moses MA, Neligan PC, Forrest CR, Lipa JE, Pang CY. Postconditioning for salvage of ischemic skeletal muscle from reperfusion injury: efficacy and mechanism. Am J Physiol Regul Integr Comp Physiol 295: R681–R689, 2008. First published May 28, 2008; doi:10.1152/ajpregu.90303.2008.—We tested our hypothesis that postischemic conditioning (PostC) is effective in salvage of ischemic skeletal muscle from reperfusion injury and the mechanism involves inhibition of opening of the mitochondrial permeability transition pore (mPTP). In bilateral 8 × 13 cm pig latissimus dorsi muscle flaps subjected to 4 h ischemia, muscle infarction increased from 22 ± 4 to 41 ± 1% between 2 and 24 h reperfusion and remained unchanged at 48 (38 ± 6%) and 72 (40 ± 1%) h reperfusion (P < 0.05; n = 4 pigs). PostC induced by four cycles of 30-s reperfusion/reocclusion at the onset of reperfusion after 4 h ischemia reduced muscle infarction from 44 ± 2 to 22 ± 2% at 48 h reperfusion. This significant protective effect of PostC was mimicked by intravenous injection of the mPTP opening inhibitor cyclosporin A or NIM-811 (10 mg/kg) at 5 min before the end of 4 h ischemia and was abolished by intravenous injection of the mPTP opener atracyloside (10 mg/kg) at 5 min before PostC (P < 0.05; n = 4–5 pigs). PostC or intravenous cyclosporin A injection at 5 min before reperfusion caused a decrease in muscle myeloperoxidase activity and mitochondrial free Ca2+ concentration and an increase in muscle ATP content after 4 h ischemia and 2 h reperfusion compared with the time-matched controls. These effects of PostC were abolished by intravenous injection of atracyloside at 5 min before PostC (P < 0.05; n = 6 pigs). These observations support our hypothesis that PostC is effective in salvage of ischemic skeletal muscle from reperfusion injury and the mechanism involves inhibition of opening of the mPTP.

skeletal muscle postconditioning; mitochondrial permeability transition pore; neutrophil accumulation; mitochondrial calcium content; 5′-adenosine triphosphate synthesis

There are many surgical procedures in which skeletal muscle is subjected to warm (room temperature) global ischemia. For example, in elective musculoskeletal and vascular reconstructive surgery, single or multiple skeletal muscles are subjected to global ischemia under vascular clamp or tourniquet control; and, in trauma surgery, multiple skeletal muscles are subjected to global ischemia as a result of amputation, vascular obstruction or compression. Human skeletal muscle is known to withstand up to ~2.5 h of warm global ischemia (5, 10, 26). However, the ischemic insult may be prolonged by unpredictable complications (e.g., vasospasm, thrombosis) occurring perioperatively in elective surgery and by a delay in surgical intervention in trauma surgery. Protracted ischemic insult can cause skeletal muscle ischemia-reperfusion (I/R) injury, ranging from loss of function or infarction in a single skeletal muscle to life-threatening acidosis, hyperkalemia, and myoglobinuria in the case of massive skeletal muscle infarction (5, 42). Intervention strategies focusing on prevention of thrombosis and free radical injury have not been proven to be of clinical benefit in prevention of I/R injury in skeletal muscle (36).

In 1986, Murry et al. (28) reported that ischemic preconditioning (IPC) of dog myocardium with four cycles of 5 min coronary artery occlusion/reperfusion immediately before sustained ischemia induced robust myocardial protection against infarction. Using this local IPC technique, we demonstrated that preconditioning pig latissimus dorsi (LD) and gracilis muscle flaps with three cycles of 10-min reperfusion/reocclusion, by occlusion of the vascular pedicle with a vascular clamp, reduced muscle infarction by 44 and 62%, respectively, when these muscle flaps were subsequently subjected to 4 h of ischemia and 48 h of reperfusion (32). However, in trauma surgery such as replantation of amputated hand, foot, and limb; decompression of compartment syndrome; or thrombolysis, there is no opportunity for preischemic treatment as in elective surgery because the sustained ischemia in traumatic injury occurs before the patient arrives at the hospital for surgical intervention. In 2003, Zhao et al. (46) demonstrated in the dog myocardium that instigation of three cycles of 30-s reperfusion/reocclusion in the coronary artery immediately after 60 min of sustained ischemia reduced myocardial infarction, coronary artery endothelial dysfunction, and neutrophil accumulation in the area at risk. This phenomenon was termed postconditioning (PostC), and the infarct protective effect was similar in extent to local IPC. The myocardial infarct protective effect of PostC was subsequently demonstrated in mice (20), rats (24), rabbits (45), pigs (22), and humans (38). The pathophysiology of PostC has been reviewed recently (15, 48). There is evidence to indicate that opening of the mitochondrial permeability transition pore (mPTP) and mitochondrial Ca2+ accumulation are key events in necrotic and apoptotic cell death in myocardial reperfusion injury (16, 48). Specifically, cytosolic Ca2+ overload and overproduction of reactive oxygen species by accumulating neutrophils and ischemic endothelial and myocardial cells can induce opening of the mPTP, which can cause efflux of cytochrome C and other proapoptotic factors to cause apoptotic cell death. Opening of the mPTP can...
Postischemic conditioning against muscle infarction

