Remote Ischemic Preconditioning Preserves Mitochondrial Function and Influences Myocardial MicroRNA Expression in Atrial Myocardium During Coronary Bypass Surgery

Katrine Hordnes Slagsvold, Øivind Rognmo, Morten Høydal, Ulrik Wisløff, Alexander Wahba

**Rationale:** Remote ischemic preconditioning (RIPC) has been suggested to induce cardioprotection during cardiac surgery. Maintaining proper atrial function is imperative in preventing arrhythmia and thrombus formation. Mitochondria have been proposed as key targets in conveying RIPC mechanisms and effects. MicroRNA (miR) is emerging as an important regulator of mitochondrial function, arrhythmia, and protection from ischemia and reperfusion.

**Objective:** This study aimed to evaluate the effect of RIPC on mitochondrial respiration and miR expression in human atrial tissue.

**Methods and Results:** Sixty patients undergoing coronary artery bypass graft surgery were randomized to RIPC (n=30) or control (n=30). RIPC was performed preoperatively by inflating a blood pressure cuff on the upper arm to 200 mmHg for 3x5 minutes, with 5 minutes reperfusion intervals. Biopsies were obtained from the right atrial appendage before and after aortic cross-clamping. Mitochondrial respiration was measured in situ and miR assessed by commercial miR array and quantitative reverse transcription polymerase chain reaction. Postoperative atrial fibrillation occurrence was monitored by biotelemetry. Maximal mitochondrial respiration was preserved throughout surgery after RIPC but significantly reduced (−28%; P<0.05) after aortic cross-clamping in control. Incidence of postoperative atrial fibrillation was lower after RIPC versus control (14% versus 50%; P<0.01). Myocardial expression of miR-133a and miR-133b increased after aortic cross-clamping in both RIPC and control, whereas miR-1 was upregulated in control only. MiR-338-3p expression was higher in RIPC versus control after aortic cross-clamping.

**Conclusions:** RIPC preserves mitochondrial respiration and prevents upregulation of miR-1 in the right atrium during coronary artery bypass graft.

Clinical Trial Registration: URL: http://www.clinicaltrials.gov. Unique identifier: NCT01308138

(Circ Res. 2014;114:851-859.)

Key Words: coronary artery bypass ■ ischemic preconditioning ■ microRNAs ■ mitochondria

Remote ischemic preconditioning (RIPC) has been advocated as a feasible, harmless, and efficient way of protecting the myocardium during cardiac surgery. It has previously been demonstrated that clinical use of RIPC may ameliorate postoperative release of cardiac markers, reduce inotrope requirement, lower airway resistance, and reduce postoperative release of inflammatory markers. A recent study found that RIPC improved the long-term prognosis of patients undergoing elective coronary artery bypass graft (CABG) surgery. It has also been hypothesized that RIPC may affect the incidence of postoperative atrial fibrillation (POAF), and a multicenter study was initiated. However, other studies have been unable to confirm a significant protective effect of RIPC. In contrast, experimental models have convincingly demonstrated that ischemic preconditioning and RIPC can prevent ischemia-induced impairment of mitochondrial respiratory function in muscle tissue, reduce production of reactive oxygen species, and better preserve myocardial ATP levels. MicroRNAs (miRs) have been implicated as important influential factors in mitochondrial function, in cardiac arrhythmia, and in ischemia–reperfusion injury of the heart. The aim of this study was to investigate whether RIPC has a protective effect on mitochondrial function in human atrial myocardium in a clinical setting. As secondary
Methods

We performed a single-center, randomized-, prospective-, double-blinded study. Sixty patients referred for isolated CABG surgery at St. Olav’s University Hospital, Norway were included in the study. Patients with severe hepatic, renal or pulmonary disease, and peripheral vascular disease affecting the upper limbs were excluded. Patients were randomized with an Internet-based randomization database through the Unit for Applied Clinical Research of St. Olav’s Hospital. A blood pressure cuff was placed around the upper arm before anesthesia in all patients. Patients allocated to RIPC underwent intermittent limb ischemia after induction of anesthesia by inflating the blood pressure cuff to 200 mmHg for 5 minutes, repeated in 3 cycles interrupted by 5 minute long reperfusion intervals. For patients allocated to the control group, the blood pressure cuff remained deflated. Patients, surgeon, personnel in postoperative intensive care and laboratory personnel were blinded to which group the patients were randomized. Group allocations were revealed after data collection, and analyses were completed.

Ethical Aspects

The study was performed in compliance with the Declaration of Helsinki and was approved by the Regional Committee for Medical Research Ethics of Norway (REK 2010/461–9). All patients gave written informed consent before inclusion.

Study Design

Timing of sampling and procedures is outlined in Figure 1. All pre-, per-, and postsurgical procedures were performed according to standard routines of the department, including presurgical preparations, anesthetics, drug administration, surgical technique, and postoperative care. Premedication in the form of acetaminophen and morphine-scopolamine was administered 1 to 3 hours before surgery. Anesthetic procedures included use of thiopental, fentanyl, propofol, and cisatracurium intravenously for all patients. Isoflurane anesthetic gas was administered during ventilation before and after cardiopulmonary bypass (CPB) but not during CPB. CPB was performed at mild hypothermia (34°C) using a membrane oxygenator. Standard St. Thomas (Martindale Pharmaceuticals, Essex, United Kingdom) crystalloid or blood cardioplegia was administered for cardiac protection every 20 minutes. Protamine was administered to reverse heparinization after CPB. Distal coronary anastomoses were constructed under aortic cross-clamping (ACC). All patients were monitored with biotelemetric assessment of heart rhythm by electrocardiography intraoperatively and for a minimum of 3 days postoperatively. ECGs were further assessed daily until hospital discharge. One patient in RIPC and 2 in control group had a previous history of atrial fibrillation, and these were excluded from the analyses on POAF.

