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Desflurane-induced and ischaemic postconditioning against myocardial infarction are mediated by Pim-1 kinase

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Background: Anaesthetic-induced (APOST) and ischaemic postconditioning (IPOST) against myocardial infarction are mediated via phosphatidylinositol-3-kinase/Akt. Pim-1 kinase is acting downstream of Akt and has recently been demonstrated to enhance cardiomyocyte survival. We tested the hypothesis that both APOST and IPOST are mediated by Pim-1 kinase.
Methods: Pentobarbital-anaesthetized male C57BL/6 mice were subjected to 45-min coronary artery occlusion (CAO) and 3-h reperfusion. Animals received either no intervention, the Pim-1 kinase inhibitor II (10 µg/g intraperitoneally) or its vehicle dimethy sulfoxide (10 µl/g intraperitoneally). Three minutes prior to the end of CAO, 1.0 minimum alveolar concentration desflurane was administered for 18 min alone or in combination with Pim-1 kinase inhibitor II. IPOST was induced by three cycles of each 10-s ischaemia/reperfusion, and animals received either IPOST alone or in combination with Pim-1 kinase inhibitor II. Infarct size was determined with triphenyltetrazolium chloride and area at risk with Evans blue. Protein expression of Pim-1 kinase, Bad, phospho-Bad^{Ser112} and B-cell lymphoma 2 was determined using Western immunoblotting analysis.
Results: Infarct size in control animals (CON) was 46 ± 3%. Dimethylsulfoxide (47 ± 3%) and Pim-1 kinase inhibitor II (44 ± 5%) did not significantly reduce infarct size. Desflurane (16 ± 2%*; *$P < 0.05$ vs. CON) and IPOST (21 ± 2%*) significantly reduced infarct size compared with CON. Inhibition of Pim-1 kinase abolished desflurane-induced postconditioning (46 ± 4%) and IPOST (44 ± 5%). Western blot analysis revealed that only desflurane enhances phosphorylation of Bad at serine 112 that was abrogated by Pim-1 kinase inhibitor II.
Conclusion: These data suggest that Pim-1 kinase mediates both desflurane-induced postconditioning and IPOST in mice.

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Although early myocardial reperfusion is regarded the most effective strategy to treat acute myocardial ischaemia, considerable evidence has accumulated that myocardial reperfusion itself causes cardiomyocyte death.[1,2] This is known as lethal reperfusion injury. Myocardial reperfusion is estimated to cause 50% of myocardial infarct size (IS) as a result of ischaemia/reperfusion injury.[2] Further support to this concept was provided by the study of Zhao et al., who demonstrated that three cycles of each 30-s reperfusion/ischaemia immediately after coronary artery occlusion (CAO)_ reduced myocardial IS up to 40% in dogs.[3] This phenomenon was termed ischaemic postconditioning (IPOST).[3] IPOST has been demonstrated to reduce myocardial IS among others in dogs,[3] rabbits[4] and mice.[5–7] In humans, IPOST has been shown to reduce myocardial IS as assessed 6 months after acute myocardial infarction and to improve left ventricular ejection fraction after 1 year.[8]

Cardioprotective properties of volatile anaesthetics during early reperfusion are known for more than a decade since the first description of Schlack's group.[9] Later, this phenomenon was termed anaesthetic-induced postconditioning (APOST).[10] APOST reduces myocardial IS,[5,10,11] improves left ventricular function after myocardial ischaemia[12] and has been demonstrated to occur in humans.[13]

Isoflurane, sevoflurane and desflurane (DES) are equally effective to reduce myocardial IS in mice in vivo.[5]

Both IPOST and APOST afford their cardioprotective properties via reduction of reperfusion injury and are mediated via phosphatidylinositol-3-kinase (PI3K)/Akt[10,14,15] as a part of the reperfusion injury salvage kinase (RISK) pathway.[16]

The serine/threonine kinase Pim-1 belongs to the family of calcium/calmodulin-dependent protein kinases (CaMK).[17] DES-induced postconditioning is mediated by CaMKII in rabbits in vivo.[11] Pim-1 kinase has recently been described to act downstream of PI3K/Akt in cardiac myocytes.[18] Genetic-induced overexpression of Pim-1 confers anti-apoptotic[18] and antihypertrophic effects,[19] and reduces myocardial IS.[18] Moreover, genetic engineering of cardiac progenitor cells with Pim-1 kinase has been described to improve cellular viability, proliferation and regeneration after acute myocardial infarction.[20] More recently, our group demonstrated a crucial role for Pim-1 kinase in both DES-induced and ischaemic preconditioning (IPC) against myocardial infarction.[21]

However, whether Pim-1 kinase is involved in APOST and IPOST against myocardial infarction has not been investigated to date. Therefore, we tested the hypothesis that APOST and IPOST are mediated via Pim-1 kinase in mice in vivo.

Methods

Ethical approval

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,[22] and conformed to the Guiding Principles in the Care and Use of Animals of the American Physiological Society.