also cause collapse of mitochondrial membrane potential, matrix swelling, and uncoupling of the respiratory chain, causing the F0F1 ATPase to hydrolyze instead of synthesize ATP, thus causing cell necrosis (12, 29). Conversely, there is evidence to indicate that PostC reduces cell death when cardiomyocytes are subjected to sustained ischemia and reperfusion, and this protective effect of PostC is associated with attenuation of neutrophil accumulation and intracellular and mitochondrial Ca2+ overload (39). Of particular interest to us was the recent report that superoxide anion could also open the mPTP during reoxygenation of hypoxic rat skeletal muscle (30). However, the ischemic tolerance and blood supply are different between cardiac and skeletal muscle. It is not known if PostC can confer infarct protective effect in skeletal muscle, and, if so, it is not known if the protocol and mechanism are similar in cardiac and skeletal muscle. Therefore, we planned to investigate the efficacy and mechanism of PostC in salvage of ischemic skeletal muscle from reperfusion injury. Specifically, the objective of this research project was to use the clinically relevant in vivo pig latissimus dorsi muscle flap model to test the hypothesis that PostC of ischemic skeletal muscle with brief cycles of reperfusion/reocclusion at the onset of reperfusion is effective in salvage of ischemic skeletal muscle from infarction and the mechanism involves inhibition of opening of the mPTP, which is associated with attenuation of neutrophil accumulation, prevention of mitochondrial Ca2+ overload, and preservation of ATP synthesis during the early phase of reperfusion. Understanding the efficacy and mechanism of PostC in protection of ischemic skeletal muscle from infarction will provide important insights into the identification of a pharmacological therapy to mimic the infarct protective effect of PostC in salvage of ischemic skeletal muscle from infarction in clinical trauma surgery.

MATERIALS AND METHODS

Animal Management

Castrated Yorkshire pigs [19.1 ± 1.5 (SD) kg] were housed in a temperature-controlled (22°C) and light-controlled (0700-1900) pig holding room. All pigs were offered the same commercial diet and tap water ad libitum, but food was withheld the evening before surgery. The animal management and experimental surgery were approved by the Animal Care and User Committee of the Hospital for Sick Children Research Institute (Toronto, Canada). Similar animal management and animal surgical procedure were used previously (1, 27, 32).

Experimental Surgery

Anesthesia. Experimental surgery and in vivo experiments were performed under general anesthesia induced by intramuscular ketamine (25 mg/kg) and intravenous pentobarbitone sodium (12–15 mg/kg). After endotracheal intubation, the pig was mechanically ventilated with O2 and N2O (1:1 volume) to a tidal volume of 15 ml/kg. Body fluid and general anesthesia throughout surgery were maintained by intravenous infusion of isotonic saline (2 ml/min) containing pentobarbitone sodium (0.5–1.0 mg·kg−1·min−1). Rectal temperature was monitored and kept within normal range (38–39°C) by warming the pig with a heating blanket.

Muscle flap surgery. In clinical reconstructive surgery, LD muscle flaps are routinely used in autogenous muscle transplantation for wound coverage because the LD muscle is not essential for locomotion or arm function. In research, the pig island LD muscle flap model has been well established in our laboratory for the study of the infarct protective effect of local and remote IPC (1, 27, 32). In the following studies, bilateral 8 × 13 cm island LD muscle flaps were raised, based on the thoracodorsal neurovascular bundle and a 1-cm wide muscle tendon for support. The muscle tendon was ligated with 1-0 silk ties; thus, blood supply to the island LD muscle flap was entirely from the thoracodorsal artery and drained by two thoracodorsal veins. As in clinical autogenous muscle transplantation, the thoracodorsal nerve was transected. The skin overlying the LD muscle flap was closed with 3-0 prolene sutures, leaving a small opening in the axilla so that the vascular pedicle could be accessed and clamped to induce global ischemia in the LD muscle flaps.

