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Effects of Nitrous Oxide on the Rat Heart In Vivo

Another Inhalational Anesthetic That Preconditions the Heart?

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Background: **For nitrous oxide, a preconditioning effect on the heart has yet not been investigated. This is important because nitrous oxide is commonly used in combination with volatile anesthetics, which are known to precondition the heart. The authors aimed to clarify (1) whether nitrous oxide preconditions the heart, (2) how it affects protein kinase C (PKC) and tyrosine kinases (such as Src) as central mediators of preconditioning, and (3) whether isoflurane-induced preconditioning is influenced by nitrous oxide.**

Methods: **For infarct size measurements, anesthetized rats were subjected to 25 min of coronary artery occlusion followed by 120 min of reperfusion. Rats received nitrous oxide (60%), isoflurane (1.4%) or isoflurane–nitrous oxide (1.4%/60%) during three 5-min periods before index ischemia (each group, n = 7). Control animals remained untreated for 45 min. Additional hearts (control, 60% nitrous oxide alone%, and isoflurane–nitrous oxide [0.6%/60%, in equianesthetic doses]) were excised for Western blot of PKC-ε and Src kinase (each group, n = 4).**

Results: **Nitrous oxide had no effect on infarct size (59.1 ± 15.2% of the area at risk *vs.* 51.1 ± 10.9% in controls). Isoflurane (1.4%) and isoflurane–nitrous oxide (1.4%/60%) reduced infarct size to 30.9 ± 10.6 and 28.7 ± 11.8% (both $P < 0.01$). Nitrous oxide (60%) had no effect on phosphorylation (2.3 ± 1.8 *vs.* 2.5 ± 1.7 in controls, average light intensity, arbitrary units) and translocation (7.0 ± 4.3 *vs.* 7.4 ± 5.2 in controls) of PKC-ε. Src kinase phosphorylation was not influenced by nitrous oxide (4.6 ± 3.9 *vs.* 5.0 ± 3.8; 3.2 ± 2.2 *vs.* 3.5 ± 3.0). Isoflurane–nitrous oxide (0.6%/60%, in equianesthetic doses) induced PKC-ε phosphorylation (5.4 ± 1.9 *vs.* 2.8 ± 1.5; $P < 0.001$) and translocation to membrane regions (13.8 ± 13.0 *vs.* 6.7 ± 2.0 in controls; $P < 0.05$).**

Conclusions: **Nitrous oxide is the first inhalational anesthetic without preconditioning effect on the heart. However, isoflurane-induced preconditioning and PKC-ε activation are not influenced by nitrous oxide.**

EVERY volatile anesthetic currently in clinical use studied so far has been recognized to mimic the strong cardioprotection against ischemia reperfusion damage exerted by ischemic preconditioning.[1] This so-called anesthetic-induced preconditioning by desflurane, isoflurane, or sevoflurane reduced infarct size to the same extent as ischemic preconditioning in rats,[2–4] rabbits, and dogs.[1,5–10] However, nitrous oxide as an anesthetic supplement for these volatile anesthetics has never been subject of any cardiac preconditioning study. This seems to be of critical importance because most anesthetics in cardiac risk patients are given for noncardiac surgery, and nitrous oxide is still in widespread clinical use and finds strong advocates among anesthetists. If preconditioning and cardioprotection by anesthetics is becoming an accepted clinical concept in the future, it would be important to know whether a substance has these protective properties or even might block them like ketamine,[11] especially when it is used in combination with cardioprotective agents such as isoflurane.

Besides the volatile anesthetics, the chemically "inert" gas xenon also induces pharmacologic preconditioning in the rat heart *in vivo*.[3] Xenon shares the same anesthetic properties as the volatile anesthetics, but as a noble gas, it has a complete different molecular structure compared with the halogenated fluorocarbons. Therefore, the results of the previous study were surprising, leading us to the hypothesis that anesthetic preconditioning itself is mediated not only by volatile anesthetics but by all anesthetic gases. The molecular structure of nitrous oxide is also different from that of the halogenated agents, and in the current study, we tested the hypothesis that nitrous oxide has a preconditioning effect.

Regarding the underlying molecular mechanisms of anesthetic-induced preconditioning, there are complex different signal transduction pathways under current discussion. Activation of key enzymes such as protein kinase C (PKC) and protein tyrosine kinases (PTKs, such as Src kinase) have been shown to be involved in anesthetic preconditioning.[2,3,12] For the inert gas xenon and the volatile anesthetic isoflurane, we could recently demonstrate that cardioprotection was mediated *via* increased activation of PKC isoform ε *in vivo*.[3,4] PKC isoforms have been shown to be mainly regulated *via* translocation to different cell compartments and subsequent phosphorylation at three phosphorylation sites, resulting in their activation.[13] PKC-ε translocates from cytosolic to membrane regions upon different stimuli[14] and affects several downstream targets leading to the observed myocardial protection.

The implication of PTKs in anesthetic-induced preconditioning is still a matter of controversy.[12,15] In contrast, in ischemic preconditioning, both enzyme classes have been shown to be clearly involved in mediating cardioprotection.[16,17]

In contrast to PKC, the Src kinase, a member of the

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PTK family, is regulated by tyrosine phosphorylation at two sites with opposing effects. Phosphorylation of tyrosine 416 in the activation loop of the kinase domain up-regulates the enzyme activity, and phosphorylation of tyrosine 527 in the C-terminal tail renders the enzyme less active (for review, see Roskoski).[18]

The current study aimed to determine in an *in vivo* rat model (1) whether nitrous oxide preconditions the heart; (2) how it affects PKC and Src, both putative key enzymes of anesthetic preconditioning; (3) whether isoflurane-induced preconditioning is influenced by nitrous oxide; and (4) whether PKC-ε activation by isoflurane is still detectable in the presence of nitrous oxide.

