Helium-induced cardioprotection: in sickness and in health, for better or for worse?
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Hypoxia induced late preconditioning in the rat heart in vivo

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INTRODUCTION

Ischemic heart disease and consequent heart failure are among the leading causes of morbidity and mortality in the western world\textsuperscript{1,2}, and are of significant relevance in perioperative medicine. Perioperative myocardial infarction occurs in about 4% of patients with either an established diagnosis or risk for coronary artery disease, who undergo major non-cardiac surgery\textsuperscript{3,4}. In patients undergoing elective vascular surgery, myocardial infarction is the most common fatal complication accounting for about 40% of postoperative fatalities within the first four years\textsuperscript{5}. These data suggest that particularly in high-risk patients the choice of an anesthetic regimen that preserves myocardial function may improve the postoperative outcome.

Myocardial damage results from an insufficiency of oxygen supply to cardiac cells to meet metabolic demands. It is well established that survival of myocardial tissue subjected to ischemia can be increased by prior exposure to repeated brief episodes of sublethal ischemia induced by transient coronary artery occlusion and reperfusion, a phenomenon known as ischemic preconditioning (PC)\textsuperscript{6}. Two phases of preconditioning are discriminated. An early phase (EPC), which develops within a few minutes and lasts for 2-3 hours, and a late phase (LPC), which develops more slowly (requiring 6-12 hours) but lasts for 2-3 days\textsuperscript{7}. A variety of other stimuli, e.g. the volatile anesthetic sevoflurane and the noble gas xenon, are also known to offer protection against ischemia-reperfusion injury\textsuperscript{8,9}. The clinical relevance of these processes has been demonstrated by Garcia and co-workers, showing that preconditioning with sevoflurane reduced the incidence of cardiac events during the first year after coronary artery bypass surgery from 17% to 3\textsuperscript{10}.

Several in vitro studies indicate that hypoxic LPC might be another powerful tool to protect against myocardial ischemia-reperfusion injury\textsuperscript{11-13}. However, there are no in vivo studies yet investigating the effect and concentration-dependency of hypoxia-induced LPC on the magnitude of myocardial ischemia-reperfusion injury. Based on the foregoing issues, the goal of the present study was to investigate whether different concentrations of sustained hypoxia induce LPC in the rat heart in vivo, and how potent this effect is compared to the well established effect of EPC with sevoflurane. We also tested whether the combination of hypoxic LPC with sevoflurane EPC is more effective than each stimulus alone. The signaling pathway involved in hypoxic LPC was investigated with special emphasis on the protein kinase C (PKC) isoforms PKC-epsilon and PKC-alpha, and on the expression of the hypoxia-inducible factor (HIF)-1alpha-dependent genes heme oxygenase-1 (HO-1) and vascular endothelial growth factor (VEGF), respectively, which have previously been implicated in the genesis of hypoxic...
and ischemic preconditioning\textsuperscript{14-18}.

**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 number 85-23, revised 1996), and was performed after approval of the Animal Ethics Committee of the University of Amsterdam, The Netherlands.

**Materials and Animals**

Hypoxic gas mixtures were purchased from Linde Gas (Linde Gas Benelux BV, Dieren, The Netherlands). The PKC-alpha antibody was purchased from Millipore (Billerica, MA, USA), and the PKC-epsilon antibody from Upstate Biotechnology (Lake Placid, NY, USA). All other chemicals were purchased from Sigma-Aldrich Chemie B.V. (Zwijndrecht, The Netherlands). Male Wistar rats (323±23 g) were obtained from Harlan (The Hague, The Netherlands), and kept with free access to standard food and water.

**Hypoxic exposure**

Rats were placed in a plexiglass chamber (Heidelberg, Germany) and randomly exposed to different concentrations of normobaric hypoxia (16%, 12%, and 8% oxygen, respectively) for 4 hours by using prepared gas mixtures with the corresponding oxygen concentrations balanced with nitrogen. Normoxic control experiments were conducted identically, except that room air (21% oxygen) instead of nitrogen was introduced into the chamber. The fresh gas flow was kept at 3 liter/minute to ensure that no accumulation of carbon dioxide occurs, which in turn might affect ventilation and thus oxygenation. The oxygen concentration within the chamber was controlled continuously (Datex Capnomac Ultima, Division of Instrumentarium Corp., Helsinki, Finland). After 4 hours of hypoxic exposure all rats were exposed to room air for 24 hours.

