Development of an in vitro model for study of the efficacy of ischemic preconditioning in human skeletal muscle against ischemia-reperfusion injury

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Ischemia-reperfusion (I/R) injury causes skeletal muscle infarction and ischemic preconditioning (IPC) augments ischemic tolerance in animal models. To date, this has not been demonstrated in human skeletal muscle. This study aimed to develop an in vitro model to investigate the efficacy of simulated IPC in human skeletal muscle. Human skeletal muscle strips were equilibrated in oxygenated Krebs-Henseleit-HEPES buffer (37°C). Aerobic and reperfusion phases were simulated by normoxic incubation and reoxygenation, respectively. Ischemia was simulated by hypoxic incubation. Energy store, cell viability, and cellular injury were assessed using ATP, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), and lactate dehydrogenase (LDH) assays, respectively. Morphological integrity was assessed using electron microscopy. Studies were designed to test stability of the preparation (n = 5–11) under normoxic incubation over 24 h; the effect of 1, 2, 3, 4, or 6 h hypoxia followed by 2 h of reoxygenation; and the protective effect of hypoxic preconditioning (HPC: 5 min of hypoxia/5 min of reoxygenation) before 3 h of hypoxia/2 h of reoxygenation. Over 24 h of normoxic incubation, muscle strips remained physiologically intact as assessed by MTT, ATP, and LDH assays. After 3 h of hypoxia/2 h of reoxygenation, MTT reduction levels declined to 50.1 ± 5.5% (P < 0.05). MTT reduction levels in HPC (82.3 ± 10.8%) and normoxic control (81.3 ± 10.2%) groups were similar and higher (P < 0.05) than the 3 h of hypoxia/2 h of reoxygenation group (45.2 ± 5.8%). Ultrastructural morphology was preserved in normoxic and HPC groups but not in the hypoxia/reoxygenation group. This is the first study to characterize a stable in vitro model of human skeletal muscle and to demonstrate a protective effect of HPC in human skeletal muscle against hypoxia/reoxygenation-induced injury.

hypoxia/reoxygenation-induced injury; lactate dehydrogenase release; dimethylthiazol diphenyltetrazolium bromide reduction; ATP content

AUTOGENOUS MUSCLE TRANSPLANTATION (i.e., muscle free-flap surgery) represents one of the most popular reconstructive options used by plastic surgeons. It can provide well-vascularized coverage for wounds and limb salvage (4) or may be transferred as innervated, functional units to restore function as in reconstruction for facial paralysis or upper extremity trauma (15). Considering that tolerance of skeletal muscle to warm ischemia is limited to <2.5 h (4, 11, 34) and that unpredictable thrombosis or vasospasm during the operation can cause excessive ischemia, one of the most common complications in autogenous muscle transplantation is skeletal muscle ischemic necrosis. This occurs secondary to ischemia-reperfusion (I/R) injury, often as a result of thrombosis of the vascular pedicle with subsequent restoration of tissue perfusion. This type of injury can range from loss of function to infarction of the transferred muscle. In the case of peripheral vascular surgery, massive muscle infarction can occur after excessive limb ischemia due to unpredictable complications and subsequent revascularization, causing systemic acidosis, hyperkalemia, myoglobinuria, and eventually, irreversible extremity muscle injury and loss of function (2, 36). Although previous research strategies have focused on pharmacological approaches toward prevention or treatment of skeletal muscle I/R injury, no clinical benefit has been shown to date (29).

Ischemic preconditioning (IPC) with brief cycles of ischemia and reperfusion before a subsequent sustained ischemic insult was first demonstrated to provide protection against infarction in the canine myocardium (19). Since then, this myocardial protective effect of IPC has been well documented in small (35, 39) and large (13, 30) animal models in vivo as well as in cultured human cardiomyocytes (7, 8) and myocardium preparations (5, 14), using a variety of protocols with respect to number and duration of IPC cycles.

