

# Hypoxia Induces Late Preconditioning in the Rat Heart *In Vivo*

Marc M. Berger, M.D.,\* Ragnar Huhn, M.D., Ph.D.,† Gezina T. Oei, B.Sc., M.Sc.,‡  
 André Heinen, M.D., Ph.D.,§ Andreas Winzer, M.D.,\* Inge Bauer, Ph.D.,||  
 Benedikt Preckel, M.D., M.A., D.E.A.A.,# Nina C. Weber, Ph.D.,\*\*  
 Wolfgang Schlack, M.D., D.E.A.A.,†† Markus W. Hollmann, M.D., Ph.D., D.E.A.A.‡‡

## ABSTRACT

**Background:** Although hypoxic late preconditioning (LPC) limits ischemia-reperfusion injury *in vitro*, its cardioprotective effect is not established *in vivo*.

**Methods:** In part 1, rats were exposed to 4 h of hypoxia (16%, 12%, 8% oxygen) before 24 h of reoxygenation. In part 2, normoxic rats received early preconditioning with sevoflurane (1 minimum alveolar concentration [MAC] for 3 × 5 min), continuous administration of 1 MAC sevoflurane, or 11 mg · kg · h propofol. Thereafter, all rats underwent 25 min of regional myocardial ischemia and 120 min of reperfusion. After reperfusion, hearts were excised for infarct staining. The expression of protein kinase C (PKC)α and PKCε was assessed by Western blot analysis and the expression of heme oxygenase-1 and vascular endothelial growth factor by reverse transcriptase polymerase chain reaction.

**Results:** In normoxic control rats, infarct size was 62 ± 6% of the area at risk. Hypoxic LPC reduced infarct size (LPC16: 36 ± 11%, LPC12: 38 ± 10%, LPC8: 39 ± 11%; each  $P < 0.001$ ) to approximately the same magnitude as sevoflurane-preconditioning (40 ± 8%;  $P < 0.001$ ). Combined LPC16 and sevoflurane preconditioning was not superior to either substance alone. Continuous sevoflurane or propofol was not protective. The PKC inhibitor calphostin C abolished the cardioprotective effects of LPC16. PKCε, but not PKCα, expression was increased 6 and 28 h after hypoxic LPC. Heme oxygenase-1 and vascular endothelial growth factor were transiently up-regulated after 6 h.

**Conclusion:** Hypoxic LPC at 8%, 12%, and 16% oxygen reduces infarct size in the rat heart *in vivo*. This effect is as powerful as sevoflurane-preconditioning. PKCε is a key player in mediating hypoxic LPC.

\* Staff Anesthesiologist, Department of Anesthesiology, University Hospital of Heidelberg, Heidelberg, Germany. † Resident, ‡ Research Scientist, Department of Anesthesiology, § Resident, Department of Hemostasis and Transfusion Medicine, University Hospital of Duesseldorf, Duesseldorf, Germany. ‡ Doctoral Student, # Associate Professor, †† Professor and Chair, Department of Anesthesiology, \*\* Assistant Professor, ‡‡ Professor and Chair, Department of Experimental and Clinical Experimental Anesthesiology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands.

Received from the Department of Anesthesiology, University Hospital of Heidelberg, Heidelberg, Germany; Departments of Anesthesiology and Hemostasis and Transfusion Medicine, University Hospital of Duesseldorf, Duesseldorf, Germany; and Department of Anesthesiology, Laboratory of Experimental Intensive Care and Anesthesiology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands. Submitted for publication April 5, 2010. Accepted for publication September 13, 2010. Supported by the European Association of Cardiothoracic Anaesthesiologists (Dublin, Ireland) (to Dr. Huhn). Drs. Berger and Huhn contributed equally to this work. Presented in part at the Annual Meeting of the European Society of Anaesthesiology, Euroanaesthesia, Milan, Italy, June 7, 2009, and at the Annual Meeting of the American Society of Anesthesiologists, New Orleans, Louisiana, October 18, 2009.

Address correspondence to Dr. Huhn: Department of Anesthesiology, University Hospital Duesseldorf, Moorenstrasse 5, 40225, Duesseldorf, Germany. ragnar.huhn@med.uni-duesseldorf.de. Information on purchasing reprints may be found at [www.anesthesiology.org](http://www.anesthesiology.org) or on the masthead page at the beginning of this issue. ANESTHESIOLOGY's articles are made freely accessible to all readers, for personal use only, 6 months from the cover date of the issue.

## What We Already Know about This Topic

- ❖ Hypoxic late preconditioning (LPC) is a cardioprotective strategy that increases resistance to ischemia and reperfusion by eliciting innate protective mechanisms.

## What This Article Tells Us That Is New

- ❖ The signaling enzyme, PKCε, is essential mediating hypoxia-induced LPC in the rat heart *in vivo*.
- ❖ Hypoxia-induced LPC is as potent as sevoflurane-induced preconditioning in reducing infarct size.

**I**SCHEMIC heart disease and heart failure are among the leading causes of morbidity and mortality in the Western world<sup>1,2</sup> and of significant relevance to perioperative medicine. Perioperative myocardial infarction occurs in approximately 4% of patients with either an established diagnosis or risk for coronary artery disease undergoing major noncardiac

Supplemental digital content is available for this article. Direct URL citations appear in the printed text and are available in both the HTML and PDF versions of this article. Links to the digital files are provided in the HTML text of this article on the Journal's Web site ([www.anesthesiology.org](http://www.anesthesiology.org)).

surgery.<sup>3,4</sup> In patients undergoing elective vascular surgery, myocardial infarction is the most common fatal complication, accounting for approximately 40% of postoperative fatalities within the first 4 yr.<sup>5</sup> These data suggest that, particularly in high-risk patients, the choice of an anesthetic regimen that preserves myocardial function may improve 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 previous exposure to repeated brief episodes of sublethal ischemia induced by transient coronary artery occlusion and reperfusion, a phenomenon known as ischemic preconditioning.<sup>6</sup> Two phases of preconditioning are delineated, an early phase (EPC), which develops within a few minutes and lasts for 2–3 h, and a late phase (LPC), which develops more slowly (requiring 6–12 h) but lasts for 2–3 days.<sup>7</sup> 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.<sup>8,9</sup> The clinical relevance of these processes has been demonstrated by Garcia *et al.*,<sup>10</sup> who showed that preconditioning with sevoflurane reduced the incidence of cardiac events during the first year after coronary artery bypass surgery to 3% from 17%.