Induction of I/R injury in LD muscle flaps. Under general anesthesia, two vascular clamps (2 × 8 mm; Weck) were placed on the vascular pedicle to render the LD muscle flap totally ischemic. Global ischemia in LD muscle flaps was verified by intravenous injection of fluorescein dye (15 mg/kg), as described previously (1, 27, 32). Global ischemia in LD muscle flaps was confirmed by the absence of yellow fluorescence in LD muscle flaps observed under ultraviolet light at 10–15 min after dye injection. The LD muscle flaps were subjected to 4 h of sustained global ischemia at operating room temperature (24°C). This room temperature was similar to that in clinical autogenous muscle transplantation. Reperfusion in LD muscle flaps after removal of the vascular clamps at the end of 4 h of ischemia was confirmed by a second injection of fluorescein dye and the immediate appearance of yellow fluorescence in the muscle flap (32). The skin wounds were closed with 3-0 prolene sutures, and the pig was allowed to wake up and was returned to the pig holding room.

Instigation of IPC and PostC. In IPC, LD muscle flaps were subjected to three cycles of 10-min occlusion/reperfusion immediately before 4 h of sustained ischemia by application of a vascular clamp (2 × 8 mm; Weck) on the vascular pedicle (9). In PostC, LD muscles were subjected to four or six cycles of 30-s reperfusion/reocclusion immediately at the beginning of reperfusion after 4 h of sustained ischemia by application of a vascular clamp on the vascular pedicle.

Assessment of muscle infarct size in LD muscle flaps. LD flaps were subjected to 4 h of ischemia and 2, 24, 48, or 72 h of reperfusion. At the end of reperfusion, pigs were killed with an overdose of intravenous pentobarbitone sodium (100 mg/kg). The LD muscle flaps were immediately excised and cut transversely into 13 1 × 8 cm segments for assessment of muscle infarction, using the nitroblue tetrazolium dye (NBT) staining technique. The viable areas of muscle were stained blue, and the nonviable areas remained red in color in all 13 segments of muscle (Fig. 1). A digital photograph was taken (Konica Minolta DiMage XG), and digital imaging software (Adobe Photoshop CS) was used to calculate the amount of viable and nonviable muscle and muscle viability. This method was well established by us and was found to be closely related to the template technique (1, 9, 32). Histochemical and radioisotopic techniques have been used to document the accuracy of the NBT dye staining technique for assessment of dog skeletal muscle after 48 h of reperfusion (25). We also observed previously that there was no infarction in 8 × 13 cm LD muscle flaps in the pig without sustained ischemic insult (32). Therefore, a nonischemic control group was not planned in the present studies to avoid unnecessary killing of pigs.

Chemical Analysis

Collection of muscle biopsies. Muscle biopsies (1 × 1 cm) were taken from the bilateral LD muscle flaps in each pig. Muscle biopsies for assay of mitochondrial free calcium concentration (mito[Ca2+]i) were taken from the left LD muscle flap immediately before and at 2 and 4 h of sustained ischemia and 2 h of reperfusion. Muscle biopsies for assay of muscle ATP content and myeloperoxidase (MPO) activity were taken from the right LD muscle flap immediately before and at 2 and 4 h of sustained ischemia and 2 h of reperfusion. Muscle biopsies (1 × 1 cm) were taken sequentially from the thick dorsal edge of the muscle flaps, starting at 7 cm from the vascular pedicle of...
the LD muscle flap and continuing proximally as described previously (32). Each biopsy was immediately rinsed with cold (4°C) isotonic saline. Fresh biopsies were used for assay of free mito[Ca\(^{2+}\)]. Biopsies for assay of MPO activity and ATP content were immediately frozen in liquid nitrogen and stored at −80°C.

**Measurement of MPO activity.** The technique was described by us previously (31). Briefly, frozen muscle biopsies (~200 mg) were weighed and homogenized. The resulting supernatant was assayed for neutralophilic MPO activity using a spectrophotometry technique. One unit of enzyme activity was defined as the amount of MPO activity that produced an absorbance change of 1.0 optical density unit of 1 ml of cold (4°C) saline. The suspension was then centrifuged at 4°C for 10 min two times. The pellet was discarded. The resulting supernatant was assayed for ATP content using a FL-AA bioluminescent assay kit (Sigma, Oakville, Ontario, Canada). ATP content was determined by the Bradford Method (Bio-Rad, Hercules, CA).

