Noninvasive remote ischemic preconditioning for global protection of skeletal muscle against infarction

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There are many clinical situations in which single or multiple skeletal muscles are subjected to warm (room temperature) global ischemia. For example, a muscle flap is subjected to warm global ischemia during transfer and microsurgical vascular anastomosis in autogenous muscle transplantation, and multiple skeletal muscles are subjected to warm global ischemia in musculoskeletal and vascular reconstructive surgery under vascular clamp or tourniquet control. Clinically, tolerance of skeletal muscle to warm global ischemia is limited to <2.5 h (8, 18, 39). Excessive ischemia due to unpredictable complications can cause ischemia-reperfusion (I/R) injury, resulting in skeletal muscle ischemic necrosis (infarction). Life-threatening acidosis, hyperkalemia, and myoglobinurea may occur if the muscle infarction is massive (42). In the past, intervention strategies focused on pharmacological treatments for the prevention of microvascular dysfunction, thrombosis, and reperfusion injury but have not been proven of clinical benefit (33). In 1986, Murry et al. (23) reported that brief cycles of I/R by occlusion of the coronary artery provided robust myocardial protection against infarction in the dog. Using this local acute ischemic preconditioning (IPC) technique, we demonstrated that preconditioning pig latissimus dorsi (LD) and gracilis (GC) muscle flaps with three cycles of 10-min I/R 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 global ischemia and 48 h of reperfusion (28). Subsequently, other investigators have reported that local acute IPC augmented ischemic tolerance in rat skeletal muscle and musculocutaneous flaps (3, 19, 22, 46) and attenuated vascular dysfunction in the skeletal muscle of the rat (17) and capillary no reflow in the skeletal muscle of the rat (43) and dog (16). Despite its potent infarct-protective effect, the technique of local IPC may not be acceptable to most surgeons because it requires a prolonged operative time and, more importantly, there is always the risk of damaging the vascular pedicle due to repeated clamping. Subsequently, we demonstrated that local intraarterial infusion of adenosine, the nonspecific ATP-

Addison, Patrick D., Peter C. Neligan, Homa Ashrafpour, Asim Khan, Anguo Zhong, Michael Moses, Christopher R. Forrest, and Cho Y. Pang. Noninvasive remote ischemic preconditioning for global protection of skeletal muscle against infarction. Am J Physiol Heart Circ Physiol 285: H1435–H1443, 2003. First published June 5, 2003; 10.1152/ajpheart.00106.2003.—The aim of this study was to investigate the efficacy and mechanism of action of a noninvasive remote ischemic preconditioning (IPC) technique for the protection of multiple distant skeletal muscles against ischemic necrosis (infarction). It was observed in the pig that three cycles of 10-min occlusion and reperfusion in a hindlimb by tourniquet application reduced the infarction of latissimus dorsi (LD), gracilis (GC), and rectus abdominis (RA) muscle flaps by 55%, 60%, and 55%, respectively, compared with their corresponding control (n = 6, P < 0.01) when they were subsequently subjected to 4 h of global ischemia and 48 h of reperfusion. This infarct-protective effect of remote IPC in LD muscle flaps was abolished by an intravenous bolus injection of the nonselective opioid receptor antagonist naloxone (3 mg/kg) 10 min before remote IPC and a continuous intravenous infusion (3 mg/kg) during remote IPC and by an intravenous bolus injection of the selective δ-opioid receptor antagonist 7-benzylideneatrexone maleate (3 mg/kg). However, this infarct-protective effect of remote IPC was not affected by an intravenous bolus injection of the ganglionic blocker hexamethonium chloride (20 mg/kg) or the nonspecific adenosine receptor antagonist 8-(p-sulfophenyl)theophylline (10 mg/kg) or by a local intra-arterial injection of the adenosine receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (3 mg/muscle flap) given 10 min before remote IPC. It was also observed that this remote IPC of skeletal muscle against infarction was associated with a slower rate of muscle ATP depletion during the 4 h of sustained ischemia and a reduced muscle neutrophil myeloperoxidase activity after 1.5 h of reperfusion. These observations led us to speculate that noninvasive remote IPC by brief cycles of occlusion and reperfusion in a pig hindlimb is effective in global protection of skeletal muscle against infarction. This infarct-protective effect is most likely triggered by the activation of opioid receptors in the skeletal muscle, and remote IPC is associated with an energy-sparing effect during sustained ischemia and attenuation of neutrophil accumulation during reperfusion. ischemic preconditioning; humoral mediator; ATP; lactate; myeloperoxidase

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sensitive K⁺ (K_{ATP}) channel opener lemakalin, or the mitochondrial K_{ATP} channel opener diazoxide mimicked the infarct-protective effect of local acute IPC in the pig skeletal muscle (25–27). However, pharmacological preconditioning may also have clinical limitations because there is the risk of damaging the vascular pedicle during local intra-arterial drug infusion, and an effective systemic dose of these drugs causes hypotension in laboratory animals, which is contraindicated in autogenous muscle transplantation and musculoskeletal reconstructive surgery.