Instrumentation and surgical procedures

Male C57BL/6 mice (10–12 weeks old) were purchased from Harlan laboratories (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. Instrumentation and surgical procedures were performed, as described previously.[23] Briefly, mice were anaesthetized with an intraperitoneal injection of 60 µg/g sodium pentobarbital (Merial, Hallbergmoos, Germany), and repeated intraperitoneal injections were given as needed to maintain anaesthesia. Rectal temperature was maintained at 37.0 ± 0.1 °C using a servo-controlled heating-pad (FMI, Seeheim, Germany). After intubation of the trachea, animals were ventilated with 50%/50% air-oxygen mixture using a small rodent ventilator (SAR-P 830, CWE, Inc., Ardmore, PA, USA) operating in pressure-controlled mode. A three-lead needle-probe electrocardiogram (ECG) was attached to continuously monitor heart rate and ST segment elevation. Saline-filled polyethylen (PE) 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 µl/g/h). A left thoracotomy at the fourth intercostal space was performed, and the left anterior descending coronary artery (LAD) was exposed. CAO was achieved using the hanging weight system[23,24] and was verified by ECG ST segment elevation and paleness of the myocardial area at risk (AAR). Adequate reperfusion was verified by epicardial hyperaemia and reversion of ECG changes.

Experimental protocol

Animals were randomly assigned to seven groups to investigate the role of Pim-1 kinase in ischaemic and DES-induced postconditioning. Group size was $n = 8$ in each group. The experimental protocol is illustrated in Figure 1.

After completion of surgical procedures, all mice were allowed a 30-min equilibration period. Myocardial ischaemia was induced by 45-min CAO followed by 3 h of reperfusion. Control animals (CON) received no additional treatment. In group 2 [dimethyl sulfoxide (DMSO)], the vehicle DMSO (10 µl/g) was injected intraperitoneally 5 min prior to the end of CAO. In group 3 [Pim-1 kinase inhibitor II (PIM-Inh.II)], the selective[25] Pim-1 kinase inhibitor 2-hydroxy-3-cyano-4-phenyl-6-(3-bromo-6-hydroxyphenyl)pyridine (PIM-Inh.II, 10 µg/g, dissolved in DMSO; Merck, Darmstadt, Germany) was administered intraperitoneally 5 min prior to the end of CAO. DES was administered at a concentration of 1.0 minimum alveolar concentration (MAC, 7.5 Vol%[26]) for 18 min starting 3 min prior to the onset of reperfusion. This type of administration was chosen because the first minute of reperfusion after CAO is most probably critical for postconditioning's success.[27] Initiation of DES administration before reperfusion guaranteed an effective level of 1.0 MAC DES with the onset of reperfusion.

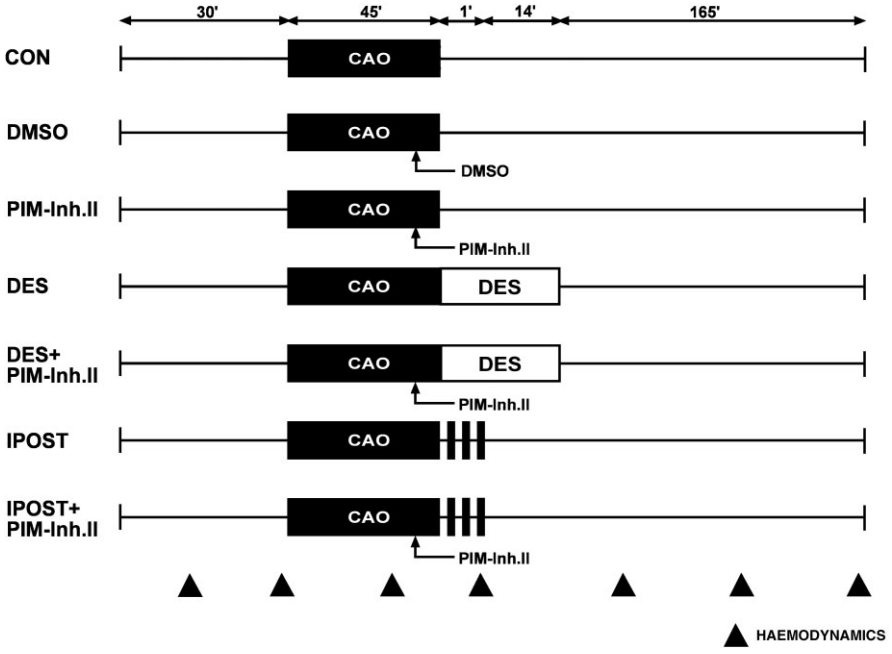

Fig. 1. Schematic diagram illustrating the experimental protocol of the study. CAO, coronary artery occlusion; CON, control group; DMSO, dimethyl sulfoxide (10 µl/g) intraperitoneal 5 min prior to the onset of reperfusion; PIM-Inh.II, Pim-1 kinase inhibitor II (10 µg/g) intraperitoneal 5 min prior to the onset of reperfusion; DES, desflurane (1.0 minimum alveolar concentration DES for 18 min starting 3 min prior to the end of CAO); DES+PIM-Inh.II, PIM-Inh.II (10 µg/g) intraperitoneal 5 min prior to the end of CAO and DES (1.0 minimum alveolar concentration for 18 min); IPOST, ischaemic postconditioning (three cycles of each 10 s ischaemia/reperfusion immediately after the end of CAO); IPOST+PIM-Inh.II, PIM-Inh.II (10 µg/g) 5 min prior to IPOST. Triangles indicate time points of analysis of haemodynamic parameters, as presented in Table 1.

