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Aprotinin Abolishes Sevoflurane Postconditioning by Inhibiting Nitric Oxide Production and Phosphorylation of Protein Kinase C-δ and Glycogen Synthase Kinase 3β

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Background: **It remains controversial whether aprotinin use during cardiac surgery is cardioprotective or detrimental. In contrast, volatile anesthetics may offer cardioprotection perioperatively. Increased nitric oxide, protein kinase C activation, and glycogen synthase kinase 3*β* inhibition play a role in sevoflurane-induced cardioprotection. The authors investigated whether aprotinin affects sevoflurane postconditioning.**

Methods: **Isolated guinea pig hearts underwent 30 min of global ischemia and 120 min of reperfusion (control [CTL]). Postconditioning was elicited with sevoflurane (2%) for 2 min at reperfusion onset (POST). Aprotinin (250 kallikrein inhibitor units/ml) was administered for 5 min at reperfusion onset (POST + APRO and CTL + APRO). In additional experiments, both sevoflurane and aprotinin were given before ischemia and throughout the reperfusion period (SEVO + APRO (throughout)) to mimic clinical conditions. Left ventricular developed and end-diastolic pressures and infarct size were measured. Western blot analysis determined phosphorylated protein kinase C-*δ*, protein kinase C-*δ*, Akt, and glycogen synthase kinase 3*β* expression. Nitric oxide production during reperfusion was measured by nitric oxide sensor.**

Results: **After ischemia–reperfusion, POST had significantly higher left ventricular developed (56 ± 11 *vs.* 26 ± 8 mmHg [mean ± SD]) and lower end-diastolic pressures (20 ± 9 *vs.* 47 ± 15 mmHg) and reduced infarct size (15 ± 3% *vs.* 41 ± 10%) *versus* CTL. Aprotinin abolished these improvements. Expressions of phospho-Akt (activated), phospho–protein kinase C-*δ* (activated), and phospho–glycogen synthase kinase 3*β* (inhibited) were significantly increased in POST. Aprotinin attenuated these increased expressions. Nitric oxide production after reperfusion was higher in POST than in CTL, but not in POST + APRO.**

Conclusions: **Aprotinin abolishes sevoflurane postconditioning, associated with inhibited phosphorylation of Akt, protein kinase C-*δ*, and glycogen synthase kinase 3*β* and reduced nitric oxide production.**

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APROTININ is an antifibrinolytic serine protease inhibitor that reduces perioperative bleeding and decreases need for blood transfusion. Despite numerous studies, it remains controversial whether the use of aprotinin during cardiac surgery is cardioprotective or detrimental. Several studies found that aprotinin reduced cardiac reperfusion injury by attenuating inflammatory responses.[1–3] Mouton *et al.*[4] showed from retrospective analysis that aprotinin seems to be safe in terms of postoperative renal function except in patients taking angiotensin-converting enzyme inhibitor during off-pump cardiac surgery. Another study found that aprotinin did not affect survival after cardiac surgery.[5] However, recent studies by Mangano *et al.*[6,7] have questioned the safety of aprotinin in patients undergoing cardiac surgery. Their data suggested that aprotinin use was associated with myocardial, cerebral, and renal adverse events. Other studies suggest that aprotinin increased renal events and mortality when compared with tranexamic acid, which is also used to reduce bleeding during cardiac surgery.[8,9] Therefore, it remains controversial whether the use of aprotinin during cardiac surgery is beneficial or detrimental.

Volatile anesthetics protect ischemic myocardium in a manner similar to ischemic preconditioning and postconditioning.[10–14] Increased nitric oxide production,[15–17] activation of protein kinase C (PKC)[18,19] and Akt, and inhibition of glycogen synthase kinase 3*β* (GSK3*β*)[20] have been implicated as mediators of volatile anesthetic–induced cardioprotection. In contrast, aprotinin has been shown to inhibit nitric oxide synthase (NOS) in rat coronary endothelial cells.[21] Reduction of nitric oxide derived from endothelial NOS could result in platelet activation and thrombosis formation. One study found that aprotinin promoted arterial thrombosis in pigs.[22] These experimental findings are consistent with a clinical report of a higher prevalence of perioperative myocardial infarction and graft thrombosis in cardiopulmonary bypass patients receiving aprotinin.[23]

We hypothesized that sevoflurane postconditioning would be abolished by aprotinin. We studied the effect of aprotinin on nitric oxide production and phosphorylation of PKC-*δ*, PKC-*ε*, Akt, and GSK3*β*, known mediators of volatile anesthetic postconditioning. A detrimental effect of aprotinin on volatile anesthetic postconditioning would further support the limited use of aprotinin in cardiac surgery.

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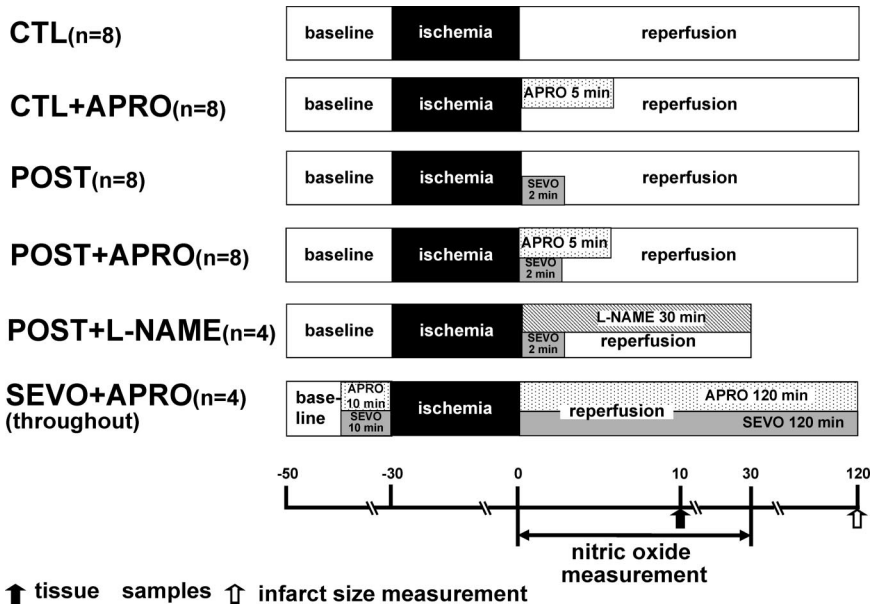

Fig. 1. Schematic illustration of the experimental protocol. All hearts were subjected to 30 min of global ischemia followed by 120 min of reperfusion. Anesthetic postconditioning was elicited by administration of sevoflurane (SEVO; 2% or 1 minimum alveolar concentration) for 2 min at the onset of reperfusion (postconditioning [POST]). Aprotinin (APRO; 250 kallikrein inhibitor units/ml) was administered for 5 min at the onset of reperfusion in POST (POST + APRO) and control (CTL + APRO) groups. In SEVO + APRO (throughout), both sevoflurane (2%) and aprotinin were given before ischemia and throughout the ischemia–reperfusion periods. Tissue samples were obtained at 10 min after reperfusion. Nitric oxide measurements were performed for the first 30 min after reperfusion. L-NAME = *N*-nitro-L-arginine methylester.