Przyklenk et al. (32) reported that the infarct-protective effect of IPC by intermittent occlusion of the circumflex artery not only protected the myocardium perfused by the circumflex artery but also protected the myocardium perfused by the left anterior descending coronary artery in the dog. This phenomenon was termed “intraorgan” remote IPC. Subsequently, other investigators reported the phenomenon of in vivo interorgan remote IPC (13). Specifically, a brief occlusion of the mesenteric artery in the rat (11, 20, 29, 31, 34) or the renal artery in the rabbit (30, 38) protected the myocardium against infarction. The mediators of remote IPC are unclear. Both neuronal (11, 20, 34) and humoral (7, 24, 29) factors have been implicated. In addition, there is evidence to indicate that remote IPC is associated with a reduced rate of ATP depletion during sustained myocardial ischemia in the rabbit (38). Furthermore, Birnbaum et al. (2) reported that reduction of femoral artery blood flow by 55–60% for 30 min with concomitant electrical stimulation of the gastrocnemius muscle also preconditioned the myocardium against infarction in the rabbit. Liew et al. (21) reported that 5 h of lethal ischemia by occlusion of the vascular pedicle of a GC muscle in the dog attenuated the infarction of the contralateral GC muscle subjected to 5 h of sustained ischemia 48 h later. However, the remote IPC techniques discussed thus far are invasive or lethal. Of particular interest to us was the observation that 10 min of hindlimb ischemia by tourniquet application preconditioned the heart against reperfusion tachyarrhythmia in the rat (24). We proposed to use this technique to study for the first time the efficacy and mechanism of noninvasive remote IPC for global protection of skeletal muscle against infarction. Specifically, we proposed to test the following hypotheses: 1) brief cycles of hindlimb occlusion and reperfusion by tourniquet application are effective in preconditioning LD, GC, and rectus abdominis (RA) muscle flaps against infarction when these muscle flaps are subsequently subjected to 4 h of sustained warm ischemia and 48 h of reperfusion; and 2) the infarct-protective effect of this remote IPC is mediated by a humoral mechanism. Pig LD, GC, and RA muscle flaps were used in the present study because the anatomy of these muscle flaps is similar to that of the human. In addition, LD, GC, and RA muscle are not essential for locomotion, and they are routinely used for autogenous muscle transplantation (muscle free flap surgery) for wound coverage. If proven effective, this technique will have potential clinical applications for the protection of skeletal muscle from infarction in autogenous skeletal muscle transplantation and in musculoskeletal and vascular reconstructive surgery because it is drug free, noninvasive, and capable of protecting multiple skeletal muscles against infarction.

**MATERIALS AND METHODS**

**Experimental Surgery**

Castrated Yorkshire pigs (20.9 ± 1.2 kg, mean ± SD) were used. The experimental protocols used in the following studies were approved by the Animal Care Committee of the Hospital for Sick Children Research Institute and were in compliance with the guidelines of the Canadian Council of Animal Care.

**Anesthesia.** All experimental surgery and in vivo experiments described in the following protocols were performed under general anesthesia induced by intramuscular ketamine (25 mg/kg) and intravenous pentobarbital sodium (10–15 mg/kg). The pig was intubated after intramuscular injection of atropine (0.04 mg/kg) and 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 isotonic saline (2 ml/min) containing pentobarbital sodium (0.5 mg·kg⁻¹·min⁻¹). Rectal temperature was monitored and kept within the normal range of 38–39°C by warming the pig with a heating blanket.

**Muscle flap surgery.** The design of island LD, GC, and RA muscle flaps was similar to that used in clinical autogenous muscle transplantation, and standard sterile procedures were observed in muscle flap surgery.