Animals of group 5 received PIM-Inh.II in combination with DES (DES+PIM-Inh.II). IPOST was induced by three cycles of each 10-s ischaemia and reperfusion immediately after CAO. In group 7, animals received PIM-Inh.II in combination with IPOST (IPOST+PIM-Inh.II).

Measurement of myocardial IS

Myocardial IS and AAR were determined using methods described previously.[23] Briefly, after 3 h of reperfusion, the LAD was re-occluded 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 µg/g), the heart was rapidly excised. The left ventricle was separated and cut into 7–8 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 weighted and digitally photographed. Photographs were analysed using Adobe Photoshop CS 8.0.1 (Adobe Systems, Inc., San Jose, CA, USA), and the normal zone, AAR and IS were determined gravitoplanimetrically by an investigator blinded to the experimental protocol. Animals with an AAR of less than 20% were excluded from the study.

Western immunoblotting

In a second set of experiments, myocardial tissue was extracted after 1 h of reperfusion to investigate effects on the expression of Pim-1 kinase, Bad and B-cell lymphoma 2 (Bcl-2), and to investigate phosphorylation of Bad at serine 112 as a surrogate for Pim-1 activity using Western immunoblotting technique.

Mice ($n = 4$ in each group) received saline (CON), PIM-Inh.II, DES alone or in combination with PIM-Inh.II (DES+PIM-Inh.II), and IPOST alone (IPOST), or in combination with PIM-Inh.II (IPOST+PIM-Inh.II). One hour after the onset of reperfusion, hearts were rapidly excised, and the left ventricle was shock frozen in liquid nitrogen and stored at –80 °C until further usage. The samples were homogenized in ice-cold radioimmunoprecipitation assay buffer [phosphate-buffered saline (PBS) (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, NaF 20 mM, sodium vanadate 1 mM in addition) and centrifuged at 12,000g at 4 °C. Cytosolic and particulate cell fractions were left unseparated for further analysis.

Proteins were loaded on 15% polyacrylamide sodium dodecylsulfate polyacrylamide gels and were subsequently blotted on nitrocellulose membranes (Protran, Whatman GmbH, Dassel, Germany). Nonspecific background was blocked using 2.5% nonfat milk powder combined with 2.5% albumin powder from bovine serum in PBS-Tween 20 (1 h at room temperature). Membranes were then

incubated with the following antibodies: antiPim-1 kinase 1 : 100 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), antiBad 1 : 100 (Abcam, Cambridge, Great Britain), antiphospho-Bad^{Ser112} 1 : 500 (Cell Signaling, Danvers, MA, USA), antiBcl-2 1 : 500 (Abcam), mouse antiglyceraldehyde-3-phosphate dehydrogenase (Millipore, Billerica, MA, USA) and mouse anti-αB-crystallin 1 : 3000 (Assay Designs, Ann Arbor, MI, USA), respectively.

The protein bands were detected using enhanced chemiluminescent® detection reagent (GE Healthcare, Buckinghamshire, UK) and visualized on an X-ray film. The films were scanned and optical density was determined using ImageJ software (NIH, Bethesda, MD, USA). Optical density of the target protein was normalized to its loading control expression. Glyceraldehyde-3-phosphate dehydrogenase served as the loading control for Bad, phospho-Bad^{Ser112} and Bcl-2, and αB-crystallin served as the loading control for Pim-1 kinase, respectively. The control group ratio was set as 100%.

Data acquisition and statistical analysis

ECG, systemic haemodynamic parameters and body temperature were continuously recorded and analysed on a personal computer (Fujitsu Siemens, Augsburg, Germany) using a haemodynamic data acquisition and analysis software (Notocord® hem 3.5, Croissy-sur-Seine, France).

Concluding from other studies on the same experimental model,[21,23,28] 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 15% with a power of 0.8 at a α-level of 0.05. Statistical analyses were done by analysis of variance (ANOVA), 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 and AAR/left ventricle were performed using one-way ANOVA, including the factor treatment (CON vs. DMSO vs. PIM-Inh.II vs. DES vs. DES+PIM-Inh.II vs. IPOST vs. IPOST+PIM-Inh.II) and post hoc Duncan's test for significant main effects and interactions. Analysis for densitometry was also performed using one-way ANOVA including the factor treatment (CON vs. PIM-Inh.II vs. DES vs. DES+PIM-Inh.II vs. IPOST vs. IPOST+PIM-Inh.II) and post hoc Duncan's test for significant main effects and interactions. Analysis of haemodynamic data was performed by a 7×7 ANOVA for repeated measures, including the between factor treatment (CON vs. DMSO vs. PIM-Inh.II vs. DES vs. DES+PIM-Inh.II vs. IPOST vs. IPOST+PIM-Inh.II) and the within factor time point (baseline vs. PreCAO vs. CAO vs. POST 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 ANOVAs were conducted for each group and each time point, where a Bonferroni correction of α was performed. Statistical analysis of data was performed using SPSS 16.0 software (The Apache Software Foundation, Forest Hill, MD, USA). Changes in means were considered statistically significant when $P < 0.05$. Data are presented as mean ± standard error of the mean.

