Na⁺/H⁺ exchange inhibitor cariporide attenuates skeletal muscle infarction when administered before ischemia or reperfusion

Sandra E. McAllister,1,2 Michael A. Moses,1,2 Kunaal Jindal,1 Homay Ashrafpour,1 Neil J. Cahoon,1,2 Ning Huang,1 Peter C. Neligan,1,2 Christopher R. Forrest,1,2 Joan E. Lipa,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

Submitted 8 August 2008; accepted in final form 10 November 2008

Na⁺/H⁺ exchange inhibitor cariporide attenuates skeletal muscle infarction when administered before ischemia or reperfusion. J Appl Physiol 106: 20–28, 2009. First published November 20, 2008; doi:10.1152/japplphysiol.91069.2008.—Administration of Na⁺/H⁺ exchange isoform-1 (NHE-1) inhibitors before ischemia has been shown to attenuate myocardial infarction in several animal models of ischemia-reperfusion injury. However, controversy still exists as to the efficacy of NHE-1 inhibitors in protection of myocardial infarction when administered at the onset of reperfusion. Furthermore, the efficacy of NHE-1 inhibition in protection of skeletal muscle from infarction (necrosis) has not been studied. This information has potential clinical applications in prevention or salvage of skeletal muscle from ischemia-reperfusion injury in elective and trauma reconstructive surgery. The objective of this research project is to test our hypothesis that the NHE-1 inhibitor cariporide is effective in protection of skeletal muscle from infarction when administered at the onset of sustained ischemia or reperfusion and to study the mechanism of action of cariporide. In our studies, we observed that intravenous administration of cariporide 10 min before ischemia (1 or 3 mg/kg) or reperfusion (3 mg/kg) significantly reduced infarction in pig latissimus dorsi muscle flaps compared with the control, when these muscle flaps were subjected to 4 h of ischemia and 48 h of reperfusion (P < 0.05; n = 5 pigs/group). Both pres ischemic and posts ischemic cariporide treatment (3 mg/kg) induced a significant decrease in muscle myeloperoxidase activity and mitochondrial-free Ca2⁺ content and a significant increase in muscle ATP content within 2 h of reperfusion (P < 0.05; n = 4 pigs/group). Posts ischemic and posts hemorrhagic cariporide treatment (3 mg/kg) also significantly inhibited muscle NHE-1 protein expression within 2 h of reperfusion after 4 h of ischemia, compared with the control (P < 0.05; n = 3 pigs/group). These observations support our hypothesis that cariporide attenuates skeletal muscle infarction when administered at the onset of ischemia or reperfusion, and the mechanism involves attenuation of neutrophil accumulation and mitochondrial-free Ca2⁺ overload and preservation of ATP synthesis in the early stage of reperfusion.

ischemia-reperfusion injury; mitochondrial-free Ca2⁺ overload; ATP synthesis

SKELETAL MUSCLE IS SUBJECT TO SUSTAINED WARM (ROOM TEMPERATURE) GLOBAL ISCHEMIA IN ELECTIVE MUSCULOSKELETAL AND VASCULAR RECONSTRUCTIVE SURGERY PERFORMED UNDER TOUNIQUET OR VASCULAR CLAMP CONTROL, AS WELL AS IN THE AMPUTATED FOOT, HAND, AND LIMB AND COMPARTMENT SYNDROME IN TRAUMA SURGERY. CLINICALLY, WARM ISCHEMIC TOLERANCE IN HUMAN SKELETAL MUSCLE IS ~2.5 h (3, 13, 30). SKELETAL MUSCLE ISCHEMIA-REPERFUSION (I/R) INJURY SOMETIMES OCCURS AS A RESULT OF PROLONGED ISCHEMIA DUE TO UNPREDICTABLE COMPLICATIONS, SUCH AS THROMBOSIS AND/OR VASOSPASM OCCURRING PERIOPERATIVELY IN SELECTIVE RECONSTRUCTIVE SURGERY OR DELAYED SURGICAL INTERVENTION IN TRAUMA SURGERY. SKELETAL MUSCLE I/R INJURY OCCURS, RANGING FROM LOSS OF FUNCTION OR MUSCLE NECROSIS (INFARCTION) IN A SINGLE MUSCLE TO LIFE-THREATENING ACIDOSIS, HYPERKALEMIA, AND MYOGLOBINURIA, IF MUSCLE INFARCTION IS MASSIVE (30, 59). IN THE PAST, RESEARCH ON INTERVENTION STRATEGIES OF I/R INJURY IN SKELETAL MUSCLE WAS FOCUSED ON PREVENTION OF THROMBOSIS AND REPERFUSION INJURY INDUCED BY FREE RADICALS FROM NEUTROPHILS, BUT NONE OF THESE HAS REACHED THE STAGE OF CLINICAL INVESTIGATION (27, 40, 46). THE ROLE OF NEUTROPHILS IN THE PATHOGENESIS OF I/R IN OTHER TISSUES, SUCH AS THE MYOCARDIUM, IS ALSO HIGHLY CONTROVERSIAL (54, 58). SPECIFICALLY, CLINICAL STUDIES WITH SCAVENGERS AND DRUGS DESIGNED TO SUPPRESS FREE RADICALS FOR PROTECTION OF MYOCARDIAL I/R INJURY WERE NOT EFFECTIVE (15, 56). FURTHERMORE, MONOCLONAL ANTIBODY TO ICAM-1 AND ANTI-CD18 ANTIBODIES WERE EFFECTIVE IN PROTECTION OF MYOCARDIUM AGAINST I/R INJURY IN LABORATORY ANIMALS (42, 65), BUT CLINICAL TRIALS WITH THESE AGENTS YIELDED NEGATIVE RESULTS (14, 48). MORE RECENTLY, Ca2⁺ OVERLOAD HAS BEEN PROPOSED TO CAUSE CELL DEATH IN THE REPERFUSION HEART (39). A RECENT REVIEW INDICATES THAT PREISCHEMIC ADMINISTRATION OF A L-TYPE Ca2⁺ CHANNEL ANTAGONIST OR MgSO₄ (AN ENDOSKELETAL Ca2⁺ ANTAGONIST) REDUCES MYOCARDIAL INFARCTION IN LABORATORY ANIMALS, BUT RESULTS FROM CLINICAL TRIALS THUS FAR FAIL TO SUPPORT THE INFARCT PROTECTIVE EFFECT OF THESE DRUGS (10).

