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Role of nuclear factor-κB in volatile anaesthetic preconditioning with sevoflurane during myocardial ischaemia/reperfusion

Chen Wang, Hong Xie, Xia Liu, Qin Qin, Xuemei Wu, Hong Liu and Chunfeng Liu

Background and objective Anaesthetic preconditioning (APC) protects against myocardial ischaemia/reperfusion injury. Nuclear factor-κB (NF-κB) has been implicated in APC-induced myocardial protection *in vitro*. Our study tested the hypothesis that in-vivo APC with sevoflurane is triggered by NF-κB through downregulation of inflammatory mediators and upregulation of antiapoptosis factors to prevent myocardial injury during ischaemia/reperfusion.

Methods In this in-vivo study, rats were anaesthetized and maintained with sodium pentobarbital throughout the experiment. Rats were exposed to 30 min of 2.5% sevoflurane followed by 15 min washout (APC group) or no inhalation anaesthetics (ischaemia/reperfusion group) before ischaemia/reperfusion. In the sevoflurane group, rats were exposed to 30 min sevoflurane followed by a 165 min washout period. The NF-κB inhibitor parthenolide (PTN) was used before or after exposure to sevoflurane (PTN+APC group and APC+PTN group). Left ventricular samples were obtained to measure infarct size, pro-inflammation and apoptosis. A P value less than 0.05 was considered significant.

Results APC reduced infarct size ($34 \pm 6\%$) compared with ischaemia/reperfusion ($53 \pm 6\%$, $P<0.05$). PTN administered before or after APC abolished the cardioprotection (53 ± 5 and $52 \pm 7\%$, respectively, $P < 0.05$). APC decreased the myocardium apoptosis compared with the ischaemia/reperfusion only group (6 ± 1 vs.$19 \pm 3\%$, $P < 0.05$); PTN administered before or after sevoflurane preconditioning abolished this effect. APC induced upregulation of NF-κB p50/p65 before ischaemia ($51 \pm 4/26 \pm 3\%$ vs. $15 \pm 1/11 \pm 1\%$ in the control group, $P < 0.05$). After reperfusion, NF-κB was upregulated in the ischaemia/reperfusion and APC groups, but it was lower in the APC group than in the ischaemia/reperfusion group. PTN administered before and after APC inhibited the expressions. Before ischaemia, Bcl-2 was increased in the APC and sevoflurane groups (94 ± 3 and $102 \pm 4\%$, respectively) compared with the control group ($68 \pm 2\%$, $P < 0.05$). After reperfusion, intercellular adhesion molecule-1, tumour necrosis factor-α and caspase-3 expressions were significantly increased in the ischaemia/reperfusion group (92 ± 5, 115 ± 4 and $65 \pm 2\%$ compared with the control group, $P < 0.05$); these increases were blunted in the APC group.

Conclusion APC with sevoflurane produced myocardial protection against ischaemia/reperfusion *in vivo*. NF-κB acted not only as a trigger but also as a mediator that played an important role in APC through upregulation of NF-κB and the antiapoptosis protein Bcl-2 during the preconditioning period and then through downregulation of the inflammatory proteins intercellular adhesion molecule-1 and tumour necrosis factor-α during reperfusion, ultimately decreasing caspase-3 expression and apoptosis.

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Introduction

Volatile anaesthetic preconditioning (APC) has been shown to be cardioprotective against ischaemia/reperfusion in experimental animal models and humans with coronary artery disease.[1,2] Our previous study suggested that extracellular signal-related kinase 1 and 2 (Erk1/2) played an important role in isoflurane preconditioning.[3] Accumulating evidence suggests that apoptosis, the production of reactive oxygen species (ROS), calcium overload and the inflammatory reaction process contribute to myocardial cell death during ischaemia/reperfusion.[4,5] The inhibition of myocardium apoptosis is thought to be beneficial for myocardial protection during ischaemia/reperfusion.[6–8] Others, and our previous in-vitro studies, have reported that APC protects the myocardium against ischaemia/reperfusion injury by attenuating the activation of nuclear factor-kappa B (NF-κB) and subsequently suppressing the expression of NF-κB-regulated inflammatory genes during reperfusion.[3,9–11]

NF-κB is a transcription factor that regulates the expression of a large number of genes. Many are critical to apoptosis and inflammation, such as cytokines, chemokines and adhesion molecules in ischaemia/reperfusion injury.[12–14] NF-κB, composed of two subunits (p50 and p65), will dissociate from its inhibitors, such as IκB-α, and translocate into the nucleus. Various stimuli such as ROS

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and interleukin-1 (IL-1) can activate IκB kinase and cause phosphorylation of its inhibitors, which is followed by its ubiquitination and subsequent degradation. Then the two subunits will translocate into the nucleus and further bind to consensus sites in promoter or enhancer regions of the target genes to initiate transcription.[9,15] However, whether the two subunits have different roles is still uncertain. Our previous study demonstrated that in-vitro APC with sevoflurane attenuated NF-κB p50/p65 activation and reduced inflammatory gene expression.[9] APC also reduced infarct size and creatine kinase release, and improved myocardial left ventricular developed pressure during ischaemia/reperfusion *in vitro*. There are data showing that the APC with sevoflurane protects against myocardial ischaemia/reperfusion injury *in vivo*.[16,17] However, few data are available on the relation between NF-κB and antiapoptosis in APC. In this study, we tested the hypothesis that in-vivo APC with sevoflurane has myocardial protective effects, and the two subunits of NF-κB (p50/p65) play important roles in this mechanism. APC with sevoflurane suppresses the expression of inflammatory mediators and increases the upregulation of antiapoptosis factors.

Methods

All animals were cared for according to the *Guide for the Care and Use of Laboratory Animals* (NIH Publication vol. 25 no. 28, revised 1996) and policies of Soochow University. The Committee for Experimental Animals of the Medical College of Soochow University approved the experimental procedures and protocols used in this investigation.