Materials and Methods

The study was performed in accord with the regulations of the German Animal Protection Law and was approved by the Bioethics Committee of the District of Düsseldorf (Düsseldorf, Germany). A total of 40 male Wistar rats were used (200–250 g; 7 per group for infarct size measurement and 4 per group for Western blot).

Materials

Monoclonal mouse anti–α-tubulin and rabbit polyclonal anti–actin antibody were purchased from Sigma (Taufkirchen, Germany). The enhanced chemoluminescence protein detection kit was purchased from Santa Cruz (Heidelberg, Germany). Total PKC-ε rabbit polyclonal and phospho PKC-ε rabbit antibody were from Upstate (Charlottesville, VA). Peroxidase-conjugated goat anti-rabbit and donkey anti-mouse antibodies were from Jackson Immunolab (Dianova, Hamburg, Germany). Anti–phospho Src (Tyr 416) and phospho Src (Tyr 527) rabbit polyclonal antibodies were from Cell Signaling (Frankfurt/M, Germany). All other materials were purchased from either Sigma or Merck-Eurolab (Munich, Germany).

Animal Preparation

Male Wistar rats (200-250 g) were anesthetized by intraperitoneal *S*-ketamine injection (150 mg/kg). It has been demonstrated previously that the *S*-enantiomer of ketamine does not influence myocardial preconditioning.[11] Animals had free access to food and water at all times before the start of the experiments.

All animals were left untreated for 10 min before the start of the respective preconditioning protocol (figs. 1A and B).

Further preparation and infarct size measurement by triphenyltetrazolium chloride (TTC) staining were performed as described previously.[3] In summary, after tracheal intubation, the lungs were ventilated with oxygen-enriched air and a positive end-expiratory pressure of 2–3 cm H_2O. Respiratory rate was adjusted to maintain

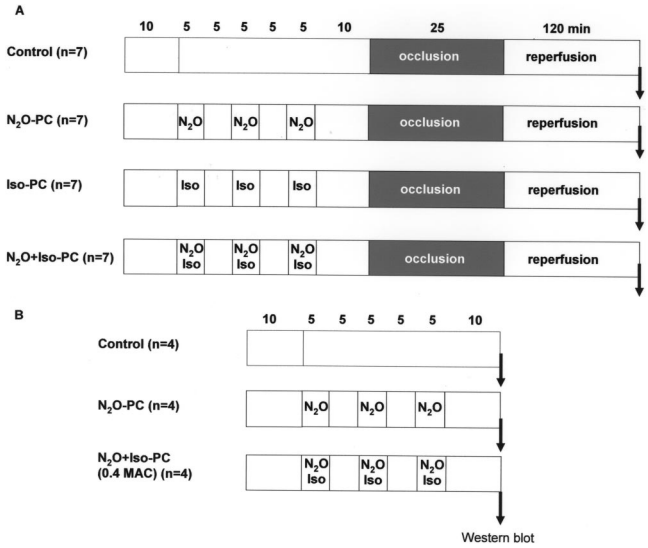

Fig. 1. (*A*) Experimental protocol for infarct size measurement. (*B*) Experimental protocol for Western blot assay and immunofluorescence staining. Iso = isoflurane; MAC = minimum alveolar concentration; N_2O = nitrous oxide; PC = preconditioning.

partial pressure of carbon dioxide within physiologic limits. Body temperature was maintained at 38°C by the use of a heating pad. The right jugular vein was cannulated for saline and drug infusion, and the left carotid artery was cannulated for measurement of aortic pressure. Anesthesia was maintained by continuous α-chloralose infusion. A lateral left-sided thoracotomy followed by pericardiotomy was performed, and a ligature (5-0 Prolene®; Ethicon GmbH, Norderstedt, Germany) was passed below the main branch of the left coronary artery. The ends of the suture were threaded through a propylene tube to form a snare, and the coronary artery was occluded by tightening the snare. Successful coronary artery occlusion was verified by epicardial cyanosis.

Infarct Size Measurement/TTC Staining

After 120 min of reperfusion, the heart was excised and mounted on a modified Langendorff apparatus for perfusion with ice-cold normal saline *via* the aortic root at a perfusion pressure of 40 cm H_2O to wash out intravascular blood. After 5 min of perfusion, the coronary artery was reoccluded, and the remainder of the myocardium was perfused through the aortic root with 0.2% Evans blue in normal saline for 10 min. Intravascular Evans blue was then washed out by perfusion for 5 min with normal saline. This treatment identified the area at risk as unstained. The heart was then cut into transverse slices, 2 mm thick. The slices were stained with 0.75% TTC solution for 10 min at 37°C and fixed in 4% formalin solution for 6 h at room temperature. The area of risk and the infarcted area were determined by planimetry using SigmaScan Pro 5® computer software (SPSS Science Software, Chicago, IL) and corrected for dry weight.

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Experimental Protocols for Infarct Size Measurement

Rats were divided into four groups (fig. 1A):

Control Group (n = 7). Rats received 20% oxygen plus 80% nitrogen during three 5-min periods before they were subjected to 25 min of left coronary artery occlusion, followed by 2 h of reperfusion. During the washout times, rats received 50% oxygen and 50% nitrogen.

