Xenon Induces Late Cardiac Preconditioning In Vivo: A Role for Cyclooxygenase 2?

Nina C. Weber, PhD*
Jan Fräßdorf, MD*
Christoph Ratajczak, CAND, MED†
Yvonne Grueber, BS†
Wolfgang Schlack, MD, DEAA*
Markus W. Hollmann, MD, PhD, DEAA*
Benedikt Preckel, MD, MA, DEAA*

BACKGROUND: Xenon induces early myocardial preconditioning of the rat heart in vivo, but whether xenon induces late cardioprotection is not known. Cyclooxygenase-2 (COX-2) has been shown to be an important mediator in the signal transduction of myocardial ischemic late preconditioning (i-LPC). We investigated whether xenon induces late preconditioning (Xe-LPC) and whether COX-2 activity and/or expression are involved in mediating this effect.

METHODS: Anesthetized male Wistar rats were instrumented with a coronary artery occluder. After 7 d of recovery, animals were randomized to 1 of 5 groups each containing 8 animals. The i-LPC group underwent 5 min of coronary occlusion to induce i-LPC. Xe-LPC was achieved by administration of xenon (70 volume%) for 15 min. Additional rats were pretreated with the COX-2 inhibitor NS-398 (5 mg kg⁻¹ body weight i.p.) with and without Xe-LPC. A group of sham operated animals not undergoing i-LPC or Xe-LPC served as controls (Con). After 24 h, all animals were anesthetized and underwent 25 min of myocardial ischemia induced by tightening of the coronary artery occluder followed by 2 h of reperfusion. Myocardial infarct size was assessed by triphenyltetrazolium chloride staining. In additional experiments, hearts were excised at different time points after preconditioning to investigate COX-2 mRNA and protein expression by polymerase chain reaction and infrared Western blot, respectively.

RESULTS: Both i-LPC and Xe-LPC reduced myocardial infarct size (% of the area at risk) compared with Con (i-LPC: 29 ± 7%; Xe-LPC 31 ± 8%, both P < 0.05 vs Con 64 ± 6%). NS-398 abolished the cardioprotective effect of Xe-LPC (61 ± 6%, P < 0.05 vs Xe-LPC). COX-2 mRNA and protein expression was only increased in the i-LPC group, but not in the Xe-LPC group.

CONCLUSION: Xenon induces late myocardial preconditioning that is abolished by functional blockade of COX-2 activity. In contrast to i-LPC, Xe-LPC did not lead to an increased expression of COX-2 mRNA and protein. These data suggest differences in COX-2 regulation in i-LPC and Xe-LPC.


Brief episodes of myocardial ischemia render the myocardium resistant to subsequent longer periods of ischemia.¹ This phenomenon, known as ischemic preconditioning, consists of 2 time windows: an early phase which begins within a few minutes after an ischemic stimulus and lasts for 2 to 3 h, and a late phase which occurs 12 to 24 h after the preconditioning stimulus and lasts for 3 to 4 d.² Drugs including volatile anesthetics mimic myocardial ischemic preconditioning and are able to induce early preconditioning in different animal species³,⁴ as well as in humans.⁵ Volatile anesthetics have been found to induce late preconditioning,⁶⁻⁸ although this effect may be species dependent.⁹ In addition to volatile anesthetics, the noble gas xenon has been demonstrated to induce early myocardial preconditioning,¹⁰ via activation of several signal transduction kinases.¹¹,¹² However, in contrast to volatile anesthetics, whether xenon also produces late myocardial preconditioning is not known.

Cyclooxygenase (COX) is a prostaglandin synthetase that mediates the conversion of arachidonic acid to prostaglandin H₂. In contrast to the constitutively expressed isoform COX-1, COX-2 is inducible in response to proinflammatory stimuli and is considered to be an important mediator of inflammation.¹³ Both ischemic late preconditioning (i-LPC) and volatile anesthetic-mediated late preconditioning have been shown to involve COX-2 pathways.¹⁴,¹⁵ Moreover, i-LPC...
increases COX-1 and 2 expression and enhances myocardial concentrations of prostaglandins prostaglandin E2 and 6-keto-PGF1α after 24 h.16

In the present study, we tested the hypothesis that xenon inhalation 24 h before myocardial ischemia induces late myocardial preconditioning in rat hearts in vivo. We further sought to evaluate the role of COX-2 in mediating late xenon myocardial preconditioning (Xe-LPC).

METHODS

The study was performed in accordance with the regulations of the German Animal Protection Law and was approved by the Bioethics Committee of the District of Düsseldorf, Germany.

