

# Helium-induced Preconditioning in Young and Old Rat Heart

## Impact of Mitochondrial $\text{Ca}^{2+}$ -sensitive Potassium Channel Activation

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**Background:** The noble gas helium induces cardiac preconditioning. Whether activation of mitochondrial  $\text{K}^+$  channels is involved in helium preconditioning (He-PC) is unknown. The authors investigated whether He-PC (1) is mediated by activation of  $\text{Ca}^{2+}$ -sensitive potassium channels, (2) results in mitochondrial uncoupling, and (3) is age dependent.

**Methods:** Anesthetized Wistar rats were randomly assigned to six groups ( $n = 10$  each). Young (2–3 months) control (Con) and aged (22–24 months) control animals (Age Con) were not treated further. Preconditioning groups (He-PC and Age He-PC) inhaled 70% helium for  $3 \times 5$  min. The  $\text{Ca}^{2+}$ -sensitive potassium channel blocker iberiotoxin was administered in young animals, with and without helium (He-PC+Ibtx and Ibtx). Animals underwent 25 min of regional myocardial ischemia and 120 min of reperfusion. In additional experiments, cardiac mitochondria were isolated, and the respiratory control index was calculated (state 3/state 4).

**Results:** Helium reduced infarct size in young rats from  $61 \pm 7\%$  to  $36 \pm 14\%$  ( $P < 0.05$  vs. Con). Infarct size reduction was abolished by iberiotoxin ( $60 \pm 11\%$ ;  $P < 0.05$  vs. He-PC), whereas iberiotoxin alone had no effect ( $59 \pm 8\%$ ; not significant vs. Con). In aged animals, helium had no effect on infarct size (Age Con:  $59 \pm 7\%$  vs. Age He-PC:  $58 \pm 8\%$ ; not significant). Helium reduced respiratory control index in young animals ( $2.76 \pm 0.05$  to  $2.43 \pm 0.15$ ;  $P < 0.05$ ) but not in aged animals (Age Con:  $2.87 \pm 0.17$  vs. Age He-PC:  $2.87 \pm 0.07$ ; not significant). Iberiotoxin abrogated the helium effect on respiratory control index ( $2.73 \pm 0.15$ ;  $P < 0.05$  vs. He-PC) but had no effect itself on mitochondrial respiration ( $2.75 \pm 0.05$ ; not significant vs. Con).

**Conclusion:** Helium causes mitochondrial uncoupling and induces preconditioning in young rats via  $\text{Ca}^{2+}$ -sensitive potassium channel activation. However, these effects are lost in aged rats.

ISCHEMIC heart disease, with its clinical consequences of acute myocardial infarction, sudden cardiac death, arrhythmias, and heart failure, is the leading cause of

morbidity and mortality in industrialized nations. Several studies demonstrated tissue-protective effects of preconditioning during ischemia-reperfusion interventions, both in animals<sup>1–3</sup> and in humans.<sup>4,5</sup>

However, most of these studies were conducted in young and healthy animals. The morbidity and mortality of myocardial infarction is increased with increasing age,<sup>6–8</sup> possibly partly because of an aging-related loss of the protective potency of cardioprotective strategies, e.g., preconditioning.<sup>9–12</sup> The underlying reason for this loss of cardioprotection in the senescent heart is unknown. Lee *et al.*<sup>11</sup> demonstrated a loss of protection in patients older than 65 yr undergoing coronary angioplasty compared with patients younger than 55 yr. Because a prolonged period of ischemia and the mitochondrial adenosine triphosphate-sensitive potassium ( $\text{K}_{\text{ATP}}$ ) channel activator nicorandil were able to (re)initiate a preconditioning state in the older patients, the authors concluded that the impaired preconditioning response is caused by some defects in signal transduction of activation of  $\text{K}_{\text{ATP}}$  channels with aging. In addition to activation of  $\text{K}_{\text{ATP}}$  channels,<sup>13</sup> preconditioning seems to be mediated also by another class of  $\text{K}^+$  channels:  $\text{Ca}^{2+}$ -sensitive potassium ( $\text{K}_{\text{Ca}}$ ) channels.<sup>14,15</sup> These channels have recently been described in mitochondria, although their identity with surface membrane channels remains to be proven.<sup>16</sup> It is proposed that activation of  $\text{K}^+$  channels in the inner mitochondrial membrane with the consequence of  $\text{K}^+$  influx into the mitochondrial matrix causes alterations in mitochondrial function.<sup>13,17</sup> Recently, we discovered that the effect of activation of mitochondrial  $\text{K}_{\text{Ca}}$  ( $\text{mK}_{\text{Ca}}$ ) channels on mitochondrial function is age dependent.<sup>18</sup>

A recent study demonstrated that the cardioprotective effect of preconditioning can be mimicked by the noble gas helium.<sup>19</sup> Helium might be a perfect alternative as an organ-protective gas: In contrast to volatile anesthetics or the noble gas xenon, helium is a nonanesthetic gas that might be administered to patients who are subjected to organ ischemia (vascular surgery, organ transplantation, cardiac surgery) or who recently underwent regional ischemia (stroke, angina pectoris, myocardial infarction, organ transplantation) without the “side effect” of being anesthetized.<sup>20</sup> Like xenon, whose clinical use on a routine basis has been, until now, limited because of its high costs and difficulties in application,

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Received from the Department of Anesthesiology, Laboratory of Experimental Intensive Care and Anesthesiology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands. Submitted for publication April 22, 2008. Accepted for publication July 14, 2008. Supported by a Research Starter Grant (to Dr. Heinen) from the Society of Cardiovascular Anesthesiologists, Richmond, Virginia, and M.D.-medical research trainee grant No. 92003450 (to Dr. Heinen) from the Netherlands Organisation for Health Research and Development, Den Haag, The Netherlands. Drs. Heinen and Huhn contributed equally to this work.

