Sevoflurane postconditioning reduces myocardial reperfusion injury in rat isolated hearts via activation of PI3K/Akt signaling and modulation of Bcl-2 family proteins[*]

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Abstract: Sevoflurane postconditioning reduces myocardial infarct size. The objective of this study was to examine the role of the phosphatidylinositol-3-kinase (PI3K)/Akt pathway in anesthetic postconditioning and to determine whether PI3K/Akt signaling modulates the expression of pro- and antiapoptotic proteins in sevoflurane postconditioning. Isolated and perfused rat hearts were prepared first, and then randomly assigned to the following groups: Sham-operation (Sham), ischemia/reperfusion (Con), sevoflurane postconditioning (SPC), Sham plus 100 nmol/L wortmannin (Sham+Wort), Con+Wort, SPC+Wort, and Con+dimethylsulphoxide (DMSO). Sevoflurane postconditioning was induced by administration of sevoflurane (2.5%, v/v) for 10 min from the onset of reperfusion. Left ventricular developed pressure (LVDP), left ventricular end-diastolic pressure (LVEDP), maximum increase in rate of LVDP ($+\mathrm{d}P/\mathrm{d}t$), maximum decrease in rate of LVDP ($-\mathrm{d}P/\mathrm{d}t$), heart rate (HR), and coronary flow (CF) were measured at baseline, R30 min (30 min of reperfusion), R60 min, R90 min, and R120 min. Creatine kinase (CK) and lactate dehydrogenase (LDH) were measured after 5 min and 10 min reperfusion. Infarct size was determined by triphenyltetrazolium chloride staining at the end of reperfusion. Total Akt and phosphorylated Akt (phospho-Akt), Bax, Bcl-2, Bad, and phospho-Bad were determined by Western blot analysis. Analysis of variance (ANOVA) and Student-Newman-Keuls' test were used to investigate the significance of differences between groups. The LVDP, $\pm\mathrm{d}P/\mathrm{d}t$, and CF were higher and LVEDP was lower in the SPC group than in the Con group at all points of reperfusion ($P<0.05$). The SPC group had significantly reduced CK and LDH release and decreased infarct size compared with the Con group [$(22.9\pm8)\%$ vs. $(42.4\pm9.4)\%$, respectively; $P<0.05$]. The SPC group also had increased the expression of phospho-Akt, Bcl-2, and phospho-Bad, and decreased the expression of Bax. Wortmannin abolished the cardioprotection of sevoflurane postconditioning. Sevoflurane postconditioning may protect the isolated rat heart. Activation of PI3K and modulation of the expression of pro- and antiapoptotic proteins may play an important role in sevoflurane-induced myocardial protection.

Key words: Sevoflurane, Postconditioning, Cardioprotection, Akt, Bcl-2, Bad
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1 Introduction

Using volatile anesthetics in early reperfusion to protect against myocardial reperfusion injuries was first proposed in the 1990s (Schlack *et al.*, 1998) and in vitro (Preckel *et al.*, 1998; 1999). In recent years the method has been called "anesthetic postconditioning

(APO)" (Chiari *et al*., 2005). Postconditioning is an effective therapeutic strategy for attaining myocardial protection against ischemia/reperfusion injuries, and from a clinical point of view, postconditioning is particularly promising to provide effective protection, as no previous information of the ischemic event is required.

Research has been focused on the cardioprotective mechanisms of pharmacological-induced postconditioning. However, the mechanisms underlying anesthetic postconditioning are complex and are not yet well understood. Previous research has mainly dealt with the following aspects: release of reactive oxygen species (ROS) (Tsutsumi *et al*., 2007), reduction of Ca^{2+} overload (Obal *et al*., 2005), attenuation of myocardial apoptosis (Venkatapuram *et al*., 2006), modulation of the phosphatidylinositol-3-kinase (PI3K)/Akt (Chiari *et al*., 2005) and its downstream component glycogen synthase kinase 3β (GSK3β) (Pagel *et al*., 2006), activation of extracellular signal-regulated kinase (ERK1/2) pathway (Krolikowski *et al*., 2006), opening of mitochondrial adenosine triphosphate (ATP)-sensitive potassium channels (mitoK$_{ATP}$), and inhibition of mitochondrial permeability transition pore (mPTP) opening (Krolikowski *et al*., 2005).

Due to its low tissue and blood/gas partition coefficient, sevoflurane, a new type of volatile anaesthetic, provides more controllable depth of anaesthesia and more rapid recovery than other volatile anaesthetics. Research has demonstrated that anesthetic postconditioning reduces ischemia/reperfusion myocardial infarction and attenuates cardiocyte apoptosis (Obal *et al*., 2001; 2005; Krolikowski *et al*., 2005; Weihrauch *et al*., 2005; Wang *et al*., 2006). Many genes have been reported to be linked to the regulation of apoptosis under physiological and pathological conditions. Among such genes, the Bcl-2 family has been suggested as a major one controlling the pathway to apoptotic cell death, especially the roles of antiapoptotic protein Bcl-2 and pro-apoptotic protein Bax. The antiapoptotic prosurvival kinase signaling cascade PI3K/Akt (protein kinase B) pathway has been demonstrated to provide protection against ischemia/reperfusion injuries (Fujio *et al*., 2000), and has also been proposed to play a central role in reducing apoptosis by modulating the Bcl-2 family (Takatani *et al*., 2004). Activation of the

