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Sevoflurane confers additive cardioprotection to ethanol preconditioning associated with enhanced phosphorylation of glycogen synthase kinase-3β and inhibition of mitochondrial permeability transition pore opening.

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Sevoflurane Confers Additive Cardioprotection to Ethanol Preconditioning Associated with Enhanced Phosphorylation of Glycogen Synthase Kinase 3β and Inhibition of Mitochondrial Permeability Transition Pore Opening


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ABSTRACT

**Objective:** The purpose of this study was to investigate whether sevoflurane enhances moderate dose ethanol preconditioning, and whether this additional cardioprotection is associated with glycogen synthase kinase 3β (GSK3β), Akt, mammalian target of rapamycin (mTOR), 70-KDa ribosomal s6 kinase 1 (p70s6K), and/or mitochondrial permeability transition pore (MPTP) opening.

**Design:** In vitro study using an isolated heart Langendorff preparation.

**Setting:** University research laboratory.

**Participants:** Male guinea pigs (n=170).

**Interventions:** Isolated perfused guinea pig hearts underwent 30 min ischemia and 120 min reperfusion (control:CTL). The ethanol group (EtOH) received 5% ethanol in their drinking water for 8 weeks. Anesthetic preconditioning was elicited by 10 min exposure to sevoflurane (2%) in ethanol (EtOH+SEVO) or non-ethanol (SEVO) hearts. Inhibition of GSK3β phosphorylation and mTOR was achieved with LY294002 and rapamycin, respectively. GSK3β, Akt, mTOR and p70s6K expression were determined by Western blot. Calcium-induced MPTP opening was assessed in isolated calcein-loaded mitochondria.

**Measurements and Main Results:** After ischemia-reperfusion, EtOH, SEVO and EtOH+SEVO had higher left ventricular developed pressure recovery and lower end-diastolic pressure versus CTL. Infarct size was reduced in EtOH and SEVO versus CTL. EtOH+SEVO further reduced infarct size. Phosphorylation of GSK3β and Akt, but not mTOR and p70s6K, was increased in EtOH and SEVO. Phosphorylation of GSK3β, but not mTOR and p70s6K was further increased in EtOH+SEVO. EtOH and SEVO reduced calcium-induced MPTP opening. EtOH+SEVO further reduced MPTP opening.
**Conclusions:** Sevoflurane and chronic ethanol preconditioning offer additive cardioprotection. This effect is associated with enhanced GSK3β phosphorylation and inhibition of MPTP opening.

Key words: sevoflurane, preconditioning, ethanol, glycogen synthase kinase 3β, mitochondrial permeability transition pore
INTRODUCTION

Epidemiological studies have shown that mortality rates for people who regularly drink ethanol in moderation are lower than in abstainers, primarily due to decreased fatal ischemic heart disease.¹ Further, moderate ethanol consumers have lower rates of myocardial infarction compared with abstainers.² These beneficial cardiac effects may be due to pleiotropic effects of ethanol on lipids,³ platelets, and fibrinolytic activity.⁴,⁵ In addition, experimental studies have revealed that light to moderate regular ethanol consumption renders hearts more tolerant to ischemia-reperfusion injury (ethanol preconditioning)⁶-¹², similar to ischemic preconditioning¹³ in which brief episodes ischemia and reperfusion dramatically limit infarct size following prolonged ischemia. A recent study suggests that light to moderate regular ethanol consumption in the year prior to myocardial infarction is associated with reduced mortality following myocardial infarction.¹⁴ These findings suggest that light to moderate ethanol consumption not only prevents myocardial infarction but also improves survival after myocardial infarction.

