

Differential Role of Pim-1 Kinase in Anesthetic-induced and Ischemic Preconditioning against Myocardial Infarction

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Background: Ischemic preconditioning (IPC) and anesthetic-induced preconditioning against myocardial infarction are mediated *via* protein kinase B. Pim-1 kinase acts downstream of protein kinase B and was recently shown to regulate cardiomyocyte survival. The authors tested the hypothesis that IPC and anesthetic-induced preconditioning are mediated by Pim-1 kinase.

Methods: Pentobarbital-anesthetized male C57Black/6 mice were subjected to 45 min of coronary artery occlusion and 3 h of reperfusion. Animals received no intervention, Pim-1 kinase inhibitor II (10 µg/g intraperitoneally), its vehicle dimethyl sulfoxide (10 µl/g intraperitoneally), or 1.0 minimum alveolar concentration desflurane alone or in combination with Pim-1 kinase inhibitor II (10 µg/g intraperitoneally). IPC was induced by three cycles of 5 min ischemia-reperfusion each, and animals received IPC either alone or in combination with Pim-1 kinase inhibitor II (10 µg/g intraperitoneally). Infarct size was determined with triphenyltetrazolium chloride, and area at risk was determined with Evans blue (Sigma-Aldrich, Taufkirchen, Germany). Protein expression of Pim-1 kinase, Bad, phospho-Bad^{Ser112}, and cytosolic content of cytochrome *c* were measured using Western immunoblotting.

Results: Infarct size in the control group was 47 ± 2%. Pim-1 kinase inhibitor II (44 ± 2%) had no effect on infarct size. Desflurane (17 ± 3%) and IPC (19 ± 2%) significantly reduced infarct size compared with control (both $P < 0.05$ vs. control). Blockade of Pim-1 kinase completely abrogated desflurane-induced preconditioning (43 ± 3%), whereas IPC (35 ± 3%) was blocked partially. Desflurane tended to reduce cytosolic content of cytochrome *c*, which was abrogated by Pim-1 kinase inhibitor II.

Conclusion: These data suggest that Pim-1 kinase mediates at least in part desflurane-induced preconditioning and IPC against myocardial infarction in mice.

ISCHEMIC heart disease is still the leading cause of death in industrialized nations. Strategies to enhance cardiomyocyte tolerance to ischemic periods are of enormous interest to improve patient outcome after acute myocardial ischemia. Cardioprotection by ischemic preconditioning (IPC)¹ and

anesthetic-induced preconditioning²⁻⁵ is a well-known phenomenon and has been demonstrated in various species.^{1,3,5} Both types of preconditioning are mediated *via* activation of phosphatidylinositol 3-kinase/protein kinase B.⁶⁻¹⁰

Pim-1 kinase was recently identified as a new downstream mediator of protein kinase B/Akt activity in cardiomyocytes.¹¹ Pim-1 kinase was shown to reduce myocardial infarct size (IS)¹¹ and cardiomyocyte apoptosis^{11,12} and to exert profound antihypertrophic properties.¹² The same properties have been ascribed to Akt kinase before.^{13,14}

The serine/threonine kinase Pim-1 belongs to the family of calcium/calmodulin-regulated kinases.¹⁵ Pim-1 kinase expression can be induced by various cytokines and growth factors,¹⁵ consistent with an important role for this kinase in regulating cell growth and proliferation. Pim-1 kinase phosphorylates several downstream targets, including the proapoptotic molecule Bad specifically on residue serine 112.¹⁶ This phosphorylation results in inactivation of Bad.¹⁶ Therefore, a direct role for Pim-1 in preventing cell death was suggested because inactive Bad enhances Bcl-2 activity, thereby promoting cell survival.¹⁶

Whether Pim-1 kinase plays a role in the signal transduction cascade of IPC and anesthetic-induced preconditioning has not been investigated. Therefore, we tested the hypothesis that activation of Pim-1 kinase is necessary for IPC and desflurane-induced preconditioning against myocardial infarction in mice *in vivo*.

Materials and Methods

Animals

Male C57Black/6 mice (10-12 weeks old) were purchased from Harlan (Horst, The Netherlands). Animals were housed under controlled conditions (22°C, 55-65% humidity, 12-h light-dark cycle) and were allowed free access to water and a standard laboratory chow. All experimental procedures used in this investigation were reviewed and approved by the Animal Care and Use Committee of the Government of Lower Franconia, Bavaria, Germany. All experiments were in accordance with the *Guide for the Care and Use of Laboratory Animals*¹⁷ and conformed to the Guiding Principles in the Care and Use of Animals of the American Physiologic Society.

Instrumentation and Surgical Procedures

Instrumentation and surgical procedures were performed as described previously.¹⁸ Briefly, mice were

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anesthetized with an intraperitoneal injection of 60 $\mu\text{g/g}$ sodium pentobarbital (Merial, Hallbergmoos, Germany), and repeated intraperitoneal injections were given as needed to maintain anesthesia. Rectal temperature was maintained at $37.0^\circ \pm 0.1^\circ\text{C}$ using a servocontrolled heating pad (FMI, Seeheim, Germany). After intubation of the trachea, animals were ventilated with a 50%/50% air-oxygen mixture using a small rodent ventilator (SAR-P 830; CWE Inc., Ardmore, PA) operating in pressure-controlled mode. A three-lead needle-probe electrocardiogram was attached to continuously monitor heart rate and ST-segment elevation. Saline-filled polyethylene catheters were placed into the right common carotid artery for measurement of mean arterial blood pressure and into the right jugular vein for continuous fluid administration ($20 \mu\text{l} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$). A left thoracotomy at the fourth intercostal space was performed, and the left anterior descending coronary artery was exposed. The ligature was realized as described previously.¹⁹ Coronary artery occlusion was achieved using the hanging weight system¹⁹ and was verified by ST-segment elevation in the electrocardiogram and paleness of the myocardial area at risk (AAR). Adequate reperfusion was verified by epicardial hyperemia and reversion of electrocardiogram changes.