Materials and Methods

This study was conducted in accordance with the Guidelines for Animals Research at Osaka Dental University and with the approval of the Animal Experiment Committee of Osaka Dental University, Osaka, Japan. These guidelines conform to those laid out in the *Guide for the Care and Use of Laboratory Animals*, available from the National Academy of Science. Male Hartley guinea pigs were fed Lab Diet (RC4; Oriental Yeast, Tokyo, Japan) and given water *ad libitum*. Aprotinin (10,000 kallikrein inhibitor units [KIU]/ml) was purchased from MP Biomedicals, LLC (Solon, OH).

Isolated Heart Perfusion and Measurement of Function

Male guinea pigs weighing 650–700 g (12–13 weeks old) were given heparin (1,000 units intraperitoneally) and then anesthetized with pentobarbital (60 mg/kg intraperitoneally). Hearts were excised and immediately arrested in cold iso-osmotic saline containing 20 mM KCl. The aorta was cannulated, and the isolated hearts were perfused at 70 mmHg on a nonrecirculating isovolumic perfused heart apparatus, using a Krebs-Henseleit perfusate: 118 mM NaCl, 4.0 mM KCl, 2.52 mM $CaCl_2$, 24.8 mM $NaHCO_3$, 1.7 mM $MgSO_4$, 1.2 mM KH_2PO_4, 11.0 mM glucose, 0.5 mM EDTA, and 8 units/l insulin. The perfusate was insufflated continuously with 95% O_2–5% CO_2 (pH, 7.42 ± 0.04; partial pressure of oxygen, 521 ± 12 mmHg; partial pressure of carbon dioxide, 38.6 ± 1.2 mmHg) and was filtered through cellulose acetate membranes with a pore size of 4.0 μm to remove particulate matter. Hearts were paced at 240 beats/min using platinum-tipped electrodes connected to a Grass Instruments SD-5 stimulus generator (Grass Instruments, Quincy, MA). Left ventricular developed pressure (LVDP; mmHg) was measured using a 2.5-French, high-fidelity micromanometer (Nihon-Kohden, Tokyo, Japan) passed into a compliant latex balloon, inserted into the left ventricle, and recorded on a PowerLab 2/20 Data Recording System (ADInstruments, Hayward, Australia). The balloon was connected to a Y-adapter with one end used to advance the micromanometer and the other used to fill the left ventricular balloon with bubble-free water to an end-diastolic pressure (LVEDP) of 10 mmHg. The maximum rate of increase of left ventricular pressure (dP/dt_{max}) and the minimum rate of decrease of left ventricular pressure (dP/dt_{min}) were calculated using Chart 5 (ADInstruments). Coronary flow was measured by collecting effluent and was normalized to heart weight ($ml \cdot min^{-1} \cdot g^{-1}$). Global ischemia was achieved by clamping the aortic inflow line. During ischemia, hearts were maintained at 37°C by enclosure in a water-jacketed air chamber. Warmed perfusate kept in the lower part of the chamber saturated the air with humidity and prevented cooling by evaporation. Heart temperature was continuously monitored by a digital thermometer (PTW-100A; Unique Medical, Tokyo, Japan).

Experimental Protocol

Animals were assigned to one of six groups (n = 8; fig. 1). After a 20-min equilibration, baseline LVDP, LVEDP, and coronary flow were recorded, and then isolated hearts were subjected to 30 min of global ischemia and 120 min of reperfusion (control [CTL]). Anesthetic postconditioning was elicited by administration of sevoflurane (2%; 1 minimum alveolar concentration [MAC]) for 2 min upon reperfusion (POST). To investigate the effects of aprotinin on sevoflurane postconditioning, aprotinin (250 KIU/ml) was administered for 5 min at the onset of reperfusion in POST (POST + APRO) and CTL (CTL + APRO) groups. To more fully mimic the clinical

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setting, additional experiments were performed in which both sevoflurane and aprotinin were given before ischemia and throughout the ischemia–reperfusion periods (n = 4; SEVO + APRO (throughout)). In another set of experiments, the nonselective NOS inhibitor *N*-nitro-L-arginine methylester (L-NAME; 100 μM; Sigma, St. Louis, MO) was administered during early reperfusion in POST to confirm that nitric oxide production was blocked (n = 4; POST + L-NAME). Sevoflurane was insufflated by passing the 95% O_2–5% CO_2 gas mixture through a calibrated vaporizer (ACOMA, Tokyo, Japan). Samples of coronary perfusate were collected anaerobically from the aortic cannula for measurement of sevoflurane concentration by an organic vapor sensor (O.S.P. Inc., Saitama, Japan). We performed experiments in the following order: first a CTL, followed by POST, POST + APRO, CTL + APRO, and so forth until the first series of experiments were completed. This sequence was repeated a total of eight times.

Determination of Myocardial Infarct Size

At the end of experiments, the hearts were quickly frozen at −80°C for 15 min and then sliced into 2-mm-thick transverse sections from apex to base (6 slices/heart). After removing the right ventricle and defrosting, each slice was weighed and incubated at 37°C with 1% triphenyltetrazolium chloride (Sigma Chemicals) in phosphate buffer (pH 7.4) for 10 min and then fixed in 10% formalin for at least 5 h to distinguish red-stained viable tissue from pale, unstained necrotic tissue.[24] Each slice was photographed, and the necrotic area was determined using Adobe Photoshop CS (Adobe, San Jose, CA), multiplied by the weight of the slice and then expressed as a fraction of left ventricle.