Blood Samples

Blood samples were taken from an arterial catheter at 4 different time points; preoperative (T1), 3 hours (T2) and 6 hours after removal of ACC (T3), and on the first postoperative day (T4; see Figure 1 for overview). All blood sample analyses were performed according to standard procedures at the laboratory of St. Olav’s Hospital. Concentrations of creatine kinase-myocardial band (CK-MB) and cardiac troponin T (cTnT) were measured by electrochemiluminescence immunoassay (Roche Modular E-170, Roche, Basel, Switzerland) according to manufacturer’s instructions.

Atrial Biopsies

Myocardial tissue samples were excised from the right atrial appendage before atrial cannulation (pre ACC) and after decannulation (post ACC). Biopsies were divided into 3 parts: 1 sample was immersed into a mitochondria-preserving storage solution and kept on ice for mitochondrial analyses, 1 was put on RNAlater (Ambion) for RNA analyses, and 1 sample immediately snap-frozen in liquid nitrogen. Both the sample on RNAlater and the snap-frozen sample were thereafter kept at −80°C until further analyses.

Mitochondrial Respiration In Situ

Mitochondrial respiration was measured in situ after careful removal of connective tissue, fine dissection under microscope, and saponin permeabilization as thoroughly described and reviewed by

---

**Nonstandard Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACC</td>
<td>aortic cross-clamping</td>
</tr>
<tr>
<td>CABG</td>
<td>coronary artery bypass grafting</td>
</tr>
<tr>
<td>CK-MB</td>
<td>creatine kinase-myocardial band</td>
</tr>
<tr>
<td>CPB</td>
<td>cardiopulmonary bypass</td>
</tr>
<tr>
<td>cTnT</td>
<td>cardiac troponin T</td>
</tr>
<tr>
<td>miR</td>
<td>microRNA</td>
</tr>
<tr>
<td>POAF</td>
<td>postoperative atrial fibrillation</td>
</tr>
<tr>
<td>RIPC</td>
<td>remote ischemic preconditioning</td>
</tr>
</tbody>
</table>

---

Figure 1. Timeline of main procedures and samples (illustration of sequence, not indicating accurate time intervals). ACC indicates aortic cross-clamping; CPB, cardiopulmonary bypass; and RIPC, remote ischemic preconditioning.
other authors.\textsuperscript{14–19} Respiration rates were assessed at 22°C in respiration solution using a Clark-type microcathode oxygen electrode (Strathkelvin Instruments, Glasgow, United Kingdom) with a fluorinated ethylene propylene membrane. Respiratory rates are given as micromoles O\textsubscript{2} per minute per gram dry weight of myocardial tissue (μmol O\textsubscript{2}/min per gram dry weight). Basal respiration rate (V\textsubscript{B}) was assessed in the presence of glutamate and malate (substrates for complex I in the electron transport chain) without ADP. A subsaturating amount of 0.1 mmol/L ADP was added (V\textsubscript{ADP}) before the addition of 20 mmol/L creatine (V\textsubscript{ creatine}) followed by addition of saturating amount of ADP (2 mmol/L). Maximal respiration rate (V\textsubscript{max}) was measured while assessing the maximal respiration rate of the respiratory chain from complexes I through IV with glutamate and malate as substrates. Succinate (10 mmol/L) was added to supplement complex II of the electron transport chain (V\textsubscript{succinate}). This was followed by amytal (1 mmol/L amobarbital) that blocks complex I (NADH dehydrogenase) and allows assessment of complex II (V\textsubscript{ amytal}). Subsequent addition of ascorbate (0.5 mmol/L) and N,N,N′,N′-tetramethyl-p-phenylenediamine (0.5 mmol/L) induces respiration in complex IV, measuring V\textsubscript{ ascorbate N,N,N′,N′-tetramethyl-p-phenylenediamine}. The acceptor control ratio was calculated as the ratio of V\textsubscript{max}/V\textsubscript{p}, hereby quantifying to what degree the rate of oxidation is controlled by phosphorylation of ADP into ATP. ADP sensitivity ratio was calculated by V\textsubscript{ADP}/V\textsubscript{max} to estimate the mitochondrial sensitivity to ADP. The percentage of creatine-induced increase in respiration rates was evaluated (IRR Cr). The ratio of V\textsubscript{ADP}/V\textsubscript{max} quantifies excess respiration of the cytochrome oxidase complex. The apparent constant of Michaelis for ADP was calculated as the ratio of V\textsubscript{max}/V\textsubscript{0}, hereby quantifying to what degree the mitochondrial sensitivity to ADP. The percentage of creatine-induced increase in respiration rates was evaluated (IRR Cr). The ratio of V\textsubscript{ADP}/V\textsubscript{max} quantifies excess respiration of the cytochrome oxidase complex. The apparent constant of Michaelis for ADP was calculated as the ratio of V\textsubscript{max}/V\textsubscript{0}, hereby quantifying to what degree the mitochondrial sensitivity to ADP. The ratio of RR Cr). The acceptor control ratio was calculated as the ratio of V\textsubscript{max}/V\textsubscript{p}, hereby quantifying to what degree the rate of oxidation is controlled by phosphorylation of ADP into ATP. ADP sensitivity ratio was calculated by V\textsubscript{ADP}/V\textsubscript{max} to estimate the mitochondrial sensitivity to ADP. The percentage of creatine-induced increase in respiration rates was evaluated (IRR Cr). The ratio of V\textsubscript{ADP}/V\textsubscript{max} quantifies excess respiration of the cytochrome oxidase complex. The apparent constant of Michaelis for ADP was calculated as the ratio of V\textsubscript{max}/V\textsubscript{0}, hereby quantifying to what degree the mitochondrial sensitivity to ADP. The percentage of creatine-induced increase in respiration rates was evaluated (IRR Cr).