**LD muscle flaps.** An island LD muscle flap (8 × 13 cm) based on the thoracodorsal neurovascular bundle and a 1-cm-wide muscle tendon was raised and returned to its original site and sutured with 3-0 dexon sutures (25–28). The muscle tendon used to support the neurovascular bundle 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. The thoracodorsal nerve was transected as in autogenous muscle transplantation. The skin overlying the muscle flap was closed with 3-0 prolene sutures leaving a small opening in the axilla for access to the vascular pedicle for clamping to induce total muscle flap ischemia.

**GC muscle flaps.** The entire GC muscle was used for construction of an island GC muscle flap as described previously (28). The GC muscle flap was raised based on the major neurovascular pedicle and a 1-cm-wide muscle tendon in the cranial portion and sutured to its site with 3-0 dexon sutures. The muscle tendon was ligated with 1-0 silk ties, and all nerves and minor blood vessels were also ligated and cut; thus the artery of the major vascular pedicle was the only source of blood supply to the GC muscle flap. The overlying skin was closed with 3-0 prolene sutures, leaving a small opening for access to the vascular pedicle.

**RA muscle flap.** An island RA muscle flap (6 × 30 cm) was raised based on the inferior epigastric vascular pedicle, together with the anterior rectus sheath for support. All motor nerves and lateral blood vessels were transected. The inferior tendinous insertion was ligated with 2-0 silk sutures, and the RA muscle flap was sutured to its original site using 3-0 dexon sutures. The overlying skin was closed with 3-0 prolene sutures, leaving a small opening for access to the vascular pedicle.

**Ischemia and reperfusion in muscle flaps.** Two microvascular clamps (2 × 8 mm, Weck) were used to occlude the...
vascular pedicle of each muscle flap, rendering it totally ischemic; thus the entire muscle flap was at risk. Ischemia was confirmed by intravenous injection of fluorescein dye (15 mg/kg). Absence of yellow fluorescence in the muscle flap observed under ultraviolet light at 10 min after dye injection verified total ischemia. The muscle was subjected to 4 h of sustained warm (surgical room temperature, 24°C) ischemia. The temperature was similar to that during clinical autogenous muscle transfer and microvascular anastomosis. Reperfusion after removal of vascular clamps at the end of 4 h of sustained ischemia was ascertained by a second dye injection and the appearance of yellow fluorescence in the muscle flap. The skin wound was closed with 3-0 prolene sutures. Anesthesia was withdrawn, and the pig was allowed to recover and returned to an animal holding room with controlled light (07:00–19:00) and temperature (22°C). The pig was killed 2 days later with an overdose of pentobarbital sodium. The muscle flaps were immediately excised and cut transversely into 1-cm-thick segments for assessment of muscle infarction, using the nitroblue tetrazolium dye staining technique and calculation previously described for pig and dog muscle flaps (25). Our previous observations (28) in the present preliminary study indicate that there was no infarction in our LD, GC, and RA muscle flap models without sustained ischemic insult. Therefore, nonischemic control groups were not planned in the present study to avoid the unnecessary death of pigs.

It has been reported that there is no difference in ischemic tolerance between type I and II muscle fibers at rest in the rabbit (37). Therefore, although the LD, GC, and RA muscle flaps used in the present study may have different fiber type compositions, it is unlikely that there would be a significant difference in ischemic tolerance among these muscle flaps because they were denervated and noncontractile throughout the experiment.

Remote noninvasive IPC of pig LD, GC, and RA muscle flaps. Remote IPC, instigated by three cycles of 10-min occlusion and reperfusion in a hindlimb by tourniquet application (~300 mmHg), was performed immediately before 4 h of sustained ischemia. The hindlimb used for remote IPC was contralateral to the GC muscle flap. We used this protocol for remote IPC in the present study because we have previously observed that acute local IPC of pig LD and GC muscle flap with three cycles of 10-min I/R reduced the infarct size by 44% and 62%, respectively, when these muscle flaps were subjected to 4 h of ischemia and 48 h of reperfusion (28).

Muscle Biopsies

Muscle biopsies (0.5 x 0.5 cm) for assays of ATP and lactate, protein contents, and myeloperoxidase (MPO) activity were taken sequentially 1 cm from the dorsal edge of the LD muscle flap. Each biopsy was immediately rinsed with cold (4°C) saline, frozen in liquid nitrogen, and stored at -80°C. It was documented previously that muscle infarction and the protective effect of IPC occurred consistently in this biopsied region of the LD muscle flap, and harvesting of each biopsy would not have a significant effect on the blood supply to the remaining muscle flap (28).

