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Efficacy and mechanism of hypoxic postconditioning in salvage of ex vivo human rectus abdominis muscle from hypoxia/reoxygenation injury

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ABSTRACT

In reconstructive surgery, skeletal muscle may endure protracted ischemia before reperfusion, which can lead to significant ischemia/reperfusion injury. Ischemic postconditioning induced by brief cycles of reperfusion/reocclusion at the end of ischemia has been shown to salvage skeletal muscle from ischemia/reperfusion injury in several animal models. However, ischemic postconditioning has not been confirmed in human skeletal muscle. Using an established in vitro human skeletal muscle hypoxic conditioning model, we tested our hypothesis that hypoxic postconditioning salvages ex vivo human skeletal muscle from hypoxia/reoxygenation injury and the mechanism involves inhibition of opening of the mitochondrial permeability transition pore (mPTP) and preservation of ATP synthesis. Muscle strips (~0.5×0.5×15 mm) from human rectus abdominis muscle biopsies were cultured in Krebs–Henseleit–HEPES buffer, bubbled with 95%N_2/5%CO_2 (hypoxia) or 95%O_2/5%CO_2 (reoxygenation). Samples were subjected to 3 h hypoxia/2 h reoxygenation. Hypoxic postconditioning was induced by one or two cycles of 5 min reoxygenation/5 min hypoxia after 3 h hypoxia. Muscle injury, viability and ATP synthesis after 2 h of reoxygenation were assessed by measuring lactate dehydrogenase (LDH) release, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) reduction and ATP content, respectively. Hypoxic postconditioning or treatment with the mPTP-opening inhibitors Cyclosporine A (CsA, 5×10^{-6} M) or N-Methyl-4-isoleucine Cyclosporine (NIM811, 5×10^{-6} M) 10 min before reoxygenation decreased LDH release, increased MTT reduction and increased muscle ATP content ($n=7$ patients; $P<0.05$). Conversely, treatment with the mPTP opener Atractyloside (5×10^{-6} M) 10 min before hypoxic postconditioning abolished its protective effect ($n=7$ patients; $P<0.05$). We conclude that hypoxic postconditioning effectively salvages human skeletal muscle from hypoxia/reoxygenation injury by inhibition of mPTP opening and preservation of ATP synthesis during reoxygenation.

1. Introduction

In reconstructive surgery, skeletal muscle is transferred or replanted in order to restore and enhance form and function, or to reconstruct defects and cover wounds. Although human skeletal muscle can withstand ~2.5 h of warm (room temperature) global ischemia (Blaisdell, 2002; Eckert and Schnackerz, 1991; Larsson and Hultman, 1979; Tountas and Bergman, 1977), unpredictable intra- and postoperative complications such as thrombosis or vasospasm can occur, leading to a prolonged ischemic insult. When blood flow is reestablished, ischemia/reperfusion injury can occur. Intervention strategies focusing on prevention of thrombosis and free radical injury have not been proven to be of clinical benefit in prevention of ischemia/reperfusion injury in skeletal muscle (Rubin et al., 1996). However, ischemic preconditioning, first reported by Murry et al. (1986) using short cycles of coronary artery occlusion/reperfusion prior to sustained ischemia in canine hearts, provided robust myocardial protection against ischemia/reperfusion injury. Subsequently, ischemic preconditioning was also reported to be effective in small (Guo et al., 1998; Liu et al., 1991; Yellon et al., 1992) and other large (Mizumura et al., 1995; Schulz et al., 1994) myocardial animal models, cultured human cardiomyocytes (Ikonomidis et al., 1994) as well as human right atrial muscle strips (Ghosh et al., 2000). Furthermore, we demonstrated the infarct protective effect of ischemic preconditioning in pig latissimus dorsi muscle flaps in vivo (Mounsey et al., 1992; Pang et al., 1995) and also in human skeletal muscle in vitro (Martou et al., 2006). Subsequently, other investigators reported the efficacy of ischemic preconditioning against ischemia/reperfusion injury in skeletal muscle of the rat (Carroll et al., 1997) and dog (Jerome et al., 1995).