miR Expression

Ten tissue samples from each experimental group, both pre ACC and post ACC, were randomly selected for miR analyses. The entire process involving miR analyses, including RNA isolation, miR array, and quantitative reverse transcription polymerase chain reaction, was performed by Exiqon Services, Vedbaek, Denmark. The tissue samples were transported from St. Olav’s Hospital, Norway, to Exiqon Services on dry ice transportation medium within 24 hours. RNA quality was controlled by use of Agilent 2100 bioanalyzer (Agilent Technologies, Inc, Santa Clara, CA). For miR array, samples were labeled using Exiqon miRCURY LNA microRNA Hi-Power Labeling Kit, Hy3/Hy5 and hybridized on the miRCURY LNA microRNA Array (sixth generation; Exiqon, Denmark). Background correction of the quantified signals was performed by application of the global Lowess regression algorithm. For miR reverse transcription polymerase chain reaction, the miRCURY LNA Universal RT miRNA PCR (Exiqon, Denmark) pick and mix panel was used, and the average of 3 designated normalization assays detected in all samples was applied for normalization. Level of miR expression is presented as normalized crossing point, which was calculated by subtracting the crossing point of the investigated miR from the average crossing point of normalization miRs.

Statistical Analysis

We estimated that a sample of 27 patients per group was required for 80% statistical power based on previous reports on RIPC and postoperative release of cardiac markers after CABG surgery.\textsuperscript{1} Statistics was performed by the use of SPSS 19.0 for Mac (IBM SPSS Statistics, Chicago, IL). Unpaired and paired Students t tests were conducted to compare between-group and within-group differences, respectively. A 2-tailed P<0.05 was considered significant. Pearson χ\textsuperscript{2} test and the Fisher exact test were used for categorical data. Graphics were produced by the use of GraphPad Prism 5 (GraphPad Software, Inc, San Diego, CA).

Results

Sixty patients were included in the study; there was no 30-day mortality (see Figure 2 for Consolidated Standards of Reporting Trials diagram of inclusion process). Patient characteristics were similar in the 2 groups regarding preoperative assessment scores, age, sex distribution, body mass index, creatinine clearance, and preoperative evaluation of left ventricular function. Medication was comparable with the exception of calcium channel blocker, which was used more in the control group. Anesthetic regimens and intraoperative parameters were similar in the 2 groups, including time on CPB, duration of ACC, amount of cardioplegic solution used and number of grafts. There was no significant difference in length of stay in postoperative intensive care (RIPC 1.03±0.2 versus control 1.07±0.3 days). See Table 1 for details on demographics and perioperative parameters.

Biochemical Markers

We found no difference in pre- or postoperative release of cTnT, CK-MB, or N-terminal probrain natriuretic peptide regarding maximal values, area under the curve, or at any 1 specific time point (Table 2). Pre- and postoperative creatinine, C-reactive protein, electrolytes, hemoglobin, platelets, or leukocyte values were not different between the groups (data not shown).

Postoperative Atrial Fibrillation

We found a significantly lower incidence of POAF in RIPC (4 of 29 patients, 14%) compared with control (14 of 28 patients, 50%; P<0.01).

---

Figure 2. CONSORT diagram of inclusion process. RIPC indicates remote ischemic preconditioning.
### Table 1. Patient Characteristic and Perioperative Parameters