Chemical Analysis of Muscle Samples

Sample extraction and ATP, lactate, and protein assays. Frozen muscle samples (200–250 mg) were weighed and homogenized in 2 ml of ice-cold trichloroacetic acid (2.5% vol/vol). The supernatant obtained after 10-min centrifugation at 1,000 g and 4°C was neutralized with 1 M Tris base (120 µl/mL supernatant). The resultant supernatant was used for assays of ATP content (FL-AA bioluminescent assay kit, Sigma) and enzymatic assay of lactate content (catalog no. 735-10, Sigma). The pellet was neutralized with 1 ml of 0.5 M NaOH, and the protein content was determined by the Bradford method (Bio-Rad). The muscle contents of ATP and lactate are expressed as micromoles per gram of protein.

Assay of MPO activity. Frozen muscle samples (~200 mg) were weighed and homogenized, and the resulting supernatant was collected and assayed for neutrophilic MPO activity using a spectrophotometry technique as described by us previously (14, 25, 27). One unit of enzyme activity was defined as the amount of MPO activity that produced an absorbance change of 1.0 optical density units min^-1 g muscle wet wt^-1 at 37°C.

Experimental Protocols

Protocol 1: investigation of the efficacy of brief cycles of hindlimb occlusion and reperfusion in remote IPC of multiple skeletal muscles against infarction. Our animal protocol only allowed a maximum of two muscle flaps to be constructed in each pig. Therefore, pigs with contralateral LD and GC, LD and RA, or GC and RA muscle flaps were assigned to control and treatment groups (n = 6). In the treatment groups, all pigs underwent remote IPC instigated by three cycles of 10-min occlusion and reperfusion in a hindlimb by tourniquet application (~300 mmHg). All control pigs underwent sham manipulation. All muscle flaps were subjected to 4 h of sustained ischemia, and muscle infarction was assessed 48 h after reperfusion as described above.

Protocol 2: investigation of the role of a neurogenic or humoral pathway in remote IPC of skeletal muscle against infarction with brief cycles of hindlimb occlusion and reperfusion. Pigs with bilateral LD muscle flaps were assigned to one of nine groups (n = 6): 1) ischemic control; 2) remote IPC; 3) intravenous injection of the ganglionic blocker hexamethonium chloride (20 mg/kg) 10 min before remote IPC; 4) intravenous injection of the non-specific adenosine receptor antagonist 8-(p-sulphophenyl) theophylline (SPT; 10 mg/kg) 10 min before remote IPC; 5) local intra-arterial injection of the adenosine_1 receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX; 3 mg/muscle flap) 10 min before remote IPC; 6) intravenous injection of the non-specific opioid receptor antagonist naloxone (3 mg/kg) 10 min before remote IPC and continuous intravenous infusion (3 mg/kg) during remote IPC due to the short half-life; 7) intravenous injection of the selective δ-opioid receptor antagonist 7-benzylidenaltrexone maleate (BNTX; 3 mg/kg) 10 min before remote IPC; 8) intravenous injection of naloxone (3 mg/kg), followed by 60 min of intravenous infusion (3 mg/kg) before sustained ischemia without remote IPC; and 9) intravenous injection of BNTX (3 mg/kg) 60 min before 4 h of sustained ischemia without remote IPC. All muscle flaps were subjected to 4 h of sustained ischemia, and muscle infarction was assessed after 48 h of reperfusion.

Additional anesthetized pigs were used to study the effect of remote IPC and drug treatment on mean arterial blood pressure monitored through a femoral artery catheter connected to a blood pressure recorder (model 770, Hewlett-Packard recorder). After 30 min of stabilization, the mean arterial blood pressure was recorded before and at 5, 10, 15, 20, 30, 45, 60, and 90 min after the beginning of remote IPC or intravenous treatment of hexamethonium, SPT, naloxone, or BNTX (n = 4) as described above.

