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Remote ischemic preconditioning preserves mitochondrial function and activates pro-survival protein kinase Akt in the left ventricle during cardiac surgery: A randomized trial☆

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ABSTRACT

Background: Understanding the intracellular mechanisms induced by remote ischemic preconditioning (RIPC) in the human left ventricle opens new possibilities for development of pharmacological cardioprotection against ischemia and reperfusion injury. In this study we investigated the effects of RIPC on mitochondrial function, activation of pro-survival protein kinase Akt and microRNA expression in left ventricular biopsies from patients undergoing coronary artery bypass surgery (CABG).

Methods: Sixty patients were randomized to control (n = 30) or RIPC (n = 30). A blood pressure cuff was applied to the arm of all patients preoperatively. The cuff remained deflated in control group, whereas RIPC was performed by 3 cycles of cuff inflation to 200 mm Hg for 5 min, separated by 5 min deflation intervals. Left ventricular biopsies were obtained before and 15 min after aortic declamping. The primary outcome was mitochondrial respiration measured in situ. Secondary outcomes were activation of protein kinase Akt, assessed by western immunoblotting, and expression of microRNAs assessed by array and real-time polymerase chain reaction.

Results: Mitochondrial respiration was preserved during surgery in patients receiving RIPC (+0.2 μmol O_2/min/g, p = 0.69), and reduced by 15% in controls (−1.5 μmol O_2/min/g, p = 0.02). Furthermore, RIPC activated protein kinase Akt before aortic clamping (difference from control +43.3%, p = 0.04), followed by increased phosphorylation of Akt substrates at reperfusion (+26.8%, p < 0.01). No differences were observed in microRNA expression.

Conclusions: RIPC preserves mitochondrial function and activates pro-survival protein kinase Akt in left ventricle of patients undergoing CABG. Modulation of mitochondrial function and Akt activation should be further explored as cardioprotective drug targets.

Clinical Trial Registration: http://www.clinicaltrials.gov, unique identifier: NCT01308138.

1. Introduction

Remote ischemic preconditioning (RIPC) has emerged as a promising strategy to reduce myocardial reperfusion injury after cardiac surgery [1]. RIPC involves exposing a tissue to brief, non-harmful periods of ischemia to induce protection against subsequent ischemic challenge in another organ. The cardioprotective effects of RIPC have been extensively investigated in animal models, revealing potential molecular targets for pharmacological cardioprotection. Previous studies have shown that regulation of mitochondrial function and activation of pro-survival protein kinase Akt are involved in the cardioprotection induced by RIPC [2,3], and that these cellular mechanisms interact [4].

Conserved mitochondrial function is required for optimal cardiac function, since it directly influences physiological processes that are essential for cardiomyocyte survival and proper contractile activity,

Abbreviations: RIPC, remote ischemic preconditioning; CABG, coronary artery bypass graft; CPB, cardiopulmonary bypass; ACC, aortic cross-clamping; cTnT, cardiac troponin T; CK-MB, creatine kinase-MB; NT-pro-BNP, N-terminal pro-brain natriuretic peptide; ADP, adenosine diphosphate; ACR, acceptor control ratio; TMPD, N,N,N′,N′-tetramethyl-p-phenylenediamine; AUC, area under the curve; ATP, adenosine triphosphate; qRT-PCR, quantitative reverse-transcription polymerase chain reaction.

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including maintenance of energy substrates (ATP), pH control and scavenging of reactive oxygen species [5]. Previous studies have shown that both local ischemic preconditioning and RIPC prevent impairment of mitochondrial respiration induced by ischemia in rat skeletal muscle [6], and that maintaining an optimal mitochondrial function plays an important role in protecting the heart against ischemia [3,7]. Moreover, mitochondrial damage has been unequivocally demonstrated as a trigger of apoptotic cardiomyocyte death [8,9]. Preclinical studies showed that local ischemic preconditioning and RIPC reduce ischemic cardiac damage by blocking apoptosis through activation of pro-survival protein kinase Akt [10]. Accordingly, inhibition of Akt signaling completely blocks the effects of RIPC in a porcine model [11], while targeted activation of Akt renders potent cardioprotection in vivo [12].

Experimental studies also reported a causal involvement of microRNAs (e.g. microRNAs 199a and 320) [13,14] in ischemia–reperfusion injury and mitochondrial physiology [15]. However, the effects of RIPC on left ventricular mitochondrial function and microRNA expression have never been explored in humans.

Despite robust preclinical evidence, intracellular mechanisms induced by RIPC in the human left ventricle are nearly unexplored. Therefore, we investigated the effects of RIPC on left ventricular mitochondrial function, microRNA expression and activation of protein kinase Akt in patients undergoing coronary artery bypass graft (CABG) surgery.