Na⁺/H⁺ exchange isoform-1 (NHE-1) seems to play a central role in the pathogenesis of I/R injury (21, 23, 50). Specifically, the NHE-1 is an ion exchange protein associated with the maintenance of intracellular pH. The NHE-1 acts as an antiporter to extrude intracellular H⁺ in exchange for extracellular Na⁺, thus contributing to the maintenance of intracellular pH and Na⁺ content (26). However, it is unclear if NHE-1 is required for the maintenance of intracellular pH in normal conditions. In ischemia, mitochondrial ATP synthesis ceases, and glycylisys takes place, resulting in an accumulation of lactate and intracellular H⁺ (9). This buildup of intracellular H⁺ stimulates increased activity of the NHE-1 antiporter, resulting in extrusion of H⁺ and accumulation of intracellular Na⁺. There is a further increase in Na⁺ accumulation because Na⁺ extrusion is limited by inactivation of energy-dependent Na⁺/K⁺-ATPase pump (16, 31). Increases in intracellular Na⁺ correlate with increases in intracellular Ca2⁺ by activation of the Na⁺/Ca²⁺ exchanger causing Ca²⁺ influx (11, 16, 51, 52, 69).
63). However, within 10–20 min of ischemia, the NHE-1 is inhibited (62) because the extracellular acidosis is more pronounced than the intracellular acidosis (57). At reperfusion, the rapid washout of the extracellular H+ reactivates the NHE-1, resulting in further accumulation of intracellular Na+, causing cytosolic Ca2+ overload through the Na+/Ca2+ activity (12, 51). Cytosolic Ca2+ overload is believed to cause mitochondrial Ca2+ overload, which impairs ATP synthesis (37, 55). Based on the above sequence of events, it can be speculated that the NHE-1 plays a pivotal role in the pathogenesis of I/R injury. Indeed, there are publications that demonstrate that pharmacological inhibition of the NHE-1 shortly before sustained ischemia is effective in protection of myocardium from I/R injury in laboratory animals (23). However, there is controversy regarding the time of drug delivery for effective cardioprotection. Specifically, there are studies that demonstrate effective cardioprotection in the pig (44), dog (19, 20), rabbit (32, 60, 61), and guinea pig (2), when NHE-1 inhibitors were given intravenously, either shortly before sustained ischemia or at the onset of reperfusion. On the other hand, there are studies in the pig (17, 28, 29) and rabbit (5, 36) that demonstrated cardioprotection only when NHE-1 inhibitors were administered intravenously shortly before sustained ischemia, but not at the onset of reperfusion. There are also findings from clinical studies to indicate that administration of NHE-1 inhibitor before sustained ischemia attenuates myocardial I/R injury (4, 35, 53), but it remains unclear if postischemic administration of NHE-1 inhibitor is effective in the salvage of ischemic myocardium from reperfusion injury in humans (47, 64). The NHE-1 is known to be present in rat and human skeletal muscle (24, 25), and this controversy is important in the development of pharmacological therapy for salvage of ischemic skeletal muscle from reperfusion injury. Specifically, in trauma surgery, such as replantation of amputated hand, foot, and limb, decompression of compartment syndrome, or thrombolysis, there is no opportunity for preischemic infant protective treatment as in elective surgery, because the sustained ischemia in traumatic injury occurs before the patient arrives at the hospital for surgical intervention. Therefore, we plan to investigate the efficacy and mechanism of NHE-1 inhibition in protection of skeletal muscle from I/R injury. Specifically, the objective of this research project was to use the clinically relevant in vivo pig latissimus dorsi (LD) muscle flap model to test the hypothesis that the selective NHE-1 inhibitor cariporide is effective in attenuation of skeletal muscle necrosis (infarction) when administered intravenously immediately before sustained ischemia or at the onset of reperfusion. The mechanism involves attenuation of neutrophil accumulation, mitochondrial-free Ca2+ overload, and preservation of ATP synthesis in the early phase of reperfusion when the NHE-1 protein expression is significantly inhibited compared with the control. The information to be obtained from this research project will provide important insights into the application of NHE-1 inhibitors for protection of skeletal muscle from I/R injury in elective and trauma surgery.

