Sevoflurane-induced Preconditioning

Impact of Protocol and Aprotinin Administration on Infarct Size and Endothelial Nitric-Oxide Synthase Phosphorylation in the Rat Heart In Vivo

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ABSTRACT

Background: Sevoflurane induces preconditioning (SevoPC). The effect of aprotinin and the involvement of endothelial nitric-oxide synthase (NOS) on SevoPC are unknown. We investigated (1) whether SevoPC is strengthened by multiple preconditioning cycles, (2) whether SevoPC is blocked by aprotinin, and (3) whether endothelial NOS plays a crucial role in SevoPC.

Methods: Anesthetized male Wistar rats were randomized to 15 groups (each n = 6) and underwent 25-min regional myocardial ischemia and 2-h reperfusion. Controls were not treated further. Preconditioning groups inhaled 1 minimum alveolar concentration of sevoflurane for 5 min (SEVO-I), twice for 5 min each (SEVO-II), three times for 5 min each (SEVO-III), or six times for 5 min each (SEVO-VI). Aprotinin was administered with and without sevoflurane. Involvement of endothelial NOS was determined with the nonspecific NOS blocker N-nitro-L-arginine-methyl-ester, the specific neuronal NOS blocker 7-nitroindazole, and the specific inducible NOS blocker aminoguanidine.

Results: SevoPC reduced infarct size in all protocols (SEVO-I, 42 ± 6%; SEVO-II, 33 ± 4%; SEVO-III, 11 ± 5%; SEVO-VI, 16 ± 4%; all $P < 0.001$ vs. control, 67 ± 3%) and was least after three and six cycles of sevoflurane ($P < 0.001$ vs. SEVO-II and -I, respectively). Aprotinin alone had no effect on infarct size but blocked SevoPC. N-nitro-L-arginine-methyl-ester abolished SevoPC (67 ± 4%; $P < 0.05$ vs. SEVO-III). Aminoguanidine and 7-nitroindazole blocked SevoPC only partially (25 ± 6 and 31 ± 6%, respectively; $P < 0.05$ vs. SEVO-III and control). SevoPC induced endothelial NOS phosphorylation, which was abrogated by aprotinin.

Conclusion: SevoPC is strengthened by multiple preconditioning cycles, and phosphorylation of endothelial NOS is a crucial step in mediating SevoPC. These effects are abolished by aprotinin.

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What We Already Know about This Topic

❖ The role of aprotinin in the setting of ischemia and reperfusion is not clear, aprotinin exhibits cardioprotective properties, but also may abolish ischemic induced preconditioning and anesthetic induced postconditioning.

What This Article Tells Us That Is New

❖ Cardioprotection from sevoflurane improves with multiple cycles of exposure and depends on phosphorylation of endothelial nitric oxide synthase.

❖ Aprotinin abolishes this cardioprotection from sevoflurane.

ISCHEMIC preconditioning is a strong protective mechanism of the heart in which brief exposure to ischemia/reperfusion markedly enhances the ability to withstand a subsequent ischemic injury. Beside ischemic stimuli, it is also possible to mimic this cardioprotective effect with volatile anesthetics, but it remains unclear whether repeated preconditioning stimuli can increase cardioprotection.[1] Volatile an-

esthetics also induce cardioprotection during cardiopulmonary bypass.[2] Besides the fact that all volatile anesthetics elicit cardioprotection by preconditioning in animals,[3] there is also strong evidence for clinical cardioprotection with these substances.[4,5] However, these cardioprotective effects are attributable to additive effects of preconditioning and postconditioning and to antiischemic effects. The evidence for a clinically significant preconditioning-only effect is weak. We have shown that in contrast to just one 5-min cycle, two 5-min cycles of 1 minimum alveolar concentration (MAC) sevoflurane before aortic crossclamping for coronary artery bypass graft surgery reduces myocardial damage in terms of troponin I release.[6] Compared with other clinical studies, which did not show a protective effect, we identified two major differences: the preconditioning protocol and the use of aprotinin.

From animal studies, it is known that multiple cycles of ischemic- and anesthetic-induced preconditioning strengthened the cardioprotective effect. A protocol consisting of two cycles of sevoflurane administration is more efficient than a single, longer period with the same concentration in guinea pig hearts *in vitro*.[7] Recently these findings were confirmed in rabbit hearts *in vivo* for desflurane.[8] The authors demonstrated that multiple cycles of preconditioning reduce the desflurane concentration that is needed to induce cardioprotection and therefore the side effects as well.[8] In addition, the use of multiple cycles of ischemic preconditioning induces additional signaling pathways and makes the cellular signaling more robust against blockade.[9]

Aprotinin was widely used in cardiac surgery to minimize blood loss. At the current time, serious concerns regarding the safety of aprotinin has limited its use. At first, an increased morbidity was observed in observational studies.[10] A randomized controlled trial was stopped early because treatment of high-risk patients with aprotinin was associated with a 50% higher mortality compared with treatment with lysine analogs.[11] However, there are still advocates who believe that aprotinin is a useful and safe drug in other patient populations (*e.g.*, in liver transplantation).[12,13]

The role of aprotinin in the setting of ischemia and reperfusion is not clear. There is evidence that aprotinin itself has cardioprotective properties.[14,15] However, there is also evidence that aprotinin abolishes ischemia-induced preconditioning[16,17] and anesthetic-induced postconditioning.[18] Despite its antifibrinolytic properties, aprotinin is known to be a competitive protein inhibitor of nitric-oxide synthase (NOS).[19] Ulker *et al.*[19] demonstrated that aprotinin down-regulates endothelial NOS (eNOS) messenger RNA and protein expression in cultured rat coronary microvascular endothelial cells. In rabbit hearts *in vivo*, administration of desflurane induces a transient activation of NOS activity. Blocking NOS activity with the unspecific blocker *N*-nitro-L-arginine methyl ester (L-NAME) abolishes desflurane-induced preconditioning.[20] It is not clear which isoform of the NOS system is involved in anesthetic induced preconditioning. It has been shown in human coronary artery endothelial cells that eNOS is activated through the 90-kd heat shock protein after isoflurane administration.[21]

The aim of the study was to go from the bedside[6] back to the bench and investigate the following: (1) Do multiple cycles of anesthetic-induced preconditioning induce a stronger cardioprotection than one cycle of sevoflurane-induced preconditioning (SevoPC)? (2) What influence does aprotinin have on SevoPC? (3) Is aprotinin blockade of eNOS responsible for the assumed aprotinin-induced SevoPC inhibition?

