Sevoflurane post-conditioning protects isolated rat hearts against ischemia-reperfusion injury via activation of the ERK1/2 pathway

Hong XIE1, #, Jing ZHANG2, #, Jiang ZHU1, Li-xin LIU3, Mario REBECCHI3, Su-mei HU1, Chen WANG1, 2, *

1Department of Anesthesiology, The Second Affiliated Hospital of Soochow University, Suzhou 215000, China; 2Institute of Neuroscience, Soochow University, Suzhou 215000, China; 3Department of Anesthesiology, Stony Brook University School of Medicine, Stony Brook, NY 11794, USA

Aim: To investigate the role of extracellular signal-regulated kinases (ERKs) in sevoflurane post-conditioning induced cardioprotection in vitro.

Methods: Isolated rat hearts were subjected to 30 min ischemia followed by 120 min reperfusion (I/R). Sevoflurane post-conditioning was carried out by administration of O2-enriched gas mixture with 3% sevoflurane (SEVO) for 15 min from the onset of reperfusion. Cardiac functions, myocardial infarct size, myocardial ATP and NAD+ contents, mitochondrial ultrastructure, and anti-apoptotic and anti-oncrosis protein levels were measured.

Results: Sevoflurane post-conditioning significantly improved the heart function, decreased infarct size and mitochondria damage, and increased myocardial ATP and NAD+ content in the I/R hearts. Furthermore, sevoflurane post-conditioning significantly increased the levels of p-ERK and p-p70S6K, decreased the levels of porimin, caspase-8, cleaved caspase-3, and cytosolic cytochrome c in the I/R hearts. Co-administration of the ERK1/2 inhibitor PD98059 (20 μmol/L) abolished the sevoflurane-induced protective effects against myocardial I/R.

Conclusion: Sevoflurane post-conditioning protects isolated rat hearts against myocardial I/R injury and inhibits cell oncosis and apoptosis via activation of the ERK1/2 pathway.

Keywords: heart; myocardium; ischemia-reperfusion injury; inhalational anesthetic; sevoflurane; post-conditioning; ERK1/2; PD98059; oncosis; apoptosis

Introduction

The prevalence of ischemic heart diseases (IHDs) is continuously increasing around the world1. Patients suffering from these diseases are often at increased risk of myocardial I/R injury, which is an important reason for increasing perioperative complications and mortality2. A series of brief episodes of ischemia and reperfusion at the onset of reperfusion can reduce myocardial infarct size, known as “ischemic post-conditioning”3. However, it is difficult to apply ischemic post-conditioning to clinical practice because well-controlled, short episodes of myocardial ischemia are almost impossible to perform. Sevoflurane is a volatile anesthetic and has been widely used in clinical practice. Anesthetic post-conditioning was first reported to protect myocardium from I/R injury in 20064. In recent years, many studies, including experimental investigations5, 6 and clinical practice7–9 have demonstrated that sevoflurane post-conditioning protects the hearts against myocardial I/R injury. Although many studies have addressed anesthetic post-conditioning in recent years, the underlying mechanisms have not been fully elucidated.

Extracellular-signal regulated kinases (ERKs), members of the subfamilies of mitogen-activated protein kinases (MAPKs), are well known to modulate cellular transcriptional activities, cell proliferation, differentiation, survival and apoptosis9. ERK1/2 pathway has previously been implicated in ischemic and anesthetic post-conditioning10, 11 and plays a critical role in myocardial protection against I/R injury. Phosphorylation of ERK up-regulates expression of its downstream target p-p70S6K. ERK1/2 pathway, activated by sevoflurane post-conditioning, has been shown to regulate mitochondrial...
permeability transition thereby inhibiting caspase activation. It is believed that the mitochondrial permeability transition pore (mPTP), which is a crucial end-effector in myocardium I/R injury, opens in the first few minutes after reperfusion. Increased mPTP opening is a major cause of cardiomyocyte apoptosis and oncosis following myocardium I/R injury\(^7\)\(^8\)\(^9\)\(^6\)\(^7\).

