Inducing late phase of infarct protection in skeletal muscle by remote preconditioning: efficacy and mechanism

Michael A. Moses,1,2 Patrick D. Addison,1,2 Peter C. Neligan,1,2 Homa Ashrafpoor,1 Ning Huang,1 Sandra E. McAllister,1,2 Joan E. LIPA,2 Christopher R. Forrest,1,2 and Cho Y. Pang1,2,3

1Research Institute, The Hospital for Sick Children; and Departments of 2Surgery and 3Physiology, University of Toronto, Toronto, Ontario, Canada MSG IX8

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Moses, Michael A., Patrick D. Addison, Peter C. Neligan, Homa Ashrafpoor, Ning Huang, Sandra E. McAllister, Joan E. LIPA, Christopher R. Forrest, and Cho Y. Pang. Inducing late phase of infarct protection in skeletal muscle by remote preconditioning: efficacy and mechanism. Am J Physiol Regul Integr Comp Physiol 289: R1609-R1617, 2005. First published September 22, 2005; doi:10.1152/ajpregu.00395.2005.—We have previously demonstrated that remote ischemic preconditioning (IPC) by instigation of three cycles of 10-min occlusion/reperfusion in a hindlimb of the pig elicits an early phase of infarct protection in local and distant skeletal muscles subjected to 4 h of ischemia immediately after remote IPC. The aim of this project was to test our hypothesis that hindlimb remote IPC also induces a late phase of infarct protection in skeletal muscle and that KATP channels play a pivotal role in the trigger and mediator mechanisms. We observed that pig bilateral lattissimus dorsi (LD) muscle flaps sustained 46 ± 2% infarction when subjected to 4 h of ischemia/48 h of reperfusion. The late phase of infarct protection appeared at 24 h and lasted up to 72 h after hindlimb remote IPC. The LD muscle infarction was reduced to 28 ± 3, 26 ± 1, 23 ± 2, 24 ± 2 and 24 ± 4% at 24, 28, 36, 48 and 72 h after remote IPC, respectively (P < 0.05; n = 8). In subsequent studies, hindlimb remote IPC or intravenous injection of the sarcolemmal KATP (sKATP) channel opener P-1075 (2 μg/kg) at 24 h before 4 h of sustained ischemia (i.e., late preconditioning) reduced muscle infarction from 43 ± 4% (ischemic control) to 24 ± 2 and 19 ± 3%, respectively (P < 0.05, n = 8). Intravenous injection of the sKATP channel inhibitor HMR 1098 (6 mg/kg) or the nonspecific KATP channel inhibitor glibenclamide (Glib; 1 mg/kg) at 10 min before remote IPC completely blocked the infarct-protective effect of remote IPC in LD muscle flaps subjected to 4 h of sustained ischemia at 24 h after remote IPC. Intravenous bolus injection of the mitochondrial KATP (mKATP) channel inhibitor 5-hydroxydecanoate (5-HD; 5 mg/kg) immediately before remote IPC and 30-min intravenous infusion of 5-HD (5 mg/kg) during remote IPC did not affect the infarct-protective effect of remote IPC in LD muscle flaps. However, intravenous Glib or 5-HD, but not HMR 1098, given 24 h after remote IPC completely blocked the late infarct-protective effect of remote IPC in LD muscle flaps. None of these drug treatments affected the infarct size of control LD muscle flaps. The late phase of infarct protection was associated with a higher (P < 0.05) muscle content of ATP at the end of 4 h of ischemia and 1.5 h of reperfusion and a lower (P < 0.05) neutrophilic activity at the end of 1.5 h of reperfusion compared with the time-matched control. In conclusion, these findings support our hypothesis that hindlimb remote IPC induces an uninterrupted long (48 h) late phase of infarct protection, and sKATP and mKATP channels play a central role in the trigger and mediator mechanism, respectively.

late phase protection; adenosine 5’-triphosphate; myeloperoxidase; muscle ischemia/reperfusion injury

As CLINICALLY, SKELETAL MUSCLE is subjected to warm (room temperature) global ischemia in autogenous muscle transplantation for wound coverage and in musculoskeletal and vascular reconstructive surgery performed under vascular clamp or tourniquet control to maintain a relatively bloodless field during surgery (14, 27). Warm ischemic tolerance of human skeletal muscle is limited to about 2.5 h (6, 12, 24, 51). Unpredictable surgical complications, such as thrombosis and vasospasm, can cause excessive and/or repeated sustained ischemia, resulting in muscle ischemic necrosis (infarction) due to ischemia/reperfusion (I/R) injury. Muscle I/R injury ranges from loss of function or muscle infarction in a single muscle, as in autogenous muscle transplantation, to massive muscle infarction in musculoskeletal and vascular reconstructive surgery, which can cause systemic acidosis, hyperkalemia, and myoglobinuria (6, 53). In the past, major research efforts were focused on pharmacological treatment for the prevention of vascular dysfunction, thrombosis, and reperfusion injury in skeletal muscle, but none has been proven to be of clinical benefit (45).