Materials and Methods

The study was performed in accordance with the guidelines laid out in the *Guide for the Care and Use of Laboratory Animals*, which is available from the U.S. National Academy of Sciences, and the regulations of the German Animal Protection Law and was approved by the District Government of Düsseldorf, Germany.

The influence of the preconditioning protocol and aprotinin on anesthetic-induced preconditioning and the influence of anesthetic-induced preconditioning and aprotinin on eNOS activity were investigated within two substudies.

Experiments for Infarct Size Determination

A total of 90 male Wistar rats were used for infarct size determination experiments (n = 6 per group; body weight [BW], 380–420 g).

Animals had free access to water and standard rat food at all times before experiments. The animal preparation and infarct size determination were performed as described previously.[22] The animals were anesthetized by intraperitoneal injection of $S(+)$-ketamine (150 mg/kg BW) and were mechanically ventilated (positive end-expiratory pressure, 2–3 cm H_2O; respiratory rate, 60 breaths/min; tidal volume, 5 ml; with oxygen-enriched air (inspired oxygen fraction, 0.4) after tracheal intubation. The respiratory rate was adjusted to maintain partial pressure of carbon dioxide within physiologic limits (end-tidal carbon dioxide, 35 ± 5 mmHg). Body temperature was maintained at 38°C by using a heating pad. After a midline cervical incision, the right jugular vein was cannulated for fluid replacement and drug administration, and the left carotid artery was cannulated for measurement of aortic pressure. Aortic pressure signals were digitized using an analog-digital converter and continuously recorded on a personal computer using the PowerLab software (PowerLab/8SP, Chart 5.0; ADInstruments Pty, Ltd., Castle Hill, Australia). Mean aortic pressure and heart rate were continuously recorded. Anesthesia was maintained by continuous α-chloralose infusion (25 mg · kg BW^{-1} · h^{-1}). After left-sided lateral thoracotomy and pericardiotomy were performed, a ligature (Prolene® 5.0; Ethicon GmbH, Nordersted, Germany) was passed below a main branch of the left coronary artery. The ends of the ligature were passed through a propylene tube to form a snare. Successful coronary occlusion was verified by epicardial cyanosis.

Experimental Protocol

After successful instrumentation, the animals were randomly allocated (using sealed envelopes) into one of the 15 groups (see fig. 1). All animals underwent 25 min of left coronary artery occlusion and 2 h of reperfusion.

CON Group. Rats in the control (CON) group received no further treatment.

SEVO-I Group. Rats received a single 5-min episode of 1 MAC sevoflurane (in rats 2.4 vol%)[22] 10 min before the 25-min left coronary artery occlusion.

SEVO-II Group. Rats received 1 MAC sevoflurane for two 5-min periods, interspersed with one 5-min washout period, 10 min before ischemia and reperfusion.

SEVO-III Group. Rats received 1 MAC sevoflurane for three 5-min periods, interspersed with two 5-min washout periods, 10 min before ischemia and reperfusion.

SEVO-VI Group. Rats received 1 MAC sevoflurane for six 5-min periods, interspersed with five 5-min washout periods, 10 min before ischemia and reperfusion.

APRO-SEVO-I, -II and -III Groups. Aprotinin (Trasylol; Bayer AG, Leverkusen, Germany; 40.000 kallikrein inhibitor units/kg BW bolus IV, followed by infusion of 40.000 kallikrein inhibitor units $\cdot$ kg BW^{-1} $\cdot$ h^{-1}) was administered continuously over a time period of 45 min starting before ischemia and reperfusion.

APRO. Rats were treated with aprotinin (Trasylol; 40.000 kallikrein inhibitor units/kg BW bolus IV, followed by infusion of 40.000 kallikrein inhibitor units $\cdot$ kg BW^{-1} $\cdot$ h^{-1}) over a time period of 45 min before ischemia and reperfusion.

To investigate whether eNOS is involved in SevoPC, we blocked NOS activity. However, because of the lack of a specific eNOS blocker, we employed the nonspecific NOS blocker L-NAME, the selective neuronal NOS (nNOS) blocker 7-nitroindazole (7-NI), or the specific inducible NOS (iNOS) blocker aminoguanidine.

L-NAME-SEVO-III Group. The nonselective NOS inhibitor L-NAME 10 mg/kg was administered as an IV infusion over 10 min starting 30 min before the SevoPC protocol.

L-NAME Group. The nonselective NOS inhibitor L-NAME 10 mg/kg was administered as an IV infusion over 10 min beginning 65 min before left coronary artery occlusion.

Aminoguanidine-SEVO-III Group. The selective iNOS inhibitor aminoguanidine 300 mg/kg was injected subcutaneously 30 min before starting the SevoPC protocol.

Aminoguanidine Group. The selective iNOS inhibitor aminoguanidine 300 mg/kg was injected subcutaneously 65 min before coronary occlusion.

7-NI-SEVO-III Group. The selective nNOS inhibitor 7-NI 50 mg/kg was injected peritoneally 30 min before starting the SevoPC protocol.

7-NI Group. The selective nNOS inhibitor 7-NI 50 mg/kg was injected peritoneally 65 min before left coronary artery occlusion.

The doses of L-NAME, aminoguanidine, and 7-NI employed were based on those in the literature.[23] After 2 h of reperfusion, the hearts were excised and infarct sizes were determined using a method described previously.[22] The area at risk and the infarct size were determined using planimetry and corrected for dry weight in each slice by using SigmaScan Pro5® (SPSS Science Software, Chicago, IL).

