J Anesth (2013) 27:251–260
DOI 10.1007/s00540-012-1494-3

ORIGINAL ARTICLE

Delayed anesthetic preconditioning protects against myocardial infarction via activation of nuclear factor-κB and upregulation of autophagy

Shigang Qiao · Hong Xie · Chen Wang · Xuemei Wu · Hong Liu · Chunfeng Liu

Received: 26 March 2012 / Accepted: 13 September 2012 / Published online: 10 November 2012

Abstract
Purpose Delayed volatile anesthetic preconditioning (APC) can protect against myocardial ischemia/reperfusion (I/R) injury; the delayed phase is called the second window of protection (SWOP), but the underlying mechanism is unclear. Nuclear factor-κB (NF-κB) is involved in the myocardial protection conferred by APC in the acute phase; autophagy has been reported to confer apoptosis inhibition and infarction reduction. We hypothesized that APC initiates delayed cardioprotection against I/R injury via the activation of NF-kB and upregulation of autophagy, thus attenuating the inflammatory response and apoptosis
Methods After a rat I/R model was set up, left ventricular samples were obtained before I/R to assess NF-κB-DNA binding activity and microtubule-associated protein 1 light chain 3 (LC3) and cathepsin B protein expression, and to examine autophagosomes with a transmission electron microscope. Infarct size and the expressions of tumor necrosis factor α (TNF-α), interleukin 1β (IL-1β), and caspase-3 were measured at the end of 2-h reperfusion.
Results The infarct size was significantly reduced in the SWOP group (30 ± 3 %) when compared with that in the I/R group (47 ± 7 %, $P < 0.05$), and this finding was associated with increased NF-κB-DNA binding activity and autophagosomes. In addition, the expressions of LC3-II and cathepsin B were also up-regulated, and the expressions of TNF-α, IL-1β, and caspase-3 were attenuated in the SWOP group when compared with the findings in the I/R group. However, this protection was abolished by the administration of parthenolide (PTN) before sevoflurane inhalation, which resulted in an infarct size that was significantly increased (47 ± 5 %, $P < 0.05$ PTN + SWOP vs. SWOP group).
Conclusion Delayed APC protected the rat heart from I/R injury. The underlying mechanisms may include NF-κB activation, upregulation of autophagy, and the attenuation of TNF-α, IL-1β, and caspase-3 expressions.

Keywords Ischemia/reperfusion injury · Autophagy · Preconditioning · Apoptosis · Nuclear factor-κB

Introduction

Volatile anesthetic preconditioning (APC) has been shown to decrease myocardial ischemia/reperfusion (I/R) injury through acute and delayed cardiac protection [1, 2]. The delayed phase, called second window of protection (SWOP), arises 12–24 h after the injury and may last for up to 72 h [3, 4]. This delayed cardiac protection may be clinically relevant in patients with coronary artery disease during the first 24–48 h after surgery when myocardial ischemia can occur [5].

An array of signaling molecules and pharmacological agents, including nitric oxide (NO) donors, protein kinase

S. Qiao and H. Xie contributed equally to this manuscript.

S. Qiao · H. Xie · C. Wang (✉) · X. Wu
Department of Anesthesiology, The Second Affiliated Hospital of Soochow University, 1055 Sanxiang Road, Suzhou 215004, People's Republic of China
e-mail: anesthesia_chen@163.com

C. Wang · C. Liu
Institute of Neuroscience, Soochow University, Suzhou 215004, People's Republic of China

H. Liu
Department of Anesthesiology and Pain Medicine, University of California Davis Health System, Davis, CA 95616, USA

C (PKC), and apoptosis signaling mediators (e.g., Bcl-2, p53) [6, 7] have been demonstrated to exhibit beneficial effects in APC. However, the underlying mechanism of the delayed phase is unclear.

Recent studies have demonstrated that nuclear factor κB (NF-κB) and autophagy participate in anti-apoptotic mechanisms in response to I/R [8–10]. NF-κB, an important inducible transcription factor, produced in response to reactive oxygen species (ROS) or nitric oxide, is released from the inhibitory protein IκB and then initiates transcription via translocating to the nucleus and binding to consensus sites in the promoter or enhancer regions of target genes, such as some anti-apoptosis proteins [11, 12]. A modest increase in NF-κB protects against myocardial injury and limits apoptosis [11, 13]. Activation of NF-κB before I/R is considered to be a critical element in the antiapoptotic effect of acute-phase APC. One reported mechanism of this effect is that APC increased Bcl-2 expression before I/R and decreased cytochrome c release and caspase-3 degradation with I/R, and then reduced apoptosis and functional impairment [10]. Autophagy is a highly regulated process that can be involved in the turnover of long-lived proteins and organelles. Part of the cytoplasm and intracellular organelles are sequestered within characteristic double- or multimembraned autophagic vacuoles (named autophagosomes) and are finally delivered to lysosomes for bulk degradation. These superfluous, damaged, or aged cells or organelles are eliminated under conditions of stress, hypoxia, and ischemia [14, 15] and autophagy can be considered as an end-effector in these processes [16]. Inhaled sevoflurane provides effective delayed myocardial protection [3]; however, it is unclear whether the delayed APC confers the protective effect through activating NF-κB or through autophagy. We hypothesized that the APC-initiated delayed cardioprotection against I/R injury occurs via the activation of NF-κB and upregulated autophagy, thus attenuating the inflammatory response and apoptosis.

Materials and methods

Study protocol

The study protocol was approved by the Committee for Experimental Animals of the Medical College of Soochow University (Suzhou, People's Republic of China; protocol number: SZULL-20090309, approved on 9 March 2009; Chairperson: Professor Zhi-mou Xue) and all experiments were conducted in accordance with "the Guide for the Care and Use of Laboratory Animals" (NIH publication vol. 25 no. 28, revised 1996) and the policies of Soochow University.

Preconditioning intervention with sevoflurane

Twenty-four hours before I/R, adult male Sprague–Dawley rats (aged 9–10 weeks, body weight 270–320 g) were allowed to spontaneously breathe an air-oxygen mixture (fractional inspired oxygen concentration = 0.33%) with or without sevoflurane (Abbott Laboratories, Shanghai, China) in an induction chamber. At the end of this exposure, arterial blood gas analysis was performed (iSTAT, Princeton, NJ, USA) to exclude hypoxia or hypercarbia. Preconditioning with sevoflurane was delivered with an anesthetic drug monitor (ARIN-0104; Dräger Primus SW2.n, Lübeck, Germany) at 3 L/min and was maintained for 2 h at a concentration of 2.5 %, the equivalent of 1 minimal alveolar concentration in rats [3, 17]. Upon recovery from sevoflurane, the animals were returned to individual cages with free access to food and water.