The dose and treatment modality of SPT and DPCPX used in the present study were effective in blocking the protective effect of acute local IPC against skeletal muscle infarction.
according to our preliminary and published results (27). Therefore, the same dose and treatment modality of SPT and DPCPX were used in the present study to investigate the role of adenosine receptors in remote IPC. The single intravenous dose of hexamethonium used in the present study was about twice as high as the effective single intravenous dose of hexamethonium used as a ganglionic blocker in the pig reported by two groups of investigators (4, 12). In our preliminary study, this high dose of hexamethonium did not have any effect on the blood pressure, and this dose of hexamethonium was also used by other investigators to block remote IPC in the rat (11, 20). Furthermore, the biological half-life of naloxone is short in the pig. Naloxone was effective in blocking the myocardial protective effect of acute local IPC in the pig by an intravenous bolus injection, followed by an intravenous infusion during IPC and sustained ischemia (36). Therefore, a similar treatment modality for naloxone was used in the present study to block remote IPC in the pig. It was also reported that a single intravenous injection of BNTX (3 mg/kg) was effective in blocking the myocardial infarct-protective effect of acute local IPC in the rat (35). This dose and treatment modality were used to block the protective effect of remote IPC in the present study.

Protocol 3: investigation of the effect of remote IPC instigated by brief cycles of hindlimb occlusion and reperfusion on energy metabolism and neutrophil accumulation during sustained ischemia and reperfusion in LD muscle flaps. Muscle biopsies (0.5 × 0.5 cm) were taken from bilateral LD muscle flaps immediately before remote IPC and ischemia and at the end of 2 and 4 h of sustained ischemia and 1.5 h of reperfusion with or without hindlimb remote IPC (n = 6 pigs). The muscle biopsies were stored at −80°C for assay of ATP, lactate, and protein contents and neutrophilic MPO activity.

Biochemicals

All chemical reagents, drugs, and assay kits were purchased from Sigma (St. Louis, MO) unless otherwise stated. Purified water (MilliQ water system) was used to make all solutions and standards.

Statistical Analysis

All values are expressed as means ± SE. The number of observations and the specific statistical tests used are stated in the figures and table. Statistical significance was set at P ≤ 0.05 for all tests.

RESULTS

Efficacy of Brief Cycles of Hindlimb Occlusion and Reperfusion in Remote IPC of Multiple Skeletal Muscles Against Infarction

Noninvasive remote IPC instigated by three cycles of 10-min occlusion and reperfusion in a hindlimb by tourniquet application reduced the muscle infarct size in LD, GC, and RA muscle flaps when subsequently subjected to 4 h of ischemia and 48 h of reperfusion (Fig. 1). Specifically, remote IPC reduced the infarct size of the LD, GC, and RA muscle flaps by 55%, 60%, and 55%, respectively, compared with their corresponding control (n = 6, P < 0.01).

Effect of Ganglionic Blocker and Humoral Mediators on Noninvasive Remote IPC of LD Muscle Flaps Against Infarction

Muscle infarction was reduced by 58% (P < 0.01; n = 6) in LD muscle flaps undergoing remote IPC before 4 h of sustained ischemia compared with the ischemic control (Fig. 2). Intravenous injection of the ganglionic blocker hexamethonium chloride (20 mg/kg) did not affect the infarct-protective effect of remote IPC as the infarct size of LD muscle flaps in this group was similar to that of LD muscle flaps undergoing remote IPC before 4 h of ischemia without any drug pretreatment (Fig. 2). This observation can be interpreted to indicate that the anti-infarction effect of remote IPC with brief cycles of hindlimb occlusion and reperfusion is unlikely to be mediated by a neurogenic pathway. Similarly, adenosine alone is unlikely to be an important mediator of the infarct-protective effect of this remote IPC because neither an intravenous injection of the nonspecific adenosine receptor antagonist SPT (10 mg/kg) nor local intra-arterial injection of the adenosine_1 receptor antagonist DPCPX (3 mg/muscle flap) before remote IPC affected the infarct size of LD muscles compared with LD muscle flaps undergoing remote IPC without any drug pretreatment (Fig. 2). Of importance was the observation that the infarct-protective effect of remote IPC was blocked by the nonspecific opioid receptor antagonist naloxone given intravenously 10 min before (3 mg/kg) and during (3 mg/kg) remote IPC and by the selective δ-opioid receptor antagonist BNTX (3 mg/kg) given as an intravenous bolus injection 10 min before remote IPC. However, the intravenous naloxone or BNTX treatment did not affect the infarct size of the ischemic LD muscle flaps without remote IPC. These observations implied that remote IPC by brief cycles of hindlimb occlusion and reperfusion mobilized and/or released endogenous opioids in the circulation, which
protected various distant skeletal muscle from I/R injury. Neither remote IPC nor intravenous injection of hexamethonium, SPT, naloxone, or BNTX had any significant effect on the mean arterial blood pressure of anesthetized pigs (Table 1).