**Assay for muscle ATP content.** Frozen muscle biopsies (~200 mg) were subjected to 4 h of ischemia and 48 h of reperfusion. Muscle infarction in LD muscle flaps was assessed at the end of reperfusion, using the nitroblue tetrazolium dye staining technique.

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four cycles of 30-s reperfusion/reocclusion (PostC) immediately after 4 h of sustained ischemia. In groups 3 and 4, pigs (n = 5) received an intravenous injection of the mPTP inhibitor cyclosporin A (CsA, 10 mg/kg) and NIM-811 (10 mg/kg), respectively, at 5 min before onset of reperfusion. In groups 5 and 6, pigs (n = 4) received an intravenous injection of the mPTP opener atracyloside (ATRAC, 10 mg/kg) at 5 min before PostC or 5 min before onset of reperfusion without PostC, respectively. Muscle infarction in all LD muscle flaps was assessed at the end of 48 h of reperfusion, using the nitroblue tetrazolium dye staining technique.

In small laboratory animals, the effective intravenous bolus dose for the mPTP inhibitors CsA and NIM-811 was 10 mg/kg (3) and for the mPTP opener ATRAC was 5–25 mg/kg (33, 43). In our preliminary study, we observed that the effective intravenous bolus dose for CsA, NIM-811, and ATRAC was 10 mg/kg, and this dose was used in this and the following study.

Study 4: Investigation of the mechanism associated with PostC in salvage of ischemic skeletal muscle from infarction. This study was designed to demonstrate that the infarct protective effect of PostC involved attenuation of neutrophil accumulation, preservation of ATP synthesis in ischemic skeletal muscle in early reperfusion. Pigs with bilateral LD muscle flaps were assigned to one control and four treatment groups (n = 6 pigs). All LD muscle flaps were subjected to 4 h of ischemia and 2 h of reperfusion. Group 1 was the ischemic control. In group 2, LD muscle flaps underwent four cycles of 30-s reperfusion/reocclusion (PostC) at the onset of reperfusion after 4 h of ischemia. Pigs in group 3 received intravenous injection of CsA (10 mg/kg) at 5 min before onset of reperfusion of LD muscle flaps after 4 h of ischemia. Pigs in groups 4 and 5 received an intravenous injection of the mPTP opener ATRAC (10 mg/kg) at 5 min before PostC or 5 min before onset of reperfusion without PostC. In the left LD muscle flaps, muscle biopsies (1 × 1 cm) were taken immediately before and at the end of 2 and 4 h of sustained ischemia and at 2 h of reperfusion, and these fresh biopsies were used for assay of muscle free mito[Ca2+]2. In the right LD muscle flaps, muscle biopsies were taken immediately before and at the end of 2 and 4 h of ischemia and 2 h of reperfusion. These biopsies were frozen immediately in liquid nitrogen and stored at −80°C for assay of MPO activity and ATP content. In our previous studies, we observed that IPC significantly increased ATP content and decreased neutrophil accumulation in pig LD muscle flaps within 2 h of reperfusion compared with the control (31, 32). In addition, our recent preliminary data indicated that PostC reduced mito[Ca2+]2 at 0.5, 1, and 2 h of reperfusion in pig LD muscle flaps compared with the time-matched control. Therefore, we chose 2 h of reperfusion to investigate the effect of PostC on ATP content, neutrophil accumulation, and mito[Ca2+]2 in pig LD muscle flaps in this study.

Statistical Analysis

All values are expressed as means ± SE. In Fig. 1–3, one-way analysis of variance followed by Neuman-Keuls test were used for multiple comparisons of means. In Figs. 4–6, treatment effect was detected using two-way analysis of variance with repeated measures. Within each time point, one-way analysis of variance and Neuman-Keuls test were used for multiple comparison of means. Statistical significance was set at P ≤ 0.05 for all tests.

RESULTS

Reperfusion Injury in Skeletal Muscle

In LD muscle flaps subjected to 4 h of ischemia, there was 22 ± 4% muscle infarction at 2 h of reperfusion (P < 0.05). The muscle infarction increased significantly (P < 0.05) to 41 ± 1% within 24 h of reperfusion, and the infarct size remained statistically unchanged at 48 h (38 ± 6%) and 72 h (40 ± 1%) of reperfusion (Fig. 2).