Apoptosis is a process of programmed cell death process, and oncosis is an important non-apoptotic mode. ATP levels determine which pathway is taken, apoptosis, with normal intracellular ATP levels or oncosis, with intracellular ATP deficiency\(^7\). Caspase-8 plays an important role in caspase activation cascades, activating downstream effector caspases such as caspase-3, -6, and -7 that lead to apoptosis\(^7\). On the other hand, oncotic cell death can be mediated by porimin, a highly glycosylated protein and member of the cell membrane-associated mucin family located on the cell surface\(^15\). For example, oncosis-like cell death can be mediated by introduction of porimin cDNA into Jurkat cells\(^7\).

The mechanisms underlying the cardioprotective actions of sevoflurane post-conditioning remain unclear. Here we test the hypothesis that activation of the ERK1/2 pathway is necessary for sevoflurane post-conditioning and involves reducing oncosis protein porimin expression and inhibiting mPTP opening in the isolated perfused rat hearts subjected to global ischemia.

**Materials and methods**

Adult, healthy male Sprague-Dawley rats, weighing 280–330 g, and of healthy grade were maintained from Soochow University School of Medicine Experimental Animal Center. The animal experiments were approved by the Committee of the Medical College of Soochow University (Suzhou, China; License Number: 2002-0008 Grade II).

**Experimental protocols**

One hundred and twenty-six isolated perfused rat hearts were randomly divided into 6 groups: control group (CON), ischemia/reperfusion group (I/R), sevoflurane postconditioning group (SEVO), DMSO group (DMSO), PD98059 group (PD), and PD98059+SEVO group (PD+SEVO). Except the CON group, each rat heart was subjected to 30 min of global ischemia followed by 2 h of reperfusion. SEVO group received 2.5% (v/v) sevoflurane for 15 min at the onset of reperfusion, then reperfusion the hearts for 105 min. PD group received the ERK1/2 specific inhibitor PD98059 (20 µmol/L) was administered for 15 min at the onset of reperfusion in the PD group. Both PD98059 in DMSO and 3% sevoflurane were administered for 15 min at the onset of reperfusion in the PD+SEVO group. In a parallel set of hearts (n=6), the experiments were terminated after 15 min of reperfusion to obtain samples for NAD\(^+\) measurements. CON, control; I/R, ischemia/reperfusion; SEVO, sevoflurane post-conditioning; DMSO, dimethyl sulfoxide; PD, PD98059; ISC, ischemia.

**Infarct size determination**

Myocardial infarct size was determined as previously described, then the hearts were quickly excised and mounted on a Langendorff apparatus via the aorta for retrograde perfusion with K-H solution at constant pressure (10 kPa)\(^1\). The pump was adjusted to maintain coronary transudes to 12 mL/min.

**Cardiac functions**

Ventricular chamber pressures were taken and maintained with a cuff pressure transducer (SIA Industrial & Trade, Beijing, China). A saline-filled latex balloon was inserted into the left ventricle through the mitral valve to monitor the heart function\(^1\). The cuff volume was adjusted to achieve a stable left ventricular end-diastolic pressure (LVEDP) of 5–10 mmHg during initial equilibration. Cardiac functions, including left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP), zd/df\(_{max}\) and heart rate (HR) were recorded at 30 min of equilibration (T\(_0\)), 30 min (T\(_1\)), 60 min (T\(_2\)), 90 min (T\(_3\)), and 120 min (T\(_4\)) after reperfusion by Med Lab 6.0 software. Rats with refractory ventricular fibrillation, frequent arrhythmia, LVSP<75 mmHg or HR<180 beats/min were excluded.
described[22]. Briefly, at the end of reperfusion, the rat hearts were removed and cut into 6 pieces in cross-section. The hearts were stained with 1% 2,3,5-triphenyltetrazolium chloride triazone (TTC, Sigma-Aldrich, St Louis, MO, USA) in pH 7.4 buffer at 37°C for 20 min and fixed overnight in 10% formalin. Because TTC stains viable tissue a deep red color, unstained tissue was presumed to be infarcted. The infarct size was calculated for each slice, and reported as the percent of infarct area divided by the total area at risk by Alpha Ease FC Imaging System.