Murry et al. (33) reported that ischemic preconditioning (IPC) of dog myocardium with four cycles of 5-min coronary artery occlusion and reperfusion provided robust myocardial protection against infarction when the preconditioned myocardium was subsequently subjected to 40 min of sustained ischemia and 4 days of reperfusion. Subsequently, other investigators observed that the myocardial infarct-protective effect of this local IPC is biphasic. Specifically, it was observed in the rabbit and dog that the early phase of the infarct-protective effect appeared immediately and lasted 1–4 h after local IPC, then disappeared completely (7, 23, 32). The late phase (second window) of myocardial infarct protection appeared between 12 and 24 h after local IPC in the rat, rabbit, and dog (3, 4, 23, 28, 56) and lasted up to 72 h in the rabbit (3). In the pig, the efficacy of the late phase of myocardial infarct protection varied with the protocol of local IPC (17, 43, 47). Brief cycles of occlusion/reperfusion in the intestine also induced myocardial infarct protection in rats and rabbits 24 h after intestinal IPC (54, 55), and this infarct-protective effect of remote IPC also lasted up to 72 h in the rabbit (55). Furthermore, it was demonstrated with pharmacological probes that the sarcolemmal KATP (sKATP) channel was involved in the trigger mechanism in the rat (40) and that the mitochondrial KATP (mKATP) channel was involved in the mediator mechanism in the rabbit (5, 32) in the late phase of myocardial infarct protection induced by local IPC.

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If the biphasic infarct-protective effect also occurs in skeletal muscle, it will be possible to use the long late phase of infarct protection for perioperative protection of skeletal muscle against infarction in reconstructive surgery. For example, sustained skeletal muscle ischemia due to unpredictable complications can occur intra- and postoperatively and most commonly within 24 h postoperatively in musculoskeletal and vascular reconstructive surgery. Thus the late phase of infarct protection can offer 48 h of uninterrupted perioperative protection of skeletal muscle against infarction if surgery is performed 24 h after IPC. So far, the efficacy of local and remote IPC for late protection of skeletal muscle against I/R injury was studied only in the rat; the results were controversial and the mechanism was not studied (2, 8, 25, 41, 42). The trigger and mediator mechanisms of late protection induced by remote IPC also have not been studied in any tissue/organ. Therefore, the aim of this research project was to test our hypothesis that hindlimb remote IPC also induces a long late phase of infarct protection in skeletal muscle and that K_{ATP} channels play a crucial role in the trigger and mediator mechanisms of the late phase of this hindlimb remote IPC. The pig bilateral latissimus dorsi (LD) muscle flaps were used in the present studies because LD muscle is used clinically for autogenous muscle transplantation, the anatomy of the pig LD muscle is similar to that of the human, and we have previously established this pig LD muscle model (1, 31, 37–39). Remote IPC of skeletal muscle against infarction in the present studies was induced by instigation of three cycles of 10-min occlusion and reperfusion by tourniquet application because this technique is simple and noninvasive and is known to induce global protection of skeletal muscle and cardiac muscle against infarction; thus this technique has potential clinical application (1, 20). Results obtained from the present studies supported our hypothesis that hindlimb remote IPC induces a late phase of 48 h of uninterrupted protection of skeletal muscle against infarction and that sK_{ATP} and mK_{ATP} channels play a crucial role in the trigger and mediator mechanism, respectively, in hindlimb remote IPC of skeletal muscle against infarction.

**Materials and Methods**

**Animal Care and Experimental Surgery**

**Animal management.** Castrated Yorkshire pigs (19.1 ± 1.5 kg; means ± SD) were housed in a temperature-controlled (22°C) and light-controlled (0700–1900) animal holding room. All pigs were offered the same commercial diet and tap water ad libitum, but food was withheld the evening before surgery. The experimental surgery and animal management described in the following studies were approved by the Animal Care and Use Committee of the Research Institute, the Hospital for Sick Children, and were in compliance with the guidelines of the Canadian Council of Animal Care. The procedure in animal care and experimental surgery were also published previously (1, 31, 37–39).

**Anesthesia.** Experimental surgery and in vivo experiments were performed under general anesthesia induced by intramuscular ketamine (25 mg/kg) and intravenous pentobarbital sodium (10–15 mg/kg). After 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 were maintained by intravenous infusion of saline (2 ml/min) containing pentobarbital sodium (0.5 mg·kg\(^{-1}\)·min\(^{-1}\)). Rectal temperature was kept within the normal range 38–39°C by warming the pig with a heating blanket.