Experimental Protocol

The experimental protocol used in this study is illustrated in figure 1. After completion of surgical procedures, mice were randomly assigned to one of the seven study groups by opening a sealed envelope containing information about the study group. Group size was $n = 9$ in each group. All mice were allowed a 30-min equilibration period. Myocardial ischemia was induced by 45 min of coronary artery occlusion (CAO) followed by 3 h of reperfusion. Control animals (CON) received no treatment before CAO. In group 2 (DMSO), the vehicle dimethyl sulfoxide (DMSO, 10 $\mu\text{l/g}$) was injected intraperitoneally 35 min before CAO. In group 3 (PIM-Inh.II), the highly selective²⁰ Pim-1 kinase inhibitor 2-hydroxy-3-cyano-4-phenyl-6-(3-bromo-6-hydroxyphenyl)pyridine (Pim-1 kinase inhibitor II, 10 $\mu\text{g/g}$, dissolved in DMSO; Merck, Darmstadt, Germany) was administered intraperitoneally 35 min before CAO. Pim-1 kinase inhibitor II was reported to have an IC_{50} for Pim-1 kinase of 0.05 μM (50 nM).²⁰ The chemical structure of Pim-1 kinase inhibitor II is illustrated in figure 2. Desflurane (DES) was administered at a concentration of 1.0 minimum alveolar concentration (MAC) (7.5 vol%²¹) for 15 min starting 30 min before CAO. Animals of group 5 received Pim-1 kinase inhibitor II in combination with desflurane (DES +

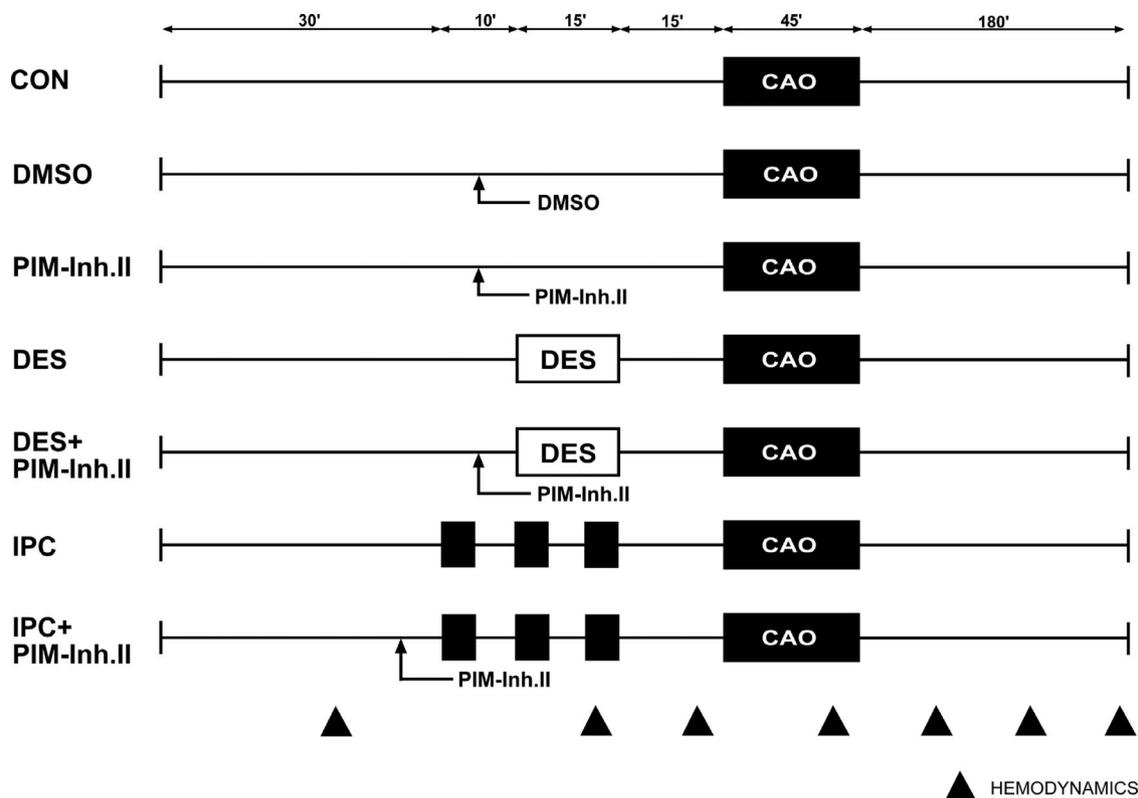


Fig. 1. Schematic diagram illustrating the experimental protocol. CAO = coronary artery occlusion; CON = control group; DMSO = dimethyl sulfoxide (10 $\mu\text{l/g}$) intraperitoneally 35 min before coronary artery occlusion; DES = 1.0 minimum alveolar concentration desflurane for 15 min starting 30 min before coronary artery occlusion; DES + PIM-Inh.II = Pim-1 kinase inhibitor II (10 $\mu\text{g/g}$) intraperitoneally 5 min before desflurane (1.0 minimum alveolar concentration for 15 min); IPC = ischemic preconditioning (three cycles of 5 min ischemia–reperfusion each); IPC + PIM-Inh.II = Pim-1 kinase inhibitor II (10 $\mu\text{g/g}$) 5 min before ischemic preconditioning; PIM-Inh.II = Pim-1 kinase inhibitor II (10 $\mu\text{g/g}$) intraperitoneally 35 min before coronary artery occlusion.