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helium lacks hemodynamic side effects. In mechanically ventilated patients with chronic obstructive pulmonary disease, hemodynamics are improved after helium inhalation.<sup>21</sup> In contrast to xenon, helium can be easily supplied *via* a tube or a facemask.

Helium confers cardioprotection *via* modulation of the mitochondrial permeability transition pore (mPTP).<sup>19</sup> It has been suggested that opening of the mPTP can be prevented by alterations in mitochondrial function.<sup>22</sup> However, it is unknown whether helium-induced preconditioning is mediated by  $K_{Ca}$  channels with the consequence of altered mitochondrial respiration or whether helium initiates preconditioning in the senescent heart. Here, we hypothesize that helium-induced preconditioning (1) is mediated by activation of  $K_{Ca}$  channels, (2) results in mitochondrial uncoupling, and (3) is abolished in the aged myocardium.

## Materials and Methods

The investigation is in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (publication No. 85-23, revised 1996) and was performed after approval of the Animal Ethics Committee of the University of Amsterdam, Amsterdam, The Netherlands.

### Materials

Helium was purchased from Linde Gas (Linde Gas Benelux BV, Dieren, The Netherlands), and KCl was purchased from EMD Chemicals (Gibbstown, NJ); all other chemicals were purchased from Sigma Chemical Co. (Taufkirchen, Germany). The polyclonal  $K_{Ca}$  channel  $\beta_1$  subunit antibody and the immunizing peptide were purchased from Abcam (Cambridge, United Kingdom).

### Surgical Preparation

Animals had free access to food and water at all times before the start of the experiments. Young (3–4 months)

male Hannover Wistar rats ( $352 \pm 15$  g) and old (22–24 months) male Hannover Wistar rats ( $621 \pm 34$  g) were anesthetized by intraperitoneal *S*-ketamine injection (150 mg/kg) and diazepam (1.5 mg/kg).

Surgical preparation was performed as described previously.<sup>23,24</sup> In brief, after tracheal intubation, the lungs were ventilated, and respiratory rate was adjusted to maintain partial pressure of carbon dioxide within physiologic limits. 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 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 min before the start of the respective experimental protocol. Aortic pressure was digitized using an analog-to-digital converter (PowerLab/8SP; ADInstruments Pty Ltd, Castle Hill, Australia) at a sampling rate of 500 Hz and was continuously recorded on a personal computer using Chart for Windows version 5.0 (ADInstruments).

### Experimental Protocol

Rats were divided into six groups (fig. 1). All animals underwent 25 min of coronary artery occlusion and 2 h of reperfusion.

**Control group** ( $n = 10$ ): After surgical preparation, rats received 30% oxygen–70% nitrogen.

**Helium preconditioned group** ( $n = 10$ ): Rats received 70% helium–30% oxygen for three 5-min periods, interspersed with two 5-min washout periods 10 min before ischemia and reperfusion.

**Helium preconditioned group with iberiotoxin** ( $n = 10$ ): Rats received 70% helium–30% oxygen for three 5-min periods, interspersed with two 5-min washout periods 10 min before ischemia and reperfusion. Ibe-

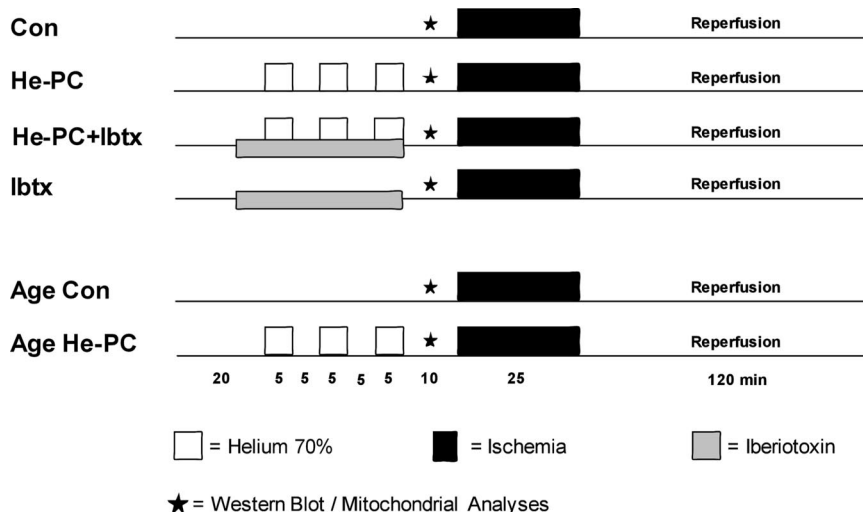


Fig. 1. Experimental protocol. Age = aged rats (22–24 months); Con = controls; He-PC = helium preconditioning; Ibtx = iberiotoxin.

riotoxin ( $6 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ ) was administered continuously over a time period of 30 min starting 5 min before the first preconditioning stimulus.

**Iberiotoxin group** ( $n = 10$ ): Rats received iberiotoxin continuously over a time period of 30 min starting 5 min before the first preconditioning stimulus.

**Aged control group** ( $n = 10$ ): After surgical preparation, rats received 30% oxygen–70% nitrogen.

**Aged helium preconditioned group** ( $n = 10$ ): Rats received 70% helium–30% oxygen for three 5-min periods, interspersed with two 5-min washout periods 10 min before ischemia and reperfusion.

