Helium-induced cardioprotection: in sickness and in health, for better or for worse?
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Helium-induced early preconditioning and postconditioning are abolished in obese zucker rats in vivo

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INTRODUCTION

Diabetes mellitus is a known risk factor for the development of ischemic heart disease and myocardial infarction\textsuperscript{1}. It was shown that acute myocardial infarction is consistently associated with an increased mortality in patients with type 2 diabetes\textsuperscript{2}. Furthermore, diabetes mellitus is associated with a loss of the protective potency of cardioprotective strategies, e.g. preconditioning, both in humans and animals\textsuperscript{2-4}. Katakam et al.\textsuperscript{4} demonstrated that both, ischemic and pharmacological preconditioning by the mitochondrial ATP-activated potassium (mK\textsubscript{ATP}) channel agonist diazoxide is abolished in Zucker obese rats, a widely used animal model of insulin resistance and type 2 diabetes.

Recently it was shown that exposure to the noble gas helium initiates a pronounced protection of the myocardium against ischemia reperfusion injury\textsuperscript{5}. Helium is easy and safe to administer, and when compared to volatile anesthetics or xenon, the absence of anesthetic effects, as well as the lack of hemodynamic side effects would make helium an optimal agent for cardioprotection\textsuperscript{6,7}. These properties might offer the possibility to use helium in various groups of patients, e.g. during the perioperative period in patients at risk for cardiac events, as well as in non-surgical patients, e.g. in patients with unstable angina or myocardial infarction. Helium is already safely used in the therapy of asthma and chronic obstructive pulmonary disease, as well as in young children with ventilation disorders\textsuperscript{7,8}.

Cardioprotective effects of helium are mediated by activation of prosurvival signaling kinases and prevention of mitochondrial permeability transition pore (mPTP) opening\textsuperscript{5}. Opening of the mPTP can be regulated by different mechanisms including alterations in mitochondrial bioenergetics or regulation of glycogen synthase kinase-3beta (GSK-3beta) activity\textsuperscript{9,10}. The underlying mechanism, however, by which helium confers cardioprotection via mPTP is unknown.

We aimed to investigate (1) if the noble gas helium initiates cardiac preconditioning in the Zucker obese rat in vivo, and (2) the underlying sub-cellular mechanism by which helium prevents mPTP opening, i.e. regulation of mitochondrial bioenergetics and/or inhibition of prosurvival kinase dependent pathways.
METHODS

All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996), and were approved by the Institutional Committee for Animal Care and Use (Academic Medical Center Amsterdam, The Netherlands).

Materials

Helium was purchased from Linde Gas (Linde Gas Benelux BV, Dieren, The Netherlands). KCl was purchased from EMD Chemicals (Gibbstown, NJ, USA); all antibodies were purchased from Cell Signaling Technology Inc. (Danvers, USA) except the anti-alpha-tubulin and the anti-actin antibodies (Sigma, Saint Louis, USA). All other chemicals were purchased from Sigma-Aldrich Chemie B.V. (Zwijndrecht, The Netherlands). Rat insulin samples were measured with a Rat Insulin ELISA from Orange Medical (Orange Medical, Tilburg, The Netherlands).

Surgical preparation

Animals had free access to food and water at all times before the start of the experiments. Male Zucker lean rats (248±5 g) and male Zucker obese rats (334±5 g) were anesthetized by intraperitoneal injection of S-ketamine (150 mg/kg) and Diazepam (1.5 mg/kg).

Surgical preparation was performed as described previously11. Briefly, after tracheal intubation, the lungs were ventilated with 30% oxygen and 70% nitrogen and a positive end-expiratory pressure of 2-3 cm H₂O. During the experiments the endtidal CO₂ (etCO₂) concentration was measured in the expiratory gas (Datex Capnomac Ultima, Division of Instrumentarium Corp., Helsinki, Finland). Respiratory rate was adjusted to maintain etCO₂ between 35 - 45 mmHg. Body temperature was maintained at 38°C by the use of a heating pad. The right jugular vein was cannulated for saline and drug infusion, and the left carotid artery was cannulated for measurement of aortic pressure. Anesthesia was maintained by continuous alpha-chloralose infusion. A lateral left sided thoracotomy followed by pericardiotomy was performed and a ligature (5-0 Prolene) was passed below a major branch of the left coronary artery.