2. Methods

2.1. Study design and participants

This single-center, randomized, prospective, double-blinded study included sixty patients admitted for urgent or elective first-time on-pump CABG surgery at St. Olav's Hospital, Trondheim University Hospital, Norway. The study was conducted in 2011. Exclusion criteria were severe hepatic, renal or pulmonary disease, and peripheral vascular disease of the upper limbs. A database provided by the Unit for Applied Clinical Research at St. Olav's Hospital was used for randomization. Randomization was accomplished immediately before performing the procedure. Patients, surgeons and personnel (both in postoperative intensive care as well as in the laboratory) were blinded to group allocations until after completion of data collection and analyses. This investigation conforms to the principles outlined in the Declaration of Helsinki and was approved by the Regional Committee for Medical Research Ethics of Norway (REK 2010/461-9). Written informed consent was obtained from all patients prior to inclusion. The study is registered at www.clinicaltrials.gov, identification number NCT01308138.

Premedication in the form of acetaminophen and morphine-scopolamine was administered 1–3 h before surgery. Intravenous thiopental, fentanyl, propofol and cisatracurium were used for anesthesia, supplemented by the volatile anesthetic isoflurane, which was administered during pulmonary ventilation before and after cardiopulmonary bypass (CPB). CPB was conducted with a membrane oxygenator at mild hypothermia of 34 °C. Cold crystalloid or blood cardioplegia with standard St. Thomas' solution (Martindale Pharmaceuticals, United Kingdom) was given every ~20 min. Distal coronary anastomoses were constructed under aortic cross-clamping (ACC). All perioperative procedures were performed according to standard routines of the department, including presurgical preparations, anesthetics, drug administration, surgical technique and postoperative care.

2.2. Intervention

All patients included in the study had a blood-pressure cuff applied to the upper arm before induction of anesthesia. For patients randomized to RIPC ($n = 30$), the cuff was inflated to 200 mm Hg for 3 cycles of 5 min ischemia and 5 min reperfusion, after the induction of anesthesia. The cuff remained deflated for an equivalent period in control patients ($n = 30$), Harris J, Barnard M, Grundy E, (Fig. 1).

2.3. Biochemical markers

Blood samples were collected preoperatively (T1), 3 h after removal of ACC (T2), 6 h after removal of ACC (T3), as well as on the first postoperative day (T4). Analyses of circulating biochemical markers (creatinine; CRP, C-reactive protein; cTnT, cardiac troponin T; CK-MB, creatine kinase-MB; and pro-BNP, N-terminal pro-brain natriuretic peptide) were performed at the laboratory of St. Olav's Hospital according to standard procedures.

2.4. Ventricular biopsies

Left ventricular samples were obtained by use of a disposable automatic 16G biopsy needle (BioPince™ Full Core Biopsy Instrument) during CPB at two separate intraoperative time-points; the first before application of ACC (pre-ACC) and the second ~15 min after removal of ACC (post-ACC). One part of the biopsy was added to ice-cold storage solution for mitochondrial analyses, one part was immersed in RNAlater® (Ambion®) and one part was immediately snap-frozen in liquid nitrogen for protein analyses. The sample in RNAlater and the snap-frozen sample were maintained frozen at −80 °C until further processing.

2.5. Mitochondrial respiration in situ

Mitochondrial respiration was measured in situ, as described [16,17]. The tissue was continuously kept at 4 °C during preparatory procedures. The myocardium was kept in storage solution from harvest until membrane permeabilization with 50 μg/mL saponin for 30 min, followed by a rinse cycle of 10 min in pure storage solution and an additional 10 min in respiration solution. The storage solution contained (in mmol/L) 2.77 CaK_2EGTA, 7.23 K_2EGTA, 6.56 $MgCl_2$ (1 mmol/L free Mg^{2+}), 20 taurine, 0.5 dithiothreitol (DTT), 20 imidazole, 50 potassium-methanesulfonate (CH_3KO_3S), 5.7 Na_2ATP, and 15 phosphocreatine (PCr) (pH 7.1 at 22 °C). The respiration solution contained (in mmol/L) 2.77 CaK_2EGTA, 7.23 K_2EGTA, 1.38 $MgCl_2$ (1 mmol/L free Mg^{2+}), 20 taurine, 0.5 dithiothreitol (DTT), 20 imidazole (pH 7.1 at 22 °C), 90 potassium-methanesulfonate (CH_3KO_3S), 10 sodium-methanesulfonate (CH_3SO_3Na), 3 K_2HPO_4, 10 glutamate, 4 malate, and 2 mg/mL bovine serum albumin. Assessment of mitochondrial function was performed in 3 mL of respiration solution at 22 °C with a fluorinated ethylene propylene membrane on a Clark-type microcathode oxygen electrode (Strathkelvin Instruments, UK). Measurement of basal respiration rate (V_0) with glutamate and malate as substrates for respiratory Complex I was followed by addition of subsaturating amount (0.1 mmol/L) of adenosine diphosphate (ADP) (measuring V_{ADP}) and subsequently 20 mmol/L creatine (measuring $V_{creatine}$). Supplement of a saturating amount of ADP (2 mmol/L) allowed assessment of respiration rates involving the entire respiratory chain including Complexes I through IV with glutamate and malate as substrates, recording maximal respiration rate (V_{max}). Complex II substrate succinate (10 mmol/L) was added to assess $V_{succinate}$, after which supplement of Complex I inhibitor amytal (1 mmol/L amobarbital) allowed assessment of Complex II (V_{amytal}). Lastly, ascorbate (0.5 mmol/L) and N,N,N′,N′-tetramethyl-p-phenylenediamine (TMPD, 0.5 mmol/L) were added to induce respiration of Complex IV ($V_{ascorbateTMPD}$). Respiratory rates are given as micromoles O_2 per minute per gram dry weight of left ventricular tissue (μmol O_2/min/g dw). The acceptor control ratio (ACR) was calculated from the ratio of V_{max}/V_0 to quantify the degree of coupling between oxidation and phosphorylation. Mitochondrial sensitivity to ADP was estimated by the ratio of V_{ADP}/V_{max}. Excess respiration of the cytochrome oxidase complex was quantified by the ratio V_{amytal}/V_{max}. Effect of creatine is given as percent increase in respiration rate after the addition of creatine (↑RR Cr). The apparent constant of Michaelis for ADP was estimated in the absence ($^{app}K_m^{(ADP-Cr)}$) and presence of creatine ($^{app}K_m^{(ADP+Cr)}$) [16].