The existence of the protective effect of IPC in skeletal muscle was first demonstrated by Pang et al. (24). Preconditioning of porcine latissimus dorsi and gracilis muscle flaps with three 10-min cycles of I/R by direct occlusion of their vascular pedicles before 4 h of global ischemia and 48 h of reperfusion reduced muscle infarction by 44 and 62%, respectively. This effect of acute local IPC was confirmed in rat skeletal muscle and musculocutaneous flaps (3, 12, 16, 40). IPC was also shown in rat skeletal muscle to attenuate vascular dysfunction (10) and capillary no reflow (37), and this effect was confirmed in dog skeletal muscle (9).

Acute local IPC, however, is an invasive procedure, requires prolonged operative time, and has the potential for damage to the vascular pedicle of a muscle free flap secondary to repetitive clamping. Pharmacological simulation of IPC with adenosine (12, 22) or KATP channel antagonists (17, 18, 23) has also been effective in increasing animal skeletal muscle tolerance to I/R injury. However, the invasive and hypotension-inducing nature of this approach is contraindicated for patients undergoing autogenous muscle transplantation in reconstructive surgery.

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Recently, Addison et al. (1) demonstrated that application of three 10-min cycles of I/R in a hindlimb of the pig by tourniquet application provided global protection of skeletal muscle against infarction. The phenomenon of remote IPC was also demonstrated in animal myocardium using different techniques (28, 32). Moses et al. (18) showed that the duration of this muscle infarct protection achieved by remote IPC was biphasic, with a short 4-h early window of protection followed by a subsequent 24- to 72-h late window of protection. Thus remote IPC could emerge as a new, noninvasive, drug-free, and inexpensive technique for the protection of skeletal muscle against I/R injury, with the potential for global protection of muscle against subsequent damage from unexpected ischemic complications for up to 48 h. Despite the fact that the early and late protective effects of IPC have also been proven to exist in the human myocardium in vitro (5, 14), animal myocardium (19, 26, 35), and animal skeletal muscle (18) in vivo, it has yet to be demonstrated in human skeletal muscle. Thus the objective of this study was to develop and characterize a novel in vitro human skeletal muscle model to investigate the existence of the infarct protective phenomenon of IPC. We hypothesized that such a model can be developed to maintain intact physiological and morphological parameters over a 24-h period and that this model can be used to test the hypothesis that that IPC reduces I/R injury in human skeletal muscle.

METHODS

Preparation of perfusion buffers and perfusion apparatus. Aerobic and ischemic conditions were simulated by normoxic and hypoxic conditions, respectively. In human skeletal muscle tissue perfusion, normoxic conditions were maintained with Krebs-Henseleit-HEPES (KH) buffer (118 mM NaCl, 4.8 mM KCl, 27.2 mM NaHCO3, 1 mM KH2PO4, 1.2 mM MgCl2, 20 mM HEPES). This buffer was supplemented with 10 mM glucose, 10% fetal bovine serum (FBS) and 0.25 U/ml insulin (DIN 00586714, Humulin R, Lilly) and was bubbled with a mixture of 95% O2-5% CO2 (19:1 vol/vol) to maintain a PO2 of 250–300 Torr and a pH between 7.36 and 7.45. For the simulation of ischemia, hypoxic conditions were created by bubbling nonsupplemented KH buffer with 95% N2-5% CO2 (19:1 vol/vol) at a vigorous rate (40–50 bubbles/s) to obtain a PO2 of 10–20 Torr. Bubbling of the nonsupplemented KH buffer with 95% N2-5% CO2 resulted in a pH of 6.8–7.0. This hypoxic KH buffer was not supplemented with FBS, glucose, or insulin, to simulate ischemic conditions in which muscle would be deprived of circulating plasma and its nutrients and endocrine factors. The PO2, PCO2, and pH in the incubation medium were monitored by intermittent analyses using an automated blood-gas analyzer (model ABL 500, Radiometer, Copenhagen, Denmark). A 2× stock solution of the KH buffer was prepared and was stored to a maximum of 1 wk at 4°C. Incubation medium, depending on the conditions desired, i.e., normoxic or hypoxic incubation with the human skeletal muscle strips and the appropriate buffer depending on the type of incubation desired, i.e., normoxic or hypoxia. Thin arrows indicate gas flow. Thick arrows indicate 3-way stopcocks for connection of perfusion tubing.