Western Blot Analysis

Separate experiments were performed (n = 4 in each group) to examine phospho–PKC-δ, PKC-ε, phospho-Akt, and phospho-GSK3β expression. Myocardial tissue samples were collected at 10 min after reperfusion and homogenized in ice-cold homogenizing buffer containing 250 mM sucrose, 20 mM HEPES (pH 7.5), 10 mM KCl, 2 mM EGTA, 2 mM $MgCl_2$, 25 mM NaF, 50 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mM PMSF, 1% Triton X, and protease inhibitor leupeptin (10 μg/ml). The homogenate was centrifuged at 1,000*g* and 4°C for 5 min. The supernatant was recentrifuged at 10,000*g* and 4°C for 15 min. The protein concentration was estimated with a Bradford assay. Equivalent amounts (50 μg) of protein samples were loaded and separated on a 5–10% SDS-PAGE gradient gel and then electrically transferred overnight to a polyvinylidene difluoride membrane (Millipore Co., Billerica, MA). After blocking with 5% skim milk in Tris-buffered saline containing 0.1% Tween-20, the membranes were incubated for 2 h or overnight at 4 °C in 0.1% Tween-20 containing 5% milk and a 1:200–1,000 dilution of rabbit primary antibody for phospho–PKC-δ (Ser643; Cell Signaling Technology, Boston, MA), phospho–PKC-ε (Ser729; Upstate, Lake Placid, NY), phospho-Akt (Ser473; Cell Signaling Technology), and phospho-GSK3β (Ser9; Cell Signaling Technology). Membranes were incubated with a 1:1,000 dilution of horseradish peroxidase–labeled anti-rabbit immunoglobulin G (NA 934V; GE Healthcare, Buckinghamshire, United Kingdom). The same blot was stripped and reblotted with antibodies to α-tubulin, total PKC-δ, total PKC-ε, total Akt (C-20, C-15, H-136; Santa Cruz Biotechnology, Santa Cruz, CA), and total GSK3β (27C10; Cell Signaling Technology) to confirm equal protein loading. Bound antibody signals were detected with enhanced chemiluminescence (Pierce Biotechnology, Rockford, IL) and visualized using a VersaDoc 5000 Imaging System (Bio-Rad, Hercules, CA). Quantitative analysis of the band densities was performed by Quantity One software (Bio-Rad), and the results are presented as the ratio of phospho–PKC-δ, phospho–PKC-ε, phospho-Akt, and phospho-GSK3β to total (including nonphosphorylated and phosphorylated protein) PKC-δ, total PKC-ε, total Akt, and total GSK3β, respectively. The average light intensity was multiplied by 10 to facilitate presentation of an *x*-fold increase.

Measurements of Nitric Oxide Released from Coronary Effluent

Nitric oxide released from the coronary effluent during the 30 min after the onset of reperfusion was measured with an inNO-T nitric oxide sensor (Innovative Instruments, Inc., Tampa, FL). The inNO-T system includes a current monitor and data acquisition software with a temperature compensation function.[25] Before nitric oxide measurements, the nitric oxide sensor was calibrated as follows: The nitric oxide sensor was immersed in a vial containing 20 ml of 0.1 M H_2SO_4 + KI solution; the solution was stirred until a stable baseline was obtained; 20, 40, and 80 μl of 100 μM $NaNO_2$ standard solution (Innovative Instruments) were added into the vial, resulting in a saturated nitric oxide solution for each $NaNO_2$ standard solution; nitric oxide was derived from the following chemical formula: $2\ KNO_2 + 2\ KI + 2\ H_2SO_4 \rightarrow 2\ NO + I_2 + 2\ H_2O + 2\ K_2SO_4$; a standard current–nitric oxide curve (pA/nM) was made; and obtained pA data during reperfusion were converted to nitric oxide content. During measurements, the nitric oxide sensor was placed at the bottom of heart chamber where effluent collected. Because nitric oxide produced after reperfusion is quickly converted to NO_X by oxygen, nitrogen gas was delivered into the heart chamber to produce an oxygen-free condition. Oxygen content in the chamber was monitored with an oxygen sensor (XO-326ALB; New Cosmos Electric Co., Ltd., Osaka, Japan). During measurement of nitric oxide, oxygen in heart chamber was not significantly detectable.

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Statistical Analysis

All data are expressed as mean ± SD. Power analysis demonstrated that a sample size of eight hearts per group was required to detect a 35% difference (SD of ±9%) in infarct size with a power of 80% and a two-tailed α of 0.05. Hemodynamic data were tested for normal distribution and subsequently analyzed by a two-factor repeated-measures analysis of variance for time and treatment. If an overall difference between the variables was observed, comparisons were performed as one-way analysis of variance followed by Tukey *post hoc* test for intergroup differences and by Dunnett test for intragroup differences with baseline values as the reference time point. Analysis of infarct size and Western blot was performed using one-way analysis of variance followed by Student *t* test with Bonferroni correction for multiple comparisons to avoid a type 1 error. For changes within and between groups, a two-tailed *P* value less than 0.05 was considered significant in advance (SPSS17 for Windows; SPSS Japan, Tokyo, Japan).

Results

Of a total of 44 infarct size experiments, six hearts were not used secondary to intractable ventricular fibrillation after reperfusion (three in CTL, two in CTL + APRO, and one in POST + APRO) and two hearts were not used because of aortic rupture. If a heart was discarded because of aortic rupture or intractable ventricular fibrillation, an additional heart was studied until each group had n = 8 successful experiments (except SEVO + APRO (throughout) and POST + L-NAME when n = 4). Thirty-six hearts (n = 4 for each group) were used for Western blot analysis and nitric oxide measurement. The concentration of sevoflurane in the perfusate collected during the first 2 min of reperfusion was 0.35 ± 0.07 mM. Sevoflurane was not detected in the effluent during the baseline and ischemic period or after discontinuation of sevoflurane during reperfusion. The heart (left ventricle) weights were not different among the groups (CTL: 1.62 ± 0.35 g, CTL + APRO: 1.48 ± 0.30 g, POST: 1.37 ± 0.10 g, POST + APRO: 1.21 ± 0.20 g, SEVO + APRO (throughout): 1.31 ± 0.12 g).

Hemodynamics

Baseline LVDP, dP/dt_{max}, dP/dt_{min}, and coronary flow were similar among the four groups (table 1). Recovery of LVDP was greater in POST compared with CTL throughout the reperfusion period (56 ± 11 *vs.* 26 ± 8 mmHg, $P < 0.05$ at 120 min of reperfusion). Recovery of LVDP in POST hearts was abolished by administration of aprotinin (POST + APRO: 37 ± 10 mmHg). Treatment with aprotinin alone did not affect the recovery of LVDP (CTL + APRO: 35 ± 11 mmHg). LVEDP increased significantly compared with baseline in CTL after ischemia–reperfusion. The increase in LVEDP was significantly less in POST compared with CTL hearts during the reperfusion period (20 ± 9 *vs.* 47 ± 15 mmHg, $P < 0.05$ at 120 min of reperfusion). LVEDP in CTL+APRO was similar to that of CTL hearts (42 ± 10 mmHg).