### Infarct Size Measurement

After 120 min of reperfusion, the heart was excised, with the occluding suture left in place, and then mounted on a modified Langendorff apparatus for perfusion with ice-cold normal saline *via* the aortic root at a perfusion pressure of 80 cm  $\text{H}_2\text{O}$  to wash out intravascular blood. After 5 min of perfusion, the coronary artery was reoccluded, and the remainder of the myocardium was perfused through the aortic root with 0.2% Evans blue in normal saline for 10 min. Intravascular Evans blue was then washed out by perfusion with normal saline for 10 min. This treatment identified the area at risk as unstained. The heart was then cut into 2-mm-thick transverse slices. The slices were stained with 0.75% triphenyltetrazolium chloride solution for 10 min at  $37^\circ\text{C}$  and fixed in 4% formalin solution for 24 h at room temperature. The area of risk and the infarcted area were determined by planimetry using SigmaScan Pro 5<sup>®</sup> computer software (SPSS Science Software, Chicago, IL).

For mitochondrial respiration and Western blot analysis, additional experiments ( $n = 8$  each) were performed. Hearts were excised 5 min before the onset of ischemia (total baseline 50 min).

### Mitochondrial Isolation

Heart mitochondria were isolated by differential centrifugation as described previously.<sup>18</sup> Briefly, atria were removed, and ventricles were placed in isolation buffer [200 mM mannitol, 50 mM sucrose, 5 mM  $\text{KH}_2\text{PO}_4$ , 5 mM 3-(*n*-morpholino) propanesulfonic acid, 1 mM EGTA, and 0.1% bovine serum albumin; pH 7.15 adjusted with potassium hydroxide] and minced into 1-mm<sup>3</sup> pieces. The suspension was homogenized for 15 s in 2.5 ml isolation buffer containing 5 U/ml protease (from *Bacillus licheniformis*, Enzyme Commission No. 3.4.21.14) and for another 15 s after addition of 17 ml isolation buffer. The suspension was centrifuged at 3,220g 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 3,220g 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  $4^\circ\text{C}$ .

### Mitochondrial Respiration

Oxygen consumption was measured polarographically at  $37^\circ\text{C}$  using a respirometric system (System S 200A; Strathkelvin Instruments, Glasgow, Scotland). Mitochondria (0.3 mg protein/ml) were suspended in respiration buffer containing 130 mM KCl, 5 mM  $\text{K}_2\text{HPO}_4$ , 20 mM 3-(*n*-morpholino) propanesulfonic acid, 2.5 mM EGTA, 1  $\mu\text{M}$   $\text{Na}_4\text{P}_2\text{O}_7$ , and 0.1% bovine serum albumin (pH 7.15 adjusted with potassium hydroxide). Mitochondrial respiration was initiated by administration of 10 mM complex II substrate succinate (+ 10  $\mu\text{M}$  complex I blocker rotenone) after 60 s. State 3 respiration was initiated after 120 s by addition of 200  $\mu\text{M}$  adenosine diphosphate. Respiration rates were recorded under state 3 conditions and after complete phosphorylation of adenosine diphosphate to adenosine triphosphate (state 4). The respiratory control index (RCI; state 3/state 4) and the ratio of phosphate incorporated into adenosine triphosphate 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 three mitochondrial samples, and the average was taken (and counted as  $n = 1$ ). Respiration rates are expressed as absolute rates in  $\text{nmol O}_2 \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$ .

### Western Blot Analysis

The content of  $\text{K}_{\text{Ca}}$  channels in the mitochondria was determined by Western blot analysis. One hundred microliters mitochondrial suspension was treated with 5  $\mu\text{l}$  Triton X-100 (10%), 20  $\mu\text{l}$  KCl (4.5 M), and protease inhibitor mix (aprotinin, leupeptin, and pepstatin); stirred; and incubated at room temperature for 5 min. After centrifugation (10,000g, 5 min), the protein concentration was determined by the Lowry method.<sup>25</sup> Subsequently, equal amounts of mitochondrial protein (30  $\mu\text{g}$ ) were mixed with loading buffer (1:1) containing Tris-HCl, glycerol, and bromphenol blue. Samples were loaded on a 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel, separated by electrophoresis and transferred to a polyvinylidene fluoride membrane by tank blotting (100 V, 2 h). Unspecific binding of the antibody was blocked by incubation with 5% skimmed milk solution in Tris-buffered saline containing Tween for 2 h. Subsequently, the membrane was incubated overnight at  $4^\circ\text{C}$  with the  $\text{K}_{\text{Ca}}$  channel  $\beta_1$  subunit antibody (1:1,000). After washing in fresh, cold Tris-buffered saline containing Tween, the blot was subjected to the appropriate horseradish peroxidase–conjugated secondary antibody for 2 h at room temperature. Immunoreactive bands were visualized by chemiluminescence and detected on x-ray film (Amersham Hyperfilm ECL; GE



Healthcare Limited, Buckinghamshire, United Kingdom) using the enhanced chemiluminescence system Santa Cruz (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The blots were quantified using a Kodak Image Station® (Eastman Kodak Comp., Rochester, NY), and the results are presented as ratio of  $K_{Ca} \beta_1$  subunit (arbitrary units) to citrate synthase activity (mU/mg). Equal loading of protein on the gel was in addition proved by Coomassie blue staining of the gels. For identification of the specific  $K_{Ca} \beta_1$  subunit band, additional blocking experiments were conducted using the immunizing peptide in a large molar excess (approximately 70-fold) for competitive inhibition of antibody-protein binding.