**Experimental protocol for infarct size determination**

Surgical preparation was performed as described previously\textsuperscript{19,20}. In brief, rats were anesthetized by intraperitoneal S-ketamine injection (150 mg/kg) followed by continuous
alpha-chloralose infusion (30 mg/kg/h), except for the groups that received sevoflurane (1 MAC) or propofol (11 mg/kg/h) continuously. After tracheal intubation, the lungs were ventilated with 30% oxygen and 70% nitrogen and a positive end-expiratory pressure of 2-3 cm H$_2$O. The right jugular vein was cannulated for saline and drug infusion, and the left carotid artery was cannulated for measurement of aortic pressure. 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 for a later occlusion of 25 minutes during the experiments. Aortic pressure and electrocardiographic signals were digitized using an analogue to digital converter (PowerLab/8SP, ADInstruments Pty Ltd, Castle Hill, Australia) and were continuously recorded on a personal computer using Chart for Windows v5.0 (ADInstruments).

After 120 minutes of reperfusion, the heart was excised and mounted on a modified Langendorff apparatus for infarct staining.$^{21}$ After staining with 0.2% Evans blue, the heart was cut into transverse slices, which were then stained with 0.75% triphenyltetrazoliumchloride solution. The area of risk and the infarcted area were determined by planimetry using SigmaScan Pro 5$^\text{®}$ computer software (SPSS Science Software, Chicago, IL).

**Study design**

The experimental protocol is shown in Figure 1. Part one of the study (all n=7-8): The purpose of this part was to investigate the effect of hypoxic LPC at different oxygen concentrations. Therefore rats were treated according to the following protocol (Fig. 1A): Twenty-four hours before the ischemia-reperfusion sequence started, rats were exposed for 4 hours to 21% oxygen (Con), 16% oxygen (LPC16), 12% oxygen (LPC12) or 8% oxygen (LPC8), respectively. To further investigate whether the effect of hypoxic LPC can be blocked by a specific PKC inhibitor, control rats and rats preconditioned with 16% oxygen received calphostin C (0.1 mg/kg) intravenously 35 minutes before myocardial ischemia was induced.

Part two of the study (all n=7-8): To compare the effect of hypoxic LPC with other preconditioning models, 3 groups of rats were subjected to the following protocol (Fig. 1B): the first group was exposed to EPC with sevoflurane (S-PC) with an end-tidal concentration of 1 minimum alveolar concentration (MAC) for 3x5 minutes interspersed with 2x5 minutes and one final 10 minutes washout period. The second group was exposed to continuous administration of 1 MAC sevoflurane (S), and the third group to continuous administration of propofol (P) in a concentration of 11 mg/kg/h. The latter two groups did not receive an anesthesia with alpha-chloralose.

Part three of the study (all n=9): The purpose of this part was to investigate whether
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Figure 1. Experimental protocol. Control = normoxic control, LPC16, -12, -8 = late preconditioning with 16%, 12% and 8% oxygen, S-PC = sevoflurane early preconditioning, Sevoflurane = sevoflurane continuously, Propofol = propofol continuously. A) study design to test the cardioprotective effect of hypoxic late preconditioning at different oxygen concentrations and the functional involvement of PKC, B) study design to test the cardioprotective effect of early preconditioning with sevoflurane and continuous administration of sevoflurane and propofol, respectively, and C) study design to test the cardioprotective effect of hypoxic late preconditioning combined with the groups from part B).
the combination of hypoxic LPC (4 hours at 16% oxygen) with either EPC with sevoflurane, or with continuous administration of sevoflurane or propofol, respectively, is more cardioprotective than each stimulus alone. Therefore, the protocols from part one and two of the study were combined, resulting in the following design (Fig. 1C): The first group of rats was exposed to hypoxic LPC combined with sevoflurane early preconditioning (LPC16+S-PC). The second group was exposed to hypoxic LPC combined with continuous administration of 1 MAC sevoflurane (LPC16+S), and the third group was subjected to hypoxic LPC combined with continuous administration of 11 mg/kg/h propofol (LPC16+P).

**Western blot analysis**

For Western Blot analysis of myocardial PKC-alpha and PKCepsilon additional experiments were performed. Six and 28 hours after the hypoxic exposure (16% oxygen for 4 hours) was initiated, hearts were excised (LPC16 6h and LPC16 28h, respectively, all n=12). Hearts from control animals (Con) were excised after exposure to 21% oxygen. Before excision of the heart rats underwent a sham operation in order to follow the same surgical protocol as in the infarct size experiments.