All animals were left untreated for 20 minutes before the start of the respective experimental protocol. Aortic pressure was digitized using an analogue to digital converter (PowerLab/8SP, ADInstruments Pty Ltd, Castle Hill, Australia) at a sampling rate of 500 Hz and were continuously recorded on a personal computer using Chart for
Experimental protocol

Rats were assigned to seven groups (Figure 1): Animals for infarct size measurements underwent 25 min of coronary artery occlusion and 2 hours of reperfusion (I/R). Zucker lean control group (ZL Con, n=8): after surgical preparation rats received 30% oxygen/70% nitrogen. Zucker lean helium preconditioned group (ZL He-PC, n=8): rats received helium 70%/30% oxygen for three 5-min periods, interspersed with two 5-min wash-out periods 10 min before ischemia and reperfusion. Zucker obese control group (ZO Con, n=8): after surgical preparation rats received 30% oxygen/70% nitrogen. Zucker obese helium preconditioned group (ZO He-PC, n=8): rats received helium 70%/30% oxygen for three 5-min periods, interspersed with two 5-min wash-out periods 10 min before ischemia and reperfusion. Zucker obese helium preconditioned group (ZO He-PC (6x), n=8): rats received helium 70%/30% oxygen for six 5-min periods.

![Figure 1](image)

**Figure 1. Experimental protocol. ZL = Zucker lean, ZO = Zucker obese, Con = Control, He-PC = helium preconditioning, He-PostC = helium postconditioning.**
interspersed with five 5-min wash-out periods 10 min before ischemia and reperfusion. In two additional groups we investigated whether we could induce cardioprotection in ZO rats by helium postconditioning. In these groups ZL and ZO rats (each n=8) received helium 70%/30% oxygen for 15 min at the onset of reperfusion.

**Infarct size measurement**

After 120 minutes of reperfusion, the heart was excised and mounted on a modified Langendorff apparatus for perfusion with ice cold normal saline via the aortic root at a perfusion pressure of 80 cm H$_2$O in order to wash out intravascular blood. After 2 minutes of perfusion, the coronary artery was re-occluded and the remainder of the myocardium was perfused through the aortic root with 0.2% Evans blue in normal saline for 10 minutes. Intravascular Evans blue was then washed out by perfusion for 10 minutes with normal saline. This treatment identified the area at risk as unstained. The heart was then cut into transverse slices, 2 mm thick. The slices were stained with 0.75% triphenyltetrazoliumchloride (TTC) solution for 10 minutes at 37°C, fixed in 4% formalin solution for 24 hours at room temperature. The area of risk and the infarcted area were determined by planimetry using SigmaScan Pro 5 computer software (SPSS Science Software, Chicago, IL) and corrected for dry weight of each slide.

To investigate the effects of helium preconditioning on mitochondrial respiration, additional experiments (n = 8 for each group) were conducted using the same preconditioning protocol except that the hearts were excised 5 min after the third helium administration (see Fig. 1). The effect of helium preconditioning on phosphorylation of the enzymes GSK-3beta (Ser9), Akt (Thr308 and Ser473) and extracellular regulated kinase (Erk1/2) (p42/p44) were determined in additional experiments in group 1-4 at four different time points (n = 4 for each time point in duplicate): time point 1 after the first helium administration, time point 2 after the third helium administration, time point 3 after 15 minutes of ischemia and time point 4 after 15 minutes of reperfusion (see Figure 1).

**Mitochondrial isolation**

Heart mitochondria were isolated by differential centrifugation as described previously. Briefly, atria were removed and ventricles were placed in isolation buffer [200 mmol/L mannitol, 50 mmol/L sucrose, 5 mmol/L KH$_2$PO$_4$, 5 mmol/L 3-(n-morpholino)propanesulfonic acid, 1 mmol/L Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid, 0.1% bovine serum albumin, pH 7.15 adjusted with KOH], and minced into 1 mm$^3$ pieces. The suspension was homogenized for 15 sec in 2.5 ml
isolation buffer containing 5 U/ml protease, and for another 15 sec after addition of 17 ml isolation buffer. The suspension was centrifuged at 3220g for 10 min, the supernatant was removed, and the pellet was resuspended in 25 ml isolation buffer and centrifuged at 800g for 10 min. The supernatant was centrifuged at 3220g for 10 min, and the final pellet was suspended in 0.5 ml isolation buffer and kept on ice. Protein content was determined by the Bradford method. All isolation procedures were conducted at 4°C.