**Muscle flap surgery.** Clinically, 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. The pig LD muscle flap model is well established for the study of infarct-protective effect of local and remote IPC (1, 16, 31, 37–39). In the present 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 so that the 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 transplant, the thoracodorsal nerve was transected. Prolene sutures (3-0) were used to close the skin wound above the muscle flaps, leaving a small opening in the axilla for access to the vascular pedicle for clamping to induce total ischemia in the LD muscle flaps.

**Noninvasive remote IPC.** Under general anesthesia, one of the hindlimbs of the pig was subjected to three cycles of 10-min occlusion/reperfusion by tourniquet application (~300 mmHg). LD muscle flaps were subjected to 4 h of ischemia starting immediately (0 h) or at 4, 6, 8, 24, 28, 36, 48, 72, or 96 h after hindlimb remote IPC. We previously observed that hindlimb remote IPC did not have any significant effect on the mean arterial blood pressure in anesthetized pigs (1, 31). Therefore, the mean arterial blood pressure was not monitored in the present studies.

**Induction of I/R in LD muscle flaps.** Under general anesthesia, the LD muscle flaps were rendered totally ischemic by occlusion of the vascular pedicle with two vascular clamps (2 × 8 mm; Weck). Global ischemia in LD muscle flaps was confirmed by intravenous injection of fluorescein dye (15 mg/kg), as described previously (1, 39). Global ischemia in LD muscle flaps was verified 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 then subjected to 4 h of sustained global ischemia at operating room temperature (24°C). This temperature was similar to that in clinical autogenous transplantation and vascular reconstruction. Reperfusion in LD muscle flaps after removal of the vascular clamp 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 flaps (39). Prolene sutures (3-0) were used to close the skin wounds. The pig was allowed to wake up and was returned to the animal holding room.

**Assessment of muscle infarct size.** Pigs were killed with an overdose of pentobarbital sodium after 48 h of reperfusion. The LD muscle flaps were excised and cut transversely into thirteen 1 × 8-cm segments, and the nitroblue tetrazolium dye staining technique was used to measure muscle infarction, as described previously for pig LD muscle flaps (1, 16, 31, 37–39).

It has been reported (48) that there is no difference in ischemic tolerance between types I and II muscle fibers at rest in the rabbit. Furthermore, we observed previously in the pig that there was no infarction in 8 × 13-cm LD muscle flaps without sustained ischemia (39). Therefore, a nonischemic control group for the study of muscle infarct size was not planned in the present studies to avoid unnecessary killing of pigs (1).

**Experimental Protocols**

**Protocol 1: To investigate the time course of hindlimb remote IPC in protection of skeletal muscle against infarction.** Pigs with bilateral (8 × 13-cm) LD muscle flaps were assigned to one control and eight treatment groups (n = 4 pigs; 8 LD muscle flaps/group). All control and treatment LD muscle flaps were subjected to 4 h of warm global ischemia and 48 h of reperfusion. In treatment groups 2–9, LD muscle flaps underwent remote IPC against infarction starting at 0 (immediately), 4, 6, 8, 24, 28, 36, 48, 72, or 96 h before 4 h of sustained ischemia. Remote IPC was induced by instigation of three cycles of 10 min of occlusion and reperfusion in a hindlimb of the pig by tourni-
quiet application. Infarction in control and treatment LD muscle flaps was assessed at the end of 48 h of reperfusion, using the nitroblue tetrazolium dye staining technique.

**Protocol 2:** To investigate the role of K_{ATP} channels in the trigger mechanism of hindlimb remote IPC in late protection of skeletal muscle against infarction. Pigs with bilateral LD muscle flaps were assigned to one control and eight treatment groups (n = 4 pigs; 8 muscle flaps) as shown in Fig. 1. All control and treatment LD muscle flaps were subjected to 4 h of sustained ischemia and 48 h of reperfusion. Remote IPC, sham remote IPC, or intravenous bolus injection of the sK_{ATP} channel opener P-1075 (2 μg/kg) was performed 24 h before 4 h of sustained ischemia (i.e., late preconditioning). The sK_{ATP} channel inhibitor HMR 1098 (6 mg/kg) and the nonspecific K_{ATP} channel inhibitor glibenclamide (Glib; 1 mg/kg) were injected intravenously at 10 min before remote IPC or sham remote IPC. The mK_{ATP} channel inhibitor 5-hydroxydecanoate (5-HD) was injected intravenously in a bolus (5 mg/kg) 10 min before remote IPC or sham remote IPC followed by 30-min intravenous infusion (5 mg/kg). Muscle viability was assessed after 48 h of reperfusion, using the nitroblue tetrazolium dye staining technique.