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However, in trauma surgery such as replantation of amputated hand, foot, arm or limb, sustained ischemia occurs before the patient arrives at the hospital for surgical intervention. Zhao et al. (2003) demonstrated for the first time that ischemic conditioning with brief cycles of occlusion/reperfusion at the end of a prolonged ischemic insult (i.e. postconditioning) could reduce canine myocardial infarction to a similar extent as ischemic preconditioning. Most of the subsequent studies on the efficacy and mechanism of ischemic postconditioning in mice (Heusch et al., 2006), rats (Kin et al., 2004; Tsang et al., 2004), rabbits (Darling et al., 2005; Yang et al., 2004, 2005), dogs (Fujita et al., 2007), pigs (Iliodromitis et al., 2006; Skyschally et al., 2009), monkeys (Yang et al., 2010) and humans (Staat et al., 2005) were also performed with cardiac muscle. We demonstrated for the first time the protective effect of ischemic postconditioning in skeletal muscle using an in vivo pig latissimus dorsi muscle flap model (McAllister et al., 2008). Currently, the molecular mechanism of the protective effect of ischemic preconditioning and postconditioning is still unclear but the provisional consensus is that lethal reperfusion injury is caused by the opening of the mitochondrial permeability transition pore (mPTP) which disrupts ATP synthesis (Granfeldt et al., 2009; Hausenloy et al., 2009; Mewton et al., 2010; Ovize et al., 2010; Zhao, 2010).

However, the efficacy and mechanism of ischemic postconditioning in preventing ischemia/reperfusion injury has not been investigated in human skeletal muscle. Here, we used the in vitro human skeletal muscle model previously developed by us to demonstrate hypoxic preconditioning (Martou et al., 2006), to investigate, for the first time, the efficacy and mechanism of hypoxic postconditioning in human skeletal muscle. We tested our hypothesis that hypoxic postconditioning protects ex vivo hypoxic human skeletal muscle from hypoxia/reoxygenation injury and that the mechanism involves inhibition of mPTP opening and preservation of ATP synthesis. Understanding the efficacy and mechanism of hypoxic postconditioning in human skeletal muscle will lead us to identify pharmacological agents to mimic the protective effect of ischemic postconditioning in salvage of human ischemic skeletal muscle from ischemia/reperfusion injury in autogenous muscle transplantation and replantation in reconstructive surgery.

2. Materials and methods

2.1. Ethical approval

The University Health Network Research Ethics Board and the Mount Sinai Hospital Research Ethics Board in Toronto, Ontario, Canada, approved this protocol. In this project, rectus abdominis muscle biopsies were obtained from consenting patients undergoing reconstructive surgery of the breast. Only the muscle that was routinely excised and discarded during the dissection was used for this project.

2.2. Patient characteristics and exclusion criteria

All patients were female and between 46 and 68 years of age (55.4 ± 7.8 years; mean $\pm$ S.D.). Patients with diabetes mellitus were excluded, because it is known that the cardioprotection against ischemia/reperfusion injury in postconditioning is lost in mice with type I or type II diabetes (Przyklenk et al., 2011). Muscle biopsies were inspected macroscopically and were excluded from the study if they were excessively bruised or traumatized, indicative of gross tissue injury.

2.3. Ex vivo human skeletal muscle model for the study of hypoxia/reoxygenation injury

We previously developed an in vitro model to study the efficacy and mechanism of hypoxic preconditioning (HPreC) of human skeletal muscle against hypoxia/reoxygenation injury (Martou et al., 2006). This was derived from a previously established model for human atrial muscle (Ghosh et al., 2000; Zhang et al., 2000). Specifically, aerobic and ischemic conditions were simulated by normoxic and hypoxic conditions, achieved by differentially supplementing incubation medium and bubbling it with oxygen or nitrogen gas, respectively. Erlenmeyer flasks (25 ml) containing 10 ml of medium were incubated at 37 °C in a water incubator shaking at 100 cycles/min (Dubnoff, Winchester, Virginia). Individual perfusion lines with a calibrated flow rate of humidified gas were used to achieve the desired oxygen and carbon dioxide tensions and pH in the culture medium.