Mitochondrial respiration

Oxygen consumption was measured polarographically at 37°C using a respirometric system (System S 200A, Strathkelvin Instruments, Glasgow, Scotland). Mitochondria (0.3 mg protein/ml) were suspended in respiration buffer containing 130 mmol/L KCl, 5 mmol/L K₂HPO₄, 20 mmol/L 3-(n-morpholino) propanesulfonic acid, 2.5 mmol/L Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid, 1 µmol/L Na₄P₂O₇, 0.1% bovine serum albumin, pH 7.15 adjusted with KOH. Mitochondrial respiration was initiated by administration of 10 mmol/L complex II substrate succinate (+10 µmol/L complex I blocker rotenone) after 60 sec. State 3 respiration was initiated after 120 sec by addition of 200 µmol/L ADP. Respiration rates were recorded under state 3 conditions and after complete phosphorylation of ADP to ATP (State 4). The respiratory control index (RCI, state 3/state 4) and the P/O ratio (phosphate incorporated into ATP to oxygen consumed) were calculated as parameter of mitochondrial coupling between respiration and oxidative phosphorylation, and mitochondrial efficiency, respectively. From each heart, respiration measurements were repeated in 3 mitochondrial samples and the average was taken (and counted as n = 1). Respiration rates are expressed as absolute rates in nmol O₂/mg/min.

Separation of cytosolic fraction

For cellular fractionation and subsequent Western blot assay, tissue specimens were prepared for protein analysis of GSK-3beta (Ser9), Akt (Thr308 and Ser473) and Erk1/2 (p42/p44), respectively. The excised hearts were frozen in liquid nitrogen. Subsequently, a cellular fractionation was performed as described previously. The frozen tissue was pulverized and dissolved in lysis buffer containing: Tris base, EGTA, NaF and Na₃VO₄ (as phosphatase inhibitors), a freshly added protease inhibitor mix (aprotinin, leupeptin and pepstatin) and DTT. The solution was vigorously homogenized on ice (Homogenisator, IKA, Staufen, Germany) and then centrifuged at 1000 g, 4°C, for 10 min. The supernatant, containing the cytosolic fraction, was centrifuged again at 16000 g, 4°C, for 15 min to clean up this fraction for further Western blot assay.
Western blot analysis

After protein concentration was determined by the Lowry method\textsuperscript{16} equal amounts of protein were prepared and loaded on a 10% SDS-PAGE gel. The proteins were separated by electrophoresis (100 V, 85 min) and then transferred to a PVDF membrane by tank blotting (100V, 1h). To prevent unspecific antibody binding the membrane was subsequently blocked with 5% skimmed milk solution in Tris buffered saline containing Tween (TBS-T) for 2 hours. Then, the membrane was incubated over night at 4°C with the respective primary antibody GSK-3beta (1:10,000), Akt(Thr308)(1:1000), or Akt(Ser473)(1:1000), Erk1/2 (p42/p44)(1:10,000). After washing in fresh, cold TBS-T, the blot was subjected to the appropriate horseradish peroxidase conjugated secondary antibody for 2 hours at room temperature. Immunoreactive bands were visualized by chemiluminescence detected on X-ray film (Amersham Hyperfilm ECL, GE Healthcare Limited, United Kingdom) using the enhanced chemiluminescence system Santa Cruz (Santa Cruz Biotechnology, Inc., Santa Cruz). The blots were quantified using a Kodak Image station\textsuperscript{®} (Eastman Kodak Comp., Rochester, NY) and the results are presented as ratio of phosphorylated protein to total protein. Equal loading of protein on the gel was additionally proved by detection of alpha-tubulin or actin, respectively, and Coomassie blue staining of the gels.