Surgical instrumentation

In this in-vivo study, male Sprague–Dawley rats (270–350 g) were anaesthetized with intraperitoneal (i.p.) injection of sodium pentobarbital (50 mg kg^{-1}) and received additional sodium pentobarbital (15–25 mg kg^{-1}) to ensure that pedal and palpebral reflexes were absent throughout the experiment. Heparin-filled catheters were inserted into the right jugular vein and the right carotid artery for fluid or drug administration and arterial blood pressure measurement. The rats were intubated via tracheostomy and ventilated with a rodent ventilator (Shanghai Alcott Biotech Co., Shanghai, China) with 33% oxygen. The heart was exposed via a left thoracotomy at the left fifth intercostal space and was suspended in a pericardial cradle. A 6–0 prolene ligature was placed around the proximal left descending coronary artery (LAD) in the area immediately below the left atrial appendage. The ends of the suture were threaded through a propylene tube to form a snare. Coronary artery occlusion was produced by clamping the snare onto the epicardial surface of the heart and was confirmed by the appearance of epicardial cyanosis. Reperfusion was achieved by loosening the snare and was verified by observing an epicardial hyperaemic response.[3] Haemodynamic data were continuously recorded on a polygraph with Medlab-U/4C501H (Nanjing Mei Yi Technology, Nanjing, China). The rate–pressure product (systolic blood pressure × heart rate (HR)/100, RPP) was used as a major determinant of myocardial oxygen consumption.

Experimental protocol

The experimental design is illustrated in Fig. 1. Rats were randomly divided into six groups ($n = 8$ in each group) for the determination of infarct size (Fig. 1a). Thirty-minute baseline data were collected before any treatment. In the ischaemia/reperfusion group, the hearts were subjected to 30 min of coronary artery occlusion followed by 120 min of reperfusion. In the APC group, rats were subjected to 30 min of 2.5% sevoflurane (Abbott Laboratories, Shanghai, China) inhalation followed by a 15 min washout (memory period) and followed by 30 min ischaemia and 120 min reperfusion. This concentration is approximately 0.9 minimum alveolar concentration (MAC) for young adult rats.[10] In the parthenolide+APC (PTN+APC) group, the NF-κB inhibitor sesquiterpene lactone PTN (Sigma-Aldrich Inc., Saint Louis, USA) 500 μg kg^{-1} in dimethylsulfoxide (DMSO) was administered i.p. 5 min before exposure to sevoflurane as described in the APC group.[18,19] This was designed to test whether NF-κB triggers the inflammatory mediators and antiapoptosis factors. In the APC+PTN group, PTN 500 μg kg^{-1} was administered i.p. 5 min after discontinuation of the sevoflurane pretreatment, as described in the APC group, to determine NF-κB as a mediator. In the PTN group, the rats received only PTN 500 μg kg^{-1} i.p. at the end of the 30 min baseline period. In the DMSO group, the rats received DMSO only i.p. at the end the of the 30 min baseline period.

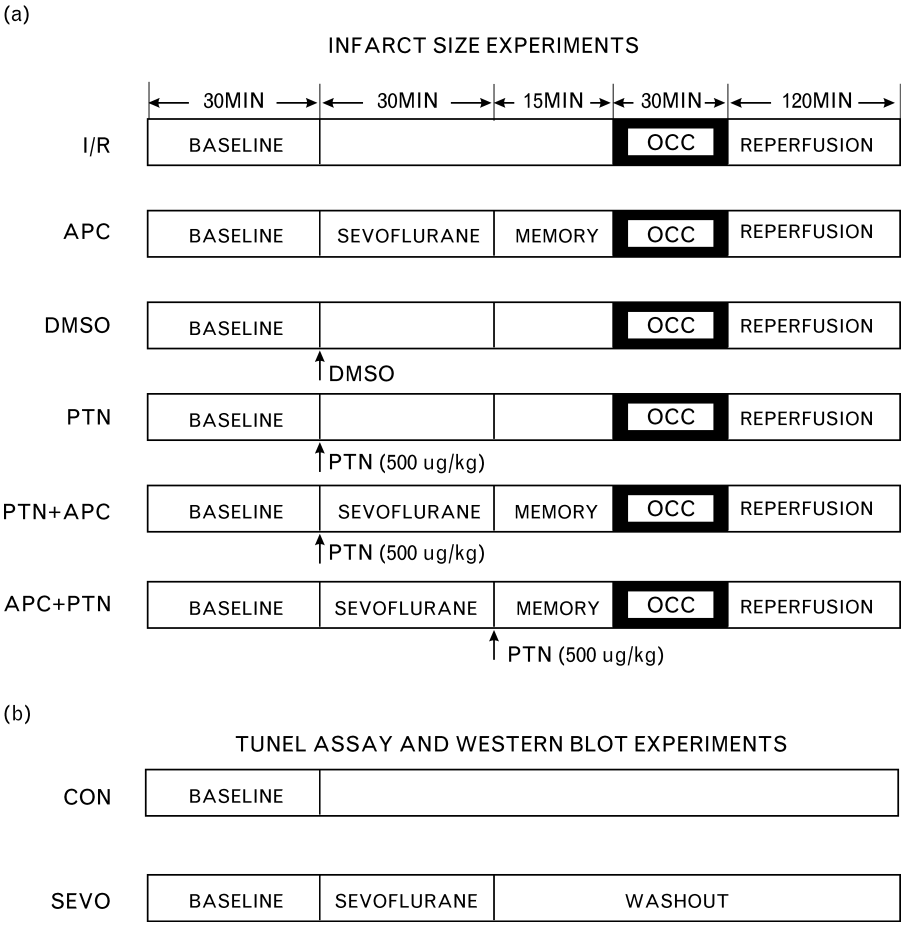
Determination of myocardial infarct size