Incubation media were prepared daily from a two-time concentrated stock Krebs–Henseleit–HEPES (KHH) buffer (118 mM NaCl, 4.8 mM KCl, 27.2 mM $NaHCO_3$, 1 mM KH_2PO_4, 1.2 mM $MgCl_2$, 1.25 mM $CaCl_2$, 20 mM HEPES), stored at 4 °C for a maximum of one week. Normoxic buffer consisted of KHH buffer supplemented with 10 mM glucose, 10% fetal bovine serum (FBS) and 0.25 U/ml insulin, and bubbled intermittently with a humidified gas mixture of 95% O_2 and 5% CO_2 to maintain a pO_2 of 250–300 mm Hg and a pH between 7.35 and 7.45. Hypoxic buffer consisted of unsupplemented KHH buffer and was bubbled continuously with a humidified gas mixture of 95% N_2 and 5% CO_2 to achieve a pO_2 of 10–20 mm Hg, and a pH of 6.8–7.0. The pO_2, pCO_2 and pH in the incubation medium were monitored by intermittent analyses using an automated blood gas analyser (model ABL 500, Radiometer, Copenhagen, Denmark).

2.4. Preparation of human skeletal muscle strips for experiments

Human rectus abdominis muscle specimens measuring ~$2.0 \times 1.0 \times 1.0$ cm (~1.2 ± 0.2 g) were taken from patients undergoing reconstructive surgery of the breast and placed in cold (4 °C), normoxic buffer for transportation from the operating room to the laboratory, where they were immediately processed. Using microsurgical scissors and under loupe magnification, strips measuring ~$0.5 \times 0.5 \times 15$ mm (about 10–20 mg each) were dissected from the specimen in 4 °C normoxic buffer, yielding approximately 50 ± 5 strips. All were equally divided into 25 ml Erlenmeyer flasks (4–5 muscle strips/flask) containing pre-bubbled normoxic buffer and equilibrated for 30 min at 37 °C. The time from biopsy harvest to equilibration, including transportation and processing, was approximately 45 minutes.

2.5. Chemical analysis

2.5.1. Lactate dehydrogenase (LDH) assay

The extent of cellular injury was assessed based on the concentration of LDH in the culture medium. LDH is a stable, cytoplasmic enzyme, rapidly released upon damage of the plasma membrane. The activity of LDH (units/g wet weight) was assayed spectrophotometrically by monitoring NADH oxidation at 500 nm absorbance (Cytotoxicity LDH Detection Kit, Roche Applied Science, Mississauga, Ontario, Canada). This technique has previously been used for assessment of hypoxia/reoxygenation injury in rat cardiomyocytes (Sun et al., 2005), human atrial muscle in vitro (Ghosh et al., 2000; Zhang et al., 2000) and human skeletal muscle strips in vitro (Martou et al., 2006).

2.5.2. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) reduction assay

The MTT assay has been previously used to quantify tissue viability in human atrial muscle (Zhang et al., 2000) and in human skeletal muscle strips in vitro (Martou et al., 2006). It is a colorimetric assay for measuring the activity of living cells that reduce MTT, a yellow tetrazole, to insoluble, purple formazan salt. At the end of each experiment, 1–2 muscle strips from each group were incubated in 15 ml Falcon tubes containing 2 ml of 3 mM MTT in phosphate buffered saline for 30 min in a shaking water bath at 100 cycles/minute at 37 °C to allow entry of MTT into the cells. Strips were then blotted dry, weighed and stored in 2 ml of DMSO at −80 °C. Later, the muscle strips in DMSO were homogenized and incubated for 30 min at 37 °C in a shaking water