Surgical instrumentation

In this in vivo study, rats were anesthetized with an intraperitoneal (i.p.) injection of sodium pentobarbital (50 mg/kg) to ensure that pedal and palpebral reflexes were absent throughout the experiment. One hour (30 min completing surgical operation and 30 min baseline period) before coronary artery occlusion (CAO), the right jugular vein was cannulated for saline and drug infusion; the right carotid artery was cannulated for arterial blood pressure measurement on a polygraph, carried out with Medlab-U/4C501H (Nanjing Mei Yi Technology, Nanjing, China). The rats were intubated via tracheostomy and ventilated with a rodent ventilator (Shanghai Alcott Biotech, Shanghai, China) at 60–65 breaths per min with 33 % O_2. Body temperature was maintained at 37 ± 0.5 °C by using a heating pad. The heart was exposed via a left thoracotomy at the left fifth intercostal space and suspended in a pericardial cradle. A 6-0 Prolene (Ethicon, Raleigh, NC, USA) ligature was placed around the proximal left anterior descending coronary artery (LAD) in the proximity of their base. Both ends of the suture were threaded through a propylene tube to make a snare for the performance of CAO [2]. The effectiveness of CAO was verified by epicardial color change and the progressive exhibition of marked arrhythmia. Reperfusion was visually confirmed by observing an epicardial hyperemic response. The rate-pressure product (RPP; systolic blood pressure × heart rate/100) was used as a determinant of myocardial oxygen consumption.

Experimental protocol

Figure 1 illustrates the experimental design used to determine the myocardial infarct size. During the 30-min

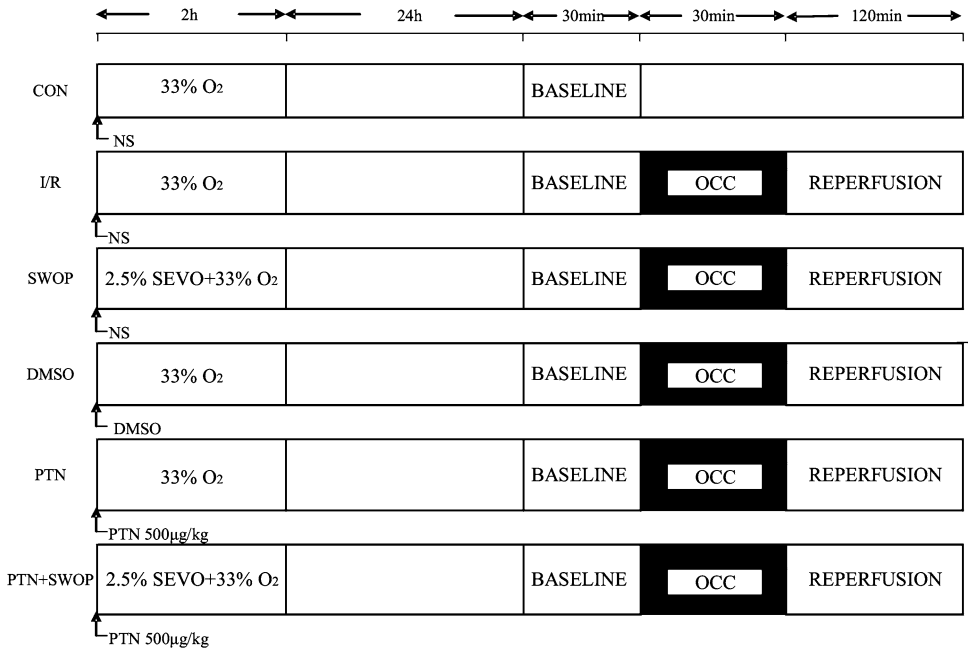

Fig. 1 Schematic illustration of the experimental protocols used in the myocardial infarct size and Western immunoblotting experiments. Twenty-four hours before I/R, rats were randomly assigned to inhale 33 % oxygen and 2.5 % sevoflurane for 2 h (SWOP group). *CON* control, *I/R* ischemia and reperfusion, *SWOP* sevoflurane preconditioning, *SEVO* sevoflurane control, *DMSO* dimethylsulfoxide, *PTN* parthenolide, *OCC* occlusion

baseline period, hemodynamics were recorded and all rats in the experimental groups underwent 30 min of CAO followed by 2-h reperfusion. In addition, 24 h before I/R, rats were randomly assigned to receive 33 % oxygen (I/R group) or 33 % oxygen + 2.5 % sevoflurane for 2 h (SWOP group). Rats that received 33 % oxygen without subsequent ischemia and reperfusion 24 h later served as the control (CON) group. The NF-κB inhibitor parthenolide was administered i.p. alone (PTN group) or before exposure to sevoflurane (PTN + SWOP group) as described in the SWOP group in a previous study [18]. In the DMSO group, the rats received DMSO only. This was designed to test whether sevoflurane triggers NF-κB and the downstream expression of this protein.

Determination of myocardial infarct size

At the end of 2-h reperfusion, the coronary artery was re-occluded ($n = 6$). Five percent Evans Blue stain (Sinopharm Chemical Reagent, Beijing, China) was administered via the right jugular vein to stain the normal region of the left ventricle (LV), followed by rapid removal of the heart. The LV was cut into 6 cross-sectional slices of 2-mm thickness. The non-stained LV area 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 M phosphate buffer, pH 7.4. The tissues were fixed overnight in 10 % formaldehyde solution, 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 LV area at risk.

Preparation of nuclear and cytosolic extracts

Apart from the determination of myocardial infarct size, an additional series of experiments was designed to determine the protein level and DNA binding activity of NF-κB, examining these features before I/R and after 2-h reperfusion ($n = 5$). The LVs from each group were frozen in liquid nitrogen. The extraction and isolation of nuclear and cytoplasmic proteins were performed according to the instructions in the Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime Institute of Biotechnology, Haimen, China). First, the LV tissues were homogenized in ice-cold Cytoplasmic Protein Extraction agent A and B with phenylmethanesulfonyl fluoride (PMSF). The samples were then centrifuged for 5 min at 1500*g* at 4 °C and the supernatant, consisting of the cytosolic components, was immediately frozen for further analysis. The sediment fractions were then resuspended in Nuclear Protein Extraction agent A and B supplemented with PMSF. After vortexing of the tubes 15–20 times for 30 min and centrifuging for 10 min at 14,000*g* at 4 °C, the supernatants containing the nuclear extracts were obtained [20]. The protein concentrations of the nuclear and cytosolic extracts were determined by the Bradford assay (Bio-Rad Laboratories, Shanghai, China).