At the end of the 120 min reperfusion, the coronary artery was reoccluded. Evans Blue stain (Sinopharm Chemical Reagent Co. Ltd., Beijing, China) was administered intravenously to stain the normal region of the left ventricle (LV), and then the heart was rapidly removed. The LV was separated and cut into six cross-sectional slices of 2 mm thickness. The nonstained left ventricular zone at risk was separated from the blue-stained normal area and incubated at 37°C for 15 min in 1% 2,3,5-triphenyltetrazolium chloride in 0.1 mol l^{-1} phosphate buffer adjusted to pH 7.4. Tissues were fixed overnight in 10% formaldehyde, and the infarcted tissue was carefully separated from the area at risk using a dissecting microscope. Infarct size was expressed as a percentage of the left ventricular area at risk. A total of 48 hearts were used for this measurement.

Terminal deoxynucleotidyl transferase dUTP nick end labelling assay

To determine myocardial apoptosis in a quantitative manner, the apoptotic cell death was evaluated by the terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) method using an apoptosis detection kit, ZK-8005 (Zhongshan Goldenbridge Biotechnology,

Fig. 1



Schematic illustration of experimental protocols used in infarct size (a) and terminal deoxynucleotidyl transferase dUTP nick end labelling assay and western immunoblotting experiments (b). For the infarct size study, the tissue samples were obtained at the end of reperfusion. For the terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay, the tissue samples were obtained at the end of the experiment. For western immunoblotting measurements, the tissue samples were obtained before ischaemia (samples were obtained at 45 min after baseline in the CON group, and 15 min after the end of sevoflurane exposure in the SEVO group) and at the end of the experiments. APC, anaesthetic preconditioning; CON, control; DMSO, dimethylsulphoxide; I/R, ischaemia and reperfusion; PTN, parthenolide; SEVO, sevoflurane.

Beijing, China). Because the TUNEL assay uses the whole LV, different rats were used for this measurement. The ischaemia/reperfusion group and DMSO group were replaced by control (CON) and sevoflurane (SEVO) groups, respectively. In the CON group, the rats received sham surgery and no other treatment for the entire experiment. In the SEVO group, rats were exposed to 2.5% sevoflurane for 30 min followed by 165 min of washout. The rats were divided into six groups (CON, APC, SEVO, PTN, PTN+APC and APC+PTN groups, Fig. 1b) and the heart samples were obtained at the end of each experiment ($n = 5$ in each group with a total of 30 hearts). The left ventricular tissues were quickly removed and washed in ice-cold phosphate-buffered saline (PBS) solution. Then the tissues were incubated with 10% paraformaldehyde overnight. The tissues were then fixed and embedded in paraffin. The sections were cut to 4 μm thickness and mounted on glass slides. Necrotic areas were confirmed from the morphological appearance of the tissue after haematoxylin and eosin staining. Immunohistochemical procedures for detecting apoptotic cardiocytes were performed according to the manufacturer's instructions. For each slide, 10 fields were randomly selected. Nuclei with brown staining indicated TUNEL-positive cells. The cell apoptosis index was expressed as a percentage of total cells counted.

Western blot

Western blot analysis was used to detect the protein expressions before ischaemia and after reperfusion. Because of the different tissue preparations, separate hearts were needed for this analysis. The groups were the same as those in the TUNEL assay, which included CON, APC, SEVO, PTN, PTN+APC and APC+PTN (Fig. 1b). Left ventricular tissue samples were obtained before ischaemia (samples were obtained at 45 min after

baseline in the CON group and 15 min after the end of sevoflurane exposure in the SEVO group, $n = 5$ in each group, total of 30 samples) and at the end of each experiment ($n = 5$, total of 30 samples).

LVs were quickly separated for analysis. Tissues were homogenized in ice-cold cell lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) containing a complete protease inhibitor, phenylmethyl sulphonylfluoride (PMSF) (Beyotime Institute of Biotechnology) to isolate total protein. Protein concentrations were determined by a protein concentration measurement kit (Beyotime Institute of Biotechnology).

Equivalent amounts of protein were mixed with loading buffer, heated at 100°C for 5 min, separated by 10–12% SDS-PAGE and then transferred onto nitrocellulose membrane (Pall Corporation, East Hills, USA). The membrane was stained with 0.1% Ponceau S (Beyotime Institute of Biotechnology) in 5% acetic acid for 5 min to verify equal loading of lanes using the method described previously.[3] Nonspecific reactivity was blocked in 5% nonfat dry milk in Tris-buffered saline with Tween (TBST) (10 mmol l^{-1} Tris-HCl, pH 7.5, 150 mmol l^{-1} NaCl and 0.05%Tween-20) for 60 min at room temperature. The membrane was then incubated overnight at 4°C with 5% milk and 1 : 500 dilution of rabbit or goat polyclonal antibody [NF-κB p65, tumour necrosis factor-α (TNF-α), Bcl-2, and caspase-3 (Cell Signaling, Danvers, USA), NF-κB p50, intercellular adhesion molecule-1 (ICAM-1; Santa Cruz Biotechnology, Santa Cruz, USA)] followed by reaction with secondary antibody (Cell Signaling). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a reference for the quantitative analysis. It is one of the most commonly used housekeeping genes in comparisons of gene expression data. Reactive protein was detected by an enhanced chemiluminescence system (ECL; Merck, Darmstadt, Germany). Quantitative analysis of the band densities was performed using Sigma pro5.0 (Sigma-Aldrich).

Statistical analysis

SAS 8.1 (SAS Institute Inc., Cary, USA) software was used for statistical analysis. Analysis of data within and between groups was performed with multiple analysis of variance (ANOVA) for repeated measures followed by the Student–Newman–Keuls test. All values are expressed as mean ± SD. All a P values were two-tailed and a P value less than 0.05 was considered significant.