Several *in vitro* studies indicate that hypoxic LPC might be another powerful tool to protect against myocardial ischemia–reperfusion injury.<sup>11–13</sup> However, there are no *in vivo* studies investigating the effect and concentration dependence of hypoxia-induced LPC on the magnitude of myocardial ischemia–reperfusion injury. Therefore, the goal of the current study was to investigate whether different concentrations of sustained hypoxia induce LPC in the rat heart *in vivo*. We also sought to determine the potency of this effect as compared with the well-established effect of EPC with sevoflurane. In addition, we 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 $\alpha$  and PKC $\epsilon$  and on the expression of the hypoxia-inducible factor-1 $\alpha$ -dependent genes heme oxygenase-1 (HO-1) and vascular endothelial growth factor (VEGF), which have previously been implicated in the genesis of hypoxic and ischemic preconditioning.<sup>14–18</sup>

## Materials and Methods

The current investigation was conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (Publication number 85-23, revised 1996) and was performed after obtaining approval from the Animal Ethics Committee of the University of Amsterdam, The Netherlands.

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

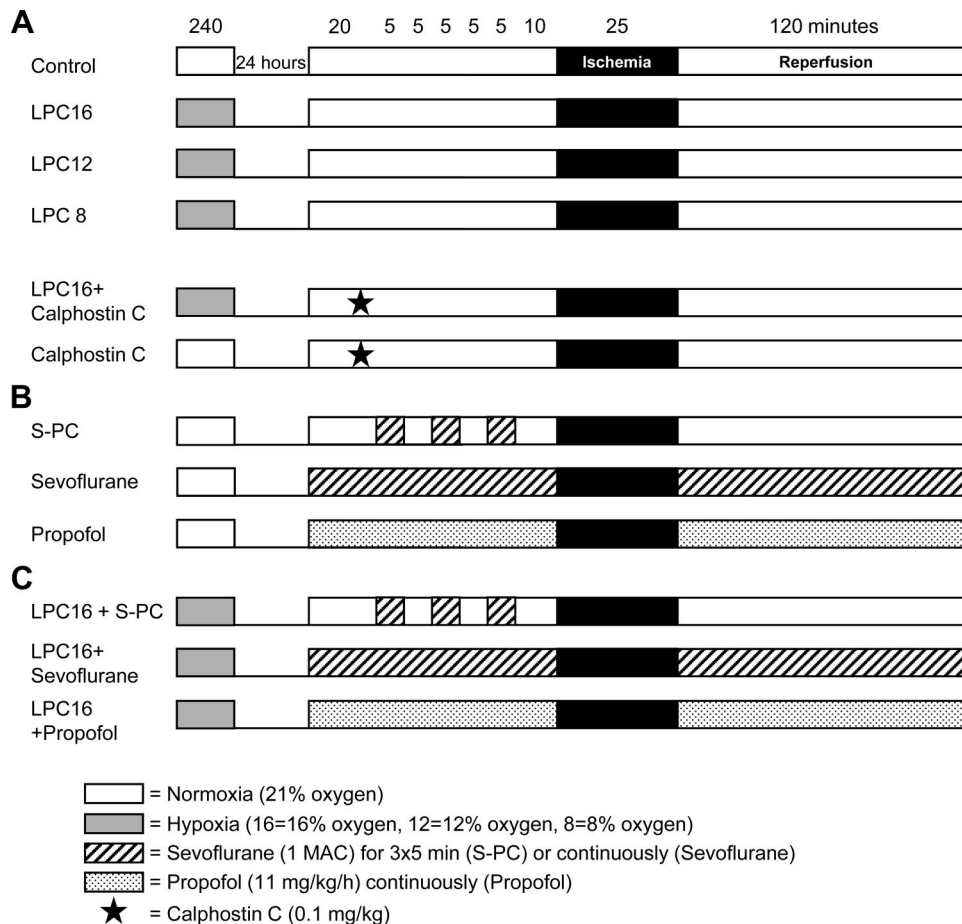
### Hypoxic Exposure

Rats were placed in a plexiglass chamber and randomly exposed to different concentrations of normobaric hypoxia (16%, 12%, and 8% oxygen, respectively) for 4 h 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. Fresh gas flow was kept at 3 l/min to ensure that no accumulation of carbon dioxide occurred, which in turn might have affected ventilation and thus oxygenation. Oxygen concentration within the chamber was controlled continuously (Capnomac Ultima; Datex-Ohmeda, Helsinki, Finland). After 4 h of hypoxic exposure, all rats were exposed to room air for 24 h.

### Experimental Protocol for Determining Infarct Size

Surgical preparation was performed as described previously.<sup>19,20</sup> In brief, rats were anesthetized by intraperitoneal S-ketamine injection (150 mg/kg) followed by continuous 30 mg  $\cdot$  kg  $\cdot$  h  $\alpha$ -chloralose infusion, except for the groups that received sevoflurane (1 minimum alveolar concentration [MAC] or continuous 11 mg  $\cdot$  kg  $\cdot$  h propofol). After tracheal intubation, the lungs were ventilated with 30% oxygen and 70% nitrogen and a positive end-expiratory pressure of 2–3 cm H<sub>2</sub>O. The right jugular vein was cannulated for saline and drug infusion; 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 min during the experiments. Aortic pressure and electrocardiographic signals were digitized using an analog-to-digital converter (PowerLab/8SP; ADInstruments Pty, Ltd., Castle Hill, Australia) and were continuously recorded on a personal computer (version 5, Chart for Windows; ADInstruments Pty, Ltd.).

After 120 min of reperfusion, the heart was excised and mounted on a modified Langendorff apparatus for infarct staining.<sup>21</sup> After staining with 0.2% Evans blue, the heart was cut into transverse slices, which were then stained with 0.75% triphenyltetrazolium chloride solution. The area of risk and the infarcted area were determined by planimetry (SigmaScan Pro 5.0®; SPSS Science Software, Chicago, IL).



**Fig. 1.** Experimental protocol. (A) Part 1. Study design to test cardioprotective effects of hypoxic LPC at different oxygen concentrations (16, 12, 8%) and the functional involvement of protein kinase C. (B) Part 2. Study design to test cardioprotective effects of early preconditioning with sevoflurane and continuous administration of sevoflurane or propofol. (C) Part 3. Study design to test cardioprotective effects of hypoxic LPC combined with early preconditioning protocols described in part 2. Control = normoxic control; LPC = late preconditioning; LPC16 = LPC, 16% oxygen; LPC12 = LPC, 12% oxygen; LPC8 = LPC, 8% oxygen; Propofol = continuous propofol early preconditioning; Sevoflurane = continuous sevoflurane early preconditioning; S-PC = sevoflurane early preconditioning.