Comparison of IPC and PostC in Protection of Skeletal Muscle from I/R Injury

The infarction in pig LD muscle flaps subjected to 4 h of ischemia and 48 h of reperfusion was 44 ± 2% (Fig. 3). Acute IPC of LD muscle flaps with three cycles of 10-min occlusion/reperfusion immediately before 4 h of ischemia and PostC of LD muscle flaps with four cycles of 30-s reperfusion/reocclusion at the onset of reperfusion immediately after 4 h of ischemia significantly (P < 0.05) reduced the muscle infarction to 23 ± 1 and 22 ± 2%, respectively, and these two mean values were not significantly different. PostC LD muscle flaps with six cycles of 30-s reperfusion/reocclusion did not further reduce muscle infarction compared with PostC with four cycles of 30-s occlusion/reperfusion (Fig. 3).

Role of the mPTP in PostC of Ischemic Skeletal Muscle Against Infarction

PostC or intravenous injection of the mPTP inhibitor CsA (10 mg/kg) or NIM-811 (10 mg/kg) at 5 min before the end of 4 h of ischemia reduced infarction in pig LD muscle flaps to a similar extent at 48 h of reperfusion (Fig. 4). Specifically, the muscle infarction in the control group was 45 ± 2%. PostC and intravenous injection of CsA or NIM-811 significantly (P < 0.05) reduced infarction to 22 ± 1, 27 ± 1, and 26 ± 1% (Fig. 4). Conversely, intravenous injection of the mPTP opener ATRAC (10 mg/kg) at 5 min before PostC completely abolished the infarct protective effect of PostC, increasing the infarction to 47 ± 2%. However, intravenous injection of ATRAC at 5 min before onset of reperfusion without subsequent PostC did not affect the infarct size of LD muscle flaps compared with the ischemic control (Fig. 4).

Effect of PostC and Intravenous Injection of CsA at 5 min Before Onset of Reperfusion on MPO Activity, Free Mito[Ca2+]2, and ATP Content in Ischemic LD Muscle Flaps During Early Reperfusion

MPO activity. There was no significant difference in neutrophilic MPO activity in LD muscle flaps within and between control and treatment groups immediately before ischemia and at the end of 2 and 4 h of ischemia (Fig. 5). However, at the end of 2 h of reperfusion, the muscle MPO activity was significantly (P < 0.05) higher in the control LD muscle flaps than in
LD muscle flaps that had been treated with PostC at the onset of reperfusion or with intravenous injection of the mPTP inhibitor CsA (10 mg/kg) at 5 min before onset of reperfusion. The ability of PostC to lower muscle MPO activity observed at 2 h of reperfusion was completely abolished by intravenous injection of the mPTP opener ATRAC (10 mg/kg) at 5 min before PostC. However, this ATRAC treatment did not have any effect on muscle MPO activity in LD muscle flaps subjected to 4 h of ischemia and 2 h of reperfusion without PostC, compared with the control (Fig. 5).

Muscle free mito[Ca^{2+}]. The muscle free mito[Ca^{2+}] was similar among control and treatment groups of LD muscle flaps before ischemia and at the end of 2 and 4 h of ischemia (Fig. 6). However, the muscle free mito[Ca^{2+}] in the ischemic control LD muscle flaps increased significantly (P < 0.05) at 2 h of reperfusion compared with the control LD muscle flaps before ischemia and at the end of 2 and 4 h of ischemia. PostC at the onset of reperfusion or intravenous injection of the mPTP inhibitor CsA (10 mg/kg) at 5 min before onset of reperfusion inhibited the significant increase in muscle free mito[Ca^{2+}] in LD muscle flaps at 2 h of reperfusion (Fig. 6). The effect of PostC in lowering muscle free mito[Ca^{2+}] in ischemic LD muscle flaps during 2 h of reperfusion was completely abolished by intravenous injection of the mPTP opener ATRAC (10 mg/kg) at 5 min before onset of reperfusion. However, this ATRAC treatment did not have any effect on muscle mito[Ca^{2+}] in LD muscle flaps subjected to 4 h of ischemia and 2 h of reperfusion without PostC, compared with the ischemic control (Fig. 6).