Results

One hundred and forty-three rats were instrumented to obtain 138 successful experiments. Two rats were excluded because of technical difficulties during instrumentation. Three rats were excluded because intractable ventricular fibrillation occurred during coronary artery occlusion. During the 30 min sevoflurane exposure in the APC, PTN+APC and APC+PTN groups, HR, mean arterial blood pressure (MAP) and RPP decreased. These changes recovered before the occlusion of the coronary artery. The MAP and RPP were decreased during reperfusion in all groups compared with baseline ($P < 0.05$; Table 1).

Infarct sizes were reduced in the APC group compared with the ischaemia/reperfusion group ($P < 0.05$), and this was blocked by PTN in both the PTN+APC and APC+PTN groups ($P < 0.05$; Table 2). In the ischaemia/reperfusion group, there was a significant increase in TUNEL-positive

Table 1 Systemic haemodynamics

					Reperfusion	
	Baseline	Sevoflurane	Preocclusion	Occlusion	1 h	2 h
HR (min^{-1})						
I/R	370 ± 55	366 ± 59	363 ± 31	369 ± 40	355 ± 32	345 ± 35
DMSO	369 ± 59	370 ± 54	367 ± 32	379 ± 33	365 ± 27	357 ± 36
PTN	367 ± 59	372 ± 49	355 ± 38	371 ± 49	340 ± 26	348 ± 36
SEVO	365 ± 37	296 ± 48*	349 ± 36	364 ± 26	362 ± 35	354 ± 41
PTN + SEVO	378 ± 60	295 ± 48*	371 ± 60	373 ± 56	361 ± 53	359 ± 53
SEVO + PTN	344 ± 39	281 ± 45*	334 ± 42	353 ± 38	355 ± 43	355 ± 46
MAP (mmHg)						
I/R	105 ± 19	110 ± 20	103 ± 18	99 ± 20	77 ± 16*	71 ± 18*
DMSO	109 ± 12	105 ± 15	117 ± 9	111 ± 7	80 ± 11*	68 ± 6*
PTN	107 ± 22	103 ± 19	110 ± 21	105 ± 21	79 ± 13*	66 ± 12*
SEVO	111 ± 16	63 ± 14*	99 ± 19	99 ± 12	70 ± 13*	67 ± 17*
PTN + SEVO	109 ± 27	71 ± 9*	121 ± 24	113 ± 27	75 ± 8*	68 ± 8*
SEVO + PTN	101 ± 25	68 ± 10*	114 ± 26	108 ± 30	76 ± 8*	69 ± 9*
RPP (min^{-1} mmHg 10^3)						
I/R	46 ± 11	47 ± 10	43 ± 7	43 ± 11	34 ± 5*	33 ± 8*
DMSO	47 ± 7	45 ± 12	51 ± 3	48 ± 4	38 ± 7*	32 ± 6*
PTN	45 ± 8	46 ± 11	46 ± 9	45 ± 11	32 ± 3*	30 ± 5*
SEVO	49 ± 9	24 ± 5*	42 ± 8	40 ± 6	33 ± 4*	31 ± 7*
PTN + SEVO	47 ± 13	26 ± 4*	52 ± 13	47 ± 10	34 ± 3*	30 ± 4*
SEVO + PTN	41 ± 11	24 ± 5*	45 ± 13	45 ± 16	34 ± 7*	30 ± 9*

Data are mean ± SD. DMSO, dimethylsulphoxide; HR, heart rate; I/R, ischaemia and reperfusion; MAP, mean arterial blood pressure; PTN, parthenolide; RPP, rate–pressure product; SEVO, sevoflurane. *Significantly ($P < 0.05$) different from baseline.

Table 2 Myocardial infarct size

	Left ventricle (mg)	Area at risk (mg)	Infarct area (mg)	Area at risk/left ventricle (%)	Infarct size (% of area at risk)
I/R	591 ± 19	215 ± 18	113 ± 17	36 ± 2	53 ± 6
DMSO	587 ± 34	206 ± 16	116 ± 11	35 ± 2	57 ± 4
PTN	629 ± 33	271 ± 60	136 ± 32	43 ± 7	50 ± 6
SEVO	558 ± 65	189 ± 27	64 ± 12*	34 ± 2	34 ± 6*
PTN + SEVO	605 ± 26	238 ± 43	125 ± 25	39 ± 6	53 ± 5
SEVO + PTN	643 ± 45	261 ± 50	139 ± 37	40 ± 5	52 ± 7