Over the last 15 years, experimental and clinical studies have indicated that the use of volatile anesthetics constitutes an additional therapeutic approach in the care of patients at risk of developing perioperative cardiac complications, known as volatile anesthetic preconditioning.¹⁵ It has been shown that preconditioned myocardium can also be protected by pharmacological agents such as erythropoietin.¹⁶ volatile anesthetics¹⁷ and ethanol.¹⁸,¹⁹ We previously demonstrated that regular ethanol consumption renders hearts more tolerant to ischemia-reperfusion injury, to a degree similar to acute ischemic preconditioning.⁶ We also reported that sevoflurane enhances low dose (2.5%) ethanol-induced preconditioning through modulation of protein kinase C (PKC), mitochondrial K_{ATP} channels (mitoK_{ATP}) and
endothelial nitric oxide synthase (eNOS). However, whether sevoflurane exerts enhanced cardioprotection at higher, clinically relevant, ethanol doses (e.g., 5%) is unknown. Furthermore, the precise mechanisms which ultimately confer this additive cardioprotection have not been elucidated. Growing evidence suggests that many cardioprotective signaling pathways converge on the mitochondria to reduce cell death. Mitochondrial permeability transition pore (MPTP) opening has been proposed as a crucial determinant of ischemia-reperfusion injury.

Ge et al. demonstrated that prevention of MPTP opening by isoflurane postconditioning is eNOS dependent. Interestingly, chronic ethanol exposure has been shown to increase eNOS expression.

Studies have shown that inactivation of glycogen synthase kinase 3β (GSK3β) by phosphorylation at Ser inhibits MPTP opening and protects cardiomyocytes. GSK3β is inactivated by phosphatidylinositol 3-kinase (PI3K)/Akt and the mammalian target of rapamycin (mTOR)/p70s6K pathways. Neznanova et al. reported that acute ethanol challenge phosphorylates GSK3β in the rat prefrontal cortex. Zhou et al. demonstrated that acute ethanol exposure to H9c2 cardiac cells prevents oxidant-induced MPTP opening through inactivation of GSK3β. Inhibition of GSK3β has been also implicated in volatile anesthetic postconditioning.

These data suggest that the additive effect of sevoflurane and ethanol preconditioning could be mediated by enhanced inhibition of MPTP opening. To date, the roles of the Akt/GSK3β and mTOR/p70s6K pathways in cardioprotection induced by chronic ethanol exposure has not been studied. Thus, we hypothesized that sevoflurane enhances moderate dose (5%) ethanol-induced preconditioning and this additive cardioprotection is associated
with 1) enhanced GSK3β inactivation, 2) Akt, mTOR or p70s6K activation and/or 3) enhanced inhibition of MPTP opening. Elucidating mechanisms underlying the additive cardioprotection of ethanol and sevoflurane could lead to a drug discovery to further reduce perioperative myocardial ischemia-reperfusion injury.
METHODS

This study was conducted in accordance with the Guidelines for Animals Research and with the approval of the Animal Experiment Committee of Osaka Dental University, 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 were fed Lab Diet (RC4, Oriental Yeast, Tokyo, Japan) and given water ad libitum.

Male guinea pigs weighing 300-350g (3-4 weeks old) were initially given 1.25% ethanol in their drinking water for 1 week, 2.5% for 1 week then 5% for 8 weeks (ethanol group: EtOH). Control group was treated with drinking water for 10 weeks (CTL). After treatment, animals (550-700g, 13-14 weeks old) were given heparin (1000 units intraperitoneally), then anesthetized with pentobarbital (60 mg/kg, intraperitoneally). Hearts were excised and immediately arrested in cold iso-osmotic saline containing 20 mM KCl. The aorta was cannulated and the isolated hearts were perfused at 70 mmHg on a nonrecirculating isovolumic perfused heart apparatus, using a Krebs-Henseleit perfusate and paced at 240 beats/min as previously described. Serum ethanol levels at time of sacrifice were measured by gas chromatography (Shimadzu, Kyoto, Japan). Liver enzymes were measured to rule out possible liver tissue damage. 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 (OSP, Saitama, Japan).
Animals were assigned to one of 8 groups (n=8 each; Figure 1). After a 20 min equilibration, baseline left ventricular developed (LVDP; mmHg) and end-diastolic (LVEDP; mmHg) pressures, and coronary flow (CF; ml·min⁻¹·g⁻¹) were recorded. Control and EtOH groups were subjected to 30 min of ischemia followed by 120 min of reperfusion. Anesthetic preconditioning was elicited by administration of sevoflurane (2%) for 10 min followed by 10 min washout before 30 min of ischemia followed by 120 min of reperfusion (SEVO). To investigate whether sevoflurane enhances ethanol preconditioning, ethanol-treated hearts were subjected to the identical protocol of SEVO group (EtOH+SEVO).