Animal Preparation

One hundred twelve male Wistar rats (200–250 g) were subjected to this study. However, 10 animals died from surgical complications after coronary artery occluder implantation. Another 10 animals died of ventricular fibrillation or uncontrollable bleeding during the preparation for the ischemic or xenon preconditioning experiments.

After the depth of anesthesia was deemed adequate (by lack of muscle movement and absence of palpebral reflex), a left thoracotomy was performed in the fourth intercostal space and the pericardium opened. The left anterior descending coronary artery was identified and encircled with 2 5–0 prolene sutures (Ethicon 5/0, 1-metric, TF, Johnson and Johnson Medical BV, Nordstedt, Germany). A silicone tube (1.0 mm internal diameter, Roth, Karlsruhe, Germany) was tunnelled subcutaneously and externalized between the scapulae. The internal end was placed close to the sutures around the coronary artery. The two sutures were then externalized through this tube and the tube was filled with petroleum gel to prevent pneumothorax. The chest wound was closed in layers and covered by a vest (rat jacket, size M, Byron, Grand Island, NY) to protect the tube and the externalized sutures. Postoperative analgesia was provided by intercostal blockade and wound infiltration with 0.5% bupivacaine. The animals received antibiotic prophylaxis with cefazolin (35 mg kg⁻¹). Postoperatively, the animals had free access to food and water and were under close observation by an animal care specialist in the animal care department of the University of Düsseldorf.

Late Preconditioning Protocols

The animals were randomly assigned to five groups using sealed envelopes (Fig. 1A). There were eight animals per group for the myocardial infarct size measurement studies and four per group for the Western Blot analyses (discussed below). The animals were anesthetized with isoflurane 7 d after the prior procedure.

Ischemic preconditioning was induced by tightening of the previously placed suture around the coronary artery for 5 min. Myocardial ischemia was verified by the immediate occurrence of electrocardiogram (ECG) ST segment elevation. At the end of the 5-min period of coronary artery occlusion, the suture was released and removed to ensure proper reperfusion. This was verified by the disappearance of the ECG changes.

Animals in the XE-LPC groups were sedated with the i.p. injection of s-ketamine (250 mg kg⁻¹). After tracheal intubation, their lungs were ventilated with 70% xenon and 30% oxygen for 15 min. The animals of the COX-2 inhibitor groups received 5 mg kg⁻¹ NS-398
(in saline) i.p. with or without subsequent xenon preconditioning. The animals were then allowed to recover. After the ischemic and xenon preconditioning studies, the animals were transferred back in their cages and were closely observed by the investigators for at least 1 h. After this period, the animals were brought back into the animal care facility for housing under the previously described conditions.

**Myocardial Infarction Size Determination**

Twenty-four hours after completing the respective preconditioning protocol, the rats were anesthetized by an i.p. injection of S (+)-ketamine (250 mg kg$^{-1}$). Surgical preparation was then performed as previously described. The right jugular vein was cannulated and a continuous infusion of $\alpha$-choloralose (25 mg kg$^{-1}$) administered. A polyethylene catheter was inserted into the descending aorta via a carotid artery and connected to a pressure transducer for measurement of aortic pressure. All animals were connected to an ECG monitor. A temperature probe was placed sub-diaphragmatically and body temperature was maintained constant at 37.9 ± 0.7°C by adjusting a heating pad and a warming lamp. Arterial blood gases were analyzed at baseline, during ischemia and reperfusion and kept within physiological range.

Twenty-five minutes of myocardial ischemia was induced by tightening the coronary artery occluder and verified by the appearance of epicardial cyanosis and ECG changes. After 120 min of reperfusion, the heart was excised and myocardial infarct size was determined as previously described. Briefly, hearts were mounted on a modified Langendorff apparatus for perfusion with ice cold normal saline via the aortic root at a perfusion pressure of 40 cm H$_2$O in order to wash out intravascular blood. After 5 min of perfusion, the coronary artery was re-occluded and the remainder of the myocardium was perfused through the aortic root with 0.2% Evans blue in normal saline for 10 min. Intravascular Evans blue was then washed out by perfusion for 5 min with normal saline. This treatment identified the area at risk (AAR) as unstained. The heart was then cut into 2 mm transverse slices. The slices were stained with 0.75% triphenyltetrazolium chloride solution for 10 min at 37°C, fixed in 4% formalin solution for 6 h at room temperature. The myocardial AAR and the infarcted area were determined by planimetry using SigmaScan Pro 5® computer software (SPSS Science Software, Chicago, IL) and corrected for dry weight.