NF-κB-DNA binding activity

An electrophoretic mobility shift assay was performed to determine the NF-κB-DNA binding activity in the CON, SEVO, SWOP, DMSO, PTN, and PTN + SWOP groups. The 3′ end of a double-stranded synthetic oligonucleotide

probe (Roche, Basel, Switzerland) for NF-κB (5′-AGT TGAGGGGACTTTCCCAGGC-3′ and 3′-TCAACTCCC CTGAAAGGGTCCG-5′) was labeled with [Γ ^{32}P]dATP using T4 polynucleotide kinase. The binding reaction of nuclear proteins to the probe was assessed by the incubation of mixtures containing 5 μg nuclear protein, 0.5 μg poly (dI · DC), and 40,000-cpm ^{32}P-labeled probe in the binding buffer (12 mM hydroxyethyl piperazineethanesulfonic acid [HEPES], pH 7.9, 35 mM NaCl, 5 mM $MgCl_2$, 1 mM ethylenediamine tetraacetic acid [EDTA], 1 mM dithiothreitol [DTT], 12 % glycerol) for 30 min at 25 °C. Samples were subjected to electrophoretic separation at room temperature on a nondenaturing 5 % acrylamide gel at 30 mA using 0.5× Tris borate EDTA buffer. The gels were dried at 80 °C for 1 h and exposed to radiography film for 6–18 h at −70 °C with intensifying screens. Specific band intensities were quantified with Scion Image 4.03 software (Scion, Frederick, MD, USA).

Western blot analysis

The expressions of microtubule-associated protein 1 light chain 3 (LC3) and cathepsin B before I/R (in the CON, SEVO, SWOP, DMSO, PTN, and PTN + SWOP groups) and the expressions of tumor necrosis factor α (TNF-α), interleukin 1β (IL-1β), and caspase-3 after reperfusion (in the CON, I/R, SWOP, DMSO, PTN, and PTN + SWOP groups) in heart samples obtained in the presence or absence of sevoflurane inhalation were determined by Western blot analysis. The disposal method of SEVO group was similar to the SWOP group except no ischemia reperfusion for the entire experiment. The samples were loaded and separated by 10–12 % sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by transblotting to an ImmunBlot nitrocellulose membrane (Pall, East Hills, NY, USA). Nonspecific reactivity was blocked in 5 % skim milk in TBST (10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.05 %Tween-20) for 2 h at room temperature. The membrane was subsequently probed with primary LC3 (Abcam, Cambridgeshire, UK), cathepsin B (Millipore, Temecula, CA, USA), IL-1β (BioVision, Palo Alto, CA, USA), TNF-α, caspase-3, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibodies (Cell Signaling Technology, Danvers, MA, USA), at a dilution of 1:1,000. Horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology) was added at a dilution of 1:5,000. Specific antigen–antibody complex was detected by an enhanced chemiluminescence system (ECL; Merck, Darmstadt, Germany). The amount of detected protein was quantified by Scion Image 4.03 software (Scion) and was expressed as the ratio to GAPDH protein.

Transmission electron microscopy

Tissue samples of the apex cordis, approximately 1 mm thick, were immediately immersed in ice-cold 2.5 % glutaraldehyde in 0.1 mol/L phosphate-buffered saline (PBS) and preserved at 4 °C for further processing. After 24 h, the tissue blocks were post-fixed in 1 % osmium tetroxide for 2 h, dehydrated in graded alcohols, and subsequently embedded in Epon 812. Areas of interest were selected from toluidine-blue-stained semithin sections, and the ultrathin sections were mounted in slot grids, covered with Formvar (Chisso Corporation Company, Shanghai, China), and double-stained in uranyl acetate and lead citrate. The sections were examined with a transmission electron microscope (TEM; Technai 10; Philips, Eindhoven, The Netherlands) at magnifications ranging from ×3,000 to ×15,000 to assess the ultrastructural features of cardiomyocytes.

Data analysis

Data are presented as means ± SD. One-way analysis of variance (ANOVA) and two-way analysis were used for the statistical analysis (GraphPad Prism, San Diego, CA, USA) of data obtained within the same group of rats and between groups of rats, respectively, followed by Tukey's test for multiple comparisons of group means. $P < 0.05$ was considered statistically significant.

Results

Systemic hemodynamic changes

One hundred rats were used to obtain 90 successful experiments, following the protocol. Three rats were excluded as a result of technical difficulties in surgical preparation, one rat in the I/R group and two rats in the DMSO group. A total of seven rats were excluded because intractable ventricular fibrillation occurred during CAO–two rats in the I/R group, two rats in the PTN group, and three rats in the PTN + SWOP group.

There were no significant differences among the five myocardial infarction study groups with respect to baseline heart rate (HR), mean arterial blood pressure (MAP), or RPP (Table 1). There were no differences in hemodynamics observed among the experimental groups at the baseline, during CAO, or at reperfusion ($n = 6$).

Area at risk and infarct size

The weight of the area at risk (AAR) and the AAR percentage of the LV mass did not significantly differ among

Table 1 Systemic hemodynamics during in vivo experiments

	Baseline	Preocclusion	Occlusion	Reperfusion	
				1 h	2 h
HR (min^{-1})					
I/R	365 ± 46	368 ± 41	358 ± 26	362 ± 39	362 ± 27
SWOP	373 ± 47	359 ± 53	369 ± 41	357 ± 36	356 ± 36
DMSO	358 ± 43	357 ± 42	367 ± 46	352 ± 43	362 ± 47
PTN	374 ± 35	357 ± 38	358 ± 31	369 ± 54	358 ± 35
PTN + SWOP	367 ± 56	368 ± 53	369 ± 60	372 ± 46	369 ± 37
MAP (mmHg)					
I/R	104 ± 8	107 ± 7	98 ± 17	77 ± 13*	73 ± 14*
SWOP	115 ± 12	109 ± 15	105 ± 15	75 ± 11*	68 ± 14*
DMSO	107 ± 14	114 ± 13	105 ± 15	78 ± 15*	64 ± 17*
PTN	106 ± 7	107 ± 14	106 ± 9	74 ± 10*	66 ± 14*
PTN + SWOP	112 ± 14	109 ± 15	115 ± 13	73 ± 15*	65 ± 16*
RPP (min^{-1} mmHg × 10^3)					
I/R	44 ± 3	44 ± 5	45 ± 15	34 ± 4*	33 ± 6*
SWOP	46 ± 4	54 ± 3	47 ± 4	37 ± 5*	34 ± 5*
DMSO	47 ± 5	48 ± 7	46 ± 8	34 ± 4*	32 ± 6*
PTN	48 ± 8	46 ± 5	42 ± 7	35 ± 5*	33 ± 5*
PTN + SWOP	45 ± 12	54 ± 12	46 ± 5	33 ± 5*	28 ± 5*