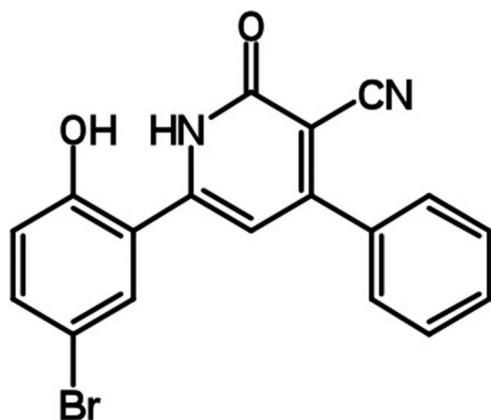


Fig. 2. Chemical structure of Pim-1 kinase inhibitor II (2-hydroxy-3-cyano-4-phenyl-6-(3-bromo-6-hydroxyphenyl)pyridine).

PIM-Inh.II). Ischemic preconditioning (IPC) was induced by three cycles of 5-min ischemia-reperfusion before CAO. In group 7, animals received Pim-1 kinase inhibitor II in combination with IPC (IPC + PIM-Inh.II).

Measurement of Myocardial Infarct Size

Myocardial IS and AAR were determined using methods described previously.¹⁸ Briefly, after 3 h of reperfusion, the left anterior descending coronary artery was reoccluded and 1 ml Evans blue (0.1 g/ml; Sigma-Aldrich, Taufkirchen, Germany) was slowly injected into the carotid artery. After intraperitoneal injection of a lethal dose of sodium pentobarbital (150 μ g/g), the heart was rapidly excised. The left ventricle was separated and cut into seven or eight transversal slices of 1-mm thickness. Slices were incubated in 2,3,5-triphenyltetrazolium chloride (20 mg/ml) for 30 min at 37°C. After overnight fixation in 10% formaldehyde, slices were weighed and digitally photographed. Photographs were analyzed using Adobe Photoshop CS 8.0.1 (Adobe Systems Inc., San Jose, CA), and the normal zone, AAR, and IS were determined gravitoplanimetrically by a blinded investigator. Animals with an AAR of less than 20% were excluded from the study.

Western Immunoblotting

In a second and a third set of experiments, myocardial tissue was extracted at two different time points in the experimental protocol. The first time point was 15 min after the end of desflurane administration and to the corresponding time points in the other experimental groups, respectively. The second time point was after 2 h of reperfusion. The aim of these additional experiments was to measure protein expression of Pim-1 kinase, Bad, and phosphorylated Bad at serine 112 at the first time point and cytosolic content of cytochrome *c* at the second time point.

In the second set of experiments, mice ($n = 6$ in each group) received saline (CON), Pim-1 inhibitor II (PIM-Inh.II), desflurane alone (DES) or in combination with

Pim-1 inhibitor II (DES + PIM-Inh.II), and IPC alone (IPC) or in combination with Pim-1 inhibitor II (IPC + PIM-Inh.II). The aim of this second set of experiments was to investigate effects on the expression of Pim-1 kinase and Bad and to investigate phosphorylation of Bad at serine 112 using a Western immunoblotting technique. Fifteen minutes after the end of desflurane administration and to the corresponding time points in the other experimental groups, respectively, hearts were rapidly excised, and the left ventricle was shock frozen in liquid nitrogen and stored at -80°C until further use. The samples were homogenized in ice-cold radioimmunoprecipitation assay buffer (phosphate-buffered saline [Na_2HPO_4 , NaH_2PO_4 , NaCl , H_2O ; pH 7.4], 1% Igepal CA-630, 0.5% sodium desoxycholic acid, 0.1% sodium dodecylsulfate polyacrylamide; for phospho samples: protease inhibitor, 20 mM NaFl, 1 mM Na-vanadate in addition) and centrifuged at 12,000g at 4°C . Cytosolic and particulate cell fractions were left unseparated for further analysis.

In the third set of experiments, myocardial tissue was extracted after 2 h of reperfusion to measure cytosolic content of cytochrome *c* as a marker of induction of the apoptotic cascade.²² The myocardial tissue was washed with ice-cold phosphate-buffered saline (Na_2HPO_4 , NaH_2PO_4 , NaCl , H_2O ; pH 7.4) and homogenized in buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EDTA). Buffer A+ (buffer A with Triton X-100 in addition) was added, and the samples were incubated on ice for 15 min. After stirring, the samples were centrifuged at 10,000g for 30 min. The supernatant containing cytosol was removed, and the pellet was frozen at -80°C until further use. Proteins were loaded on 15% polyacrylamide sodium dodecylsulfate polyacrylamide gels and were subsequently blotted on nitrocellulose membranes (Protran, Whatman GmbH, Germany). Nonspecific background was blocked using 2.5% nonfat milk powder combined with 2.5% albumin powder from bovine serum in phosphate-buffered saline-Tween 20 (1 h at room temperature). Membranes were then incubated with the following antibodies: anti-Pim-1 kinase 1:100 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Bad 1:50 (Santa Cruz Biotechnology), anti-phospho-Bad^{Ser112} 1:1,000 (Santa Cruz Biotechnology), anti-cytochrome *c* 1:300 (Santa Cruz Biotechnology), mouse anti-glyceraldehyde-3-phosphate dehydrogenase 1:3,000 (Millipore, Billerica, MA), and mouse anti- α B-crystallin 1:3,000 (Assay Designs, Ann Arbor, MI).