Western Blot Experiments

To investigate the possible effects of SevoPC on eNOS phosphorylation, additional experiments were performed. In total, 28 rats were instrumented as described above and underwent the preconditioning protocol as the animals of the CON, SEVO-I, -II, -III, -VI, APRO-SEVO-III, and APRO alone groups, respectively (n = 4 per group). After completion of the preconditioning protocol, the hearts were excised, washed in ice-cold saline to remove remaining blood, frozen in liquid nitrogen, and stored at −80°C. Cellular fractionation and subsequent Western blot analysis for phosphorylation of eNOS and cellular distribution of either total eNOS or phosphorylated eNOS was performed. Therefore, a cellular fractionation was performed as described previously.[24] The cytosolic and the membrane fractions were further used for Western blot analysis. After protein determination by the Lowry method, equal amounts of protein were mixed with loading buffer containing bromphenol blue, glycerol, and tris(hydroxymethyl)aminomethane-HCl. Samples were vortexed and heated for 5 min at 95°C before being subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (7.5%). The proteins were separated by electrophoresis and transferred to a polyvinylidene difluoride membrane by tank blotting (100 V, 1 h). Nonspecific binding of the antibody was blocked by incubation with 5% fat dry milk solution in tris(hydroxymethyl)aminomethane-buffered saline containing Tween-20 for 2 h. Thereafter, the membrane was incubated overnight at 4°C with specific antibody (phospho-eNOS antibody #9571; Cell Signaling Technology, Danvers, MA; dilution 1:1,000 in 5% bovine serum albumin), which detects phosphorylation of eNOS at the activating site Ser1177, or a specific total eNOS antibody (#9572, Cell Signaling Technology), respectively. After washing in cold tris(hydroxymethyl)aminomethane buffered saline containing Tween-20, the blot was subjected to antirabbit horseradish peroxidase-conjugated secondary antibody for 2 h at room temperature. By using chemiluminescence detected on X-ray film (Hyperfilm ECL; GE Healthcare, Freiburg, Germany) using the enhanced chemiluminescence system Santa Cruz Biotechnology (Santa Cruz, CA), the immunoreactive bands were visualized. The blots were quantified using a KODAK Image Station® (Carestream Health, Rochester, NY), and the results are presented as the ratio of phosphorylated eNOS to total eNOS or total eNOS to α-tubulin. In addition, equal loading of protein on the gel was confirmed by detection of α-tubulin and Coomassie staining of the gels.

Statistical Analysis

Sample size analysis revealed that a group size of n = 6 was necessary to detect a difference in infarct size 25% with a power of 80% and an α of 0.05 (two-tailed). Data are ex-
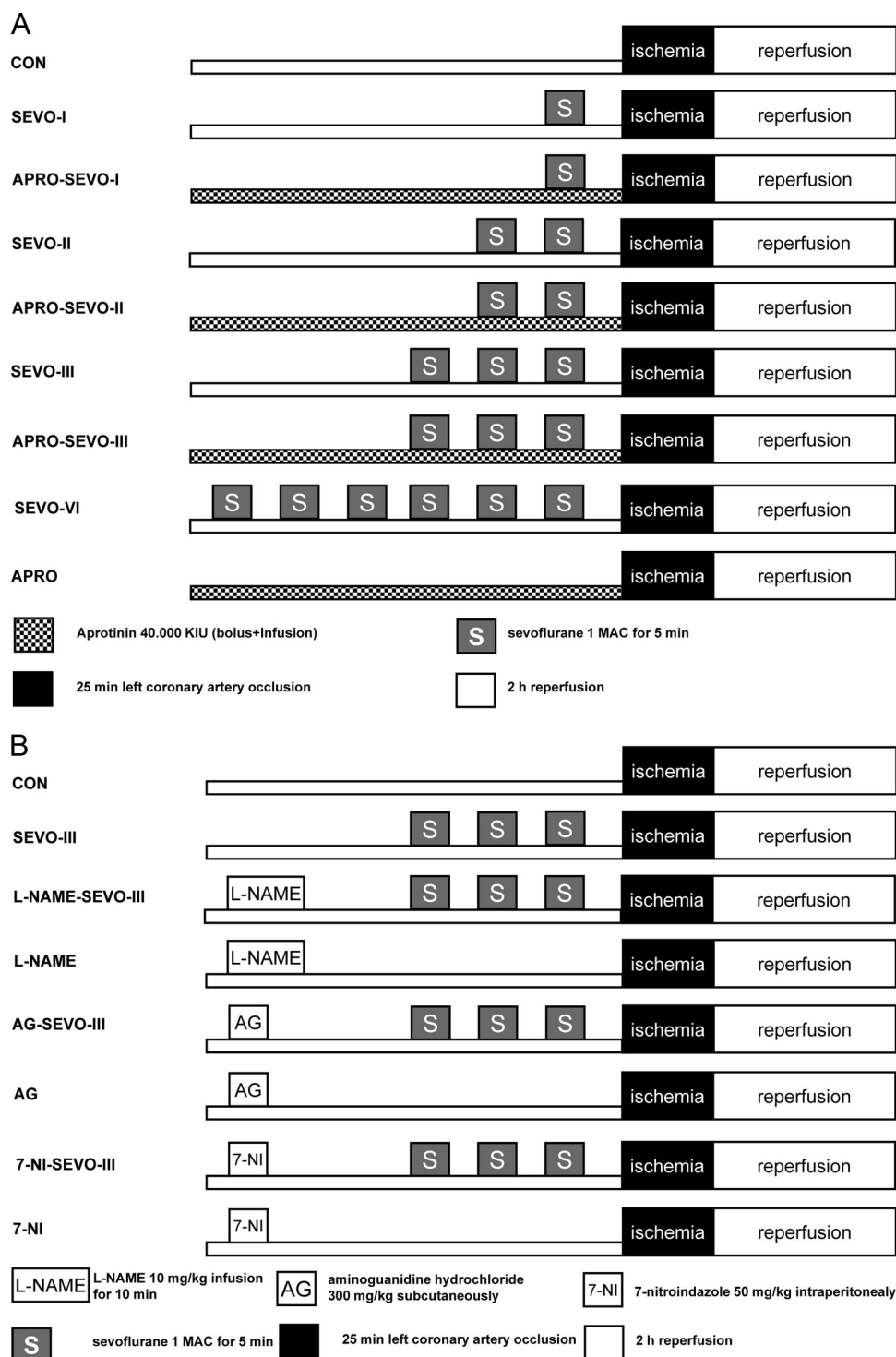
Fig. 1. Experimental protocol. (*A*) Dose effect and influence of aprotinin. (*B*) Involvement of NOS isoforms. All blockers were given 30 min before the sevoflurane preconditioning protocol or at corresponding time point in control experiments. 7-NI = 7-nitroindazole; AG = aminoguanidine; APRO = aprotinin 45 min before ischemia; APRO-SEVO-I, -II, and –III = same protocol as corresponding SEVO-group with coadministration of aprotinin, starting 45 min before ischemia; CON = control group; KIU = kallikrein inhibitor units; L-NAME = *N*-nitro-L-arginine methyl ester; L-NAME-SEVO-III, AG-SEVO-III, and 7-NI-SEVO-III = like SEVO-III with pretreatment with L-NAME (nonspecific NOS blocker), AG (specific inducible NOS blocker) or 7-NI (neuronal NOS blocker), respectively; L-NAME, AG, and 7-NI = control experiments plus NOS blocker; MAC = minimum alveolar concentration; NOS = nitric-oxide synthase; SEVO-I, -II, -III, or -VI = sevoflurane group with administration of 1 MAC sevoflurane 1, 2, 3, or 6 times, respectively, for 5 min each, interspersed with 5 min of washout, 10 min before ischemia and reperfusion.