PI3K/Akt pathway has been shown to be essential for the antiapoptotic effects of hypoxic preconditioning (Uchiyama *et al*., 2004; Hausenloy *et al*., 2005) and isoflurane preconditioning (Raphael *et al*., 2006) in the protection of cardiomyocytes. Previous studies have indicated that adoption of sevoflurane at the onset of reperfusion may protect against myocardial reperfusion injuries by activating the PI3K/Akt pathway (Li *et al*., 2008). However, it is not clear whether PI3K/Akt signaling is also essential for sevoflurane postconditioning-induced reduction of myocardial apoptosis. In this study, we have investigated the hypothesis that sevoflurane-induced postconditioning attenuates myocardial apoptosis in isolated rat hearts via the PI3K/Akt signaling and the modulation of Bcl-2 family proteins.

2 Materials and methods

All experimental procedures and protocols used in this investigation were reviewed and approved by the Animal Care and Use Committee at the School of Medicine of Zhejiang University, China, and they also conformed to the Guiding Principles in the Care and Use of Laboratory Animals published by the National Institutes of Health of the USA (NIH publication 85-23, revised in 1996).

2.1 Langendorff heart preparation

Male Sprague-Dawley rats (230–250 g) were heparinized (500 U i.p.) and anesthetized (pentobarbital sodium, 60 mg/kg i.p.). The hearts were immediately excised and perfused in a non-recirculation Langendorff apparatus with Krebs-Henseleit buffer (155 mmol/L Na^+, 5.6 mmol/L K^+, 138 mmol/L Cl^-, 2.1 mmol/L Ca^{2+}, 1.2 mmol/L PO_4^{3-}, 25 mmol/L HCO_3^-, 0.56 mmol/L Mg^{2+}, and 11 mmol/L glucose). The buffer (pH 7.4±0.5) was oxygenated with a mixture of 95% (v/v) O_2 and 5% (v/v) CO_2 [partial pressure of O_2 (PO$_2$): (381±8) mmHg; partial pressure of CO_2 (PCO$_2$): (19±2) mmHg], the temperature was maintained at (37±0.1) °C, and the perfusion pressure was kept at 80 mmHg throughout the experimental procedures. A small latex balloon connected to a pressure transducer (RM6240, Chengdu, China) was inserted into the left ventricle through the left atrium and the mitral valve. The balloon was filled with

saline to achieve a left ventricular end-diastolic pressure (LVEDP) of 4–6 mmHg and the balloon volume remained unchanged (Li *et al*., 2008). Heart temperature was maintained at 37 °C.

2.2 Experimental protocol

Each experiment lasted 190 min in total (Fig. 1), with 30 min equilibration, 40 min global ischemia, and then 120 min reperfusion in general. The prepared hearts were randomly assigned into the following seven groups (*n*=8 in each group): (1) Sham-operated group (Sham): 30 min equilibration and then direct perfusion with Krebs-Henseleit buffer for 160 min; (2) Control group (Con): global ischemia for 40 min followed by 120 min reperfusion; (3) Sevoflurane postconditioning group (SPC): administration of 2.5% (v/v) sevoflurane [1.0 minimum alveolar concentration (MAC) in rats at 37 °C] for 10 min right at the onset of reperfusion. The buffer solution was equilibrated with sevoflurane using a Sevotec 3 vaporizer (Datex-Ohmeda, Tewksbury, MA) with an air bubbler. Sevoflurane concentrations were measured in the buffer solution right before entering the aorta using a gas chromatograph [Perkin-Elmer, Norwalk, CT; (0.50±0.04) mmol/L]. All reservoirs were filled with buffer saturated with 1.0 MAC sevoflurane by adding sevoflurane to the perfusate 10 min before opening the three-way stopcock for reperfusion; (4) Sham+ wortmannin group (Sham+Wort): administration of

100 nmol/L wortmannin, a selective PI3K inhibitor dissolved in dimethylsulphoxide (DMSO), for 10 min prior to 110 min of perfusion; (5) Control+wortmannin group (Con+Wort): administration of 100 nmol/L wortmannin (Tsang *et al*., 2004) for 10 min at the onset of reperfusion; (6) Sevoflurane+wortmannin group (Sevo+Wort): administration of both 100 nmol/L wortmannin in DMSO and 2.5% (v/v) sevoflurane for 10 min at the onset of reperfusion; and (7) Control+ DMSO group (Con+DMSO): administration 0.02% (v/v) DMSO for 10 min at the onset of reperfusion. The sevoflurane was from Maruishi Pharmaceutical Co., Ltd., Osaka, Japan, and the wortmannin and DMSO were from Sigma-Aldrich, CA, USA.

2.3 Measurements of hemodynamics

The measured hemodynamic parameters were left ventricular developed pressure (LVDP), left ventricular end-diastolic pressure (LVEDP), maximum increase in the rate of LVDP ($+dP/dt$), maximum decrease in the rate of LVDP ($-dP/dt$), and heart rate (HR). Coronary flow (CF) was measured by timed collection of coronary effluent. These parameters were continuously measured throughout the experimental procedure.