**Effects of Remote IPC on ATP Depletion, Lactate Accumulation, and Neutrophilic MPO Activity in LD Muscle Flaps During Sustained Ischemia and Reperfusion**

The muscle content of ATP in LD muscle flaps was similar between the control and treatment groups before remote IPC (Fig. 3). The muscle content of ATP decreased progressively and was slower in the preconditioned than in the control LD muscle flaps over 4 h of sustained ischemia. The muscle content of ATP was higher in the remote IPC treatment group than the control by 6.8, 14.6 (P < 0.05, n = 5), and 6.3 µmol/g protein (P < 0.05; n = 5) at the end of 2 and 4 h of ischemia and 1.5 h of reperfusion, respectively, compared with the time-matched ischemic control (Fig. 3).

The muscle content of lactate was similar between the control and treatment LD muscle flaps before remote IPC (Fig. 4). The muscle content of lactate increased over 4 h of sustained ischemia in both control and preconditioned LD muscle flaps, but the lactate content was lower (P < 0.05, n = 5) in the preconditioned LD muscle flaps at the end of 4 h of ischemia compared with the time-matched control (Fig. 4).

The neutrophilic MPO activity was similar between the control and preconditioned LD muscle flaps before and during the 4 h of sustained ischemia (Fig. 5). There was a significant (P < 0.01) increase in muscle MPO activity in the control and preconditioned LD muscle flaps within 1.5 h of reperfusion, but this increase in the muscle MPO activity was higher (P < 0.01, n = 5) in the ischemic control LD muscle flaps compared with the time-matched preconditioned LD muscle flaps (Fig. 5).

**DISCUSSION**

**Major Findings in the Present Study**

For the first time, we have demonstrated a noninvasive remote IPC technique for the protection of multiple skeletal muscles, at different locations, against infarction. Specifically, three cycles of 10-min occlusion and reperfusion in a pig hindlimb preconditioned LD, GC, and RA muscle flaps against infarction when these muscle flaps were subsequently subjected to 4 h of sustained ischemia and 48 h of reperfusion. We further demonstrated that the infarct-protective effect of this

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Table 1. Effect of remote IPC and hexamethonium chloride, SPT, naloxone, or BNTX treatment on mean arterial blood pressure

<table>
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<tr>
<th>Treatment</th>
<th>Baseline</th>
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Values are means ± SE (in mmHg); n = 4 pigs/group. Hexamethonium chloride (20 mg/kg), 8-[p-sulfophenyl]theophylline (SPT; 10 mg/kg), and 7-benzylidenealtrexone (BNTX; 3 mg/kg) were given as an intravenous bolus. Naloxone (3 mg/kg) was given as an intravenous bolus followed by a continuous intravenous infusion (3 mg/kg) over 60 min. Remote ischemic preconditioning (IPC) was instigated by three cycles of 10-min occlusion of a hindlimb by tourniquet application. Remote IPC or drug treatments did not have any significant effect on mean arterial blood pressure at all time points studied compared with the control (baseline) within each group (one-way ANOVA).
remote IPC was abolished by intravenous pretreatment with the nonselective opioid receptor antagonist naloxone and the selective δ1-opioid receptor antagonist BNTX but not by the ganglionic blocker hexamethonium, the nonspecific adenosine receptor antagonist SPT, and the adenosine 1 receptor antagonist DPCPX. Finally, we observed that the infarct-protective effect of remote IPC was associated with a slower rate of ATP depletion and lactate accumulation during 4 h of sustained ischemia and a lower neutrophilic MPO activity within 1.5 h of reperfusion compared with the time-matched control. Taken together, these observations led us to speculate that brief cycles of occlusion and reperfusion in a hindlimb may mobilize and/or release endogenous opioid peptide(s) into the circulation that trigger an infarct-protective effect in all skeletal muscle in the body. In addition, remote IPC is also associated with an energy-sparing effect during sustained ischemia and a decrease in neutrophil accumulation during reperfusion.

**Global Protection of Skeletal Muscle Against Infarction by Noninvasive Remote IPC**

The phenomenon of remote IPC of skeletal muscle against infarction has been described previously. Specifically, it was reported that 5-h occlusion of the vascular pedicle of a GC muscle flap in the dog attenuated the infarction of the contralateral GC muscle flap when it was subjected to 5 h of ischemia 48 h later (21). However, this technique was invasive and lethal to the organ of preconditioning, and the mechanism may not be the same as sublethal remote IPC. Here, we demonstrate for the first time a noninvasive sublethal technique for remote IPC of multiple skeletal muscles against infarction at different locations in the body. Specifically, noninvasive remote IPC instigated by three cycles of 10-min occlusion and reperfusion in a hindlimb by tourniquet application preconditioned the LD, GC, and RA muscle against infarction (Fig. 1). Remote IPC reduced the infarct size of LD, GC, and RA muscle flaps by 55%, 60%, and 55%, respectively, compared with the control in each group. We speculate that this technique of remote IPC most likely produces a robust infarct-protective effect in all skeletal muscle, i.e., global protection of skeletal muscle against infarction.