Obtaining and processing of human skeletal muscle. Human skeletal muscle specimens measuring 2.0 × 1.5 × 1.0 cm were collected in cold (4°C) and supplemented KH buffer that had been bubbled with 95% O2-5% CO2 to the desired PO2 (250–300 Torr) and pH (7.36–7.45) for transportation. In the laboratory, muscle specimens were cut into strips on ice. Briefly, the tissue was placed on filter paper fixed to a rectangular glass plate (5 × 25 cm), and using microsurgical scissors, strips of muscles were cut along the longitudinal axis of the muscle fibers, maintaining the length of the original specimen. They were 1 mm in width and depth dimensions. The cutting apparatus and the tissue were kept moist with cold (4°C) normoxic buffer throughout the preparation stage that was no longer than 5 min.

One muscle specimen was obtained from each consented patient and was used for control and treatment groups in one experiment only. Therefore, the number of observations (n) indicates number of patients whose muscle specimen was used for the study. Multiple strips (10–30 mg strip) were prepared as outlined above from each muscle specimen obtained from each patient. Two or three muscle strips in each flask were used for each time point in the protocol.

Experimental protocols. After cutting of muscle specimens, muscle strips were blotted with filter paper and loaded into 25-ml Erlenmeyer flasks, which were already placed in the water bath (37°C) of the shaking incubator (80 cycles/min). Each flask contained 10 ml of normoxic buffer that was continuously bubbled with 95% O2-5% CO2 during the preparation stage. The muscle strips were then equilibrated in this normoxic buffer for 30 min at 37°C. At the end of equilibration (0 h), muscle strips were used in one of the three experimental protocols.

Study 1: To develop a human skeletal muscle model and culture technique and to characterize the stability of its viability and morphology over 24 h of perfusion. After the 30-min equilibration period, muscle strips were rinsed with fresh normoxic buffer (37°C) and randomly placed in clean flasks, each containing 10 ml of fresh normoxic buffer. Muscle strips underwent various periods of normoxic incubation at 37°C (Fig. 2). Muscle strips were collected after 0, 1, 4, 8, and 24 h of normoxic incubation, and viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) reduction assay. Muscle strips were also used for...
Fig. 3. Experimental protocol for study 2. Human skeletal muscle strips were equilibrated in normoxic conditions at 37°C for 30 min and were then incubated in normoxic conditions for the time intervals shown above at 37°C for up to 24 h.

assessment of their ATP content and for morphological examination by electron microscopy. Samples of the incubation buffer were also collected at these time points for assessment of cell injury by lactate dehydrogenase (LDH) leakage measurements.

**Study 2: To determine the tolerance of the human skeletal muscle to hypoxia/reoxygenation-induced injury in vitro.** In study 2, the tolerance of the muscle strips to different durations of hypoxia (simulated ischemia) was examined to determine the duration of the hypoxic insult that would result in a 50% decrease in muscle viability (Fig. 3). At the end of equilibration, muscle strips were rinsed once with hypoxic buffer, blotted, and then randomly assigned to 1, 2, 3, 4, or 6 h of hypoxia in flasks containing fresh hypoxic buffer at 37°C. At the end of each hypoxic insult, muscle strips were rinsed in normoxic buffer, blotted, and then subjected to 2 h of normoxic incubation in flasks with fresh normoxic buffer at 37°C. This period of reoxygenation was to simulate reperfusion. Muscle strips were collected at the end of reoxygenation for each of the hypoxic intervals for assessment of cell viability, using the MTT reduction assay. In assessment of cell injury, samples of the incubation buffer were collected at the end of hypoxia and reoxygenation for LDH assay.