**Protocol 3:** To investigate the role of K_{ATP} channels in the mediator mechanism of hindlimb remote IPC in late protection of skeletal muscle against infarction. Pigs with bilateral LD muscle flaps were assigned to one control and seven treatment groups (n = 4 pigs; 8 muscle flaps) as shown in Fig. 2. All control and treatment LD muscle flaps were subjected to 4 h of sustained ischemia and 48 h of reperfusion. Remote IPC or sham remote IPC was performed 24 h before 4 h of sustained ischemia. 5-HD was given in an intravenous bolus injection (5 mg/kg) at 24 h after remote IPC or sham remote IPC. HMR 1098 (6 mg/kg) or Glib (1 mg/kg) was injected intravenously at 24 h after remote IPC or sham remote IPC. Muscle infarction was assessed after 48 h of reperfusion by using the nitroblue dye staining technique.

**Protocol 4:** To investigate the effect of late phase of infarct protection in hindlimb remote IPC on energy metabolism and neutrophil accumulation in ischemic skeletal muscle. Control and treatment bilateral LD muscle flaps in the pig were subjected to 4 h of ischemia and 1.5 h of reperfusion. Treatment LD muscle flaps underwent remote IPC 24 h before sustained ischemia (late preconditioning). Multiple muscle biopsies (0.5 × 0.5 cm) were taken from control and treatment LD muscle flaps before and at the end of 2 and 4 h of sustained ischemia and 1.5 h of reperfusion. The muscle biopsies were washed with cold isotonic saline, immediately frozen with liquid nitrogen, and stored at −80°C for assay of muscle content of ATP and MPO activity.

**Chemical Analysis of Muscle Samples**

**Collection of muscle biopsies.** Muscle biopsies (0.5 × 0.5 cm) for assays of ATP and MPO activity were taken sequentially immediately before and at 2 and 4 h of sustained ischemia and 1.5 h of reperfusion. The muscle biopsies were taken from the thick dorsal edge of the muscle flap at 7, 6, 5, and 4 cm, respectively, from the vascular pedicle of the LD muscle flaps (39). Each muscle biopsy (6 mg/kg) was injected intravenously (5 mg/kg) immediately before 4 h of sustained ischemia. The specific sK_{ATP} channel inhibitor HMR 1098 (6 mg/kg) or the nonspecific K_{ATP} channel inhibitor Glib (1 mg/kg) was given as an intravenous bolus injection 10 min before 4 h of sustained ischemia. Muscle infarction was assessed after 48 h of reperfusion by using the nitroblue dye staining technique.

**Assay for muscle ATP content.** Frozen muscle samples (~200 mg) were homogenized in 2 ml of cold (4°C) trichloroacetic acid (2.5% vol/vol), and the homogenate was centrifuged for 10 min at 1,000 g at 4°C. The supernatant was neutralized with 1 M Tris base (120 μM/l

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<tr>
<th>Group 1: (Control)</th>
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<th>4 h ischemia</th>
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<td>Group 2:</td>
<td>RIPC</td>
<td>24 h delay</td>
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<td>Group 3:</td>
<td>P1075</td>
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<td>5-HD</td>
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<td>Glib</td>
<td>RIPC</td>
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<td>5-HD</td>
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<td>Group 9:</td>
<td>Glib</td>
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Fig. 1. Protocol for investigation of the trigger mechanism in hindlimb late remote ischemic preconditioning (RIPC). All control and treatment LD muscle flaps were subjected to 4 h of sustained ischemia and 48 h of reperfusion. Sham RIPC or RIPC were performed 24 h before sustained ischemia (late protection). The mK_{ATP} channel inhibitor 5-HD was given as an intravenous bolus injection (5 mg/kg) followed by 30-min intravenous infusion (5 mg/kg) immediately before 4 h of sustained ischemia. The specific sK_{ATP} channel inhibitor HMR 1098 (6 mg/kg) or the nonspecific K_{ATP} channel inhibitor Glib (1 mg/kg) was given as an intravenous bolus injection 10 min before 4 h of sustained ischemia. Muscle infarction was assessed after 48 h of reperfusion by using the nitroblue dye staining technique.
Supernatant, and was then used for assay of ATP content (FL- AA bioluminescent assay kit; Sigma, St. Louis, MO). The pellet was neutralized with 1 ml of 0.5 M NaOH, and the protein content was determined by the Bradford method (Bio-Rad, Hercules, CA). The muscle content of ATP was calculated as micromoles per gram of protein (1, 31).

**Assay for MPO activity.** Frozen muscle samples (~200 mg) were homogenized, and the resulting supernatant was collected and assayed for neutrophilic MPO activity using a spectrophotometry technique previously described by us (16, 31, 37–39). One unit of enzyme activity was defined as the amount of MPO that produced an absorbance change of 1.0 optical density unit·min⁻¹·g wet muscle⁻¹ at 37°C.

**Biochemicals**

All chemical reagents and assay kits were purchased from Sigma unless otherwise stated. P-1075 was purchased from Tocris Bioscience, and HMR 1098 was supplied by Aventis Pharma. Purified water (Milli Q water system) was used to make all solutions and standards.