### Study Design

The experimental protocol is shown in figure 1.

**Part 1 (n = 7–8): Effect of Hypoxic LPC at Different Oxygen Concentrations.** Rats were treated according to the following protocol (fig. 1A): 24 h before the ischemia–reperfusion sequence started, LPC rats were exposed for 4 h to 21% oxygen (normoxic control), 16% oxygen (LPC16), 12% oxygen (LPC12), or 8% oxygen (LPC8), respectively. To investigate further whether the effect of hypoxic LPC can be blocked by a specific PKC inhibitor, normoxic control rats and LPC16 rats received calphostin C (0.1 mg/kg) intravenously 35 min before myocardial ischemia was induced.

**Part 2 (n = 7–8): Effect of Hypoxic LPC with Other Preconditioning Models.** Rats were subjected to the following protocol (fig. 1B): The first group of EPC rats was exposed to sevoflurane with an end-tidal concentration of 1 MAC for 3 × 5 min interspersed with 2 × 5-min exposure and one final 10-min washout period. The second group was exposed to continuous administration of 1 MAC sevoflurane. The third group was exposed to continuous 11 mg · kg · h propo-

fol administration. The latter two groups did not receive an anesthesia with  $\alpha$ -chloralose.

**Part 3 (n = 9): Effect of Combined Hypoxic LPC (LPC16) and EPC.** Protocols from parts 1 and 2 of the current 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. The second group was exposed to hypoxic LPC combined with continuous administration of 1 MAC sevoflurane. The third group was subjected to hypoxic LPC combined with continuous 11 mg · kg · h propofol administration.

### Western Blot Analysis

For Western blot analysis of myocardial PKC $\alpha$  and PKC $\epsilon$ , additional experiments were performed. Six and 28 h after hypoxic exposure (LPC16) was initiated, hearts were excised (n = 12). Hearts from normoxic control animals were excised after exposure to 21% oxygen. Before heart excision, rats underwent a sham operation to follow the same surgical protocol as was used in the infarct size experiments.

After protein determination by the Lowry method,<sup>22</sup> 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. 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 to guarantee equal protein loading. Unspecific binding of the antibody was blocked by incubation with 5% fat dry milk powder solution in Tris-buffered saline for 2 h. Subsequently, the membrane was incubated overnight 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 h at room temperature. Immunoreactive bands on the membrane were visualized by a two-channel laser system, and blots were quantified (Odyssey IR Imager®; LI-COR Biosciences, Bad Homburg, Germany).

#### Semiquantitative Reverse Transcriptase–Polymerase Chain Reaction Assay

HO-1 and VEGF messenger ribonucleic acid (mRNA) expression was 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 Supplemental Digital Content 1, <http://links.lww.com/ALN/A647>. 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. Images were transferred to a computer for densitometric analysis (Gelscan; 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 and Tukey test. Western blot and reverse transcriptase–polymerase chain reaction data were analyzed by a Kruskal–Wallis and Student–Newman–Keuls test. Data obtained periodically throughout the experiment, such as hemodynamic variables, were analyzed using one-way repeated-measures analysis of variance and Holm–Sidak test. All data are presented as mean  $\pm$  SD unless otherwise specified. Changes within and between groups were

**Table 1.** Infarct Sizes Related to Area at Risk

Group	n	Mean $\pm$ SD Infarct Size, % AAR
Normoxic control	8	62 $\pm$ 6
LPC16	8	36 $\pm$ 11*
LPC12	8	38 $\pm$ 10*
LPC8	8	39 $\pm$ 11*
LPC16+Calphostin C	7	57 $\pm$ 11†
Calphostin C	8	58 $\pm$ 9
S-PC	7	40 $\pm$ 8*
Sevoflurane	7	53 $\pm$ 6
Propofol	7	56 $\pm$ 10
LPC16+S-PC	9	37 $\pm$ 12*
LPC16+Sevoflurane	9	40 $\pm$ 10*
LPC16+Propofol	9	42 $\pm$ 10*

\*  $P < 0.05$  vs. normoxic control. †  $P < 0.05$  vs. LPC16.

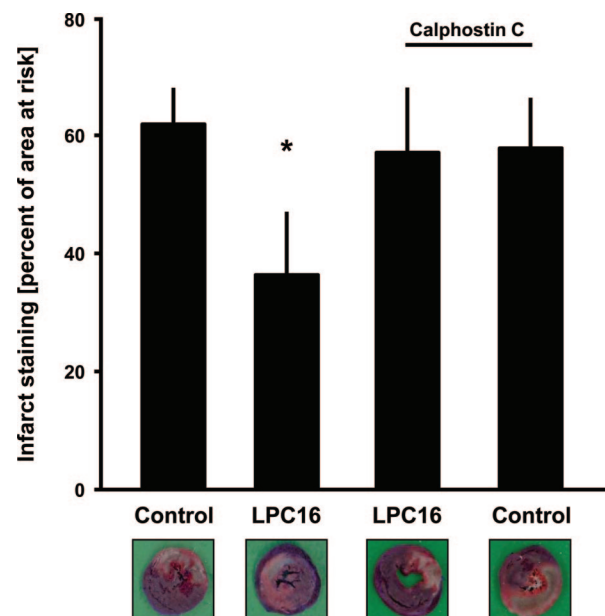
AAR = area at risk; LPC = late preconditioning; LPC16 = LPC, 16% oxygen; LPC12 = LPC, 12% oxygen; LPC8 = LPC, 8% oxygen; Sevoflurane = continuous sevoflurane early preconditioning; S-PC = sevoflurane early preconditioning; Propofol = continuous propofol early preconditioning.

considered statistically significant if  $P$  values were less than 0.05 (version 17, SPSS; SPSS Science Software).

## Results

### Infarct Size Measurement

As summarized in table 1, hypoxic LPC significantly reduced infarct size at all tested oxygen concentrations ( $P < 0.001$  vs. normoxic control). Cardioprotective effects were similar among the three LPC study groups ( $P = 0.84$ , table 1). The



**Fig. 2.** Infarct size measurement. Histogram shows the infarct size (area at risk, %) of normoxic control animals, hypoxic LPC with 16% oxygen alone (LPC16), hypoxic LPC16 combined with calphostin C, and calphostin C alone. All data are presented as mean  $\pm$  SD unless otherwise specified. \*  $P < 0.05$  vs. normoxic control. LPC = late preconditioning.