Nitrous Oxide–preconditioned Group (n = 7). Rats received 60% nitrous oxide (equivalent to 0.4 minimum alveolar concentration [MAC] in rats) plus 20% oxygen and 20% nitrogen for three 5-min periods. The preconditioning periods were interspersed with two 5-min washout periods. After the last preconditioning, a 10-min final washout was performed before the 25-min coronary artery occlusion. Ischemia was followed by 2 h of reperfusion. During the washout times, rats received 50% oxygen and 50% nitrogen.

Isoflurane-preconditioned Group (n = 7). Rats received 1.4% isoflurane (1 MAC in rats) plus 20% oxygen and 78.6% nitrogen for three 5-min periods. The preconditioning periods were interspersed with two 5-min washout periods. After the last preconditioning, a 10-min final washout was performed before the 25-min coronary artery occlusion. Ischemia was followed by 2 h of reperfusion. During the washout times, rats received 50% oxygen and 50% nitrogen.

Isoflurane in Nitrous Oxide–preconditioned Group (n = 7). Rats received a mixture of 60% nitrous oxide, 20% oxygen, and 18.6% nitrogen to which 1.4% isoflurane was added for three 5-min periods. The preconditioning periods were interspersed with two 5-min washout periods. After the last preconditioning, a 10-min final washout was performed before the 25-min coronary artery occlusion. Ischemia was followed by 2 h of reperfusion. During the washout times, rats received 50% oxygen and 50% nitrogen.

Experimental Protocols for Western Blot Assay

Rats were divided into three groups (fig. 1B):

Control Group (n = 4). Rats received 20% oxygen plus 80% nitrogen during three 5-min periods. The preconditioning periods were interspersed with two 5-min washout periods. After the last preconditioning, a 10-min final washout was performed, and the hearts were excised and shock frozen in liquid nitrogen. During the washout times, rats received 50% oxygen and 50% nitrogen.

Nitrous Oxide–preconditioned Group (n = 4). Rats received 60% nitrous oxide (equivalent to 0.4 MAC in rats) plus 20% oxygen and 20% nitrogen for three 5-min periods. The preconditioning periods were interspersed with two 5-min washout periods. After the last preconditioning, a 10-min final washout was performed, and the hearts were excised and shock frozen in liquid nitrogen. During the washout times, rats received 50% oxygen and 50% nitrogen.

Isoflurane in Nitrous Oxide–preconditioned Group (n = 4). Rats received a mixture of 60% nitrous oxide, 20% oxygen, and 19.4% nitrogen to which 0.6% isoflurane was added for three 5-min periods. The preconditioning periods were interspersed with two 5-min washout periods. After the last preconditioning, a 10-min final washout was performed, and the hearts were excised and shock frozen in liquid nitrogen. This isoflurane concentration corresponds to 0.4 MAC in rats. To compare the results with those of our previous study using a similar preconditioning protocol with xenon and isoflurane,[3] we used the same isoflurane concentration for the investigations of the molecular targets. This isoflurane concentration was shown to induce a strong PKC phosphorylation and translocation in our previous study.[3] During the washout times, rats received 50% oxygen and 50% nitrogen.

After each preconditioning protocol, the end-tidal concentration of the anesthetic was measured during the final washout period to guarantee a sufficient washout of the respective anesthetic. End-tidal concentration of the anesthetic returned to zero within 2 min during the last washout.

Measurement of Hemodynamic Variables

Aortic pressure and electrocardiographic signals were digitized using an analog-to-digital converter (PowerLab/8SP; ADInstruments Pty. Ltd., Castle Hill, Australia) at a sampling rate of 500 Hz and were continuously recorded on a personal computer using Chart for Windows version 5.0 (ADInstruments Pty. Ltd.).

Separation of Membrane and Cytosolic Fraction

Tissue specimens were prepared for protein analysis to investigate PKC-ε activation and distribution (membrane and cytosolic fraction) within the myocytes. The excised hearts were frozen in liquid nitrogen. Subsequently, a tissue fractionation was performed that was adapted from the literature.[19,20] The frozen tissue was pulverized and dissolved in lysis buffer containing Tris base, EGTA, NaF, and Na_3VO_4 (as phosphatase inhibitors), a freshly added protease inhibitor mix (aprotinin, leupeptin, and pepstatin), and dithioerythriol. The solution was vigorously homogenized on ice (Homogenizer; IKA®, Staufen, Germany) and then centrifuged at 1,000*g*, 4°C, for 10 min. This centrifugation at low speed allows a raw separation between the cytosolic fraction that still contains cellular organelles and their membranes and the membrane fraction still containing nuclear particles. The supernatant, containing cytosolic fraction, was centrifuged again at 16,000*g*, 4°C, for 15 min to clean up this fraction and to separate the mitochondria and other cellular organelles from this fraction. The remaining pellet was resuspended in lysis buffer containing 1% Triton X 100,

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Table 1. Hemodynamic Variables