The protein bands were detected using ECL[®] detection reagent (GE Healthcare, Buckinghamshire, United Kingdom) and visualized on an x-ray film. The films were scanned and optical density was determined using ImageJ software (National Institutes of Health, Bethesda, MD). Optical density of the target protein was normalized to its loading control expression (glyceraldehyde-3-

phosphate dehydrogenase and α B-crystallin, respectively). The control group ratio was set as 100%.

Data Acquisition and Statistical Analysis

Electrocardiogram, systemic hemodynamic parameters, and body temperature were continuously recorded and analyzed on a personal computer (Fujitsu Siemens, Augsburg, Germany) using a hemodynamic data acquisition and analysis software (Notocord[®] hem 3.5; Croissy sur Seine, France).

Concluding from other studies on the same experimental model,^{5,18} we expected a myocardial IS of 50% (IS/AAR). Power analysis revealed a group size of $n = 8$ to detect a reduction in means of IS from 50% to 35% with a power of 0.8 at an α level of 0.05. Statistical analyses were performed by analysis of variance, which were based on two-tailed F tests for comparison of components of the factors' total deviation. Analyses for body weight, left ventricle weight, left ventricle weight/body weight, AAR, IS, IS/left ventricle weight, and AAR/left ventricle weight were performed using one-way analysis of variance including the factor treatment (CON vs. DMSO vs. PIM-Inh.II vs. DES vs. DES + PIM-Inh.II vs. IPC vs. IPC + PIM-Inh.II) and *post hoc* Duncan test for significant main effects and interactions. Analysis for densitometry was also performed using one-way analysis of variance including the factor treatment (CON vs. PIM-Inh.II vs. DES vs. DES + PIM-Inh.II vs. IPC vs. IPC + PIM-Inh.II) and *post hoc* Duncan test for significant main effects and interactions. Analyses of hemodynamic data were performed by a 7×7 analysis of variance for

repeated measures, including the between factor treatment (CON vs. DMSO vs. PIM-Inh.II vs. DES vs. DES + PIM-Inh.II vs. IPC vs. IPC + PIM-Inh.II) and the within factor time point (baseline vs. intervention period vs. memory period vs. CAO vs. reperfusion 60 min vs. reperfusion 120 min vs. reperfusion 180 min). In case of any significant main effects or interactions, *post hoc* one-way analyses of variance were conducted for each group and each time point. Statistical analyses of data were performed using SPSS 15.0 software (The Apache Software Foundation, Forest Hill, MD). Changes in means were considered statistically significant at $P < 0.05$. Data are presented as mean \pm SEM.

Results

A total of 141 mice were included in the study. Sixty-nine mice were assigned to the ischemia-reperfusion experiments to obtain 63 successful experiments. Six animals were excluded because of pump failure during CAO (one in the control group, two in the DMSO group, one in the PIM-Inh.II group, and one in the IPC + PIM-Inh.II group) or because the AAR was less than 20% (one in the PIM-Inh.II group). Seventy-two mice were used to measure protein expression using a Western immunoblotting technique.

Hemodynamic Parameters and AAR

Hemodynamic parameters at baseline and AAR were not different among groups (tables 1 and 2). MAP was significantly decreased during CAO in four groups (CON, PIM-Inh.II, DES + PIM-Inh.II, and IPC + Pim-Inh.II).

Table 1. Systemic Hemodynamics

	BL	INTERV	MEM	CAO	Reperfusion		
					60 min	120 min	180 min
HR, min ⁻¹							
CON	457 \pm 12	466 \pm 14	459 \pm 17	456 \pm 14	446 \pm 14	446 \pm 13	452 \pm 10
DMSO	493 \pm 15	491 \pm 14	486 \pm 12	466 \pm 9	462 \pm 9	468 \pm 12	467 \pm 14
PIM-Inh.II	485 \pm 17	478 \pm 12	483 \pm 14	470 \pm 14	478 \pm 13	471 \pm 22	466 \pm 19
DES	471 \pm 12	427 \pm 14	450 \pm 31	435 \pm 16	447 \pm 13	452 \pm 12	458 \pm 12
DES + PIM-Inh.II	484 \pm 8	478 \pm 8	482 \pm 7	493 \pm 10	475 \pm 10	477 \pm 12	486 \pm 8
IPC	462 \pm 11	441 \pm 12	459 \pm 17	440 \pm 16	450 \pm 20	433 \pm 17	438 \pm 16
IPC + PIM-Inh.II	492 \pm 16	490 \pm 16	491 \pm 11	461 \pm 10	481 \pm 8	482 \pm 17	476 \pm 26
MAP, mmHg							
CON	70 \pm 3	68 \pm 4	68 \pm 2	55 \pm 3*	64 \pm 3	60 \pm 3	63 \pm 3
DMSO	72 \pm 3	68 \pm 4	63 \pm 2	56 \pm 4	58 \pm 4	58 \pm 4	60 \pm 5
PIM-Inh.II	71 \pm 2	63 \pm 4	67 \pm 4	54 \pm 4*	60 \pm 3	61 \pm 4	63 \pm 3
DES	72 \pm 2	69 \pm 4	73 \pm 3	59 \pm 3	66 \pm 2	64 \pm 4	63 \pm 3
DES + PIM-Inh.II	72 \pm 2	69 \pm 2	71 \pm 4	54 \pm 3*	63 \pm 3	59 \pm 4*	62 \pm 3
IPC	68 \pm 4	66 \pm 3	68 \pm 3	52 \pm 4	59 \pm 4	58 \pm 4	58 \pm 3
IPC + PIM-Inh.II	73 \pm 4	61 \pm 4	61 \pm 4	52 \pm 3*	61 \pm 3	59 \pm 2	61 \pm 3