<table>
<thead>
<tr>
<th></th>
<th>Control (n=30)</th>
<th>RIPC (n=30)</th>
<th>PValue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female sex, n (%)</td>
<td>7 (23)</td>
<td>3 (10)</td>
<td>0.17</td>
</tr>
<tr>
<td>Age, μ±SD</td>
<td>68.1±8.2</td>
<td>64.2±9.0</td>
<td>0.09</td>
</tr>
<tr>
<td>Euroscore II (2011), μ±SD</td>
<td>1.3±0.9</td>
<td>1.35±1.2</td>
<td>0.90</td>
</tr>
<tr>
<td>ASA score, μ±SD</td>
<td>3.6±0.6</td>
<td>3.4±0.5</td>
<td>0.23</td>
</tr>
<tr>
<td>Creatinine clearance, mL/min</td>
<td>90±25</td>
<td>102±41</td>
<td>0.19</td>
</tr>
<tr>
<td>BMI, μ±SD</td>
<td>27.7±4.0</td>
<td>28.9±7.6</td>
<td>0.48</td>
</tr>
<tr>
<td>LV EF &gt;50, n (%)</td>
<td>22 (76)</td>
<td>24 (80)</td>
<td>0.70</td>
</tr>
<tr>
<td>LV EF 31–50, n (%)</td>
<td>7 (24)</td>
<td>5 (17)</td>
<td>0.48</td>
</tr>
<tr>
<td>LV EF 21–30, n (%)</td>
<td>0 (0)</td>
<td>1 (3)</td>
<td>1.00</td>
</tr>
<tr>
<td>LV EF &lt;20, n (%)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>1.00</td>
</tr>
<tr>
<td>Comorbidity, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Previous MI &gt;2 wk</td>
<td>9 (30)</td>
<td>16 (53)</td>
<td>0.07</td>
</tr>
<tr>
<td>Previous MI &lt;2 wk</td>
<td>6 (20)</td>
<td>3 (10)</td>
<td>0.47</td>
</tr>
<tr>
<td>UAP &lt;3 d preoperative</td>
<td>3 (10)</td>
<td>0 (0)</td>
<td>0.24</td>
</tr>
<tr>
<td>Previous PCI</td>
<td>5 (17)</td>
<td>5 (17)</td>
<td>1.00</td>
</tr>
<tr>
<td>Previous cerebral insult</td>
<td>0 (0)</td>
<td>3 (10)</td>
<td>0.24</td>
</tr>
<tr>
<td>Hypertension</td>
<td>15 (50)</td>
<td>12 (40)</td>
<td>0.44</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>6 (20)</td>
<td>7 (23)</td>
<td>0.75</td>
</tr>
<tr>
<td>Current smoker (last 6 mo)</td>
<td>8 (27)</td>
<td>8 (27)</td>
<td>1.00</td>
</tr>
<tr>
<td>Previous smoker (&gt;6 mo ago)</td>
<td>21 (70)</td>
<td>20 (67)</td>
<td>0.78</td>
</tr>
<tr>
<td>COPD</td>
<td>2 (7)</td>
<td>3 (10)</td>
<td>1.00</td>
</tr>
<tr>
<td>Previous atrial fibrillation</td>
<td>2 (7)</td>
<td>1 (3)</td>
<td>1.00</td>
</tr>
</tbody>
</table>

**Drug therapy, n (%)**

- Statin                  | 27 (90)        | 25 (83)     | 0.71   |
- Calcium antagonist       | 9 (30)         | 1 (3)       | 0.01*  |
- β-Blocker               | 24 (80)        | 25 (83)     | 0.74   |
- ACE inhibitor/ATII inhibitor | 11 (37)    | 14 (47)     | 0.43   |
- Diuretics               | 4 (13)         | 7 (23)      | 0.32   |
- Acetylsalicylic acid    | 29 (97)        | 28 (93)     | 1.00   |
- Clodigorel              | 11 (37)        | 13 (43)     | 0.60   |
- Dipyridamole            | 0 (0)          | 2 (7)       | 0.49   |
- Warfarin                | 2 (7)          | 1 (3)       | 1.00   |
- Organic nitrates        | 6 (20)         | 3 (10)      | 0.47   |
- Metformin               | 4 (13)         | 3 (10)      | 1.00   |
- Sulfonilurea            | 1 (3)          | 1 (3)       | 1.00   |
- Glibenclamide           | 1 (3)          | 0 (0)       | 1.00   |
- Insulin                 | 1 (3)          | 2 (7)       | 1.00   |

**Intraoperative parameters, μ±SD**

- Bypass time, min        | 76.3±23        | 71.1±14     | 0.31   |
- Aortic cross-clamping time, min | 41.8±11 | 41.1±11 | 0.80 |
- Cardioplegia, mL         | 1000±284       | 911±257     | 0.21   |
- Distal coronary graft anastomoses | 3.27±0.7 | 3.47±1.0 | 0.31 |
- Isoflurane anesthesia, n (%) | 30 (100)   | 30 (100)    | 1.00   |

ACE inhibitor indicates angiotensin-converting enzyme inhibitor; ASA score, preoperative physical assessment score developed by the American Society of Anesthesiology; ATII inhibitor, angiotensin II receptor blocker; BMI, body mass index (kg/m²); COPD, chronic obstructive pulmonary disease; LV EF, left ventricular ejection fraction; MI, myocardial infarction; PAD, peripheral arterial disease; carotid stenosis, claudicatio intermittens; PCI, percutaneous coronary intervention; RIPC, remote ischemic preconditioning; and UAP, unstable angina pectoris.

*P<0.05.

---

**Mitochondrial Respiration**

Mitochondrial data are presented in Table 3 and Figure 3. We found no significant differences of mitochondrial respiratory rates between RIPC and control groups in samples obtained pre ACC. In samples obtained post ACC, V\textsubscript{O}\textsubscript{2} was significantly higher in RIPC compared with control (RIPC 3.2±1.1 versus control 2.5±1.1; P<0.05).

In paired comparison of mitochondrial respiration pre ACC versus post ACC, we observed that V\textsubscript{O}\textsubscript{2} was significantly reduced by an average of 28% in the control group post ACC (pre ACC 8.0±2.6 versus post ACC 5.7±2.5; P<0.001; Figure 3A), with no significant reduction through surgery within RIPC (Figure 3B). All post ACC respiratory rates in the control group were reduced in comparison with pre ACC, indicating generally impaired mitochondrial function without indications of dysfunction in any specific complex of the electron transport chain (Figure 3A). In RIPC, the majority of post ACC respiration rates were maintained at a level comparable to pre ACC (Figure 3B); however, T\textsubscript{OCR} and V\textsubscript{O}\textsubscript{2} were significantly higher pre ACC versus post ACC, and \( K\textsubscript{m} \text{ (ADP+Cr)} \) was reduced pre ACC.