**Role of Neurogenic Pathway in Remote IPC Against Infarction**

Several investigators have used hexamethonium as a probe to investigate the role of the neurogenic pathway in remote IPC against myocardial infarction, but the results available thus far are equivocal. Specifically, it was reported that 10 min of mesenteric artery occlusion in the rat preconditioned the myocardium against infarction when subsequently subjected to sustained coronary artery occlusion, and this anti-infarction effect of remote IPC was blocked by hexamethonium pretreatment (20 mg/kg iv) (11, 20). In addition, it was reported that intramesenteric infusion of bradykinin mimicked the myocardial infarct-protective ef-
fect of remote IPC induced by 15 min of mesenteric artery occlusion in the rat, and this protective effect was abolished by pretreatment with hexamethonium (20 mg/kg iv) (34). These observations were interpreted to indicate that the neurogenic pathway played an important role in remote IPC of myocardium against infarction. On the other hand, other investigators reported that the same dose of hexamethonium (20 mg/kg iv) did not attenuate the myocardial infarct-protective effect of remote IPC by 25-min occlusion and reperfusion of the mesentery artery (44) or 15-min occlusion and reperfusion of the infrarenal aorta in the rat (45).

We also used hexamethonium as a probe to investigate the role of the neurogenic pathway in remote IPC of skeletal muscle against infarction in the pig. We observed that brief cycles of occlusion and reperfusion in a hindlimb protected distant LD muscle flaps against infarction when these muscle flaps were subsequently subjected to 4 h of sustained ischemia and 48 h of reperfusion. It is important to point out that these LD muscle flaps were preconditioned against infarction, although they were surgically denervated to mimic the clinical situation of autogenous muscle transplantation. In addition, intravenous injection of hexamethonium chloride (20 mg/kg) at 10 min before remote IPC did not attenuate the infarct-protective effect of remote IPC in LD muscle flaps (Fig. 2). This observation suggested that the neurogenic pathway may not play a key role in remote IPC of pig skeletal muscle against infarction.

Role of Humoral Mediators in Remote IPC Against Infarction in Skeletal Muscle

It was demonstrated in rabbit hearts in vitro that transfusion of the effluent collected during local acute myocardial IPC protected the acceptor heart against infarction (6) and naloxone abrogated the myocardial infarct-protective effect of the transferred effluent (5). These observations suggested a humoral mechanism involving opioid receptors in the mediation of remote IPC. Subsequently, Patel et al. (29) reported that occlusion of the mesenteric artery either as a single 15-min occlusion or three cycles of 5-min occlusion afforded myocardial infarct protection in the rat, and this protective effect was blocked by intravenous pretreatment with naloxone (10 mg/kg). These investigators speculated that brief occlusions of the mesenteric artery result in mobilization of endogenous opioids, which were carried in the circulation to the myocardium where they exerted an infarct-protective effect.

Of particular interest in the present study was the observation that the nonselective opioid receptor antagonist naloxone, given 10 min before and during remote IPC (a total of 6 mg/kg), or the δ1-selective opioid receptor antagonist BNTX (3 mg/kg), given 10 min before remote IPC, abolished the infarct-protective effect of remote IPC in pig LD muscle flaps. However, naloxone or BNTX treatment did not have any effect on the infarct size of the ischemic LD muscle flaps without remote IPC (Fig. 2). These observations led us to speculate that hindlimb preconditioning may cause mobilization and release of one or more endogenous opioid peptides in the circulation, which trigger an infarct-protective effect in all skeletal muscle. Opioid peptides are known to be present in neuromuscular junctions (15). The presence of opioid receptors, especially δ-receptors (9), and the humoral action of opioid peptides in skeletal muscle have also been described previously (10).