		Preconditioning						Ischemia		Reperfusion		
	Baseline	PC-1	Wash-1	PC-2	Wash-2	PC-3	Wash-3	Occ 15	Occ 24	Rep 15	Rep 60	Rep 120
HR, min^{-1}												
Control	458 ± 33	459 ± 34	459 ± 34	451 ± 37	458 ± 36	460 ± 33	458 ± 34	462 ± 34	460 ± 33	460 ± 55	454 ± 53	441 ± 61
N_2O PC	462 ± 45	465 ± 47	439 ± 40	444 ± 36	441 ± 38	449 ± 37	440 ± 43	456 ± 28	462 ± 29	449 ± 31	456 ± 33	446 ± 42
Isoflurane PC	459 ± 36	402 ± 37*†	443 ± 39	382 ± 45*†	426 ± 37	380 ± 37*†	432 ± 42	465 ± 32	457 ± 33	449 ± 36	415 ± 47	404 ± 49*
Isoflurane + N_2O PC	460 ± 23	417 ± 29	447 ± 43	405 ± 36*	431 ± 52	399 ± 40*†	437 ± 58	447 ± 56	446 ± 47	436 ± 48	415 ± 53*	402 ± 46*
AoP_{mean}, mmHg												
Control	134 ± 11	131 ± 12	130 ± 11	127 ± 13	127 ± 12	132 ± 10	130 ± 13	108 ± 27	114 ± 22	104 ± 24*	102 ± 25*	94 ± 31*
N_2O PC	142 ± 12	126 ± 23	138 ± 10	126 ± 16	128 ± 14	122 ± 20	123 ± 15	111 ± 35	113 ± 40	101 ± 35	94 ± 27*	80 ± 20*
Isoflurane PC	147 ± 27	80 ± 17*†	140 ± 26	68 ± 11*†	133 ± 26	67 ± 15*†	128 ± 33	137 ± 15	128 ± 19*	118 ± 26*	101 ± 27*	87 ± 21*
Isoflurane + N_2O PC	144 ± 23	82 ± 25*†	148 ± 34	75 ± 24*†	142 ± 40	70 ± 21*†	142 ± 38	117 ± 39	122 ± 34	109 ± 38*	101 ± 42*	84 ± 32*

Data are presented as mean ± SD.

* $P < 0.05$ *vs.* baseline. † $P < 0.05$ *vs.* control.

AoP_{mean} = mean aortic pressure; HR = heart rate; N_2O = nitrous oxide; Occ = coronary artery occlusion; PC = preconditioning cycle; Rep = reperfusion; Wash = washout.

incubated for 60 min on ice and vortexed. The solution was centrifuged at 16,000*g*, 4°C, for 15 min, and the supernatant was collected as membrane fraction.

Western Blot Analysis

After protein determination by the Lowry method,[21] equal amounts of protein were mixed with loading buffer containing Tris-HCl, glycerol, and bromphenol blue. Samples were vortexed and boiled at 95°C before being subjected to SDS-PAGE. Samples were loaded on a 7.5% (PKC-ε) or a 10% (Src) SDS electrophoresis gel, respectively. The proteins were separated by electrophoresis and then transferred to a polyvinylidenfluorid membrane by tank blotting. Unspecific binding of the antibody was blocked by incubation with 5% fat dry milk powder solution in Tris-buffered saline containing Tween (TBS-T) 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 TBS-T, the blot was subjected to the appropriate horseradish peroxidase–conjugated secondary antibody for 2 h at room temperature. Immunoreactive bands were visualized by chemoluminescence detected on x-ray film (Hyperfilm ECL; Amersham, Freiburg, Germany) using the enhanced chemoluminescence system from Santa Cruz. The blots were quantificated using a Kodak Image station® (Eastman Kodak Co., Rochester, NY). Equal loading of the protein to the SDS-PAGE gel was ensured by Coomassie blue staining (Coomassie brilliant blue®; Serva electrophoresis GmbH, Heidelberg, Germany) of each gel, and the results are presented as ratio of phosphorylated protein to total protein, as ratio of phosphorylated protein to actin, or as ratio of phosphorylated protein to α-tubulin (as indicated in the respective figure legend) after multiplication of the average light intensity by 10 to facilitate the presentation of an X-fold increase.

Statistical Analysis Sample Size Calculation

Sample sizes were calculated using the program GraphPad StatMate version 1.01 (GraphPad Software, San Diego, CA). For infarct size with and α error of $\alpha < 0.05$ and a β error < 10%, a group size of n = 7 is necessary to detect a minimal difference in infarct size of 30% with a power of 95%.

For Western blot assay with and α error of $\alpha < 0.05$ and a β error < 10%, a group size of n = 4 is necessary to detect a minimal difference in phosphorylation state of 45% with a power of 95%.

Data are expressed as mean ± SD. Group comparisons were analyzed by Student *t* test with Welch modification (Graph Pad Prism version 4.00) followed by Bonferroni correction for multiple comparisons.

Statistical analysis of the hemodynamic variables was performed by two-way repeated-measures analysis of variance for time and treatment (experimental group) effects. If an overall significance was found, comparisons between groups were performed for each time point using one-way analysis of variance followed by Dunnett *post hoc* test with the control group as the reference group. Time effects within each group were analyzed by repeated-measures analysis of variance followed by Dunnett *post hoc* test with the baseline value as the reference time point. $P < 0.05$ was considered statistically significant.