Values are means ± SD. The hemodynamics in the five experimental groups at the baseline, during CAO, and at reperfusion showed no between-group differences

HR heart rate, *MAP* mean arterial blood pressure, RPP rate-pressure product, *I/R* ischemia and reperfusion, *SWOP* sevoflurane preconditioning, *DMSO* dimethylsulfoxide, *PTN* parthenolide

* $P < 0.05$ versus baseline

the experimental groups (Table 2). Compared with the infarct size in the I/R group (47 ± 7 %), the size was significantly reduced in the SWOP group (30 ± 3 %, $P < 0.05$, Fig. 2). This infarct-limiting effect was abrogated by pretreatment with PTN given 15 min before sevoflurane exposure (48 ± 5 %, $P > 0.05$ PTN + SWOP vs. I/R). However, DMSO or PTN alone had no effect on infarct size (51 ± 5 and 49 ± 9 %, respectively, both $P > 0.05$ vs. I/R).

Sevoflurane preconditioning induced autophagosome formation

Some animals in the CON and SWOP groups ($n = 3$/group) were used to detect the typical morphological features of autophagosomes. As shown in the images in Fig. 3, ultrastructural analysis of the myocardium in the CON group showed cells with a round shape, and they contained normal-looking organelles, nuclei, and chromatin. After sevoflurane preconditioning, the cells were found to contain many vesicles with the typical morphological features of autophagosomes; a number of isolated double-membrane structures were observed in the cytoplasm, and these membrane structures had engulfed cytoplasmic fractions and organelles to form double or multimembraned autophagosomes.

NF-κB DNA-binding activity

Five rats per group were used to detect NF-κB DNA-binding activity before I/R. As shown in Fig. 4, a significant increase in NF-κB DNA-binding activity was detected in the nuclear extracts of the SWOP (143 ± 7 %) and SEVO groups (150 ± 3 %) when compared with the activity in the CON group (100 %) ($P < 0.05$, $n = 5$/group, respectively). This NF-κB activation was blocked by PTN administered before sevoflurane exposure (36 ± 9 %, $P < 0.05$ PTN + SWOP vs. CON), though PTN (66 ± 4 %) or DMSO (87 ± 6 %) alone had significantly decreased NF-κB DNA-binding activity.

Western blot analysis

Five rats per group were used to detect the expression of proteins before I/R and at the end of 2-h reperfusion. As shown in Fig. 5, compared with the CON group (100 %),

Table 2 AAR and infarct size weights in the treatment groups

	Left ventricle (mg)	Area at risk (mg)	Infarct size (mg)	Area at risk/left ventricle (%)	Infarct size (% of area at risk)
I/R	613 ± 147	258 ± 42	122 ± 20	42 ± 6	47 ± 7
SWOP	652 ± 25	245 ± 65	72 ± 24*	37 ± 8	30 ± 3*
DMSO	586 ± 26	226 ± 42	115 ± 21	38 ± 6	51 ± 5
PTN	646 ± 48	257 ± 25	126 ± 31	39 ± 6	49 ± 9
PTN + SWOP	612 ± 53	252 ± 46	120 ± 26	41 ± 8	48 ± 5

Values are means ± SD, $n = 6$ animals per group. The infarct size was significantly reduced in the SWOP group compared with that in the I/R group; this infarct-limiting effect was abrogated by pretreatment with PTN before sevoflurane exposure. However, DMSO or PTN alone had no effect on infarct size

I/R ischemia and reperfusion, *SWOP* sevoflurane preconditioning, *DMSO* dimethylsulfoxide, *PTN* parthenolide

* $P < 0.05$ versus I/R group in the Tukey analysis

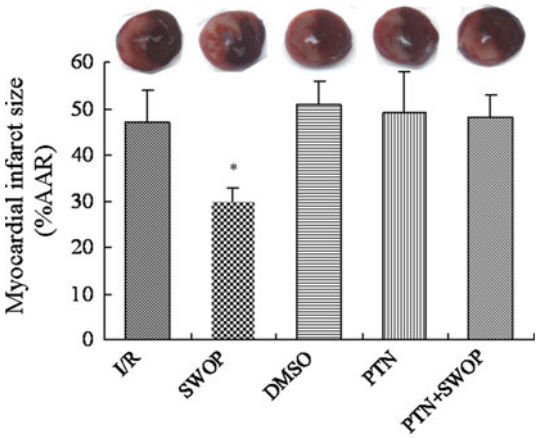

Fig. 2 The effect of delayed sevoflurane preconditioning during I/R. Infarct size (IS) is expressed as a percentage of the area at risk (*AAR*). The myocardial IS was reduced in the SWOP group compared with that in the I/R group; this infarct-limiting effect was abrogated by pretreatment with PTN (500 μg/kg, i.p.) given 15 min before sevoflurane exposure. However, DMSO or PTN alone had no effect on IS. *$P < 0.05$: statistically significant. *I/R* ischemia and reperfusion, *SWOP* sevoflurane preconditioning, *DMSO* dimethylsulfoxide, *PTN* parthenolide

rats preconditioned with sevoflurane exhibited an increase in the expression of myocardial LC3-II and cathepsin B before I/R (294 ± 33 and 161 ± 13 %, respectively, both $P < 0.05$ vs. CON). The expressions of myocardial LC3-II and cathepsin B before I/R in the SEVO group were also increased when compared with those in the CON group (318 ± 49 and 153 ± 9 %, respectively, both $P < 0.05$ vs. CON, Fig. 5a, b). After sevoflurane preconditioning, the increase in the expressions of TNF-α, IL-1β, and caspase-3 at the end of 2-h reperfusion (142 ± 15, 135 ± 8, and 242 ± 15 %) were blunted when compared with findings in the I/R group (294 ± 62, 257 ± 25,and 396 ± 62 %, respectively, all $P < 0.05$ vs. I/R); however, PTN administered before sevoflurane exposure abolished this effect (338 ± 75, 243 ± 23, and 354 ± 27 %, $P > 0.05$ vs. I/R, as shown in Figs. 5c, d, and 6).