ATP content. There was no difference in muscle ATP content among all the five groups of LD muscle flaps before ischemia and at the end of 2 and 4 h of ischemia (Fig. 7). The muscle ATP content decreased significantly (P < 0.05) to a similar extent in all groups of LD muscle flaps at the end of 4 h of reperfusion in LD muscle flaps immediately before 4 h of ischemia. PostC was induced with 4 cycles of 30-s reperfusion/reocclusion at the onset of reperfusion. The mPTP opener atractyloside (10 mg/kg) was injected iv at 5 min before onset of reperfusion. PostC was induced with 4 cycles of 30-s reperfusion/reocclusion at the onset of reperfusion. The mPTP opener atractyloside (10 mg/kg) was injected iv at 5 min before PostC or 5 min before onset of reperfusion without PostC. Values are means ± SE; n = 6 pigs. Within each time point, means with an asterisk are similar and are significantly (*P < 0.05) different from the means of the control and the rest of the treatment groups.
of ischemia. However, at the end of 2 h of reperfusion, the muscle ATP content was significantly \((P < 0.05)\) higher in LD muscle flaps that were postconditioned with four cycles of 30-s reperfusion/reocclusion at the onset of reperfusion, compared with the time-matched ischemic control. Intravenous injection of the mPTP inhibitor CsA (10 mg/kg) at 5 min before onset of reperfusion also significantly \((P < 0.05)\) increased the muscle content of ATP at the end of 2 h of reperfusion compared with the time-matched control (Fig. 7). The muscle content of ATP at 2 h of reperfusion was similar in LD muscle flaps treated with PostC or with intravenous injection of CsA at 5 min before onset of reperfusion. The effect of PostC in maintaining a significantly higher muscle content of ATP in ischemic LD muscle flaps at the end of 2 h of reperfusion compared with the time-matched control was abolished by intravenous injection of the mPTP opener ATRAC (10 mg/kg) at 5 min before onset of reperfusion. This ATRAC treatment did not have any effect on muscle ATP content in LD muscle flaps subjected to 4 h of ischemia and 2 h of reperfusion without PostC, compared with the time-matched control (Fig. 7).

**DISCUSSION**

**Important Findings from Present Studies**

Using the pig bilateral LD muscle flap model, we investigated for the first time the efficacy and mechanism of PostC in salvage of ischemic skeletal muscle from reperfusion injury. Briefly, we observed that pig LD muscle flaps sustained 22 ± 4% infarction after 4 h of warm (room temperature) ischemia and 2 h of reperfusion, and the maximal infarction (41 ± 1%) occurred within 24 h of reperfusion. Instigation of three cycles of 10-min occlusion/reperfusion immediately before 4 h of ischemia (i.e., IPC) or four cycles of 30-s reperfusion/reocclusion at the onset of reperfusion after 4 h of ischemia (i.e., PostC) reduced muscle infarction from 44 ± 2 to 23 ± 1 and 22 ± 2%, respectively, assessed at 48 h of reperfusion, and the infarct protective effect of IPC and PostC was similar in extent. The infarct protective effect of PostC was mimicked by intravenous injection (10 mg/kg) of the mPTP inhibitor CsA or NIM-811 at 5 min before onset of reperfusion and was abolished by intravenous injection of the mPTP opener ATRAC (10 mg/kg) at 5 min after PostC. The muscle infarct protective effect of PostC or intravenous injection of CsA at 5 min before onset of reperfusion was associated with a decrease in muscle neutrophilic MPO activity and in muscle free mito\([Ca^{2+}]\) and an increase in muscle ATP content after 4 h of ischemia and 2 h of reperfusion, compared with the time-matched ischemic control. Collectively, these observations indicate that PostC is effective in salvaging ischemic skeletal muscle from reperfusion injury, and the mechanism involves inhibition of mPTP opening, which is associated with attenuation of neutrophil accumulation, prevention of mitochondrial Ca\(^{2+}\) overload, and preservation of ATP synthesis.

**Time Course of Reperfusion Injury in Skeletal Muscle**

Reperfusion after sustained ischemia is a “double-edged sword.” There is no doubt that timely reperfusion is essential for salvage of ischemic tissue/organ from ischemic injury. However, uncontrolled reperfusion can cause reperfusion injury as well. For example, it was reported in dog myocardium subjected to 1 h of ischemia that the infarct size increased significantly between 6 and 24 h of reperfusion, and the infarct size remained unchanged up to 48 h of reperfusion (47). In the present study, the time course of reperfusion injury in ischemic skeletal muscle was found to be similar. Specifically, pig LD muscle flaps sustained 22 ± 4% muscle infarction within 2 h of reperfusion after 4 h of warm ischemia. Maximal infarction of 41 ± 1% occurred within 24 h of reperfusion, and the infarction remained unchanged up to 48 h (38 ± 6%) and 72 (40 ± 1%) h of reperfusion (Fig. 2). In the following studies, muscle infarction was assessed at the end of 48 h of reperfusion to ascertain the long-term infarct protective effect of ischemic and pharmacological PostC.