To investigate the role of GSK3β, its upstream, PI3K inhibitor, LY294002 (Cayman Chemical, Ann Arbor, MI) or saline as a vehicle were administered starting 5 min before sevoflurane exposure and throughout the reperfusion period in EtOH, SEVO, EtOH+SEVO and CTL groups (EtOH+LY, SEVO+LY, EtOH+SEVO+LY, CTL+LY). LY294002 was dissolved in ethanol (0.03%), and added to the KH perfusate to a final concentration of 15 μM. To investigate the involvement of mTOR and p70s6K in EtOH hearts treated with sevoflurane, rapamycin (100nM, Sigma ALDRICHI, St. Louis, MO) or saline as a vehicle were administered starting 5 min before sevoflurane exposure and throughout the reperfusion period in CTL and EtOH+SEVO groups (CTL+Rapa, EtOH+SEVO+Rapa).

These doses of LY294002 and rapamycin were previously reported to effectively inhibit PI3K and mTOR, respectively.\textsuperscript{34,35} We performed experiments in the following order (with the laboratory investigator performing the experiments blinded to the groups): first a CTL, followed by ETOH, SEVO, ETOH+SEVO, and so forth until the first series of experiments were completed. The experiments using LY294002 and rapamycin were done in the same manner.
At the end of experiments, the hearts were quickly frozen at –80 °C for 15 min, then sliced into 2 mm thick transverse sections from apex to base (6 slices/heart). Each slice was weighed and incubated at 37 °C with 1% triphenyltetrazolium chloride (Sigma ALDRICHI) in phosphate buffer (pH 7.4) for 10 min and then fixed in 10% formalin for at least 5 h to distinguish red stained viable tissue from pale unstained necrotic tissue. Infarct size was determined using Adobe Photoshop® CS4 (Adobe, San Jose, USA) as previously described.

Separate experiments were performed (n=4 in each group) to examine expression of Akt, mTOR, p70s6K and GSK3β. For this purpose, different hearts from infarct size study were used. Myocardial tissue samples were collected at 5 min after reperfusion, and homogenized in ice-cold homogenizing buffer with protein samples and membranes prepared as previously described. After blocking with 5% skim milk in Tris-buffered saline containing 0.1% Tween-20 (TBS-T), the membranes were incubated for 2 h at 4 °C in TBS-T containing 5% milk and overnight 1:500-1000 dilution of rabbit primary antibody for phospho Akt (Ser47), mTOR (Ser2448), p70s6K (Thr389) and GSK3β (Ser9) (Cell Signaling TECHNOLOGY, Boston, USA). Membranes were incubated with a 1:1000 dilution of horseradish peroxidase–labeled anti-rabbit immunoglobulin G (NA 934V, GE Healthcare, Buckinghamshire, UK). The same blot was stripped and re-blotted with antibodies to α-tubulin (to confirm equal protein loading), total Akt, mTOR, p70s6K and GSK3β (Cell Signaling TECHNOLOGY). Bound antibody signals were detected with enhanced chemiluminescence (Pierce Biotechnology, Rockford, USA) and visualized using VersaDoc 5000 Imaging System (Bio-Rad, Hercules, USA). Quantitative analysis of the band densities was performed by Quantity One software (Bio-Rad) as previously
described.\textsuperscript{37}