MATERIALS AND METHODS

Animal Management

Young, castrated Yorkshire pigs (18.6 ± 1.0 kg; mean ± SD) 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. Food was withheld the evening before surgery. The following experimental surgery and protocols were approved by the Animal Care Committee of the Hospital for Sick Children Research Institute, Toronto, Ontario, Canada. Similar pig management and surgical procedure were used previously (1, 34, 38, 41).

Experimental Surgery

Anesthesia. All surgical procedures were performed under general anesthesia induced by intramuscular ketamine (25 mg/kg) and intravenous pentobarbital sodium (10–15 mg/kg). After endotracheal intubation, the pig was mechanically ventilated with oxygen and nitrous oxide (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 monitored and kept within the normal range of 38–39°C by warming the pig with a heating blanket.

LD muscle flap model. The LD muscle flap is used clinically for autogenous muscle transplantation in reconstructive surgery. The anatomy of the pig LD muscle flap is similar to that of the human. In the pig, as in the human, the LD muscle is not vital for locomotion, thus allowing the long-term (48 h) study of reperfusion injury (1, 34, 38, 41). In the following studies, bilateral 8 × 13-cm LD muscle flaps were constructed based on the thoracodorsal neurovascular bundle. The thoracodorsal nerve was divided to mimic the clinical situation of replantation and autogenous muscle transplantation in human reconstructive surgery. A 1-cm-wide tendon was left to support the vascular pedicle of the LD muscle flap, and the tendon was ligated with 1–0 silk ties to ensure that blood supply to the LD muscle flaps was solely from the thoracodorsal artery and drained by two thoracodorsal veins. The LD muscle flap was sutured to its original site with 3–0 vicryl sutures, while 3–0 nylon sutures were used to close the skin overlying the muscle flap. A small opening was left in the axilla for access to the vascular pedicle for application of vascular clamp to induce global ischemia.

Induction of I/R injury in LD muscle flaps. Global ischemia was induced in LD muscle flaps by clamping the vascular pedicle of each flap with two 2 × 8 mm microvascular clamps (Weck). Complete occlusion of the vascular pedicle was verified by intravenous injection of fluorescein dye (15 mg/kg) in an ear vein catheter. The absence of yellow fluorescence in the muscle flap observed under ultraviolet light at 10–15 min after dye injection indicated complete occlusion of the vascular pedicle. The pig was then positioned prone on the operating table for the entire period of muscle ischemia. All flaps were subjected to 4 h of sustained global ischemia at operating room temperature (24°C). At the end of ischemia, vascular clamps were removed, at which time reperfusion was confirmed by a second injection of fluorescein dye and the immediate appearance of yellow fluorescence in the muscle flap. Skin wounds were sutured with 3–0 nylon. The pig was allowed to recover from anesthesia and was returned to the animal holding room.

After 48-h reperfusion, the pig was anesthetized again. Muscle flaps were excised and sectioned transversely into thirteen 1 × 8-cm segments for assessment of muscle infarction, using the nitroblue tetrazolium staining method, as described previously (41). Following muscle harvest, pigs were killed with an overdose of intravenous pentobarbital sodium (100 mg/kg).

It was previously observed that muscle infarction did not occur if the flap was not rendered ischemic (41), so a nonschematic control group was not included. It was also previously observed that 8 × 13-cm LD muscle flaps subjected to 4 h of warm ischemia and 48-h reperfusion experienced 38–45% infarction (1, 34, 38, 41). Therefore, the same I/R time was utilized.
Assessment of Infarct Size

A solution containing 200 mg nitroblue tetrazolium (Sigma) and 400 ml of 0.2% Tris buffer at pH 7.4 and temperature 22°C was used to stain the sectioned LD muscle flaps. The muscle flap sections were immersed in the solution for 30 min at 22°C. Areas of viable muscle stained dark blue (formazan pigment), while nonviable muscle remained pink. The muscle sections were removed from the solution, placed on paper, and digitally photographed. The areas of viable and nonviable muscle were determined by computer planimetry (Adobe Photoshop CS, Adobe Systems), as reported previously (34).

Chemical Analysis of Muscle Biopsies

Muscle biopsies (1 × 1 cm) were collected at various time points, as described in the following experimental protocols, for assay of muscle myeloperoxidase (MPO) activity, muscle-free mitochondrial Ca2+ content and muscle ATP content, and Western blot analysis of NHE-1 protein expression.

Assay of muscle MPO activity. About 200 mg of frozen muscle samples were weighed and homogenized in 2 ml of solution A (100 mM NaCl, 20 mM NaH2PO4, 15 mM EDTA, pH 4.7) for 30 s at 4°C. The homogenate was centrifuged at 12,000 g and 4°C for 20 min. After discarding the supernatant, the pellet was rehomogenized in 2 ml of solution B (50 mM KH2PO4, 0.5% hexadecyltrimethylammonium bromide, pH 5.4) for 1 min at 4°C. The homogenate was sonicated for 10 s on an ice bath, followed by three freeze-thaw cycles. After centrifugation at 12,000 g and 4°C for 20 min, the supernatant was collected for the MPO assay. The enzyme activity was measured by using the 2,2’-azino-di(3-ethylbenzothiazoline-6-sulfonic acid) (o-TMB) method (Beckman DUS5) was used to measure the generation of oxidized 3,3’,5,5’-tetramethylbenzidine. A Spectrophotometer (Beckman DUS5) was used to measure the concentration of oxidized 3,3’,5,5’-tetramethylbenzidine at 655 nm. One unit of enzyme activity that produced an absorbance change of 1.0 optical density units per minute per gram muscle wet weight at 37°C (1, 34, 38).