**MicroRNA**

There were no significant differences between the subgroups randomly selected for miR analyses, including clinical characteristics, preoperative assessment scores, duration of CPB, and ACC or mitochondrial respiratory parameters pre ACC. The only difference observed in medication regimens was a higher percentage of patients with calcium channel blockers in the control group compared with RIPC.

An increase in the myocardial expression of miR-133a and miR-133b was detected in samples obtained post ACC versus pre ACC in both groups. MiR-1, however, was increased post ACC within control samples only (Figure 4A and 4B).

In between-group comparison of RIPC versus control, no significant differences were detected in pre-ACC samples, whereas in post-ACC samples an increased expression of miR-338-3p was found in RIPC versus control (Figure 4C).

**Discussion**

Our main finding was that RIPC is associated with preserved mitochondrial respiration, reduced incidence of POAF, and prevention of miR-1 upregulation during CABG surgery. We also demonstrated that myocardial miR-133a and miR-133b are upregulated during CABG surgery.

Our findings indicate that RIPC provides mitochondrial protection of the right atrial myocardium during CABG, thus supporting the notion that early cardioprotective effects of RIPC are mediated by preservation of mitochondrial function during ischemia–reperfusion. Of particular relevance is the observation that RIPC is associated with preserved maximal mitochondrial respiratory capacity of the right atrium. The heart requires an estimated average of 30 kg ATP per day to maintain normal function, of which 90% is derived from mitochondria. In this perspective, a 28% reduction in maximal mitochondrial respiratory capacity may have significant effect in maintaining adequate ATP supply both during and in the recovery period of perioperative ischemia–reperfusion. Repeated periods of intermittent myocardial ischemia–reperfusion cycles...
have previously been linked to a reduced rate of ATP use, as well as reduced accumulation of lactate in cycles after the first ischemic period. Recently, it was also demonstrated that both the direct and remote approach to ischemic preconditioning provide mitochondrial protection of skeletal muscle in rats undergoing ACC. Disruption of mitochondrial function increases the risk of disturbing homeostasis of electrolytes and energy balance, which in turn increases risk of cardiac arrhythmia.

Although most respiratory parameters remained unaltered after surgery within RIPC, we did observe elevated RR Cr, along with lower \(\frac{\text{V}_{\text{ADP}}}{V_{\text{max}}}\) for ADP compared with post ACC, which indicates greater ADP sensitivity in the presence of creatine early in surgery compared with later in surgery. This may either indicate a brief enhancement of coupling of mitochondrial creatine kinase system in RIPC pre-ACC. Even so, the observation may be of limited significance because of its transient nature. However, ischemic preconditioning has previously been associated with preserving a low \(\frac{\text{appK}_{\text{m}}}{(\text{ADP}+\text{Cr})}\) while maintaining mitochondrial respiratory capacity in rat myocardium.

We observed a significant difference in POAF between the experimental groups. Previous studies have reported a divergent incidence of POAF after cardiac surgery varying as widely as 5% to 70% after CABG surgery. Known risk factors associated with increased incidence of POAF are age and duration of CPB or ACC, and no significant differences were found between the experimental groups on either of these parameters. An association has been observed between impaired mitochondrial function after simulated ischemia–reperfusion injury of human right atrial myocardium and development of POAF. In addition, mitochondria are closely linked to arrhythmogenesis through its involvement in reactive oxygen species production and calcium homeostasis. Although the incidence of POAF is lower than expected in RIPC-treated patients of our study, the incidence in the control group is higher than one would expect. This, along with the limited number of patients included with respect to assessing POAF, indicates that these results must be interpreted with caution. The potential effect of RIPC on POAF requires further investigation.

Insights into the roles of miR in cardiac pathophysiology are currently progressing. We found that miR-133a and miR-133b were upregulated post ACC versus pre ACC in samples of both groups, whereas miR-1 was upregulated post ACC within the control group only. Circulating levels of the muscle specific miR-133a, miR-133b, and miR-1 are all known to increase in the aftermath of a myocardial infarction. MiR-133a and miR-133b have nearly identical sequences and likely serve nearly the same biological functions. Elevated circulating levels of miR-133a have been measured as early as 2 hours after onset of chest pain in patients, indicating that alterations in miR-133a expression are a part of a rapid-onset response. Moreover, serum miR-133a was detected in these patients even when cTnT was undetectable, leading to the proposal of miR-133a as a potential marker in diagnosis of acute coronary syndrome. A correlation between circulating miR-133a levels and the extent of myocardial injury

<table>
<thead>
<tr>
<th>Table 2. Biochemical Markers</th>
<th>Preoperative (T1)</th>
<th>AUC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (n=30)</td>
<td>RIPC (n=30)</td>
</tr>
<tr>
<td>cTnT, ng/L</td>
<td>27±59</td>
<td>37±63</td>
</tr>
<tr>
<td>CK-MB, μg/L</td>
<td>3±1</td>
<td>3±1</td>
</tr>
<tr>
<td>NT-proBNP, ng/L</td>
<td>441±768</td>
<td>764±1643</td>
</tr>
</tbody>
</table>

Cardiac marker release in the control group (n=30) vs RIPC (n=30). Numbers represent mean AUC±SD for cTnT, CK-MB, and NT-proBNP. Blood samples were obtained preoperative (T1), 3 h after ACC (T2), 6 h after ACC (T3), and 1 d postoperative (T4). ACC indicates aortic cross-clamping; AUC, area under the curve; CK-MB, creatine kinase-MB; cTnT, cardiac troponin T; NT-proBNP, N-terminal probrain natriuretic peptide; and RIPC, remote ischemic preconditioning.