Transmission electron microscopy
Myocardial ultrastructural alterations were detected by transmission electron microscopy (TEM)[23]. Rat hearts were rapidly removed at the end of reperfusion and a small piece (1 mm³) of tissue was dissected from left ventricular and fixed in 2.5% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer (pH 7.4) for 4 h. Tissues were post-fixed in 1% osmium tetroxide in 1% KFe(CN)₆ dehydrated through graded concentrations of ethanol and propylene oxide, embedded in Epon812 and then sectioned using an ultramicrotome. Longitudinal sections were placed onto copper grids which were stained with uranyl acetate and lead nitrate, and visualized on an H-600 electron microscope (Hitachi Limited, Tokyo, Japan). Three fields were examined for each sample at ×8000 magnification.

Myocardial ATP content measurement
Myocardial ATP concentration was determined by using a bioluminescence method as described[24, 25]. A luminometric method based on the luciferin-luciferase reaction was used to quantify ATP in myocardium by using an ATP assay kit (Beyotime Institute of Biotechnology, Haimen, China). The concentration of phosphocreatine in myocardium was determined using the reverse phase-high performance liquid chromatography (RP-HPLC) methods described[26]. The phosphocreatine standard was from Sigma-Aldrich. The concentration of glycogen in myocardium was determined with a chemistry colorimetry method by using a glycogen detection kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Myocardial NAD⁺ content measurement
The left ventricular tissues were taken after 15 min of reperfusion for measuring nicotinamide adenine dinucleotide (NAD⁺) levels. NAD⁺ was extracted from left ventricular tissues at risk using perchloric acid[27]. Freeze-clamped tissue (30 mg) was powdered in a mortar and completely mixed with 150 mL 0.6 mol/L perchloric acid. Subsequently, the mixture was homogenized, neutralized with 3 mol/L KOH and centrifuged for 10 min. The centrifuged sediment was dissolved in 0.1 mol/L NaOH solution. The dilutions of the supernatant samples were removed and NAD⁺ concentrations were determined fluorometrically using alcohol dehydrogenase (Sigma-Aldrich, St Louis, MO, USA) at a wavelength of 460 nm in a Multi-frequency Phase SS K2 Spectrofluorimeter (ILC Technology Inc, Sunnyvale, CA, USA).

Mitochondrial and cytoplasm preparation and protein extraction
The left ventricular tissue was taken at the end of reperfusion and homogenized in ice-cold sucrose buffer, which contained 300 mmol/L sucrose, 5 mmol/L TES, 0.2 mmol/L EGTA. After the homogenate was centrifuged at 800×g for 10 min, the supernatant was further centrifuged at 10000×g for 15 min. The resulting pellet contained mitochondria. The supernatant after 10000×g centrifugation was centrifuged at 100000×g for 60 min and this supernatant contained the cytoplasm fraction. Total or mitochondrial protein extracts were obtained by homogenizing heart tissue samples or mitochondrial pellets in lysis buffer [20 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L NaVO₃, 1 mmol/L NaF, 2.5 mmol/L Na₃P₄O₁₀, 1% NP40, 0.1% SDS, 1 mmol/L DTT, 1 mmol/L PMSF]. The pellet was washed and resuspended in lysis buffer, and a complete proteinase inhibitor cocktail (Sigma, one tablet per 10 mL). The homogenates were vortexed under ultrasound and then centrifuged at 14000×g for 10 min at 4°C. BCA protein assay kit (Beyotime Institute of Biotechnology, Haimen, China) was used for determining protein concentrations in each fraction and the lysate was transferred to the same concentration.