#### Determination of Enzyme Activities

Citrate synthase activity, a mitochondrial marker, was measured according to standard spectrophotometric procedures<sup>26</sup> and served as a control for Western blot results of  $mK_{Ca}$  channels. It was shown that citrate synthase activity does not change with increasing age.<sup>27,28</sup>

#### Statistical Analysis

Data are expressed as mean  $\pm$  SD. Heart rate (in beats/min) and mean aortic pressure (in mmHg) were measured during baseline, coronary artery occlusion, and reperfusion periods. Comparisons between groups or between time points in a group were performed (SPSS Science Software, version 12.0.1) using two-way analysis of variance followed by the Tukey *post hoc* test. Infarct sizes were analyzed by one-way analysis of variance followed by the Tukey *post hoc* test. Changes within and between groups were considered statistically significant if  $P < 0.05$ . Mitochondrial respiration results and Western blot data were analyzed by one-way analysis of variance followed by the Tukey *post hoc* test.

## Results

#### Infarct Size Measurement

Helium-induced preconditioning reduced infarct size in young animals from  $61 \pm 7\%$  in controls to  $36 \pm 14\%$  ( $P < 0.05$ ; fig. 2). Administration of ibuprofen during the preconditioning period completely abolished cardioprotection ( $60 \pm 11\%$ ; not significant [NS] *vs.* controls). Ibuprofen alone had no effect on infarct size ( $59 \pm 8\%$ ; NS *vs.* controls). Infarct size in aged controls was comparable with that in young controls ( $59 \pm 7\%$ ). In contrast to young rats, helium did not reduce infarct size in aged rats ( $58 \pm 8\%$ ; NS *vs.* aged controls; fig. 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, or reperfusion. At the end of the exper-

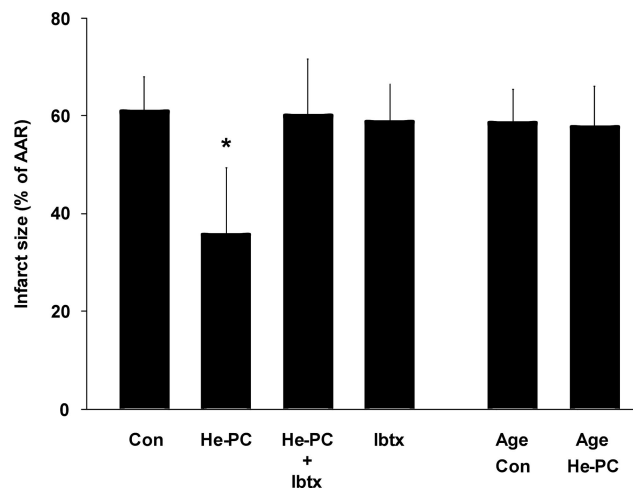


Fig. 2. Infarct size measurement. Histogram shows the infarct size (percent of area at risk [AAR]) of controls (Con), preconditioning with 70% helium (He-PC), preconditioning with 70% helium combined with ibuprofen (He-PC+Ibtx), ibuprofen alone (Ibtx), controls in aged rats (Age Con), and preconditioning in aged rats with 70% helium (Age He-PC). Data are presented as mean  $\pm$  SD. \*  $P < 0.05$  *versus* control group.

iments, mean aortic pressure and heart rate were significantly decreased compared with baseline in all groups.

#### Mitochondrial Function

The respiratory control indices are shown in figure 3. There was no significant difference in RCI between young ( $n = 8$ ) and aged ( $n = 8$ ) control rats ( $2.76 \pm 0.05$  *vs.*  $2.87 \pm 0.10$ ; NS). Helium preconditioning reduced the RCI in young rats ( $n = 8$ ;  $2.43 \pm 0.12$ ;  $P < 0.05$  *vs.* controls) but had no effect on RCI in aged rats ( $n = 8$ ;  $2.87 \pm 0.09$ ; NS *vs.* aged controls). RCI reduction was completely abolished by administration of the  $mK_{Ca}$  channel blocker ibuprofen ( $2.73 \pm 0.15$ ; NS *vs.* controls), whereas ibuprofen itself had no effect on RCI ( $2.75 \pm 0.05$ ; NS *vs.* controls). There was no difference between any groups in the efficiency of oxidative phosphorylation, as demonstrated by no changes in the ratio of phosphate incorporated into adenosine triphosphate to oxygen consumed.

#### Western Blot Analysis

Figure 4 shows that there was no difference of  $K_{Ca} \beta_1$  subunit expression in mitochondrial lysates from young and aged rat heart mitochondria (normalized to citrate synthase activity; young:  $143 \pm 23$  arbitrary units, old:  $153 \pm 12$  arbitrary units; NS). The analysis of citrate synthase activity showed no difference between young and old mitochondria (young:  $1,012 \pm 109$  mU/mg, aged:  $1,065 \pm 61$  mU/mg; NS).

## Discussion

The main findings of our study are that helium-induced preconditioning (1) is mediated by activation of  $K_{Ca}$

Table 1. Hemodynamic Variables

	Baseline	Washout 3	Ischemia	Reperfusion	
			15 min	30 min	120 min
Heart rate, beats/min					
Con	445 ± 27	428 ± 35	424 ± 35	397 ± 36	369 ± 33*
He-PC	448 ± 12	443 ± 21	449 ± 27	397 ± 36*	374 ± 32*
He-PC+Ibtx	435 ± 29	417 ± 31	426 ± 28	390 ± 23*	361 ± 25*
Ibtx	461 ± 31	439 ± 27	459 ± 20	429 ± 27	385 ± 40*
Age Con	421 ± 28	405 ± 24	410 ± 28	374 ± 37*	323 ± 44*
Age He-PC	409 ± 27	407 ± 28	402 ± 29	367 ± 33*	334 ± 34*
Mean aortic pressure, mmHg					
Con	127 ± 18	112 ± 20	101 ± 22	91 ± 23*	68 ± 11*
He-PC	133 ± 25	125 ± 23	108 ± 31	95 ± 23*	76 ± 19*
He-PC+Ibtx	117 ± 21	119 ± 17	101 ± 25	88 ± 21*	72 ± 9*
Ibtx	127 ± 24	129 ± 16	124 ± 16	94 ± 18*	65 ± 17*
Age Con	114 ± 26	114 ± 18	115 ± 24	98 ± 21	78 ± 21*
Age He-PC	119 ± 21	120 ± 20	111 ± 25	92 ± 16*	77 ± 19*

Data are mean ± SD.