Data are mean \pm SEM. $n = 9$ per group. Data were analyzed at the end of the baseline period (BL), at the end of the intervention period (INTERV; desflurane [DES], ischemic preconditioning [IPC], or corresponding time point), before (memory period [MEM]) and at the end of coronary artery occlusion (CAO), and 60, 120, and 180 min after the onset of reperfusion, as indicated by triangles in figure 1.

* Significantly ($P < 0.05$) different from BL.

CON = control; DMSO = dimethyl sulfoxide; HR = heart rate; MAP = mean arterial pressure; PIM-Inh.II = Pim-1 kinase inhibitor II.

Table 2. Body Weight and Planimetry

	n	BW, g	LV, mg	LV/BW, %	AAR, mg	IS, mg	IS/LV, %	AAR/LV, %
CON	9	23.6 ± 0.4	65.2 ± 1.7	0.28 ± 0.01	22.6 ± 3.5	10.6 ± 1.8	16.2 ± 2.7	34.7 ± 5.2
DMSO	9	25.3 ± 0.8	72.0 ± 2.3	0.28 ± 0.01	26.0 ± 2.1	12.2 ± 1.7	16.6 ± 1.8	35.8 ± 2.0
PIM-Inh.II	9	24.7 ± 0.6	68.0 ± 3.1	0.27 ± 0.01	24.7 ± 2.6	10.7 ± 1.0	15.9 ± 1.3	36.7 ± 3.9
DES	9	25.4 ± 1.0	72.6 ± 3.6	0.29 ± 0.01	23.4 ± 1.8	4.1 ± 0.8*	5.7 ± 1.2*	32.5 ± 2.4
DES + PIM-Inh.II	9	25.7 ± 0.7	73.6 ± 2.7	0.29 ± 0.01	24.1 ± 2.1	10.8 ± 1.6	14.4 ± 1.8	32.4 ± 2.1
IPC	9	23.1 ± 0.4	65.8 ± 1.4	0.29 ± 0.01	22.5 ± 2.0	4.3 ± 0.5*	6.6 ± 0.8*	34.3 ± 3.0
IPC + PIM-Inh.II	9	25.6 ± 0.5	72.9 ± 2.3	0.29 ± 0.01	26.0 ± 1.6	9.1 ± 1.0	12.5 ± 1.2	35.7 ± 2.1

Data are mean ± SEM.

* Significantly ($P < 0.05$) different from control.

AAR = area at risk; BW = body weight; CON = control; DES = desflurane; DMSO = dimethyl sulfoxide; IPC = ischemic preconditioning; IS = infarct size; LV = left ventricle; PIM-Inh.II = Pim-1 kinase inhibitor II.

Myocardial Infarct Size

Myocardial infarct size (IS/AAR) was $47 \pm 2\%$ in the control group (fig. 3). Pim-1 kinase inhibitor II alone (PIM-Inh.II; $44 \pm 2\%$) and its vehicle DMSO alone (DMSO; $46 \pm 4\%$) did not reduce myocardial IS. The administration of 1.0 MAC desflurane (DES; $17 \pm 3\%$; $P < 0.05$) significantly reduced myocardial IS compared with the control group. The blockade of Pim-1 kinase with Pim-1 kinase inhibitor II completely abolished desflurane-induced preconditioning (DES + PIM-Inh.II; $43 \pm 3\%$). Three cycles of 5 min ischemia-reperfusion reduced IS to a similar extent as desflurane-induced preconditioning (IPC; $19 \pm 2\%$; $P < 0.05$). IPC was abolished in part by application of Pim-1 kinase inhibitor II (IPC + PIM-Inh.II; $35 \pm 3\%$; $P < 0.05$ vs. CON, PIM-Inh.II, and IPC).