Western blot analyses of proteins
Equivalent amounts (30 µg each) of protein from cytosol and mitochondria were thoroughly mixed with 2xLaemmeli buffer and then heated at 97.0°C for 5 min. Subsequently, denatured protein extracts were electrophoresed on 10% or 15% SDS-PAGE gels and then transferred to a polyvinylidene fluoride (PVDF) membrane. After blocking with 5% non-fat milk for 2 h, PVDF membranes were incubated with following primary antibodies at 4°C overnight: anti-ERK1/2 (1:1000, Cell Signaling Technology, Danvers, MA, USA), anti-p-ERK1/2 (1:1000, Cell Signaling Technology), anti-p70S6K (1:1000, Cell Signaling Technology, anti-p-p70S6K (1:1000, Cell Signaling Technology), anti-Porimin (1:1000, Sigma-Aldrich), anti-cytochrome c (1:1000, Santa Cruz, Dallas, TX, USA), anti-caspase-8 (1:1000, Santa Cruz) and anti-cleaved caspase-3 (1:1000, Santa Cruz). The primary antibody binding was detected with a secondary anti-mouse or rabbit antibody (1:5000) and visualized with enhanced chemiluminescence. Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:2000, AG019, Beyotime Institute of Biotechnology) and anti-prohibitin (PHB) (1:1000) (Cell Signaling Technology) were used as internal control for cytoplasm and mitochondria, respectively. Semiquantitative analysis of the optical densities of protein bands was performed using Image J software. Band densities obtained from p-ERK1/2 and p-p70S6K were normalized against the concentrations of t-ERK1/2 and t-p70S6K in the same samples.

Statistical analysis
Graphpad Prism 4.00 statistical software were used for statistical processing. Measurement data were expressed as mean±standard deviation (mean±SD). Data were analyzed by
Results
Cardiac functions results
Baseline hemodynamics among all groups were similar (P>0.05). Compared with equilibration, in addition to the CON group, LVSP and HR, ±dp/dt_max obviously decreased, LVEDP significantly increased during reperfusion among the experimental groups (P<0.05). LVSP, HR, and ±dp/dt_max significantly decreased and LVEDP significantly increased in the other groups compared with the CON group at 30, 60, 90, and 120 min of reperfusion (P<0.05). LVSP, HR, and ±dp/dt_max increased, LVEDP significantly decreased in the SEVO group, relative to the I/R group (P<0.05). The DMSO, PD, and PD+SEVO groups were similar to the I/R group (P>0.05) (Table 1).

Myocardial infarct size
Myocardial infarct size in the SEVO group (23.44%±1.98%) was decreased compared with the I/R group (51.97%±1.63%).

Table 1. Hemodynamic parameters.