<table>
<thead>
<tr>
<th>Table 3. Mitochondrial Respiratory Parameters of the Right Atrium</th>
<th>Control (n=30)</th>
<th>RIPC (n=30)</th>
<th>(P) Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>RR, mean±SD</td>
<td>Pre ACC</td>
<td>Post ACC</td>
<td>(P) Value</td>
</tr>
<tr>
<td>(\frac{V_{\text{ADP}}}{V_{\text{max}}})</td>
<td>0.91±0.3</td>
<td>0.94±0.5</td>
<td>0.82</td>
</tr>
<tr>
<td>(\frac{V_{\text{ADP}}}{V_{\text{max}}})</td>
<td>0.47±0.2</td>
<td>0.48±0.2</td>
<td>0.59</td>
</tr>
<tr>
<td>ACR</td>
<td>4.02±1.5</td>
<td>3.70±1.5</td>
<td>0.33</td>
</tr>
<tr>
<td>(\frac{\text{appK}_{\text{m}}}{(\text{ADP}+\text{Cr})}), μmol/L</td>
<td>133±83</td>
<td>130±72</td>
<td>0.92</td>
</tr>
<tr>
<td>(\frac{\text{appK}_{\text{m}}}{(\text{ADP}+\text{Cr})}), μmol/L</td>
<td>50±35</td>
<td>66±83</td>
<td>0.41</td>
</tr>
<tr>
<td>(\frac{\text{RR} \text{ Cr}, %}{%})</td>
<td>56±47</td>
<td>52±45</td>
<td>0.78</td>
</tr>
</tbody>
</table>

ACC indicates aortic cross-clamping; ACR, acceptor control ratio; RR Cr, increase in respiration rate after addition of creatine; \(\frac{V_{\text{ADP}}}{V_{\text{max}}}\) quantification of excess respiration of the cytochrome oxidase complex; \(\frac{V_{\text{ADP}}}{V_{\text{max}}}\) ADP sensitivity ratio; \(\frac{\text{appK}_{\text{m}}}{(\text{ADP}+\text{Cr})}\), apparent Michaelis–Menten constant for ADP (μmol/L) in the presence of creatine; \(\frac{\text{appK}_{\text{m}}}{(\text{ADP}+\text{Cr})}\), apparent Michaelis–Menten constant for ADP (μmol/L) in the absence of creatine. *P<0.05.
has been demonstrated in patients undergoing ST-segment-elevation myocardial infarction. The exact source of miR-133a within the myocardium, as well as pinpointing precisely what stimuli induces its release has not been established, but experiments in mice have revealed reduced tissue levels within infarcted areas, as well as in the surrounding border zone 24 hours after onset of hypoxia. Because our post ACC samples were obtained only ≈25 minutes after onset of reperfusion, this correlates with the notion that expression of miR-133a is induced as part of an immediate myocardial response in exposure to ischemia–reperfusion stimuli. Because upregulation of miR-133a and miR-133b were observed in both groups, their expression does not seem to be influenced by RIPC.

Increased miR-1 expression has been associated with a greater extent of myocardial injury in ischemia–reperfusion injury, and it has been demonstrated that ischemic preconditioning reduces the level of miR-1 expression along with myocardial infarction size in rats. Consequently, the increase in miR-1 expression in samples from control patients post ACC may indicate greater tissue damage toward the end of CABG in this patient group compared with the preconditioned patients, where no increase in miR-1 expression could be detected. Moreover, overexpression of miR-1 has been implicated in promoting arrhythmogenesis, correlating with a higher occurrence of POAF in the group with increased miR-1 expression in our material. Intriguingly, it has previously been illustrated that stimulation of the β-adrenergic signaling pathway can increase miR-1 expression and that the beneficial effects of β-blockers seem to be because of, at least partly, the downregulation of miR-1. In addition, there is evidence of miR-1 having a proapoptotic role in cardiomyocytes.

Expression of miR-338-3p was higher in myocardial samples from RIPC versus control post ACC, a difference not present in pre ACC samples. MiR-338-3p is implicated in the influence of mitochondrial function in neurons, specifically altering axonal expression of cytochrome c oxidase IV and ATP5G1 expression. Its precise role in myocardial tissue remains to be resolved.
It has been suggested that several anesthetic agents including isoflurane, opioids, and propofol have preconditioning effects that may interfere with RIPC. However, results are inconsistent and reduced cardiac enzyme release after RIPC has been demonstrated in cardiac surgery both with and without isoflurane. Yet other studies found no effect of RIPC with or without isoflurane or with the administration of propofol. In this study, patients in both groups received isoflurane, propofol, and fentanyl peroperatively. Thus a preconditioning effect may have been present in all patients, and it cannot be excluded that this may have attenuated the effect of RIPC. In particular, the administration of isoflurane may have masked the protective effects of RIPC in our study. However, this should not affect the observed between-group differences.

Limitations
Our study constitutes, to our knowledge, the first study investigating the effect of RIPC on mitochondrial respiration in human atrial myocardium in the setting of CABG surgery. These findings need to be verified through additional research, and it must be established whether this is applicable to a general patient population undergoing heart surgery or if specific subgroups of patients may benefit.