**Table 2.** Mean  $\pm$  SD Hemodynamic Variables

	Baseline	Washout 3	Ischemia 15 min	Reperfusion	
				30 min	120 min
<b>Heart rate, bpm</b>					
Normoxic control	410 $\pm$ 27	403 $\pm$ 28	399 $\pm$ 19	365 $\pm$ 18§	322 $\pm$ 28§
LPC16	419 $\pm$ 34	411 $\pm$ 31	407 $\pm$ 35	380 $\pm$ 30	350 $\pm$ 35§
LPC12	415 $\pm$ 27	408 $\pm$ 23	407 $\pm$ 20	390 $\pm$ 22§	361 $\pm$ 34§
LPC8	426 $\pm$ 33	414 $\pm$ 23	407 $\pm$ 32	364 $\pm$ 35§	314 $\pm$ 34§
LPC16+Calphostin C	423 $\pm$ 16	415 $\pm$ 14	428 $\pm$ 15	377 $\pm$ 15§	324 $\pm$ 13§
Calphostin C	418 $\pm$ 18	411 $\pm$ 21	420 $\pm$ 21	373 $\pm$ 17§	325 $\pm$ 17§
S-PC	368 $\pm$ 37	333 $\pm$ 22*§	320 $\pm$ 28*†§	283 $\pm$ 17*†§	311 $\pm$ 22§
Sevoflurane	355 $\pm$ 25*	359 $\pm$ 33	339 $\pm$ 33*	341 $\pm$ 39	330 $\pm$ 17§
Propofol	399 $\pm$ 35	380 $\pm$ 54	408 $\pm$ 24	376 $\pm$ 24	360 $\pm$ 31
LPC16+S-PC	414 $\pm$ 32	368 $\pm$ 24§	377 $\pm$ 24§	343 $\pm$ 15§	299 $\pm$ 21§
LPC16+Sevoflurane	359 $\pm$ 24†	348 $\pm$ 27†	351 $\pm$ 36†	349 $\pm$ 15	331 $\pm$ 35§
LPC16+Propofol	428 $\pm$ 24	418 $\pm$ 35	392 $\pm$ 57	398 $\pm$ 51	360 $\pm$ 27§
<b>Mean aortic pressure, mmHg</b>					
Normoxic control	140 $\pm$ 16	135 $\pm$ 25	111 $\pm$ 32	89 $\pm$ 26§	80 $\pm$ 29§
LPC16	130 $\pm$ 19	127 $\pm$ 20	114 $\pm$ 20	95 $\pm$ 23§	89 $\pm$ 21§
LPC12	140 $\pm$ 9	128 $\pm$ 11	117 $\pm$ 17§	103 $\pm$ 19§	101 $\pm$ 28§
LPC8	124 $\pm$ 19	121 $\pm$ 23	108 $\pm$ 27	88 $\pm$ 21§	72 $\pm$ 17§
LPC16+Calphostin C	138 $\pm$ 18	136 $\pm$ 19	113 $\pm$ 24§	100 $\pm$ 18§	78 $\pm$ 9§
Calphostin C	143 $\pm$ 5	132 $\pm$ 5§	128 $\pm$ 7§	88 $\pm$ 14§	72 $\pm$ 6§
S-PC	119 $\pm$ 18	86 $\pm$ 17*§	70 $\pm$ 12*§	65 $\pm$ 7§	65 $\pm$ 10§
Sevoflurane	100 $\pm$ 21*	116 $\pm$ 25	113 $\pm$ 25	105 $\pm$ 15	96 $\pm$ 9
Propofol	144 $\pm$ 13	134 $\pm$ 20	136 $\pm$ 10	116 $\pm$ 17§	107 $\pm$ 27§
LPC16+S-PC	126 $\pm$ 10	107 $\pm$ 10§	99 $\pm$ 15§	74 $\pm$ 7§	62 $\pm$ 4§
LPC16+Sevoflurane	108 $\pm$ 19	126 $\pm$ 13§	125 $\pm$ 12§	113 $\pm$ 11	103 $\pm$ 28
LPC16+Propofol	131 $\pm$ 25	121 $\pm$ 27	100 $\pm$ 41§	101 $\pm$ 23§	116 $\pm$ 18§

\*  $P < 0.05$  vs. normoxic control. †  $P < 0.05$  vs. LPC16. ‡  $P < 0.05$  vs. LPC16+S-PC. §  $P < 0.05$  vs. baseline.

LPC = late preconditioning; LPC16 = LPC, 16% oxygen; LPC12 = LPC, 12% oxygen; LPC8 = LPC, 8% oxygen; Sevoflurane = continuous sevoflurane early preconditioning; S-PC = sevoflurane early preconditioning; Propofol = continuous propofol early preconditioning.

PKC inhibitor calphostin C abolished the preconditioning effect of LPC16 ( $P < 0.001$ ) but had no effect on infarct size when it was administered to normoxic control animals ( $P = 0.30$ , table 1 and fig. 2).

Early preconditioning with intermittent administration of sevoflurane reduced infarct size ( $P < 0.01$  vs. normoxic control) to approximately the same magnitude as hypoxic LPC ( $P = 0.87$ , table 1). In contrast, continuous administration of sevoflurane and propofol was not cardioprotective ( $P = 0.77$  and  $P = 0.96$ , respectively, vs. normoxic control, table 1).

As shown in table 1, the combination of LPC16 with sevoflurane EPC did not provide cardioprotective effects superior to those of each substance alone ( $P < 0.05$  vs. normoxic control;  $P = 0.87$  vs. LPC16;  $P = 0.85$  vs. sevoflurane EPC). The cardioprotective effects of LPC16 were not affected by the two nonprotective 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 or propofol ( $P < 0.01$  vs. normoxic control).

### Hemodynamic Variables

In all groups, heart rate decreased at the end of the experiment compared with baseline values ( $P < 0.05$ , table 2).

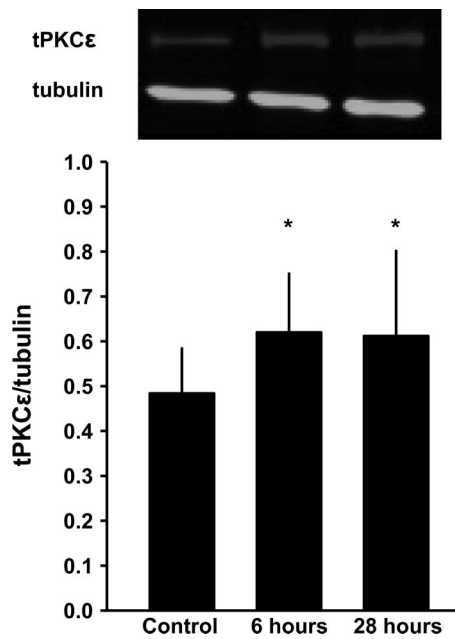
Decrease in heart rate failed to reach statistical significance only when rats were exposed to continuous administration of propofol ( $P = 0.05$ ). Decrease in heart rate was paralleled by a decrease in mean aortic pressure ( $P < 0.05$ ). However, continuous administration of sevoflurane, alone or in combination with LPC16, prevented a decrease in mean blood pressure.