Assay for muscle mitochondrial-free Ca2+ content. Our laboratory previously assayed mitochondrial-free Ca2+ content ([Ca2+]o,ret) in pig skeletal muscle (34), using the method described by Rousseau et al. (45). Mitochondria were isolated from fresh muscle samples by differential centrifugation. About 1.5 g of tissue were homogenized in 25 ml of buffer A (50 mM Tris-HCl, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol) and solubilized at 95°C for 10 min and then centrifuged at 14,000 rpm for 10 min. All preparations were carried out at 4°C. Protein concentration was determined by the RC-DC protein assay (Bio-Rad). For Western blotting, samples containing 100 μg of protein were separated by 8% SDS-polyacrylamide gel electrophoresis, and the separated proteins were electrophotographically transferred at 4°C to polyvinylidene fluoride membrane (immobilon-P, Millipore, MA) at a constant voltage (35 V) overnight. After blocking with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 h at room temperature, the membrane was incubated overnight with rabbit NHE-1 polyclonal antibody (Chemicon) at a 1:500 dilution at 4°C. After rinsing in TBST, the membrane was incubated for 1 h at room temperature, in a 1:3,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Bio-Rad). After six washes with TBST, the blots were developed by the enhanced chemiluminescence system (Hy Glo, Denville Scientific) on high-performance chemiluminescence film (Amersham, Quebec). The band densities were quantified using scanning laser densitometry (Fluoroochem Software, Alpha Innotech, San Leandro, CA). The Western blots were probed with an anti-β-actin antibody for standardization of protein loading.

Biochemicals

All chemicals and assay kits were purchased from Sigma (Oakville, Ontario, Canada). Cariporide (HOE 642, MW 379.46) was donated by Aventis Pharma, Germany. Cariporide solutions were prepared in normal saline within 30 min of injection.

Experimental Protocols

Study 1: To investigate the efficacy of the selective NHE-1 inhibitor cariporide in the protection of skeletal muscle from IR injury when administered before or after sustained ischemia. Pigs with bilateral 8 × 13-cm LD muscle flaps were assigned to one control and three treatment groups. There were five pigs (10 muscle flaps) in the control and in each treatment group. All 10 LD muscle flaps in each group were subjected to 4 h of ischemia and 48 h of reperfusion. Pigs in the control group received intravenous injection of saline (10 ml) at 10 min before 4 h of ischemia and at 10 min before reperfusion. Pigs in the three treatment groups received one of the following drug injections: 1) intravenous injection of 10 ml of normal saline containing a low dose of cariporide (1 mg/kg) at 10 min before 4 h of ischemia; 2) intravenous injection of 10 ml of normal saline containing a high dose of cariporide (3 mg/kg) at 10 min before ischemia; and 3) intravenous injection of 10 ml of normal saline containing a high dose of cariporide (3 mg/kg) at 10 min before reperfusion in an ear vein catheter. After 48 h of reperfusion, all pigs were killed with an overdose of intravenous pentobarbitone sodium (100 mg/kg) for assessment of infarct size in LD muscle flaps. We chose these doses with 1 M Tris base (120 μl/ml supernatant). The supernatant after spinning at 1,000 g and 4°C for 5 min was used for assay of ATP content (FL-AA triphosphate bioluminescence assay kit, Sigma, Oakville, Ontario, Canada). The pellet was homogenized in 1 ml of 0.5 M NaOH, and the protein content was determined by the Bradford method (Bio-Rad). The muscle ATP content is expressed as micromoles per gram of protein, as previously reported by our laboratory (1, 34, 38).

Protein extraction and Western blot analysis for the study of NHE-1 protein expression. The methods were similar to those previously reported for cardiac muscle of laboratory animals (8, 49).

Frozen muscle biopsies (~200 mg) were cut into small pieces and homogenized for 30 s in 2 ml of ice cold lysis buffer containing 50 mM Tris·HCl (pH 7.4), 50 mM EDTA, 150 mM NaCl, 0.1% Triton 100, 1 mg/ml pepstatin, 200 mM phenylmethyl sulfonyl fluoride, and 1 mg/ml leupeptin. The homogenates were mixed with loading buffer (50 mM Tris·HCl, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol) and solubilized at 95°C for 10 min and then centrifuged at 14,000 rpm for 10 min. All preparations were carried out at 4°C. Protein concentration was determined by the RC-DC protein assay (Bio-Rad). For Western blotting, samples containing 100 μg of protein were separated by 8% SDS-polyacrylamide gel electrophoresis, and the separated proteins were electrophotographically transferred at 4°C to polyvinylidene fluoride membrane (immobilon-P, Millipore, MA) at a constant voltage (35 V) overnight. After blocking with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 h at room temperature, the membrane was incubated overnight with rabbit NHE-1 polyclonal antibody (Chemicon) at a 1:500 dilution at 4°C. After rinsing in TBST, the membrane was incubated for 1 h at room temperature, in a 1:3,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Bio-Rad). After six washes with TBST, the blots were developed by the enhanced chemiluminescence system (Hy Glo, Denville Scientific) on high-performance chemiluminescence film (Amersham, Quebec). The band densities were quantified using scanning laser densitometry (Fluorochem Software, Alpha Innotech, San Leandro, CA). The Western blots were probed with an anti-β-actin antibody for standardization of protein loading.
of cariporide in our study because it was reported that intravenous injection of cariporide at the dose of 1 mg/kg at 15 min after onset of 60 min of ischemia in the pig myocardium significantly reduced myocardial infarct size compared with the control after 24 h of reperfusion (28). In addition, results from other investigators also indicate that a much higher dose of NHE-1 inhibitor is needed for salvage of ischemic cardiac muscle from reperfusion injury if the NHE-1 inhibitor is given at the onset of reperfusion (29, 44).