After protein determination by the Lowry method\(^2\) equal amounts of the cytosolic protein fraction were mixed with loading buffer containing Tris-hydrochloric acid, glycerol and bromphenol blue. Samples were vortexed and boiled at 95°C before being subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis. Samples were loaded on a sodium dodecyl sulfate electrophoresis gel. The proteins were separated by electrophoresis and then transferred to a polyvinylidene membrane by tank blotting. The respective gels were stained with coomassie brilliant blue solution in order to guarantee equal loading of protein. Unspecific binding of the antibody was blocked by incubation with 5% fat dry milk powder solution in Tris buffered saline for 2 hours. Subsequently, the membrane was incubated over night at 4 °C with the respective first antibody at indicated concentrations. After washing in fresh, cold Tris buffered saline, the blot was subjected to the appropriate Alexa fluor 688 or Alexa fluor 788 infrared conjugated secondary antibody for 2 hours at room temperature. Immunoreactive bands on the membrane were visualised by the two channel laser system of the Odyssey system. The blots were quantified using the Odyssey IR Imager\(^a\) (LI-COR Biotechnology, Bad Homburg, Germany).

**Semi-quantitative reverse transcriptase polymerase chain reaction assay**

HO-1 and VEGF messenger ribonucleic acid (mRNA) expression were measured by
reverse transcriptase polymerase chain reaction assay. Total RNA was isolated with Trizol according to the manufacturer’s instructions (Invitrogen, Darmstadt, Germany). Two micrograms total RNA were reverse transcribed (Promega, Mannheim, Germany). To quantify VEGF, HO-1 and glyceraldehyde 3-phosphate dehydrogenase mRNAs, gene-specific primers were used. Details about the primer sequences and reverse transcriptase polymerase chain reaction conditions are provided in the Supplemental Digital Content 1. Amplified products were resolved by electrophoresis on 2% agarose gels and stained with ethidium bromide. Gels were photographed under ultraviolet transillumination with a digital camera, and the images were transferred to a computer for densitometric analysis (Gelscan Software, BioSciTec, Frankfurt, Germany). Final results were expressed as the ratio of HO-1- and VEGF-mRNA to glyceraldehyde 3-phosphate dehydrogenase-mRNA for each sample.

**Statistical Analysis**

Normal distribution of the data was tested using the Kolmogorov-Smirnov test. Infarct sizes were analyzed by a one-way analysis of variance (ANOVA) followed by Tukey’s post-hoc test. Western blot and reverse transcriptase polymerase chain reaction data were analyzed by a Kruskal-Wallis one-way ANOVA followed by Student-Newman-Keuls post-hoc test. Data that were obtained at multiple time-points throughout the experiment, such as heart rate and blood pressure, were analyzed using one-way repeated measures ANOVA, followed by Holm-Sidak post-hoc test. Data are expressed as mean ± SD. Changes within and between groups were considered statistically significant if P<0.05. Statistics were performed using SPSS Science Software, version 17.0.
CHAPTER 3

RESULTS

Infarct size measurement

As summarized in Table 1, hypoxic LPC significantly reduced infarct size at all tested oxygen concentrations (all P<0.001 vs. normoxic controls). The cardioprotective effect was similar between 16%, 12% and 8% oxygen, respectively (P=0.84, Table 1). The PKC inhibitor calphostin C completely abolished the preconditioning effect of hypoxic LPC at 16% oxygen (p<0.001), but had no effect on infarct size when it was administered to normoxic controls (p=0.30, Table 1 and Figure 2).

Early preconditioning with intermittent administration of sevoflurane (S-PC) reduced infarct size (P<0.01 vs. normoxic controls) to about the same magnitude as hypoxic LPC (P=0.87, Table 1). In contrast, continuous administration of sevoflurane (S) and propofol (P) were not cardioprotective (P=0.77 and P=0.96, respectively, vs. normoxic controls, Table 1). As shown in Table 1, the combination of LPC16 with sevoflurane early preconditioning (LPC16+S-PC) was not superior to the cardioprotective effect of each substance alone (P<0.05 vs. normoxic control, P=0.87 vs. LPC16, P=0.85 vs. S-PC). The cardioprotective effect of LPC16 was not affected by the two non-protective interventions with continuous administration of sevoflurane or propofol. In other words, hypoxic LPC was still able to reduce infarct size when it was combined with the continuous administration of sevoflurane (LPC16+S) or propofol (LPC16+P), respectively (P<0.01 vs. control).