Results

A total of 86 mice were included to the study. Sixty-two mice were assigned to the ischaemia-reperfusion experiments to obtain 56 successful experiments. Six animals were excluded because of pump failure during CAO (one in the DMSO group, one in the PIM-Inh.II group, one in the DES+PIM-Inh.II group, one in the IPOST group, two in the IPOST+PIM-Inh.II group). Twenty-four mice were used to measure protein expression using Western immunoblotting technique.

Haemodynamic parameters, AAR

Haemodynamic parameters at baseline and AAR were not different among groups (Tables 1 and 2). MAP was significantly decreased compared with baseline values during CAO in two groups (DMSO and DES). In the PIM-Inh.II group, MAP was significantly decreased compared with the control group after 120 min of reperfusion.

Myocardial IS

Myocardial IS (IS/AAR) was 46% ± 3% in control group (Fig. 2). PIM-Inh.II (44% ± 5%) alone and its vehicle DMSO (47% ± 3%) alone did not reduce myocardial IS. A 15-min administration of 1.0 MAC DES during early reperfusion (DES; 16% ± 2%; $P < 0.05$ vs. control group) significantly reduced myocardial IS compared with control group. Inhibition of Pim-1 kinase using PIM-Inh.II completely abolished DES-induced postconditioning (DES+PIM-Inh.II; 46% ± 4%). IPOST induced by three cycles of each 10-s ischaemia and reperfusion reduced myocardial IS to 21% ± 2% (IPOST; $P < 0.05$ vs. control group, PIM-Inh.II and IPOST+PIM-Inh.II). Similar to DES-induced post-

Table 1

Systemic haemodynamics.

HR (min^{-1})	BL	PreCAO	CAO	POST	Reperfusion		
					60 min	120 min	180 min
CON	463 ± 19	462 ± 20	474 ± 21	473 ± 16	473 ± 11	455 ± 20	470 ± 10
DMSO	486 ± 17	477 ± 11	471 ± 14	483 ± 16	467 ± 11	463 ± 6	463 ± 18
PIM-Inh.II	471 ± 13	465 ± 25	458 ± 8	480 ± 19	482 ± 12	480 ± 14	489 ± 8
DES	451 ± 7	461 ± 7	457 ± 14	434 ± 5	439 ± 6	446 ± 10	460 ± 15
DES+PIM-Inh.II	479 ± 13	466 ± 14	471 ± 10	478 ± 13	493 ± 10	484 ± 10	484 ± 8
IPOST	455 ± 12	443 ± 10	436 ± 6	447 ± 9	434 ± 14	452 ± 15	447 ± 14
IPOST+PIM-Inh.II	467 ± 15	452 ± 15	470 ± 14	488 ± 16	445 ± 30	480 ± 16	494 ± 11
MAP (mmHg)							
CON	72 ± 3	66 ± 5	60 ± 2	70 ± 4	71 ± 4	68 ± 4	66 ± 6
DMSO	71 ± 5	70 ± 6	63 ± 5*	67 ± 7	70 ± 5	67 ± 4	64 ± 3
PIM-Inh.II	70 ± 5	67 ± 5	58 ± 6	58 ± 6	59 ± 3	54 ± 1†	56 ± 3
DES	69 ± 2	68 ± 2	58 ± 3*	63 ± 4	65 ± 4	64 ± 3	68 ± 3
DES+PIM-Inh.II	73 ± 2	68 ± 3	67 ± 2	70 ± 2	64 ± 4	64 ± 3	72 ± 6
IPOST	71 ± 5	66 ± 4	62 ± 3	67 ± 3	68 ± 4	65 ± 3	66 ± 5
IPOST+PIM-Inh.II	74 ± 4	71 ± 4	63 ± 4	60 ± 4	66 ± 3	68 ± 4	67 ± 4

Data are mean ± SEM.
Data were analysed during baseline period (BL), before (PreCAO) and during (CAO) CAO, during early reperfusion period (POST) 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.
†Significantly ($P < 0.05$) different from CON.
HR, heart rate; MAP, mean arterial pressure; CON, control; DMSO, dimethyl sulfoxide; PIM-Inh.II, Pim-1 kinase inhibitor II; DES, desflurane; IPOST, ischaemic postconditioning; CAO, coronary artery occlusion; SEM, standard error of the mean.