Statistical Analysis

Data are expressed as mean ± standard error of the mean (SE). Heart rate (in bpm) and mean aortic pressure (in mmHg) were measured during baseline, coronary artery occlusion, and reperfusion period. Comparisons of hemodynamics between groups or between time points in a group were performed (SPSS Science Software, version 12.0.1) using two-way analysis of variance (ANOVA) followed by Tukey’s post hoc test. Infarct sizes were analyzed by one-way ANOVA followed by Tukey’s post-hoc test. Data from mitochondrial and Western blot experiments were analyzed by Student’s t-test with Bonferroni’s correction for multiple comparisons. Changes within and between groups were considered statistically significant if p<0.05.
RESULTS

Infarct size measurement

Helium preconditioning reduced infarct size in ZL rats from 52 ± 3 % in controls to 32 ± 2 % (p<0.05; Figure 2). In ZO control rats, infarct size was similar to ZL controls (54 ± 3 %, n.s. vs. ZL Con). In contrast to the protection seen in ZL rats, in ZO rats did helium not reduce infarct size (56 ± 3 %, n.s.; Figure 2).

Figure 2. Histogram shows the infarct sizes as percent of area at risk (AAR). ZL = Zucker lean, ZO = Zucker obese, Con = Control, He-PC = helium preconditioning, He-PostC = helium postconditioning. Data are presented as mean ± SE, *p < 0.05 vs. ZL Con.
Furthermore, an increased preconditioning stimulus by 6 cycles of helium could not protect the ZO rat heart (57 ± 4 %, n.s.; Figure 2). Helium postconditioning reduced infarct size in ZL rats (37 ± 2 %, p<0.05; Figure 2). This effect was also completely abolished in ZO rats (51 ± 3 %, n.s.; Figure 2).

Hemodynamic variables

Hemodynamic variables are summarized in Table 1. No significant differences in heart rate and aortic pressure were observed between the experimental groups during baseline, ischemia and reperfusion. At the end of the experiments, mean aortic pressure was significantly decreased compared with baseline in all groups with the exception of the ZO control group.

<table>
<thead>
<tr>
<th></th>
<th>Heart Rate (BPM)</th>
<th>Mean AOP (mmHg)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Baseline Washout</td>
<td>Ischemia</td>
</tr>
<tr>
<td>ZL Con</td>
<td>419 ± 9</td>
<td>428 ± 9</td>
</tr>
<tr>
<td>ZL He-PC</td>
<td>417 ± 12</td>
<td>420 ± 11</td>
</tr>
<tr>
<td>ZL He-PostC</td>
<td>407 ± 12</td>
<td>389 ± 5</td>
</tr>
<tr>
<td>ZO Con</td>
<td>414 ± 12</td>
<td>397 ± 11</td>
</tr>
<tr>
<td>ZO He-PC</td>
<td>416 ± 14</td>
<td>410 ± 8</td>
</tr>
<tr>
<td>ZO He-PC (6x)</td>
<td>407 ± 7</td>
<td>389 ± 13</td>
</tr>
<tr>
<td>ZO He-PostC</td>
<td>400 ± 7</td>
<td>392 ± 3</td>
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</table>

Table 1. Hemodynamic variables. Data are Mean ± SE. ZL = Zucker lean; ZO = Zucker obese; Con = control group; He-PC = helium preconditioning, He-PostC = helium postconditioning. *p<0.05 vs. baseline.
Weights and blood glucose levels

The body weights (g) of ZO rats were significantly higher than in ZL rats (Table 2). Blood glucose levels were not different between groups (Table 2). Insulin levels were significantly higher in ZO compared to ZL rats (Table 2).

Mitochondrial respiration

Mitochondrial respiration results are summarized in Figure 3. There was no significant difference in the RCI between ZL (n = 8) and ZO (n = 7) control rats (2.51 ± 0.03 vs. 2.52 ± 0.03, n.s.). Helium preconditioning reduced the RCI in ZL rats (2.27 ± 0.03; n = 8; p<0.05 vs. ZL Con), but had no effect on the RCI in ZO rats (2.52 ± 0.04; n = 8; n.s. vs. ZO Con). The reduction in the RCI in ZL He-PC was caused by an increase in state 4 respiration (155 ± 4 nmol O₂/mg/min vs. 139 ± 3 nmol O₂/mg/min, p<0.05); state 3 respiration was not affected by helium preconditioning in both ZL and ZO rats.

There was no difference between all groups in the efficiency of oxidative phosphorylation as demonstrated by no changes in the P/O ratio.