**Western Blot Assay**

Animals in the Western Blot portion of the study were assigned to one of three groups as shown in Fig. 1B. To exclude any confounding effect of anesthesia on Western Blot assay, the rats were decapitated. At the respective time points ($n = 4$ for each time period), the hearts were quickly excised (Sham, 0 h, 2 h, 4 h, 8 h, 12 h, 24 h) after the respective treatment of Xe- or i-LPC. The excised hearts were washed in ice cold saline solution and frozen in liquid nitrogen. The frozen tissue was pulverized and dissolved in lysis buffer containing the following ingredients: Tris base, ethylene glycol tetraacetic acid, sodium fluoride and sodium orthovanadate, as phosphatase inhibitors (all from Sigma-Aldrich Chemicals, Taufkirchen, Germany), and a freshly added protease inhibitor mix [aprotinin, leupeptin and pepstatin] and dithioerythriol (all from Merck-Eurolab, Munich, Germany). The solution was vigorously homogenized on ice (Homogenizer, IKA) and then centrifuged at 1000 g, 4°C, for 10 min. The supernatant, containing cytosolic fraction, was centrifuged again at 16,000 g, 4°C, for 15 min to purify this fraction.

COX-2 expression in the myocardial tissue was determined using infrared Western Blot analysis as previously described. After protein determination by the Lowry et al. method, equal amounts of the homogenate were mixed with mercaptoethanol and sodium dodecyl sulfate (SDS) polyacrylamid and boiled at 95°C with loading buffer containing Tris–hydrochloride, glycerol and bromphenol blue. Using SDS as an anionic detergent produces proteins covered with a negative charge. These proteins than can be separated by SDS gel electrophoresis according to their size. The negatively charged proteins were transferred to an Immobilon–fluorescence membrane (Millipore, Schwabach, Germany) using an electric current. Using the electric current enables the proteins to migrate from the gel to the membrane. COX-2 and $\alpha$ tubulin were detected using specific antibodies binding to these two enzymes on the membrane (Monoclonal mouse anti-$\alpha$-tubulin antibody, Sigma-Aldrich, Taufkirchen, Germany; total COX-2 rabbit polyclonal antibody, Biomol, Hamburg, Germany). Nonspecific binding of the antibody was blocked by a 2 h incubation with 5% fat dry milk powder solution in Tris buffered saline containing Tween.

After washing the membrane in fresh, cold Tris buffered saline containing Tween, secondary antibodies conjugated with infrared-dye 700 or infrared-dye 800 (Biomol, Hamburg, Germany) were applied. Protein bands on the membrane were visualized and quantified by the Odyssey infrared imager® (Licor Biosciences, Bad Homburg, Germany).

Equal loading of the protein to the SDS gel electrophoresis gel was ensured by Coomassie blue staining (Coomassie brilliant blue®, Serva Electrophoresis GmbH, Heidelberg, Germany) of each gel. The results are presented as ratio of total protein to $\alpha$-tubulin, which was used as internal standard.

**RNA Isolation**

RNA analysis was performed using a RNeasy® RNA isolation Mini kit (Qiagen, Hilden, Germany). Reverse transcription polymerase chain reaction (PCR) was performed using a one-step PCR kit from Qiagen. Reverse transcription PCR experiments
were performed with primers for the respective target gene COX-2 (sense: 5’-CTGTATCCGCGCTGC
TGTG-3’, anti-sense: 5’-GAGGCACCTTGCGTGTGCTGT-
3’) and were standardized on Glyceraldehyde 3-phosphate dehydrogenase (sense: 5’-TCCCTCAAGA
TTGTCAAGCA-3’, anti-sense: 5’-AGATCCACTCAGGA
TACATT-3’). The conditions were as follows: COX-2:
50°C 30 min, 15 min 95°C, [24 s 93°C
–30 s 55°C-1 min 73°C] × 30 cycles, 10 min 73°C; Glyceraldehyde 3-phosphate dehydrogenase: 30 min 50°C, 15 min 95°C, [30 s 93°C
–30 s 55°C-1 min 73°C] × 30 cycles, 10 min 73°C. The
PCR products were separated by agarose gel elec-
trophoresis, ethidium bromide staining, and densitometric
analysis using a Kodak Image station® (Eastman Kodak
Comp., Rochester, NY).

