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Short title: mitochondrial permeability transition pore in sevoflurane preconditioning

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Abstract

Purpose: To assess whether sevoflurane preconditioning is associated with inhibition of mitochondrial permeability transition pore (MPTP), the effects of sevoflurane were compared with those of cyclosporine A (CsA), a known inhibitor of MPTP opening.

Methods: Isolated perfused guinea pig hearts underwent 30 min global ischemia and 120 min reperfusion (control:CTL). Sevoflurane preconditioning was elicited by administration of 2% sevoflurane for 10 min with 10 min washout before ischemia (SEVO). A preconditioning-like cardioprotection was also induced by administering CsA (0.2 μ M) for 15 min, starting 5 min before ischemia and for 10 min after the onset of reperfusion (CsA). Left ventricular developed (LVDP), end-diastolic (LVEDP) pressures, coronary flow and infarct size were measured. Expressions of Akt and glycogen synthase kinase 3 β (GSK3 β), known mediators of inhibition of MPTP opening, were determined by Western blot analysis. GSK3 β inhibition was achieved with LY294002. The effects of sevoflurane and CsA on calcium-induced MPTP opening in isolated calcein-loaded mitochondria were assessed.

Results: After ischemia-reperfusion, SEVO and CsA had higher LVDP versus CTL. Infarct size was significantly reduced in SEVO and CsA versus CTL. This was abolished by LY294002 in SEVO, but not in CsA. Akt and GSK3 β phosphorylation after reperfusion

were significantly increased in SEVO and CsA versus CTL. Ca^{2+} -induced reduction in calcein fluorescence was significantly attenuated in SEVO and CsA.

Conclusions: Preconditioning agents, sevoflurane and CsA increase the threshold of calcium-induced MPTP opening to a similar extent. This effect by sevoflurane, but not CsA is at least partially mediated by GSK3 β inactivation.

Key words: sevoflurane, mitochondrial permeability transition pore, calcein, cyclosporine A, Akt, glycogen synthase kinase 3 β

Introduction

Myocardial reperfusion is a prerequisite for salvaging ischemic myocardium in acute myocardial infarction. However, reperfusion induces burst production of reactive oxygen species (ROS) and calcium overload resulting in reperfusion injury. In this setting, mitochondria play a crucial role in myocyte survival and death (Gustafsson and Gottlieb, 2008). Recently, opening of the mitochondrial permeability transition pore (MPTP) has been identified as a crucial determinant of myocardial ischemia-reperfusion injury (Hausenloy et al., 2009; Huhn et al., 2008). Under physiological conditions, MPTP is predominantly in a closed state. The opening of MPTP is favored by calcium overload due to ischemia, ATP depletion, accumulation of inorganic phosphate and burst production of ROS upon reperfusion (Kowaltowski et al., 2001; Weiss et al., 2003). Using pharmacological inhibitors such as cyclosporine A (CsA) and NIM811, inhibition of MPTP opening during the early reperfusion period has been shown to confer cardioprotection against myocardial ischemia-reperfusion injury (Argaud et al., 2005a). CsA, an immunosuppressive drug, has been shown to attenuate MPTP opening and improve mitochondrial respiratory function in cardiomyocytes isolated from failing hearts (Sharov et al., 2007). Recently, Piot et al. reported that administration of CsA at the time of reperfusion reduced the release of creatine kinase and infarct tissue on magnetic resonance imaging in patients with acute myocardial