We (31) previously observed that hindlimb IPC and intravenous bolus injection of Glib (1 mg/kg) and 5-HD (10 mg/kg) did not have any significant effect on the mean arterial blood pressure in pigs monitored for 60 min under pentobarbital anesthesia. In a preliminary study, we observed that intravenous injection of P-1075 (1 μg/kg) or HMR 1098 (10 mg/kg) also did not affect the mean arterial blood pressure in pigs monitored for 60 min under pentobarbital anesthesia.

**Statistical Analysis**

All values are expressed as means ± SE. The specific statistical tests used in the present studies are stated in the legends of the figures. Statistical significance was set at \( P < 0.05 \) for all tests.

**RESULTS**

**Time Course of Hindlimb Remote IPC in Protection of Skeletal Muscle Against Infarction**

There was 46 ± 2% infarction in control LD muscle flaps subjected to 4 h of warm ischemia and 48 h of reperfusion (Fig. 3). This muscle infarction was reduced \( (P < 0.05) \) to 22 ± 3% in LD muscle flaps undergoing hindlimb remote IPC immediately before 4 h of sustained ischemia. However, the muscle infarct-protective effect of hindlimb remote IPC waned, and the muscle infarction increased to 33 ± 3% at 4 h after hindlimb remote IPC. This early phase of muscle infarct-protective effect of hindlimb remote IPC completely disappeared at 6 and 8 h after remote IPC. Muscle infarction was 44 ± 6 and 47 ± 5%, respectively, and these extents of muscle infarction were similar to that of the ischemic control (Fig. 3). Of importance was the observation that the infarct protective effect of remote IPC reappeared 24 h after hindlimb remote IPC and lasted for 48 h. Specifically, the extent of infarction in LD muscle flaps subjected to 4 h of ischemia starting at 24, 28, 36, 48, or 72 h after hindlimb remote IPC (i.e., late phase of protection in remote IPC) were 28 ± 3, 26 ± 1, 23 ± 2, 24 ± 2, and 24 ± 4%, respectively. These muscle infarct sizes were similar to that of LD muscle flaps subjected to 4 h of ischemia starting immediately after hindlimb remote IPC (22 ± 3%) and were significantly \( (P < 0.05) \) smaller than that of the ischemic control group (Fig. 3). However, the infarction in LD muscle flaps at 96 h after hindlimb remote IPC was 38 ± 4%, and this infarct size was similar to that of the ischemic control. Taken together, these observations indicate that the late phase of infarct-protective effect of hindlimb remote IPC occurred between 24 and 72 h and disappeared between 72 and 96 h after remote IPC.

**Role of \( K_{ATP} \) Channels in the Trigger Mechanism of Late Phase of Infarct Protection in Skeletal Muscle Induced by Hindlimb Remote IPC**

Hindlimb remote IPC or intravenous bolus injection of the \( sK_{ATP} \) channel opener P-1075 (2 μg/kg) 24 h before subjecting LD muscle flaps to 4 h of sustained ischemia and 48 h of reperfusion significantly \( (P < 0.05) \) reduced the LD muscle infarction from 43 ± 5% (ischemic control) to 24 ± 2 and 19 ± 3%, respectively (Fig. 4). This observation is consistent with the phenomenon of late protection of LD muscle against infarction by remote IPC seen in the preceding study. Here, we
also observed that intravenous bolus injection (5 mg/kg) of the mK_{ATP} channel inhibitor 5-HD immediately before remote IPC followed by 30 min of intravenous infusion of 5-HD (5 mg/kg) did not have any effect on the late infarct-protective effect of remote IPC (Fig. 4). On the other hand, intravenous bolus injection of the sK_{ATP} channel inhibitor HMR 1098 (6 mg/kg) or the nonspecific K_{ATP} channel inhibitor Glib (1 mg/kg) at 10 min before remote IPC completely abolished the late phase of infarction induced by remote IPC, and the LD muscle infarct size in these two treatment groups was similar to that of the ischemic control. In addition, intravenous bolus injection of HMR 1098 (6 mg/kg) or Glib (1 mg/kg) at 10 min before sham remote IPC or intravenous bolus injection of 5-HD (5 mg/kg) 10 min before and 30 min of intravenous infusion of 5-HD (5 mg/kg) during sham remote IPC did not affect the LD muscle infarct size compared with the ischemic control when subjected to 4 h of ischemia and 48 h of reperfusion (Fig. 4). Taken together, these observations indicate that sK_{ATP} but not the mK_{ATP} channels are involved in the trigger mechanism of hindlimb remote IPC for late protection of skeletal muscle against infarction.