| Group     | Baseline (T0) | 30 min (T1) | 60 min (T2) | 90 min (T3) | 120 min (T4) |
|-----------|---------------|-------------|-------------|-------------|--------------|
|           | HR (min⁻¹)    |             |             |             |              |
| CON       | 267±27        | 262±24      | 260±29      | 251±31      | 246±13       |
| I/R       | 269±12        | 228±13      | 205±13      | 178±27      | 171±12       |
| SEVO      | 259±20        | 249±20      | 219±14      | 183±11      | 181±16       |
| DMSO      | 249±27        | 205±17      | 192±23      | 184±20      | 172±12       |
| PD        | 265±30        | 214±16      | 210±18      | 180±7       | 174±22       |
| PD+SEVO   | 272±30        | 208±24      | 197±17      | 188±23      | 176±24       |
| LVEDP (mmHg) |            |             |             |             |              |
| CON       | 105±6         | 104±7       | 98±10       | 94±5        | 94±9         |
| I/R       | 115±14        | 59±9 mnemonic| 49±8 mnemonic| 46±6 mnemonic| 36±10 mnemonic|
| SEVO      | 114±5         | 90±7 mnemonic| 78±8 mnemonic| 68±9 mnemonic| 66±11 mnemonic|
| DMSO      | 116±13        | 61±3 mnemonic| 55±10 mnemonic| 44±6 mnemonic| 40±8 mnemonic|
| PD        | 113±6         | 66±7 mnemonic| 50±12 mnemonic| 39±5 mnemonic| 37±12 mnemonic|
| PD+SEVO   | 116±7         | 74±13 mnemonic| 56±14 mnemonic| 49±9 mnemonic| 37±6 mnemonic|
| LVSP (mmHg) |            |             |             |             |              |
| CON       | 6.7±1.4       | 7.0±1.3    | 8.4±1.4     | 8.5±1.7     | 8.9±1.4      |
| I/R       | 7.0±0.8       | 42.9±9.1 mnemonic| 44.5±10.7 mnemonic| 46.9±10.3 mnemonic| 50.1±9.1 mnemonic|
| SEVO      | 6.7±0.9       | 20.8±2.2 mnemonic| 23.4±5.7 mnemonic| 25.3±10.7 mnemonic| 27.9±4.3 mnemonic|
| DMSO      | 6.4±1.2       | 39.7±6.8 mnemonic| 50.8±9.1 mnemonic| 52.3±7.5 mnemonic| 54.1±9.4 mnemonic|
| PD        | 6.1±1.3       | 41.2±4.5 mnemonic| 42.4±10.6 mnemonic| 48.4±5.4 mnemonic| 52.5±10.0 mnemonic|
| PD+SEVO   | 6.3±0.8       | 41.8±6.8 mnemonic| 43.7±14.7 mnemonic| 44.6±7.8 mnemonic| 51.6±7.8 mnemonic|
| ±dp/dt_max (mmHg/s) | | | | | |
| CON       | 2785±173      | 2734±168   | 2673±105    | 2581±149    | 2589±124     |
| I/R       | 2770±105      | 2121±184 mnemonic| 1873±210 mnemonic| 1397±146 mnemonic| 1135±163 mnemonic|
| SEVO      | 2728±156      | 2499±155 mnemonic| 2123±105 mnemonic| 1902±149 mnemonic| 1823±59 mnemonic|
| DMSO      | 2726±135      | 2042±167 mnemonic| 1882±99 mnemonic| 1381±167 mnemonic| 1067±47 mnemonic|
| PD        | 2840±198      | 2059±234 mnemonic| 1903±144 mnemonic| 1240±120 mnemonic| 1100±138 mnemonic|
| PD+SEVO   | 2799±227      | 2136±165 mnemonic| 1814±196 mnemonic| 1366±83 mnemonic| 1172±127 mnemonic|
| -dp/dt_max (mmHg/s) | | | | | |
| CON       | -2684±177     | -2633±137  | -2608±232   | -2573±653   | -2509±216   |
| I/R       | -2747±180     | -1912±292 mnemonic| -1615±199 mnemonic| -1236±191 mnemonic| -1025±205 mnemonic|
| SEVO      | -2644±125     | -2498±247 mnemonic| -2095±149 mnemonic| -1884±169 mnemonic| -1616±235 mnemonic|
| DMSO      | -2633±112     | -1951±296 mnemonic| -1649±74 mnemonic| -1251±194 mnemonic| -963±216 mnemonic|
| PD        | -2683±191     | -1982±149 mnemonic| -1591±130 mnemonic| -1167±190 mnemonic| -836±159 mnemonic|
| PD+SEVO   | -2731±196     | -1821±290 mnemonic| -1660±255 mnemonic| -1178±163 mnemonic| -999±223 mnemonic|

Values are mean±SD (n=6) for each group. *P<0.05 versus T0. **P<0.05 versus CON group. ***P<0.05 versus I/R group. HR, heart rate; LVSP, left ventricular peak pressure; LVEDP, left ventricular end diastolic pressure; CON, control; I/R, ischemia/reperfusion; SEVO, sevoflurane postconditioning; DMSO, the PD98059 solvent dimethyl sulfoxide; PD, the ERK1/2 specific inhibitor PD98059.
(P<0.05); however, this was abolished by using PD98059 at PD+SEVO group (49.32%±1.82%). Infarct size in the DMSO, PD, and SEVO+PD groups were 49.60%±1.40%, 49.70%±1.39%, and 49.32%±1.82%, respectively, with no statistical differences compared to the I/R group (P>0.05) (Figure 2).