Results

Hemodynamics

Table 1 shows the time course of heart rate and mean aortic pressure during the experiments. No significant differences in heart rate and aortic pressure were observed between the experimental groups during baseline, ischemia, and reperfusion periods. Isoflurane and isoflurane plus nitrous oxide transiently reduced mean aortic pressure during preconditioning cycles, but the

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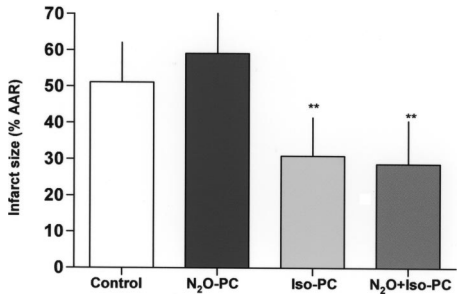

Fig. 2. Infarct size measurement. *Histogram* shows the infarct size (percent of area at risk [AAR]) of controls, nitrous oxide preconditioning (N_2O-PC), isoflurane preconditioning (Iso-PC), and nitrous oxide preconditioning plus isoflurane preconditioning (N_2O+Iso-PC) groups. Data are presented as mean ± SD. ** $P < 0.01$ *versus* control group.

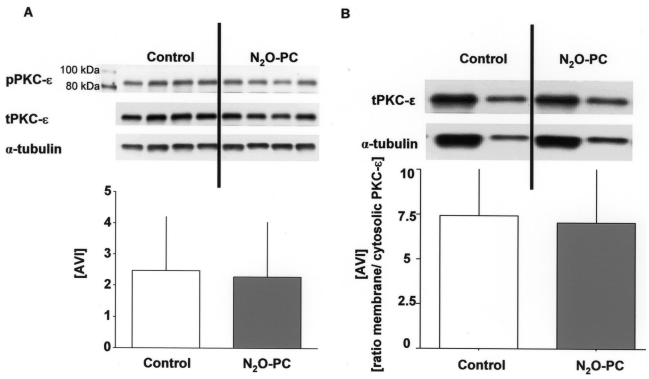

Fig. 3. (*A*) Phosphorylation of protein kinase C (PKC)-ε. One representative Western blot experiment showing phosphorylation state of PKC-ε in control and nitrous oxide–treated hearts (N_2O-PC) (each n = 4) is presented. *Upper panel* shows phosphorylated form of PKC-ε; *middle panel* shows total PKC-ε. The *histogram* presents densitometric evaluation as average light intensity (AVI). Data show ratio of phosphorylated (pPKC-ε) *versus* total PKC-ε (tPKC-ε) (mean ± SD). Equal loading of the protein was confirmed by additional α-tubulin detection (*lower panel*). (*B*) Translocation of PKC-ε in Western blot assay. Membrane and cytosolic fraction of control (*white bar*) and N_2O-PC hearts (*gray bar*) (each n = 4) were immunoblotted using antibodies against PKC-ε (*upper panel*) or α-tubulin (*lower panel*). The densitometric evaluation as AVI is shown in the *histogram*. Data are mean ± SD, and the ratio of membrane PKC-ε to cytosolic PKC-ε is presented.

values recovered to baseline during the washout periods. Isoflurane significantly reduced heart rate during the preconditioning periods compared with baseline conditions (preconditioning 1: 402 ± 37, preconditioning 2: 382 ± 45, preconditioning 3: 380 ± 37 *vs.* baseline: 459 ± 36) and with the control group (control: preconditioning 1: 459 ± 34, preconditioning 2: 451 ± 37, preconditioning 3: 460 ± 33). For isoflurane plus nitrous oxide, a difference of heart rate compared with the control group was only found during the third preconditioning cycle (preconditioning 3: 399 ± 37) and disappeared after the final washout. The respective changes disappeared after the final washout.

Infarct Size Measurement

Nitrous oxide had no effect on infarct size compared with controls (59.1 ± 15.2% of the area at risk *vs.* 51.1 ± 10.9%; fig. 2). In contrast, isoflurane significantly reduced infarct size to 30.9 ± 10.6%; $P < 0.01$). A similar infarct size reduction could be observed for the combined application of isoflurane and N_2O (28.7 ± 11.8%; $P < 0.01$ *vs.* controls).

Effects of Nitrous Oxide on PKC-ε Phosphorylation

Direct influence of nitrous oxide administration on PKC-ε was determined by the use of a phospho-specific antibody against PKC-ε. Nitrous oxide had no effect on phosphorylation of PKC-ε (2.3 ± 1.8 *vs.* 2.5 ± 1.7 in controls; fig. 3A). Total PKC-ε protein amount was equal in the different slots because the Western blot (fig. 3A, middle Western blot) showed a uniform distribution. Equal loading of protein samples was confirmed by detection of α-tubulin on the respective blot (fig. 3A, lowest Western blot).

Effects of Nitrous Oxide on PKC-ε Translocation

Protein kinase C is activated not only by phosphorylation, but also by translocation to the membrane. Both activation steps can occur in parallel and also independently of each other.[22] Therefore, the absence of an effect of nitrous oxide on PKC-ε phosphorylation does not necessarily exclude a possible effect of nitrous oxide on PKC-ε translocation to membrane regions.

Western blot assay of fractionated tissue (fig. 3B) clearly revealed that nitrous oxide had no effect on translocation of PKC-ε to membrane regions (ratio membrane to cytosol PKC-ε: 7.0 ± 4.3 *vs.* 7.4 ± 5.2 in controls; fig. 3B). α-Tubulin detection served as loading control (fig. 3B, lower Western blot).