2.4 Measurements of creatine kinase (CK) and lactate dehydrogenase (LDH) release

The severity of myocardial injuries can be indicated by concentrations of CK and LDH in the perfused buffer (He *et al*., 2008). To evaluate the myocardial injuries, 1 ml of effluent was first collected after 5 and 10 min reperfusion. The samples were analyzed by spectrophotometry to determine the CK and LDH (Cao *et al*., 2004). LDH activity was expressed as U/L (Li *et al*., 1999).

2.5 Determination of myocardial infarct size

After 120 min reperfusion, myocardial infarct size was determined by 2,3,5-triphenyltetrazolium chloride staining, as described by Feng *et al*. (2005). Hearts were frozen at −20 °C for 2 h and subsequently sliced as six 2-mm cross-sections across the long axis. The sections were incubated at 37 °C for 5 min with 1% (w/v) of 2,3,5-triphenyltetrazolium chloride in 0.1 mol/L phosphate buffer (pH 7.4), then fixed for 24 h in 10% (v/v) formaldehyde, and scanned. Tissues that were stained brick red were
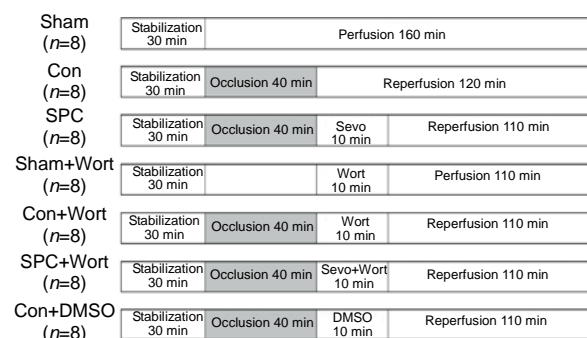
Fig. 1 Experimental protocol

Except the Sham and Sham+Wort groups, which were perfused constantly for 190 min, the remaining hearts were subjected to 40 min of global ischemia followed by 120 min of reperfusion. 1.0 MAC of sevoflurane was administered for 10 min at the onset of reperfusion. Wortmannin (100 nmol/L) was also administered for 10 min at the onset of reperfusion with sevoflurane absence or presence. Sevo: sevoflurane; Wort: wortmannin; DMSO: dimethylsulphoxide (vehicle of wortmannin)

taken as viable; tissues that appeared pale or white were deemed to be necrotic. Planimetric analysis was performed using the ImageJ 1.38 analysis software. The size of the infarcted myocardium was calculated as an area ratio (necrotic area/total area of the myocardium$\times$100%).

2.6 Western blot analysis

Seven similar experimental groups of rats (n=10 in each group) were subjected to the same experimental protocol. Left ventricular tissues were obtained and immediately frozen in liquid nitrogen (-70 °C) after 10 min (for total Akt and phosphorylated Akt (phospho-Akt) proteins, n=5) and 120 min (for Bcl-2 family proteins, n=5) reperfusion. Subsequently, the tissue samples were powdered by liquid nitrogen in mortars and homogenized in ice-cold lysis buffer (Beyotime Institute of Biotechnology, China) for 30 min. The lysis buffer contained 20 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 1% (v/v) Triton X-100, 2.5 mmol/L sodium pyrophosphate, β-glycerophosphate, 1 mmol/L Na_3VO_4, 1 mmol/L ethylenediaminetetraacetic acid (EDTA), leupeptin, 1 mmol/L phenylmethanesulfonyl fluoride (PMSF; Beyotime Institute of Biotechnology, China), and 1 mmol/L complete proteinase inhibitor cocktail (Sigma, St. Louis, MO, USA). The powdered samples were centrifuged at 13000$\times g$ for 15 min at 4 °C to remove cellular debris and to isolate total proteins. The BCA protein assay kit (Beyotime Institute of Biotechnology, China) was used to determine protein concentrations in the supernatants. The protein samples were mixed with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample-loading buffer and denatured at 100 °C for 5 min. Equivalent amounts (80 μg) of protein samples were loaded and separated on 12% (v/v) SDS-PAGE and then electrophoretically transferred to a polyvinylidene difluoride membrane (Millipore Co., Billerica, MA, USA). In addition, gels were stained with Coomassie blue to confirm that equal amounts of proteins had been loaded onto each lane. The membranes were blocked with 5% (w/v) nonfat dry milk in phosphate-buffered saline containing 0.1% (v/v) Tween 20 (PBST) for 1 h at room temperature, and subsequently incubated overnight at 4 °C with the following primary antibodies: rabbit monoclonal anti-Akt, phospho-Akt (at Ser[473]), Bcl-2; mouse

monoclonal anti-Bax, Bad, phospho-Bad (Ser[136]) (Cell Signaling Technology, Beverly, MA), 1:1000 dilution with 5% (w/v) bovine serum albumin in Tris-HCl buffered saline containing 0.1% (v/v) Tween 20 (TBST). The β-actin (1:2000 dilution; Protein Tech Group, Inc., Chicago, USA) was also detected as a loading control for protein quantity. After washing three times with PBST for 15 min, the membranes were subsequently incubated for 2 h in 5% (w/v) bovine serum albumin in TBST containing the appropriate secondary antibody of either a goat anti-rabbit or goat anti-mouse immunoglobulin G (IgG) conjugated to horseradish peroxidase (Beyotime Institute of Biotechnology, China) at 1:1000 dilution. Immune complexes were detected using the infrared imaging system (Odyssey, USA). Scanned images were imported into quantity one analyzer (Bio-Rad Laboratories, CA). Scanning densitometry was used for semi-quantitative analysis.