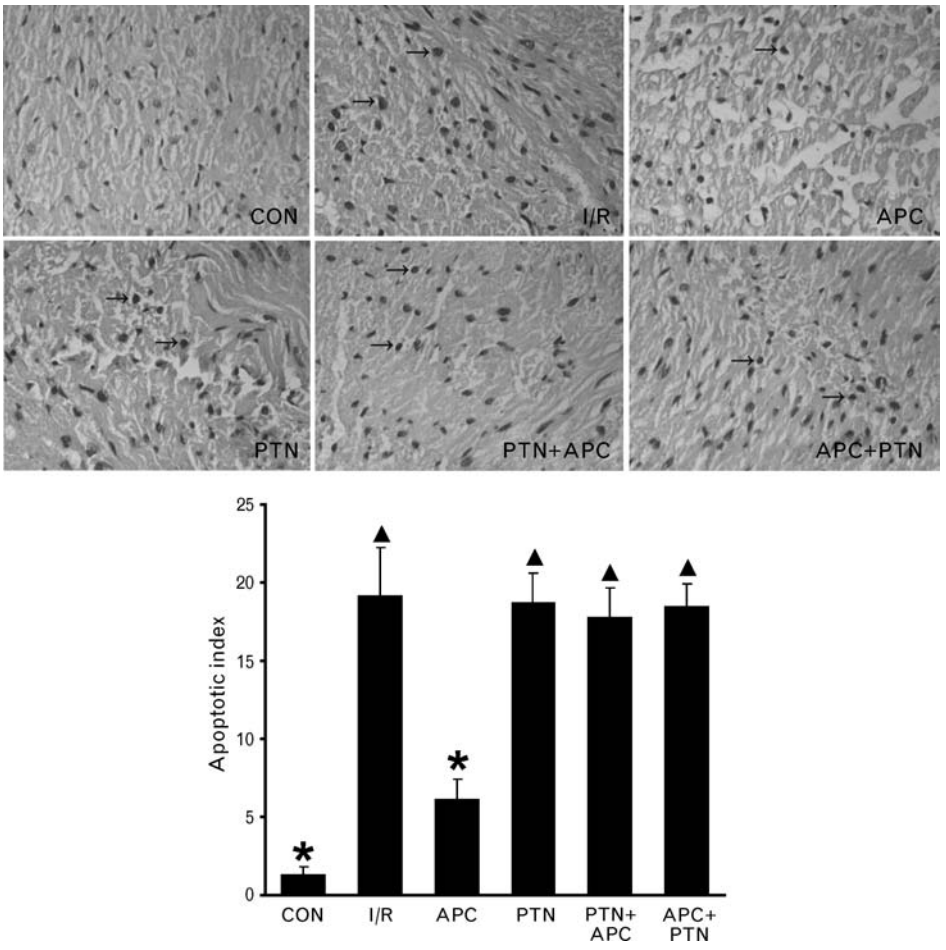
Data are presented as mean ± SD. DMSO, dimethylsulphoxide; I/R, ischaemia/reperfusion; PTN, parthenolide; SEVO, sevoflurane. * Significantly ($P < 0.05$) different from the I/R group.

cardiocytes in the periinfarct zone compared with the CON group ($P < 0.05$). APC significantly decreased it and this effect was abolished by PTN administered before or after exposure to sevoflurane. (Fig. 2)

Compared with the CON group, NF-κB p50/p65 proteins were upregulated in the SEVO group and the APC group before ischaemia ($P < 0.05$), but were lower in the SEVO group compared with the APC group ($P < 0.05$). PTN administered alone and before or after exposure to APC abolished the upregulation ($P < 0.05$). At the end of reperfusion, NF-κB p50/p65 proteins were upregulated in both the ischaemia/reperfusion and APC groups compared with the CON group, but they were lower in the APC group than in the ischaemia/reperfusion group ($P < 0.05$; Fig. 3).

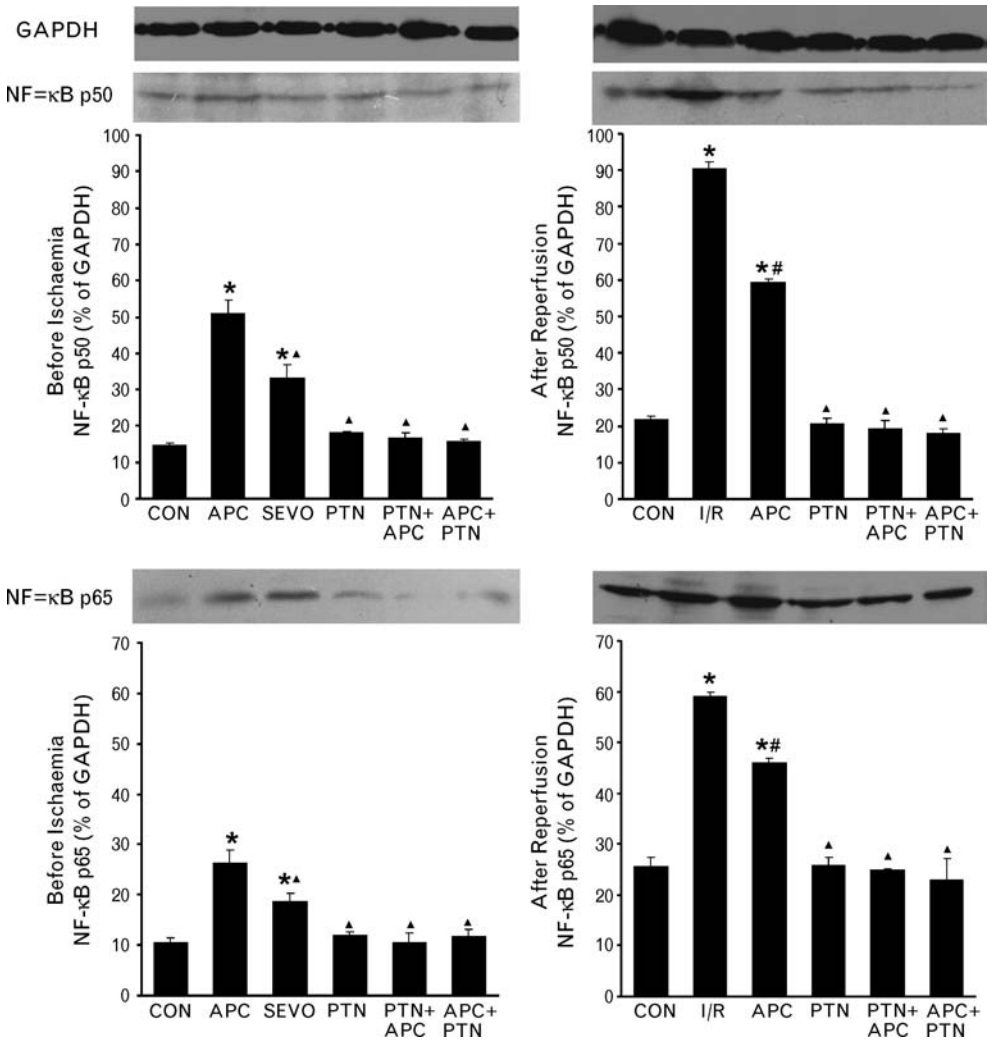
At the end of 120 min reperfusion, ICAM-1 and TNF-α were upregulated in all groups except for the CON group

Fig. 2



Representative photomicrographs of the terminal deoxynucleotidyl transferase dUTP nick end labelling assay (40×objective). Cell apoptosis index values are mean ± SD. *Significantly ($P < 0.05$) different from the I/R group. ▲Significantly ($P < 0.05$) different from the SEVO group. APC, anaesthetic preconditioning; CON, control; I/R, ischaemia and reperfusion; PTN, parthenolide; SEVO, sevoflurane.