Figure 2. Infarct size measurement. Histogram shows the infarct size (percent of area at risk) of controls, hypoxic late preconditioning with 16% oxygen (LPC16), hypoxic late preconditioning with 16% oxygen combined with Calphostin C (LPC+Calphostin C) and Calphostin C alone. Data are presented as mean ± SD, *p < 0.05 vs. control group.
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Hemodynamic variables

Hemodynamic variables are summarized in Table 2. In all groups heart rate was decreased at the end of the experiment compared to the baseline values (P<0.05). Only when rats were exposed to continuous administration of propofol (P) the decrease in heart rate just failed to reach significance (P=0.05). The decrease in heart rate was paralleled by a decrease in mean aortic pressure (P<0.05). However, continuous administration of sevoflurane, both alone or in combination with LPC16, prevented the decrease in mean blood pressure.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Infarct Size (% of AAR)</th>
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<tbody>
<tr>
<td>Con</td>
<td>8</td>
<td>62 ± 6</td>
</tr>
<tr>
<td>LPC16</td>
<td>8</td>
<td>36 ± 11</td>
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<tr>
<td>LPC12</td>
<td>8</td>
<td>38 ± 10</td>
</tr>
<tr>
<td>LPC8</td>
<td>8</td>
<td>39 ± 11</td>
</tr>
<tr>
<td>LPC16+Calphostin C</td>
<td>7</td>
<td>57 ± 11</td>
</tr>
<tr>
<td>Calphostin C</td>
<td>8</td>
<td>58 ± 9</td>
</tr>
<tr>
<td>S-PC</td>
<td>7</td>
<td>40 ± 8</td>
</tr>
<tr>
<td>S</td>
<td>7</td>
<td>53 ± 6</td>
</tr>
<tr>
<td>P</td>
<td>7</td>
<td>56 ± 10</td>
</tr>
<tr>
<td>LPC16+S-PC</td>
<td>9</td>
<td>37 ± 12</td>
</tr>
<tr>
<td>LPC16+S</td>
<td>9</td>
<td>40 ± 10</td>
</tr>
<tr>
<td>LPC16+P</td>
<td>9</td>
<td>42 ± 10</td>
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</table>

Table 1. Infarct sizes related to area at risk (AAR). Data are Mean ± SD. Con = normoxic control group; LPC16, -12, -8 = late preconditioning with 16%, 12% and 8% oxygen; Calphostin C = specific inhibitor of protein kinase C; S-PC = sevoflurane preconditioning; S = sevoflurane continuously; P = propofol continuously; *p<0.05 vs. Con, #p<0.05 vs. LPC16.
### Table 2. Hemodynamic variables. Data are Mean ± SD. Con = control group; LPC16, -12, -8 = late preconditioning with 16%, 12% and 8% oxygen; Calphostin C = specific inhibitor of protein kinase C; S-PC = sevoflurane preconditioning; S = sevoflurane continuously; P = propofol continuously. *p<0.05 vs. Con, #p<0.05 vs. LPC16, $p<0.05 vs. LPC16+S-PC, §p<0.05 vs. baseline