infarction (Piot et al., 2008). Studies have shown that inactivation of glycogen synthase kinase 3 β (GSK3 β) by phosphorylation at Ser9 inhibits MPTP opening and protects cardiomyocytes (Juhaszova et al., 2004; Tong et al., 2002). GSK3 β is inactivated by phosphatidylinositol 3-kinase (PI3K)/Akt pathway and extracellular signal-regulated kinase 1/2 (Cohen and Frame, 2001). Recent studies have found that the volatile anesthetics such as isoflurane and sevoflurane induce cardioprotection by preconditioning and postconditioning (Bouwman et al., 2010; Inamura et al., 2010; Inamura et al., 2009; Kaneda et al., 2008; Okusa et al., 2009; Weber et al., 2005). Pravdic et al. demonstrated that isoflurane preconditioning delays MPTP opening using the tetramethyl-rhodamine ethyl ester (TMRE) technique in rat cardiomyocytes (Pravdic et al., 2009). Also, it has been reported that atractyloside, a MPTP opener, abolished cardioprotection by isoflurane postconditioning (Krolkowski et al., 2005). To date, no studies have examined whether sevoflurane preconditioning is associated with inhibition of MPTP opening.

The aim of the present study was to determine whether inhibition of MPTP opening is involved in cardioprotection by sevoflurane preconditioning and to examine the effects of sevoflurane preconditioning on calcium-induced MPTP opening in isolated calcein-loaded mitochondria from guinea pig hearts. In addition, to obtain the cardioprotective effects of sevoflurane were compared with those of CsA, a powerful inhibitor of MPTP opening.

Materials and methods

This study was conducted in accordance with the Guidelines for Animals Research at Osaka Dental University, and with the approval of the Animal Experiment Committee of Osaka Dental University (No. 10-04008), Osaka, Japan. These guidelines conform to those laid out in the Guide for the Care and Use of Laboratory Animals, available from the National Academy of Science. Male Hartley guinea pigs (Keari Co., Ltd., Osaka, Japan) initially weighing 210-260g were fed Lab Diet guinea pig food (RC4TM; Oriental Yeast, Tokyo, Japan) and given water *ad libitum* for 8 weeks.

Isolated heart perfusion and measurement of function

Male guinea pigs weighing 650-700g (12-13 weeks old) were given heparin (1000 units, intraperitoneally), then anesthetized (pentobarbital 60 mg/kg, intraperitoneally). Hearts were quickly excised and immediately arrested in cold iso-osmotic saline containing 20 mmol/L KCl. The isolated hearts were cannulated via the aorta and perfused at 70 mmHg on a nonrecirculating isovolumic perfused heart apparatus, using a Krebs-Henseleit (KH) perfusate (mmol/L): 118 NaCl, 4.0 KCl, 2.52 CaCl₂, 24.8 NaHCO₃, 1.7 MgSO₄, 1.2 KH₂PO₄, 11.0 glucose, 0.5 ethylenediamine-N,N,N',N',-tetraacetic acid (EDTA) and 8 units/L. The perfusate was insufflated continuously with 95% O₂/5% CO₂ (pH: 7.40±0.07,

PO_2 : 515 ± 20 mmHg, PCO_2 : 38.2 ± 1.8 mmHg), and was filtered through stainless steel membranes with a pore size of $4.0 \mu\text{m}$ to remove particulate matter. Hearts were paced at $240 \text{ beats} \cdot \text{min}^{-1}$ using two Needle Electrodes (NE-224S; Nihon-Kohden, Tokyo, Japan) connected to stimulus generator (SD-5; Grass Instruments, Quincy, MA, USA). Left ventricular developed pressure (LVDP; mmHg) was measured using a 2.5 French, Mikro-Tip[®] catheter transducer (SPR-524; Millar Instruments, Inc., Houston, TX, USA) passed into a compliant latex balloon. The LV balloon was connected to a Y-adaptor, one end of which was used to advance the micromanometer to the latex balloon. The other end of the Y-adaptor was used to fill the LV balloon with bubble-free water to set the left ventricular end-diastolic pressure (LVEDP) at 10 mmHg. LV pressure was recorded on a PowerLab 2/20 Data Recording System (ADInstruments, Hayward, Australia). Coronary flow (CF) was measured by collecting effluent from the right ventricular outflow tract. Global ischemia was achieved by clamping the aortic inflow line. Ventricular pacing was discontinued during ischemia and was resumed after 2 min of reperfusion. Temperature of the heart was continuously monitored by a digital thermometer (PTW-100A; Unique Medical, Tokyo, Japan). During ischemia, hearts were maintained at 37°C by enclosure in a water-jacketed air chamber. Warmed perfusate kept in the lower part of the chamber saturated the air with humidity and prevented cooling by evaporation.