### Expression of PKC $\alpha$ and PKC $\epsilon$

Figure 3 shows that LPC16 was associated with an increased myocardial expression of PKC $\epsilon$  at 6 h after the hypoxic stimulus was initiated ( $n = 12$ ;  $P = 0.01$  vs. normoxic control). At 28 h, PKC $\epsilon$  expression remained elevated and was of approximately the same magnitude as after 6 h ( $n = 12$ ;  $P = 0.91$  vs. 6 h;  $P = 0.01$  vs. normoxic control). In contrast, at the same time points, myocardial expression of PKC $\alpha$  was not affected by hypoxic LPC16 (PKC $\alpha$ /tubulin ratios: normoxic control,  $0.49 \pm 0.08$ ; LPC16, 6 h,  $0.47 \pm 0.05$ , 28 h,  $0.50 \pm 0.08$ ;  $P = 0.81$ ; data not shown).

### Expression of HO-1 and VEGF

Hypoxic preconditioning with 16% oxygen transiently up-regulated the myocardial expression of the hypoxia-inducible factor-1 $\alpha$ -dependent genes HO-1 and VEGF (fig. 4 and fig. 5). Six h after hypoxic stimulus was initiated, expression of



**Fig. 3.** Protein kinase C $\epsilon$  (tPKC $\epsilon$ ) expression in rat myocardium after hypoxic late preconditioning with 16% oxygen. Representative Western blot analysis experiments of a time course (normoxic control, 6 and 28 h poststimulus). Summarized data presenting ratio of total enzyme to tubulin are shown. All data are presented as mean  $\pm$  SD unless otherwise specified. \*  $P < 0.05$  vs. normoxic control.

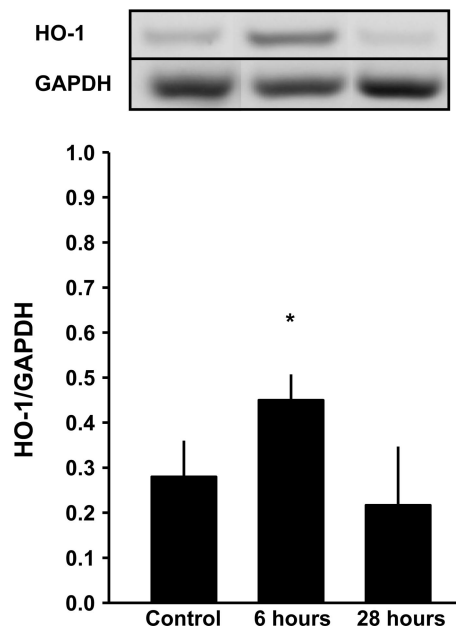
HO-1 and VEGF increased by approximately 60% and 28%, respectively (each  $n = 6$ ,  $P < 0.05$ ). However, at 28 h, expression of HO-1 ( $P = 0.50$ ) and VEGF ( $P = 0.75$ ) had returned to baseline values.

## Discussion

Ischemic preconditioning represents a powerful and reproducible tool of cardioprotection against myocardial ischemia,<sup>23</sup> yet this knowledge has not been translated into routine clinical use. Clinical applicability of this principle is limited by the nature of the fact that invasive procedures are required to induce ischemia. Although application might be possible in some situations (*e.g.*, intermittent aortic cross-clamping during coronary artery bypass surgery),<sup>24</sup> it simply is not in many others (*e.g.*, nonthoracic surgery). Much effort has, therefore, been focused on identifying clinically relevant preconditioning stimuli that are noninvasive and easy to administer.

Among various other stimuli (*e.g.*, nitric oxide, volatile anesthetics<sup>23,25</sup>), hypoxia may represent a potent preconditioning tool. Hypoxia resembles the stress that can ultimately lead to myocardial infarction (*i.e.*, cellular hypoxia/anoxia). One advantage of hypoxic rather than ischemic preconditioning is that systemic administration affects multiple organ systems, potentially offering protection against ischemic insults to the brain, liver, kidney, and other organ systems.<sup>26–28</sup>

As indicated by several *in vitro* studies,<sup>11,13</sup> intermittent exposure to 10% oxygen for 4 h induced late cardioprotec-

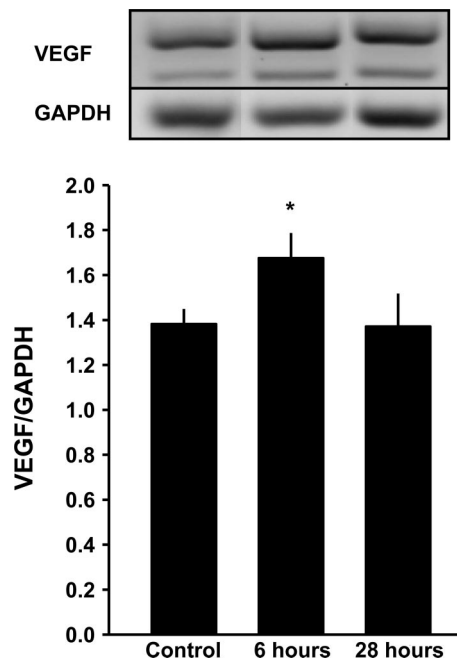


**Fig. 4.** Heme oxygenase-1 (HO-1) messenger ribonucleic acid expression in rat myocardium after hypoxic late preconditioning with 16% oxygen. Representative reverse transcriptase–polymerase chain reaction analysis experiments of a time course (normoxic control, 6 and 28 h poststimulus). Summarized data presenting ratio of heme oxygenase-1 to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are shown. All data are presented as mean  $\pm$  SD unless otherwise specified. \*  $P < 0.05$  vs. normoxic control.

tion in the isolated rat heart. Moreover, continuous hypoxic exposure to 10% oxygen for 4 h followed by 24 h of reoxygenation reduced infarct size in the isolated mouse heart, whereas shorter exposure times (30 min and 2 h, respectively) were ineffective.<sup>12</sup> In contrast, another study<sup>11</sup> found that 10% oxygen for 4 h had no cardioprotective effects. Although different protocols of hypoxia-induced myocardial preconditioning have proven effective *in vitro*, protective effects remains to be established *in vivo*.