**Efficacy of PostC in Salvage of Skeletal Muscle from Reperfusion Injury**

The first study to demonstrate the myocardial protective effects of PostC was performed in dogs (46). Specifically, it was reported that instigation of three cycles of 30-s reperfusion/reocclusion of the coronary artery after 1 h of sustained ischemia reduced the infarct size by 44% at 3 h of reperfusion, compared with the ischemic control, and this extent of infarct protection was similar to that of IPC in the dog myocardium. Subsequently, the infarct protective effect of PostC was demonstrated in mice (20), rats (24), rabbits (45), pigs (22), and humans (38). However, the duration of cycles of reperfusion/reocclusion used for induction of PostC in the myocardium seems to vary with species of animals: mice and rats (10 –15 s), pigs and dogs (30 s), and humans (60 s). The number of brief cycles required to induce the myocardial infarct protective effect of PostC also seems to vary with species. For example, PostC-induced infarct protection could be demonstrated in dog myocardium with three cycles of 30-s reperfusion/reocclusion. However, more than four cycles of 30-s reperfusion/reocclusion in the pig myocardium were required to elicit the infarct protective effect of PostC (22, 37). Additionally, it was reported that the myocardial infarct protective effect of PostC in the rat was not as robust as that reported for the dog (24). Here, we demonstrated that PostC is effective in salvage of ischemic...
skeletal muscle from reperfusion injury. Specifically, PostC of skeletal muscle with four cycles of 30-s reperfusion/reoclusion at the onset of reperfusion after 4 h of sustained ischemia reduced the muscle infarct size from 44 ± 2% (ischemic control) to 22 ± 2% at 48 h of reperfusion (Fig. 3). There was no further increase in infarct protection when six cycles of 30-s reperfusion/reoclusion were used for induction of PostC. The skeletal muscle infarct protective effect of PostC was similar to that of IPC induced by instigation of three cycles of 10-min occlusion/reperfusion immediately before 4 h of ischemia followed by 48 h of reperfusion (Fig. 3). We also demonstrated previously that IPC with three cycles of 10 min occlusion/reperfusion immediately before 4 h of ischemia induced maximal infarct protection in the pig LD muscle flaps (32). Taken together, these observations indicate that PostC offers robust protection of ischemic skeletal muscle from reperfusion injury.

Role of the mPTP in PostC

Other investigators have reported observations that lead us to believe that the mPTP also plays a pivotal role in PostC in salvage of ischemic skeletal muscle from reperfusion injury. Specifically, it was demonstrated in rat myocardium that the mPTP remained closed during sustained ischemia (14, 23) and that mPTP opening was an important causative event in postischemic reperfusion injury (8). Furthermore, instigation of brief cycles of occlusion/reperfusion in myocardium immediately before sustained ischemia (i.e., IPC) or reperfusion/reoclusion at the onset of reperfusion (i.e., PostC) inhibited opening of the mPTP through the reperfusion injury salvage kinase (RISK) pathways (19) and protected myocardium from I/R injury in rats and rabbits (2, 4, 6, 18, 23). Additionally, important observations were reported that CsA was a nonspecific and NIM-811 was a specific inhibitor of the mPTP from opening (2–4, 17, 18, 23, 41), and ATRAC was a specific opener of the mPTP (18). Here, we used these pharmacological probes to demonstrate that the mPTP also plays a pivotal role in salvage of ischemic skeletal muscle from reperfusion injury in PostC. Specifically, there was a 46 ± 2% infarction in pig LD muscle flaps subjected to 4 h of ischemia and 48 h of reperfusion. Instigation of four cycles of 30-s reperfusion/reoclusion at the onset of reperfusion (i.e., PostC) reduced the infarction to 22 ± 1% (Fig. 4). This extent of infarct protective effect of PostC in pig LD muscle flaps was also achieved by intravenous injection of the mPTP inhibitor CsA or NIM-811 at 5 min before onset of reperfusion and abolished by intravenous injection of the mPTP opener ATRAC at 5 min before PostC, probably because ATRAC opened the mPTP, which was closed by PostC (Fig. 4). However, ATRAC treatment did not affect infarct size of ischemic pig LD muscle flaps without PostC because the specific action of ATRAC was to open the mPTP, and the mPTP was already open in the ischemic LD muscle flaps. Collectively, these observations supported our hypothesis that inhibition of mPTP opening is also central to PostC in salvage of ischemic skeletal muscle from reperfusion injury.