Experimental Protocol

Figure 1 shows the experimental protocol (n=8 for each group). Animals were randomly assigned to three groups. Hearts were isolated and perfused as described above. After a 20 min equilibration period, baseline LVDP, LVEDP and CF were recorded. Hearts were then subjected to 30 min global ischemia followed by 120 min reperfusion (Control group: CTL). Anesthetic preconditioning was elicited by administration of sevoflurane for 10 min (2%) with a 10 min washout period before ischemia (Sevoflurane group: SEVO). Sevoflurane was insufflated by passing the 95%O₂/5%CO₂ gas mixture through a calibrated vaporizer (ACOMA, Tokyo, Japan). Samples of coronary perfusate were collected anaerobically from the aortic cannula for measurement of sevoflurane concentration by an organic vapor sensor (VOC-101H; O.S.P. Inc., Saitama, Japan). To investigate the effects of cyclosporine A (CsA; Cayman chemical, Ann Arbor, MI, USA), CsA (0.2μM) was administered for 15 min, starting 5 min before ischemia and for 10 min after reperfusion (CsA group: CsA). To investigate the role of GSK3β, its upstream- PI3K inhibitor, LY294002 (Cayman Chemical, Ann Arbor, MI) was administered before and throughout the reperfusion period as shown in Figure 1 (CTL+LY, SEVO+LY, CsA+LY). LY294002 was dissolved in ethanol (0.03%), and added to the KH perfusate to a final concentration of 15 μM.

Determination of infarct size

At the end of 120 min reperfusion, the hearts were quickly removed from the Langendorff apparatus and were frozen at -80°C for 15 min. They were then sliced into 2 mm thick transverse sections from apex to base (6 slices/heart). After removing the right ventricle and defrosting, each slice was weighed and incubated at 37°C with 1% 2,3,5-triphenyltetrazolium chloride (TTC) (Sigma, St. Louis, MO, USA) in phosphate buffer at pH 7.4 for 10 min and was fixed in 10% formalin for at least 5 h to distinguish clearly red-stained viable tissue from pale, unstained necrotic tissue (Fishbein et al., 1981). Each slice was photographed and the area of the necrotic myocardium was determined using digital imaging software (Adobe Photoshop[®] CS; Adobe, CA, USA). The area was then multiplied by the weight of the slice, then expressed as a fraction of the left ventricle of each heart.

Western blot analysis

To examine Akt, GSK3 β and their phosphorylation state over time, separate experiments were performed on 5 groups of 4 animals each. For this purpose, the myocardial tissue samples were collected at 5 min after reperfusion. They were frozen in liquid nitrogen and stored at

-80°C until use. Tissue samples were homogenized in ice-cold homogenizing buffer containing in mM: 250 sucrose, 20 N-(2-hydroxyethyl)-PiPerazine-N'-2-ethanesulfonic acid (HEPES) (pH 7.5), 10 KCl, 2 O,O'-Bis(2-aminoethyl)ethylene-glycol-N,N,N',N',-tetraacetic acid (EGTA), 2 MgCl₂, 25 NaF, 50 β-glycerophosphate, 1 Na₃VO₄, 1 phenylmethanesulfonyl fluoride (PMSF), 1% Triton X and protease inhibitor leupeptin (10 μg/ml). The homogenate was centrifuged at 1,000g and 4°C for 5 min to obtain the cytosolic fraction of the tissue. The supernatant, containing the cytosolic fraction was centrifuged a second time at 10,000g and 4°C for 15 min. The protein concentration of the supernatants was estimated using a Bradford assay (Smart SpecTM 3000; Bio-Rad, Hercules, CA, USA). Bovine serum albumin was used as a standard. Equivalent amounts (50 μg) of protein samples were loaded and separated on a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (Ready GELTM; Bio-Rad) and then electrically transferred at 4°C to a polyvinylidene difluoride membrane (Immobilon-P; Millipore Co., Billerica, MA, USA). Protein transfer was routinely ensured by staining the membrane with Ponceau. After blocking with 20% Blocking One[®] (Nacalai Tesque Inc., Kyoto, JAPAN) in Tris-buffered saline containing 0.1% Tween-20 (TBS-T), the membranes were incubated overnight at 4°C in TBS-T containing 20% Blocking One[®] and a dilution of primary antibody. The following antibodies (Cell Signaling Technology, Danvers, MA, USA) were used; phospho-GSK3β (Ser9; 1:500), GSK3β (1:1000),