Table 2

Body weight and planimetry.

	n	BW [g]	LV [mg]	LV/BW [%]	AAR [mg]	IS [mg]	IS/LV [%]	AAR/LV [%]
CON	8	25.4 ± 0.7	70.3 ± 2.8	0.28 ± 0.01	25.1 ± 2.6	11.4 ± 1.2	16.0 ± 1.2	35.3 ± 2.6
DMSO	8	26.6 ± 1.0	77.3 ± 3.1	0.29 ± 0.01	25.0 ± 2.1	11.9 ± 1.3	15.4 ± 1.6	32.5 ± 2.6
PIM-Inh.II	8	26.8 ± 1.4	75.9 ± 5.2	0.28 ± 0.01	23.5 ± 1.8	10.5 ± 1.7	13.7 ± 1.7	31.1 ± 1.5
DES	8	24.5 ± 1.2	68.7 ± 5.4	0.28 ± 0.01	21.6 ± 2.3	3.5 ± 0.7*†	5.1 ± 0.9*†	31.3 ± 1.9
DES+PIM-Inh.II	8	25.9 ± 1.1	76.6 ± 4.2	0.30 ± 0.01	26.7 ± 2.8	12.1 ± 1.7	16.2 ± 2.7	36.0 ± 5.1
IPOST	8	26.0 ± 0.4	72.9 ± 2.2	0.28 ± 0.01	21.6 ± 2.5	4.3 ± 0.5*‡	6.0 ± 0.6*‡	29.6 ± 3.2
IPOST+PIM-Inh.II	8	26.3 ± 1.1	75.6 ± 3.7	0.29 ± 0.01	23.0 ± 1.2	10.3 ± 1.4	13.5 ± 1.5	30.7 ± 1.7

Data are mean ± SEM.
*Significantly ($P < 0.05$) different from CON. †Significantly ($P < 0.05$) different from DES+PIM-Inh.II. ‡Significantly ($P < 0.05$) different from IPOST+PIM-Inh.II.
BW, body weight; LV, left ventricle; AAR, area at risk; IS, infarct size; CON, control; DMSO, dimethyl sulfoxide; DES, desflurane; PIM-Inh.II, Pim-1 kinase inhibitor II; IPOST, ischaemic postconditioning; SEM, standard error of the mean.

conditioning, IPOST was completely abolished by application of PIM-Inh.II (IPOST+PIM-Inh.II; 44% ± 5%).

Western immunoblotting

Total Pim-1 kinase protein expression (Fig. 3) as well as total Bad expression (Fig. 4A) was not affected by any of the study drugs. DES did not significantly increase phosphorylation of Bad at serine 112. DES-induced phosphorylation of Bad at serine 112 was significantly reduced by PIM-Inh.II (Fig. 4B). Expression of Bcl-2 protein was not affected by any intervention (Fig. 5).

Discussion

This study investigated the role of Pim-1 kinase in the signalling cascade of ischaemic and DES-induced postconditioning in a murine in vivo model of acute myocardial infarction. Both 15-min administration of 1 MAC DES during early reperfusion and three cycles of each 10-s reperfusion/ischaemia immediately after prolonged myocardial ischaemia exerted remarkable cardioprotective effects, represented by a pronounced reduction in myocardial IS. These results are in line with findings from various other investigations regarding either APOST[5,11,29] or

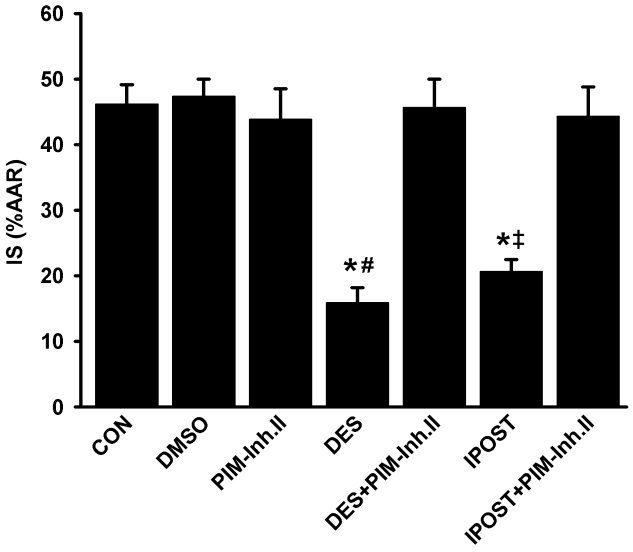

Fig. 2. Cardioprotection by desflurane (DES)-induced and ischaemic postconditioning (IPOST) is completely abolished by pharmacological blockade of Pim-1 kinase. Myocardial infarct size (IS) expressed as percentage of left ventricular area at risk (AAR). Values are mean ± standard error of the mean. n = *8 in each group. *significantly* (P < *0.05) different from control (CON) and Pim-1 kinase inhibitor II (PIM-Inh.II). #significantly* (P < *0.05) different from DES+PIM-Inh.II. ‡Significantly* (P < *0.05) different from IPOST+PIM-Inh.II. Mice received either no intervention (CON), dimethyl sulfoxide (DMSO), PIM-Inh.II), DES alone or in combination with PIM-Inh.II (DES+PIM-Inh.II), and IPOST either alone or in combination with PIM-Inh.II (IPOST+PIM-Inh.II).*