## Hypoxia Induces LPC In Vivo

In the current study, we demonstrate that continuous exposure to 4-h hypoxia followed by 24 h of normoxic reoxygenation induces LPC in the rat heart *in vivo*, reducing infarct size by nearly 40% when compared with nonpreconditioned myocardium. It is noteworthy that the extent of cardioprotection induced by 16% oxygen was comparable with that offered by 12% and 8% oxygen. However, because infarct size increased slightly with a decrease in oxygen concentration (*i.e.*, area at risk, %: LPC16, 36; LPC12, 38; LPC8, 39), our results do not exclude the possibility of less pronounced cardioprotective effects with decreased oxygen concentrations. Indeed, Béguin *et al.*<sup>11</sup> showed that intermittent hypoxia with 10% oxygen for 4 h significantly reduced infarct size in a rat model whereas the same protocol with 5% oxygen significantly increased infarct size. This observation suggests that severe hypoxia renders the myocardium more sen-



**Fig. 5.** Vascular endothelial growth factor (VEGF) messenger ribonucleic acid expression in rat myocardium after hypoxic late preconditioning with 16% oxygen. Representative reverse transcriptase–polymerase chain reaction analysis experiments of a time course (normoxic control, 6 and 28 h poststimulus). Two major bands of VEGF messenger ribonucleic acid represent polymerase chain reaction analysis fragments amplified from VEGF<sub>188</sub> and VEGF<sub>164</sub> messenger ribonucleic acids. Summarized data presenting ratio of VEGF to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are shown. All data are presented as mean  $\pm$  SD unless otherwise specified. \*  $P < 0.05$  vs. normoxic control.

sitive to ischemic injury. The reason for this result was not explored, but it is conceivable that low oxygen tension compromises oxygen supply to the tissue, inducing cell damage. 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 LPC

We next investigated the potency of the observed cardioprotective effects of hypoxic LPC compared with the well-established effects of EPC with sevoflurane. In these original experiments, EPC with sevoflurane reduced infarct size by approximately 38%, in line with previous findings.<sup>29</sup> The cardioprotective effects of sevoflurane EPC and hypoxic LPC are 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 finding is in accord those of Bein *et al.*,<sup>30</sup> who showed that continuous administration of 1 MAC sevoflurane from induction to start of cardiopulmonary bypass did not result in cardioprotective effects when compared with the normoxic control group. However, when presurgical sevoflurane administration was interrupted for 10 min, improved myocardial performance and decreased postoperative

troponin T values were observed. These data, in conjunction with our findings, suggest that interrupted administration of anesthetics 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 effects.

These findings are of special interest for clinical scenarios where myocardial ischemia occurs by chance and cannot be timed, as 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 anesthetics (*i.e.*, when sevoflurane and propofol were not cardioprotective in our study). In such situations, clinical applicability of EPC is clearly limited. Thus, as noted elsewhere, LPC is likely of greater clinical relevance than EPC because it lasts 30–40 times longer and can easily be initiated before an expected ischemic event occurs.<sup>23</sup> 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 result may be especially relevant in conjunction with previous data indicating that LPC, but not EPC, protects against reversible postischemic contractile dysfunction (*i.e.*, myocardial “stunning”).<sup>23</sup>

#### Hypoxic LPC Cannot be Enhanced by Modified Anesthetic Techniques

A study by Müllenheim *et al.*<sup>31</sup> showed that combining ischemic LPC with ischemic EPC led to enhanced cardioprotection compared with each stimulus alone. The same group showed that cardioprotection by ischemic LPC could be increased by sevoflurane-induced EPC.<sup>29</sup> Therefore, we investigated whether the cardioprotective effects of hypoxic LPC could be enhanced by EPC with sevoflurane or by continuous administration of sevoflurane or propofol. It is noteworthy that, to the best of our knowledge, there is no study investigating the cardioprotective effects 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, our results also demonstrated 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.

#### Signaling Cascade of Hypoxic LPC

Myocardial hypoxia triggers a wide range of profound cellular responses, including regulation of gene expression. In several studies, PKC, a ubiquitous intracellular mediator, has been shown to play a pivotal role in the signaling pathway of LPC, as elicited by a variety of agents.<sup>23,32</sup> Notably, the isoform PKC $\epsilon$  seems to be responsible for the development of delayed cardioprotection,<sup>33,34</sup> although, in some studies,<sup>14,15</sup> 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 effects 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 been shown to inhibit the novel isoforms of PKC, including PKC $\epsilon$ , more efficiently than conventional ones.<sup>35</sup> Moreover, a previous study<sup>36</sup> characterizing the expression of PKC isoforms in rat ventricular myocytes found that the dominant isoform was PKC $\epsilon$ . Indeed, we found that 6 h after the preconditioning stimulus was initiated, the expression of PKC $\epsilon$  was increased, and this increase remained stable until myocardial ischemia was induced (*i.e.*, after 28 h). 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 effects of hypoxic LPC in the rat heart *in vivo*. This finding is in line with a series of previous studies<sup>34,37,38</sup> showing that translocation and activation of PKC $\epsilon$ —but not of the PKC isoforms  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ ,  $\zeta$ ,  $\iota$ ,  $\lambda$ , and  $\mu$ —are necessary for ischemic LPC *in vivo*. The reasons for the conflict between study findings regarding the involvement of PKC $\alpha$  in mediating LPC are unclear but may be related to species differences (canine, rabbit, humans, and rat) and type of preconditioning (ischemic *vs.* hypoxic). However, we cannot exclude the possibility that PKC isoforms not studied in the current investigation may contribute to the cardioprotection elicited by hypoxic LPC in the rat.

Previous studies suggest that PKC plays a pivotal role in the up-regulation of the hypoxia-inducible factor-1 $\alpha$ -dependent genes HO-1 and VEGF,<sup>39,40</sup> both of which have previously been implicated in mediating hypoxic LPC.<sup>16–18,41</sup> HO-1 degrades heme and generates carbon monoxide and bilirubin, which may have antioxidant and/or antiinflammatory effects.<sup>42</sup> Indeed, mice overexpressing cardiac-specific HO-1 show a significant reduction in infarct size after ischemia reperfusion.<sup>18</sup> VEGF regulates local oxygen supply and may be important in ischemia reperfusion because it mediates neovascularization during myocardial ischemia.<sup>43,44</sup> Our finding of the transient up-regulation 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 seems conceivable that inhibition of PKC prevents the HO-1- and VEGF-mediated reduction in infarct size. Indeed, the observation that calphostin C abolished the cardioprotective effects of hypoxic LPC is consistent with this hypothesis. Moreover, our results related to calphostin C support the assumption that PKC lies upstream in the signaling pathway of HO-1 and VEGF expression in the rat myocardium, as proposed by others.<sup>16</sup>

### Limitations of the Study

One limitation of the current study is that we did not determine the oxygen threshold below which hypoxia starts to exert cardioprotective effects. We cannot exclude the possi-

bility that oxygen concentrations higher than 16%, but lower than 21%, are also cardioprotective.