2.7 Statistical analysis

All data are presented as mean±standard error of the mean (SEM). The statistical analysis was performed using SPSS 16.0 (Chicago, USA). The analysis of variance (ANOVA) was used and followed by post-hoc Tukey test analysis for multiple comparisons to investigate the significance of differences between groups. The differences were considered significant when P<0.05.

3 Results

3.1 Hemodynamic parameters

The measured hemodynamic data are summarized in Table 1. Baseline hemodynamics among all experimental groups were similar (P>0.05), and there were no differences during the experiment in the Sham and Sham+Wort groups. After 40 min global ischemia, the LVDP, $\pm$dP/dt, and CF were higher and the LVEDP was lower in the SPC group than in the Con group throughout reperfusion (P<0.05), and though the HR of the SPC group was higher than that of the Con group, this did not reach statistical significance (P>0.05). The recovery of myocardial function due to sevoflurane postconditioning was completely abolished by wortmannin, which did not influence postischemic recovery of myocardial

Table 1 Hemodynamic parameters of all experimental groups

Group	LVEDP (mmHg)	LVDP (mmHg)	+dP/dt (mmHg/s)	−dP/dt (mmHg/s)	HR (beat/min)	CF (ml/min)
Baseline						
Sham	5±1	97±4	3374±137	2077±85	320±18	8.3±0.3
Con	5±1	98±6	3164±163	1964±108	292±16	7.9±0.4
SPC	5±1	91±5	3196±188	2010±137	305±16	8.1±0.5
Sham+Wort	5±1	98±3	3414±139	2185±116	309±19	8.1±0.3
Con+Wort	5±1	89±6	2931±247	1916±74	319±21	8.4±0.3
SPC+Wort	5±1	87±6	2930±290	1985±140	299±22	7.8±0.3
Con+DMSO	5±1	98±4	2929±181	1902±94	291±18	8.2±0.5
R30 min						
Sham	9±1	87±7	2677±156	1428±92	292±21	7.8±0.2
Con	50±3^	38±4^	1034±170^	673±92^	251±14	5.2±0.4^
SPC	31±1^*	62±4^*	2242±182*	1082±50*	273±13	7.4±0.4*
Sham+Wort	15±2*#	70±4*	2558±127*	1327±76*	289±14	7.6±0.2*
Con+Wort	52±3^#Δ	32±6^#Δ	982±187^#Δ	660±108^#Δ	253±15	5.2±0.3^#Δ
SPC+Wort	50±3^#Δ	34±5^#Δ	1067±202^#Δ	672±50^#Δ	251±14	5.0±0.2^#Δ
Con+DMSO	49±3^#Δ	32±6^#Δ	890±182^#Δ	593±97^#Δ	251±18	5.1±0.3^#Δ
R60 min						
Sham	11±2	84±7	2365±162	1367±139	280±21	6.8±0.3
Con	46±2^	37±4^	1248±137^	691±57^	242±13	4.6±0.4^
SPC	26±1^*	63±5*	2064±127*	1095±43*	268±16	6.7±0.4*
Sham+Wort	15±2*#	65±4*	2309±116*	1152±68*	276±13	6.8±0.2*
Con+Wort	44±3^#Δ	38±5^#Δ	1214±158^#Δ	676±73^#Δ	244±13	4.5±0.3^#Δ
SPC+Wort	43±4^#Δ	40±5^#Δ	1243±156^#Δ	765±47^#Δ	243±15	4.6±0.2^#Δ
Con+DMSO	42±2^#Δ	38±4^#Δ	1248±193^#Δ	706±57^#Δ	242±12	4.5±0.3^#Δ
R90 min						
Sham	13±2	78±6	2158±150	1191±103	278±21	6.2±0.2
Con	42±3^	36±2^	1177±112^	655±49^	238±17	4.0±0.4^
SPC	25±1^*	59±5*	1949±112*	1009±45*	264±16	6.0±0.3*
Sham+Wort	15±2*#	61±3*	2052±120*	1054±57*	269±11	6.1±0.2*
Con+Wort	41±3^#Δ	33±5^#Δ	1142±146^#Δ	631±66^#Δ	240±7	4.0±0.2^#Δ
SPC+Wort	40±3^#Δ	36±5^#Δ	1145±155^#Δ	685±47^#Δ	239±13	4.1±0.2^#Δ
Con+DMSO	39±1^#Δ	39±5^#Δ	1170±164^#Δ	674±57^#Δ	239±10	4.1±0.3^#Δ
R120 min						
Sham	14±2	72±5	1948±142	1109±117	269±19	5.5±0.2
Con	41±2^	33±3^	1094±118^	565±52^	240±17	3.4±0.4^
SPC	25±1^*	56±5*	1839±115*	958±49*	265±16	5.1±0.2*
Sham+Wort	14±1*#	59±3*	1868±119*	943±53*	266±9	5.5±0.3*
Con+Wort	39±3^#Δ	30±5^#Δ	1039±141^#Δ	586±75^#Δ	235±5	3.5±0.2^#Δ
SPC+Wort	38±2^#Δ	33±5^#Δ	1198±167^#Δ	602±37^#Δ	232±14	3.4±0.3^#Δ
Con+DMSO	36±1^#Δ	36±4^#Δ	1153±196^#Δ	628±57^#Δ	233±9	3.4±0.3^#Δ