Study 2: To investigate the mechanism associated with preischemic and postischemic administration of the selective NHE-1 inhibitor cariporide in the protection of skeletal muscle from I/R injury. Pigs with bilateral 8 × 13-cm LD muscle flaps were assigned to one control and two treatment groups. There were four pigs (eight muscle flaps) in each group. All LD muscle flaps were subjected to 4 h of ischemia and 2 h of reperfusion. Pigs in the control group received intravenous injection of normal saline (10 ml) at 10 min before 4 h of ischemia. Pigs in the two treatment groups received intravenous injection of cariporide (3 mg/kg) in 10 ml of normal saline at 10 min before onset of 4 h of ischemia or before onset of 2 h of reperfusion.

Muscle biopsies were taken from the control and treatment LD muscle flaps immediately before and at 2 h and 4 h of 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 (41). Each biopsy was immediately rinsed with cold (4°C) normal saline. Fresh biopsies were used for assay of free [Ca\textsuperscript{2+}]m. Biopsies for assay of MPO activity and ATP content were immediately frozen in liquid nitrogen and stored at −80°C. There were eight biopsies for each time point in each group (eight LD muscle flaps/group).

Study 3: To investigate the effect of preischemic and postischemic intravenous administration of the selective NHE-1 inhibitor cariporide on NHE-1 protein expression. The experimental design was similar to that of study 2, except that there were three pigs in the control and in each of the two treatment groups, and all muscle biopsies were immediately frozen in liquid nitrogen and stored at −80°C for Western blot analysis of NHE-1 protein expression.

Statistics

In Fig. 1, one-way analysis of variance and Newman-Keuls test were used for multiple comparison of means. In Figs. 2–4, treatment effect was detected by two-way analysis of variance with repeated measures. Within each time point, one-way analysis of variance and Newman-Keuls test were used for multiple comparison of means. All values are expressed as means ± SE, and statistical significance was set at P < 0.05; n = number of pigs per group.

RESULTS

Efficacy of the NHE-1 Inhibitor Cariporide in the Protection of LD Muscle Flaps From Infarction When Administered Intravenously 10 min Before Ischemia or Reperfusion

There was 43.7 ± 2.2% infarction in control LD muscle flaps subjected to 4 h of ischemia and 48-h reperfusion (n = 5 pigs) (Fig. 1). Intravenous injection of a low dose (1 mg/kg) or a high dose (3 mg/kg) of cariporide 10 min before ischemia significantly (P < 0.05) reduced the muscle infarction to 18.3 ± 1.6 and 18.0 ± 2.4%, respectively (n = 5 pigs). Intravenous injection of cariporide (3 mg/kg) 10 min before reperfusion also significantly (P < 0.05; n = 5 pigs) reduced the muscle infarction to 24.7 ± 3.0%.

Effect of Intravenous Injection of the NHE-1 Inhibitor Cariporide 10 min Before Ischemia or Reperfusion on MPO Activity, Free [Ca\textsuperscript{2+}]m, and ATP Content in Bilateral LD Muscle Flaps Subjected to 4 h of Ischemia and 2 h of 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. 2). The muscle MPO activity in the control and treatment groups increased significantly at 2 h of reperfusion compared with the preischemic muscle MPO activity (Fig. 2). However, at 2 h of reperfusion, the muscle MPO activity was significantly (P < 0.05) higher in the control (2.1 ± 0.2 U/g wet muscle; n = 4 pigs) than in the treatment groups receiving intravenous cariporide injection 10 min before sustained ischemia (0.8 ± 0.2 U/g wet muscle; n = 4 pigs) or 10 min before onset of reperfusion (0.6 ± 0.2 U/g wet muscle; n = 4 pigs).

Muscle-free [Ca\textsuperscript{2+}]m. The muscle-free [Ca\textsuperscript{2+}]m in pig LD muscle flaps was similar among control and treatment groups of LD muscle flaps immediately before ischemia and at the end of 2 and 4 h of ischemia (Fig. 3). However, the muscle-free [Ca\textsuperscript{2+}]m 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. Intravenous administration of the NHE-1 inhibitor cariporide (3 mg/kg) at 10 min before onset of ischemia or reperfusion attenuated the increase in muscle-free [Ca\textsuperscript{2+}]m in LD muscle flaps at 2 h of reperfusion (Fig. 3).

Specifically, the muscle-free [Ca\textsuperscript{2+}]m at 2 h of reperfusion was 534 ± 41 nmol/mg mitochondrial protein for the control and 369 ± 38 and 283 ± 52 nmol/mg mitochondrial protein for the treatment groups receiving intravenous cariporide at 10 min before ischemia or reperfusion, respectively.