Mechanism Associated with Inhibition of mPTP Opening in Salvage of Ischemic Skeletal Muscle from Reperfusion Injury in PostC

Attenuation of neutrophil accumulation. The role of neutrophils in reperfusion injury and in PostC against reperfusion injury is still unclear. On one hand, it was reported that reperfusion injury and PostC against reperfusion injury occurred in neutrophil-free systems such as isolated perfusion rat hearts and cultured rat myocardial cells (39, 40). On the other hand, there is evidence to indicate that neutrophils may be an important contributor in reperfusion injury and PostC. Specifically, it was reported that, in dog myocardium undergoing 60 min of ischemia and 3 or 24 h of reperfusion, neutrophil accumulation and myocardial infarction increased between 3 and 24 h of reperfusion and PostC reduced neutrophil accumulation and myocardial infarction between 3 and 24 h of reperfusion (29). Here, we observed in pig skeletal muscle that I/R injury was also associated with neutrophil accumulation and muscle infarction, and PostC attenuated neutrophil accumulation and reperfusion injury at 2 h of reperfusion after 4 h of ischemia. Future in vivo studies are required to clarify if the reactive oxygen species produced by the accumulated neutrophils are causally related to opening of the mPTP or cell injury during reperfusion or if the accumulation of neutrophils is merely an inflammatory response to injury.

Prevention of mitochondrial Ca2+ overload. Cytosolic Ca2+ loading during reperfusion immediately after sustained ischemia is mainly caused by activation of the Na+/H+ exchanger by the high cytosolic concentration of H+. Subsequently, the high cytosolic Na+ concentration activates the Na+/Ca2+ exchanger, causing extrusion of Na+ and influx of Ca2+, resulting in cytosolic Ca2+ overload (16). A robust release of reactive oxygen species (ROS) by accumulated neutrophils at early reperfusion may also contribute to increase in cytosolic Ca2+ overload through increasing Na+/H+ exchange (34), Ca2+ influx through L-type channels (11, 13), and decreasing Ca2+ sequestration by sarcoplasmic reticular Ca2+-ATPase (21). Cytosolic Ca2+ overload promotes opening of the mPTP, resulting in mitochondrial Ca2+ overload (16, 18, 39). This results in uncoupling of oxidative phosphorylation and an inability to synthesize ATP, causing cell necrosis (16, 48). Recently, it was observed in rats and rabbits that IPC and PostC were effective in inhibition of the mPTP from opening and thus protected myocardium from reperfusion injury (3, 4, 6, 18, 23, 44). Here, we demonstrated that PostC salvaged ischemic pig skeletal muscle from reperfusion injury and the mechanism involved inhibition of opening of the mPTP and prevention of mitochondrial Ca2+ overload. Specifically, we observed that the muscle free mito[Ca2+] in LD muscle flaps remained unchanged at the end of 2 and 4 h of ischemia compared with the preischemic control (Fig. 6). This observation was in line with previous reports by other investigators that the myocardial mPTP remained closed during sustained ischemia thus preventing influx of Ca2+ in the mitochondria (14, 23). On the other hand, we observed that the muscle free mito[Ca2+] increased by 100% at the end of 2 h of reperfusion, compared with the preischemic control (Fig. 6). This observation was also consistent with previous observations reported by other investigators that the myocardial mPTP opened during reperfusion, allowing influx of cytosolic Ca2+ in the mitochondria (14, 23). Of particular importance is our observation that PostC of pig ischemic LD muscle at the onset of reperfusion after 4 h of ischemia or intravenous injection of the mPTP opening inhibitor CsA at
observed that the effect of PostC in inhibiting the increase in muscle free mito[Ca^{2+}] during early reperfusion was abolished by intravenous injection of the mPTP opener ATRAC at 5 min before onset of reperfusion after 4 h of ischemia (Fig. 6). Taken together, these observations indicate that the mechanism of PostC in salvage of ischemic skeletal muscle from infarction involved inhibition of opening of the mPTP and attenuation of mitochondrial Ca^{2+} overload.

**Perspectives**

In trauma surgery, such as replantation of hand, foot and limb; decompression of compartment syndrome; and thrombolysis, prolonged sustained ischemic insult may occur in the skeletal muscle before the patient arrives at the surgical room for surgical intervention, and there is no opportunity for preischemic treatment for prevention of ischemic skeletal muscle from reperfusion injury. The information obtained from this research project provides important insights into the development of a pharmacological therapy with inhibitors of mPTP opening for salvage of ischemic skeletal muscle from reperfusion injury in trauma surgery.

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