Data are presented as mean±SEM, n=8 in each group. ^ P<0.05 compared with the Sham group; * P<0.05 compared with the Con group; # P<0.05 compared with the SPC group; Δ P<0.05 compared with the Sham+Wort group. Sham: Sham-operation group; Con: ischemia/ reperfusion group; SPC: sevoflurane postconditioning group; Sham+Wort: Sham+wortmannin group; Con+Wort: Con+wortmannin group; SPC+Wort: SPC+wortmannin group; Con+DMSO: Con+dimethylsulphoxide group. LVDP: left ventricular developed pressure; LVEDP: left ventricular end-diastolic pressure; +dP/dt: maximum increase in rate of LVDP; −dP/dt: maximum decrease in rate of LVDP; HR: heart rate; CF: coronary flow

function when given alone ($P>0.05$). DMSO had no effect on cardiac variables ($P>0.05$).

3.2 Effects on CK and LDH release and myocardial infarct size

The measurements of CK and LDH release and the myocardial infarct size were shown in Figs. 2 and 3, respectively. After 120 min of reperfusion, the SPC group significantly reduced the infarct size compared with the Con group [(22.9±8)% vs. (42.4±9.4)%, $P<0.05$]. The PI3K inhibitor alone did not influence infarct size [(43.8±3.1)% in the Con+Wort group), but eliminated the cardioprotection produced by sevoflurane [(42.3±4)%, $P<0.05$ compared with the SPC group). No myocardial infarctions were measured in

the Sham and Sham+Wort groups. The reduction of CK and LDH release was consistent with the decrease of the myocardial infarct size.

3.3 Effects on expression of total Akt and phospho-Akt

As shown in Fig. 4, the expression of total Akt and its activated, phosphorylated form (phospho-Akt) at Ser^{473} was measured by Western blot after 10 min reperfusion. There were no differences in total Akt expression observed among groups, whereas the phospho-Akt expression was significantly increased in the SPC group compared with the Con group. Wortmannin inhibited the increased expression of phospho-Akt in the SPC group.
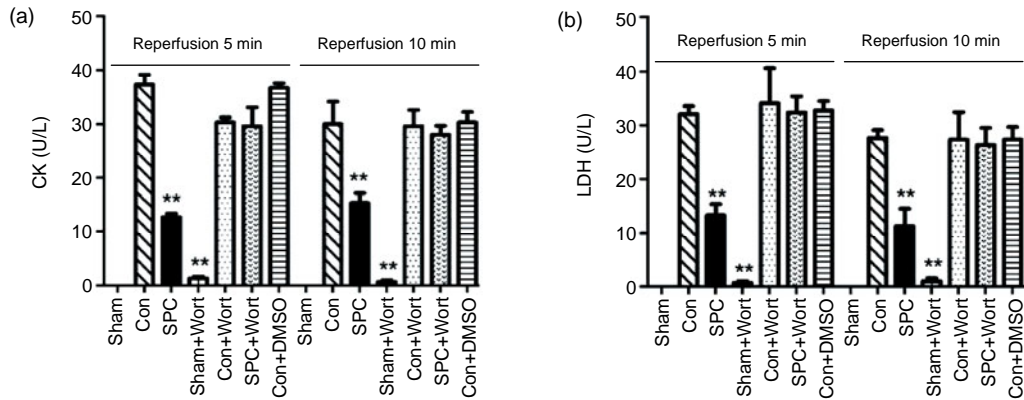

Fig. 2 Measurements of CK (a) and LDH (b) release during reperfusion 5 and 10 min in each experimental group
Data are presented as mean±SEM. $^{**}P<0.01$ compared with the Con group
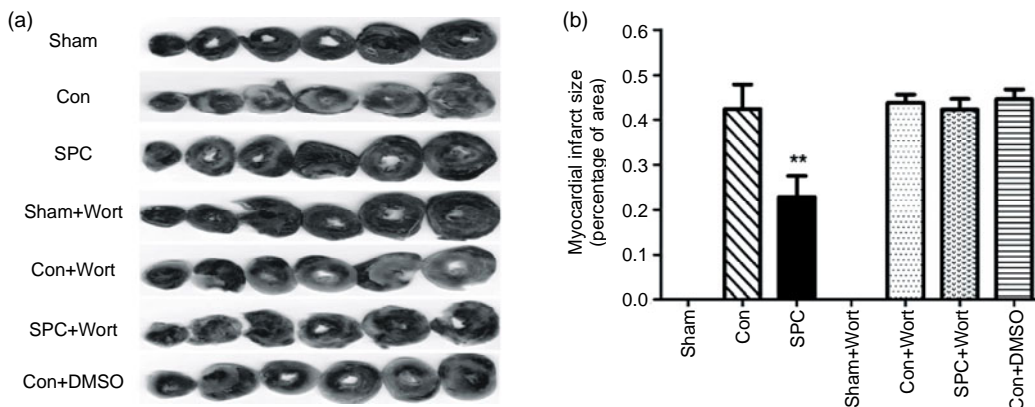