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Fig. 2. Infarct size measurement. Infarct size (IS) in percentage of the area at risk (AAR). Control animals underwent 25 min of regional myocardial ischemia, followed by 2 h of reperfusion. All blockers were given 30 min before the sevoflurane precon-ditioning protocol or at corresponding time point in control experiments. (*A*) Dose effect and influence of aprotinin. *** $P < 0.001$ *versus* CON. ††† $P < 0.001$ *versus* SEVO-I and SEVO-II. ### $P < 0.001$ *versus* corresponding SEVO-group, n = 6 per group, all data are mean ± SD. (*B*) Involvement of NOS isoforms. *** $P < 0.001$ *versus* CON. ‡ $P < 0.05$ *versus* SEVO-III. ‡‡‡ $P < 0.001$ *versus* SEVO-III. ### $P < 0.001$ *versus* corresponding SEVO group, n = 6 per group, all data are mean ± SD. 7-NI = 7-nitroindazole; AG = aminoguanidine; APRO = aprotinin 45 min before ischemia; APRO-SEVO-I, -II, and –III = same protocol as corresponding SEVO-group with coadministration of aprotinin, starting 45 min before ischemia; CON = control group; KIU = kallikrein inhibitor units; L-NAME = *N*-nitro-L-arginine methyl ester; L-NAME-SEVO-III, AG-SEVO-III, and 7-NI-SEVO-III = like SEVO-III with pretreatment with L-NAME (nonspecific NOS blocker), AG (specific inducible NOS blocker) or 7-NI (neuronal NOS blocker), respectively; L-NAME, AG, and 7-NI = control experiments plus NOS blocker; MAC = minimum alveolar concentra-tion; NOS = nitric-oxide synthase; SEVO-I, -II, -III. or -VI = sevoflurane group with administration of 1 MAC sevoflurane 1, 2, 3, or 6 times, respectively, for 5 min each, interspersed with 5 min of washout, 10 min before ischemia and reperfusion.
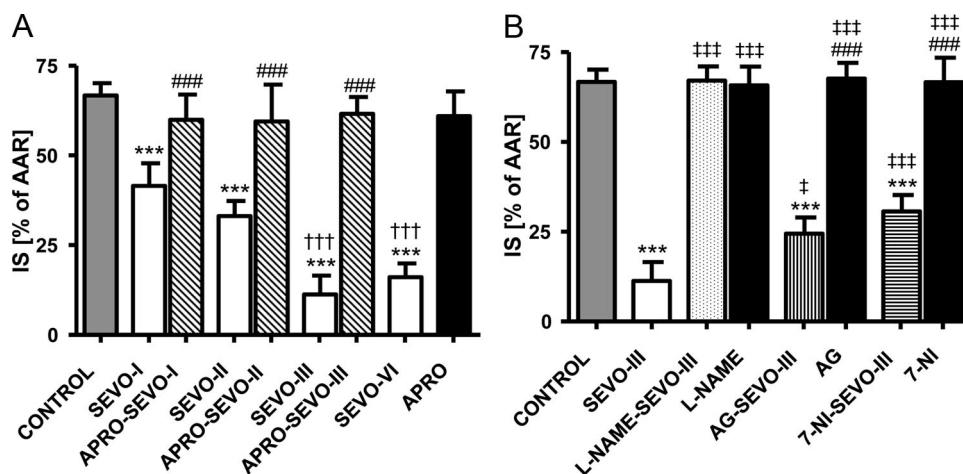
pressed as mean ± SD. Statistical analysis of infarct size and eNOS measurements was performed by ANOVA with Bon-ferroni's multiple comparison test (two-tailed; Prism ver. 4.00; GraphPad Software, San Diego, CA). Statistical anal-ysis of the hemodynamic variables was performed by two-way ANOVA for time and treatment effects. If an overall significance was found, comparisons between groups were done for each time point using ANOVA followed by Dun-nett *post hoc* test with the control group as reference group. Time effects within each group were analyzed by repeated-measures ANOVA followed by two-tailed Dunnett *post hoc* test with the baseline value as the reference time point. *P* less than 0.05 was considered statistically significant.