To investigate the involvement of MPTP in enhanced cardioprotection, mitochondria were isolated from guinea pig hearts (n=4 for each group). For this purpose, different hearts from infarct size study were used. After perfused and exposed to sevoflurane and inhibitors, isolated hearts were removed from the Langendorff apparatus and homogenized in ice-cold MSTEB buffer as previously described.\textsuperscript{37} The extracted mitochondria were diluted in ice-cold respiratory buffer and incubated with 1μM calcein-AM (Invitrogen Molecular Probes, Carlsbad, 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 as previously described.\textsuperscript{37} Calcein-loaded mitochondria were treated with 50 and 150 μM Ca\textsuperscript{2+} per milligram of protein, and were incubated for 10 min at room temperature. Then mitochondrial fluorescences were acquired. Flow cytometric analysis was performed on FACS calibur\textsuperscript{TM} (Becton Dickinson, Franklin lakes, USA). Mitochondria labeled with calcein-AM were analyzed by flow cytometry in an instrument equipped with a 488 nm excitation source.

Statistical power analysis revealed that a sample size of n=8 would provide sufficient power (0.8) to detect a difference between mean infarct size indices of 15 % (SD=9, \(\alpha=0.05\)). A group size of n=4 was used for Western blot and calcein studies to provide a power of 0.8 to detect a difference between means of 20% (SD=10, \(\alpha=0.05\)). 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 and by Dunnett's for intra-group differences with baseline values as the reference time point. Analysis of infarct size, Western blot and mitochondrial calcein fluorescence was performed using one-way ANOVA followed by Student's t-test with Bonferroni's correction for multiple comparisons to avoid type I error. For changes within and between groups a two-tailed p value less than 0.05 was considered significant in advance. (SPSS17 for Windows, SPSS Japan, Tokyo, Japan).
RESULTS

Of a total of 170, 10 hearts were not used secondary to intractable ventricular fibrillation after reperfusion (mortality rate was not different among groups) and one heart was not used due to aortic rupture. Additional hearts were studied until each group had n=8 successful experiments. There was no significant difference in body weight among groups. Serum ethanol level at time of sacrifice was 3.5±2.7 mM. As a comparison, the minimum blood alcohol concentration associated with intoxication in humans is 8 mM (40 mg/dl or 0.04%). Ethanol was not detected in the effluent of the 10 EtOH hearts sampled after 50 min washout. There was no difference in liver enzymes levels between EtOH and CTL animals. The concentration of sevoflurane in the coronary perfusate after 10 min of exposure was 0.27±0.02 mM.

Myocardial infarct size in EtOH and SEVO were significantly reduced compared with CTL (EtOH:24±8%, SEVO:23±6% vs. CTL:41±6%, p<0.05). The administration of sevoflurane in EtOH hearts further decreased infarct size compared with EtOH alone (EtOH+SEVO:10±4%, vs. EtOH: 24±8%, p<0.05). This reduction of myocardial infarct size in EtOH+SEVO was abolished by LY294002, but not by rapamycin (EtOH+SEVO+LY:31±3%, p=0.188, EtOH+SEVO+Rapa:17±5%, p<0.05, vs. CTL). Treatment with LY294002 alone did not affect the infarct size compared with CTL, though treatment with rapamycin alone did reduce infarct size (CTL+Rapa: 27±9%, p<0.05, vs. CTL) (Fig 2).