**Role of K_{ATP} Channels in the Mediator Mechanism of Late Phase of Infarct Protection in Skeletal Muscle Induced by Hindlimb Remote IPC**

In this study, hindlimb remote IPC performed 24 h before subjecting LD muscle flaps to 4 h of ischemia and 48 h of reperfusion significantly reduced the LD infarction from 40 ± 2% (ischemic control) to 25 ± 2% (Fig. 5). Intravenous bolus injection (5 mg/kg) and 30 min intravenous infusion (5 mg/kg) of the mK_{ATP} channel inhibitor 5-HD or intravenous bolus injection of the nonspecific K_{ATP} channel inhibitor Glib (1 mg/kg) at 24 h after remote IPC completely blocked the late infarct-protective effect of remote IPC when the LD muscle flaps were subsequently subjected to 4 h of ischemia and 48 h of reperfusion. However, intravenous injection of the sK_{ATP} channel inhibitor HMR 1098 (6 mg/kg) 24 h after remote IPC had no effect on the late infarct-protective effect of remote IPC. The infarct size of LD muscle flaps was not significantly different between sham remote IPC pigs with or without intravenous 5-HD, Glib, or HMR 1098 treatment given 24 h after sham remote IPC (Fig. 5). Collectively, these observations indicate that mK_{ATP} channels play a central role in the mediator mechanism of late protection of skeletal muscle against infarction in hindlimb remote IPC.

**Effect of Late Phase of Infarct Protection Induced by Hindlimb Remote IPC on ATP Content and Neutrophilic MPO Activity in Skeletal Muscle**

LD muscle flaps in the control and treatment group were subjected to 4 h of ischemia and 1.5 h of reperfusion. LD muscle flaps in the treatment group underwent hindlimb remote IPC at 24 h before sustained ischemia. Within the control and treatment groups, the ATP content in LD muscle flaps decreased during the 4 h of ischemia compared with the muscle ATP content before ischemia, and this decrease in muscle ATP content was significant (P < 0.05) at the end of 4 h of ischemia and 1.5 h of reperfusion (Fig. 6). More importantly, the muscle ATP content was significantly (P < 0.05) higher in the ischemic preconditioned LD muscle flaps compared with the time-matched control at the end of 4 h of sustained ischemia and 1.5 h of reperfusion (Fig. 6).

There was no difference in muscle neutrophilic MPO activity within and between the control and preconditioned LD muscle flaps before and at the end of 4 h of sustained ischemia (Fig. 7). The muscle MPO activity was significantly (P < 0.05) higher at the end of 1.5 h of reperfusion than before or after 4 h of ischemia in both control and treatment groups. However, the muscle neutrophilic MPO activity was significantly (P < 0.05) higher in the control than the preconditioned LD muscle flaps at the end of 1.5 h of reperfusion (Fig. 7).
ANOVA and Newman-Keuls multiple comparison test. significantly (P < 0.05) different from the time-matched control; two-way ANOVA and Newman-Keuls multiple comparison test.

DISCUSSION

Important Findings From This Research Project

This is the first report on the time course and mechanism of remote IPC for late protection of skeletal muscle against infarction. Specifically, we observed that instigation of three cycles of 10-min occlusion/reperfusion in a hindlimb of the pig by tourniquet application elicited a biphasic pattern of infarction protection in remote bilateral LD muscle flaps. An early phase of infarct protection started immediately after hindlimb remote IPC, waned within 4 h after remote IPC, and completely disappeared before 6 h of remote IPC. The late phase of infarct protection (re)appeared within 24 h after hindlimb remote IPC and lasted up to 72 h. Using pharmacological probes, we also demonstrated that the sKATP channel is a trigger, whereas the mKATP channel is a mediator of remote IPC for late protection of skeletal muscle against infarction. Furthermore, we observed that the late phase of infarct protection in hindlimb remote IPC was associated with a higher muscle content of ATP at the end of 4 h of sustained ischemia and 1.5 h of reperfusion and an attenuation of neutrophil accumulation at the end of 1.5 h of reperfusion, compared with the time-matched control. The information obtained from this project provides important insight into the development of pharmacological or noninvasive remote IPC preconditioning for 48 h of uninterrupted perioperative protection of skeletal muscle against infarction in musculoskeletal and vascular reconstructive surgery.

Time Course of Skeletal Muscle Infarct Protection Induced by Hindlimb Remote IPC

So far, the efficacy of IPC for late protection of skeletal muscle against I/R injury was studied in rats only, and the results were controversial. One group of investigators (25) failed to observe infarct protection in rat gastrocnemius muscle undergoing local or hindlimb remote IPC with two cycles of 10-min occlusion/15-min reperfusion at 24 h before subjecting the rat gastrocnemius muscle to 2 h of sustained ischemia and 24 h of reperfusion. In this study, a rubber band tourniquet was used and the pressure was not reported. Other investigators (2, 24) reported that local IPC of rat LD muscle flaps with three cycles of 10-min occlusion/reperfusion or two cycles of 30-min occlusion/reperfusion of the thoracodorsal arteries and veins elicited potent late phase of infarct protection in rat LD muscle flaps subsequently subjected to 4 h of ischemia at 24 h after local IPC. It is unclear how the differences in IPC protocol and technique could have affected the induction of late phase of infarct protection by local or remote IPC in the rat skeletal muscle. Here, we were the first to demonstrate successfully the entire time course of acute and late protection of skeletal muscle against infarction in the pig by remote IPC. Remote IPC was induced by three cycles of 10 min of occlusion (∼300 mmHg) and reperfusion by tourniquet application in a hindlimb of the pig. In general, the time course of biphasic skeletal muscle infarct protection elicited by hindlimb remote IPC observed in the present study (Fig. 3) is similar to the time course of myocardial resilience to ischemia induced by local IPC in the rat, rabbit, and dog (7, 23, 33). More importantly, hindlimb remote IPC also induces an uninterrupted infarct protection in skeletal muscle subjected to 4 h of sustained ischemia between 24 and 72 h after remote IPC.