Results

Infarct Size Measurement
All SevoPC protocols in the absence of aprotinin led to an infarct size reduction *versus* control (SEVO-I, 42 ± 6%, SEVO-II, 33 ± 4%; SEVO-III, 11 ± 5%; SEVO-VI, 16 ± 4% *vs.* CON, 67 ± 3%; for all four SevoPC groups, *P* less than 0.001 *vs.* CON; see fig. 2A). The strongest infarct size reduction was observed after administration of three or six sevoflurane cycles in the SEVO-III and -VI groups (*P* < 0.001 *vs.* SEVO-II and -I, respectively). Each sevoflurane-induced myocardial protection was blocked by coadminis-tration of aprotinin during the preconditioning protocol (see fig. 2). Aprotinin alone had no effect on infarct size (APRO, 61 ± 7%; *P* > 0.05 *vs.* CON).

L-NAME completely blocked SevoPC after three cycles of preconditioning (L-NAME-SEVO-III, 67 ± 4%; *P* < 0.001 *vs.* SEVO-III) but had no effect on infarct size alone (L-NAME, 66 ± 5%; *P* > 0.05 *vs.* CON). Aminoguanidine and 7-NI partially inhibited the cardioprotective effect of SevoPC (aminoguanidine-SEVO-III, 25 ± 5%; *P* < 0.05 *vs.* SEVO-III, *P* < 0.001 *vs.* CON; 7-NI-SEVO-III, 31 ± 5%; *P* < 0.001 *vs.* SEVO-III and CON, respectively). The block-ers alone had no effect on infarct size (aminoguanidine, 68 ± 4%; 7-NI, 67 ± 7%; *P* > 0.05 *vs.* CON, see fig. 2B).

Hemodynamic Measurement
SevoPC led to a decrease in mean aortic pressure and heart rate (see table 1) during the preconditioning protocol. How-ever, at the last washout before ischemia these hemodynamic changes disappeared. Heart rate was reduced only in the aminoguanidine-SEVO-III and 7-NI-SEVO-III groups dur-ing ischemia compared with CON. During reperfusion, heart rate was reduced in the SEVO-II and SEVO-III groups compared with CON.

Immunoblotting of Phosphorylated eNOS
In the cytosolic fraction (P1), we could observe no changes in total or phosphorylated eNOS, respectively (data not shown). In the membrane fraction (P2), there was no change in total eNOS content, but we observed a 2.4–3.2-fold increase in eNOS phosphorylation after SEVO-I, -II, -III, and -VI, respectively. In the SEVO-III

Table 1. Global Hemodynamics

	Baseline				Preconditioning			
	Base	Post INT	APC-1	Wash-1	APC-2	Wash-2	APC-3	Wash-3
HR, beats/min								
CON	434 ± 24	434 ± 25	433 ± 25	429 ± 30	434 ± 30	434 ± 22	434 ± 23	434 ± 11
SEVO-I	430 ± 16	441 ± 25	446 ± 32	442 ± 26	440 ± 24	440 ± 25	392 ± 37	419 ± 51
APRO-SEVO-I	451 ± 36	444 ± 42	437 ± 47	449 ± 47	441 ± 44	438 ± 40	394 ± 57	431 ± 66
SEVO-II	431 ± 16	434 ± 15	418 ± 23	424 ± 10	386 ± 19	392 ± 23	374 ± 18†	387 ± 18
APRO-SEVO-II	425 ± 22	416 ± 30	406 ± 29	405 ± 30	368 ± 22	399 ± 35	361 ± 20*	380 ± 47
SEVO-III	440 ± 20	415 ± 16	365 ± 18*†	392 ± 24	343 ± 27†	372 ± 38†	339 ± 6*†	364 ± 16†
APRO-SEVO-III	412 ± 21	396 ± 30	362 ± 34*†	392 ± 28	354 ± 31†	383 ± 30	350 ± 32*†	372 ± 24
APRO	429 ± 27	384 ± 42*	365 ± 69*	377 ± 28*	390 ± 30	386 ± 29	374 ± 34*	374 ± 47
L-NAME-SEVO-III	402 ± 43	388 ± 27	317 ± 55*	324 ± 72	310 ± 56	307 ± 68	336 ± 57*	319 ± 59
L-NAME	400 ± 52	390 ± 38	374 ± 42	368 ± 40	365 ± 39	364 ± 39*	367 ± 32*	370 ± 33
AG-SEVO-III	350 ± 24	319 ± 44	306 ± 34	315 ± 39	288 ± 33	293 ± 37	278 ± 28	285 ± 38
AG	396 ± 36*	375 ± 29	368 ± 36*	368 ± 40	359 ± 52	361 ± 51*	352 ± 62*	365 ± 32
7-NI-SEVO-III	364 ± 34	350 ± 32	321 ± 29	335 ± 38*	314 ± 33	318 ± 36	305 ± 32	313 ± 51
7-NI	398 ± 56*	389 ± 50	388 ± 50	388 ± 49	389 ± 52	388 ± 50	389 ± 44	383 ± 43
AoPmean, mmHg								
CON	135 ± 15	135 ± 15	134 ± 15	136 ± 9	130 ± 15	133 ± 14	131 ± 15	130 ± 16
SEVO-I	115 ± 28	124 ± 24	114 ± 26	117 ± 23	119 ± 23	119 ± 25	74 ± 23*	109 ± 28
APRO-SEVO-I	126 ± 35	116 ± 45	109 ± 41	113 ± 43	113 ± 49	108 ± 39	81 ± 35*	115 ± 44
SEVO-II	120 ± 19	129 ± 22	121 ± 30	133 ± 30	94 ± 27	128 ± 24	85 ± 17*	117 ± 29
APRO-SEVO-II	137 ± 17	123 ± 26	117 ± 19	114 ± 19	89 ± 32	119 ± 37	84 ± 30*	112 ± 42
SEVO-III	122 ± 16	121 ± 14	82 ± 23*†	113 ± 22	69 ± 24*†	97 ± 24	64 ± 25*†	116 ± 14
APRO-SEVO-III	119 ± 11	93 ± 12*	78 ± 9*†	102 ± 22	71 ± 5*†	105 ± 19	73 ± 7*†	106 ± 12
APRO	117 ± 14	92 ± 21*	88 ± 21*	87 ± 29*	89 ± 31	88 ± 37	86 ± 29*	92 ± 28
L-NAME-SEVO-III	120 ± 29	109 ± 40	112 ± 34	160 ± 20	124 ± 31	143 ± 27	109 ± 25	143 ± 36
L-NAME	126 ± 34	147 ± 44†	182 ± 22*	179 ± 25	173 ± 26	166 ± 27	163 ± 25	163 ± 22
AG-SEVO-III	135 ± 18	137 ± 28	93 ± 19†	125 ± 29	78 ± 14*†	113 ± 27	71 ± 11†	119 ± 27
AG	108 ± 20	120 ± 28	124 ± 28	128 ± 30	126 ± 29	127 ± 27	127 ± 25	129 ± 26
7-NI-SEVO-III	144 ± 21	147 ± 17	101 ± 24†	133 ± 18	88 ± 23†	113 ± 21	74 ± 13*†	112 ± 27
7-NI	116 ± 26	121 ± 37	121 ± 38	119 ± 34	119 ± 35	122 ± 25	119 ± 29	113 ± 27