Regulation of GSK-3beta, Akt and Erk1/2 phosphorylation during helium preconditioning

Figure 4 shows that there were no differences at any time point in Akt and Erk1/2 phosphorylation during the experiments in ZL and ZO rats. Helium reduced GSK-3beta phosphorylation during ischemia in ZL rats compared with respective controls (0.49 ± 0.07 vs. 0.72 ± 0.07, p<0.05; Figure 4A3).
<table>
<thead>
<tr>
<th>Group</th>
<th>Blood sugar (mmol l⁻¹)</th>
<th>Insulin levels (nmol l⁻¹)</th>
<th>Body weight (g)</th>
<th>Heart dry weight (g)</th>
<th>Area at risk (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZL Con</td>
<td>6.7 ± 0.1</td>
<td>0.24 ± 0.05</td>
<td>244 ± 10</td>
<td>0.176 ± 0.007</td>
<td>24 ± 5</td>
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<tr>
<td>ZL He-PC</td>
<td>6.3 ± 0.6</td>
<td>0.26 ± 0.06</td>
<td>262 ± 10</td>
<td>0.172 ± 0.008</td>
<td>26 ± 3</td>
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<tr>
<td>ZL He-PostC</td>
<td>6.0 ± 0.1</td>
<td>0.20 ± 0.05</td>
<td>238 ± 4</td>
<td>0.174 ± 0.006</td>
<td>21 ± 3</td>
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<tr>
<td>ZO Con</td>
<td>6.9 ± 0.4</td>
<td>2.20 ± 0.48*,#,*†</td>
<td>315 ± 12*,#,*</td>
<td>0.182 ± 0.007</td>
<td>23 ± 3</td>
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<tr>
<td>ZO He-PC</td>
<td>7.8 ± 0.8</td>
<td>2.18 ± 0.42*,#,*†</td>
<td>330 ± 10*,#,*</td>
<td>0.186 ± 0.008</td>
<td>23 ± 5</td>
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<td>ZO He-PC (6x)</td>
<td>6.4 ± 0.2</td>
<td>2.65 ± 0.38*,#,*†</td>
<td>343 ± 4*,#,*</td>
<td>0.194 ± 0.002</td>
<td>20 ± 2</td>
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<tr>
<td>ZO He-PostC</td>
<td>6.2 ± 0.2</td>
<td>2.64 ± 0.68*,#,*†</td>
<td>348 ± 4*,#,*</td>
<td>0.186 ± 0.005</td>
<td>18 ± 3</td>
</tr>
</tbody>
</table>

Table 2: Blood glucose levels and weights and heart dry weights. Data are Mean ± SD. ZL = Zucker lean; ZO = Zucker obese; Con = control group; He-PC = Helium preconditioning. *p<0.05 vs. ZL Con; #p<0.05 vs. ZL He-PC.
Figure 3. Summarized data for the effects of helium preconditioning on mitochondrial respiration. ZL = Zucker lean, ZO = Zucker obese, Con = Control, He-PC = helium preconditioning. RCI = respiratory control index, a parameter for the coupling between mitochondrial respiration and oxidative phosphorylation. P/O ratio = ratio between phosphate incorporated into ATP and oxygen consumed; a parameter for the efficiency of oxidative phosphorylation. Data are presented as mean ± SE, *p < 0.05 vs. ZL Con.

<table>
<thead>
<tr>
<th></th>
<th>ZL Con</th>
<th>ZL He-PC</th>
<th>ZO Con</th>
<th>ZO He-PC</th>
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<tr>
<td>state 3</td>
<td>349±6</td>
<td>349±7</td>
<td>344±8</td>
<td>348±5</td>
</tr>
<tr>
<td>state 4</td>
<td>139±3</td>
<td>155±4*</td>
<td>136±5</td>
<td>139±2</td>
</tr>
<tr>
<td>P/O ratio</td>
<td>1.54±0.02</td>
<td>1.53±0.03</td>
<td>1.56±0.02</td>
<td>1.55±0.03</td>
</tr>
</tbody>
</table>

Figure 4. Effects of Helium-induced preconditioning on GSK-3beta (Ser9) (panel A), Akt (Thr308 and Ser473) (panel B and C) and Erk1/2 (panel D and E) phosphorylation. Summarized data presenting ratio of phosphorylated enzyme to total enzyme are shown. Time point 1 after the first helium administration (A1 - E1 respectively), time point 2 after the third helium administration (A2 - E2 respectively), time point 3 after 15 min of ischemia (A3 - E3 respectively), time point 4 after 15 min of reperfusion (A4 - E4 respectively), LC = Zucker lean control, OC = Zucker obese control, LH = Zucker lean helium preconditioning, OH = Zucker obese helium preconditioning. Data are presented as mean ± SE, *p < 0.05 vs. LC.
Helium conditioning in the obese Zucker rat
DISCUSSION

The main findings of our study are that the cardioprotective effect of helium-induced preconditioning a) is abolished in the prediabetic rat heart, and b) is mediated in the non-diabetic heart rather by regulation of mitochondrial respiration, i.e. mild mitochondrial uncoupling, than by activation of prosurvival signaling kinases. We also demonstrate that helium induces postconditioning, but that this protection is abolished in the prediabetic rat.