Another limitation of our study is that pharmacologic inhibitors such as 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. Other kinases, such as protein kinase A (half-maximal inhibitory concentration more than 50  $\mu$ M), p60<sup>V-src</sup> (half-maximal inhibitory concentration more than 50  $\mu$ M), and protein kinase G (half-maximal inhibitory concentration more than 25  $\mu$ M) are inhibited only at higher concentrations.

### Conclusion

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 approximately the same magnitude as moderate (12% oxygen) and severe (8% oxygen) hypoxia; (3) the cardioprotective effects of hypoxia-induced LPC are as powerful as those of EPC with sevoflurane; and (4) PKC $\epsilon$  is the dominant isoform involved in the cardioprotective effects of hypoxic LPC in the rat heart *in vivo*. These findings may provide a conceptual framework for developing novel therapeutic strategies aimed at powerful cardioprotection, especially in situations where myocardial ischemia occurs by chance and early preconditioning strategies are not applicable.

The authors thank Yvonne Grüber, M.T.A. (Technician, Department of Anesthesiology, University Hospital of Duesseldorf, Duesseldorf, Germany), and Elmar Menold, M.T.A. (Technician, Department of Internal Medicine VII, Division of Sports Medicine, University of Heidelberg, Heidelberg, Germany), for excellent technical support.

### References

- Murray CJ, Lopez AD: Global mortality, disability, and the contribution of risk factors: Global Burden of Disease Study. *Lancet* 1997; 349:1436–42
- Lloyd-Jones DM, Hong Y, Labarthe D, Mozaffarian D, Appel LJ, Van Horn L, Greenlund K, Daniels S, Nichol G, Tomaselli GF, Arnett DK, Fonarow GC, Ho PM, Lauer MS, Mousoudi FA, Robertson RM, Roger V, Schwamm LH, Sorlie P, Yancy CW, Rosamond WD, American Heart Association Strategic Planning Task Force and Statistics Committee: Defining and setting national goals for cardiovascular health promotion and disease reduction: The American Heart Association's strategic Impact Goal through 2020 and beyond. *Circulation* 2010; 121:586–613
- Ashton CM, Petersen NJ, Wray NP, Kiefe CI, Dunn JK, Wu L, Thomas JM: The incidence of perioperative myocardial infarction in men undergoing noncardiac surgery. *Ann Intern Med* 1993; 118:504–10
- Mangano DT, Browner WS, Hollenberg M, Li J, Tateo IM: Long-term cardiac prognosis following noncardiac surgery. The Study of Perioperative Ischemia Research Group. *JAMA* 1992; 268:233–9
- McFalls EO, Ward HB, Santilli S, Scheffel M, Chesler E, Doliszny KM: The influence of perioperative myocardial infarction on long-term prognosis following elective vascular surgery. *Chest* 1998; 113:681–6