Fig. 3 Myocardial infarct size in each experimental group
(a) Representative photographs of heart slices with 1% (w/v) triphenyltetrazolium chloride staining in each group; (b) Average infarct size in six hearts for each group. The size of the infarcted myocardium was calculated as an area ratio (necrotic area/total area of the myocardium×100%). $^{**}P<0.01$ compared with the Con group
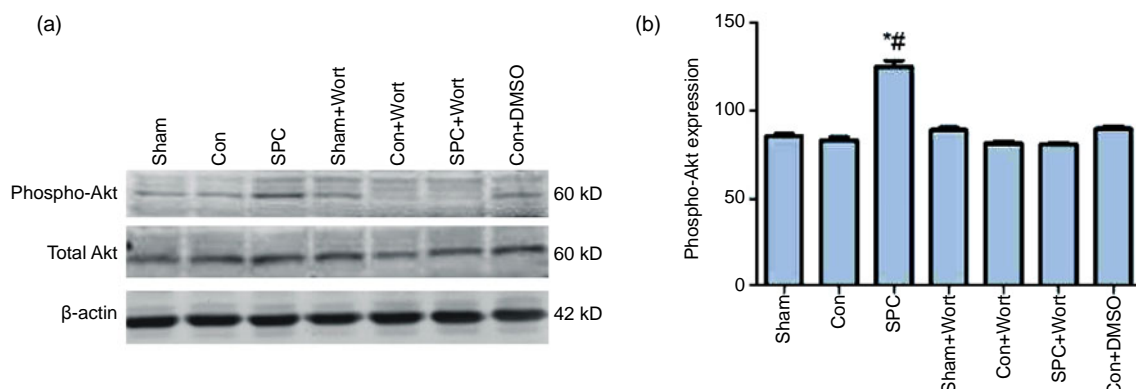
Fig. 4 Western blot analysis of total Akt and phospho-Akt at Ser^{473}
(a) Western blotting analysis of phospho-Akt at Ser^{473} and total Akt. β-actin was used to demonstrate equal protein loading;
(b) Graphic presentation of phospho-Akt normalized against total Akt expression. Values in the graphs are presented as mean±SEM. $^{*}P<0.05$ compared with the Con group; $^{#}P<0.05$ compared with the SPC+Wort group

3.4 Effects on expression of Bcl-2 and Bax proteins

The expression of Bcl-2 and Bax proteins extracted by Western blot analysis from left ventricular necrotic myocardia is shown in Fig. 5. The expression of the antiapoptotic protein Bcl-2 was significantly decreased and that of the pro-apoptotic protein Bax was significantly increased in the Con group compared with the Sham group. The SPC group significantly increased the expression of Bcl-2 and decreased the expression of Bax ($P<0.05$ compared with the Con group) to a level similar to that of the Sham group. Wortmannin counteracted the effects of up-regulation of the Bcl-2 and the down-regulation of the Bax due to the sevoflurane postconditioning. It also decreased the Bcl-2 expression and increased the Bax expression to a degree similar to that of the Con group.

3.5 Effects on expression of total Bad and phospho-Bad

No significant difference among all the groups was found in the expression of total Bad. However, the phospho-Bad expression at Ser^{136} was significantly decreased in the Con group compared with the SPC group ($P<0.05$). Wortmannin abolished the effect of sevoflurane postconditioning on the phospho-Bad and caused the phospho-Bad expression to decrease to a level similar to that of the Con group (Fig. 6).
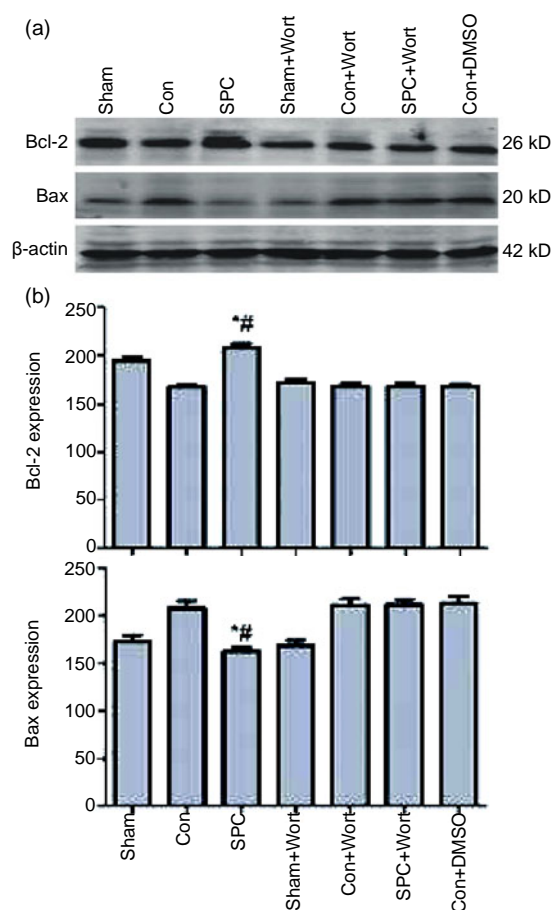