Statistical Analysis-Sample Size Calculation

Sample size calculations were performed using
GraphPad StatMate™ Version 1.01 (GraphPad Soft-
ware, San Diego, CA). A sample size of eight animals
in each group allowed for the detection of a difference
in myocardial infarct size of 30% between control and
the preconditioning groups or the preconditioning
groups and the NS-398 COX-2 inhibitor group, respec-
tively, with a power of 95% and an α < 0.05. For PCR
and Western Blot assay experiments, four animals in
each group allows for the detection of a difference in
protein expression between groups of 30% with a
power of 95%, a standard deviation of 10, and an
α < 0.05.

Statistical analysis of the hemodynamic variables
was performed by two-way repeated measures analy-
sis of variance (ANOVA) for time and treatment
(experimental group) effects. If an overall significance
was found, comparisons between groups were made
for each time point using one-way ANOVA followed by
Dunnett’s post hoc test with the control group as the
reference group. Time effects within each group were
analyzed by repeated-measures ANOVA followed by
Dunnett’s post hoc test with the baseline value as the
reference time point. PCR and Western Blot data were
analyzed by one-way ANOVA followed by Dunnett’s
post hoc test with the control group as the reference
group (Graph Pad Prism version 4.00). Differences in
myocardial infarct size were analyzed by ANOVA
followed by Student’s t-test with Bonferroni correction
for multiple testing. Data are expressed as mean ± sd
and P < 0.05 was considered statistically signif-

RESULTS

Hemodynamic Results

Table 1 shows the heart rate and mean aortic
pressure during the different experimental protocols.
There were no differences between the experimental
groups at baseline or during myocardial isch-
emia. At the end of the reperfusion period, heart rate
and mean aortic pressure were lower in the groups
receiving the COX-2 inhibitor NS-398 compared with
baseline values. Heart rate was slower in the animals
receiving NS-398 without xenon, compared with the
control animals after 60 and 120 min of reperfusion.

Myocardial Infarct Size

Myocardial infarct size as a percentage of the AAR
in the experimental groups is shown in Fig. 2. Xe-LPC
(31 ± 8%) and i-LPC (29 ± 7%) reduced myocardial
infarct size, compared with the control animals (64 ±
6%, P < 0.0001). Administration of the COX-2 inhibi-
tor NS-398 blocked the reduction in myocardial infar-
cion size observed with Xe-LPC (NS-398 + Xe-LPC,
61 ± 6%, P = 0.0001 vs Xe-LPC), but it had no effect on
myocardial infarct size when administered alone (NS-
398 61 ± 7%, P = 1.0 vs control).

COX-2 Messenger Ribonucleic Acid
and Protein Expression

Results for the COX-2 messenger ribonucleic acid
(mRNA) and protein expression experiments are
shown in Fig. 3. i-LPC increased the expression of
COX-2 mRNA in a time-dependent manner (Sham:
0.3 ± 0.25, 0 h: 0.8 ± 0.5; 2 h: 2.5 ± 1.6; 4 h: 1.8 ± 0.1;
8 h 2.0 ± 1.2; 12 h: 1.8 ± 1.4; 24 h: 0.8 ± 0.4, all average light intensity, Fig. 3A) with a maximum increase occurring 2 h after i-LPC (P = 0.038 vs Controls). In contrast, there was no increase in COX-2 mRNA expression in the Xe-LPC group (Con: 0.3 ± 0.1; 0 h: 0.25 ± 0.1; 2 h: 0.5 ± 0.3; 4 h: 0.2 ± 0.1; 8 h: 0.3 ± 0.2; 12 h: 0.3 ± 0.2; 24 h: 0.3 ± 0.2; P = 0.979, 0.374, 0.723, 0.999, 1.0, 0.999, respectively, Fig. 3B).

Similar to the results of for the mRNA analysis, there was a time-dependent increase in COX-2 protein expression after i-LPC with a maximum increased level occurring 4 h after myocardial ischemia (P = 0.417 vs Controls, Fig. 4A). There was no time-dependent change in COX-2 protein expression in Xe-LPC (Fig. 4B).

**DISCUSSION**

The present data show that the noble gas xenon induces a late phase of myocardial preconditioning in rat hearts *in vivo*. The magnitude of protection is similar to that resulting from late myocardial ischemic preconditioning. Furthermore, we demonstrated that COX-2 blockade abolished the cardioprotective effect of Xe-LPC (Fig. 2). Moreover, i-LPC increased the expression of COX-2 mRNA and protein in a time-dependent manner, but there was no difference in COX-2 mRNA and protein expression observed after xenon late myocardial preconditioning.