In this study, no significant changes in cardiac markers were found. Several clinical studies investigating the effects of RIPC in cardiac surgery have focused on the release of cardiac markers. However, results are inconsistent. The release of cardiac markers indicates myocardial necrosis. Serum concentrations after coronary surgery are highly variable and depend on intraoperative variables, such as quality of anastomosis and possible intraoperative myocardial infarction, myocardial dissection to access intramural arteries, and ACC. Even the mere use of extracorporeal circulation, independent of myocardial ischemia or necrosis, has been shown to increase the release of cardiac markers. Thus, cardiac markers such as CK-MB and cTnT are presumably a poor measure of myocardial ischemia in the context of RIPC. This is supported by a recent publication on myocardial perfusion imaging. In patients undergoing primary revascularization with angioplasty after myocardial infarction, it was demonstrated that ischemic conditioning increases ischemic salvage without detectable differences in cardiac marker release. We suggest that the observed differences in mitochondrial respiration between RIPC-treated patients and controls are a more sensitive marker of ischemia–reperfusion injury than serum levels of CK-MB or cTnT.

cTnT and CK-MB levels were not assessed past the first 24 hours postoperatively in our study. However, in previous studies, the largest difference of cTnT release between RIPC and control has been observed within the first 24 hours after cardiac surgery, and there are also studies measuring cardiac markers 48 to 72 hours postoperatively, where no difference in cardiac marker release is observed.

The relatively short duration of ACC and consequent global myocardial ischemia compared with other publications may have contributed to a lack of difference in cardiac marker release between the groups.

In this study we chose to focus our investigation on atrial myocardium. Although differences in oxidative capacities exist between atria and ventricles because of a larger number of mitochondria in the ventricles, it has been demonstrated that alterations in mitochondrial quality are identical in all 4 cardiac chambers of patients with heart failure. Therefore it seems unlikely that qualitative differences between atrial and ventricular tissue exist, although this cannot entirely be ruled out. Direct measurement of cytochrome c oxidase for quantification of mitochondrial content was precluded because the amount of atrial tissue was limited.

Because slowed ATP depletion rates have been demonstrated in preclinical studies of animals undergoing ischemia, direct assessment of ATP would have been valuable to establish whether this occurs in human myocardium after RIPC. Unfortunately, this was not possible because of limited amount of tissue.

The duration of a mitochondrial fusion/fission cycle of adult mice cardiomyocytes has been estimated to last 16 days. This indicates that although the number of mitochondria was not assessed in our study, the alterations in mitochondrial respiration rates are likely to be because of functional status of the mitochondria because the samples were obtained within a relatively short time interval from the same localization in each patient pre- and post-ACC.

With regards to the incidence of POAF, it must be kept in mind that our study included a limited number of patients from a single treatment center, and larger patient populations must be investigated to clarify a potential association between RIPC and POAF.

By coincidence, the number of patients on calcium channel blockers was different between groups. However, there is no indication that this should influence the incidence of POAF or the effects on RIPC and mitochondrial function. Moreover we found no significant differences in mitochondrial function between patients on calcium antagonists and others.

The overall understanding of functions of miR in human myocardium, in particular in the setting of RIPC, is not yet fully understood. Thus our findings describe an association between mitochondrial respiration, miR, and atrial fibrillation without giving evidence for a causal relationship.

Conclusions
RIPC preserves mitochondrial function and influences myocardial miR expression of the right atrium in patients undergoing CABG surgery. These findings implicate that RIPC induces myocardial protection of the human atrium even when differences in release of cardiac markers cannot be detected. Consequently, it seems that RIPC should not be discarded as a measure of cardioprotection, and continued research into the effects of RIPC on mitochondrial function and miR expression constitutes a novel approach to further improving cardiac protection in clinically encountered ischemia–reperfusion.

Acknowledgments
We are particularly grateful to the Department of Cardiothoracic surgery, St. Olav’s Hospital, Trondheim University Hospital, Norway, for providing biopsies.

Sources of Funding
This study was supported by Liaison Committee between the Central Norway Regional Health Authority and the Norwegian University of Science and Technology (NTNU), Unimed Innovation’s Research
Disclosures

None.

References


---

**Novelty and Significance**

What Is Known?

- Remote ischemic preconditioning (RIPC) involves subjecting a part of the body to brief periods of ischemia and reperfusion to protect another organ during a subsequent ischemic insult.
- In animal models, RIPC has been shown to protect mitochondrial respiratory function after ischemia–reperfusion.
- MicroRNAs (miRs) are small noncoding RNA that influence several biological processes including mitochondrial function, arrhythmia, and ischemia–reperfusion.
- Postoperative atrial fibrillation is a common complication of coronary artery bypass graft surgery.

What New Information Does This Article Contribute?

- RIPC preserves mitochondrial respiration in right atrial myocardium of patients undergoing coronary artery bypass graft surgery.
- The expression of miR-133a and miR-133b increased after aortic cross-clamping in both RIPC and control groups.
- miR-1 was upregulated after aortic cross-clamping in patients in the control group but not in RIPC.
- Incidence of postoperative atrial fibrillation was significantly higher in control than in RIPC.