Discussion

This study demonstrated that delayed APC with sevoflurane conferred a significant cardioprotective effect in an in vivo rat model by decreasing myocardial infarct size and reducing inflammation and apoptosis. This beneficial effect may have been mediated by the activation of the transcription factor NF-κB and the upregulation of autophagy, as evidenced by increased NF-κB DNA-binding activity and the promotion of autophagosome formation. Administration of the NF-κB inhibitor PTN before sevoflurane exposure not only blocked its DNA-binding activity, but also prevented autophagic protein expression and abolished the delayed cardioprotection. This indicates that the activation of NF-κB and upregulation of autophagy before I/R are essential steps for the development of sevoflurane-induced delayed APC.

NF-κB is an important inducible transcription factor that can drive a variety of protein expressions to attenuate apoptosis [21]. Pharmacological activation of NF-κB, via opiods [22], isoflurane [23], or adenosine [24], has been shown to be protective with subsequent I/R. ROS and nitric oxide have been demonstrated to mediate the phosphorylation of IκB and the activation of NF-κB before I/R, and when the generation of ROS and nitric oxide was inhibited, NF-κB binding activity was abrogated and myocardial function was impaired [10, 23]. Consistent with our hypothesis, the present data show that the delayed APC activated NF-κB, and that the use of the pharmacological inhibitor PTN, which can inhibit IκB kinase (IKK) activity [25] and the nuclear translocation of NF-κB [19], abolished the activation of NF-κB-DNA binding activity. In our study, when NF-κB activation was inhibited, the infarct size was significantly increased compared with that in the SWOP group, and the effect of delayed APC was abrogated, though sevoflurane exposure alone blunted the increase in inflammation mediators and apoptosis compared with findings in the I/R group. Our results support the

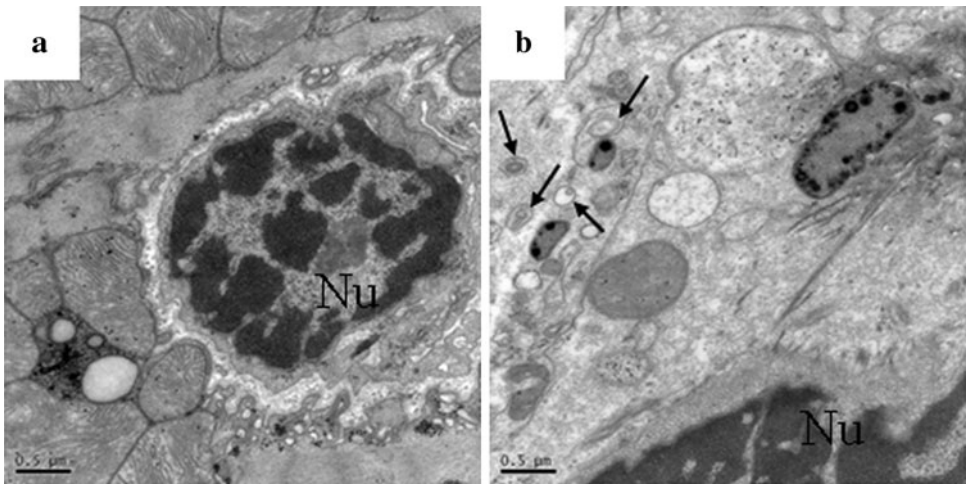

Fig. 3 The delayed sevoflurane preconditioning induced autophagosome formation before I/R. Electron microscopy images show normal ultrastructural features of control heart (**a**), and heart preconditioned with sevoflurane (**b**). **b** The characteristic ultrastructural morphology of autophagy is shown: isolated double-membraned and multimembraned autophagosomes have engulfed the cytoplasm fraction, and organelles are distributed throughout the cytoplasm. *Nu* nucleus. Autophagosomes are indicated by *arrows*

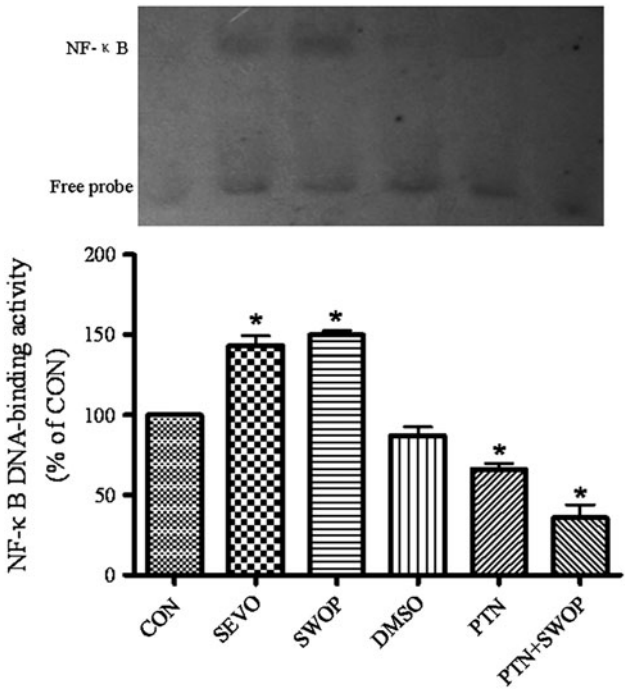

Fig. 4 Effect of delayed sevoflurane preconditioning on nuclear factor kappa B (*NF-κB*)-DNA binding activity before I/R. Results of electrophoretic mobility shift assay showing NF-κB-DNA binding activity in nuclear extracts of myocardial samples obtained 24 h after exposure to sevoflurane in the presence or absence of PTN. The figure shows that the NF-κB-DNA binding activity in the SWOP and SEVO groups was significantly increased when compared with that in the CON group. This NF-κB activation was blocked by PTN administered before sevoflurane exposure. Values are means ± SD, $n = 5$ animals per group. $^*P < 0.05$: statistically significant. *CON* control, *SWOP* sevoflurane preconditioning, *SEVO* sevoflurane control, *DMSO* dimethylsulfoxide, *PTN* parthenolide

hypothesis that NF-κB activation is a critical element in delayed APC-induced cardioprotection. Also, in concert with prior data showing that the acute phase of APC could activate NF-κB [18], our present findings further demonstrate that the activation of NF-κB plays an important role in the delayed phase of APC-induced cardioprotection. This effect may be related to the increased expression of the antiapoptotic protein Bcl-2 and a resultant decrease in cytochrome c release and caspase-3 degradation; as we know, modest NF-κB activation promotes Bcl-2 synthesis [10, 18].