\*  $P < 0.05$  vs. baseline.

Age = aged rats; Con = controls; He-PC = helium preconditioning; Ibtx = ibuprofen.

channels, (2) is accompanied by alterations in mitochondrial respiration, and (3) is abolished in the senescent heart.

In a recent study, the noble gas helium, a gas without anesthetic properties, was found to mimic the cardioprotective effect of preconditioning.<sup>19</sup> The results of the current study are in line with these previous findings that helium confers cardioprotection *in vivo* as seen by a strong infarct size reduction in the helium preconditioning group compared with control hearts.<sup>19</sup> It was beyond the scope of the current study to unravel the

complete mechanism of helium-induced preconditioning. However, our results demonstrate that activation of  $K_{Ca}$  channels is critically involved in the signal transduction pathway because the infarct size-reducing effect of helium was completely abrogated by the  $K_{Ca}$  channel antagonist ibuprofen. A central role of  $K_{Ca}$  channels in preconditioning has been shown by several studies demonstrating that either pharmacologic activation of these channels initiates cardioprotection or pharmacologic preconditioning can be blocked by  $K_{Ca}$  channel antagonists.<sup>14,15,29-31</sup> In 2002, Xu *et al.*<sup>15</sup> reported evidence for the existence of  $K_{Ca}$  channels in the inner mitochondrial membrane of ventricular myocytes, and the authors also

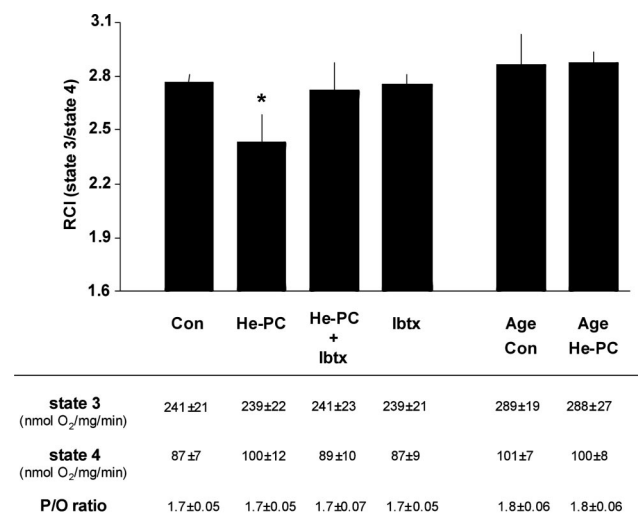


Fig. 3. Mitochondrial respiration. Summarized data for the effects of helium-induced preconditioning on mitochondrial respiration. Age = aged rats; Con = controls; He-PC = helium preconditioning; Ibtx = ibuprofen; P/O ratio = ratio between phosphate incorporated into adenosine triphosphate and oxygen consumed, a parameter for the efficiency of oxidative phosphorylation; RCI = respiratory control index, a parameter for the coupling between mitochondrial respiration and oxidative phosphorylation. Data are presented as mean ± SD. \*  $P < 0.05$  versus control group.

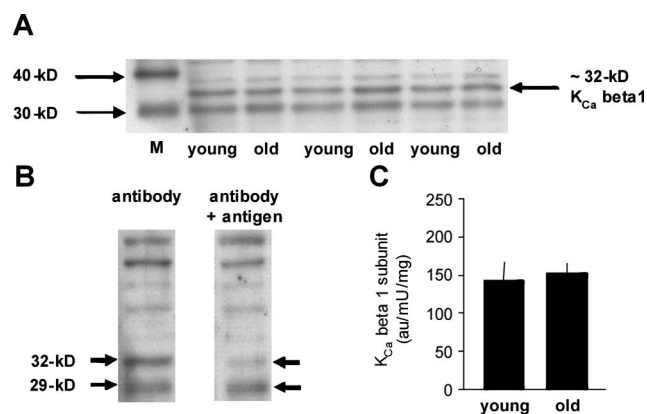


Fig. 4. Western blot analysis. (A) Representative Western blot ( $Ca^{2+}$ -sensitive potassium [ $K_{Ca}$ ] channel  $\beta_1$  subunit) showing two major bands at approximately 32 and 29 kD, respectively, in mitochondrial lysate from both young and old aged heart mitochondria. (B) Identification of specific band by immunizing peptide blocking experiment. The arrows denote positions of the 32- and 29-kD bands. Blocking the antibody with the antigen demasks the specific band (32 kD) by strongly reducing the intensity of the band (right), while the intensity of the 29-kD band (and other nonspecific bands) remains unchanged. (C) Summarized data of the Western blot analysis of  $K_{Ca}$  channel  $\beta_1$  subunit normalized to citrate synthase activity.

demonstrated a cardioprotective potency of  $mK_{Ca}$  channel activation. Recently, we showed that activation of  $mK_{Ca}$  channels increases mitochondrial state 4 respiration and reduces the respiratory control index in isolated guinea pig heart mitochondria.<sup>32</sup> In the current study, helium-induced preconditioning not only reduced infarct size, but also caused a significant reduction in the mitochondrial respiratory control index. Furthermore, helium-induced reduction in the respiratory control index was completely abolished by coadministration of iberiotoxin. We conclude from these data that helium confers cardioprotection by activation of  $mK_{Ca}$  channels, with the consequence of mild mitochondrial uncoupling. A mild mitochondrial uncoupling during the trigger phase of preconditioning may represent a common characteristic of mitochondria in a "preconditioned" state.<sup>15,33-35</sup> From our data, we cannot state whether  $mK_{Ca}$  channels exclusively mediate helium-induced preconditioning or whether other mitochondrial potassium channels (e.g.,  $mK_{ATP}$ ) are also involved. Interestingly, both the infarct size-reducing effect and the mitochondrial uncoupling were completely blocked by iberiotoxin. This suggests a crucial role of  $mK_{Ca}$  channels in helium-induced preconditioning.