Role of KATP Channels in Hindlimb Remote IPC for Late Protection of Skeletal Muscle Against Infarction

We (31) previously observed that mKATP channels play a crucial role in the trigger mechanisms of hindlimb remote IPC for acute protection of pig skeletal muscle against infarction. In that study, pig LD muscle flaps were subjected to 4 h of sustained ischemia immediately after remote IPC. The role of KATP channels in the trigger and mediator mechanism of remote IPC for late infarct protection against infarction has not been studied in any tissue or organ. Here, LD muscle flaps were subjected to 4 h of ischemia at 24 h after remote IPC. We observed that sKATP, but not mKATP channels, were involved in the trigger mechanism of hindlimb remote IPC for late protection of pig skeletal muscle against infarction. Specifically, hindlimb remote IPC or intravenous bolus injection of the sKATP channel opener P-1075 at 24 h before subjecting pig LD muscle flaps to 4 h of sustained ischemia and 48 h of reperfusion reduced the muscle infarction from 43 ± 5% (ischemic control) to 24 ± 2 and 19 ± 3%, respectively (Fig. 4). There is evidence to indicate that P-1078 may open both sKATP and mKATP channels in rabbit myocytes (13, 35, 46). However, intravenous bolus injection of the sKATP channel inhibitor HMR 1098 or the nonspecific KATP channel inhibitor Glib at 10 min before hindlimb remote IPC completely blocked the late infarct-protective effect of hindlimb remote IPC. In addition, intravenous bolus injection of the mKATP channel inhibitor 5-HD (5 mg/kg) immediately before hindlimb remote IPC followed by 30 min of intravenous infusion of 5-HD did not attenuate the late infarct-protective effect of remote IPC in pig LD muscle flaps subjected to 4 h of ischemia and 48 h of reperfusion (Fig. 4). It is important to note that this dose of 5-HD was effective in blocking the trigger mechanism of remote IPC for acute protection of pig LD muscle flaps against infarction (31). Taken together, we have demonstrated that...
**sK<sub>ATP</sub>** channels play a central role in the trigger mechanism of hindlimb remote IPC for late protection of pig skeletal muscle against infarction. It is of interest to point out that sK<sub>ATP</sub> but not mK<sub>ATP</sub> channels are also involved in the trigger mechanism of local hypoxic IPC for late protection of human right atrial tissue against I/R injury (26). These observations indicate the possible existence of species difference in the trigger mechanism in local IPC for late protection against myocardial infarction. Here, we observed that sK<sub>ATP</sub> channels play a key role in the trigger mechanism of remote IPC in pig skeletal muscle. Future study is required to investigate the role of sK<sub>ATP</sub> and mK<sub>ATP</sub> channels in the trigger mechanism of late protection of human skeletal muscle against infarction induced by remote IPC.

There is also convincing evidence from the present study to indicate that mK<sub>ATP</sub> channels play a pivotal role in the mediator mechanism of hindlimb remote IPC for late protection of pig LD muscle against infarction. Specifically, intravenous bolus injection of the nonspecific K<sub>ATP</sub> channel inhibitor Glib or intravenous bolus injection of the mK<sub>ATP</sub> channel inhibitor 5-HD followed by 30 min of intravenous infusion 24 h after hindlimb remote IPC completely blocked the late infarct-protective effect in pig LD muscle flaps subsequently subjected to 4 h of ischemia and 48 h of reperfusion (Fig. 5). However, intravenous injection of the sK<sub>ATP</sub> channel inhibitor HMR 1098 given 24 h after remote IPC did not attenuate the infarct-protective effect of late remote IPC. This intravenous treatment of HMR 1098, Glib, or 5-HD given 24 h after sham remote IPC did not aggravate infarction in the ischemic control LD muscle flaps. Collectively, these observations indicate that mK<sub>ATP</sub> channels play a key role in the mediator mechanism of hindlimb remote IPC in late protection of skeletal muscle against infarction.