Effects of Nitrous Oxide on Src Phosphorylation

Nitrous oxide had no effect on phosphorylation of Src-kinase (Tyr 416/527) (4.6 ± 3.9 *vs.* 5.0 ± 3.8 and 3.2 ± 2.2 *vs.* 3.5 ± 3.0; figs. 4A and B). Actin detection served as internal standard (fig. 4A, lower Western blot), and the ratio of phosphorylated protein to actin is shown. In addition, protein on the electrophoresis gels were stained with Coomassie blue to show equal protein loading on the respective gel (fig. 4A).

Influence of Nitrous Oxide on Isoflurane-induced Phosphorylation and Translocation of PKC-ε

We could recently show that the volatile anesthetic isoflurane (0.4 MAC) enhanced phosphorylation as well as translocation of PKC-ε and that blockade of PKC *in vivo* by calphostin C completely abolished isoflurane-induced cardioprotection.[3] Therefore, we aimed to clarify whether this increased phosphorylation and translocation of a key enzyme in isoflurane-induced preconditioning is influenced in the presence of nitrous oxide in an equianesthetic dose (0.4 MAC). The combined administration of isoflurane plus nitrous oxide (0.4

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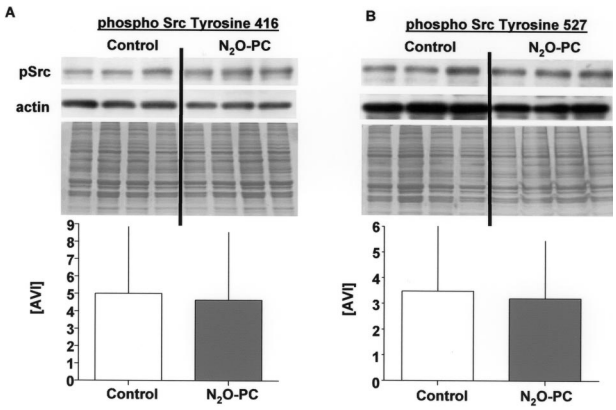

Fig. 4. Influence of nitrous oxide on Src kinase phosphorylation (two phosphorylation sites). The phosphorylation state of Src kinase (tyrosine 416 [*A*] and tyrosine 527 [*B*]) in control and nitrous oxide–treated (N_2O-PC) hearts (each n = 4) is demonstrated. *Upper panel* shows respectively phosphorylated form of Src (pSrc); *middle panel* shows actin distribution. The *histogram* presents densitometric evaluation as average light intensity (AVI). Data demonstrate ratio of phosphorylated Src *versus* actin (means ± SD). Equal loading of protein on the gel was confirmed by Coomassie blue staining of the gel (respective *lower panel*).

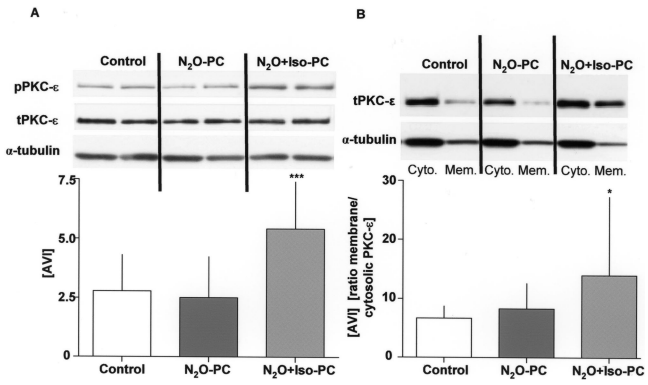

Fig. 5. (*A*) Phosphorylation of protein kinase C (PKC)-ε after combined nitrous oxide–isoflurane preconditioning. The phosphorylation state of PKC-ε in control, nitrous oxide–treated (N_2O-PC), or nitrous oxide–isoflurane-treated (N_2O+Iso-PC) hearts (each n = 4) is depicted. *Upper panel* is the phosphorylated form of PKC-ε (pPKC-ε); *middle panel* is the total PKC-ε (tPKC-ε). The densitometric evaluation as average light intensity (AVI) is summarized in the *histogram*. Data show ratio of pPKC-ε *versus* tPKC-ε (mean ± SD). Equal loading of the protein was confirmed by additional α-tubulin detection (*lower panel*). *** $P < 0.01$ *versus* control group. (*B*) Translocation of PKC-ε after combined N_2O+Iso-PC. Membrane and cytosolic fraction of control (*white bar*) and N_2O-PC hearts (*gray bar*) and N_2O+Iso-PC (each n = 4) were immunoblotted using antibodies against PKC-ε (*upper panel*) or α-tubulin (*lower panel*). One representative Western blot experiment is shown. The *histogram* presents densitometric evaluation as AVI. Data are mean ± SD, and the ratio of membrane PKC-ε to cytosolic PKC-ε is shown. * $P < 0.05$ *versus* control group.

MAC/0.4 MAC) induced PKC-ε phosphorylation (5.4 ± 1.9 *vs.* 2.8 ± 1.5; $P < 0.001$; fig. 5A) and translocation (ratio membrane to cytosol PKC-ε: 13.8 ± 13.0 *vs.* 6.7 ± 2.0 in controls; $P < 0.05$; fig. 5B) similar to that seen in the previous study with isoflurane (0.4 MAC) alone,[3,4] demonstrating that PKC-ε is still activated by isoflurane in the presence of nitrous oxide.

Discussion

We investigated whether nitrous oxide preconditions the rat heart *in vivo* and whether nitrous oxide affects the two key mediator enzymes of anesthetic-induced preconditioning, PKC and PTKs. We also investigated whether isoflurane-induced preconditioning and activation of PKC isoform ε is influenced by nitrous oxide.