phospho-Akt (Ser473; 1:500), and Akt (1:1000). Membranes were then washed three times with TBS-T for 10 min and subsequently incubated with a 1:1000 dilution of horseradish peroxidase-labeled anti-rabbit immunoglobulin G (NA 934V; GE Healthcare, Little Chalfont, NA, UK) in TBS-T containing 20% Blocking One[®]. The same blot was stripped and reblotted with antibodies to α -tubulin to confirm equal protein loading. Bound antibody signals were detected with enhanced chemiluminescence (Super Signal[®]; Pierce Biotechnology, Rockford, IL, USA) and visualized using a cooled-CCD imaging system (VersaDoc 5000[®]; Bio-Rad, Hercules, CA, USA). Quantitative analysis of the band densities was performed by image analysis software (Quantity One[®]; Bio-Rad). The average light intensity was multiplied by 100 to facilitate presentation of an x -fold increase.

Studies in isolated cardiac mitochondria

To assess MPTP opening, mitochondria were isolated from guinea pig hearts (n=4 for each group). After perfused and exposed to sevoflurane, CsA or LY294002, isolated hearts were removed from the Langendorff apparatus, and homogenized in ice-cold MSTEB buffer containing 210 mM mannitol, 70 mM sucrose, 10 mM Tris/HCl (pH7.4), 1mM EGTA 0.5mg/mL bovine serum albumin, pH7.5. After centrifugation (450g, 5 min) two-thirds of the supernatant was decanted into fresh, pre-chilled tubes. The mitochondria were isolated by

further centrifugation (5800g, 10 min). The mitochondrial pellet was re-suspended in cold MST buffer containing 210 mM mannitol, 70 mM sucrose, 10 mM Tris/HCl (pH7.4), pH7.5 and the previous centrifugation step repeated. The extracted mitochondria were diluted in ice-cold respiratory buffer (5 mg/ml) containing 250 mM sucrose, 10 mM Hepes, 2 mM K_2HPO_4 , 80 mM KCl, 2 mM Mg acetate, pH7.5 and incubated with 1 μ M calcein-AM (Invitrogen Molecular Probes, Carlsbad, CA, USA) for 15 min at room temperature. Calcein-AM readily enters the mitochondria and is trapped in the matrix in its free form, which is fluorescent. After calcein was trapped in mitochondria, the mitochondria were washed by KCl buffer containing 120 mM KCl, 5 mM N-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES), 0.1 mM $MgCl_2$, 0.2 mM Adenosine 5'-triphosphate (ATP), 10 mM sodium succinate. Calcein-loaded mitochondria were treated with 50, 150 and 250 μ M Ca^{2+} per milligram of protein, and were incubated for 10 min at room temperature. Mitochondrial fluorescences were acquired. Flow cytometric analysis was performed on FACS calibur™ (Becton Dickinson, Franklin lakes, NJ, USA). Mitochondria labeled with calcein-AM were analyzed by flow cytometry in an instrument equipped with a 488 nm excitation source.