Western Immunoblotting

Pim-1 Kinase, Bad, and P-Bad^{Ser112}. Total Pim-1 kinase protein expression (fig. 4) and total Bad expres-

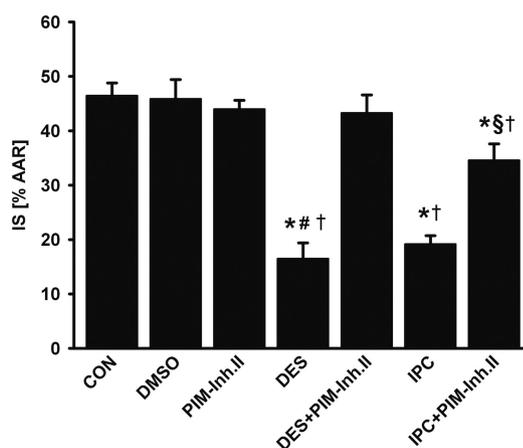


Fig. 3. Myocardial infarct size (IS) expressed as percentage of left ventricular area at risk (AAR). Values are mean ± SEM. n = 9 in each group. Mice received no intervention (CON), dimethyl sulfoxide (DMSO), desflurane (DES), ischemic preconditioning (IPC), Pim-1 kinase inhibitor II alone (PIM-Inh.II) or in combination with desflurane (DES + PIM-Inh.II), or ischemic preconditioning (IPC + PIM-Inh.II). * Significantly ($P < 0.05$) different from CON. # Significantly ($P < 0.05$) different from DES + PIM-Inh.II. § Significantly ($P < 0.05$) different from IPC. † Significantly ($P < 0.05$) different from PIM-Inh.II.

sion (fig. 5A) were not affected by any of the study drugs. Desflurane and IPC tended to result in increased phosphorylation of Bad at serine 112. Desflurane-induced phosphorylation of Bad at serine 112 was significantly reduced by Pim-1 kinase inhibitor II, whereas IPC-induced phosphorylation was not affected (fig. 5B).

Cytochrome c. Desflurane and IPC tended to result in reduced cytosolic content of cytochrome c. The desflurane-induced reduction was abolished by Pim-1 kinase inhibitor II (fig. 6). The IPC-induced reduction of cytosolic cytochrome c was not affected by pharmacologic inhibition of Pim-1 kinase.

Discussion

In the current study, we tested the hypothesis that desflurane-induced preconditioning and IPC against myocar-

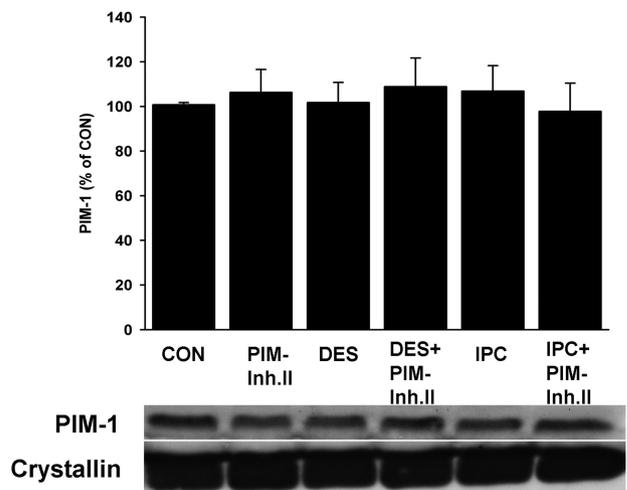


Fig. 4. Western blot analysis of myocardial expression of Pim-1 kinase. Results are presented as representative original immunoblottings and average densitometric results as percentage of control (n = 6 per group). CON = control group; DES = desflurane (1.0 minimum alveolar concentration); DES + PIM-Inh.II = Pim-1 kinase inhibitor II in combination with desflurane; IPC = ischemic preconditioning (three cycles of 5 min ischemia-reperfusion each); IPC + PIM-Inh.II = Pim-1 kinase inhibitor II in combination with ischemic preconditioning; PIM-Inh.II = Pim-1 kinase inhibitor II (10 µg/g).