The mechanism by which  $mK_{Ca}$  channel activation mediates cardioprotection is still incompletely understood. Opening of  $mK_{Ca}$  channels is capable of causing a slight increase in mitochondrial reactive oxygen species generation.<sup>32</sup> Stowe *et al.*<sup>31</sup> demonstrated that the cardioprotective effect of the  $K_{Ca}$  channel agonist NS1619 requires superoxide radical generation during the preconditioning stimulus. Furthermore, the authors demonstrated that preconditioning by NS1619 reduces mitochondrial calcium overload and mitochondrial reactive oxygen species production during the subsequent period of ischemia and early reperfusion.<sup>31</sup> Such a reduction in mitochondrial calcium overload and reactive oxygen species generation has been suggested to prevent mPTP opening.<sup>22,36</sup> Pagel *et al.*<sup>19</sup> demonstrated that the infarct size-reducing effect of helium was abolished by coadministration of the mPTP opener atracyloside, thereby showing that modulation of the mPTP is involved in helium-induced preconditioning. Furthermore, helium-induced preconditioning was initiated by acting on prosurvival signaling kinases including phosphatidylinositol-3-kinase, extracellular signal-regulated kinase, and 70-kd ribosomal protein s6 kinase. To our knowledge, there is no evidence that "prosurvival kinases" activate  $mK_{Ca}$  channels to regulate mitochondrial function. Recently, it was shown that adrenomedullin treatment before ischemia reduces infarct size *via* protein kinase A-mediated activation of  $mK_{Ca}$  channels.<sup>37</sup> This effect was independent of phosphatidylinositol-3-kinase. In the current study, we did not test whether "prosurvival kinases" or protein kinase A are involved in helium effects on  $K_{Ca}$  channel activation.

In the current study, helium-induced preconditioning did not reduce infarct size in the aged rat heart. There is strong evidence that the infarct size-reducing effects of cardioprotective interventions are diminished in the aged myocardium.<sup>12,38</sup> Tani *et al.*<sup>10</sup> demonstrated that ischemic preconditioning is not effective in middle-aged (50 weeks) hearts from Fischer 344 rats compared with hearts from young adults (12 weeks). Furthermore, in middle-aged rat hearts, direct activation of  $mK_{ATP}$  channels by diazoxide reduced infarct size, whereas protein kinase C activation by 1,2-dioctanoyl glycerol was ineffective in reducing infarct size. The authors concluded that the loss of protection by ischemic preconditioning in middle-aged hearts is partially caused by the failure to activate protein kinase C. Recently, Mio *et al.*<sup>39</sup> demonstrated that isoflurane-induced preconditioning is attenuated in aged human cardiomyocytes. Isoflurane caused a better preservation of mitochondrial respiratory capacity after hypoxia in mitochondria obtained from middle-aged compared with aged myocardium, and the authors concluded that the aging-related attenuation of isoflurane-induced preconditioning is caused by alterations in mitochondrial function. The underlying reason for the age-related loss of the cardioprotective potency of helium-induced preconditioning is yet unknown.

In the current study, we show not only that the infarct size-reducing effect of helium is lost in the senescent rat heart, but also that the helium-induced effect on mitochondrial respiration before the onset of lethal ischemia is abolished. Based on these data, we suggest that the aging-related blockade of helium-induced preconditioning is related to defects at the level of the  $mK_{Ca}$  channel or its upstream signaling cascade. Previously, we demonstrated that the effects of  $mK_{Ca}$  channel activation by NS1619 on mitochondrial respiration were reduced in isolated cardiac mitochondria from aged rats.<sup>18</sup> It has been demonstrated that cardiac mitochondria exist in two functionally distinct populations, subsarcolemmal and interfibrillar mitochondria.<sup>40</sup> Differences between these populations exist with regard to calcium handling and susceptibility to ischemic damage.<sup>41</sup> Fannin *et al.*<sup>42</sup> demonstrated that aging selectively decreased oxidative capacity in interfibrillar mitochondria, whereas respiration rates of subsarcolemmal mitochondria remained unchanged. Therefore, a limitation of the current study is that we investigated only a mixed population of both subsarcolemmal and interfibrillar mitochondria and did not test for differences between these mitochondrial subpopulations.

Furthermore, there is evidence that aging is associated with a decrease in  $K_{Ca}$  channel  $\beta_1$  subunit expression in the plasma membrane of coronary myocytes,<sup>43</sup> but it is unknown whether  $mK_{Ca}$  channel expression is also changed with increasing age. In the current study, we found that aging was without effect on  $mK_{Ca}$  channel  $\beta_1$  subunit expression, suggesting that the aging-related loss



of helium-induced cardioprotection is not caused by a decrease in  $mK_{Ca}$  channel density. However, this conclusion has to be proven in further studies, because the Western blot analysis of  $K_{Ca}$  channel  $\beta_1$  subunit expression was conducted from mitochondria that were isolated by differential centrifugation without further purification. Therefore, it might be possible that the mitochondrial preparation is contaminated with plasma membrane and that the Western blot band that we detected resulted at least partially from a possible contamination with sarcolemmal  $K_{Ca}$   $\beta_1$  subunit.