In animal studies, RIPC is associated with improved mitochondrial protection during ischemia–reperfusion, but its effect on mitochondrial function has not been investigated in the human atrium. In this study, we demonstrate that RIPC is associated with preserved right atrial mitochondrial respiration in patients undergoing coronary artery bypass graft surgery. RIPC also prevented upregulation of miR-1, which has been previously associated with increased myocardial injury and arrhythmia. In contrast, the expression of miR-133a and b was elevated after aortic cross-clamping in both patients groups, underscoring their significance as markers of myocardial damage. Moreover, in comparison with controls, the incidence of postoperative atrial fibrillation was significantly lower after RIPC. These findings suggest a potential link among miR-1, mitochondrial respiration, and postoperative atrial fibrillation. Further insight into the mechanisms underlying RIPC would help in optimizing cardioprotection during surgery.
Remote Ischemic Preconditioning Preserves Mitochondrial Function and Influences Myocardial MicroRNA Expression in Atrial Myocardium During Coronary Bypass Surgery

Katrine Hordnes Slagsvold, Øivind Rognmo, Morten Høydal, Ulrik Wisløff and Alexander Wahba

*Circ Res.* 2014;114:851-859; originally published online December 26, 2013;
doi: 10.1161/CIRCRESAHA.114.302751

*Circulation Research* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2013 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/114/5/851

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2013/12/26/CIRCRESAHA.114.302751.DC1

**Permissions:** Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Circulation Research* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

**Reprints:** Information about reprints can be found online at:
http://www.lww.com/reprints

**Subscriptions:** Information about subscribing to *Circulation Research* is online at:
http://circres.ahajournals.org/subscriptions/
Supplemental Material

Detailed Methods

Mitochondrial respiration in situ

Mitochondrial respiration was measured in situ as thoroughly described and reviewed by other authors [1-5]. The tissue sample for mitochondrial analyses was placed into an ice-cold storage solution immediately after harvesting. All visible connective tissue was removed under a microscope, leaving only myocardial tissue. The myocardium was then finely dissected with forceps transforming the dense tissue to a fine net. It was subsequently submersed in storage solution with 50 μg/ml saponin for 30 minutes at 4 °C under continuous shaking to permeabilize the cell membranes, while leaving the inner mitochondrial membranes intact. Next, the sample was placed back into pure storage solution and shaken for at least 10 minutes to remove any residue of saponin, followed by another rinse in respiration solution for 10 minutes. Subsequently the permeabilized myocardial tissue was placed in 3 ml of respiration solution inside a water-jacketed respiration cell. A Clark-type microcathode oxygen electrode (Strathkelvin Instruments, Glasgow, UK) with a fluorinated ethylene propylene (FEP) membrane was used for respiration measurement. The storage solution contained 2.77 mM CaK2EGTA, 7.23 mM K2EGTA (100 nM free Ca2+), 6.56 mM MgCl2 (1 mM free Mg2+), 20 mM taurine, 0.5 mM dithiothreitol (DTT), and 20 mM imidazole 50 mM potassium-methanesulfonate (CH3K03S), 5.7 mM Na2ATP, 15 mM phosphocreatine (PCr) (pH 7.1 at 22°C). The respiration solution contained 2.77 mM CaK2EGTA, 7.23 mM K2EGTA (100 nM free Ca2+), 1.38 mM MgCl2 (1 mM free Mg2+), 20 mM taurine, 0.5 mM dithiothreitol (DTT), and 20 mM imidazole (pH 7.1 at 22°C), 90 mM potassium-methanesulfonate (CH3K03S), 10 mM sodium-methanesulfonate (CH3SO3Na), 3 mM K2HPO4, 10 mM glutamate, 4 mM malate, and 2 mg/ml bovine serum albumin. Respiration rates were measured at 22°C. Basal respiration rate (V0) was assessed in the presence of glutamate and malate (substrates for complex I in the electron transport chain) without adenosine diphosphate (ADP). A subsaturating amount of 0.1 mM ADP was added (VADP) prior to the addition of 20 mM creatine (Vcreatine). Next, a saturating amount of ADP (2 mM) was added, assessing the maximal respiration rate of the respiratory chain from complex I through IV with glutamate and malate as substrates (Vmax). Subsequently 10 mM succinate was added, supplementing complex II of the electron transport chain, assessing Vsuccinate. This was followed by the supplement of amytal (1mM amobarbital) which blocks complex I (NADH dehydrogenase), allowing assessment of complex II (Vamytal). Subsequent addition of ascorbate (0.5 mM) and N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD, 0.5 mM) induces respiration in complex IV, measuring VascorbateTMPD. The tissue samples were dried in a heat-centrifuge at 60 °C for 75 minutes before weighing. Respiratory rates are given as micromoles O2 per minute per gram dry weight myocardial tissue (μmol O2/min/g dw). The acceptor control ratio (ACR) was calculated as the ratio of Vmax/V0, representing the degree of coupling between oxidation and phosphorylation. ADP sensitivity ratio was calculated by VADP/Vmax to estimate the mitochondrial sensitivity to ADP. The percentage of creatine-induced increase in respiration rates was evaluated. The ratio of Vamytal/Vmax quantifies excess respiration of the cytochrome oxidase complex. The apparent constant of Michaelis in the absence of creatine (km(app)(ADP-Cr)) was estimated as follows: km(app)(ADP-Cr)= 100 μM(Vmax/VADP-1), and the apparent km in the presence of creatine (km(app)(ADP+C r)), km(app)(ADP+C r)= 100 μM(Vmax/Vcreatine-1) [4].
References