Baseline LVDP and CF were similar among the 8 groups. Administration of sevoflurane or treatment with LY294002 and rapamycin did not significantly affect LVDP or CF before ischemia. After 120 min reperfusion, recovery of LVDP was greater in EtOH,
SEVO, EtOH+SEVO and CTL+Rapa compared with CTL. Recovery of LVDP in EtOH+SEVO hearts was abolished by administration of LY294002, but not rapamycin. Treatment with LY294002 or rapamycin alone did not adversely affect recovery of LVDP. LVEDP increased to 600% of baseline in CTL after ischemia-reperfusion. The increase in LVEDP was significantly less in EtOH, SEVO, EtOH+SEVO and CTL+Rapa compared with CTL. Of note, LVEDP in LY294002 treated groups was lower compared with CTL, but not compared with EtOH, SEVO or EtOH+SEVO. There was no significant difference in CF among all groups throughout the experiment. This suggests that changes in CF could not account for the improved contractile recovery of EtOH, SEVO, ETOH+SEVO hearts (Table 1).

Total Akt and GSK3β were comparable in all samples. The ratio of phospho Akt to total Akt and phospho GSK3β to total GSK3β was significantly increased in EtOH and SEVO compared with CTL. This increase was not caused by unequal loading of the western blot, as shown by the detection of α-tubulin. The combination of ethanol and sevoflurane enhanced this increased expression of phospho GSK3β in EtOH+SEVO. Administration of LY294002, but not rapamycin, abolished this enhanced expression of phospho GSK3β in EtOH+SEVO. Treatment with rapamycin alone increased phospho Akt and phospho GSK3β expressions. The ratio of phospho mTOR to total mTOR and phospho p70s6K to total p70s6K was not increased in CTL, EtOH and SEVO. However, it was significantly increased in EtOH+SEVO compared with other groups. This increase was abolished by rapamycin (Fig 3).

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 μM Ca\(^{2+}\) did not induce MPTP opening in any groups (Fig 4A). However, exposure of mitochondria to 150μM Ca\(^{2+}\) induced MPTP opening, represented by a reduction in calcein fluorescence of -59.3±12.1% in CTL. This Ca\(^{2+}\)-induced reduction was attenuated in EtOH and SEVO (-37.9±11.9%; p<0.05, -26.9±8.1%; p<0.05, respectively, vs. CTL) (Fig 4B). This effect was enhanced by the combination of ethanol and sevoflurane (EtOH+SEVO: -3.0±9.9%; p<0.05 vs. EtOH and SEVO), which was abrogated by treatment with LY294002 (-60.4±9.3%), but not rapamycin (-16.4±19.2%; p<0.05 vs. CTL). Treatment with rapamycin alone slightly attenuated reductions in fluorescence of CTL, but this effect did not reach statistical significance (CTL+Rapa: -48.5±9.4%) (Fig 4C).
DISCUSSION

Sevoflurane confers additive cardioprotection to moderate dose ethanol-induced preconditioning. Further, this cardioprotective effect is associated with enhanced phosphorylation/inhibition of GSK3β and inhibition of MPTP opening. We previously demonstrated that exposure to 2.5% (low dose) ethanol for 6 weeks reduces ischemia-reperfusion injury to the same degree as 5%, 10%, and 20% ethanol for 6 to 12 weeks. We also found that sevoflurane enhances low dose ethanol-induced preconditioning. The present study suggests that sevoflurane enhances cardioprotection afforded by ethanol preconditioning at a higher level of exposure, equivalent to moderate ethanol consumption. It has been reported that the combination of two preconditioning stimuli enhances cardioprotection afforded by either stimulus alone. The present study demonstrated that the combination of moderate ethanol consumption and sevoflurane exposure enhanced inhibition of MPTP opening associated with enhanced GSK3β phosphorylation through both PI3K-Akt and mTOR/p70s6K pathways.

Nishihara et al. demonstrated that erythropoietin affords additional cardioprotection to ischemic preconditioning by enhanced phosphorylation of GSK3β in vivo rabbit hearts, but involvement of MPTP opening was not investigated. Zhou et al. demonstrated that acute ethanol exposure to H9c2 cardiac cells prevented oxidant-induced MPTP opening through inactivation of GSK3β.