Fig. 3



Representative immunoblots (top panel) of nuclear factor-κB p50/p65 in rats before and after reperfusion in each group. Histograms illustrating nuclear factor-κB (NF-κB) p50/p65 correspond to the pictures. All data are mean ± SD. *Significantly ($P < 0.05$) different from the CON group. ▲Significantly ($P < 0.05$) different from the SEVO group. #Significantly ($P < 0.05$) different from the I/R group. APC, anaesthetic preconditioning; CON, control; I/R, ischaemia and reperfusion; PTN, parthenolide; SEVO, sevoflurane.

($P < 0.05$). Compared with the ischaemia/reperfusion group, ICAM-1 and TNF-α were lower in the APC group. PTN administered alone and before or after exposure to APC also limited ICAM-1 and TNF-α expressions compared with the ischaemia/reperfusion group (Fig. 4).

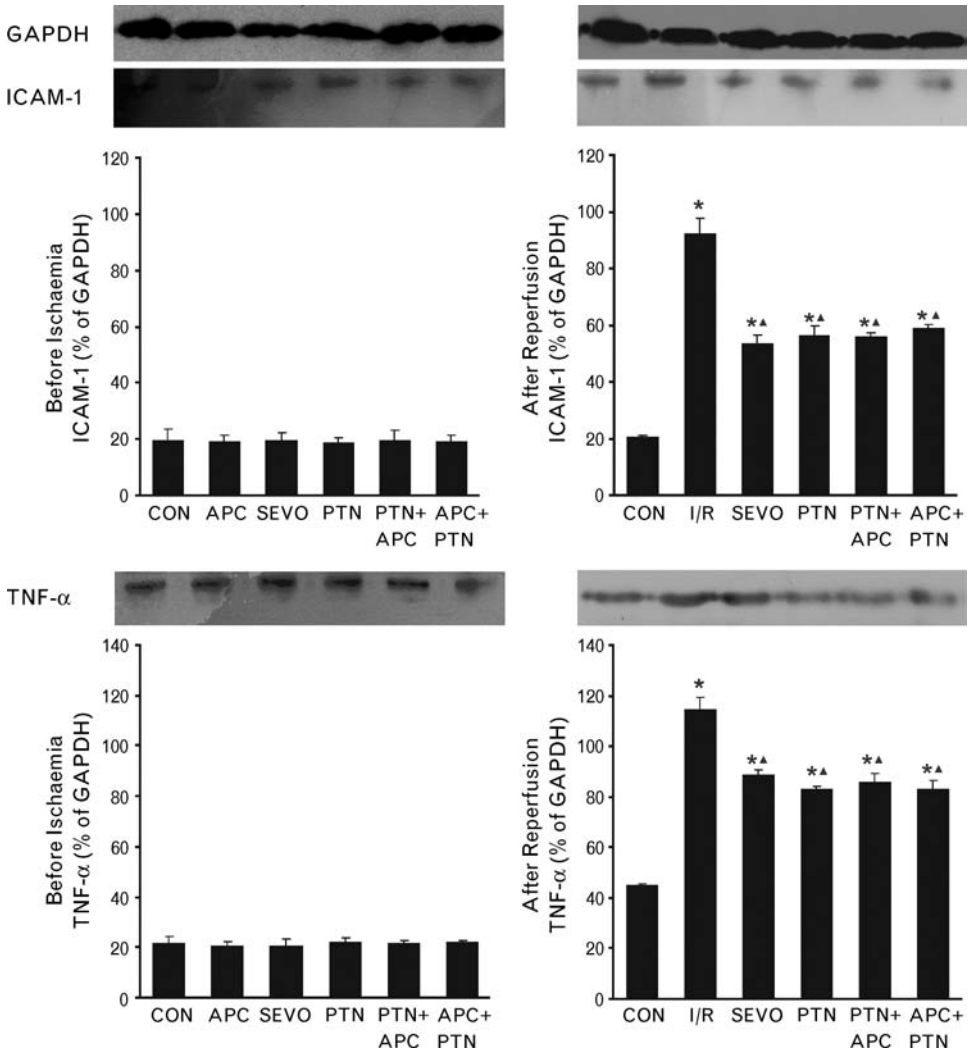
In the APC group prior to ischaemia and in the SEVO group, Bcl-2 was increased compared with the CON group ($P < 0.05$). At the end of reperfusion, Bcl-2 was downregulated in the ischaemia/reperfusion group compared with the CON group. APC blocked the Bcl-2 downregulation and the downregulation was abolished by PTN administered before or after exposure to APC ($P < 0.05$). Caspase-3 was increased in the ischaemia/reperfusion group compared with the CON group. APC blunted its increase compared with the ischaemia/reperfusion group ($P < 0.05$). This effect was also abolished by PTN administered before or after APC ($P < 0.05$; Fig. 5).

Discussion

Results from this in-vivo study showed that APC with sevoflurane significantly reduced myocardial ischaemia/reperfusion injury by decreasing the infarct size and reducing inflammation, apoptosis and myocardial oxygen consumption. Administration of the NF-κB inhibitor PTN before or after APC abolished its cardioprotective effects.