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Washout 3</th>
<th>Ischemia 15 min</th>
<th>Ischemia 30 min</th>
<th>Ischemia 120 min</th>
<th>Reperfusion 15 min</th>
<th>Reperfusion 30 min</th>
<th>Reperfusion 120 min</th>
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<tr>
<td><strong>Heart Rate (BPM)</strong></td>
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<tr>
<td>Con</td>
<td>410 ± 27</td>
<td>403 ± 28</td>
<td>399 ± 19</td>
<td>365 ± 18&lt;sup&gt;i&lt;/sup&gt;</td>
<td>322 ± 28&lt;sup&gt;i&lt;/sup&gt;</td>
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<tr>
<td>LPC16</td>
<td>419 ± 34</td>
<td>411 ± 31</td>
<td>407 ± 35</td>
<td>380 ± 30</td>
<td>350 ± 35&lt;sup&gt;j&lt;/sup&gt;</td>
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<tr>
<td>LPC12</td>
<td>415 ± 27</td>
<td>408 ± 23</td>
<td>407 ± 20</td>
<td>390 ± 22&lt;sup&gt;i&lt;/sup&gt;</td>
<td>361 ± 34&lt;sup&gt;i&lt;/sup&gt;</td>
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<tr>
<td>LPC8</td>
<td>426 ± 33</td>
<td>414 ± 23</td>
<td>407 ± 32</td>
<td>364 ± 35&lt;sup&gt;i&lt;/sup&gt;</td>
<td>314 ± 34&lt;sup&gt;i&lt;/sup&gt;</td>
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<tr>
<td>LPC16+Calphostin C</td>
<td>423 ± 16</td>
<td>415 ± 14</td>
<td>428 ± 15</td>
<td>377 ± 15&lt;sup&gt;i&lt;/sup&gt;</td>
<td>324 ± 13&lt;sup&gt;i&lt;/sup&gt;</td>
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<tr>
<td>Calphostin C</td>
<td>418 ± 18</td>
<td>411 ± 21</td>
<td>420 ± 21</td>
<td>373 ± 17&lt;sup&gt;i&lt;/sup&gt;</td>
<td>325 ± 17&lt;sup&gt;i&lt;/sup&gt;</td>
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<tr>
<td>S-PC</td>
<td>368 ± 37</td>
<td>333 ± 22&lt;sup&gt;ii&lt;/sup&gt;</td>
<td>320 ± 28&lt;sup&gt;iii&lt;/sup&gt;</td>
<td>283 ± 17&lt;sup&gt;ii&lt;/sup&gt;</td>
<td>311 ± 22&lt;sup&gt;i&lt;/sup&gt;</td>
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<tr>
<td>S</td>
<td>355 ± 25&lt;sup&gt;*&lt;/sup&gt;</td>
<td>359 ± 33</td>
<td>339 ± 33&lt;sup&gt;*&lt;/sup&gt;</td>
<td>341 ± 39</td>
<td>330 ± 17&lt;sup&gt;i&lt;/sup&gt;</td>
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<tr>
<td>P</td>
<td>399 ± 35</td>
<td>380 ± 54</td>
<td>408 ± 24</td>
<td>376 ± 24</td>
<td>360 ± 31</td>
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<tr>
<td>LPC16+S-PC</td>
<td>414 ± 32</td>
<td>368 ± 24&lt;sup&gt;ii&lt;/sup&gt;</td>
<td>377 ± 24&lt;sup&gt;i&lt;/sup&gt;</td>
<td>343 ± 15&lt;sup&gt;i&lt;/sup&gt;</td>
<td>299 ± 21&lt;sup&gt;i&lt;/sup&gt;</td>
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<tr>
<td>LPC16+S</td>
<td>359 ± 24&lt;sup&gt;ii&lt;/sup&gt;</td>
<td>348 ± 27&lt;sup&gt;ii&lt;/sup&gt;</td>
<td>351 ± 36&lt;sup&gt;ii&lt;/sup&gt;</td>
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<td>331 ± 35&lt;sup&gt;i&lt;/sup&gt;</td>
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<tr>
<td>LPC16+P</td>
<td>428 ± 24</td>
<td>418 ± 35</td>
<td>392 ± 57</td>
<td>398 ± 51</td>
<td>360 ± 27&lt;sup&gt;i&lt;/sup&gt;</td>
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<table>
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<tr>
<th><strong>Mean aortic pressure (mmHg)</strong></th>
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<tbody>
<tr>
<td>Con</td>
</tr>
<tr>
<td>LPC16</td>
</tr>
<tr>
<td>LPC12</td>
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<tr>
<td>LPC8</td>
</tr>
<tr>
<td>LPC16+Calphostin C</td>
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<tr>
<td>Calphostin C</td>
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<td>S-PC</td>
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<td>S</td>
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<tr>
<td>LPC16+S-PC</td>
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<tr>
<td>LPC16+S</td>
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<tr>
<td>LPC16+P</td>
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Table 2. Hemodynamic variables. Data are Mean ± SD. Con = control group; LPC16, -12, -8 = late preconditioning with 16%, 12% and 8% oxygen; Calphostin C = specific inhibitor of protein kinase C; S-PC = sevoflurane preconditioning; S = sevoflurane continuously; P = propofol continuously. *p<0.05 vs. Con, #p<0.05 vs. LPC16, $p<0.05 vs. LPC16+S-PC, §p<0.05 vs. baseline
Expression of PKC-alpha and PKC-epsilon