Fig. 5 Western blot analysis of Bcl-2 and Bax
(a) Western blotting analysis of Bcl-2 and Bax. β-actin was used to demonstrate equal protein loading; (b) Graphic presentations of Bcl-2 and Bax expression. Values are expressed as mean±SEM. $^{*}P<0.05$ compared with the Con group; $^{#}P<0.05$ compared with the SPC+Wort group
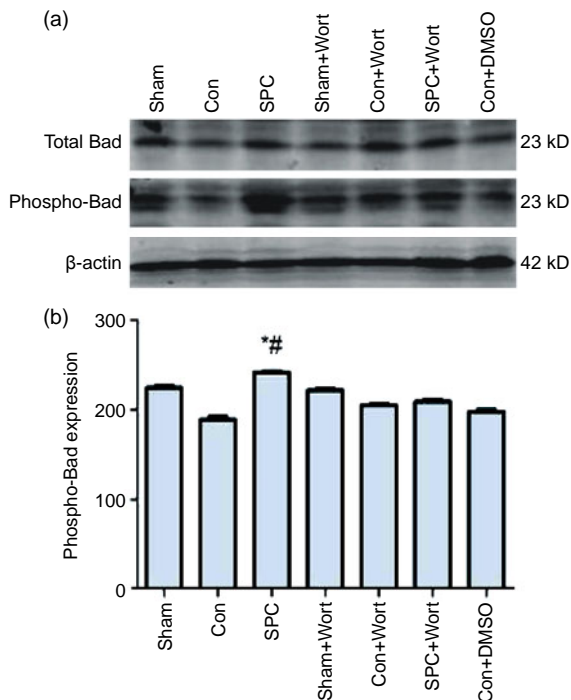
(a)

Fig. 6 Western blot analysis of total Bad and phospho-Bad
(a) Western blotting analysis of total Bad and phospho-Bad at Ser[136]. β-actin was used to demonstrate equal protein loading; (b) Graphic presentation of phospho-Bad normalized against total Bad expression. Values are expressed as mean±SEM. [*] $P<0.05$ compared with the Con group; [#] $P<0.05$ compared with the SPC+Wort group

4 Discussion

The current study has shown several salient findings in isolated rat hearts. Firstly, sevoflurane postconditioning improves the recovery of myocardial function, attenuates myocardial apoptosis, and reduces infarct size after ischemia/reperfusion injuries. Secondly, the activation of the PI3K/Akt pro-survival pathway plays an important role on cardioprotection contributed by the sevoflurane postconditioning. Thirdly, the cardiocyte apoptosis inhibited by sevoflurane postconditioning may influence the modulation of the expression of pro- and antiapoptotic proteins, specifically Bcl-2, Bax, and phospho-Bad. The PI3K/Akt signaling pathway may participate in the modulation of the expression of pro- and antiapoptotic proteins in sevoflurane postconditioning.

The purpose of using the isolated rat heart model in the current study is to exclude the effects of neuroendocrine system on the hearts. Studies have re-

ported that infarct size did not decrease when administrating 0.5 MAC volatile anaesthetic at the onset of reperfusion and the cardioprotective effects were greater when administrating 1.0 MAC volatile anaesthetic after ischemia (Obal *et al.*, 2001; Krolikowski *et al.*, 2005; Weihrauch *et al.*, 2005; Wang *et al.*, 2006; Deyhimy *et al.*, 2007).

The pathology associated with myocardial ischemia and reperfusion is cardiocyte necrosis and apoptosis. Apoptosis is also called programmed cell death. Its chief morphologic features include cell shrinkage, nuclear chromatin condensation, DNA fragmentation, formation of cytoplasmic blebs, and apoptotic bodies, no loss of membrane integrity and no inflammatory response (Bartling *et al.*, 1998; Freude *et al.*, 1998; Haunstetter and Izumo, 1998; van Heerde *et al.*, 2000). Apoptosis mainly appears in myocardia subjected to a brief period of ischemia followed by reperfusion, and it does not appear in ischemic tissues without reperfusion (Freude *et al.*, 2000; Zhao *et al.*, 2000). Research has demonstrated that anesthetic postconditioning reduces ischemia/reperfusion myocardial infarction and attenuates cardiocyte apoptosis (Obal *et al.*, 2001; 2005; Krolikowski *et al.*, 2005; Weihrauch *et al.*, 2005; Wang *et al.*, 2006).

Mechanisms that the Bcl-2 may adopt to regulate the antiapoptotic pathway have been proposed as: direct antioxidant effect, inhibition of release of pro-apoptotic proteins, and attenuation of cytotoxic effect of pro-apoptotic regulators such as Bax (Freude *et al.*, 1998; Haunstetter and Izumo, 1998). Misao *et al.* (1996) showed that increased Bcl-2 expression occurs in salvaged myocytes in acute infarction, while Bax was expressed only in the infarcted area in matured myocardial infarction, which suggests that Bcl-2 may play an important role in salvage of ischemic tissue. Kirshenbaum and de Moissac (1997) demonstrated that the Bcl-2 was expressed in isolated rat myocytes to prevent apoptosis. Wang *et al.* (2006) reported that inhibition of the Bcl-2 activity decreased the protective effects against myocardial infarction produced by isoflurane postconditioning that occurred as a result of indirect modulation of mitochondrial permeability transition pore (mPTP) by Bcl-2 in vivo, which suggests that the Bcl-2 family proteins regulate apoptosis by modulating mitochondrial permeability.