Statistical analysis

All data are expressed as mean \pm SD. Hemodynamic data were tested for normal distribution and subsequently analyzed by a two-factor repeated-measures analysis of variance for time and treatment. If an overall difference between the variables was observed, comparisons were performed as one-way ANOVA followed by Tukey's post-hoc test for inter-group differences. Analysis of infarct size and Western blot was performed using one-way ANOVA followed by Student's t-test with Bonferroni's correction for multiple comparisons to avoid type I error. A value of $p < 0.05$ was considered statistically significant (SPSS17 for Windows, SPSS Japan, Tokyo, Japan).

Results

A total of 85 guinea pigs (48 for infarct size study, 12 for western blot and 20 for study of mitochondrial calcein fluorescence) were used in the present study. Five hearts were not used secondary to intractable ventricular fibrillation after reperfusion (two in CTL, two in SEVO+LY and one in CTL+LY). One heart was not used because of aortic rupture. If a heart was not used, an additional heart was studied. There was no significant difference in body weight among groups. The concentration of sevoflurane was 0.28 ± 0.02 mM. Sevoflurane was not detected in the effluent during the baseline, nor after discontinuation of sevoflurane. The heart (left ventricle) weights were not different among the groups.

Hemodynamics

Baseline LVDP, LVEDP and CF were similar among groups (Table 1). Administration of LY294002 transiently decreased LVDP and LVEDP (data not shown) during baseline period. Recovery of LVDP was greater in SEVO and CsA compared with CTL throughout the reperfusion period (52 ± 8 , 55 ± 10 vs. 29 ± 6 mmHg, $p < 0.01$ at 120 min of reperfusion). Administration of LY294002 abolished the improved recovery of LVDP in SEVO, but not in CsA (SEVO+LY: 34 ± 6 mmHg vs. SEVO, $p < 0.001$, CsA+LY: 44 ± 11 mmHg vs. CsA, $p = 0.17$). LVEDP significantly increased after ischemia-reperfusion compared to baseline in CTL. This

increase in LVEDP was significantly less in SEVO and CsA during reperfusion period (23 ± 5 , 28 ± 3 vs. 59 ± 15 mmHg, $p<0.01$ at 120 min of reperfusion). There was no significant difference in CF among all groups throughout the experiment. This suggests that changes in coronary flow could not account for the improved contractile recovery of SEVO and CsA (Table 1).

Infarct size

Myocardial infarct size in SEVO and CsA group was significantly reduced by approximately 50 % compared with control hearts (SEVO: $25\pm 6\%$, CsA: $27\pm 6\%$ vs. CTL: $48\pm 11\%$, $p<0.001$) (Fig. 2). Myocardial infarct size of SEVO group treated with LY294002 did not differ compared to CTL hearts (SEVO+LY: $42\pm 10\%$, $p=0.68$ vs. CTL), suggesting LY294002 abolished the infarct size limiting effect of sevoflurane preconditioning. In contrast, LY294002 did not abolish the reduction of infarct size in CsA (CsA+LY: $21\pm 8\%$, vs. CsA, $p=0.53$). LY294002 alone did not affect infarct size in CTL (CTL+LY: $37\pm 10\%$, vs. CTL, $p=0.41$).

Western blot analysis

The phosphorylation state of GSK3 β and upstream Akt at 5 min after reperfusion is

illustrated by a representative Western blot in Figure 3. Total Akt and GSK3 β were comparable in all samples. The ratio of phospho Akt to total Akt and phospho GSK3 β to total GSK3 β was significantly increased in SEVO and CsA compared with CTL. **This increase was abolished by PI3K inhibitor, LY294002 in SEVO, but not in CsA.** The increase of phospho Akt and phospho GSK3 β was not caused by unequal loading of Western blot, as shown by the detection of α -tubulin (Fig. 3B).