Many studies demonstrated that APC with sevoflurane not only protects against ischaemia/reperfusion injury, but also improves functional recovery, decreases post-ischaemic myocardial stunning and attenuates myocardial apoptosis. These protective effects were observed

Fig. 4



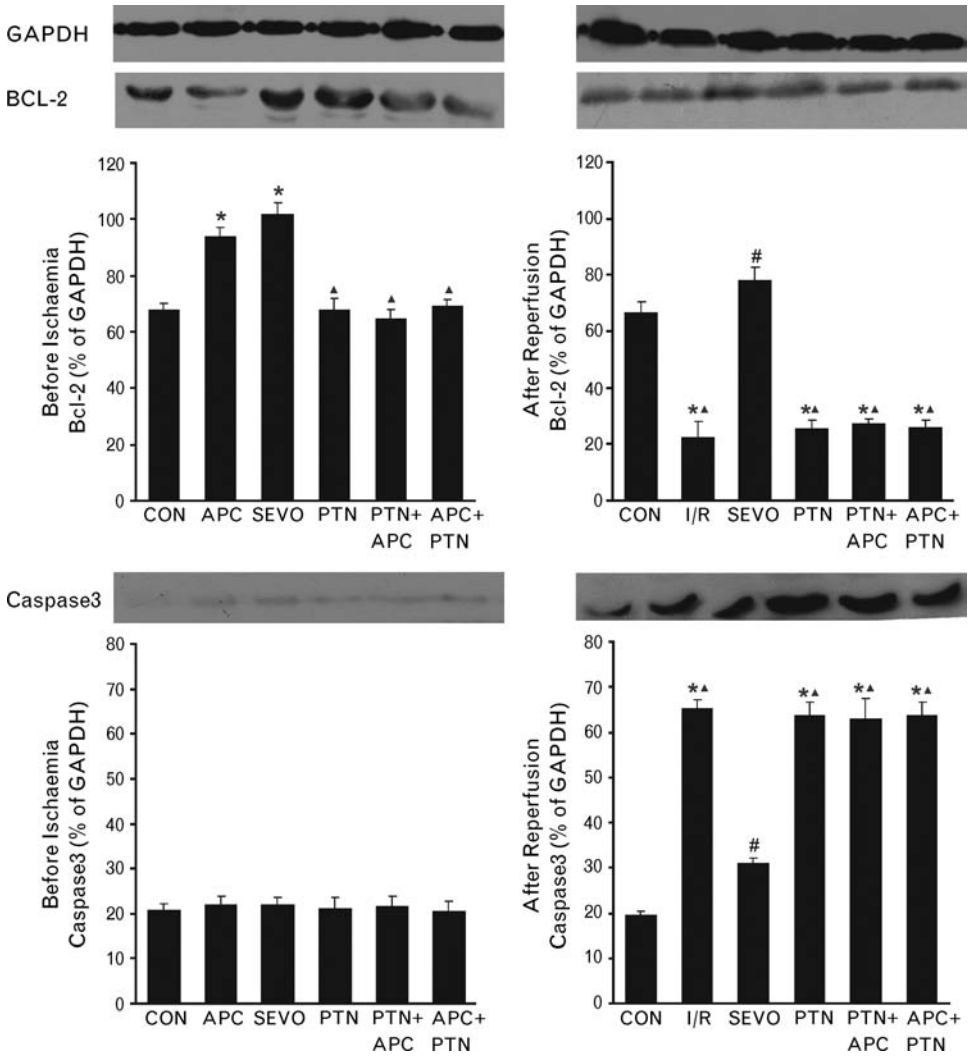
Representative immunoblots (top panel) of intercellular adhesion molecule-1 and tumour necrosis factor-α in rats before and after reperfusion in each group. Histograms illustrating intercellular adhesion molecule-1 (ICAM-1) and tumour necrosis factor-α (TNF-α) correspond to the pictures. All data are mean ± SD. *Significantly ($P < 0.05$) different from the CON group. ▲Significantly ($P < 0.05$) different from the I/R group. APC, anaesthetic preconditioning; CON, control; I/R, ischaemia and reperfusion; PTN, parthenolide; SEVO, sevoflurane.

with sevoflurane, isoflurane and desflurane preconditioning.[4,10,20,21] However, there is also a report, with conflicting results, that found APC with sevoflurane *in vivo* fails to reduce myocardial injury at the end of reperfusion.[22] The results of our study are consistent with most published results that APC with sevoflurane blunts the ischaemia/reperfusion-induced myocardial injury by decreasing infarct size and reducing inflammation. The mechanisms of myocardial protection are thought to be associated with downregulation of inflammatory factors and upregulation of antiapoptosis,[8] especially the intracellular signal transduction pathways related to the process.[3,23,24]

It has been reported that PTN protects the myocardium against ischaemia/reperfusion injury and is associated with inhibition of IκB kinase (IKK) activity, enhancing stability of IκBa and inhibition of nuclear translocation of NF-κB. Konia *et al.*[10] reported that PTN+APC protected hearts more than either intervention alone. We did not find that PTN has a cardioprotective effect, in contrast to others.[10] This may be due to in-vitro vs. in-vivo animal models, as well as acute vs. delayed preconditioning mechanisms.