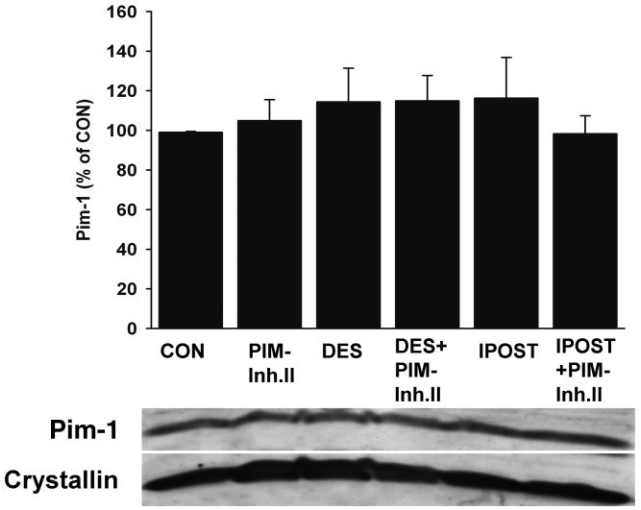

Fig. 3. Myocardial expression of Pim-1 kinase is not affected by DES-induced and IPOST. Western blot analysis of myocardial expression of Pim-1 kinase. Results are presented as representative original immunoblottings and average densitometric results as percentage of control (n = *4 per group). CON, control group; PIM-Inh.II, Pim-1 kinase inhibitor II (10 μg/g); DES, desflurane (1.0 minimum alveolar concentration); DES+PIM-Inh.II, PIM-Inh.II in combination with DES; IPOST, ischaemic postconditioning (three cycles of each 10-s ischaemia/reperfusion); IPOST+PIM-Inh.II, PIM-Inh.II in combination with IPOST.*

IPOST,[5–7,29] and provide further evidence that APOST and IPOST are promising strategies to alleviate cardiac ischaemia/reperfusion injury. In this study, DES and IPOST reduced myocardial IS to a similar extent. Our results confirm results from other investigations either in rabbits[29] or mice.[5,6]

A major finding of the present study is that blockade of Pim-1 kinase using the highly selective[25] PIM-Inh.II completely abolishes DES-induced postconditioning and IPOST against myocardial infarction. The dosage of 10 μg/g body weight has recently been demonstrated to inhibit Pim-1 kinase activity effectively in mice in vivo.[21] Our results demonstrate a possible role for the serine/threonine kinase Pim-1 in the signalling pathway of APOST and IPOST. Regarding DES, the present results are similar to the previous finding that blockade of Pim-1 kinase completely abolishes DES-induced preconditioning,[21] indicating a similar signal transduction pathway of DES-induced preconditioning and DES-induced postconditioning. In contrast with IPC that was only partially blocked by Pim-1 inhibition,[21] IPOST is completely abolished by Pim-1 blockade. This might imply differences in the exact role of Pim-1 kinase in the signalling cascades of IPC and IPOST. Further studies are required to reveal these possible differences.

Considerable evidence has accumulated that the serine/threonine kinase Pim-1 exerts relevant cardioprotective properties. Muraski et al. demonstrated a crucial role for Pim-1 in the regulation of cardiomyocyte survival downstream of Akt,[18] which itself is a well-known mediator of ischaemic[14] and APOST[10] as a part of the RISK pathway.[16] Transgenic animals with myocardial overexpression of Pim-1 kinase exhibit less myocardial ISs[18] and are protected from remodelling following transaortic constriction.[19] More recently, the same group demonstrated that female mice that receive cardiac progenitor cells overexpressing Pim-1 kinase show improved ventricular function and reduced myocardial IS.[20] Additionally, Pim-1 kinase increases expression of the sarco/endoplasmatic reticulum Ca^{2+}-adenosine triphosphatase,[18] which might be involved in the prevention of cardiomyocyte calcium overload during myocardial reperfusion. Furthermore, it has been reported that the highly sequence homologous and structurally similar