Figure 3 shows that hypoxic preconditioning with 16% oxygen was associated with an increased myocardial expression of PKC-epsilon at 6 hours after the hypoxic stimulus was initiated (n=12; P=0.01 vs. control). At 28 hours the PKC-epsilon expression remained elevated and was of about the same magnitude as after 6 hours (n=12; P=0.91 vs. 6 hours, P=0.01 vs. control). In contrast, at the same time points the myocardial expression of PKC-alpha was not affected by hypoxic preconditioning with 16% oxygen (PKC-alpha/tubulin ratio for control: 0.49±0.08; for LPC16 at 6 hours: 0.47±0.05, for LPC16 at 28 hours: 0.50±0.08; P=0.81; not shown).

Expression of HO-1 and VEGF

Hypoxic preconditioning with 16% oxygen transiently upregulated the myocardial expression of the HIF-1-alpha-dependent genes HO-1 and VEGF (Fig. 4 and Fig. 5). Six hours after the hypoxic stimulus was initiated the expression of HO-1 and VEGF was increased by about 60% and 28%, respectively (each n=6; both P<0.05). However, at 28 hours the expression of both HO-1 and VEGF had returned to baseline values again (each n=6; P=0.50 and P=0.75 vs. control; Fig. 4 and Fig. 5).
Figure 4. Heme oxygenase (HO)-1 mRNA expression in rat myocardium. Representative reverse transcriptase polymerase chain reaction analysis experiments of a time course (Control, 6 hours, 28 hours after the hypoxic stimulus was initiated). Summarized data presenting ratio of HO-1 to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) are shown. Data are presented as mean ± SD. *p < 0.05 vs. Control.

Figure 5. Vascular endothelial growth factor (VEGF) mRNA expression in rat myocardium. Representative reverse transcriptase polymerase chain reaction analysis experiments of a time course (Control, 6 hours, 28 hours after the hypoxic stimulus was initiated). Two major bands of VEGF mRNA represent polymerase chain reaction analysis fragments amplified from VEGF188 and VEGF164 mRNAs. Summarized data presenting ratio of VEGF to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) are shown. Data are presented as mean ± SD. *p < 0.05 vs. Control.
DISCUSSION

It is well established that ischemic preconditioning represents a powerful and reproducible tool of cardioprotection against myocardial ischemia\textsuperscript{23}, but yet it has not been translated into routine clinical use. One of the reasons that limits the clinical applicability of ischemic preconditioning of the heart is that an invasive procedure is required to induce ischemia. Although this might be possible in some situations, e.g. by intermittent aortic cross clamping during coronary artery bypass surgery\textsuperscript{24}, it is not applicable in many others, e.g. during non-thoracic surgery. Much effort has therefore been focused on identifying preconditioning stimuli that are non-invasive, easy to administer and thus clinically more relevant.

Among various other stimuli (e.g. nitric oxide and volatile anesthetics\textsuperscript{23,25}), hypoxia may represent a potent preconditioning tool. The attraction of using hypoxia as preconditioning stimulus is that it very much resembles the stress that ultimately leads to myocardial infarction (i.e. cellular hypoxia/anoxia). An advantage of hypoxic preconditioning compared to ischemic preconditioning is that its systemic administration does not only exert a local effect but rather affects multiple organ systems which thus may also be protected against ischemic insults as it has been shown for the brain, liver, kidney, and other organ systems\textsuperscript{26-28}.

As indicated by several in vitro studies, intermittent exposure to 10% oxygen for 4 hours induced late cardioprotection in the isolated rat heart\textsuperscript{11,13}. Moreover, continuous hypoxic exposure to 10% oxygen for 4 hours followed by 24 hours of reoxygenation reduced infarct size in the isolated mouse heart, whereas shorter periods (30 minutes and 2 hours, respectively) remained uneffective\textsuperscript{12}. In contrast, in one study 10% oxygen for 4 hours had no cardioprotective effect\textsuperscript{11}. However, although different protocols of hypoxia-induced myocardial preconditioning have been proven to be effective in vitro its protective effect remains to be established in vivo.