Mitochondrial calcein fluorescence

The mitochondrial calcein fluorescence values after treatment with Ca²⁺ were taken as the values from which any reduction in fluorescence was measured. Exposure of mitochondria to 50 μ M Ca²⁺ did not induce MPTP opening in all groups (data not shown). However, exposure of mitochondria to 150 μ M Ca²⁺ induced MPTP opening, represented by a reduction in calcein fluorescence of -54 \pm 7 % in CTL. This Ca²⁺-induced reduction in calcein fluorescence was attenuated in SEVO and CsA (-31 \pm 4%, -27 \pm 5%, respectively) (Fig. 4B). This effect was abrogated by treatment with LY in SEVO, but not in CsA (SEVO+LY:-62 \pm 14%, CTL+LY: -66 \pm 7%, CsA+LY: -27 \pm 7% vs. CTL p<0.01). Exposure of mitochondria to 250 μ M Ca²⁺ induced MPTP opening in all groups (data not shown).

Discussion

The present study confirmed the preconditioning-like effect of sevoflurane elicited by 2% sevoflurane for 10 min with 10 min washout before ischemia, and demonstrated the direct evidence for inhibition of calcium-induced MPTP opening in sevoflurane-treated isolated mitochondria. This effect was mediated by inhibition of GSK3 β through Akt activation. These effects of sevoflurane preconditioning were comparable to those of CsA, a potent inhibitor of MPTP opening, administered before ischemia and during early reperfusion period. In isolated mitochondria, the reduction of mitochondrial calcein fluorescence, which indicates calcium-induced MPTP opening, was similarly attenuated in sevoflurane and CsA treated mitochondria. Thus, it appears that sevoflurane preconditioning inhibits MPTP opening to a similar extent as CsA.

Inhibition of MPTP opening has been implicated in the mechanisms of cardioprotection by volatile anesthetics (Feng et al., 2005; Pagel et al., 2006). Griffiths et al. first described that MPTP remains closed during prolonged myocardial ischemia, but subsequently opens upon reperfusion (Griffiths and Halestrap, 1993). Ca²⁺ overload due to ischemia, ATP depletion, accumulation of inorganic phosphate and burst production of reactive oxygen species upon reperfusion favors MPTP opening in the inner membrane (Kowaltowski et al., 2001; Weiss et al., 2003). Studies have shown that inactivation of GSK3 β by phosphorylation at Ser9

inhibits MPTP opening and protects cardiomyocytes against ischemia-reperfusion injury (Juhaszova et al., 2004; Tong et al., 2002). Pharmacological inhibition of GSK3 β was induced with isoflurane and this effect was abolished by atractyloside, MPTP opener (Krolkowski et al., 2005). However, the evidence that MPTP is specifically involved in cardioprotection by volatile anesthetics remains indirect and there was no evidence of inhibition of MPTP opening by sevoflurane. In the present study, exposure of mitochondria to 150 μ M Ca²⁺ induced MPTP opening in CTL, represented by reduction in calcein fluorescence. This was significantly attenuated in sevoflurane and CsA-treated mitochondria, suggesting the direct evidence that sevoflurane and CsA similarly increase the threshold for calcium-induced MPTP opening. These findings in CTL and CsA are similar to previously reported results. For example, Argaud et al. demonstrated that in isolated rabbit mitochondria subjected to 10 min of ischemia, Ca²⁺ overload required for MPTP opening was 83 μ M Ca²⁺ (Argaud et al., 2005b). Hausenloy et al. reported that exposure of untreated mitochondria to 500 μ M Ca²⁺ opened MPTP, but not CsA-treated mitochondria in rat hearts (Hausenloy et al., 2002). The new finding in the present study is that exposure of mitochondria to 150 μ M Ca²⁺ induced MPTP opening in CTL, but not in sevoflurane preconditioning mitochondria (similar to and CsA-treated mitochondria). Exposure of mitochondria to 250 μ M Ca²⁺ induced MPTP opening in all groups.