**Study 3: To determine whether hypoxic preconditioning protects human skeletal muscle from hypoxia/reoxygenation-induced injury in vitro.** The effect of hypoxic preconditioning (HPC) on the protection of human skeletal muscle against hypoxia/reoxygenation-induced injury was tested in study 3 (Fig. 4). After equilibration, HPC was induced by subjecting muscle strips to 5 min of hypoxia/5 min of reoxygenation. Muscle strips then underwent 3 h hypoxia and 2 h reoxygenation (simulated reperfusion). The 3-h hypoxic insult was chosen because this duration of hypoxia reduced muscle viability by 50% in study 2. At the end of 2 h reoxygenation, muscle strips were collected for assessment of cell viability (MTT assays) and morphological examination (electron microscopic study). Samples of the incubation buffer were also collected for assessment of cell injury (LDH assay). Normoxic control muscle strips were incubated in normoxic buffer, for the entire duration of the study (Fig. 4) whereas the hypoxic control group was subjected to 3 h hypoxia and 2 h of reoxygenation. All incubations were carried out at 37°C.

**MTT assay for assessment of tissue viability.** Muscle strip viability was assessed using the MTT (Sigma M5655–16) assay reported previously for human myocardial tissue (41). In this assay, the yellow MTT powder was reduced to a blue formazan product by mitochondria of viable tissue. Briefly, at the end of the different incubation periods, muscle strips were blotted dry, weighed, and placed in 15-ml Falcon conical tubes containing 3 mM MTT in 2 ml of phosphate-buffered saline (0.05 M). They were then incubated for 60 min at 37°C, blotted dry and placed in the same type of 15-ml Falcon tubes containing 2 ml of DMSO (D136-1, Fischer) and stored at −80°C. At the end of each study, when all samples were collected, they were thawed and finely minced in the DMSO solution using microsurgery scissors, which aided in the dissolution of the formazan product. They were then incubated for 30 min at 37°C to allow for complete dissolution of the blue formazan product, and samples of the supernatant (0.2 ml) were dispensed into 96-well flat-bottom microtiter plate (model 821581, Sarstedt), and the absorbance at 550 nm was measured on a plate reader (model 0013199, VersaMax Molecular Devices).

Results were expressed as absorbance at 550 nm per milligram wet weight and converted to percent viability levels. Specifically, viability of the fresh surgical specimens was considered to be 100%, whereas the remaining samples from 0 h onward were expressed as a percentage of the viability of fresh surgical specimens.

**Assessment of muscle ATP content.** Muscle strips were frozen in liquid nitrogen at the end of the experiment. The frozen muscle strips were stored at −80°C. ATP content was determined using a quantitative bioluminescent assay technique in which the light emitted on oxidation of luciferin is proportional to the ATP that is present (FLAA ATP Bioluminescent Assay Kit, Sigma) (18). Results were expressed as micromoles per milligram of protein and subsequently converted to a percentage of ATP content compared with that of the fresh surgical specimen. Protein content was determined by the Bradford method (Bio-Rad, Hercules, CA).

**LDH assay for assessment of cellular injury.** Degree of cellular injury was assessed based on the activity of LDH in the incubation medium, which was assessed spectrophotometrically by monitoring...
NADH oxidation at absorbance at 550 nm (Cytotoxicity Detection Kit 1-644793, Roche) and expressed as units per gram of wet weight of sample (41).

Morphological examination (electron microscopy). Muscle strips were collected after completion of each experiment and were fixed in 4% paraformaldehyde. Tissue processing was carried out at the Electron Microscopy Laboratory of The Hospital for Sick Children, Toronto, Canada.

Statistical analysis. Data are expressed as means ± SE. One-way ANOVA followed by the post hoc Tukey test were used for multiple comparisons of means. Statistical significance was set at \( P \leq 0.05 \). Microsoft Excel and GraphPad Prism software were used.

RESULTS

Study 1: Stability of viability and morphology of the human skeletal muscle over 24 h of normoxic incubation. MTT reduction by the skeletal muscle strips was used as an index of mitochondrial function and tissue viability. At the end of 30 min of equilibration (i.e., 0 h), viability of muscle strips decreased to 87.0 ± 4.8% \((n = 11)\) compared with the viability of the fresh surgical specimens (99.8 ± 4.0). This decrease was not statistically significant (Fig. 5). Viability of muscle strips at the end of 1 h (86.9 ± 6.7%), 4 h (83.9 ± 4.8%), 8 h (84.0 ± 8.7%), and 24 h (79.1 ± 10.1%) of normoxic incubation were not significantly different from muscle strip viability at the end of equilibration \((n = 11)\) or viability of fresh surgical specimens. These results indicate the stability of viability of the human skeletal muscle over a 24-h period of normoxic incubation (Fig. 5).