Comparison of Local and Remote IPC Against Infarction in Pig Skeletal Muscle

The potency of the infarct-protective effect in skeletal muscle was quite similar between local and remote IPC. Specifically, we (28) previously observed that three cycles of 10-min occlusion of the local vascular pedicle of the LD and GC muscle flaps reduced the infarction by 44% and 60%, respectively, when they were subjected to 4 h of ischemia and 48 h of reperfusion. Here, we demonstrated that remote IPC by three cycles of 10-min occlusion and reperfusion of a hindlimb reduced the infarction of LD and GC muscle flaps by 55% and 60%, respectively, when these muscle flaps were subjected to the same duration of sustained ischemia and reperfusion (Fig. 1).

We (27) demonstrated previously that adenosine receptors play a key role in acute local IPC of skeletal muscle. In the present study, neither intravenous injection of the nonspecific adenosine receptor antagonist SPT nor the same dose of a local intraarterial injection of the adenosine1 receptor antagonist DPCPX abolished the infarct-protective effect of acute local IPC in pig LD muscle flaps. These observations were interpreted to indicate that adenosine receptors play a role in remote local IPC of skeletal muscle. In the present study, neither intravenous injection of the nonspecific adenosine receptor antagonist SPT nor the same dose of a local intraarterial injection of the adenosine1 receptor antagonist DPCPX blocked the infarct-protective effect of remote IPC of LD muscle flaps (Fig. 2). These observations indicated that adenosine receptors did not play an important role in remote IPC induced by three cycles of 10-min occlusion and reperfusion in a hindlimb of the pig. However, it is important to point out that other investigators have reported that 10 min of renal artery occlusion preconditioned against myocardial infarction in the rabbit and this infarct protection was abolished by pretreatment with SPT (30). Therefore, there is the possibility that the role of adenosine receptors in remote IPC may vary with the species of animal or organs and technique (e.g., number of cycles of occlusion/reperfusion) used for remote IPC.

We (14, 25–27) also demonstrated previously that the mechanism of acute local IPC against infarction in pig skeletal muscle involved adenosine1 receptor-protein kinase C-KATP channel-linked events. In a recent preliminary study (1), we observed that intravenous injection of the KATP channel inhibitor 5-hydroxydecanoate (5 mg/kg) 10 min before remote IPC by three cycles of 10-min occlusion/reperfusion in a hindlimb of
ATP depletion and lactate accumulation during 4 h of occlusion in a hindlimb was associated with a slower rate of reperfusion injury. The mechanism of remote IPC is unclear. Observations made from our previous studies indicated that energy preservation associated with acute local IPC was unlikely dependent on an increase in muscle blood flow or preischemic reserve of phosphocreatine and/or ATP in our totally denervated, and noncontractile LD muscle flap model (25, 27, 28). There is the possibility that IPC may cause a reduction in mitochondrial ATPase activity, thereby reducing cellular energy demand in preconditioned myocardium, but this concept remains equivocal at the present time (40, 41).

Finally, we observed that remote IPC reduced neutrophil accumulation as indicated by the neutrophilic MPO activity within 1.5 h of reperfusion in pig LD muscle flaps subjected to 4 h of sustained ischemia (Fig. 5). We (25, 27) also previously observed this attenuation of neutrophil accumulation in acute local IPC of pig LD muscle flaps subjected to 4 h of sustained ischemia. Neutrophils are known to participate in reperfusion injury, but the mechanism for the reduction of neutrophil accumulation by IPC is not known. Our present and previous observations (25, 27) led us to speculate that the reduction in ischemic injury due to the protective effect of IPC decreases cellular inflammation, and this in turn decreases local neutrophil activation and accumulation and cell injury at reperfusion.

In summary, we demonstrated for the first time that a noninvasive remote IPC protocol instigated by brief cycles of occlusion and reperfusion in a hindlimb of the pig protected various distant skeletal muscles against infarction when these skeletal muscles were subjected to 4 h of sustained ischemia and 48 h of reperfusion. This infarct-protective effect of noninvasive remote IPC was most likely triggered by activation of opioid receptors in the skeletal muscle, and remote IPC was associated with an energy-sparing effect and attenuation of lactate accumulation during sustained ischemia and attenuation of neutrophil accumulation during early reperfusion. This simple, drug-free, and noninvasive technique has potential clinical applications as a prophylactic procedure for the global protection of skeletal muscle against ischemic necrosis (infarction) in reconstructive surgery under control of vascular clamps or tourniquets.

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DISCLOSURES

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REFERENCES


BRIEF LIMB ISCHEMIA INDUCES DISTANT ISCHEMIC TOLERANCE

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