**Effect of Hindlimb Remote IPC on ATP Content and Neutrophilic MPO Activity in Ischemic and Reperfused Skeletal Muscle**

Other investigators reported that early local IPC of rabbit and pig myocardium (30, 50), late local IPC of rabbit myocardium (50), or early remote (renal) IPC of rat myocardium (44) induced a higher myocardial ATP content during sustained ischemia and reperfusion. In addition, we (1, 39) reported previously that early local or remote hindlimb IPC of pig skeletal muscle maintained a higher muscle ATP content at the end of 4 h of ischemia and 1.5 h of reperfusion compared with the time-matched control. Here, we report for the first time that late hindlimb remote IPC also elicited a higher muscle ATP content at the end of 4 h of sustained ischemia and 1.5 h of reperfusion compared with the time-matched control (Fig. 6). The mechanism of IPC in delaying the decrease of tissue ATP content during sustained ischemia is unclear. There is evidence to indicate that IPC may induce the following activities during sustained ischemia: 1) reduction in ATP demand or consumption (18, 34); 2) inhibition of mitochondrial ATP synthase activity (52); or 3) enhanced glucose uptake and anaerobic glycolysis during sustained ischemia (21, 29).

Neutrophilic MPO activity is commonly used for assessment of neutrophil accumulation in tissue subjected to ischemia and reperfusion (22, 29, 54). It is well known that neutrophil accumulation begins within an hour after the onset of reperfusion and continues for ~24 h (11, 57). There is evidence to indicate that neutrophils generate reactive oxygen species, which can cause tissue injury (19, 58), and reduction in neutrophilic MPO activity or accumulation is associated with reduction in myocardial I/R injury (9, 15), and that depletion of neutrophils is associated with attenuation of I/R injury in the pig skeletal muscle (10). More recently, it was reported that the infarct protective effect of early local IPC in rat and dog myocardium (22, 29) and late remote (intestine) IPC in rat myocardium was associated with a lower myocardial MPO activity compared with the control (54). We also previously observed (1, 39) that the protective effect of early local and hindlimb remote IPC of pig LD muscle flaps against infarction was associated with a decrease in muscle MPO activity. Now, we report for the first time that the late infarct-protective effect of hindlimb remote IPC in pig LD muscle flaps was also associated with a lower muscle MPO activity after 4 h of ischemia and 1.5 h of reperfusion compared with the control (Fig. 7). We speculate that late remote IPC attenuates ischemic injury, resulting in a decrease in inflammation in LD muscle flaps. Decrease in inflammation, in turn, decreases local neutrophil adhesion and accumulation, thus reducing cell injury induced by reactive oxygen species at reperfusion.

In summary, we investigated for the first time the efficacy and mechanism of noninvasive hindlimb remote IPC for late protection of skeletal muscle against infarction. We observed that the skeletal muscle infarct-protective effect of hindlimb remote IPC was biphasic in the pig skeletal muscle. The early phase of infarct protection started immediately after remote IPC, but began to wane at 4 h of reperfusion, and disappeared before 6 h of reperfusion. The late phase of muscle infarct protection reappeared at 24 h and lasted up to 72 h after remote IPC. sK<sub>ATP</sub> and mK<sub>ATP</sub> channels play a central role in the trigger and mediator mechanism, respectively, in late protection of pig skeletal muscle against infarction induced by hindlimb remote IPC. The late infarct-protective effect of remote IPC was associated with maintenance of a higher muscle ATP content at the end of 4 h of ischemia and 1.5 h of reperfusion, and a lower muscle neutrophil accumulation at the end of 1.5 h of reperfusion, compared with the time-matched ischemic control.

**PERSPECTIVES**

Autogenous muscle transplantation and musculoskeletal and vascular reconstructive surgery are performed under vascular clamp or tourniquet control to maintain a relatively bloodless field during surgery. Human skeletal muscle is known to withstand ~2.5 h of warm global ischemia. Unpredictable complications, such as thrombosis and vasospasm, can cause additional and excessive sustained skeletal muscle ischemia intra- and postoperatively and most commonly within 24 h postoperatively. Of clinical importance is our present finding that hindlimb remote IPC induced early and late biphasic infarct protection in the pig skeletal muscle, with the late phase of infarct protection lasting between 24 and 72 h after remote IPC. In addition, we observed that intravenous bolus injection...
of the sK\textsubscript{ATP} channel opener P-1075 mimicked the late infarct protective effect of remote IPC in the pig skeletal muscle. These findings provide an important insight into the design of noninvasive ischemic or pharmacological preconditioning for 48 h of uninterrupted perioperative skeletal muscle infarct protection in reconstructive surgery. Specifically, hindlimb remote IPC or oral intake of a K\textsubscript{ATP} channel opener at 24 h before surgery may induce 48 h of uninterrupted perioperative protection of skeletal muscle against infarction.

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REFERENCES


32. Pang CY, Addison PD, Ashraftour H, Forrest CR, Zhong A, and Neligan PC. Global protection against skeletal muscle infarction by...