All groups had reduced dP/dt_{max} after reperfusion compared with baseline. Recovery of dP/dt_{min} was significantly greater in POST compared with CTL hearts, but not in aprotinin-treated hearts. Changes of dP/dt_{min} during the reperfusion period were similar to those of dP/dt_{max}.

There was no significant difference in coronary flow among all groups throughout the experiment. This suggests that changes in coronary flow could not account for the improved contractile recovery of POST hearts (table 1).

Myocardial Infarct Size

As shown in figure 2, myocardial infarct size in the POST group was significantly reduced by approximately 60% compared with control hearts (POST: 15 ± 3% *vs.* CTL: 41 ± 10%, $P < 0.001$). Myocardial infarct size in the hearts in the POST group treated with aprotinin did not differ compared with CTL hearts (POST + APRO: 34 ± 9%, $P = 0.25$; SEVO + APRO (throughout): 38 ± 10%, $P = 0.78$ *vs.* CTL, respectively), suggesting that aprotinin abolished the infarct size–limiting effect of postconditioning. Treatment with aprotinin alone did not affect the infarct size compared with the CTL group ($P = 0.67$ *vs.* CTL).

Western Blot Analysis

The phosphorylation state of PKC-δ, PKC-ε, GSK3β, and upstream Akt at 10 min after reperfusion is illustrated by a representative Western blot in figures 3 and 4. Total PKC-δ, PKC-ε, Akt, and GSK3β were comparable in all samples. The ratio of phospho–PKC-δ to total PKC-δ, phospho-Akt to total Akt, and phospho-GSK3β to total GSK3β was significantly increased in POST compared with CTL (figs. 3 and 4). This increase was not caused by unequal loading of the Western blot, as shown by the detection of α-tubulin. Administration of aprotinin upon reperfusion abolished this increased expressions of phospho–PKC-δ, phospho-Akt, and phospho-GSK3β in the POST + APRO group. There was no significant difference in expression of phospho–PKC-ε among groups.

Nitric Oxide Released from Coronary Effluent

There was no significant difference in nitric oxide concentration among groups during the baseline period. As shown in figure 5, nitric oxide concentration in POST was higher than in other groups at 20 and 30 min of reperfusion. This was abolished by aprotinin administration in the POST + APRO group. Similarly, the nonselective NOS inhibitor L-NAME (100 μM) blocked increased nitric oxide in the POST + L-NAME group.

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Table 1. Hemodynamic Variables

		Reperfusion		
	Baseline	30 min	60 min	120 min
LVDP, mmHg				
CTL	114 ± 17	40 ± 5	34 ± 7	26 ± 8
CTL + APRO	103 ± 13	36 ± 12	37 ± 12	35 ± 11
POST	96 ± 11	67 ± 12*†	63 ± 11*†	56 ± 11*†
POST + APRO	101 ± 12	38 ± 12	40 ± 11	37 ± 10
SEVO + APRO (throughout)	101 ± 9	27 ± 11	29 ± 9	32 ± 12
LVEDP, mmHg				
CTL	10 ± 0	44 ± 14	44 ± 16	47 ± 15
CTL + APRO	10 ± 0	49 ± 13	47 ± 12	42 ± 10
POST	10 ± 0	18 ± 7*†	18 ± 7*†	20 ± 9*
POST + APRO	10 ± 0	34 ± 7*	31 ± 7*	29 ± 7*
SEVO + APRO (throughout)	10 ± 0	51 ± 16	45 ± 17	44 ± 16
CF, $ml \cdot min^{-1} \cdot g^{-1}$				
CTL	22 ± 10	16 ± 7	16 ± 6	15 ± 7
CTL + APRO	23 ± 7	14 ± 5	13 ± 6	13 ± 7
POST	19 ± 4	15 ± 3	14 ± 4	13 ± 5
POST + APRO	23 ± 4	18 ± 4	16 ± 3	15 ± 3
SEVO + APRO (throughout)	25 ± 3	16 ± 4	15 ± 4	14 ± 4
dp/dt_{max}, mmHg/s				
CTL	1,829 ± 252	871 ± 138	725 ± 137	604 ± 119
CTL + APRO	1,656 ± 192	761 ± 179	783 ± 133	736 ± 126
POST	1,559 ± 162	1,194 ± 146*†	1,214 ± 132*†	1,090 ± 96*†
POST + APRO	1,674 ± 131	722 ± 171	860 ± 198	827 ± 193
SEVO + APRO (throughout)	1,678 ± 351	688 ± 17	803 ± 41	806 ± 114
dp/dt_{min}, mmHg/s				
CTL	−1,460 ± 269	−592 ± 162	−562 ± 151	−471 ± 108
CTL + APRO	−1,496 ± 242	−615 ± 142	−616 ± 128	−574 ± 137
POST	−1,435 ± 203	−1,009 ± 102*†	−961 ± 107*†	−898 ± 78*†
POST + APRO	−1,428 ± 93	−686 ± 122	−669 ± 181	−662 ± 122
SEVO + APRO (throughout)	−1,396 ± 55	−542 ± 20	−586 ± 116	−576 ± 108

Data are presented as mean ± SD. n = 8 for each group except SEVO + APRO (throughout) (n = 4).

* $P < 0.05$ *vs.* CTL. † $P < 0.05$ *vs.* POST + APRO.

APRO = aprotinin; CF = coronary flow; CTL = control; dP/dt_{max} = maximum rate of increase of left ventricular pressure; dP/dt_{min} = minimum rate of increase of left ventricular pressure; LVDP = left ventricular developed pressure; LVEDP = left ventricular end-diastolic pressure; POST = postconditioning; SEVO = sevoflurane.

Discussion

The current study provides novel data demonstrating that aprotinin abolishes cardioprotection by sevoflurane postconditioning. Further, this detrimental effect is potentially mediated by inhibition of nitric oxide production and phosphorylation of PKC-δ, Akt, and GSK3β, previously shown to play a role in postconditioning cardioprotection by volatile anesthetics.[26,27] Postconditioning is a potent strategy of myocardial protection.[14] Sevoflurane (1 MAC) given for the first 2 min of postischemic reperfusion was shown to effect cardioprotection in an *in vivo* rat heart model.[28] In the current *in vitro* study, this postconditioning cardioprotection by a volatile anesthetic was abolished by administration of aprotinin. This finding suggests that aprotinin use is not beneficial for salvaging ischemic myocardium.