The new findings of the current study are as follows: First, nitrous oxide is the first inhalational anesthetic that does not precondition the heart *in vivo*. Second, nitrous oxide did not influence the activation (phosphorylation and translocation) of PKC-ε or Src tyrosine kinase. Third, isoflurane-induced preconditioning and activation of PKC-ε by isoflurane were not affected by nitrous oxide.

The results obtained from the current study were surprising because not only all volatile anesthetics but also the inert gas xenon (0.4 MAC) had a strong cardioprotective effect on the rat heart *in vivo*.[3] Moreover, the preconditioning effect was for a long time regarded to be a specific action of inhalational anesthetics. Therefore, nitrous oxide seems to be an exception to this rule.

Because nitrous oxide is commonly used in combination with volatile anesthetics such as isoflurane, a potential interaction between both anesthetics might influence the cardioprotection of volatile anesthetics. However, we could show that the presence of nitrous oxide had no effect on isoflurane-induced preconditioning, because the combined administration of nitrous oxide and isoflurane (0.4 MAC/1 MAC) reduced infarct size up to 30%. These data suggest that there is no interaction between both anesthetics with regard to the preconditioning effect of the volatile anesthetic.

The data from infarct size measurement were strongly supported by the findings on the molecular target PKC-ε, which has been identified as mediator of isoflurane-induced preconditioning in our *in vivo* rat model.[3,4] Isoflurane-induced phosphorylation and translocation of PKC-ε were not influenced by nitrous oxide. Moreover, nitrous oxide alone did not affect the phosphorylation and translocation state of this enzyme.

The phenomenon of anesthetic-induced preconditioning has raised a lot of attention in the past years, and preconditioning by different inhalational anesthetics has been demonstrated *in vitro* and *in vivo* in different animal species and in humans.

Ischemic and anesthetic-induced preconditioning share several steps in the signaling cascade. One of these steps is the activation of different PKC isoforms by phosphorylation and translocation. PKC plays a central role in both preconditioning forms.[23] We previously demonstrated that PKC-ε phosphorylation was increased after three 5-min periods of xenon (0.4 MAC) and isoflurane

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(0.4 MAC) inhalation and that the PKC inhibitor calphostin C abolished both the anesthetic-induced infarct size reduction and the increased phosphorylation and translocation of PKC isoform ε.[3] Similarly to xenon and isoflurane-induced preconditioning, desflurane preconditioning involves the ε isoform of PKC.[2] Moreover, the activation of PKC-ε seems to be initiated only by low doses of isoflurane because 0.4 MAC but not 1 MAC significantly increased PKC-ε phosphorylation and translocation.[4] This is in contrast to findings of Ludwig *et al.*,[12] who found that 1 MAC isoflurane given for 30 min before index ischemia induced translocation of PKC-ε. However, there are two aspects that should be taken into account regarding the discrepancy between these results. First, Ludwig *et al.* used a different preconditioning model in comparison to our study. They induced preconditioning by 30 min of isoflurane administration followed by a 15-min washout phase. In our study, 15 min of isoflurane (1 MAC) and a 10-min washout were used. Second, in the former study, hearts for tissue preparation and immunohistochemistry were excised directly after the preconditioning phase and not after the washout phase. As it is know from ischemic preconditioning and another study of our group, the translocation and phosphorylation of PKC follow a sensitive time course pattern.[2] Therefore, these differences in time course pattern and preconditioning duration might explain the different results.

However, identification of the PKC isoform mainly responsible for the cardioprotection by anesthetics is still a matter of controversy. For example, Uecker *et al.*[22] identified PKC-δ activation rather than PKC-ε as a key step in isoflurane-induced preconditioning. They did not find an increased phosphorylation of PKC-ε after 15 min of isoflurane (1.5 MAC) administration in the isolated rat heart.[22] However, as in our previous studies, they reported an increased translocation of PKC-ε to membrane regions.[22] In contrast to these two studies, Stowe *et al.*[24] identified PKC-ε as key mediator of preconditioning with sevoflurane in guinea pigs. These divergent data clearly indicate that there is a complex signaling cascade of anesthetic preconditioning depending on the choice of anesthetic, the species, and the preconditioning protocol. Moreover, there is obviously a pronounced discrepancy between the *in vitro* and *in vivo* situations.

In recent preconditioning studies using the same experimental model as in the current study, PKC-ε could be identified as the central mediator of anesthetic-induced preconditioning *in vivo*.[2–4] Therefore, we focused on PKC-ε as one potential target of nitrous oxide and demonstrated that this inhalational agent does not influence PKC-ε activation.

Besides the activation of PKC, a variety of additional kinases are involved in ischemic as well as in anesthetic preconditioning. The activation of different PKC isoforms is closely related to PTK phosphorylation. Evidence has been reported that PTKs can be upstream of,[25,26] parallel to,[17,27,28] or downstream of PKC.[29,30] Regarding the signal transduction cascade of ischemic preconditioning, the blockade of PTK has been shown to inhibit cardioprotection by ischemic preconditioning.[29,31] Moreover, PTKs were identified as triggers rather than mediators of ischemic preconditioning.[32] Vahlhaus *et al.*[28] demonstrated in pigs that a combined inhibition of PKC and TK is needed to sufficiently block cardioprotection of ischemic preconditioning. Fryer *et al.*[16] partially blocked ischemic preconditioning by the use of genistein and lavendustin A. The same group reported a successful blockade of cardioprotection induced by one 5-min cycle of preconditioning ischemia using genistein.[17] However, genistein did not abrogate preconditioning by three 5-min cycles of ischemia in rats.[17] In contrast to these studies, Kitakaze *et al.*[33] could not show an implication of PTKs in the infarct size limiting effect of ischemic preconditioning in the canine heart. In summary, the role of PTK activation and its relation to PKC seem to depend on species and the choice of the ischemic preconditioning protocol.