It is well known that besides brief periods of ischemia, also pharmacological interventions can initiate cardiac preconditioning to enhance the resistance of the myocardium against ischemia and reperfusion injury. Helium is a non-anesthetic gas without significant hemodynamic side effects. These properties would make helium an ideal agent for cardioprotection in patients with cardiovascular disease not only in the perioperative setting like during cardiac surgery, but also for interventional procedures like during percutaneous coronary interventions.

There is evidence that the protective potency of both ischemic and pharmacological preconditioning is diminished in the diabetic heart. In the present study, we show that besides the rabbit heart also the rat heart can be protected by helium induced pre- and postconditioning. Furthermore, we demonstrate that the cardioprotective effect of helium is abolished in the prediabetic rat heart. Kristiansen et al. demonstrated in obese Zucker diabetic fatty and lean Goto-Kakizaki rats, two widely used rat models of type 2 diabetes, that ischemic preconditioning does not reduce infarct size. In contrast, Tsang et al. showed that preconditioning can be induced in hearts from Goto-Kakizaki rats, but the threshold that is required to achieve preconditioning is elevated in diabetic compared with non diabetic hearts. In our study, even six cycles of helium preconditioning did not result in infarct size reduction in ZO rats (Figure 2). The experimental diabetes models used in the two earlier cited studies were characterized by a significant hyperglycemia. Katakam et al. demonstrated that both, ischemic and pharmacological preconditioning induced by the mK<sub>ATP</sub> channel agonist diazoxide, are abolished in Zucker obese rats. The experiments were conducted in 10-12 weeks old rats. At this age, ZO rats are hyperinsulinemic and normoglycemic, representing a prediabetic state of type 2 diabetes. In the present study, we used 10-12 weeks old ZO rats that were also normoglycemic and hyperinsulinemic (Tab. 2), and our results are in line with the earlier findings showing that cardioprotection is abolished in the prediabetic heart (Fig. 2).

The mechanism by which helium-induced preconditioning is blocked in the prediabetic heart is unknown. Ischemic and pharmacological preconditioning failed to
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protect the diabetic myocardium, possibly caused by dysfunctional potassium channels in the inner mitochondrial membrane. Alterations in mitochondrial function caused by potassium channel activation have been proposed to protect the myocardium by reducing mPTP opening. Helium-induced preconditioning is abrogated by administration of the mPTP opener atractyloside. It has also been suggested that preconditioning prevents mPTP opening by regulation of prosurvival signaling kinases including Erk1/2, Akt and GSK-3beta, and/or by regulation of mitochondrial bioenergetics, i.e. mild uncoupling of mitochondrial respiration. Pagel et al. demonstrated that helium preconditioning is abolished by 5-hydroxydecanoate (5-HD), a mKATP channel blocker. Interestingly, activation of the KATP channel with Diazoxide could not induce preconditioning in the Zucker obese rat heart, suggesting that the blockade of Diazoxide-induced preconditioning is related to defects at the level of the mKATP channel or its downstream signaling cascade.

Very recently we could demonstrate that opening of another mitochondrial potassium channel, namely the mitochondrial calcium sensitive potassium channel (mKCa) is involved in helium-induced preconditioning. This study, together with the study from Pagel suggest a crucial role of mitochondrial potassium channels in helium preconditioning. mKCa channel opening causes a slight increase in mitochondrial reactive oxygen species generation. Stowe et al. demonstrated that the cardioprotective effect of the mKCa channel agonist 1,3-Dihydro-1-[2-hydroxy-5-(trifluoromethyl)phenyl]-5-(trifluoromethyl)-2H-benzimidazol-2-one (NS1619) requires superoxide radical generation during the preconditioning stimulus. Furthermore, preconditioning by NS1619 reduces mitochondrial calcium overload and mitochondrial reactive oxygen species production during the subsequent period of ischemia and early reperfusion. Such a reduction in mitochondrial calcium overload and reactive oxygen species generation has been suggested to prevent mPTP opening. Both, mKATP and mKCa channel activation trigger preconditioning independent from each other and by involvement of the mPTP.