The stability and physiological condition of the muscle preparation were further studied by assessing the ATP contents of the human skeletal muscle strips. The mean ATP level of the fresh surgical specimens was 7.9 ± 1.1 \(\mu\)mol/mg protein. This value was expressed as 100%. The ATP contents in human skeletal muscle strips at the different normoxic incubation time points were expressed as a percentage of the ATP content of the fresh surgical specimens \((n = 9)\). The ATP contents did not change significantly by the end of the equilibration period \((113.2 ± 13.9\%)\) compared with the fresh surgical specimens (Fig. 6). Furthermore, there was no significant decrease in ATP content at 1 h \((92.2 ± 12.4\%)\), 4 h \((83.2 ± 12.0\%)\), 8 h \((80.0 ± 6.4\%)\), and 24 h \((81.2 ± 15.3\%)\) of normoxic incubation (Fig. 6).

Net release of LDH into the incubation medium by muscle strips was calculated as the difference in LDH levels between two adjacent time points divided by the time of incubation in hours \((U \cdot g^{-1} \cdot h^{-1})\). There was a high level of net LDH release during the equilibration period \((83.1 ± 21.2 U \cdot g^{-1} \cdot h^{-1})\). There was a significant decrease \((P < 0.05)\) in LDH release 1 h after equilibration, and this release of LDH remained low and stable at 1, 4, 8, and 24 h of normoxic perfusion, indicating the absence of a significant degree of cellular injury over 24 h of normoxic incubation (Fig. 7).

Study 2: To determine of the tolerance of human skeletal muscle to hypoxia/reoxygenation-induced injury in vitro. The viability of human skeletal muscle strips \((n = 7)\) at the end of 8 h of normoxic (control) incubation \((85.0 ± 3.9\%)\) was not significantly different from that of muscle strips undergoing 1 h of hypoxia and 2 h of reoxygenation \((75.3 ± 7.2\%)\) or 2 h of hypoxia and 2 h of reoxygenation \((70.5 ± 5.3\%)\) (Fig. 8). A significant \((P < 0.05)\) decrease in viability of muscle strips was...
ANOVA followed by Tukey test; a letter are significantly different (1-way ANOVA followed by Tukey test; a letter).

Release in human skeletal muscle strips subjected to 4 or 6 h of hypoxia and 2 h of reoxygenation. There was a significant decrease in viability compared with the normoxic control for samples subjected to 3, 4, or 6 h of hypoxia. Values are means ± SE; n = 7. Means without a common letter are significantly different (1-way ANOVA followed by Tukey test; a > b, P < 0.05).

LDH release in the normoxic control muscle strips was 28.9 ± 2.9 U/g (n = 6) (Fig. 9). Increasing duration of hypoxia resulted in a time-related increase in LDH release, which became statistically significant (P < 0.05) after 4 h of hypoxia and 2 h of reoxygenation (154.0 ± 18.7 U/g). After 6 h of hypoxia and 2 h of reoxygenation, LDH release was 207.4 ± 75.5 U/g (Fig. 9).

Study 3: Determination of HPC in protection of human skeletal muscle against hypoxia/reoxygenation-induced injury. Muscle viability was assessed by MTT reduction assay, and viability was calculated as a percentage of the viability in fresh surgical specimens. Viability of the human skeletal muscle strips (n = 7) undergoing 5 h of normoxic incubation was 81.3 ± 10.3% (Fig. 10). In muscle strips subjected to 3 h of hypoxia and 2 h of reoxygenation, the viability decreased to 45.2 ± 5.8% (P < 0.05). HPC prevented decrease in viability induced by 3 h of hypoxia and 2 h of reperfusion, and the viability (82.3 ± 10.8%) was similar to that of the normoxic control (Fig. 10).