Aprotinin is a naturally occurring serine protease inhibitor isolated from bovine lung which inhibits kallikrein and plasmin, and is used to reduce blood loss and the need for perioperative blood transfusion. Although it is well established that aprotinin reduces blood loss during cardiac surgery, reports suggest that aprotinin exacerbates ischemic myocardial damage and attenuates the protective effects afforded by ischemic preconditioning in *in vivo* sheep hearts.[29] Confusing the issue, aprotinin has been shown to reduce myocardial reperfusion injury after regional ischemia and cardioplegic arrest in pigs.[2] Furthermore, a recent study by Carter *et al.*[3] suggests that aprotinin reduces reperfusion injury by attenuating apoptosis and polymorphonuclear leukocyte adhesion to coronary endothelium. Therefore, it remains controversial whether aprotinin is protective or detrimental in the setting of cardiac ischemia–reperfusion injury.

Postconditioning with volatile anesthetics may be a potent strategy to salvage ischemic myocardium during cardiac surgery.[14] Among the potential cellular mechanisms responsible for postconditioning cardioprotection, pathway signaling seems to converge on the mitochondria. Opening of mitochondrial adenosine triphosphate–sensitive potassium channels and prevention of mitochondrial permeability transition pore (mPTP) opening are key events in both preconditioning and postconditioning

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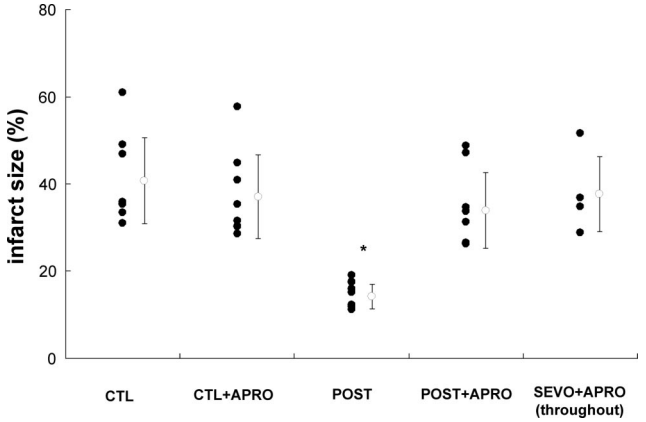

Fig. 2. Infarct size as a percentage of left ventricle. Treatment with sevoflurane (SEVO; 1 minimum alveolar concentration) reduced infarct size compared with control (postconditioning [POST]: 15 ± 3% *vs.* control [CTL]: 41 ± 10%, $P < 0.001$). This cardioprotective effect was abolished by aprotinin (POST + APRO: 34 ± 9%, $P = 0.25$; SEVO + APRO (throughout): 38 ± 10%, $P = 0.78$ *vs.* CTL, respectively). Aprotinin treatment alone did not affect infarct size (CTL + APRO: 40 ± 9%, $P = 0.67$ *vs.* CTL). Data are presented as mean ± SD. * $P < 0.05$: POST *versus* CTL, CTL + APRO, POST, POST + APRO, and SEVO + APRO (throughout).

cardioprotection.[10,12,20,30] Under physiologic conditions, mPTP is predominantly in a closed state. Calcium overload due to ischemia induces mPTP opening.[31] Postconditioning has been shown to reduce calcium-induced mPTP opening in ischemic myocardium.[32] A recent study suggests that phosphorylation activates Akt and inhibits GSK3β during early reperfusion, playing a crucial role in the prevention of mPTP opening in isoflurane postconditioning.[20,33,34] In the current study, administration of aprotinin upon reperfusion inhibited phosphorylation of Akt and GSK3β, associated with a loss of sevoflurane postconditioning cardioprotection.

Nitric oxide has been found to play a role in the signal transduction of ischemic and volatile anesthetic postconditioning.[15,27,35] Endothelial NOS-derived nitric oxide may inhibit mPTP opening in ischemic postconditioning.[36] Endogenous nitric oxide has been shown to contribute to myocardial contractile function after ischemia–reperfusion.[37] One study suggested that aprotinin protects against ischemia–reperfusion injury through inducible NOS-derived nitric oxide.[38] However, a study by Ülker *et al.*[39] demonstrated that aprotinin inhibits nitric oxide release from coronary endothelial cells. Although we did not determine which NOS isozyme produced the increased nitric oxide associated with sevoflurane postconditioning during early reperfusion, aprotinin inhibited this increase in nitric oxide, similar to the nonselective NOS inhibitor L-NAME. It has been reported that the functional recovery after postischemic reperfusion is attenuated by administration of L-NAME compared with untreated control hearts.[40] The precise mechanisms by which nitric oxide produces postconditioning by volatile anesthetics remain unclear. Costa *et al.*[41] demonstrated that nitric oxide opens mitochondrial adenosine triphosphate–sensitive potassium channels in a PKC-ε–dependent mechanism in isolated mitochondria. Although phosphorylation of PKC-ε was not evident with sevoflurane postconditioning in the current study, phosphorylation of PKC-δ at serine 643 but not PKC-ε was shown to be important in transferring the isoflurane preconditioning stimulus to mitochondrial adenosine triphosphate–sensitive potassium channels.[18] In addition, nitric oxide is known to play a role in inhibition of platelet aggregation and adhesion.[42]

Clinically, a full dose protocol of aprotinin administration (280-mg intravenous loading, 280-mg pump prime, and 70-mg/h intravenous constant infusion) results in plasma concentration of around 250 KIU/ml[43] and has been shown to have an antiplasmin and antikallikrein effects.[44,45] The concentration used in the current study is equivalent to this clinical dose. Further, aprotinin at this concentration has been found to selectively inhibit endothelial NOS messenger RNA and protein expression in cultured microvascular endothelial cells.[21]