2.6. MicroRNA expression

Random selection of pre-ACC and post-ACC ventricular samples from 10 patients in RIPC and 10 patients in control group was used for microRNA analyses. Samples were transferred on dry ice transportation-medium from St. Olav's Hospital, Trondheim University Hospital, Norway to Exiqon Services, Denmark (<24 h), where microRNA analyses were performed. RNA samples were reverse transcribed into complementary DNA in triplicate. Agilent 2100 bioanalyzer (Agilent Technologies, Inc., Santa Clara, California) was used to evaluate RNA quality. Samples were labeled for microRNA array with miRCURY LNA™ microRNA Hi-Power Labeling Kit, Hy3™/Hy5™ (Exiqon, Denmark), and hybridization was performed on the miRCURY LNA™ microRNA Array (6th gen) (Exiqon, Denmark) including capture probes for all human miRs registered in the miRBASE 16.0. miRCURY LNA™ Universal RT miRNA PCR (Exiqon, Denmark) human pick and mix panel was used for microRNA quantitative reverse-transcription polymerase chain reaction (qRT-PCR). qRT-PCR was performed for microRNA-1, -125b-1*, -129*, -133a, -133b, -185, -191, -199a-3p, -199a-5p, -208b, -21, -210, -23a, -299-5p, -320a, -320b, -338-3p, -423-3p, -494, -525-5p, -630, -92a, and miR-943. Average values from three out of four preselected normalization assays were applied for normalization.

2.7. Western immunoblotting

Biopsies were kept stored at −80 °C and untouched until all samples were collected. All biopsies were prepared for analysis on the same day to guarantee identical handling procedures. Samples were homogenized in ice-cold RIPA buffer (150 mmol/L NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 50 mmol/L Tris, pH 8.0; Sigma-Aldrich, Germany) supplemented with protease and phosphatase inhibitor cocktails (Sigma-Aldrich, Germany). Lysates were centrifuged (12,000 *g*, 4 °C, 15 min) to remove pelleted debris. Total protein concentration in the lysates was measured in triplicate by the BCA method (Thermo Scientific, Norway). Total protein yield did not differ between control and RIPC groups. Sample loading buffer supplemented with reducing agent was added to the lysates and samples were heated at 95 °C for 10 min in a heating block for protein denaturation. Ten micrograms of total proteins was loaded into polyacrylamide Bis-Tris gels (4–20% or 10% acrylamide, Bolt Precast Gels, Life Technologies, Norway) and subjected to electrophoresis at 165 V. Gels were removed from cassettes and proteins were blotted to nitrocellulose membranes by dry transfer (P0 protocol with iBlot system, Life Technologies, Norway). Effective and even transfer was verified by Ponceau staining (Sigma-Aldrich, Norway) of the membrane (5 min at room temperature). Membranes were blocked for 1 h at room temperature (5% BSA in PBS plus 0.05% Tween-20), followed by incubation with primary antibody (1:1000 dilution in blocking buffer, overnight at 4 °C). Primary antibodies against total Akt, total P70-S6k, total GSK3β, total ribosomal S6 protein, phosphorylated Akt (serine 473), phosphorylated targets of Akt, phosphorylated GSK3-beta (serine 9) and phosphorylated ribosomal S6 protein (Serine 240/244) were