Cellular injury was again assessed by LDH release by muscle strips. LDH release at the end of 5 h of normoxic perfusion was (25.7 ± 2.5 U/g). LDH release was increased (P < 0.05) at the end of 3 h of hypoxia/2 h of reoxygenation (156.4 ± 37.4 U/g). HPC prevented increase in LDH release (47.1 ± 10.6 U/g) induced by 3 h of hypoxia/2 h of reoxygenation (Fig. 11).

Morphological assessment. Representative high-resolution transmission electron micrographs of human skeletal muscle (Fig. 12) were prepared from fresh surgical specimens (A) and muscle strips collected after 30 min of equilibration (B), 3 h of hypoxia and 2 h of reoxygenation (C), or with HPC followed by 3 h of hypoxia and 2 h of reoxygenation (D). Normal morphology can be seen in the fresh surgical specimen (Fig. 12A) and muscle strips collected after equilibration (Fig. 12B). Specifically, there were intact nuclei, compact myofibrils with...
uniform staining of nucleoplasm, and well-defined rows of mitochondria between myofibrils. After 3 h of hypoxia and 2 h of reoxygenation, the muscle strips were characterized by reduced staining of cytoplasmic organelles, disintegrated nuclei, wavy myofibrils, and overall disorganized architecture reflecting the hypoxia/reoxygenation-induced injury (Fig. 12C). Normal cell morphology in muscle strips was maintained by the end of 30 min of equilibration (B) and in muscle strips subjected to HPC followed by 3 h of hypoxia/2 h of reoxygenation (D). There was deterioration of myofibril, mitochondrial, and nuclear structure in the muscle strips subjected to 3 h of hypoxia/2 h of reoxygenation (C). Scale bar = 500 nm (applies to each panel); n, nucleus; mf, myofibril; m, mitochondria.

**DISCUSSION**

Skeletal muscle infarction in I/R injury remains a common complication in autogenous muscle transplantation in reconstructive surgery. Although IPC is a proven effective method in protection of skeletal muscle against infarction caused by I/R injury in the rat (3, 10, 16, 37, 40), pig (24), and dog (9), this phenomenon has not been demonstrated in human skeletal muscle. For the first time, we have established a novel in vitro model of human skeletal muscle culture that maintains the morphological and physiological parameters of human skeletal muscle over 24 h of normoxic incubation. Furthermore, we have utilized this model to mimic the effect of I/R injury by subjecting human skeletal muscle strips to various time intervals of simulated ischemia (i.e., hypoxia) followed by 2 h of simulated reperfusion (i.e., reoxygenation). Last but not least, we have demonstrated for the first time the efficacy of hypoxic preconditioning (with 5 min hypoxia and 5 min reoxygenation) for protection of human skeletal muscle strips from irreversible injury, when subsequently subjected to 3 h of hypoxia and 2 h of reoxygenation.

Stability of human skeletal muscle strips over 24 h of normoxic incubation. The results from study 1 indicate that the muscle strip preparation was stable. Specifically, the muscle viability (Fig. 5) and ATP contents (Fig. 6) over 24 h of normoxic incubation remained unchanged and were similar to those in fresh muscle specimens. LDH release rates remained low and stable (Fig. 7) over 24 h of normoxic incubation. During equilibration, a minor decrease in viability was appropriately mirrored by a decrease in MTT reduction and a rise in LDH release (Figs. 5 and 7). However, although the decrease in viability during equilibration was not statistically significant, the LDH levels were. This was likely because cells that sustained mechanical injury during preparation would release LDH but could still be viable. Cell injury was unlikely to be secondary to hypoxic injury because processing was undertaken in normoxic incubation at 4°C and was no more than 5–7 min in duration.

As noted by Zhang et al. (41) in the development of an in vitro model of human myocardial (atrial) tissue culture, the viability of such tissue preparations was affected by a number of factors. These included composition and temperature of the incubation buffer used for collection, preparation, and incuba-
tion of the muscle preparation; the thickness of tissue sections; and the PO₂ of the buffer, which depended on the incubation conditions (e.g., normoxic or hypoxic). The contribution of each of these factors was examined in our preliminary studies (data not shown) of the stability of the human skeletal muscle preparation. Similar to previous findings with animal and human myocardial tissues (25, 27, 41), short preparation time (<5 min) under hypothermic (4°C) conditions and a PO₂ of 250–300 Torr were important factors in ensuring viability of the human skeletal muscle preparation.