	Base		APC-1	Wash-1	APC-3	Wash-3	APC-6	Wash-6
HR, beats/min								
SEVO-VI	381 ± 29		336 ± 30	358 ± 34	316 ± 32†	334 ± 35	291 ± 25†	326 ± 30†
AoPmean, mmHg	144 ± 11		100 ± 11*†	137 ± 10	77 ± 8*†	123 ± 20	68 ± 11*†	101 ± 41†

Data are mean ± SD.

* $P < 0.05$ *vs.* CON. † $P < 0.05$ *vs.* Base.

7-NI = 7-nitroindazole; AG = aminoguanidine; AoP_{mean} = mean aortic pressure; APC-1, −2, −3, −6 = anesthetic-induced preconditioning (sevoflurane administration); APRO = aprotinin; APRO-Sevo-I, APRO-Sevo-II, APRO-Sevo-III = aprotinin + 1, 2, 3 cycles of sevoflurane preconditioning, respectively; Base = baseline measurement; CON = control group; HR = heart rate; L-NAME = N-nitro-L-arginine methyl ester; Occ 15 and Occ 24 = left coronary artery occlusion at 15 and 24 min, respectively; Post INT = measurement after administration of blocker; Rep 15, Rep 60, and Rep 120 = after 15, 60, and 120 min of reperfusion, respectively; SEVO-I, SEVO-II, SEVO-III, SEVO-VI = 1-, 2-, 3-, or 6-cycle sevoflurane preconditioning, respectively; Wash-1, −2, −4, −6 = wash out after sevoflurane administration.

protocol, the increased phosphorylation was abolished by aprotinin. However, aprotinin alone had no effect on eNOS phosphorylation (see table 2).

Discussion

The aim of the study was to investigate whether multiple cycles of SevoPC are superior to a single cycle of SevoPC. Furthermore, we determined in which way aprotinin interferes with SevoPC and whether eNOS is involved.

In our model, multiple cycles SevoPC led to increased cardioprotection in rat hearts *in vivo*. Even using a proto-col wherein maximal protection is achieved, aprotinin completely abolished SevoPC. L-NAME likewise entirely abolished SevoPC, indicating the involvement of NOS. In contrast, 7-NI and aminoguanidine led to a partial block-ade of SevoPC. This indicates that nNOS and iNOS are partially involved in SevoPC.

Each SevoPC protocol employed led to a similar in-crease of eNOS phosphorylation. Treatment with aproti-nin before SevoPC completely abolished this phosphory-lation, indicating that eNOS phosphorylation seems to be necessary for SevoPC.

Table 1. Continued

Ischemia		Reperfusion		
Occ 15	Occ 24	Rep 15	Rep 60	Rep 120
437 ± 33	433 ± 41	446 ± 38	430 ± 18	390 ± 56
405 ± 22	420 ± 22	406 ± 18	411 ± 52	405 ± 41
424 ± 74	422 ± 55	401 ± 53	409 ± 70	407 ± 84
399 ± 21	388 ± 22	378 ± 28†	349 ± 44†	329 ± 68†
389 ± 57	375 ± 75	374 ± 47	381 ± 39	365 ± 40
352 ± 37*†	346 ± 35*†	351 ± 39*†	325 ± 36†	302 ± 39*†
383 ± 15	386 ± 22	372 ± 30	372 ± 29	361 ± 32†
381 ± 52	372 ± 51	361 ± 53*	330 ± 34†	330 ± 16†
305 ± 63	313 ± 64	319 ± 65	296 ± 68	351 ± 106
370 ± 32	362 ± 35	332 ± 80	294 ± 88	314 ± 55
301 ± 36	296 ± 40	286 ± 32	286 ± 24	284 ± 23
352 ± 49*	361 ± 34	331 ± 39	312 ± 24	349 ± 42
338 ± 47*	330 ± 42	320 ± 42	287 ± 47†	296 ± 34†
388 ± 47	385 ± 47	370 ± 48*	351 ± 52	339 ± 32
103 ± 28†	98 ± 25†	102 ± 18†	101 ± 14†	94 ± 13†
81 ± 27	78 ± 22	96 ± 30	84 ± 28	77 ± 31
98 ± 40	91 ± 37	94 ± 49	89 ± 59	99 ± 52
108 ± 30	93 ± 27	84 ± 17	70 ± 15†	59 ± 13†
96 ± 48	99 ± 49	91 ± 25	101 ± 38	85 ± 41
71 ± 23†	74 ± 31†	78 ± 25†	67 ± 18†	62 ± 19†
105 ± 11	104 ± 18	99 ± 18	107 ± 16	90 ± 15†
87 ± 37	91 ± 31	85 ± 28	64 ± 9†	59 ± 13†
118 ± 37	111 ± 43	132 ± 38	99 ± 27	87 ± 21
125 ± 29	131 ± 22	119 ± 45	85 ± 47	72 ± 42
97 ± 26†	91 ± 28†	88 ± 22†	74 ± 10†	77 ± 7†
107 ± 38	97 ± 38	85 ± 30	70 ± 25	91 ± 30
102 ± 22	92 ± 16†	91 ± 17†	78 ± 9†	74 ± 8†
86 ± 37	79 ± 31	76 ± 16	59 ± 14†	60 ± 18†
Occ 15	Occ 24	Rep 15	Rep 60	Rep 120
337 ± 32	329 ± 31	313 ± 31†	300 ± 31†	283 ± 40†
101 ± 23†	93 ± 20†	88 ± 12†	79 ± 13†	72 ± 15†