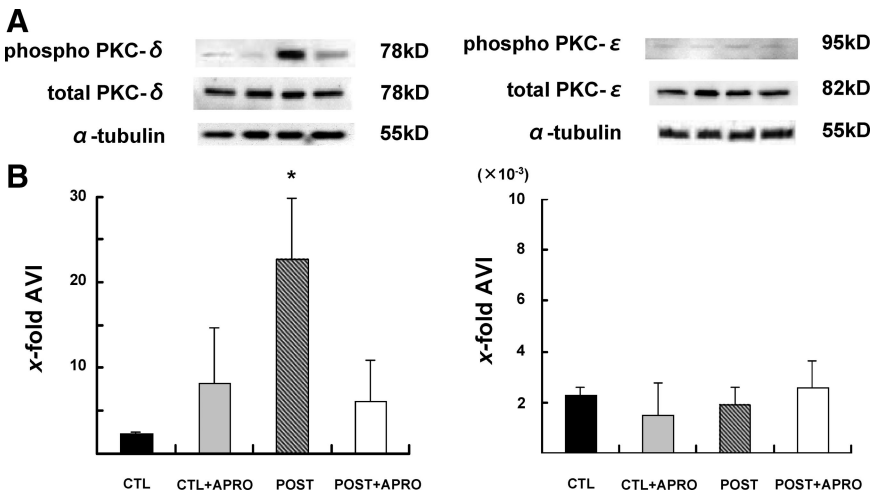

Fig. 3. (*A*) Representative Western blot of phospho–protein kinase C (PKC)-δ and phospho–PKC-ε from left ventricular samples acquired at 10 min after reperfusion (n = 4 for each group). Expression of phospho–PKC-δ was significantly increased in postconditioning (POST). Administration of aprotinin (APRO) upon reperfusion abolished increased expression of phospho–PKC-δ in POST + APRO. There was no significant difference in expression of phospho–PKC-ε among groups. (*B*) Densitometric evaluation of four experiments as the *x*-fold increase in average light density (AVI) *versus* control (CTL). The results are presented as the ratio of phospho–PKC-δ and phospho–PKC-ε to total PKC-δ and total PKC-ε, respectively. The average light intensity was multiplied by 10 to facilitate presentation of an *x*-fold increase. Data are mean ± SD. * $P < 0.05$: POST *versus* CTL, CTL + APRO, and POST + APRO.

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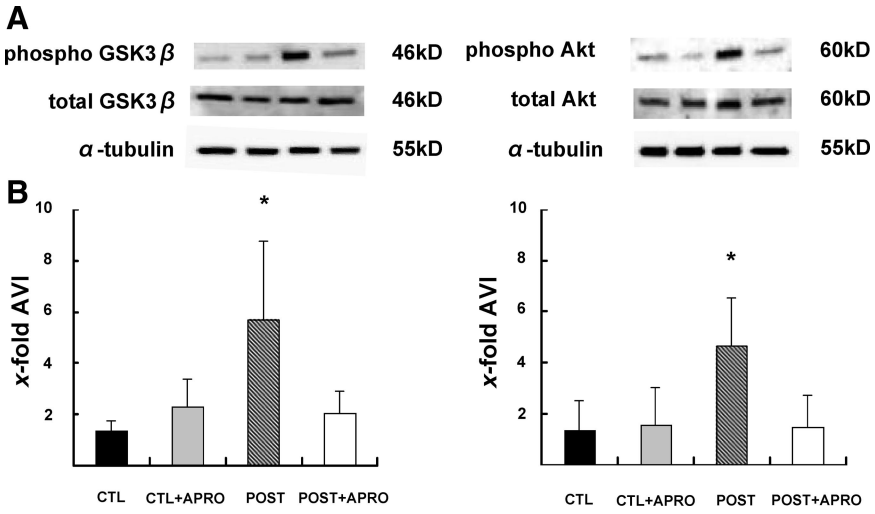

Fig. 4. (*A*) Representative Western blot analysis of phospho–glycogen synthase kinase 3β (GSK3β) and phospho-Akt from left ventricular samples acquired at 10 min after reperfusion (n = 4 for each group). Expression of phospho-GSK3β and phospho-Akt were significantly increased in postconditioning (POST). Administration of aprotinin (APRO) upon reperfusion abolished increased expression of phospho-GSK3β and phospho-Akt in POST + APRO. (*B*) Densitometric evaluation of four experiments as the *x*-fold increase in average light density (AVI) *versus* control (CTL). The results are presented as the ratio of phospho-GSK3β and phospho-Akt to total GSK3β and total Akt, respectively. The average light intensity was multiplied by 10 to facilitate presentation of an *x*-fold increase. Data are mean ± SD. * $P < 0.05$: POST *versus* CTL, CTL + APRO, and POST + APRO.

The following study limitations should be acknowledged. First, the experimental protocol used in the current study does not exactly mimic the clinical scenario in which aprotinin and sevoflurane are given before ischemia and throughout the ischemia–reperfusion periods. However, we performed additional experiments administering both sevoflurane and aprotinin before ischemia and throughout the ischemia–reperfusion periods. The results were the same as those when using our protocol where sevoflurane and aprotinin are administered upon reperfusion. We elected to focus on the early postischemic reperfusion period because mPTP opening occurs within 3–10 min after reperfusion, but not before or during ischemia, in buffer-perfused rat hearts.[46] We administered sevoflurane for the first 2 min of postischemic reperfusion because this protocol has been shown to be as effective for postconditioning as 5- or 10-min administration.[28] Further, Obal *et al.*[47] demonstrated that 1 MAC sevoflurane is sufficient to protect against myocardial reperfusion injury, and increasing the sevoflurane concentration above 1 MAC resulted in no further protection. We did not study higher concentrations of sevoflurane to determine whether the loss of postconditioning protection by aprotinin could be overcome. Although this could prove to be the case, we studied a clinically relevant dose of sevoflurane to mimic the clinical setting. A second limitation to be acknowledged is that, with a crystalloid-perfused heart model, leukocyte–endothelial cell adhesive interactions are not a factor. Therefore, the current study could not address potential antiinflammatory effects of aprotinin against ischemia reperfusion injury. Conversely, we were able to assess the effects of aprotinin directly on the myocardium and vasculature. Third, we demonstrated that aprotinin inhibited sevoflurane postconditioning, which was associated with decreased nitric oxide production and increased phosphorylation of PKC-δ, Akt, and GSK3β during early reperfusion. Nevertheless, the possibility that aprotinin directly inhibited other protein kinases or mediators involved in the postconditioning signaling cascade cannot be ruled out. Fourth, when measuring nitric oxide, dissolved oxygen in the Krebs-Henseleit perfusate can affect the obtained current. However, it has been demonstrated that the sensor has high selectivity to nitric oxide compared with dissolved oxygen (over 25,000 times).[25]

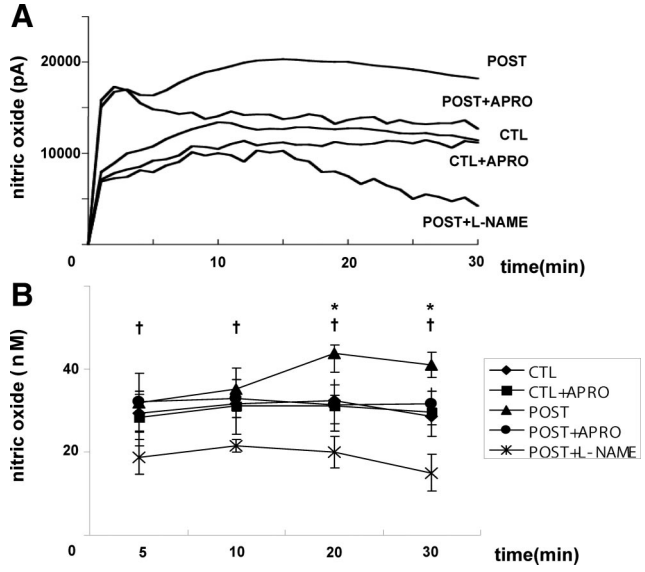

Fig. 5. Time course of nitric oxide concentration in the coronary effluent for 30 min after the onset of reperfusion. (*A*) Representative time course of the measured current. (*B*) Time course of nitric oxide concentration measured corresponding to the current in five groups (n = 4 for each group). * $P < 0.05$: POST *versus* control (CTL), CTL + aprotinin (APRO), postconditioning (POST) + APRO, and POST + *N*-nitro-L-arginine methylester (L-NAME). † $P < 0.05$: POST + L-NAME *versus* CTL, CTL + APRO, POST, and POST + APRO.