When we observed the morphological features of cardiocytes with a transmission electron microscope (TEM), we occasionally found that more autophagosomes had formed in sevoflurane-preconditioned cardiocytes than in unpreconditioned cardiocytes. As mentioned before, autophagy can also affect the death of cells, and, in order to further confirm whether sevoflurane modulated autophagy, we examined the levels of LC3-II and cathepsin B protein expression. LC3-II has been suggested as a marker for autophagy induction and cathepsin B is a lysosome-related protein known to mediate autophagy [26]. In the present study, LC3-II and cathepsin B expressions were significantly increased in the SWOP group when compared with findings in the CON group. As shown with the TEM, many autophagosomes had formed after sevoflurane exposure, and as autophagosomes inside cells can be used as an indicator to analyze the induction of autophagy, this finding supports the idea that sevoflurane exposure promotes the occurrence of autophagy. Autophagic protein expression was effectively blocked by injecting PTN before sevoflurane exposure, indicating that NF-κB modulates the

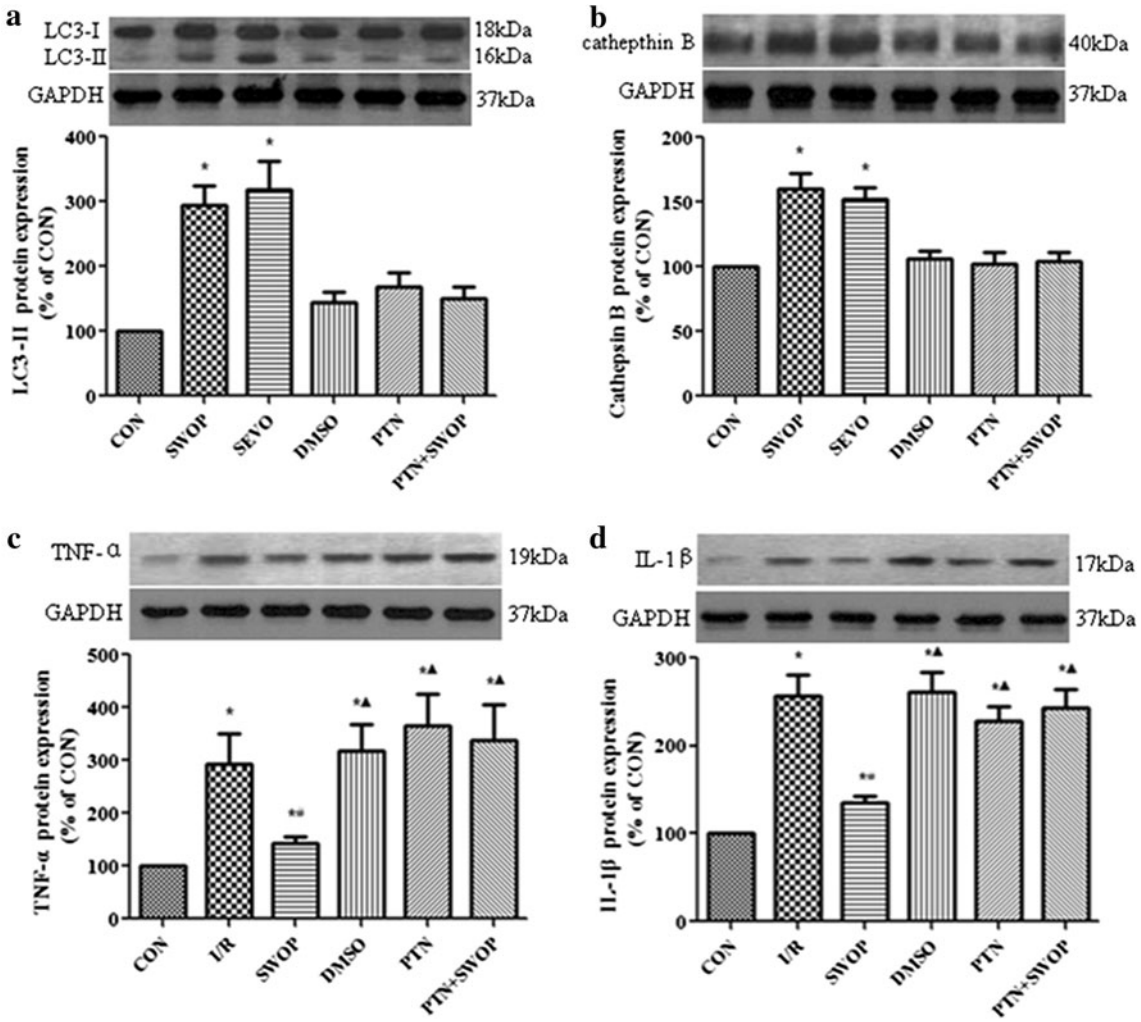

Fig. 5 Representative immunoblots of LC3, cathepsin B in rats before I/R and *TNF-α*, *IL-1β* in rats after reperfusion in each group. The figure shows that sevoflurane preconditioning increased the expression of myocardial LC3-II (**a**) and cathepsin B (**b**) before I/R compared with the CON group, but after sevoflurane preconditioning, the expressions of TNF-α (**c**) and IL-1β (**d**) at the end of 2 h reperfusion were blunted their increase when compared with I/R group. However, PTN administered before sevoflurane exposure reverseded this effect. Values are mean ± SD, $n = 5$ animals per group. $^{*}P < 0.05$ versus CON group in the Tukey analysis. $^{\blacktriangle}P < 0.05$ versus SWOP group. $^{\#}P < 0.05$ versus I/R group. $P < 0.05$: statistically significant. *CON* control, *I/R* ischemia and reperfusion, *SEVO* sevoflurane control, *SWOP* sevoflurane preconditioning, *DMSO* dimethylsulfoxide, *PTN* parthenolide

autophagic protein expression and that autophagy may play an important role in reducing infarct size and apoptosis. Recently Comb et al. [27] have shown that mammalian autophagy required the induction of IKK; autophagy was not induced in the absence of one of the subunits of IKK catalysis. Likely, IKK plays multiple roles in regulating NF-κB-dependent antiapoptosis, as well as in the induction of autophagy-related genes; however, IKK activity, but not NF-κB, induces the expression of the proautophagic gene LC3. PTN is an inhibitor that plays a role in inhibiting IKK activity; similar to the findings of the study by Comb et al. [27], we found that PTN inhibited the NF-κB binding activity, along with its inhibition of the induction of autophagy.