It is now widely recognized that MPTP opening is a major cause of myocardial cell death after reperfusion and a target for cardioprotection. Under physiological conditions, MPTP is predominantly in a closed state. The opening of MPTP is increased by calcium overload due to ischemia, ATP depletion, accumulation of inorganic phosphate and burst
production of reactive oxygen species (ROS) immediately after reperfusion. This MPTP opening results in depolarization of the mitochondrial membrane potential and matrix swelling.\textsuperscript{39,40} Although acute ethanol exposure to H9c2 cardiac cells has been shown to prevent ROS-induced MPTP opening,\textsuperscript{29} whether chronic ethanol consumption prevents calcium-induced MPTP opening has not been investigated. The present study addressed the susceptibility of MPTP opening after \( \text{Ca}^{2+} \) loading in isolated mitochondria from hearts that were chronically exposed to moderate ethanol. Furthermore, acute sevoflurane exposure to ethanol-treated mitochondria enhanced the inhibition of MPTP opening. Exposure of mitochondria to 150 \( \mu \text{M} \) \( \text{Ca}^{2+} \), not 50 \( \mu \text{M} \) \( \text{Ca}^{2+} \), induced MPTP opening in control hearts, represented by reduction in calcein fluorescence. This reduction was attenuated in mitochondria from ethanol and sevoflurane exposed hearts, and to an even greater degree in mitochondria from hearts exposed to both moderate ethanol and sevoflurane.

The threshold for MPTP opening is elevated by phosphorylation of GSK3\( \beta \) at Ser\textsuperscript{9}, which inactivates this kinase.\textsuperscript{26} In turn, GSK3\( \beta \) has been shown to be inactivated by PI3K/Akt and mTOR/p70s6K pathways.\textsuperscript{27} In the present study, ethanol or sevoflurane alone increased GSK3\( \beta \) phosphorylation through Akt activation, but not mTOR/p70s6K. Interestingly, the combination of ethanol and sevoflurane activated both pathways, leading to enhanced phosphorylation of GSK3\( \beta \). The role of mTOR in cardioprotection remains controversial. Studies have demonstrated a protective effect of rapamycin, an mTOR inhibitor.\textsuperscript{41} A recent study demonstrated that cardioprotection by ischemic preconditioning involves both mTOR and Wnt pathways, placing mTOR downstream of GSK3\( \beta \).\textsuperscript{42} In the present study, phosphorylation of both GSK3\( \beta \) and mTOR was seen in only EtOH+SEVO, which was abolished by LY294002. This suggests that PI3K/Akt and GSK3\( \beta \) are important
mediators of this additive cardioprotection. Rapamycin failed to abolish the enhanced inhibition of MPTP opening and reduction of infarct size in EtOH+SEVO. However, rapamycin has been shown to protect the heart against ischemia-reperfusion injury through Akt activation.\textsuperscript{35,41} This is consistent with the present study as seen in CTL+Rapa group. The combination of EtOH and sevoflurane may activate additional cardioprotective signaling pathways and mTOR may not be essential for inhibition of MPTP. The exact relationship between GSK3β and mTOR remains unclear, as there is currently no drug available to directly inhibit phosphorylation/inactivation of GSK3β. mTOR consists of two functionally distinct complexes, that is, mTOR complex 1 and complex 2. A component of mTOR complex 2, Rictor (rapamycin-insensitive associated protein of mTOR) has been implicated in activating cell survival.\textsuperscript{43} Recently, ethanol has been shown to increase the activity of mTOR complex 2 in myoblast.\textsuperscript{44} The potential mechanisms of enhanced cardioprotection by the combination of ethanol and sevoflurane found in this study are summarized in Figure 5.