In summary, aprotinin abolishes sevoflurane postconditioning. This loss of cardioprotection is associated with inhibited nitric oxide production, and phosphorylation of PKC-δ, Akt, and GSK3β, known mediators of volatile anesthetic postconditioning. Future research will be needed to determine whether this detrimental effect of aprotinin on

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volatile anesthetic postconditioning contributes to the adverse events associated with aprotinin during cardiac surgery.

References

1. Bull DA, Maurer J: Aprotinin and preservation of myocardial function after ischemia-reperfusion injury. Ann Thorac Surg 2003; 75:S735–9
2. Khan TA, Bianchi C, Voisine P, Feng J, Baker J, Hart M, Takahashi M, Stahl G, Sellke FW: Reduction of myocardial reperfusion injury by aprotinin after regional ischemia and cardioplegic arrest. J Thorac Cardiovasc Surg 2004; 128: 602–8
3. Carter J, Buerke U, Rossner E, Russ M, Schubert S, Schmidt H, Ebelt H, Pruefer D, Schlitt A, Werdan K, Buerke M: Anti-inflammatory actions of aprotinin provide dose-dependent cardioprotection from reperfusion injury. Br J Pharmacol 2008; 155:93–102
4. Mouton R, Finch D, Davies I, Binks A, Zacharowski K: Effect of aprotinin on renal dysfunction in patients undergoing on-pump and off-pump cardiac surgery: A retrospective observational study. Lancet 2008; 371:475–82
5. Hausenloy DJ, Pagano D, Keogh B: Aprotinin: Still courting controversy. Lancet 2008; 371:449–50
6. Mangano DT, Tudor IC, Dietzel C: The risk associated with aprotinin in cardiac surgery. N Engl J Med 2006; 354:353–65
7. Mangano DT, Miao Y, Vuylsteke A, Tudor IC, Juneja R, Filipescu D, Hoeft A, Fontes ML, Hillel Z, Ott E, Titov T, Dietzel C, Levin J: Mortality associated with aprotinin during 5 years following coronary artery bypass graft surgery. JAMA 2007; 297:471–9
8. Karkouti K, Beattie WS, Dattilo KM, McCluskey SA, Ghannam M, Hamdy A, Wijeysundera DN, Fedorko L, Yau TM: A propensity score case-control comparison of aprotinin and tranexamic acid in high-transfusion-risk cardiac surgery. Transfusion 2006; 46:327–38
9. Shaw AD, Stafford-Smith M, White WD, Phillips-Bute B, Swaminathan M, Milano C, Welsby IJ, Aronson S, Mathew JP, Peterson ED, Newman MF: The effect of aprotinin on outcome after coronary-artery bypass grafting. N Engl J Med 2008; 358:784–93
10. Kersten JR, Schmeling TJ, Pagel PS, Gross GJ, Warltier DC: Isoflurane mimics ischemic preconditioning *via* activation of K_{ATP} channels: Reduction of myocardial infarct size with an acute memory phase. ANESTHESIOLOGY 1997; 87: 361–70
11. Preckel B, Schlack W, Comfere T, Obal D, Barthel H, Thamer V: Effects of enflurane, isoflurane, sevoflurane and desflurane on reperfusion injury after regional myocardial ischaemia in the rabbit heart *in vivo*. Br J Anaesth 1998; 81:905–12
12. Zaugg M, Lucchinetti E, Uecker M, Pasch T, Schaub MC: Anaesthetics and cardiac preconditioning, I: Signalling and cytoprotective mechanisms. Br J Anaesth 2003; 91:551–65
13. Kaneda K, Miyamae M, Sugioka S, Okusa C, Inamura Y, Domae N, Kotani J, Figueredo VM: Sevoflurane enhances ethanol-induced cardiac preconditioning through modulation of protein kinase C, mitochondrial KATP channels, and nitric oxide synthase, in guinea pig hearts. Anesth Analg 2008; 106:9–16
14. Pagel PS: Postconditioning by volatile anesthetics: salvaging ischemic myocardium at reperfusion by activation of prosurvival signaling. J Cardiothorac Vasc Anesth 2008; 22:753–65
15. Amour J, Brzezinska AK, Weihrauch D, Billstrom A, Zielonka J, Krolikowski JG, Bienengraeber MW, Warltier DC, Philip FJ, Kersten JR: Role of heat shock protein 90 and endothelial nitric oxide synthase during early anesthetic and ischemic preconditioning. ANESTHESIOLOGY 2009; 110:317–25
16. Novalija E, Fujita S, Kampine JP, Stowe DF: Sevoflurane mimics ischemic preconditioning effects on coronary flow and nitric oxide release in isolated hearts. ANESTHESIOLOGY 1999; 91:701–12
17. Riess ML, Kevin LG, McCormick J, Jiang MT, Rhodes SS, Stowe DF: Anesthetic preconditioning: The role of free radicals in sevoflurane-induced attenuation of mitochondrial electron transport in Guinea pig isolated hearts. Anesth Analg 2005; 100:46–53
18. Uecker M, Da Silva R, Grampp T, Pasch T, Schaub MC, Zaugg M: Translocation of protein kinase C isoforms to subcellular targets in ischemic and anesthetic preconditioning. ANESTHESIOLOGY 2003; 99:138–47
19. Okusa C, Miyamae M, Sugioka S, Kaneda K, Inamura Y, Onishi A, Domae N, Kotani J, Figueredo VM: Acute memory phase of sevoflurane preconditioning is associated with sustained translocation of PKC-α and ε, but not δ in isolated guinea pig hearts. Eur J Anaesthesiol 2009; 26:582–8
20. Feng J, Lucchinetti E, Ahuja P, Pasch T, Perriard JC, Zaugg M: Isoflurane postconditioning prevents opening of the mitochondrial permeability transition pore through inhibition of glycogen synthase kinase 3β. ANESTHESIOLOGY 2005; 103:987–95
21. Ülker S, McKeown PP, Bayraktutan U: Aprotinin impairs coronary endothelial function and down-regulates endothelial NOS in rat coronary microvascular endothelial cells. Cardiovasc Res 2002; 55:830–7