Muscle ATP content. There was no significant difference in muscle ATP content among the control and treatment groups immediately before ischemia and at 2 and 4 h of ischemia (Fig. 4). However, at 2 h of reperfusion, the muscle ATP contents
were significantly \( (P < 0.05) \) higher in the treatment groups receiving intravenous cariporide injection at 10 min before sustained ischemia \((22.5 \pm 2.6 \text{ mmol/g protein})\) or 10 min before reperfusion \((21.6 \pm 1.5 \text{ mmol/g protein})\) compared with the time-matched ischemic control \((9.0 \pm 3.0 \text{ mmol/g protein})\).

Effect of Intravenous Injection of the NHE-1 Inhibitor Cariporide 10 min Before Ischemia or Reperfusion on NHE-1 Exchanger Protein Expression in Bilateral LD Muscle Flaps Subjected to 4 h of Ischemia and 2 h of Reperfusion

There was no significant difference in NHE-1 exchanger protein expression among the control and treatment groups immediately before ischemia and at 2 and 4 h of ischemia (Fig. 5). However, at 2 h of reperfusion, the protein expressions of NHE-1 exchanger were significantly \( (P < 0.05; \ n = 3 \text{ pigs/group}) \) lower in the treatment groups receiving intravenous cariporide injection at 10 min before 4 h of ischemia or 10 min before reperfusion, compared with the time-matched ischemic control (Fig. 5).

DISCUSSION

Important Findings From Present Studies

Using the clinically relevant pig LD muscle flap model, we investigated for the first time the efficacy and mechanism of preischemic and postischemic administration of NHE-1 inhibitor in protection of skeletal muscle from infarction. We observed that intravenous injection of the selective NHE-1 inhibitor cariporide at the onset of sustained ischemia \((1 \text{ or } 3 \text{ mg/kg})\) or reperfusion \((3 \text{ mg/kg})\) protected pig LD muscle flaps from infarction compared with the control, when these LD muscle flaps were subjected to 4 h of ischemia and 48 h of reperfusion. Both preischemic and postischemic cariporide treatment induced a significant decrease in muscle MPO activity and free

Fig. 2. Effect of the NHE-1 inhibitor cariporide on myeloperoxidase activity in bilateral LD muscle flaps subjected to 4 h of ischemia and 2 h of reperfusion. Cariporide was administered intravenously 10 min before ischemia or reperfusion. Values are means \( \pm \text{SE}; \ n = 4 \text{ pigs/group}. * \text{Results are similar within each time point and are significantly different from the control} (P < 0.05). \)

Fig. 3. Effect of the NHE-1 inhibitor cariporide on mitochondrial-free \( \text{Ca}^{2+} \) content \((\text{[Ca}^{2+}]_{m})\) in bilateral LD muscle flaps subjected to 4 h of ischemia and 2 h of reperfusion. Cariporide was administered intravenously 10 min before ischemia or reperfusion. Values are means \( \pm \text{SE}; \ n = 4 \text{ pigs/group}. * \text{Results are similar within each time point and are significantly different from the control} (P < 0.05). \)

Fig. 4. Effect of the NHE-1 inhibitor cariporide on ATP content in bilateral LD muscle flaps subjected to 4 h of ischemia and 2 h of reperfusion. Cariporide was administered 10 min before ischemia or reperfusion. Values are means \( \pm \text{SE}; \ n = 4 \text{ pigs/group}. * \text{Results are similar within each time point and are significantly different from the control} (P < 0.05). \)

Fig. 5. Effect of the NHE-1 inhibitor cariporide on NHE-1 exchanger protein expression in pig LD muscle flaps subjected to 4 h of ischemia and 2 h of reperfusion. Cariporide was administered intravenously 10 min before ischemia or reperfusion. Time points are as follows: 1, before ischemia; 2, 2-h ischemia; 3, 4-h ischemia; 4, 2-h reperfusion. Values are means \( \pm \text{SE}; \ n = 3 \text{ pigs/group}. * \text{Results are similar within each time point and are significantly different from the control} (P < 0.05). \)
[Ca\(^{2+}\)]_m and a significant increase in ATP synthesis at the end of 2 h of reperfusion compared with the time-matched control. The preischemic and postischemic cariporide treatment also attenuated NHE-1 protein expression within 2 h of reperfusion after 4 h of ischemia, compared with the time-matched control. These findings provide important insights into the application of pharmacological therapy for prevention or salvage of skeletal muscle from infarction in reconstructive surgery.

Efficacy of Intravenous Injection of the NHE-1 Inhibitor Cariporide at the Onset of Ischemia or Reperfusion for the Protection of Skeletal Muscle from Infarction