The following study limitations should be acknowledged. First, in order to more fully mimic the clinical setting, sevoflurane should be administered throughout experiments. However, our previous study has shown that a brief exposure to the heart is as effective as continued administration of sevoflurane.\textsuperscript{32} Second, we utilized isolated mitochondria exposed to high concentrations of Ca\textsuperscript{2+} to evaluate MPTP opening susceptibility in vitro. A previous study demonstrated that the amount of Ca\textsuperscript{2+} required to induce MPTP opening depends on the experimental conditions such as the presence of adenine nucleotides in the medium.\textsuperscript{45} Although we cannot the possibility that our results may not accurately reflect the conditions experienced by in vivo mitochondria during ischemia-reperfusion,\textsuperscript{46} our primary purpose was to assess the susceptibility of MPTP opening in isolated mitochondria exposed
to ethanol and sevoflurane. Third, GSK3β is a substrate for multiple pro-survival protein kinases, including Akt, ε-PKC, extracellular signal-regulated kinase 1/2 and protein kinase G. We cannot exclude the possibility that kinases other than PI3K/Akt and mTOR/p70s6K pathways may contribute to enhanced phosphorylation of GSK3β by ethanol and sevoflurane. Fourth, Krenz et al. demonstrated that acute (as opposed to chronic) ethanol exposure fails to exert cardioprotection when ethanol is present during ischemia-reperfusion. Although not withdrawn from the drinking water before sacrifice in the present study, serum ethanol levels were low (3.5 mM) at the time of sacrifice, and no ethanol was present in the perfusate at the time of ischemia-reperfusion.

In conclusion, sevoflurane enhances cardiac preconditioning induced by moderate, as well as, low dose ethanol consumption. This effect may be mediated by preventing MPTP opening through convergence of protective signaling on GSK3β phosphorylation/inhibition. Because volatile anesthetics can be administrated with relatively low toxicity, elucidating the mechanisms underlying ethanol and volatile anesthetic-induced preconditioning could hopefully lead to therapies to reduce perioperative myocardial ischemia-reperfusion injury.
REFERENCES


**Figure legends**

Figure 1: Schematic illustration of the experimental protocol of this study. All hearts were subjected to 30 min global ischemia followed by 120 min reperfusion. Anesthetic preconditioning was elicited by administration of sevoflurane (2% or 1 MAC) for 10 min with a 10 min washout period. Ethanol-treated animals were initially given 1.25% ethanol in their drinking water for 1 week, 2.5% for 1 week then 5% for 8 weeks. Tissue samples were obtained at 10 min after reperfusion. CTL=control; EtOH=ethanol; SEVO=sevoflurane; LY=LY294002, PI3/Akt inhibitor; Rapa=rapamycin, mammalian target of rapamycin (mTOR) inhibitor.

Figure 2: Infarct size as a percentage of LV in eight groups. Treatment with sevoflurane (1 MAC) and 5% ethanol for 8 weeks equally reduced infarct size compared with control. The combination of sevoflurane and 5% ethanol conferred a further reduction of infarct size. This additional cardioprotective effect was abolished by LY294002, but not by rapamycin, in SEVO+EtOH. LY204002 treatment alone did not affect infarct size. Data are presented as mean ± SD. #p<0.05 vs. CTL, †p<0.05 vs. EtOH and SEVO

Figure 3: (A, B) Representative western blot of phospho Akt and phospho GSK3β from left ventricular samples acquired at 5 min after reperfusion (n=4 for each group). Expression of phospho Akt and phospho GSK3β was significantly increased in EtOH and SEVO. The combination of ethanol and sevoflurane enhanced this increased expression of phospho GSK3β in EtOH+SEVO. Administration of LY294002 abolished this enhanced expression of phospho GSK3β in EtOH+SEVO. However, administration of rapamycin failed to abolish this
enhanced expression. (C, D) Representative western blot of phospho mTOR and p70s6K from left ventricular samples acquired at 5 min after reperfusion (n=4 for each group). Expression of phospho mTOR and phospho p70s6K was not increased in CTL, EtOH and SEVO. However, the combination of ethanol and sevoflurane increased phospho mTOR and phospho p70s6K. Both LY294002 and rapamycin abolished this enhanced expression of phospho mTOR and p70S6K in EtOH+SEVO.