NF-κB is a pivotal inducible transcription factor that regulates the expression of many genes involved in important biological processes of inflammation and apoptosis. Depending on the time and degree of activation, NF-κB can be either protective or harmful. NF-κB also is a critical element that reduces apoptosis and functional impairment in APC by increasing Bcl-2 expression before ischaemia/reperfusion. Interventions that cause a mild

Fig. 5



Representative immunoblots (top panel) of Bcl-2 and caspase-3 in rats before and after reperfusion in each group. Histograms illustrating Bcl-2 and caspase-3 correspond to the pictures. All data are mean ± SD. *Significantly ($P < 0.05$) different from the CON group. ▲Significantly ($P < 0.05$) different from the SEVO group. #Significantly ($P < 0.05$) different from the I/R group. APC, anaesthetic preconditioning; CON, control; I/R, ischaemia and reperfusion; PTN, parthenolide; SEVO, sevoflurane.

increase in NF-κB activation before ischaemia/reperfusion protect the myocardium from ischaemia/reperfusion injury.[25] A previous in-vitro study demonstrated that APC protects the myocardium against ischaemia/reperfusion injury by attenuating the activation of NF-κB and subsequently suppressing the expression of NF-κB-regulated inflammatory genes during reperfusion.[9] Ischaemia/reperfusion results in activation of NF-κB, with deleterious consequences, by inducing inflammatory cytokines and cleavage of procaspases. But NF-κB activation at a lower level prior to ischaemia/reperfusion could be protective. In this study, NF-κB p65/p50 were upregulated slightly before ischaemia by APC. Although we could not tell exactly how low is protective and how high is deleterious, this finding still suggested that the degree of activation is vital. Our study also demonstrated that the upregulation of NF-κB p65/p50 continued at least 165 min after sevoflurane exposure even without ischaemia. Studies showed that an early NF-κB responsive gene encodes a ubiquitin-editing protein that is involved in the negative feedback regulation of NF-κB signalling and it is rapidly induced by a large number of stimuli, which trigger the binding of NF-κB to two specific NF-κB-binding sites in its promoter.[26,27] We presume this could be the protective mechanism of APC with sevoflurane against ischaemia/reperfusion injury.

NF-κB activation is a double-edged sword. More than 150 NF-κB target genes have been identified and the expressions of inflammatory mediators, such as cytokines (e.g. TNF-α, IL-1), adhesion molecules (e.g. ICAM-1, vascular cell adhesion molecule-1) and proinflammatory

enzymes [e.g. inducible nitric oxide synthase (iNOS), cyclooxygenase2 (COX2)] can be regulated by NF-κB.[28–30] In order to test whether NF-κB triggers the expressions of the mediators during APC, PTN was administered before (PTN+SEVO group) and after (SEVO+PTN group) APC. The result shows that PTN administered before and after sevoflurane abolished the cardioprotection of APC. But in the PTN alone group, the infarct size did not decrease compared with the ischaemia/reperfusion group. The possible explanation is that there might be some other mechanisms involved in the expression of ICAM-1 and TNF-α that PTN could not inhibit. In a similar study, IKκb inhibition decreased serum levels of TNF-α, a prototypical downstream effector of NF-κB activity. The inhibition of IKκb can provide both acute and delayed cardioprotection following ischaemia/reperfusion. It demonstrated that, by inhibiting NF-κB, the TNF-α expression was significantly reduced.[31]

The antiapoptotic process is another myocardial protective mechanism of APC. Our results demonstrated that APC with sevoflurane stimulates upregulation of Bcl-2 expression, independent of ischaemia/reperfusion. This upregulation of Bcl-2 in the preconditioning period was abolished by PTN, providing evidence that activation of NF-κB by APC stimulates the production of Bcl-2. This was further supported by a study that showed that the antiapoptosis protein Bcl-2 can be induced by NF-κB during the period of hypoxic preconditioning.[32] In a different study using transgenic mice with a defect in activation of NF-κB, the authors suggested that the cytoprotective effects of NF-κB are mediated, at least in part, by Bcl-2.[33] At the same time, caspase-3, another marker for apoptosis, was also significantly reduced in the APC group at the end of reperfusion. The results are consistent with our hypothesis that APC-induced cardioprotection could be triggered by NF-κB through upregulation of antiapoptosis factors in the preconditioning period and resulted in the decrease in apoptosis during reperfusion, and this could be one of the mechanisms of APC. These findings are consistent with results reported by Grünenfelder *et al.*[34] that blockade of caspase-3 can ameliorate reperfusion injury by upregulating Bcl-2 and inhibiting TNF-α. Results from TUNEL assays and gene expressions further strengthened findings in myocardial function and histology.

There are some limitations in this study. We used only sevoflurane and we do not know whether all the volatile anaesthetics are equal in efficacy with regard to APC. We did not assess dose–response to sevoflurane. There are many published studies using different concentrations of sevoflurane. Although several studies have demonstrated the efficacy of APC in human surgical conditions, it is not known whether NF-κB activation occurs in human APC. The results of this study should not extrapolate to other species. In addition, these findings are applicable to early APC, not delayed APC in which protection against ischaemia/reperfusion occurs 24–48 h following anaesthetic exposure. In this study, we did not find any difference between the proteins of the two subunits of NF-κB (p50 and p65). Whether the gene expression is modulated differently is still unknown. Although there are many NF-κB-regulated inflammatory and antiapoptotic genes, we tested only two of the most common ones (TNF-α and ICAM-1) for inflammation and three of the most common ones for apoptosis (cell apoptosis index, Bcl-2 and caspase-3) in this study. Finally, we did not investigate the pharmacokinetics and pharmacodynamics of PTN. We based the dosing and timing of PTN on previously published studies.

Conclusion

The results of this in-vivo study demonstrated that APC with sevoflurane produced a protective effect against myocardial ischaemia/reperfusion. Furthermore, our results indicated that NF-κB contributed significantly to the protective process of APC. NF-κB acted not only as a trigger but also as a mediator through upregulating the antiapoptosis protein of Bcl-2 in the period of preconditioning; and downregulating inflammatory proteins of ICAM-1 and TNF-α in the period of reperfusion. Ultimately, this decreased apoptosis during ischaemia/reperfusion.

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