Multiple-Cycle Preconditioning

In the early studies, ischemic preconditioning looked like an "all or nothing" phenomenon. In some studies, increasing the duration or the number of ischemic stimuli did not increase the protection against ischemia–reperfusion injury.[25,26] However, increasing the number of stimuli seems to activate more cardioprotective signaling pathways compared with a single-cycle protocol. The strength of the achievable cardioprotection depends on the experimental conditions.[9] Riess *et al.*[7] compared several protocols in guinea pig hearts *in vitro*. Using a concentration of 0.4 mM sevoflurane (approximately 2.8 vol%) for one 15-min or two 5-min treatments or a concentration of 0.2 mM sevoflurane (approximately 1.4 vol%), the authors found the strongest infarct size-reducing effect in the group treated with the high concentration given two times for 5 min.[7] These results indicated for the first time that a multiple cycle regimen is superior to a single cycle protocol and that there seems to be a dose dependency in anesthetic-induced preconditioning. This dose dependency has later been confirmed for desflurane.[8] In the same study, Lange *et al.*[8] demonstrated in rabbit hearts *in vivo* that a single-cycle protocol, consisting of 0.5 MAC desflurane for 30 min, did not induce cardioprotection, whereas the same concentration given three times for 10 min each did. One

Table 2. Western Blot

	Membrane Fraction (P2)	
	Total eNOS	Phospho eNOS
CON	0.41 ± 0.15	0.22 ± 0.13
SEVO-I	0.39 ± 0.10	0.55 ± 0.22*
SEVO-II	0.40 ± 0.10	070 ± 0.25†
SEVO-III	0.49 ± 0.14	0.53 ± 0.16‡
SEVO-VI	0.45 ± 0.10	0.62 ± 0.17†
APRO-SEVO-III	0.51 ± 0.16	0.26 ± 0.16§
APRO alone	0.31 ± 0.10	0.29 ± 0.18

Data are mean ± SD.

* $P < 0.05$ *vs.* CON. † $P < 0.01$ *vs.* CON. ‡ $P < 0.001$ *vs.* CON. § $P < 0.01$ *vs.* Sevo-III.

APRO alone = control group plus aprotinin treatment; APRO-Sevo-III = aprotinin pretreatment plus Sevo-III protocol; CON = control group; eNOS = endothelial nitric-oxide synthase; phospho eNOS = phosphorylated eNOS; SEVO-I, -II, -III, -IV = sevoflurane preconditioning with 1, 2, 3, or 6 cycles, respectively.

might ask whether the longer administration time in a multiple-cycle protocol is responsible for the observed enhancement of cardioprotection. We cannot answer this question from our data, but from experimental[7,8] and clinical data,[27] it is obvious that a pulsed administration is necessary to improve the effect of preconditioning. We could clearly demonstrate that increasing the number of preconditioning cycles improves cardioprotection in terms of infarct size reduction with a maximum after three cycles. The use of a multiple-cycle protocol was one of the identified differences from our clinical study.[6] We conclude that this effect could be responsible, at least in part, for the observed cardioprotection in our clinical trial.[6]

Aprotinin Abolished SevoPC

Aprotinin has possible direct cardioprotective properties.[14,15] On the other hand, aprotinin has been shown to abolish ischemic-induced preconditioning in sheep *in vivo*.[16] Inamura *et al.*[18] demonstrated that sevoflurane-induced postconditioning is abolished in the presence of aprotinin in guinea pig hearts *in vitro*. When given throughout the entire experimental protocol (starting 10 min before ischemia until the end of reperfusion), aprotinin also abolished the observed cardioprotection. Because many of the signaling pathways of ischemic- and anesthetic-induced preconditioning are similar, we investigated whether aprotinin also blocks SevoPC. In our model, we could detect no direct cardioprotective properties of aprotinin (fig. 2), but we could clearly show that aprotinin abolished SevoPC in the rat heart *in vivo*. This blockade is independent on the preconditioning protocol used. In contrast, in multiple cycles of ischemic-induced preconditioning, it was shown that blocking only one step of the signaling pathway (protein kinase C or adenosine 3′,5′-cyclic monophosphate activity) was not sufficient to abolish the cardioprotective effect.[28] Whether this difference is caused by differences in the signaling pathways of ischemic- and anesthetic-induced preconditioning, by the chosen target

(protein kinase C, adenosine 3′,5′-cyclic monophosphate, eNOS) or by a substance-specific effect of aprotinin cannot be answered from our data and needs further clarification.