Hypoxia induces late preconditioning in vivo

In the present study we demonstrate that continuous exposure to 4 hours hypoxia followed by 24 hours of normoxic reoxygenation induces LPC in the rat heart in vivo and reduces infarct size by nearly 40% compared to non-preconditioned myocardium. Interestingly, the extent of cardioprotection induced by 16% oxygen was comparable to the cardioprotection induced by 12% or 8% oxygen. However, because the infarct size slightly increased with the decrease in oxygen concentration (i.e. infarct size related to area at risk at 16% oxygen = 36%, at 12% oxygen = 38%, and at 8% = 39%), the results
do not exclude the possibility of as less pronounced cardioprotection with decreased oxygen concentrations. Indeed, a previous study by Béguin and co-workers\textsuperscript{11} showed that in a rat model intermittent hypoxia with 10% oxygen for 4 hours significantly reduced infarct size whereas the same protocol with 5% oxygen significantly increased infarct size. This observation suggests that to severe hypoxia renders the myocardium more sensitive to ischemic injury. The reason for this was not explored but is conceivable that a very low oxygen tension compromises oxygen supply to the tissue too much and itself induces cell damage with detrimental consequences. However, our results do not exclude the possibility that doses higher than 16% but lower than 21% oxygen also induce LPC.

**Potency of hypoxia-induced late preconditioning**

We next investigated, for the first time, how potent the observed cardioprotective effect of hypoxic LPC is compared to the well established effect of EPC with sevoflurane. In these experiments EPC with sevoflurane reduced infarct size by about 38%, which is in line with previous findings\textsuperscript{29}. This finding indicates that the cardioprotective effect elicited by sevoflurane EPC and hypoxic LPC is comparable. In contrast, when sevoflurane was administered continuously without a washout period before myocardial ischemia was induced, no reduction in infarct size was observed. This is in accordance with a previous clinical study from Bein and co-workers showing that continuous administration of 1 MAC sevoflurane from induction to start of cardiopulmonary bypass did not result in any protection compared with the control group\textsuperscript{30}. However, when the administration of sevoflurane before cardiopulmonary bypass was interrupted for 10 minutes, an improved myocardial performance and decreased postoperative troponin T values were observed. These data, in conjunction with our findings, suggest that interrupted administration of the anesthetic may be an essential feature for the occurrence of clinically relevant cardioprotective effects. This hypothesis is supported by our observation that continuous administration of propofol also exerted no cardioprotective effect.

These findings are of special interest for the clinical scenario when myocardial ischemia occurs by chance and cannot be timed, as it is possible, for example, in coronary artery bypass surgery. In the situation where a washout phase cannot be planned myocardial ischemia likely occurs during the administration of the anesthetics, i.e. when sevoflurane and propofol were not cardioprotective in our study. In these situations the clinical applicability of EPC is clearly limited. Thus, as pointed out elsewhere, LPC is likely to be of greater clinical relevance than EPC because it lasts 30-40 times longer and can easily be initiated before the expected ischemic event may occur\textsuperscript{23}. Because our results show that LPC with hypoxia was as cardioprotective as
EPC with sevoflurane, this finding underlines the potential relevance of hypoxia as LPC stimulus. This might especially be true in conjunction with previous data indicating that late but not early preconditioning protects against reversible postischemic contractile dysfunction (myocardial “stunning”).

**Hypoxic late preconditioning cannot be enhanced by modified anesthetic techniques**

A study by Müllenheim et al. showed that combining ischemic LPC with ischemic EPC led to enhanced cardioprotection compared to each stimulus alone. The same group showed that cardioprotection by ischemic LPC could be increased by sevoflurane-induced EPC. Therefore, we investigated whether the cardioprotective effect of hypoxic LPC could be enhanced by EPC with sevoflurane or by continuous administration of the two clinically used anesthetics sevoflurane and propofol. Interestingly, to the best of our knowledge, there is no study yet investigating the cardioprotective effect of LPC combined with continuous administration of sevoflurane or propofol. Our results demonstrate that none of the tested combinations was superior to the cardioprotection elicited by hypoxic LPC alone. However, the results also demonstrate that hypoxic LPC was not impaired by continuous administration of sevoflurane or propofol, highlighting the applicability and potential relevance of hypoxic LPC in the clinical setting.

**Signalling cascade of hypoxic late preconditioning**

Myocardial hypoxia triggers a wide range of profound cellular responses, including regulation of gene expression. In several studies protein kinase C (PKC), an ubiquitous intracellular mediator, has been shown to play a pivotal role in the signaling pathway of LPC, elicited by a variety of agents. Notably, the isoform PKC-epsilon appears to be responsible for the development of delayed cardioprotection, but in some studies PKC-alpha has also been implicated in provoking ischemic preconditioning of the heart.