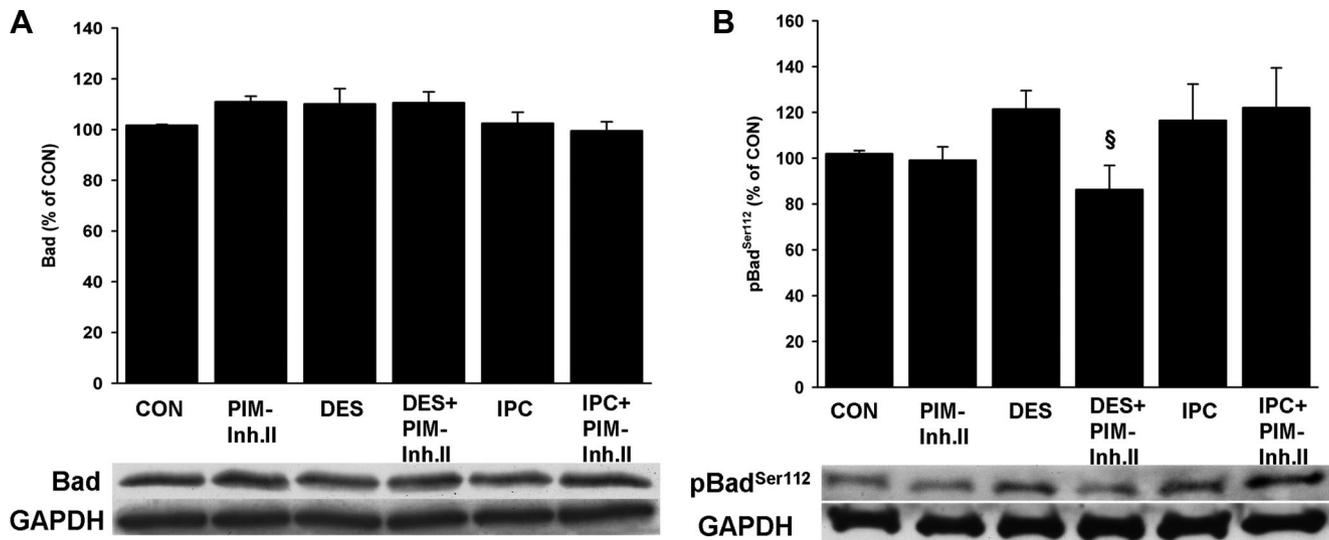


Fig. 5. Western blot analysis of myocardial expression of Bad (A) and phosphorylation of Bad (B) at serine 112. Results are presented as representative original immunoblottings and average densitometric results as percentage of control ($n = 6$ per group). CON = control group; DES = desflurane (1.0 minimum alveolar concentration); DES + PIM-Inh.II = Pim-1 kinase inhibitor II in combination with desflurane; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; IPC = ischemic preconditioning (three cycles of 5 min ischemia-reperfusion each); IPC + PIM-Inh.II = Pim-1 kinase inhibitor II in combination with ischemic preconditioning; PIM-Inh.II = Pim-1 kinase inhibitor II (10 $\mu\text{g/g}$). \S Significantly ($P < 0.05$) different from DES.

dial infarction are mediated by Pim-1 kinase. Therefore, we used a murine *in vivo* model of acute myocardial infarction. Our results demonstrate that both desflurane and three cycles of 5-min ischemia-reperfusion induce preconditioning in the murine *in vivo* model of acute myocardial

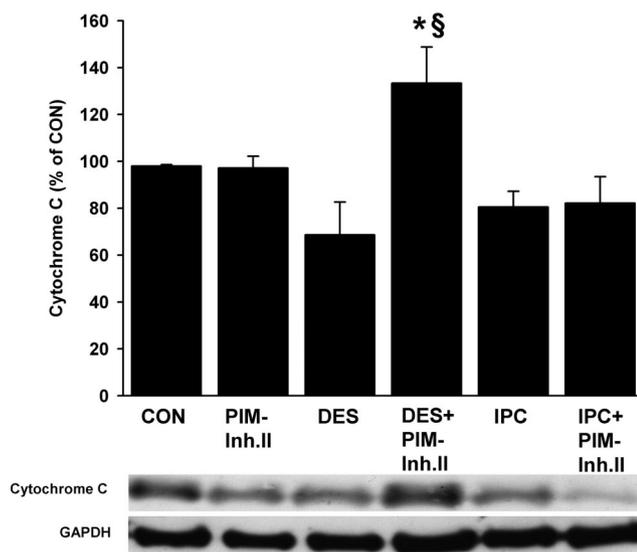


Fig. 6. Western blot analysis of cytosolic content of cytochrome *c*. Results are presented as representative original immunoblottings and average densitometric results as percentage of control ($n = 6$ per group). CON = control group; DES = desflurane (1.0 minimum alveolar concentration); DES + PIM-Inh.II = Pim-1 kinase inhibitor II in combination with desflurane; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; IPC = ischemic preconditioning (three cycles of 5 min ischemia-reperfusion each); IPC + PIM-Inh.II = Pim-1 kinase inhibitor II in combination with ischemic preconditioning; PIM-Inh.II = Pim-1 kinase inhibitor II (10 $\mu\text{g/g}$). * Significantly ($P < 0.05$) different from CON. \S Significantly ($P < 0.05$) different from DES.

infarction, represented by a pronounced reduction in myocardial IS. These results confirm findings from various other studies regarding either IPC or volatile anesthetic-induced preconditioning.^{3-5,23,24}

The first major finding of this study is that pharmacologic inhibition of Pim-1 kinase completely abolishes desflurane-induced preconditioning against myocardial infarction, whereas IPC is only partially blocked. Therefore, our results demonstrate a possible involvement of the serine/threonine kinase Pim-1 in the signaling cascade of both IPC and desflurane-induced preconditioning. There were no differences in basal expression of Pim-1 kinase among groups that could influence the observed results. Pim-1 kinase was described as a crucial mediator of cardiomyocyte survival acting downstream of Akt.¹¹ Inhibition of phosphatidylinositol 3-kinase/Akt using either wortmannin or LY294002 partially abrogated IPC against myocardial infarction in isolated rat hearts.¹⁰ In contrast, anesthetic-induced preconditioning by 30-min administration of 1 MAC isoflurane was completely blocked by inhibition of phosphatidylinositol 3-kinase/Akt using wortmannin in rabbits.⁷ Together with our results, these findings indicate a differential role for the phosphatidylinositol 3-kinase/Akt pathway in mediating anesthetic-induced preconditioning and IPC against myocardial infarction.

Pim-1 kinase belongs to the family of calcium/calmodulin-regulated kinases.¹⁵ Recently, our group reported an important role for calcium/calmodulin regulated kinase II in mediating both anesthetic-induced preconditioning and postconditioning in rabbits *in vivo*.^{25,26} The current results are in accord with these previous findings suggesting a pivotal role for the protein family of calcium/

calmodulin-regulated kinases in the signal transduction cascade of anesthetic induced cardioprotection.