6. Murry CE, Jennings RB, Reimer KA: Preconditioning with ischemia: A delay of lethal cell injury in ischemic myocardium. *Circulation* 1986; 74:1124-36
7. Bolli R: The late phase of preconditioning. *Circ Res* 2000; 87:972-83
8. Ferdinandy P, Schulz R, Baxter GF: Interaction of cardiovascular risk factors with myocardial ischemia/reperfusion injury, preconditioning, and postconditioning. *Pharmacol Rev* 2007; 59:418-58
9. Weber NC, Frässdorf J, Ratajczak C, Grueber Y, Schlack W, Hollmann MW, Preckel B: Xenon induces late cardiac preconditioning in vivo: A role for cyclooxygenase 2? *Anesth Analg* 2008; 107:1807-13
10. Garcia C, Julier K, Bestmann L, Zollinger A, von Segesser LK, Pasch T, Spahn DR, Zaugg M: Preconditioning with sevoflurane decreases PECAM-1 expression and improves one-year cardiovascular outcome in coronary artery bypass graft surgery. *Br J Anaesth* 2005; 94:159-65
11. Béguin PC, Joyeux-Faure M, Godin-Ribuot D, Lévy P, Ribuot C: Acute intermittent hypoxia improves rat myocardium tolerance to ischemia. *J Appl Physiol* 2005; 99:1064-9
12. Xi L, Tekin D, Gursoy E, Salloum F, Levasseur JE, Kukreja RC: Evidence that NOS2 acts as a trigger and mediator of late preconditioning induced by acute systemic hypoxia. *Am J Physiol Heart Circ Physiol* 2002; 283:H5-12
13. Béguin PC, Belaidi E, Godin-Ribuot D, Lévy P, Ribuot C: Intermittent hypoxia-induced delayed cardioprotection is mediated by PKC and triggered by p38 MAP kinase and Erk1/2. *J Mol Cell Cardiol* 2007; 42:343-51
14. Hassouna A, Matata BM, Galiñanes M: PKC-epsilon is upstream and PKC-alpha is downstream of mitoKATP channels in the signal transduction pathway of ischemic preconditioning of human myocardium. *Am J Physiol Cell Physiol* 2004; 287:C1418-25
15. Kitakaze M, Funaya H, Minamino T, Node K, Sato H, Ueda Y, Okuyama Y, Kuzuya T, Hori M, Yoshida K: Role of protein kinase C-alpha in activation of ecto-5'-nucleotidase in the preconditioned canine myocardium. *Biochem Biophys Res Commun* 1997; 239:171-5
16. Pagé EL, Robitaille GA, Pouysselgur J, Richard DE: Induction of hypoxia-inducible factor-1alpha by transcriptional and translational mechanisms. *J Biol Chem* 2002; 277:48403-9
17. Thirunavukkarasu M, Juhasz B, Zhan L, Menon VP, Tosaki A, Otani H, Maulik N: VEGFR1 (Flt-1+/-) gene knockout leads to the disruption of VEGF-mediated signaling through the nitric oxide/heme oxygenase pathway in ischemic preconditioned myocardium. *Free Radic Biol Med* 2007; 42:1487-95
18. Yet SF, Tian R, Layne MD, Wang ZY, Maemura K, Solovyeva M, Ith B, Melo LG, Zhang L, Ingwall JS, Dzau VJ, Lee ME, Perrella MA: Cardiac-specific expression of heme oxygenase-1 protects against ischemia and reperfusion injury in transgenic mice. *Circ Res* 2001; 89:168-73
19. 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
20. Obal D, Preckel B, Scharbatke H, Müllenheim J, Höterkes F, Thämer V, Schlack W: One MAC of sevoflurane provides protection against reperfusion injury in the rat heart *in vivo*. *Br J Anaesth* 2001; 87:905-11
21. Heinen A, Huhn R, Smeele KM, Zuurbier CJ, Schlack W, Preckel B, Weber NC, Hollmann MW: Helium-induced preconditioning in young and old rat heart: Impact of mitochondrial Ca(2+)-sensitive potassium channel activation. *ANESTHESIOLOGY* 2008; 109:830-6
22. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951; 193:265-75
23. Bolli R: Preconditioning: A paradigm shift in the biology of myocardial ischemia. *Am J Physiol Heart Circ Physiol* 2007; 292:H19-27
24. Alkhulaifi AM, Yellon DM, Pugsley WB: Preconditioning the human heart during aorto-coronary bypass surgery. *Eur J Cardiothorac Surg* 1994; 8:270-5
25. De Hert SG, Preckel B, Hollmann MW, Schlack WS: Drugs mediating myocardial protection. *Eur J Anaesthesiol* 2009; 26:985-95
26. Carini R, De Cesaris MG, Splendore R, Vay D, Domenicotti C, Nitti MP, Paola D, Pronzato MA, Albano E: Signal pathway involved in the development of hypoxic preconditioning in rat hepatocytes. *Hepatology* 2001; 33:131-9
27. Wacker BK, Park TS, Gidday JM: Hypoxic preconditioning-induced cerebral ischemic tolerance: Role of microvascular sphingosine kinase 2. *Stroke* 2009; 40:3342-8
28. Yeh CH, Hsu SP, Yang CC, Chien CT, Wang NP: Hypoxic preconditioning reinforces HIF-alpha-dependent HSP70 signaling to reduce ischemic renal failure-induced renal tubular apoptosis and autophagy. *Life Sci* 2010; 86:115-23
29. Müllenheim J, Ebel D, Bauer M, Otto F, Heinen A, Frässdorf J, Preckel B, Schlack W: Sevoflurane confers additional cardioprotection after ischemic late preconditioning in rabbits. *ANESTHESIOLOGY* 2003; 99:624-31
30. Bein B, Renner J, Caliebe D, Hanss R, Bauer M, Fraund S, Scholz J: The effects of interrupted or continuous administration of sevoflurane on preconditioning before cardiopulmonary bypass in coronary artery surgery: Comparison with continuous propofol. *Anaesthesia* 2008; 63:1046-55
31. Müllenheim J, Schlack W, Frässdorf J, Heinen A, Preckel B, Thämer V: Additive protective effects of late and early ischaemic preconditioning are mediated by the opening of KATP channels *in vivo*. *Pflugers Arch* 2001; 442:178-87
32. Pravdic D, Sedlic F, Mio Y, Vladoic N, Bienengraeber M, Bosnjak ZJ: Anesthetic-induced preconditioning delays opening of mitochondrial permeability transition pore *via* protein Kinase C-epsilon-mediated pathway. *ANESTHESIOLOGY* 2009; 111:267-74
33. Gray MO, Karliner JS, Mochly-Rosen D: A selective epsilon-protein kinase C antagonist inhibits protection of cardiac myocytes from hypoxia-induced cell death. *J Biol Chem* 1997; 272:30945-51
34. Qiu Y, Ping P, Tang XL, Manchikalapudi S, Rizvi A, Zhang J, Takano H, Wu WJ, Teschner S, Bolli R: Direct evidence that protein kinase C plays an essential role in the development of late preconditioning against myocardial stunning in conscious rabbits and that epsilon is the isoform involved. *J Clin Invest* 1998; 101:2182-98
35. Keenan C, Goode N, Pears C: Isoform specificity of activators and inhibitors of protein kinase C gamma and delta. *FEBS Lett* 1997; 415:101-8
36. Bogoyevitch MA, Parker PJ, Sugden PH: Characterization of protein kinase C isotype expression in adult rat heart. Protein kinase C-epsilon is a major isotype present, and it is activated by phorbol esters, epinephrine, and endothelin. *Circ Res* 1993; 72:757-67
37. Ping P, Zhang J, Qiu Y, Tang XL, Manchikalapudi S, Cao X, Bolli R: Ischemic preconditioning induces selective translocation of protein kinase C isoforms epsilon and eta in the heart of conscious rabbits without subcellular redistribution of total protein kinase C activity. *Circ Res* 1997; 81:404-14
38. Ping P, Zhang J, Zheng YT, Li RC, Dawn B, Tang XL, Takano H, Balafanova Z, Bolli R: Demonstration of selective protein kinase C-dependent activation of Src and Lck tyrosine kinases during ischemic preconditioning in conscious rabbits. *Circ Res* 1999; 85:542-50
39. Kawata H, Yoshida K, Kawamoto A, Kurioka H, Takase E,

- Sasaki Y, Hatanaka K, Kobayashi M, Ueyama T, Hashimoto T, Dohi K: Ischemic preconditioning upregulates vascular endothelial growth factor mRNA expression and neovascularization via nuclear translocation of protein kinase C epsilon in the rat ischemic myocardium. *Circ Res* 2001; 88:696-704
40. Ryter SW, Alam J, Choi AM: Heme oxygenase-1/carbon monoxide: From basic science to therapeutic applications. *Physiol Rev* 2006; 86:583-650
41. Semenza GL: Surviving ischemia: Adaptive responses mediated by hypoxia-inducible factor 1. *J Clin Invest* 2000; 106:809-12
42. Lee SS, Gao W, Mazzola S, Thomas MN, Csizmadia E, Otterbein LE, Bach FH, Wang H: Heme oxygenase-1, carbon monoxide, and bilirubin induce tolerance in recipients toward islet allografts by modulating T regulatory cells. *FASEB J* 2007; 21:3450-7
43. Banai S, Shweiki D, Pinson A, Chandra M, Lazarovici G, Keshet E: Upregulation of vascular endothelial growth factor expression induced by myocardial ischaemia: Implications for coronary angiogenesis. *Cardiovasc Res* 1994; 28:1176-9
44. Hashimoto E, Ogita T, Nakaoka T, Matsuoka R, Takao A, Kira Y: Rapid induction of vascular endothelial growth factor expression by transient ischemia in rat heart. *Am J Physiol* 1994; 267:H1948-54