There is general consensus that intravenous administration of the NHE-1 inhibitor at the onset of sustained ischemia is effective in protection of myocardium from infarction in laboratory animals. Contrary to preischemic treatment, the effect of postischemic treatment with the NHE-1 inhibitor for protection of myocardial infarction is controversial. Specifically, there are studies demonstrating effective myocardial infarct protection in the pig (44), dog (19, 20), and rabbit (32, 60, 61) when the NHE-1 inhibitor was given intravenously at the onset of reperfusion. On the other hand, there are studies showing failure of NHE-1 inhibitors to attenuate myocardial infarction in the pig (17, 28, 29) and rabbit (5, 36) when given intravenously at the onset of reperfusion. The density of collateral circulation in the myocardium varies among laboratory animals, and the order from high to low is as follows: dog, cat, rat, ferret, rabbit, and pig (33). Collateral blood flow may facilitate delivery of NHE-1 inhibitors during ischemia and early reperfusion. Indeed, preischemic or postischemic intravenous injection of the NHE-1 inhibitor EMD 85131 (3 mg/kg) or BBIB 513 (3 mg/kg) was effective in attenuation of myocardial infarction in dogs (19, 20). However, collateral blood flow for drug delivery may not be the only key factor that facilitates postischemic NHE-1 treatment for attenuation of myocardial infarction, because several NHE-1 inhibitors were effective in attenuation of myocardial infarction in pigs (HOE 694, 7 mg/kg) and rabbits (SM-19738, 0.017 mg/kg bolus and 0.028 mg·kg\(^{-1}\)·h\(^{-1}\) for 5 h) when administered intravenously at the onset of reperfusion (32, 44, 60, 61). Pig and rabbit myocardium are known to have little or no collateral blood flow. Drug dose may be another important factor affecting the efficacy of postischemic NHE-1 inhibitor treatment in protection of myocardium from infarction. For example, Klein et al. (29) reported that intravenous injection of the NHE-1 inhibitor HOE 694 at a dose of 3 mg/kg at 10 min before ischemia significantly reduced myocardial infarction in the pig myocardium subjected to 45 min of ischemia and 24 h of reperfusion. However, the same dose of HOE 694 when injected intravenously at 10 min before the onset of reperfusion induced a slight but insignificant reduction in myocardial infarction in the pig (29). By increasing the intravenous dose of HOE 694 to 7 mg/kg, Rohmann et al. (44) were able to demonstrate that administration of HOE 694 at 15 min before sustained ischemia or before reperfusion induced significant attenuation of myocardial infarction when the pig myocardium was subjected to 60 min of ischemia and 2 h of reperfusion. However, a longer reperfusion period (~24 h) is required to ensure a maximum myocardial infarct protection is achieved.

In the present study, collateral circulation was not an important factor in drug delivery because we used the clinically relevant island LD muscle flap model, which underwent global ischemia, and the reperfusion was derived entirely from the thoracodorsal artery. In this model, it was well established that the LD muscle underwent 38–45% infarction when subjected to 4 h of ischemia and 24–48 h of reperfusion (1, 34, 38, 41). Here, we observed in the pig that intravenous injection of the NHE-1 inhibitor cariporide (3 mg/kg) at 10 min before ischemia or reperfusion reduced the LD muscle infarction to 18.0 ± 2.4 and 24.7 ± 3%, respectively, compared with the ischemic control (43.7 ± 2.2%; P < 0.05, n = 5 pigs) when the pig LD muscle flaps were subjected to 4 h of ischemia and 48 h of reperfusion (Fig. 1). These findings support our hypothesis that administration of the NHE-1 inhibitor cariporide at the onset of ischemia or reperfusion is effective in protection of skeletal muscle from infarction caused by I/R injury.

Mechanism Associated with Preischemic and Postischemic Intravenous Injection of the NHE-1 Inhibitor Cariporide for Attenuation of Infarction in Skeletal Muscle

Attenuation of neutrophil activation and accumulation. The role of neutrophils in reperfusion injury and in NHE-1 inhibitor treatment for attenuation of myocardial infarction is unclear. On one hand, it was reported that myocardial reperfusion injury and NHE-1 treatment for attenuation of reperfusion injury occurred in neutrophil-free systems, such as isolated, perfused rat hearts and cultured rat myocardiocytes (7, 22). On the other hand, there is evidence to indicate that neutrophils may be an important factor in reperfusion injury and in attenuation of myocardial infarction by the NHE-1 inhibitor. Specifically, in an in vivo canine model of myocardial I/R injury, intravenous infusion of the selective NHE-1 inhibitor BIIB-513 before 60 min of ischemia and 3 h of reperfusion resulted in reduction of myocardial infarction associated with attenuation of neutrophil activation and accumulation (18). In a rat inflammatory model, NHE exchange inhibition with intravenous injection of cariporide at 5 min before reperfusion was seen to reduce neutrophil rolling, adhesion, and extravasation and suppression of the cell adhesion molecule P-selectin (6). Using the intravital microscopy technique, it was observed in the rat cremaster muscle that preischemic intravenous injection of the NHE-1 inhibitor cariporide reduced neutrophils from rolling, adhesion, and extravasation (43). Here, we observed for the first time in skeletal muscle that preischemic or postischemic intravenous administration of the NHE-1 inhibitor cariporide reduced the neutrophil accumulation at early reperfusion (Fig. 2). Neutrophil accumulation can be confirmed by immunohistochemical analysis in future studies. Future in vivo studies are also required to clarify if the reactive oxygen species produced by the accumulated neutrophils are causally related to reperfusion injury in ischemic skeletal muscle.