Concerning anesthetic-induced preconditioning, there is much more controversy regarding the implication of PTKs in cardioprotection. Stadnicka *et al.*[34] showed in an *in vitro* model of guinea pig ventricular myocytes that PTK may modulate the responsiveness of sarcolemmal adenosine triphosphate–sensitive potassium channels to isoflurane. In contrast to the sarcolemmal adenosine triphosphate–sensitive potassium channels, the mitochondrial adenosine triphosphate–sensitive potassium channels are activated independently of PKC and PTKs by isoflurane as reported by a later study of the same group.[35] These *in vitro* data are supported by a recent study of Ludwig *et al.*,[12] who found that the translocation of PKC-ε and -δ and activation of Src PTK mediate isoflurane-induced preconditioning *in vivo*. They used an *in vivo* rat model and the PTK blocker lavendustin A and PP1 in combination with 30 min of isoflurane (1 MAC) preconditioning. A recent study of our group using an *in vivo* rabbit model and a 15-min desflurane preconditioning stimulus demonstrated that the administration of two structurally different PTK inhibitors could not block cardioprotection by desflurane.[15]

These data again indicate that the implication of PTKs strongly depends on the choice of the anesthetic agent, the intensity of the preconditioning stimulus, and the species. In addition, a parallel pathway of PTKs and PKC, dependent on the force of the stimulus as already suggested for ischemic preconditioning by Fryer, seems a possible explanation for the above described discrepancies.

Nevertheless, besides PKC and adenosine triphosphate–sensitive potassium channels, PTKs seem to be one of the most supposable targets of preconditioning phenomena. Hence, we choose Src kinase phosphoryla-

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tion as a further cellular target to evaluate effects of nitrous oxide on preconditioning mediators. In agreement with the results from PKC-ε phosphorylation, we found no effect of nitrous oxide on either phosphorylation site of Src kinase.

Preconditioning indicates that the cardioprotective stimulus is not present during ischemia. Nitrous oxide has a low blood:gas partition coefficient of only 0.47 and shows an extremely rapid onset and offset of its action. A sufficient washout of nitrous oxide and isoflurane was guaranteed by a high fresh gas flow during the washout period. Moreover, the end-tidal concentration of anesthetics was measured continuously and returned to zero within 2 min during the last washout.

In the current study, we could not detect remarkable differences in the hemodynamic variables between the groups. During administration of the anesthetics, there was a transient reduction in mean aortic pressure and heart rate. Hemodynamics rapidly recovered to baseline conditions during the washout period. Most important, there were no significant differences between the groups *during ischemia*, and this would have been the only time period of critical importance in our experimental setting because major changes in this period might modify the ischemic injury. Regarding discussion of hemodynamic effects in our experimental setting, we would like to remark that the classic endpoint of cardioprotection by preconditioning is lethal cell damage, *i.e.*, infarct size which was measured directly in our study.

Hemodynamics were measured as a potential confounder of this target variable (*i.e.*, major changes of hemodynamics during ischemia might modify ischemic injury) but not as an endpoint (*i.e.*, stunning).

Major changes in hemodynamics cannot be expected from the salvage of tissue if one takes into account the total mass of the infarcted tissue; it seems very unlikely that one can see changes in hemodynamic variables during resting conditions, *i.e.*, when all compensatory mechanisms can still be used to keep the cardiac output at its normal resting value.

This is also what we had observed in all previous studies and in different animal models ranging from rat and rabbit to dogs. The overall size of the ischemic area is only around 25–31% of the total left ventricular contractile mass. In this area, 60–29% are infarcted. Even a reduction of the infarct size by 50% thus only gives 12–15% more of left ventricular contractile mass. With regard to healthy animals with a low resting cardiac output during anesthesia, such a small change in contractile mass is far away from bringing the left ventricle out of its compensatory range.

It should also be considered that we used only one concentration of nitrous oxide, and our results must be limited to this concentration. However, we aimed to investigate the most clinically relevant dose of both anesthetics alone and in combination. Therefore, we choose 1 MAC isoflurane and 0.4 MAC (corresponding to 60 vol%) nitrous oxide.

In general, several doses, preferably different by the factor of 10 in the form of a dose–response curve, should be investigated. Therefore, investigating a relevant further increase in the dose would result in severe technical problems because hypoxic conditions occur in the *in vivo* rat model. The only alternative would be to repeat all experiments under hyperbaric conditions to further increase the nitrous oxide dose, but this would not lead to clinically relevant results.

In summary, 60% nitrous oxide had no effect on two important mediators of anesthetic-induced preconditioning, PKC-ε and the PTK Src. In accord with these results, on the molecular level, nitrous oxide alone had no preconditioning effect on the rat heart *in vivo*. Therefore, in contrast to all other inhalational anesthetics, nitrous oxide is the first one that has no preconditioning effect. Because nitrous oxide is commonly used in combination with volatile anesthetics, it is of special importance that isoflurane-induced preconditioning and PKC-ε activation after isoflurane are not influenced by nitrous oxide.

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