The second difference between our clinical trial[6] and others has a major impact on SevoPC: the use of aprotinin. However, extrapolation of data from animal studies into the clinical setting should always be done with great caution.

eNOS, Aprotinin, and SevoPC

In human coronary artery cells, isoflurane-induced preconditioning has been shown to be mediated through the 90-kd heat shock protein-eNOS pathway.[21] Using the unspecific NOS blocker L-NAME, it was shown that desflurane-induced myocardial preconditioning is mediated by NOS activity.[29] Sevoflurane-induced postconditioning leads to an increased nitric oxide production *in vitro*, and this increase is reduced by aprotinin[18] and abolished by the nonselective NOS inhibitor L-NAME. Until now there has been no direct evidence that eNOS plays a crucial role in the signaling pathway of anesthetic-induced cardioprotection. We observed a profound increase in eNOS phosphorylation (see table 2) after SevoPC that was blocked in the presence of aprotinin. Aprotinin was clinically used as an antifibrinolytic agent; in experimental laboratories, it is widely used as protease inhibitor. Ulker *et al.*[19] demonstrated that aprotinin down-regulates eNOS messenger RNA and protein expression in cultured rat coronary microvascular endothelial cells. We detected no differences in total eNOS amount in our study. Increase in gene expression and subsequent *de novo* protein biosynthesis requires time. Ulker *et al.*[19] treated their cells overnight with aprotinin gathering the required time for the protein *de novo* synthesis. In our setting, the duration of aprotinin treatment was most likely too short for *de novo* synthesis.[19] On the other hand, phosphorylation is a fast and short-lived reaction. Therefore, we investigated whether a difference in eNOS phosphorylation at the different time points of our protocol exists. In all of our experimental protocols, we detected a similar increase in phosphorylation. This indicates that one-cycle SevoPC is sufficient to achieve a maximal phosphorylation of eNOS in the myocardium. On the other hand, this raises the question of whether eNOS phosphorylation is the only signaling pathway involved in SevoPC. As mentioned above in ischemic PC, increasing the number of cycles increases the intracellular signaling pathways, leading to stronger and more robust signaling. In addition, aprotinin is reported as the first competitive protein inhibitor of NOS activity.[30] L-NAME, like aprotinin, completely abolishes SevoPC. In contrast, 7-NI and aminoguanidine did not abolish SevoPC. However, we observed a partial inhibition of cardioprotection in that the infarct sizes in these groups were at the level of two cycles of SevoPC. Isoflurane has been shown to induce postconditioning (a cardioprotective mechanism sharing signaling pathways with preconditioning) is mediated by preventing mitochondrial permeability transition pore opening *via* an eNOS-dependent mechanism.[31] Another approach to explain NOS-me-

diated cardioprotection is based on the theory that the mitochondria itself are able to generate nitric oxide from NOS. Here, nNOS could be one of the possible sources preventing opening of the mitochondrial permeability transition pore.[32] However, the mechanism by which NOS is mediating preconditioning remains unclear. Taking all data together, we conclude that aprotinin inhibits eNOS phosphorylation in SevoPC in the rat heart *in vivo*, which is a crucial step in the signal transduction cascade.

Limitations of the Study

Because of the lack of an available eNOS blocker, we cannot directly show that eNOS mediates SevoPC. However, using specific blockers of nNOS and iNOS, we were able to show indirectly that eNOS is most likely to be the involved isoform of NOS. Because the nNOS and iNOS blockers themselves also impaired the cardioprotective effect, it is possible that these isoforms are at least partially involved in this phenomenon. On the other hand, aminoguanidine and 7-NI are not 100% specific blockers of nNOS[33] and iNOS,[34] respectively. Therefore, it is most likely that these substances partially blocked eNOS, leading to a partial blockade of the preconditioning effect.

We did not investigate four or five cycles of SevoPC, longer administration, or higher concentrations. One of these protocols could theoretically have led to stronger cardioprotection.

Global hemodynamics can influence myocardial oxygen consumption. During sevoflurane administration, we observed a decrease in mean aortic pressure and heart rate. However, this decrease was reversed immediately before index ischemia. During ischemia, we found a solely statistically significant decrease in heart rate in animals in the SEVO-III group. However, that this decrease in heart rate should be responsible for the observed cardioprotection seems highly unlikely. Animals in the L-NAME-SEVO III group had an even lower heart rate but had infarct sizes in the same order as animals in the control group.

Premenopausal women, compared with men of the same age, possess a reduced risk for cardiovascular disease.[35] This tolerance to ischemia–reperfusion is mediated by estrogen.[36] The expression of iNOS and eNOS is stimulated by 17β-estradiol in rats *in vivo*.[37] In some studies, premenopausal female animals showed reduced ischemia–reperfusion injury.[38,39] However, these results are inconsistent; no sex differences were found in *in vivo* rat models or *in vitro* mouse models. Wang *et al.*[40] investigated sex-specific differences in isoflurane-induced late preconditioning. Female rabbits had a smaller infarct size compared with male rabbits.[40] However, administration of isoflurane 24 h before ischemia and reperfusion reduced infarct size in male rabbits only. This male-specific cardioprotective effect was abolished by the nonspecific NOS blocker L-NAME, whereas specific pharmacological blockade of nNOS or iNOS had no influence on infarct size.[40] It remains unclear whether employing a different (pulsed) protocol in female subjects could lead to anesthetic-induced late preconditioning. No data exist

on sex-specific differences in anesthetic induced early preconditioning. In isolated hearts taken from female mice, ischemia-induced early preconditioning could not be induced. Again, hearts from female mice had decreased infarct sizes compared with hearts from male counterparts.[41] Despite sex-specific differences, it is also possible that species specific differences for the involvement of NOS in cardioprotection exist. In rats, nNOS plays a substantial role in nitric-oxide mediated protection against ischemia–reperfusion–induced ventricular fibrillation, whereas in rabbits and marmosets, it does not.[42,43] However, in patients with unstable angina compared with patients without angina, increased concentrations of eNOS are reported.[44]

Our results indicate for the first time that eNOS phosphorylation is a crucial step in mediating cardioprotection by sevoflurane. We did not confirm this result with an established eNOS blocker, but we could clearly show that aprotinin blocks eNOS phosphorylation and sevoflurane-induced infarct size reduction.

In conclusion, we demonstrate that multiple cycles of SevoPC are superior to single-cycle protocol. Aprotinin abolishes this cardioprotection independent of the protocol used.

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