22. Samama CM, Mazoyer E, Bruneval P, Ciostek P, Bonnin P, Bonneau M, Roussi J, Bailliart O, Pignaud G, Viars P, Caen JP, Drouet LO: Aprotinin could promote arterial thrombosis in pigs: A prospective randomized, blind study. Thromb Haemost 1994; 71:663–9
23. Cosgrove DM III, Heric B, Lytle BW, Taylor PC, Novoa R, Golding LA, Stewart RW, McCarthy PM, Loop FD: Aprotinin therapy for reoperative myocardial revascularization: A placebo-controlled study. Ann Thorac Surg 1992; 54: 1031–6
24. Fishbein MC, Meerbaum S, Rit J, Lando U, Kanmatsuse K, Mercier JC, Corday E, Ganz W: Early phase acute myocardial infarct size quantification: Validation of the triphenyl tetrazolium chloride tissue enzyme staining technique. Am Heart J 1981; 101:593–600
25. Mochizuki S, Himi N, Miyasaka T, Nakamoto H, Takemoto M, Hirano K, Tsujioka K, Ogasawara Y, Kajiya F: Evaluation of basic performance and applicability of a newly developed *in vivo* nitric oxide sensor. Physiol Meas 2002; 23:261–8
26. Feng J, Fischer G, Lucchinetti E, Zhu M, Bestmann L, Jegger D, Arras M, Pasch T, Perriard JC, Schaub MC, Zaugg M: Infarct-remodeled myocardium is receptive to protection by isoflurane postconditioning: Role of protein kinase B/Akt signaling. ANESTHESIOLOGY 2006; 104:1004–14
27. Tessier-Vetzel D, Tissier R, Waintraub X, Ghaleb B, Berdeaux A: Isoflurane inhaled at the onset of reperfusion potentiates the cardioprotective effect of ischemic postconditioning through a NO-dependent mechanism. J Cardiovasc Pharmacol 2006; 47:487–92
28. Obal D, Scharbatke H, Barthel H, Preckel B, Müllenheim J, Schlack W: Cardioprotection against reperfusion injury is maximal with only two minutes of sevoflurane administration in rats. Can J Anaesth 2003; 50:940–5
29. Bukhari EA, Krukenkamp IB, Burns PG, Gaudette GR, Schulman JJ, al-Fagih MR, Levitsky S: Does aprotinin increase the myocardial damage in the setting of ischemia and preconditioning? Ann Thorac Surg 1995; 60:307–10
30. Tsutsumi YM, Yokoyama T, Horikawa Y, Roth DM, Patel HH: Reactive oxygen species trigger ischemic and pharmacological postconditioning: *in vivo* and *in vitro* characterization. Life Sci 2007; 81:1223–7
31. Weiss JN, Korge P, Honda HM, Ping P: Role of the mitochondrial permeability transition in myocardial disease. Circ Res 2003; 93:292–301
32. Argaud L, Gateau-Roesch O, Raisky O, Loufouat J, Robert D, Ovize M: Postconditioning inhibits mitochondrial permeability transition. Circulation 2005; 111:194–7
33. Chiari PC, Bienengraeber MW, Pagel PS, Krolikowski JG, Kersten JR, Warltier DC: Isoflurane protects against myocardial infarction during early reperfusion by activation of phosphatidylinositol-3-kinase signal transduction: Evidence for anesthetic-induced postconditioning in rabbits. ANESTHESIOLOGY 2005; 102:102–9
34. Krolikowski JG, Bienengraeber M, Weihrauch D, Warltier DC, Kersten JR, Pagel PS: Inhibition of mitochondrial permeability transition enhances isoflurane-induced cardioprotection during early reperfusion: the role of mitochondrial KATP channels. Anesth Analg 2005; 101:1590–6
35. Penna C, Cappello S, Mancardi D, Raimondo S, Rastaldo R, Gattullo D, Losano G, Pagliaro P: Post-conditioning reduces infarct size in the isolated rat heart: Role of coronary flow and pressure and the nitric oxide/cGMP pathway. Basic Res Cardiol 2006; 101:168–79
36. Heusch G, Boengler K, Schulz R: Cardioprotection: nitric oxide, protein kinases, and mitochondria. Circulation 2008; 118:1915–9
37. Heusch G, Post H, Michel MC, Kelm M, Schulz R: Endogenous nitric oxide and myocardial adaptation to ischemia. Circ Res 2000; 87:146–52
38. Bruda NL, Hurlbert BJ, Hill GE: Aprotinin reduces nitric oxide production *in vitro* and *in vivo* in a dose-dependent manner. Clin Sci (Lond) 1998; 94:505–9
39. Ülker S, Cinar MG, Bayraktutan U, Evinc A: Aprotinin impairs endothelium-dependent relaxation in rat aorta and inhibits nitric oxide release from rat coronary endothelial cells. Cardiovasc Res 2001; 50:589–96
40. Yoshida K, Mizukami Y, Kitakaze M: Nitric oxide mediates protein kinase C isoform translocation in rat heart during postischemic reperfusion. Biochim Biophys Acta 1999; 1453:230–8
41. Costa AD, Garlid KD: Intramitochondrial signaling: Interactions among mitoKATP, PKC-ε, ROS, and MPT. Am J Physiol Heart Circ Physiol 2008; 295: H874–82
42. Nathan C, Xie QW: Nitric oxide synthases: Roles, tolls, and controls. Cell 1994; 78:915–8
43. Hardy JF, Desroches J: Natural and synthetic antifibrinolytics in cardiac surgery. Can J Anaesth 1992; 39:353–65
44. Fritz H, Wunderer G: Biochemistry and applications of aprotinin, the kallikrein inhibitor from bovine organs. Arzneimittelforsch 1983; 33:479–94
45. Hoffmann H, Siebeck M, Thetter O, Jochum M, Fritz H: Aprotinin concentrations effective for the inhibition of tissue kallikrein and plasma kallikrein *in vitro* and *in vivo*. Adv Exp Med Biol 1989; 247B:35–42
46. Halestrap AP, Clarke SJ, Javadov SA: Mitochondrial permeability transition pore opening during myocardial reperfusion: A target for cardioprotection. Cardiovasc Res 2004; 61:372–85
47. Obal D, Preckel B, Scharbatke H, Mullenheim J, Hoterkes F, Thamer V, Schlack W: One MAC of sevoflurane provides protection against reperfusion injury in the rat heart *in vivo*. Br J Anaesth 2001; 87:905–11

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