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 phosphorylation state to total protein. The average light intensity was multiplied by 100 to facilitate presentation of an x-fold increase. Data are mean±SD. *p<0.05 vs. CTL, #p<0.05 vs. EtOH and SEVO

Figure 4: Representative flow cytometric profile of isolated cardiac mitochondria loaded with calcein showing the effects of calcium on mPTP opening as demonstrated by reductions in mitochondrial calcein fluorescence. (A) Exposure of mitochondria to 50 μM Ca$^{2+}$: Reductions in mitochondrial calcein fluorescence were not seen in any groups. (B, C) Exposure of mitochondria to 150μM Ca$^{2+}$ induced MPTP opening in CTL. This Ca$^{2+}$-induced reduction in calcein fluorescence was attenuated in EtOH and SEVO. This effect was enhanced in EtOH+SEVO, which was abrogated by treatment with LY, but not rapamycin. (D) Effect of calcium (150μM) on MPTP opening as demonstrated by reductions in mitochondrial calcein fluorescence. Mean±SD percent change from control in the presence or absence of LY (15 μM) and rapamycin (100nM). *p<0.05 vs. CTL, #p<0.05 vs. EtOH, SEVO, n=4 for each group
Figure 5: Schematic diagram of the preconditioning cardioprotection conferred by ethanol and sevoflurane. Phosphorylation of GSK3β by PI3-Akt or mTOR-p70s6K pathway inhibits MPTP opening. Treatment with ethanol or sevoflurane alone activates the PI3-Akt pathway, but not the mTOR-p70s6K pathway. Simultaneous activation of these two pathways is achieved by the combination of ethanol and sevoflurane, but not by ethanol or sevoflurane alone. This activation of dual pathways enhances GSK3β phosphorylation and elevates the threshold of MPTP opening achieved by ethanol or sevoflurane alone. Rapamycin failed to abolish enhanced cardioprotection by the combination of ethanol and sevoflurane. This may be due to mTOR complex 2 which is rapamycin-insensitive. PI3K = phosphatidylinositol 3-kinase; Akt = protein kinase B; GSK3β = glycogen synthase kinase 3β; mTOR; mammalian target of rapamycin; p70S6K = 70-KDa ribosomal s6 kinase; MPTP = mitochondrial permeability transition pore.
Figure 1
Figure 2

#p<0.05 vs. CTL
Figure 3

A: GSK3β

Phospho-GSK3β, Total-GSK3β, α-tubulin

46KD

55KD

Phospho/total GSK3β

CTL  EtOH  SEVO  EtOH  EtOH  CTL  EtOH  SEVO  EtOH  +SEVO  +SEVO  +Rapa  +LY  +LY  +LY  +SEVO  +LY
B: Akt

Phospho-Akt, total-Akt, α-tubulin

60KD
60KD
55KD

Phospho/total Akt

CTL  EtOH  SEVO  EtOH  EtOH  CTL  CTL  EtOH  SEVO  EtOH
+SEVO +SEVO +Rapa +LY +LY +LY +LY +SEVO +LY
C: mTOR

Phospho-mTOR
Total-mTOR
α tubulin

Phospho/total mTOR

CTL  EtOH  SEVO  EtOH  EtOH  CTL  CTL  EtOH  SEVO  EtOH
+SEVO +SEVO +Rapa +LY  +LY  +LY  +LY  +SEVO +LY
D: p70s6K

[Diagram showing protein levels and a bar graph comparing CTL, EtOH, SEVO, EtOH + SEVO, EtOH + SEVO + Rapa, CTL + Rapa, CTL + LY, EtOH + LY, SEVO + LY, EtOH + SEVO + LY, with the y-axis labeled as 'Phospho/total p70s6K' and the x-axis listing different conditions.]