CsA has been shown to reduce infarct size following a prolonged ischemic insult (Griffiths and Halestrap, 1993; Weinbrenner et al., 1998). The mechanism underlying this cardioprotective effect has not been completely understood. However, inhibition of MPTP opening is thought to play a pivotal role. MPTP is formed by adenine nucleotide translocase (ANT) in the inner membrane and the voltage-dependent anion channel (VDAC). Binding of cyclophilin D (CyP-D), a matrix protein, to ANT increases sensitivity of ANT to Ca^{2+} which is a trigger of MPTP opening (Juhaszova et al., 2008). CsA prevents the interaction of CyP-D with ANT resulting in inhibition of MPTP opening. However, this drug is not specific for mitochondrial CyP-D and also binds to the cytosolic cyclophilin-A which could play a protective role against ischemia-reperfusion injury by interacting with several molecular targets within the cellular survival pathways since antisense knockout of cyclophilin makes myocytes more tolerant to hypoxic damage (Crompton, 1999; Doyle et al., 1999). Yang et al. demonstrated that CsA increases both heat shock protein 70 and ERK, but decreases p38 mitogen activated protein kinase (MAPK) and jun N-terminal kinase (JNK) expression, in reducing renal ischemia-reperfusion injury (Yang et al., 2001; Yang et al., 2003). Weinbrenner et al. demonstrated that the infarct size limiting effect of CsA may be associated with inhibition of the protein phosphatase calcineurin (Weinbrenner et al., 1998). **It has been reported that CsA treatment lead to an increase in myocardial Hsp90 expression promoting**

the recruitment of Akt (Rezzani et al., 2003). In addition, Wnt/GSK3 β pathway has been implicated in the development of T cell resistance to CsA in chronic coronary bypass graft rejection (Murphy and Hughes, 2002). In the present study, phosphorylation of Akt and GSK3 β was similarly increased in both sevoflurane and CsA treated hearts, which was abolished by LY294002 in sevoflurane, but not CsA. Similarly, LY294002 abolished the infarct size limiting effect of sevoflurane preconditioning, but not CsA-induced cardioprotection. This suggests that prevention of the interaction of Cyp-D with ANT by CsA in mitochondria rather than inhibition of GSK3 β through PI3/Akt pathway in the cytoplasm plays a major role in inhibition of MPTP opening by CsA. In contrast, cardioprotection by sevoflurane is mainly mediated by inhibition of GSK3 β by phosphorylation through PI3/Akt pathway during early reperfusion period, which was abolished by PI3k/Akt inhibitor, LY294002. Sevoflurane postconditioning has been shown to protect against myocardial reperfusion injury by activating the PI3K/Akt pathway (Li et al., 2008) and ERK (Chen et al., 2008). How inactivation of GSK3 β by phosphorylation at Ser9 increases the threshold for MPTP opening remains unclear. Nishihara et al. demonstrated that binding of phospho-GSK3 β to ANT suppresses the interaction of ANT to Cyp-D in ischemic preconditioning (Nishihara et al., 2007). However, whether this mechanism is involved in sevoflurane preconditioning has not been elucidated. Further studies are needed.

Administration of LY294002 slightly decreased LVDP and LVEDP before ischemia, and LY294002 treated hearts showed relatively low pressures at 120 min of reperfusion despite of reduction of infarct size. Recently, PI3K (inhibited by LY294002) has been shown to play an important role in maintenance of blood pressure in rats (Logan et al., 2011). This could account for the low pressure in LY294002 treated hearts in the present study. However, the effects by LY294002 did not reach statistical significance.

The following study limitations should be acknowledged. The method of using isolated mitochondria exposed to high concentrations of Ca^{2+} evaluates MPTP opening susceptibility in vitro. A prior study demonstrated that the amount of Ca^{2+} required to induce MPTP opening depends on the experimental condition such as the presence of adenine nucleotides in the medium (Chalmers and Nicholls, 2003). Although there is a possibility that our results may not correctly reflect the conditions experienced by mitochondria during ischemia-reperfusion in vivo (Di Lisa and Bernardi, 2005), the primary purpose of this study was to assess the susceptibility of MPTP opening in isolated mitochondria exposed to sevoflurane versus CsA. Also, mitochondrial Ca^{2+} overload after reperfusion is not the only trigger of MPTP opening. Other triggers such as ROS during early reperfusion and accumulation of inorganic phosphate also open MPTP (Hausenloy et al., 2004). The effects of sevoflurane on these triggers for the susceptibility of MPTP opening should be addressed

in the future study.