In various animal species, xenon has been shown to have cardioprotective effects including early myocardial preconditioning in rats and a reduction of myocardial reperfusion injury (postconditioning effect) in rabbits *in vivo*. Possible mechanisms for xenon-induced early cardioprotection like protein kinase C, p38 mitogene activated protein kinase, heat shock protein 27, K<sub>ATP</sub> channels and phosphatidyl inositol dependent kinase 1 have been identified.

However, there are no prior data on whether xenon exposure leads to late myocardial preconditioning and, if so, the potential mediators of this effect.
It has been shown that cardioprotection by i-LPC and volatile anesthetic induced late preconditioning are mediated by complex pathways including COX-2. However, the literature also suggests that involvement of COX-2 might be differentially regulated in ischemic and anesthetic-induced late preconditioning.

i-LPC increases COX-1 and 2 expression and enhances myocardial concentrations of prostaglandins E2 and 6-keto-PGF1α in rabbits. In isoflurane-induced late preconditioning in rabbits, the COX-2 inhibitor celecoxib effectively blocked the infarct size-reducing effect of the volatile anesthetic. However, an effect of isoflurane on COX-1 and COX-2 protein expression was not observed. These data are in line with our findings showing that the myocardial infarct size-limiting effect of Xe-LPC can be blocked by inhibition of COX-2 activity independently of an up-regulation of COX-2 mRNA and protein. These results suggest that pharmacological late preconditioning depends more on an increased activity of existing COX-2 rather than a transcriptional upregulation of COX-2 mRNA. In contrast, these results suggest that i-LPC requires upregulation of COX-2 mRNA and protein expression. Nonetheless, it must be considered that early anesthetic myocardial preconditioning involves a time-dependent upregulation of intracellular enzymes that could also exist in late anesthetic preconditioning. In rabbits, the signaling pathways of i-LPC differ 24 or 72 h after the preconditioning stimulus. Only 72 h after the preconditioning ischemia, myocardial levels of COX-2 mRNA, protein and cardioprotective prostaglandins were found to be increased. In our experiments, we did not observe an increase in COX-2 mRNA or protein 0 to 24 h after xenon preconditioning, but we did not measure COX-2 mRNA and protein level 72 h after preconditioning.

In addition to COX-2, inducible nitric oxide synthase (iNOS) and mitochondrial KATP channels have been shown to be involved in i-LPC. Mitochondrial KATP channels have been shown to be also involved in xenon-induced early preconditioning. Most importantly, nitric oxide, produced by iNOS, induces preconditioning via activation of COX-2 and prostaglandins generated by COX-2 may reduce infarct size by activating the opening of KATP channels. These data indicate that the protective effects of ischemic preconditioning on the myocardium via COX-2 is a complex interaction between iNOS, nitric oxide, prostaglandins and KATP channel opening. However, which other pathways surrounding COX-2 might also play a role in Xe-LPC cannot be answered from our results. Moreover, COX-2 implementation in Xe-LPC must rely on indirect evidence from the pharmacological blockade of COX-2.

NS398 alone had remarkable hemodynamic effects during the reperfusion period. This is thought to be a random effect rather than a specific pharmacological side effect of NS398, a highly selective COX-2 blocker. In this study only a single concentration of xenon was used and our results must be restricted to this concentration. Notably, xenon preconditioning may occur independent of any anesthetic effect, as other noble gases without anesthetic properties, such as helium, are also able to display cardioprotective effects. In our study, xenon cardioprotection was only

![Figure 4. Cyclooxygenase (COX)-2 protein expression in ischemic late preconditioning (i-LPC) and xenon-induced late preconditioning (Xe-LPC) myocardium. Representative infrared Western blot experiments of the time course (Con, 0 h, 2 h, 4 h, 8 h, 12 h, 24 h, each time point n = 4 animals) showing COX-2 protein expression (70 kDa) in ischemic late preconditioned (i-LPC, panel A) or xenon late preconditioned (Xe-LPC, panel B) myocardium. Upper panel shows total COX-2 protein, lower panel shows α-tubulin. The histogram presents densitometric evaluation of all experiments. Data show ratio of COX-2 normalized to α-tubulin (means ± sd).](image-url)
examined after 25 min of myocardial ischemia. Whether xenon cardioprotection occurs after longer periods of occlusion cannot be answered from this study.

In summary, the present results show that the noble gas xenon induces late myocardial preconditioning in rat hearts in vivo. The results further indicate differences in the involvement of COX-2 between i-LPC and Xe-LPC. Although the latter involves changes in the activity of existing COX-2, the former appears to depend on the upregulation of COX-2 mRNA and protein expression.

REFERENCES