There are some possibilities that sevoflurane preconditioning induces autophagy via the AMP kinase (AMPK) signal pathway and the Bcl-2 family. The AMPK pathway is one of the most important approaches in the upregulation of autophagy [28, 29]; moreover, it has been proven that the AMPK pathway is involved in sevoflurane-induced cardioprotection [30], indicating that sevoflurane preconditioning activates AMPK; thus, AMPK may activate the occurrence of autophagy. In addition, sevoflurane preconditioning may also upregulate Bcl-2 before I/R [10]. However, Bcl-2 interacts with beclin-1 and eventually inhibits autophagosome formation [31]. A possible reason for this inhibition is that autophagy induction relies on the extent of the regulation of different proteins.

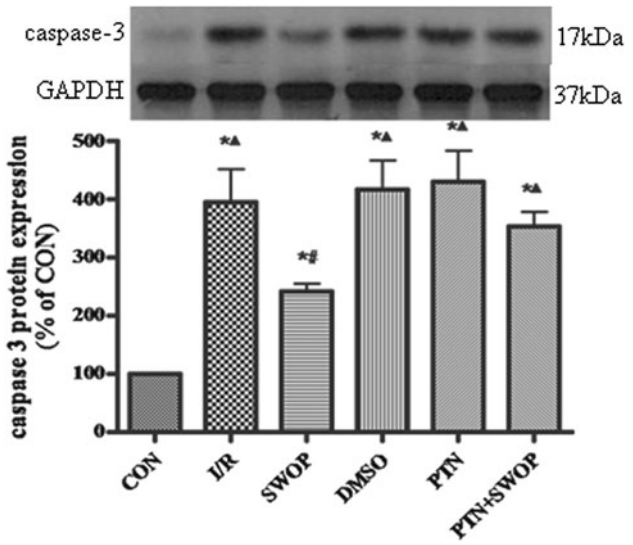

Fig. 6 Representative immunoblots of cleaved caspase-3 in in each group of rats after reperfusion. After sevoflurane preconditioning, the increase in the expression of caspase-3 at the end of 2-h reperfusion was blunted when compared with that in the I/R group. Values are means ± SD, $n = 5$ animals per group. $^{*}P < 0.05$ versus CON group in the Tukey analysis. $^{\blacktriangle}P < 0.05$ versus SWOP group. $^{\#}P < 0.05$ versus I/R group. $P < 0.05$: statistically significant. *CON* control, *I/R* ischemia and reperfusion, *SWOP* sevoflurane preconditioning, *DMSO* dimethylsulfoxide, *PTN* parthenolide

Of note, we found that, in parallel with NF-κB activation and the increase of autophagic protein expression by delayed APC, inflammatory mediators (TNF-α, IL-1β) and a marker of apoptosis [caspase-3 degradation]) were reduced and restored by the transient inhibition of NF-κB. As we know, I/R causes an intense increase in the production of proinflammatory cytokines which impair myocardial function [32]. The mechanisms of this impairment include direct cytotoxicity, oxidant stress, calcium dyshomeostasis, and cell apoptosis [33]. The present study demonstrates that NF-κB activation before I/R has a profound anti-inflammatory and antiapoptotic effect, and this finding parallels the findings in the acute phase of APC [18, 32]. Also, the role of autophagy upregulation in the antiapoptotic effect may be seen through the recycling of amino acids and the removal of damaged organelles, thus reducing oxidative stress and allowing cellular remodeling for survival, preventing or delaying the occurrence of cell apoptosis [8].

There are some limitations of this study. First of all, we did not specifically use the inhibitor of autophagy, 3-MA, because the pharmacokinetics and pharmacodynamics of 3-MA are not well known, and it is difficult to exclude the influence of this inhibitor itself. Second, we did not assess the degree of NF-κB activation on reperfusion, because prior studies have shown that sevoflurane preconditioning and PTN alone reduced NF-κB activation during I/R [32, 34]. In addition, we focused solely on the genes of two inflammatory mediators (TNF-α and IL-1β) for inflammation, and caspase-3 for apoptosis; though there are many NF-κB-regulated inflammatory and proapoptotic genes, these are the most common ones [32].

Conclusions

In conclusion, our study demonstrates that delayed sevoflurane preconditioning protected the rat heart from I/R injury, and the underlying mechanism may include NF-κB activation, upregulation of autophagy before I/R, and attenuation of the expressions of TNF-α, IL-1β, and caspase-3 at the end of reperfusion.

Acknowledgments This work was supported by Grant No. 30872453 (to Dr. Wang) from the National Natural Science Foundation of China, and by Grants No. SYS201130 (to Dr. Wang) and No. SYS201038 (to Dr. Xie) from the Technology Bureau of Suzhou, China. Dr. Wang also received support from the Revitalizing the Key Talent's Subsidy Project in Science and Education, Jiangsu Province, China).

Conflict of interest None of the authors has any conflict of interest.

References

1. Kersten JR, Schmeling TJ, Pagel PS, Gross GJ, Warltier DC. Isoflurane mimics ischemic preconditioning via activation of K(ATP) channels: reduction of myocardial infarct size with an acute memory phase. Anesthesiology. 1997;87:361–70.
2. Wang C, Weihrauch D, Schwabe DA, Bienengraeber M, Warltier DC, Kersten JR, Pratt PF Jr, Pagel PS. Extracellular signal-regulated kinases trigger isoflurane preconditioning concomitant with upregulation of hypoxia-inducible factor-1alpha and vascular endothelial growth factor expression in rats. Anesth Analg. 2006;103:281–8 (table of contents).
3. Lutz M, Liu H. Inhaled sevoflurane produces better delayed myocardial protection at 48 versus 24 hours after exposure. Anesth Analg. 2006;102:984–90.
4. Siracusano L, Girasole V, Alvaro S, Chiavarino ND. Myocardial preconditioning and cardioprotection by volatile anaesthetics. J Cardiovasc Med (Hagerstown). 2006;7:86–95.
5. Mangano DT, Wong MG, London MJ, Tubau JF, Rapp JA. Perioperative myocardial ischemia in patients undergoing noncardiac surgery-II: incidence and severity during the 1st week after surgery. The Study of Perioperative Ischaemia (SPI) Research Group. J Am Coll Cardiol. 1991;17:851–7.
6. Marinovic J, Bosnjak ZJ, Stadnicka A. Preconditioning by isoflurane induces lasting sensitization of the cardiac sarcolemmal adenosine triphosphate-sensitive potassium channel by a protein kinase C-delta-mediated mechanism. Anesthesiology. 2005;103:540–7.
7. Venkatapuram S, Wang C, Krolikowski JG, Weihrauch D, Kersten JR, Warltier DC, Pratt PF Jr, Pagel PS. Inhibition of apoptotic protein p53 lowers the threshold of isoflurane-induced cardioprotection during early reperfusion in rabbits. Anesth Analg. 2006;103:1400–5.