**Myocardial NAD⁺ content**
The NAD⁺ content in the other groups was much lower than the CON group (P<0.05). However, NAD⁺ loss was reduced in the SEVO group. There were no significant differences among the other groups: I/R, DMSO, PD, and PD+SEVO (P>0.05) (Figure 5).

**Western blot**
Compared with the CON group, the levels of p-ERK1/2 and levels of p-p70S6K at the end of reperfusion in the I/R group were increased (P<0.05). Compared with the I/R group, the ratios of p-ERK1/2/total and levels of p-p70S6K/total in the SEVO group were increased further (P<0.05); whereas, the ratios of p-ERK1/2/total and p-p70S6K/total in the I/R and the DMSO groups were similar (P>0.05) (Figure 6).

The levels of porimin, caspase-8 and cleaved caspase-3 in the other groups were significantly greater than that of the CON group (P<0.05). The expression of porimin, caspase-8 and cleaved caspase-3 in the SEVO group was reduced compared to the other I/R groups (P<0.05). There were no significant differences among the other groups: I/R, DMSO, PD, and PD+SEVO (P>0.05) (Figure 7 and 8).

**Discussion**
Recently, many other researchers have demonstrated that anesthetic post-conditioning is mediated through several signaling elements, including mitochondrial ATP-sensitive potassium channels (mito-KATP), mitochondrial permeability transition pore (mPTP), phosphatidylinositol-3-kinase (PI3K)-ERK1/2 signal pathway[14]. In the present study, we chose 3% sevoflurane to post-condition the isolated rat hearts in a Langendorff perfusion system[28]. Intracellular mitogen-activated protein kinases (MAPKs) signaling cascades play a key role in the pathogenesis of cardiac and vascular disease. ERK1/2, a member of the MAPKs, is concerned with the regulation of cell proliferation, differentiation, survival and apoptosis. Although most researchers have maintained that the phosphorylation of ERK1/2 played an important role in protecting against myocardial I/R injury, others have shown that ERK1/2 phosphorylation may aggravate myocardial cell injury[29]. ERK phosphorylation not only up-regulates its downstream target p-p70S6K and down-regulates the oncosis and apoptosis related protein expression, but also reduces the release of Cyt c from mitochondria into the cytosol and

**Figure 2.** Myocardial infarct sizes of all groups expressed as the percentage of infarct size normalized to the area at risk of isolated rat hearts after 2 h of reperfusion. Infarct size was determined using 1% triphenyltetrazolium chloride staining. The size of the infarcted myocardium was calculated as an area ratio (pale area/total area of the myocardium at risk). Data are presented as means±SD. n=6. bP<0.05, versus CON. CON, control; I/R, ischemia/reperfusion; SEVO, sevoflurane post-conditioning; DMSO, dimethyl sulfoxide; PD, PD98059.

**Morphology of mitochondrion with transmission electron microscopy analysis**
Using transmission electron microscopy (TEM), we found that mitochondrial volume density was similar within each groups. However, left ventricular myocytes exhibit significant morphological defects and oncosis, including altered cristae density and architecture in the other groups, relative to the CON group. Most mitochondria exhibited typical morphology in the CON group. The SEVO group had less mitochondria damage, swelling of the matrix and vacuolization of cristae. Magnification was set at ×8000 (Figure 3).

**Myocardial ATP content**
The ATP content in the CON group was significantly greater than that of the other groups (P<0.05). The ATP loss was reduced in the SEVO group compared to the I/R group (P<0.05). There were no statistical differences among the I/R, DMSO, PD, and PD+SEVO groups (P>0.05) (Figure 4).
inhibits mPTP opening.