It is also important to point out that the human skeletal muscle fibers are, unlike cardiac muscle, long and cylindrical (33). Therefore, the muscle specimen was cut with a pair of microsurgery scissors along the entire longitudinal length of the specimen (~1.5–2.0 cm) to preserve the maximum number of intact muscle fibers. Each muscle strip was ~1 mm in diameter. Finally, in the absence of circulatory perfusion, maintaining a high PO₂ (250–300 Torr) to ensure adequate O₂ diffusion to the muscle strips of the appropriate thickness was also integral to the stability of the muscle preparation.

Tolerance of human skeletal muscle to hypoxia/reoxygenation-induced injury in vitro. Warm ischemic tolerance of human skeletal muscle is limited to ~2.5 h, and it is generally recognized that skeletal muscle subjected to >4 h ischemia will suffer permanent and irreversible damage or necrosis (2, 4). Injury of muscle strips in our in vitro model was induced by hypoxia in a time-related manner. Specifically, LDH release levels (Fig. 9) indicated significant damage after 4 h of hypoxia/h of reoxygenation, and most of the muscle preparation underwent necrosis by 6 h of hypoxia/2 h of reoxygenation. Furthermore, 3 h of hypoxia/h of reoxygenation resulted in a 50% decrease in viability (P < 0.05) as measured by MTT reduction (Fig. 8), and this duration of hypoxic insult was chosen for the study of efficacy of HPC in protection of human skeletal muscle strips against hypoxia/reoxygenation-induced injury. It was hypothesized that skeletal muscle exposed to this duration of hypoxic/reoxygenation incubation could be protected from injury if HPC was effective.

Efficacy of HPC. Results from study 3 demonstrate for the first time the existence of the simulated infarct protective effect of IPC in human skeletal muscle. The HPC protocol (5 min of hypoxia/5 min of reoxygenation) prevented a decrease in MTT reduction (viability; Fig. 10) and increase in LDH release (cell injury; Fig. 11) in human muscle strips when subsequently subjected to 3 h of hypoxia and 2 h of reoxygenation-induced injury. In a preliminary study, there was no difference in the protective effect of HPC with one or two cycles of 5 min of hypoxia/5 min of reoxygenation (unpublished data). It is not known whether different durations of cycles of hypoxia/reoxygenation may induce different efficacies of HPC, and this model can be utilized to investigate this in the future.

It is recognized that the main limitation of the present study is the simulation of ischemia and reperfusion by hypoxic and normoxic incubation, respectively, in the absence of arterial perfusion. Results obtained from this in vitro model are less physiologically relevant than in vivo study of IPC. However, the advantage of the present study is that it demonstrates the efficacy of simulated IPC against simulated I/R injury in human skeletal muscle without vascular effects, neuroendocrine circulating elements, and blood cells (41).

Another limitation of the present study is the types of skeletal muscle from which specimens were obtained. The muscles used for reconstructive surgery consist of types I and II fibers. However, it has been demonstrated that the type of muscle fiber does not determine resistance to ischemic injury in laboratory animals (31).

In summary, this is the first study to demonstrate the efficacy of simulated IPC in protection of human skeletal muscle against simulated I/R injury using a novel in vitro HPC model. Human skeletal muscle strips can be easily prepared and are stable with respect to viability and morphological parameters up to 24 h of normoxic incubation. In the future, this model can be used to develop an HPC protocol that provides an optimal protective effect, and it can be used to study the mechanism of HPC against hypoxia/reoxygenation-induced injury in human skeletal muscle when HPC is given before or after hypoxia and when reoxygenation is extended to 48 h. Specifically, this model can be used to investigate the role of K₅₇₉ channels and mitochondrial permeability transition pores in HPC given before or after hypoxia.

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