Pim-1 kinase has been demonstrated to phosphorylate Bad specifically at serine 112, leading to its inactivation and thereby preventing apoptotic cell death.¹⁶ Furthermore, Pim-1 kinase prolongs survival and reduces apoptotic-induced mitochondrial dysfunction in murine hematopoietic cells, which was at least in part dependent on activity of the antiapoptotic protein Bcl-2.²⁷ Inactive Bad enhances Bcl-2-activity, thereby promoting antiapoptotic properties.¹⁶ Our results demonstrate that desflurane-induced preconditioning and IPC tend to result in an increased phosphorylation of Bad at serine 112, consistent with an activation of Pim-1 kinase. The desflurane-induced phosphorylation of Bad at serine 112 was significantly reduced by pharmacologic inhibition of Pim-1 kinase. However, IPC-induced phosphorylation of Bad was not affected by Pim-1 kinase inhibitor II. These results suggest a possible differential role for Pim-1 kinase in mediating desflurane-induced preconditioning and IPC. Consistent with our data, an increased phosphorylation of Bad at serine 112 in response to isoflurane preconditioning was reported.⁸ Akt is known to phosphorylate Bad predominantly on serine 136.²⁸ Based on these results and our data, we suggest that the observed⁸ phosphorylation of Bad at serine 112 is more likely an effect of Pim-1 kinase than a direct effect of Akt.

Cardiac myocyte apoptosis has been demonstrated to play a crucial role in the development of ischemia-reperfusion injury. It was demonstrated that inhibition of apoptosis led to an IS reduction up to 60% in mice.²⁹ Activation of Akt was shown to reduce myocardial IS and cardiac myocyte apoptosis after ischemia-reperfusion, to prevent cardiac dysfunction and to prevent hypoxia induced abnormalities in calcium handling.³⁰ Transgenic mice with cardiac specific overexpression of Bcl-2 showed a 50% reduction in myocardial IS and fewer apoptotic changes after ischemia-reperfusion injury.³¹ In another study, overexpression of Bcl-2 in cardiac myocytes reduced ischemia-induced necrosis as well as apoptosis. Overexpression of Bcl-2 results in a 20% decrease in 2,3,5-triphenyltetrazolium chloride-measured (necrotic) and a 3% decrease in terminal dUTP nick-end labeling (TUNEL)-measured (apoptotic) cell death.³² Therefore, the reduction in cell death cannot be attributed solely to a reduction in apoptotic cell death. These data suggest that Bcl-2 leads to a decrease in necrotic cell death as well. The mitochondrial permeability transition pore as a putative end effector of protection against ischemia-reperfusion injury is involved in both apoptosis and necrosis.³³ Furthermore, there is increasing evidence from other studies about crosstalk between apoptosis and necrosis³⁴ and autophagy,³⁵ respectively. However, the exact fraction of each form of cell death may differ in the *in vivo* versus the *in vitro* setting.

Cytochrome *c* is located in the mitochondrial intermembrane space and released into the cytosol in conse-

quence to an apoptotic stimulus. Activation of Bcl-2 prevents release of cytochrome *c* from the mitochondrial intermembrane space into the cytosol.²² Release of cytochrome *c* was shown to be an early event in the induction of the apoptotic cascade, acting upstream of caspase activation and mitochondrial depolarization.^{22,36,37} However, the exact molecular mechanism of cytochrome *c* release is still unclear and remains to be investigated. Our data demonstrate that desflurane-induced preconditioning and IPC tend to result in reduced release of cytochrome *c*. Desflurane-induced reduction of cytochrome *c* release was completely abrogated by Pim-1 kinase inhibitor II. However, we could not detect any effect of Pim-1 blockade on IPC-induced reduction of cytosolic content of cytochrome *c*. Based on our data, we suggest a possible difference in the role of Pim-1 kinase pathway in mediating the antiapoptotic properties of desflurane-induced preconditioning and IPC. In another study, isoflurane and sevoflurane were shown to induce apoptosis in human T lymphocytes, whereas desflurane did not exert any proapoptotic effects in this study.³⁸ Our results regarding IPC are in accord with previous findings from another group demonstrating that IPC by six cycles of 4-min ischemia-4-min reperfusion each led to a reduced release of cytochrome *c* to the cytoplasm in mice.³⁹

The results of the current study should be interpreted within the constraint of several potential limitations. The size of myocardial AAR and the amount of coronary collateral blood flow were shown to be crucial determinants of myocardial IS. However, the AAR was not different among groups. Coronary collateral blood flow was not measured in this study. However, small rodents are reported to have little if any coronary collateral blood flow.⁴⁰ Thus, it is highly improbable that AAR and coronary collateral blood flow contribute to differences in myocardial IS. Myocardial oxygen consumption was not directly quantified in this study. Therefore, we cannot completely exclude that changes in myocardial oxygen supply/demand ratio might contribute to our results. Pim-1 kinase inhibitor II was shown to be selective to Pim-1 kinase²⁰ with high selectivity over Pim-2 and Pim-3 kinase. Nevertheless, potential effects of Pim-1 kinase inhibitor II on Pim-2 and Pim-3 kinase and other kinases involved in signal transduction cascade of either IPC or desflurane-induced preconditioning cannot be completely excluded. Pim-1 kinase was demonstrated to specifically phosphorylate Bad on serine 112.¹⁶ However, it cannot be excluded that other kinases involved in preconditioning cascades, particularly Akt, might phosphorylate Bad on the same residue. However, it was shown that Akt phosphorylates Bad predominantly at serine 136.²⁸

In summary, our results lead to the assumption that desflurane-induced preconditioning and IPC against myocardial infarction are mediated, at least in part, *via* activation of Pim-1 kinase. Activation of Pim-1 kinase is

suggested to be involved in the antiapoptotic properties of desflurane-induced preconditioning. Cardioprotective signaling *via* Pim-1 kinase seems to have a differential role in desflurane-induced preconditioning and IPC in mice *in vivo*.

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