Prevention of free mitochondrial Ca\(^{2+}\) overload and preservation of ATP synthesis at early reperfusion. Observations made from our studies indicate that the infarct protective effect of the NHE-1 inhibitor cariporide administered at the onset of ischemia or reperfusion involves attenuation of mitochondrial-free Ca\(^{2+}\) overload and restoration of ATP synthesis during early reperfusion, but has no significant effect during sustained ischemia. Specifically, the muscle-free [Ca\(^{2+}\)]_m and ATP con-
tent were similar among the control and cariporide-treated groups immediately before ischemia and during sustained ischemia (Figs. 3 and 4). In the control group, muscle-free [Ca^{2+}]_m significantly increased at 2 h of reperfusion. However, at this time point, the muscle ATP content in the control group remained depleted to the same extent as at the end of 4 h of ischemia. Intravenous administration of the NHE-1 inhibitor cariporide at 10 min before sustained ischemia or reperfusion significantly attenuated free [Ca^{2+}]_m (Fig. 3) and increased muscle ATP content at 2 h of reperfusion (Fig. 4) compared with the time-matched control. There is an explanation in the literature of ischemic myocardium to support our observations. Specifically, in sustained ischemia, mitochondrial ATP synthesis ceases and glycolysis ensues, resulting in a net breakdown of ATP and an accumulation of lactate and intracellular H^+ (9). This increase in intracellular H^+ activates the NHE-1 exchanger, resulting in the extrusion of H^+ and accumulation of intracellular Na^+. There is a further increase in Na^+ accumulation because Na^+ extrusion is decreased due to inactivation of the Na^+/K^-ATPase pump (16, 31). Elevation of intracellular Na^+ causes an increase in intracellular Ca^{2+} by either inhibition or reversal of the Na^+/Ca^{2+} exchanger causing Ca^{2+} influx (11, 16, 51, 52, 63). If these events continue, the cytosolic Ca^{2+} will be overloaded, and significant uptake of Ca^{2+} from the cytosol to the mitochondria will occur, resulting in [Ca^{2+}]_m overload (55), which can cause cell necrosis (37). However, within 10–20 min of ischemia, the NHE-1 is inhibited (48) because the extracellular acidosis is more pronounced than the intracellular acidosis (20). The above information may explain our observation that the muscle-free [Ca^{2+}]_m remained unchanged in the control and treatment groups during 4 h of sustained ischemia (Fig. 3), but the muscle ATP contents were significantly reduced in both the control and treatment groups (Fig. 4). At reperfusion, the rapid washout of extracellular H^+ after restoration of blood supply may reactivate NHE-1 and further result in accumulation of intracellular Na^+ and Ca^{2+} ion influx through reversal of NHE-1, causing intracellular and mitochondrial Ca^{2+} overload (11, 16, 51, 52, 63), which impairs ATP synthesis at the early stage of reperfusion (37, 55). Indeed, there are publications that demonstrate effective cardioprotection in the pig (44), dog (19, 20), and rabbit (5, 36) when NHE-1 inhibitor is administered intravenously at the onset of ischemia or reperfusion. The dose of cariporide used for preischemic and posts ischemic treatment in the present study significantly attenuated NHE-1 protein expression within 2 h of reperfusion after 4 h of ischemia (Fig. 5). This indicates that an effective dose of cariporide was used in our studies. This information lends support to our observation that intravenous administration of the NHE-1 inhibitor cariporide at the onset of sustained ischemia or reperfusion attenuates mitochondrial Ca^{2+} (Fig. 3) overload and preserves ATP synthesis (Fig. 4) at the early stage of reperfusion.

In summary, we have investigated for the first time the efficacy and mechanism of the selective NHE-1 inhibitor cariporide in the protection of pig skeletal muscle from I/R injury. Specifically, we observed that intravenous administration of the selective NHE-1 inhibitor cariporide (3 mg/kg) at 10 min before sustained ischemia or reperfusion significantly attenuated infarction in pig LD muscle flaps subjected to 4 h of ischemia and 48 h of reperfusion. The mechanism involved attenuation of muscle neutrophil accumulation, prevention of mitochondrial-free Ca^{2+} overload, and preservation of ATP synthesis during the early stage of reperfusion of the ischemic LD muscle flaps. The dose of cariporide used for the present preischemic and posts ischemic treatments significantly inhibited NHE-1 protein expression within 2 h of reperfusion after 4 h of ischemia, thus indicating that an effective dose of cariporide was used in our studies.

**Perspectives**

In elective surgery, such as musculoskeletal and vascular reconstructive surgery, unpredictable complications (e.g., thrombosis, vasospasm) can occur intraoperatively, causing lethal ischemic insult to skeletal muscle. In trauma surgery, such as replantation of amputated hand, foot, and limb, thrombolysis, and decompression of compartment syndrome, prolonged ischemic insult in the skeletal muscle may occur before the patient arrives at the hospital for surgical intervention, and there is no chance for prophylactic treatment. The information obtained from this research project provides insights into the development of pharmacological therapy with NHE-1 inhibitors to be administered at the onset of sustained ischemia or reperfusion for prevention or salvage of ischemic skeletal muscle from reperfusion injury.

**ACKNOWLEDGMENTS**

The authors thank Dianne McIntyre for performing the word processing, and Kim Tsoi and Gerry Kwok for assistance in animal surgery and biochemical assays. Cariporide was a gift from Aventis Pharma, Germany.

Present addresses: J. Lipa, Division of Plastic and Reconstructive Surgery, David Geffen School of Medicine at UCLA, Los Angeles, CA 90048; P. Neligan, Division of Plastic and Reconstructive Surgery, University of Washington, Seattle, WA 98195.

**GRANTS**

This research project was supported by an operating grant from the Canadian Institutes of Health Research (MOP 118149) to C. Y. Pang. S. E. McAllister and M. A. Moses were supported by a postdoctoral fellowship from the Wharton Endowment Fund. N. Cahoon was supported by a postdoctoral fellowship from the University Health Network in Toronto. K. Jindal was a recipient of a student stipend from the Heart and Stroke Foundation of Manitoba and Great West Life Insurance Company.

**REFERENCES**