In conclusion, sevoflurane protects the heart against ischemia-reperfusion injury by increasing the threshold of MPTP opening in a fashion similar to CsA. In sevoflurane preconditioning, this effect is mediated by GSK3 β inactivation through Akt activation.

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Figure Legends

Figure 1: Schematic illustration of the experimental protocol. All hearts were subjected to 30 min global ischemia followed by 120 min reperfusion. Anesthetic preconditioning was elicited by administration of sevoflurane (2%) for 10 min with a 10 min washout (SEVO). To block the PI3/Akt pathway, LY294002 (15 $\mu\text{mol/L}$) was administered 5 min before ischemia and throughout the reperfusion period in control, sevoflurane and CsA treated hearts (CTL+LY, SEVO+LY, CsA+LY). Tissue samples were obtained at 5 min after reperfusion. n=8 for each group; CTL=control, SEVO=sevoflurane, CsA=cyclosporine A (MPTP inhibitor), LY=LY294002 (PI3K inhibitor)

Figure 2: Infarct size as a percentage of LV. Treatment with sevoflurane and CsA reduced infarct size compared with control (SEVO: $25\pm 6\%$, CsA: $27\pm 6\%$ vs. CTL: $48\pm 11\%$, $p<0.001$). This cardioprotective effect was abolished by LY294002 in SEVO, but not in CsA (SEVO+LY: $42\pm 10\%$, CsA+LY: $21\pm 8\%$). LY294002 did not affect infarct size in CTL. Data are presented as mean \pm SD. * $p<0.05$: SEVO, CsA, CsA+LY vs. CTL, CTL+LY, and SEV+LY. CTL=control, SEV=sevoglurane, CsA=cyclosporine A (MPTP inhibitor), LY=LY294002 (PI3K inhibitor) (n=8 for each group)

Figure 3: (A) Representative western blot of phospho-Akt (p-Akt) and phospho-GSK3 β (p-GSK3 β) from left ventricular samples acquired at 5 min after reperfusion (n=4 for each group). Expression of phospho-Akt and phospho-GSK3 β was significantly increased in SEVO and CsA. Administration of LY abolished this increased expression in SEVO, but not CsA. *p<0.05: SEV, CsA vs. CTL. CTL=control, SEV=sevoflurane, CsA=cyclosporine A (MPTP inhibitor), LY=LY294002 (PI3K inhibitor)

(B) Densitometric evaluation of four experiments as the *x*-fold increase in average light density (AVI) vs CTL. The results are presented as the ratio of phospho Akt and phospho GSK3 β to total Akt and total GSK3 β , respectively. The average light intensity was multiplied by 100 to facilitate presentation of an *x*-fold increase. Data are mean \pm SD. *p<0.05: vs. CTL.

Figure 4: (A) Representative flow cytometric profile of isolated cardiac mitochondria loaded with calcein showing the effects of calcium (150 μ M) on MPTP opening as demonstrated by reductions in mitochondrial calcein fluorescence. Exposure of mitochondria to 150 μ M Ca²⁺ induced MPTP opening in CTL. This Ca²⁺-induced reduction in calcein fluorescence was attenuated in SEVO and CsA. Exposure of mitochondria to 250 μ M Ca²⁺ induced MPTP opening in all groups.

(B) Effect of calcium (150 μ M) on MPTP opening as demonstrated by reductions in

mitochondrial calcein fluorescence. Mean \pm SD percent change from control in the presence or absence of LY (LY294002: 15 μ M). * p <0.05 vs. CTL, n =4 for each group