In summary, our results demonstrate that helium initiates preconditioning *via* activation of  $mK_{Ca}$  channels in the rat heart *in vivo*, but that helium's protective potency is abolished in the senescent heart.

## References

- Liu GS, Thornton J, Van Winkle DM, Stanley AW, Olsson RA, Downey JM: Protection against infarction afforded by preconditioning is mediated by A1 adenosine receptors in rabbit heart. *Circulation* 1991; 84:350-6
- Liu Y, Downey JM: Ischemic preconditioning protects against infarction in rat heart. *Am J Physiol* 1992; 263:H1107-12
- Murry CE, Jennings RB, Reimer KA: Preconditioning with ischemia: A delay of lethal cell injury in ischemic myocardium. *Circulation* 1986; 74:1124-36
- Cribier A, Korsatz L, Koning R, Rath P, Gamra H, Stix G, Merchant S, Chan C, Letac B: Improved myocardial ischemic response and enhanced collateral circulation with long repetitive coronary occlusion during angioplasty: A prospective study. *J Am Coll Cardiol* 1992; 20:578-86
- Deutsch E, Berger M, Kussmaul WG, Hirshfeld JW Jr, Herrmann HC, Laskey WK: Adaptation to ischemia during percutaneous transluminal coronary angioplasty: Clinical, hemodynamic, and metabolic features. *Circulation* 1990; 82:2044-51
- Devlin W, Cragg D, Jacks M, Friedman H, O'Neill W, Grines C: Comparison of outcome in patients with acute myocardial infarction aged > 75 years with that in younger patients. *Am J Cardiol* 1995; 75:573-6
- Haase KK, Schiele R, Wagner S, Fischer F, Burczyk U, Zahn R, Schuster S, Senges J: In-hospital mortality of elderly patients with acute myocardial infarction: Data from the MITRA (Maximal Individual Therapy in Acute Myocardial Infarction) registry. *Clin Cardiol* 2000; 23:831-6
- Maggioni AP, Maseri A, Fresco C, Franzosi MG, Mauri F, Santoro E, Tognoni G: Age-related increase in mortality among patients with first myocardial infarction treated with thrombolysis. The Investigators of the Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico (GISSI-2). *N Engl J Med* 1993; 329:1442-8
- Abete P, Ferrara N, Cioppa A, Ferrara P, Bianco S, Calabrese C, Cacciatore F, Longobardi G, Rengo F: Preconditioning does not prevent postischemic dysfunction in aging heart. *J Am Coll Cardiol* 1996; 27:1777-86
- Tani M, Suganuma Y, Hasegawa H, Shinmura K, Ebihara Y, Hayashi Y, Guo X, Takayama M: Decrease in ischemic tolerance with aging in isolated perfused Fischer 344 rat hearts: Relation to increases in intracellular  $Na^+$  after ischemia. *J Mol Cell Cardiol* 1997; 29:3081-9
- Lee TM, Su SF, Chou TF, Lee YT, Tsai CH: Loss of preconditioning by attenuated activation of myocardial ATP-sensitive potassium channels in elderly patients undergoing coronary angioplasty. *Circulation* 2002; 105:334-40
- Juhaszova M, Rabuel C, Zorov DB, Lakatta EG, Sollott SJ: Protection in the aged heart: Preventing the heart-break of old age? *Cardiovasc Res* 2005; 66:233-44
- O'Rourke B: Evidence for mitochondrial  $K^+$  channels and their role in cardioprotection. *Circ Res* 2004; 94:420-32
- Shintani Y, Node K, Asanuma H, Sanada S, Takashima S, Asano Y, Liao Y, Fujita M, Hirata A, Shinzaki Y, Fukushima T, Nagamachi Y, Okuda H, Kim J, Tomoike H, Hori M, Kitakaze M: Opening of  $Ca^{2+}$ -activated  $K^+$  channels is involved in ischemic preconditioning in canine hearts. *J Mol Cell Cardiol* 2004; 37:1213-8
- Xu W, Liu Y, Wang S, McDonald T, Van Eyk JE, Sidor A, O'Rourke B: Cytoprotective role of  $Ca^{2+}$ -activated  $K^+$  channels in the cardiac inner mitochondrial membrane. *Science* 2002; 298:1029-33
- Siemen D, Loupatatzis C, Borecky J, Gulbins E, Lang F:  $Ca^{2+}$ -activated  $K$  channel of the BK-type in the inner mitochondrial membrane of a human glioma cell line. *Biochem Biophys Res Commun* 1999; 257:549-54
- Murphy E, Steenbergen C: Preconditioning: The mitochondrial connection. *Annu Rev Physiol* 2007; 69:51-67
- Heinen A, Winning A, Schlack W, Hollmann MW, Preckel B, Frassdorf J, Weber NC: The regulation of mitochondrial respiration by opening of  $mK_{Ca}$  channels is age-dependent. *Eur J Pharmacol* 2008; 578:108-13
- Pagel PS, Krolkowski JG, Shim YH, Venkatapuram S, Kersten JR, Weihrauch D, Warltier DC, Pratt PF Jr: Noble gases without anesthetic properties protect myocardium against infarction by activating prosurvival signaling kinases and inhibiting mitochondrial permeability transition *in vivo*. *Anesth Analg* 2007; 105:562-9
- Koblin DD, Fang Z, Eger EI, Laster MJ, Gong D, Ionescu P, Halsey MJ, Trudell JR: Minimum alveolar concentrations of noble gases, nitrogen, and sulfur hexafluoride in rats: Helium and neon as nonimmobilizers (nonanesthetics). *Anesth Analg* 1998; 87:419-24