Our results suggest that regulation of mitochondrial respiration is involved in helium-induced preconditioning: helium induced a reduction of the respiratory control index in ZL rats while it had no effect on mitochondrial function in ZO rats. Furthermore, the helium-induced effect on mitochondrial respiration was abolished before the onset of lethal ischemia. Based on these data we suggest that the prediabetic related blockade of helium-induced preconditioning is related to defects at the level of the mitochondria or mitochondrial potassium channels, respectively, or its upstream signaling cascade. It has been shown that mitochondrial uncoupling is capable of inducing cardioprotection: pharmacological uncoupling by 2,4-dinitrophenol or FCCP reduced infarct size in rat heart in vitro.
The role of prosurvival signaling kinases in helium-induced preconditioning remains unclear. In the rabbit heart, the protective effect of helium was blocked by pharmacological inhibition of phosphatidylinositol-3-kinase, extracellular signal-regulated kinase, and 70-kDa ribosomal protein S6 kinase. Hausenloy et al. demonstrated for ischemic preconditioning that Akt and Erk1/2 were phosphorylated before and after the ischemic period compared to control group. In the current study, we tested time dependent phosphorylation of GSK-3beta, Akt and Erk1/2. We did not detect an effect of helium on Erk1/2 and Akt phosphorylation. GSK-3beta shows a decreased activity in Zucker lean helium treated rats after 15 minutes of ischemia compared with respective controls. The importance of Akt phosphorylation in the signal transduction of ischemic preconditioning was demonstrated by Tsang et al. In their study, preconditioning caused an increased Akt phosphorylation 5 min after the last preconditioning cycle, i.e. the same timing of tissue sampling as we used in the present study. However, to our knowledge, there is no evidence that “prosurvival kinases” activate mitochondrial $K_{Ca}$ channels to regulate mitochondrial function. Recently, it was shown that adrenomedullin treatment prior to ischemia reduces infarct size via protein kinase A mediated activation of m$K_{Ca}$ channels. This effect was independent of phosphatidylinositol-3-kinase. In the present study, we did not test whether protein kinase A is involved in helium preconditioning.

The results of the present study have to be interpreted within the scope of some limitations. First, our experiments were conducted in Zucker obese and Zucker lean rats. Zucker obese rats have a Leptin receptor mutation and develop obesity at an early age. The Zucker obese rat is described to be hyperphagic compared to lean littermates from an early age on and obese condition is evident at 5 weeks. In the present study, feeding of the Zucker obese rat was not different from Zucker lean rats. However, we did not measure differences in caloric intake. Second, in the present study we did not investigate the effect of helium on the mPTP directly. However, it has already been demonstrated that helium confers cardioprotection by prevention of mPTP opening. Therefore, the present study was designed to investigate the effect of helium on possible regulators of the mPTP (i.e. mitochondrial respiration, GSK-3beta phosphorylation). Third, we did not investigate other possible avenues of preconditioning like endothelial or inducible nitric oxide synthase and or potassium channels in the present study. It was shown that helium preconditioning is mediated by endothelial but not by inducible nitric oxide synthase. All these enzymes reported to be involved in helium preconditioning are located upstream of mitochondrial potassium channels and the mitochondria. Abolished mitochondrial uncoupling in the Zucker obese rat heart before lethal ischemia together with no effects on the expression of enzymes of the prosurvival cascade, suggest a blockade of cardioprotection in the prediabetic heart caused by yet unknown mechanisms.
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Taken together, the present study demonstrates that the noble gas helium can induce pre- and postconditioning in the rat heart in vivo. The protective effect of preconditioning could be explained by mild mitochondrial uncoupling, an alteration that is capable to prevent mPTP opening. Furthermore, the protective potency of helium-induced preconditioning is completely abrogated in the Zucker obese rat, a widely used animal model for prediabetic conditions of state 2 diabetes. Whether this cardioprotection can be re-established in the prediabetic heart by further pharmacological intervention needs further investigation.
REFERENCES


Helium conditioning in the obese Zucker rat