Our results show that the PKC inhibitor calphostin C completely blocked the cardioprotective effect of hypoxic LPC, supporting the close association between PKC activation and the infarct size limitation conferred by hypoxic LPC. Although calphostin C is not isoform selective it has previously been shown to inhibit the novel isoforms of PKC, including PKC-epsilon, more efficiently than the conventional ones. Moreover, a previous study characterizing the expression of PKC isoforms in rat ventricular myocytes found that the dominant isoform was PKC-epsilon. Indeed, we found that 6 hours after the preconditioning stimulus was initiated the expression of PKC-epsilon was increased and that this increase remained stable until myocardial ischemia was
induced (i.e. after 28 hours). In contrast, the expression of PKC-alpha was not affected by hypoxic LPC. These observations indicate that PKC-epsilon, but not PKC-alpha, is the dominant isoform involved in the cardioprotective effect of hypoxic LPC in the rat heart in vivo. This is in line with a series of previous studies showing that translocation and activation of PKC-epsilon, but not of the PKC isoforms alpha, beta, gamma, delta, zeta, iota, lamda and mu is necessary for ischemic LPC in vivo\textsuperscript{34,37,38}. The reasons for the controversy between the studies regarding the involvement of PKC-alpha in mediating LPC are unclear but may be related to differences in species (canine, rabbit, humans and rat) and type of preconditioning (ischemic and hypoxic). However, we cannot exclude that other PKC isoforms that have not been studied in this report may contribute to cardioprotection elicited by hypoxic LPC in the rat.

Previous studies suggest that PKC plays a pivotal role in the upregulation of the HIF-1alpha-dependent genes HO-1 and VEGF\textsuperscript{39,40}, both of which have previously been implicated in mediating hypoxic LPC\textsuperscript{16-18,41}. HO-1 degrades heme and generates carbon monoxide and bilirubin, which may have antioxidant and/or anti-inflammatory effects\textsuperscript{42}. Indeed, mice overexpressing cardiac-specific HO-1 show a significant reduction in infarct size following ischemia-reperfusion\textsuperscript{18}. VEGF regulates local oxygen supply, and may be important in ischemia-reperfusion because it mediates neovascularization during myocardial ischemia\textsuperscript{43,44}. Our finding of the transient upregulation of HO-1 and VEGF mRNA provides further evidence for the involvement of these two effector genes in hypoxic LPC. Along with the hypothesis that PKC is involved in HO-1 and VEGF expression, it appeared conceivable that inhibition of PKC prevents the HO-1 and VEGF-mediated reduction in infarct size. Indeed, the observation that calphostin C completely abolished the cardioprotective effect of hypoxic LPC is consistent with this hypothesis. Moreover, our calphostin C results support the assumption that in the rat myocardium PKC lies upstream in the signaling pathway of HO-1 and VEGF expression, as proposed by others\textsuperscript{16}.

**Limitations of the study**

A limitation of the study is that we did not determine the oxygen threshold below which hypoxia starts to exert its cardioprotective effects. We cannot exclude that oxygen concentration higher than 16% and lower than 21% are also cardioprotective.

Another limitation of our study is that pharmacological inhibitors like calphostin C may affect other enzymes to a certain extent. Their specificity strongly depends on the concentration used. For example, calphostin C inhibits PKC with a half maximal inhibitory concentration of 50 nM, and other kinases like protein kinase A (half maximal inhibitory concentration >50 μM), p60v-src (half maximal inhibitory concentration
>50 μM) and protein kinase G (half maximal inhibitory concentration >25 μM) are inhibited only at higher concentrations.

In summary, our results show that 1) hypoxic LPC results in profound protection against myocardial ischemia-reperfusion injury in the rat heart in vivo, as evidenced by a significant decrease in infarct size, 2) mild hypoxia (16% oxygen) induces cardioprotection of about the same magnitude as moderate (12% oxygen) and severe (8% oxygen) hypoxia, 3) the cardioprotective effect of hypoxia-induced LPC is as powerful as the cardioprotective effect of EPC with sevoflurane, and 4) PKC-epsilon is the dominant isoform involved in the cardioprotective effect of hypoxic LPC in the rat heart in vivo. These findings may provide a conceptual framework for developing novel therapeutic strategies aimed at a powerful cardioprotection especially in situations where myocardial ischemia occurs by chance and early preconditioning strategies are not applicable.
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Hypoxia induced late preconditioning in the rat heart in vivo


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