Ischemia/reperfusion decreases the expression of Bcl-2 and increases the expression of Bax in the ischemic/reperfused myocardium (Nakamura *et al.*, 2000; Zhao *et al.*, 2000). The findings of our study are consistent with the conclusions that the expression of Bcl-2 and Bax regulates the apoptotic cell death, and that the ischemic/reperfusion significantly decreases the expression of Bcl-2 and increases the expression of Bax in the left ventricular tissues of the isolated rat hearts. Sevoflurane postconditioning prevented these changes, and inhibited the decreased expression of Bcl-2 and the increased expression of Bax to a level similar to that of the Sham group. The protection accordingly led to an increased Bcl-2/Bax ratio, which has been suggested to determine the survival or death after an apoptotic stimulus. PI3K inhibitor wortmannin counteracted the effect of sevoflurane on the expression of Bcl-2 and Bax, suggesting that the reduction of myocardial apoptosis by sevoflurane postconditioning was attenuated, at least partially, by activation of PI3K/Akt signaling pathway.

Bad, another pro-apoptotic protein, is also a cell survival target of Akt. This protein can be sequestered in the cytosol if being maintained in a phosphorylated state on either of the two serine residues (Ser^{112} and Ser^{136}) embedded in the 14-3-3 consensus binding sites (Zha *et al.*, 1996). Activation of Bad by dephosphorylation results in translocation of Bad to the mitochondria with subsequent heterodimerization with Bcl-xl or Bcl-2 to promote cell death (Zha *et al.*, 1996; 1997; Adams and Cory, 1998). The translocation is inhibited by phosphorylation of Bad, leading to its cytosolic sequestration. Thus, phosphorylation of Bad may promote cell survival (Steenbergen *et al.*, 2003). Previous data have demonstrated that Akt is capable of phosphorylating Ser^{136} and thereby inactivating Bad (Datta *et al.*, 1997). Our study showed that the expression of total Bad among the various experimental groups was similar. However, the ischemia/reperfusion reduced the expression of phospho-Bad. The administration of the sevoflurane at reperfusion did maintain Bad in its nonactivated form (phospho-Bad), but prevented the decrease in Bad phosphorylation. Wortmannin abolished the effect of sevoflurane, and decreased the expression of the phospho-Bad.

Akt activation at the time of reperfusion confers cardioprotection against reperfusion-induced injuries by reducing apoptosis (Fujio *et al.*, 2000; Matsui *et al.*, 2001). The PI3K/Akt pathway participates in numerous cellular processes by phosphorylating a diverse array of substrates: PI3K converts phosphatidylinositol-4,5-bisphosphate to phosphatidylinositol-3,4,5-trisphosphate (Hausenloy and Yellon, 2004), and then phosphorylation of the serine/threonine kinase Akt by phosphoinositide-dependent kinase 1 inhibits the formation of the pro-apoptotic proteins Bad and Bax (Cantley, 2002), and, in the meantime, maintains the level of the antiapoptotic protein Bcl-2 (Uchiyama *et al.*, 2004). The results of the current study are consistent with that of Li *et al.* (2008). Further, this study has demonstrated that sevoflurane postconditioning may inhibit myocardial apoptosis, at least partially, through activating the PI3K/Akt pathway and modulating the expression of the pro- and antiapoptotic proteins.

The process of ischemia/reperfusion has been shown to activate the antiapoptotic pro-survival kinase signaling cascades: PI3K/Akt and ERK1/2, both of which are related to cellular survival and play a pivotal role in reperfusion injury salvage kinase cascade (RISK) pathway. Activating the RISK pathway at the time of reperfusion has been demonstrated to provide protection against reperfusion-induced injuries. It has been reported that ERK1/2 is involved in targeting the apoptotic components of reperfusion-induced cell death through modulation of pro- and antiapoptotic protein levels (Hausenloy and Yellon, 2004). Inamura *et al.* (2010) reported that sevoflurane postconditioning prevents the activation of caspase-3 and -9 via phosphorylation of Akt and ERK, mediators of apoptosis in ischemia/reperfusion injury. The mitogen-activated protein kinase (MAPK)/ extracellular signal-regulated kinase 1/2 (ERK1/2)/ p70S6K signaling pathway participates in the cardioprotection of anesthetic postconditioning on myocardial infarction during early reperfusion (Chen *et al.*, 2008; Yao *et al.*, 2010). However, whether ERK1/2 participates in modulating the expression of Bcl-2 family proteins is not clear. Therefore, further studies are necessary to unveil the relationship between ERK1/2 and anesthetic postconditioning-induced inhibition of cardiocyte apoptosis, and to identify other signaling elements involved in anesthetic postconditioning and the relationships among them.

The limitations of this study are as follows: Firstly, we used an isolated heart model. Secondly, we did not determine the area at risk. We determined the infarct size by the radio of necrotic area to total area of the myocardium as described previously (Feng *et al.*, 2005). Determining the necrotic area by the area at risk can be more accurate. Thirdly, we did not confirm direct morphological features of apoptotic cells.

In summary, this investigation has shown that sevoflurane postconditioning attenuates myocardial apoptosis and reduces infarct size, and the effects of sevoflurane postconditioning may be modulated by activation of the PI3K/Akt signaling pathway and modulation of the expression of pro- and antiapoptotic proteins.

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