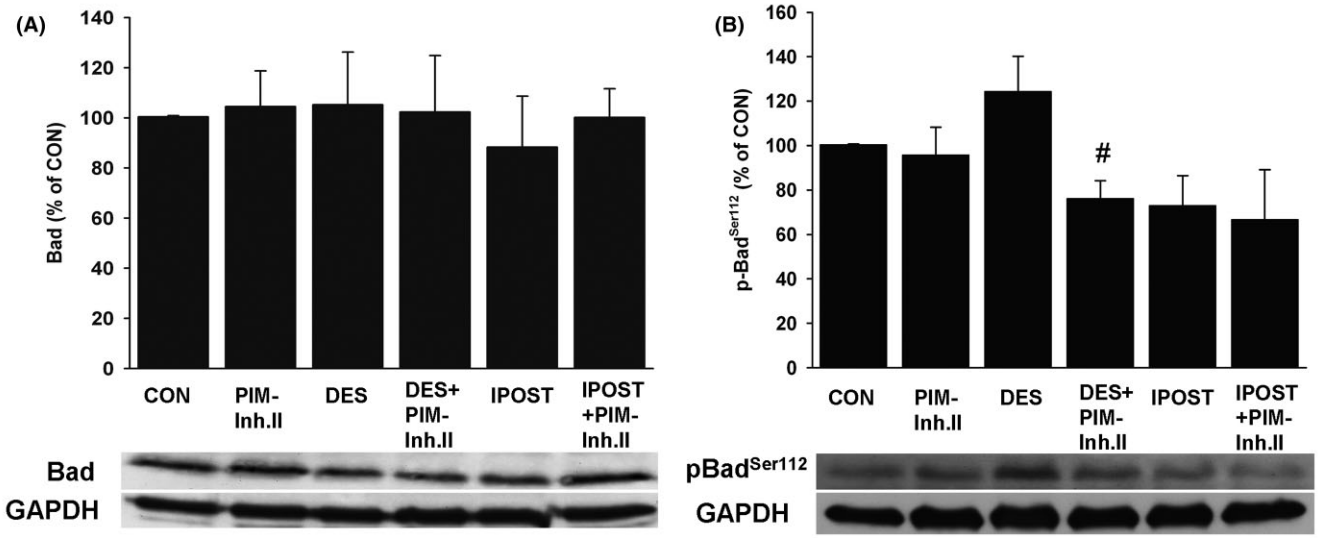

Fig. 4. DES-induced phosphorylation of Bad at serine 112 is reduced by blockade of Pim-1 kinase. Western blot analysis of myocardial expression of Bad (A) and phospho-Bad^{Ser112} (B). Results are presented as representative original immunoblottings and average densitometric results as percentage of control (n = 4 per group). #significantly (P < 0.05) different from DES. CON, control group; PIM-Inh.II, Pim-1 kinase inhibitor II (10 μg/g); DES, desflurane (1.0 minimum alveolar concentration); DES+PIM-Inh.II, PIM-Inh.II in combination with DES; IPOST, ischaemic postconditioning (three cycles of each 10-s ischaemia/reperfusion); IPOST+PIM-Inh.II, PIM-Inh.II in combination with IPOST.

Pim-3 kinase protects against cardiomyocyte apoptosis after anoxia/re-oxygenation in rat neonatal cardiac myocytes.[30] Thus, the results of the present study provide further evidence that Pim-1 kinase might be a promising target to alleviate the detrimental effects of cardiac ischaemia/reperfusion injury.

We did not observe differences in Pim-1 expression in whole cell lysates among groups that could have affected our results. These findings confirm results from our previous study.[21] A recent investigation in isolated rat hearts demonstrated a decreased Pim-1 expression in the cytosolic fraction after ischaemia/reperfusion alone.[31] This decrease was attenuated by IPOST, possibly indicating changes in the intracellular distribution of Pim-1 kinase induced by IPOST.[31] The differences between both studies might be, most probably, due to different cell fractions that were analysed (cytosolic fraction vs. whole cell lysate) or due to a different experimental model (isolated rat heart vs. in vivo mouse heart), a different I/R protocol (30-min global ischaemia/120-min reperfusion vs. 45-min LAD occlusion/60-min reperfusion) or a different postconditioning protocol (five cycles of each 10-s reperfusion/ischaemia vs. three cycles of each 10-s reperfusion/ischaemia). Thus, our study and

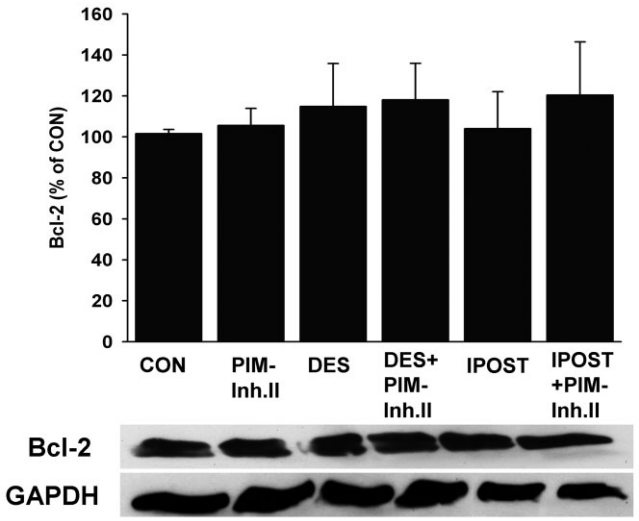

Fig. 5. Expression of B-cell lymphoma 2 (Bcl-2) is not affected by any intervention. Western blot analysis of myocardial expression of Bcl-2. Results are presented as representative original immunoblottings and average densitometric results as percentage of control (n = 4 per group). CON, control group; PIM-Inh.II, Pim-1 kinase inhibitor II (10 μg/g); DES, desflurane (1.0 minimum alveolar concentration); DES+PIM-Inh.II, PIM-Inh.II in combination with DES; IPOST, ischaemic postconditioning (three cycles of each 10-s ischaemia/reperfusion); IPOST+PIM-Inh.II, PIM-Inh.II in combination with IPOST.