Sevoflurane post-conditioning was abolished by using 20 µmol/L PD98059, a specific inhibitor of ERK. It suggests that activation of the ERK1/2 pathway is required for the myocardial protective effects of sevoflurane post-conditioning. We found sevoflurane increased p-ERK1/2 and p-p70S6K; whereas, PD98059 completely inhibited the activation of ERK1/2 and partially inhibited the activation of p70S6K. p-p70S6K plays a role in reducing cell apoptosis and regulating the activity of pro-apoptotic protein BAD to protect the hearts.

In addition to their role in cellular energy metabolism, mitochondria are now recognized as central players in cell death. By using TEM, we found myocardial mitochondrial injury, such as cristae swelling and vacuolization in the I/R group were more prominent than in the SEVO group which indicated that sevoflurane post-conditioning reduced myocardial oncosis. Myocardial NAD⁺ content is inversely proportional to mPTP opening, we found that sevoflurane post-conditioning reduced NAD⁺ release. This indicated that sevoflurane post-conditioning protects isolated rat hearts.
against I/R injury by inhibiting mPTP opening. ATP is the main source of cellular energy metabolism, and functional recovery after myocardial I/R puts further demands on energy supply. As expected, ATP content in the SEVO group was significantly higher than the other injury groups. Thus, we can conclude that sevoflurane post-conditioning protects cardiomyocytes by improving myocardial energy metabolism after ischemia and during reperfusion injury, thus inhibiting apoptosis. In addition, the release of Cyt c from mitochondria into the cytosol can trigger the activity of caspases, which is a key step in apoptosis[32].

By Western blot, we found that sevoflurane post-conditioning reduced cytosolic Cyt c, which confirmed that sevoflurane post-conditioning could reduce the caspase cascade reaction. Therefore, it is a strong possibility that ERK1/2 pathway is required for sevoflurane post-conditioning not only by suppressing apoptotic cascades, but also reducing oncosis signaling.

Poromin is a highly glycosylated protein that can be classified as a member of the cell membrane-associated mucin family and serves as a novel cell surface receptor mediating oncosis cell death[20]. Poromin is activated by various stressors, such as hypoxia, the lack of metabolic substrates, or rapid ATP depletion[33, 34]. When activated it quickly combines with its ligand anti-poromin mAb, resulting in membrane structural damage, accelerating oncosis[30]. Oncosis is an important non-apoptotic type of cell death. Cell oncosis leads to necrosis with karyolysis, characterized by cellular swelling, membrane blebbing and increased membrane permeability, and stands in contrast to apoptosis, which leads to necrosis with karyorrhexis and cell shrinkage[34]. Oncosis and apoptosis represent two different cell death pathways, but they could be produced by the same stimuli. It has been demonstrated that oncosis is a major contributor to ischemic cardiomyocyte death[35, 36]. Oncosis also occurs in myocardial I/R injury, and poromin plays an important role. By Western blot, we found that I/R injury increased poromin levels whereas sevoflurane reduced this increase, and this reduction was reversed by PD98059.

Figure 6. Western blot of p-ERK, t-ERK, p-p70S6K, and t-p70S6K from left ventricular samples acquired after the end of reperfusion. p-ERK/t-ERK and p-p70S6K/t-p70S6K ratios significantly increased in the SEVO group. PD98059 administration upon reperfusion abolished increased levels of p-ERK1/2 and p-p70S6K induced by sevoflurane. Data are presented as mean±SD. n=3. bP<0.05, versus CON. eP<0.05, versus I/R group. CON, control; I/R, ischemia/reperfusion; SEVO, sevoflurane post-conditioning; DMSO, dimethyl sulfoxide; PD, PD98059.