8. Nishida K, Yamaguchi O, Otsu K. Crosstalk between autophagy and apoptosis in heart disease. Circ Res. 2008;103:343–51.
9. Gurusamy N, Lekli I, Mukherjee S, Ray D, Ahsan MK, Gherghiceanu M, Popescu LM, Das DK. Cardioprotection by resveratrol: a novel mechanism via autophagy involving the mTORC2 pathway. Cardiovasc Res. 2010;86:103–12.
10. Lu X, Liu H, Wang L, Schaefer S. Activation of NF-kappaB is a critical element in the antiapoptotic effect of anaesthetic preconditioning. Am J Physiol Heart Circ Physiol. 2009;296: H1296–304.
11. Li C, Kao RL, Ha T, Kelley J, Browder IW, Williams DL. Early activation of IKKbeta during in vivo myocardial ischemia. Am J Physiol Heart Circ Physiol. 2001;280:H1264–71.
12. Ghosh S, May MJ, Kopp EB. NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. Annu Rev Immunol. 1998;16:225–60.
13. Petersen CA, Krumholz KA, Carmen J, Sinai AP, Burleigh BA. *Trypanosoma cruzi* infection and nuclear factor kappa B activation prevent apoptosis in cardiac cells. Infect Immun. 2006;74: 1580–7.
14. Shintani T, Klionsky DJ. Autophagy in health and disease: a double-edged sword. Science. 2004;306:990–5.
15. Rubinsztein DC, Gestwicki JE, Murphy LO, Klionsky DJ. Potential therapeutic applications of autophagy. Nat Rev Drug Discov. 2007;6:304–12.
16. Huang C, Yitzhaki S, Perry CN, Liu W, Giricz Z, Mentzer RM Jr, Gottlieb RA. Autophagy induced by ischemic preconditioning is essential for cardioprotection. J Cardiovasc Transl Res. 2010;3: 365–73.
17. Shi Y, Hutchins WC, Su J, Siker D, Hogg N, Pritchard KA Jr, Keszler A, Tweddell JS, Baker JE. Delayed cardioprotection with isoflurane: role of reactive oxygen and nitrogen. Am J Physiol Heart Circ Physiol. 2005;288(1):H175-84 (Epub 2004 Sep 23).
18. Wang C, Xie H, Liu X, Qin Q, Wu X, Liu H, Liu C. Role of nuclear factor-kappaB in volatile anaesthetic preconditioning with sevoflurane during myocardial ischaemia/reperfusion. Eur J Anaesthesiol. 2010;27:747–56.
19. Zingarelli B, Hake PW, Denenberg A, Wong HR. Sesquiterpene lactone parthenolide, an inhibitor of IkappaB kinase complex and nuclear factor-kappaB, exerts beneficial effects in myocardial reperfusion injury. Shock. 2002;17:127–34.
20. Xia Z, Godin DV, Ansley DM. Propofol enhances ischemic tolerance of middle-aged rat hearts: effects on 15-F(2t)-isoprostane formation and tissue antioxidant capacity. Cardiovasc Res. 2003;59:113–21.
21. Valen G, Yan ZQ, Hansson GK. Nuclear factor kappa-B and the heart. J Am Coll Cardiol. 2001;38:307–14.
22. Frassdorf J, Weber NC, Obal D, Toma O, Mullenheim J, Kojda G, Preckel B, Schlack W. Morphine induces late cardioprotection in rat hearts in vivo: the involvement of opioid receptors and nuclear transcription factor kappaB. Anesth Analg. 2005;101:934–41 (table of contents).
23. Chen CH, Chuang JH, Liu K, Chan JY. Nitric oxide triggers delayed anaesthetic preconditioning-induced cardiac protection via activation of nuclear factor-kappaB and upregulation of inducible nitric oxide synthase. Shock. 2008;30:241–9.
24. Zhao TC, Kukreja RC. Late preconditioning elicited by activation of adenosine A(3) receptor in heart: role of NF- kappa B, iNOS and mitochondrial K(ATP) channel. J Mol Cell Cardiol. 2002;34:263–77.
25. Hehner SP, Heinrich M, Bork PM, Vogt M, Ratter F, Lehmann V, Schulze-Osthoff K, Droge W, Schmitz ML. Sesquiterpene lactones specifically inhibit activation of NF-kappa B by preventing the degradation of I kappa B-alpha and I kappa B-beta. J Biol Chem. 1998;273:1288–97.
26. Kabeya Y, Mizushima N, Ueno T, Yamamoto A, Kirisako T, Noda T, Kominami E, Ohsumi Y, Yoshimori T. LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. EMBO J. 2000;19:5720–8.
27. Comb WC, Cogswell P, Sitcheran R, Baldwin AS. IKK-dependent, NF-kappaB-independent control of autophagic gene expression. Oncogene. 2011;30:1727–32.
28. Meley D, Bauvy C, Houben-Weerts JH, Dubbelhuis PF, Helmond MT, Codogno P, Meijer AJ. AMP-activated protein kinase and the regulation of autophagic proteolysis. J Biol Chem. 2006;281: 34870–9.
29. Samari HR, Seglen PO. Inhibition of hepatocytic autophagy by adenosine, aminoimidazole-4-carboxamide riboside, and N6-mercaptopurine riboside. Evidence for involvement of AMP-activated protein kinase. J Biol Chem. 1998;273:23758–63.
30. Lamberts RR, Onderwater G, Hamdani N, Vreden MJ, Steenhuisen J, Eringa EC, Loer SA, Stienen GJ, Bouwman RA. Reactive oxygen species-induced stimulation of 5′AMP-activated protein kinase mediates sevoflurane-induced cardioprotection. Circulation. 2009;120:S10–5.
31. Pattingre S, Tassa A, Qu X, Garuti R, Liang XH, Mizushima N, Packer M, Schneider MD, Levine B. Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. Cell. 2005;122:927–39.
32. Zhong C, Zhou Y, Liu H. Nuclear factor kappaB and anesthetic preconditioning during myocardial ischemia-reperfusion. Anesthesiology. 2004;100:540–6.
33. Meldrum DR. Tumor necrosis factor in the heart. Am J Physiol. 1998;274:R577–95.
34. Konia MR, Schaefer S, Liu H. Nuclear factor-[kappa]B inhibition provides additional protection against ischaemia/reperfusion injury in delayed sevoflurane preconditioning. Eur J Anaesthesiol. 2009;26:496–503.