Penna's study[31] are not directly comparable. More recently, Borillo et al. demonstrated an increase in mitochondrial Pim-1 levels after 30-min ischaemia and 30-min reperfusion, and a corresponding decrease in cytosolic Pim-1 levels after 30-min ischaemia and 10-min reperfusion.[32]

Pim-1 kinase is known to phosphorylate the proapoptotic downstream molecule Bad specifically on residue serine 112.[33] This phosphorylation in turn results in inactivation of Bad.[33] A direct role for Pim-1 in the prevention of cell death was suggested because inactive Bad enhances Bcl-2 activity, which promotes cell survival.[33] Transgenic overexpression of Pim-1 kinase in cardiomyocytes has been shown to prevent apoptosis.[18] In the present study, Pim-1 expression was not different among groups. Therefore, the observed differences in phospho-Bad^{Ser112} expression can be more likely attributed to different activity levels of Pim-1 kinase rather than different Pim-1 expression levels. Our results demonstrate that DES tends to increase phosphorylation of Bad at serine 112, although this increase did not reach statistical significance. These results are consistent with potential anti-apoptotic properties of the volatile agent. DES-induced phosphorylation of Bad was significantly reduced by pharmacological blockade of Pim-1 kinase. These results imply an involvement of Pim-1 kinase activation in the signalling cascade of DES-induced postconditioning. However, IPOST tends to decrease phosphorylation of Bad at serine 112, which was not affected by blockade of Pim-1 kinase, although this did not reach statistical significance again. PIM-Inh.II alone did not affect phosphorylation of Bad at serine 112. Thus, it is currently not clear whether phosphorylation of Bad at serine 112 by Pim-1 kinase is required for protection by IPOST. Further studies are needed to address this issue.

Bcl-2 protein is a key regulator of the apoptotic cascade and is activated, among others, by phospho-Bad^{Ser112}.[33] Activation of Bcl-2 prevents Cytochrome C release into the cytosol,[34] which is known to be an early event of the apoptotic cascade.[35] Overexpression of Bcl-2 in cardiac myocytes reduced ischaemia-induced necrosis as well as apoptosis.[36] Bcl-2 delays opening of the mitochondrial permeability transition pore (mPTP).[37] A crucial role for mPTP has been demonstrated for APOST[38,39] and IPOST.[40] Ischaemic and isoflurane-induced postconditioning are mediated by Bcl-2 in rabbits because pharmacological inhibition of Bcl-2 with HA14-1 abolished IS reduction by short cycles of reperfusion/ischaemia or isoflurane exposure during early reperfusion.[29] In another study, isoflurane enhanced Bcl-2 expression in neonatal rat cardiac myocytes.[41] IPC reduced apoptosis by an induction of Bcl-2 gene in isolated rat hearts.[42] Penna et al. reported a decrease of both cytosolic and mitochondrial Bcl-2 levels by ischaemia/reperfusion alone.[31] IPOST attenuated this decrease and induced a different redistribution of Bcl-2 between cytosolic and mitochondrial fraction.[31] Our data demonstrate no differences in Bcl-2 expression among groups. However, this is most probably due to our experimental setting, where myocardial tissue for Western immunoblotting was extracted after 1 h of reperfusion in contrast to 2 h in the study by Penna et al.[31] Taken together with previous results,[31,41] we suggest that this time, window is too short to detect possible differences in Bcl-2 expression.

The results of the current study should be interpreted within the constraint of several potential limitations. PIM-Inh.II has been demonstrated to be selective to Pim-1 kinase[25] with high selectivity over Pim-2 kinase. PIM-Inh.II was reported to have a half maximal inhibitory concentration (IC_{50}) for Pim-1 kinase of 50 nM, whereas the IC_{50} for Pim-2 kinase was described to be > 20 μM.[25] Nevertheless, potential effects of PIM-Inh.II on Pim-2 and Pim-3 kinase and other kinases involved in the signalling of either APOST or IPOST cannot be completely excluded. We did not measure Pim-1 activity directly. Phosphorylation of Bad at serine 112 was used as a surrogate for Pim-1 activity. Pim-1 kinase has been shown to specifically phosphorylate Bad on serine 112.[33] However, it cannot be excluded that other kinases involved in postconditioning cascades, particularly Akt, might phosphorylate Bad on the same residue. However, it has been described that Akt phosphorylates Bad predominantly at serine 136.[43]

Taken together, our results demonstrate that both DES-induced postconditioning and IPOST against myocardial infarction are mediated by the serine/threonine kinase Pim-1. DES-induced postconditioning might induce some anti-apoptotic effects, at least in part depending on Pim-1 activity. Thus, the present findings further support the concept that targeting anti-apoptotic proteins might be beneficial in the treatment of myocardial ischaemia/reperfusion injury.

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