Figure 7. Western blot of poromin from left ventricular samples were acquired at the end of reperfusion. Compared with the CON group, the expression of poromin in the other groups significantly increased. Compared with the I/R groups, the expression of poromin in the SEVO group decreased. There were no obvious differences between levels in the I/R, DMSO, PD, PD+SEVO groups. Data are presented as mean±SD. n=3. bP<0.05, versus CON. eP<0.05, versus I/R group. CON, control; I/R, ischemia/reperfusion; SEVO, sevoflurane post-conditioning; DMSO, dimethyl sulfoxide; PD, PD98059.
Thus, sevoflurane post-conditioning acting through the ERK1/2 pathway inhibits porimin expression, and thereby decreases cell oncosis, as well as apoptosis.

In our study, several limitations have to be mentioned. We only examined one inhaled anesthetic, sevoflurane. Whether other volatile anesthetics protect isolated rat hearts against myocardial I/R injury through this same post-conditioning mechanism needs further study. Although the experiment strongly supports the idea that ERK1/2 pathway is necessary for sevoflurane post-conditioning, further experimentation is needed to define the specific mechanisms that underlie its cardioprotective effect. While we chose a relatively low

**Figure 8.** Western blot of cleaved caspase-3 (A) and caspase-8 (B) from left ventricular samples were acquired at the end of reperfusion. Compared with the CON group, the expression of cleaved caspase-3 and caspase-8 in the other groups significantly increased. Compared with the I/R groups, the expression of cleaved caspase-3 and caspase-8 in the SEVO group decreased. There were no obvious differences between levels in the I/R, DMSO, PD, PD+SEVO groups. Data are presented as means±SD. n=3. aP<0.05, versus CON. bP<0.05, versus I/R group. CON, control; I/R, ischemia/reperfusion; SEVO, sevoflurane post-conditioning; DMSO, dimethyl sulfoxide; PD, PD98059.

**Figure 9.** Western blot of Cyt c in cytosol (A) and mitochondria (B) from left ventricular samples were acquired at the end of reperfusion. Compared with the CON group, the expression of Cyt c in the other groups significantly increased in cytosol and obviously decreased in mitochondria (P<0.05). Compared with the I/R group, the expression of Cyt c in the SEVO group decreased in cytosol and significantly increased in mitochondria (P<0.05). There were no statistical differences among the other groups: I/R, DMSO, PD, and PD+SEVO groups (P>0.05). Data are presented as means±SD. n=3. aP<0.05, versus CON. bP<0.05, versus I/R group. CON, control; I/R, ischemia/reperfusion; SEVO, sevoflurane post-conditioning; DMSO, dimethyl sulfoxide; PD, PD98059; Cyt c, Cytochrome c.
concentration of ERK1/2 inhibitor, we cannot eliminate the possibility that other protein kinase activities may have been affected.

In summary, our findings showed that sevoflurane post-conditioning protects isolated rat hearts against myocardial I/R injury. ERK1/2/p70S6K pathway plays an important role in sevoflurane post-conditioning against myocardial I/R injury in isolated rat hearts by inhibiting the activation of oncosis and apoptosis proteins, reducing the release of Cyt c from mitochondria into the cytosol, inhibiting mPTP opening and improving myocardial energy metabolism. The ERK1/2 pathway appears to modulate not only anti-apoptotic pathways, but also anti-oncosis pathways in the isolated rat heart I/R model. Taken as a whole, our present findings provide mechanistic insights into the cardioprotection of sevoflurane post-conditioning and underscore its protection in oncosis and apoptosis where could be part of a therapeutic strategy to treat peri-operative ischemic events.

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Author contribution
Chen WANG designed the research and provided financial support; Hong XIE collected and analyzed data, detected the protein expression; Li-xin LIU and Mario REBECCHI helped design the study and compose the manuscript; Jiang ZHU modified the paper and guided scientific research; Jing ZHANG conducted studies of the morphology of mitochondria, and measured the myocardial ATP and myocardial NAD⁺ content; Su-mei HU took part in establishing the isolated rat heart model.

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