- Lee DL, Lee H, Chang HW, Chang AY, Lin SL, Huang YC: Heliox improves hemodynamics in mechanically ventilated patients with chronic obstructive pulmonary disease with systolic pressure variations. *Crit Care Med* 2005; 33:968-73
- Halestrap AP, Clarke SJ, Khaliulin I: The role of mitochondria in protection of the heart by preconditioning. *Biochim Biophys Acta* 2007; 1767:1007-31
- Obal D, Weber NC, Zacharowski K, Toma O, Dettwiler S, Wolter JI, Kratz M, Mullenheim J, Preckel B, Schlack W: Role of protein kinase C-epsilon (PK-Cepsilon) in isoflurane-induced cardioprotection. *Br J Anaesth* 2005; 94:166-73
- Toma O, Weber NC, Wolter JI, Obal D, Preckel B, Schlack W: Desflurane preconditioning induces time-dependent activation of protein kinase C epsilon and extracellular signal-regulated kinase 1 and 2 in the rat heart *in vivo*. *ANESTHESIOLOGY* 2004; 101:1372-80
- Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951; 193:265-75
- Bergmeyer HU: Methods of enzymatic analysis. Verlag Chemie 1974; 443-4
- Moreau R, Heath SH, Doneanu CE, Harris RA, Hagen TM: Age-related compensatory activation of pyruvate dehydrogenase complex in rat heart. *Biochem Biophys Res Commun* 2004; 325:48-58
- Sample J, Cleland JG, Seymour AM: Metabolic remodeling in the aging heart. *J Mol Cell Cardiol* 2006; 40:56-63
- Cao CM, Xia Q, Gao Q, Chen M, Wong TM: Calcium-activated potassium channel triggers cardioprotection of ischemic preconditioning. *J Pharmacol Exp Ther* 2005; 312:644-50
- Ohya S, Kuwata Y, Sakamoto K, Muraki K, Imaizumi Y: Cardioprotective effects of estradiol include the activation of large-conductance  $Ca^{2+}$ -activated  $K^+$  channels in cardiac mitochondria. *Am J Physiol Heart Circ Physiol* 2005; 289:H1635-42
- Stowe DF, Aldakkak M, Camara AK, Riess ML, Heinen A, Varadarajan SG, Jiang MT: Cardiac mitochondrial preconditioning by Big  $Ca^{2+}$ -sensitive  $K^+$  channel opening requires superoxide radical generation. *Am J Physiol Heart Circ Physiol* 2006; 290:H434-40
- Heinen A, Camara AK, Aldakkak M, Rhodes SS, Riess ML, Stowe DF: Mitochondrial  $Ca^{2+}$ -induced  $K^+$  influx increases respiration and enhances ROS production while maintaining membrane potential. *Am J Physiol Cell Physiol* 2007; 292:C148-56
- Liem DA, Manintveld OC, Schoonderwoerd K, McFalls EO, Heinen A, Verdouw PD, Sluiter W, Duncker DJ: Ischemic preconditioning modulates mitochondrial respiration, irrespective of the employed signal transduction pathway. *Transl Res* 2008; 151:17-26
- Ljubkovic M, Mio Y, Marinovic J, Stadnicka A, Warltier DC, Bosnjak ZJ, Bienengraeber M: Isoflurane preconditioning uncouples mitochondria and protects against hypoxia-reoxygenation. *Am J Physiol Cell Physiol* 2007; 292:C1583-90
- Minners J, Lacerda L, McCarthy J, Meiring JJ, Yellon DM, Sack MN: Ischemic and pharmacological preconditioning in Girardi cells and C2C12 myotubes induce mitochondrial uncoupling. *Circ Res* 2001; 89:787-92
- Lim SY, Davidson SM, Hausenloy DJ, Yellon DM: Preconditioning and postconditioning: The essential role of the mitochondrial permeability transition pore. *Cardiovasc Res* 2007; 75:530-5
- Nishida H, Sato T, Miyazaki M, Nakaya H: Infarct size limitation by adenomycin: Protein kinase A but not  $PI_3$ -kinase is linked to mitochondrial  $K_{Ca}$  channels. *Cardiovasc Res* 2008; 77:398-405
- Sniescinski R, Liu H: Reduced efficacy of volatile anesthetic preconditioning with advanced age in isolated rat myocardium. *ANESTHESIOLOGY* 2004; 100:589-97
- Mio Y, Bienengraeber MW, Marinovic J, Guterman DD, Rakic M, Bosnjak ZJ, Stadnicka A: Age-related attenuation of isoflurane preconditioning in human atrial cardiomyocytes: Roles for mitochondrial respiration and sarcolemmal adenosine triphosphate-sensitive potassium channel activity. *ANESTHESIOLOGY* 2008; 108:612-20
- Lesnfsky EJ, Hoppel CL: Oxidative phosphorylation and aging. *Ageing Res Rev* 2006; 5:402-33
- Duan J, Karmazyn M: Relationship between oxidative phosphorylation and adenine nucleotide translocase activity of two populations of cardiac mitochondria and mechanical recovery of ischemic hearts following reperfusion. *Can J Physiol Pharmacol* 1989; 67:704-9
- Fannin SW, Lesnfsky EJ, Slabe TJ, Hassan MO, Hoppel CL: Aging selectively decreases oxidative capacity in rat heart interfibrillar mitochondria. *Arch Biochem Biophys* 1999; 372:399-407
- Nishimaru K, Eghbali M, Lu R, Marjic J, Stefani E, Toro L: Functional and molecular evidence of MaxiK channel beta1 subunit decrease with coronary artery aging in the rat. *J Physiol* 2004; 559:849-62