Sevoflurane Preconditioning Attenuates Myocardial Ischemia/Reperfusion Injury via Caveolin-3–Dependent Cyclooxygenase-2 Inhibition

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Background—The inhaled anesthetic sevoflurane has been demonstrated to protect against myocardial ischemia/reperfusion (MI/R) injury via mechanisms involving AMP-activated protein kinase (AMPK) and caveolin-3 (Cav-3). However, the relative contributions of AMPK and Cav-3 to sevoflurane preconditioning (SF-PreCon)–mediated cardioprotection and their precise underlying mechanisms of action remain incompletely understood.

Methods and Results—SF-PreCon (consisting of 3 cycles of 15-minute exposure to 2% sevoflurane before 30 minutes of MI) decreased MI/R injury in wild-type mice (caspase-3 activity, −29.1%; infarct size, −20.2%; and left ventricular end diastolic pressure, −33.8%). In cardiac-specific AMPKα2 dominant-negative overexpressing mice, the cardioprotective effect of SF-PreCon was largely retained (caspase-3 activity, −26.7%; infarct size, −16.7%; and left ventricular end-diastolic pressure, −25.9%; P<0.01). In contrast, SF-PreCon failed to significantly protect Cav-3 knockout mice against MI/R injury (P>0.05). SF-PreCon significantly decreased MI/R-induced superoxide generation in wild-type (−43.6%) and AMPK dominant-negative overexpressing mice (−35.5%; P<0.01) but not in Cav-3 knockout mice. SF-PreCon did not affect nicotinamide adenine dinucleotide phosphate oxidase expression but significantly inhibited cyclooxygenase-2 expression in wild-type (−38.7%) and AMPK dominant-negative overexpressing mice (−35.8%) but not in Cav-3 knockout mice.

Conclusions—We demonstrate for the first time SF-PreCon mediates cardioprotection against MI/R injury via caveolin-3–dependent cyclooxygenase-2 inhibition and antioxidative effects. (Circulation. 2013;128[suppl 1]:S121-S129.)

Key Words: caveolin ■ preconditioning ■ reperfusion injury ■ signal transduction
effect of inhaled anesthetics, such as sevoflurane, is mediated by Cav-3–dependent signaling has never been previously investigated.

Therefore, the aims of the current study were (1) to determine the cause–effect relationship (if any) between AMPK activation/Cav-3 alteration and cardioprotection after sevoflurane preconditioning (SF-PreCon); (2) to investigate the relative contribution of AMPK and Cav-3 to the cardioprotective effect of SF-PreCon; and (3) to identify the downstream signaling molecules and mechanisms responsible for sevoflurane-mediated cardioprotection.

Materials and Methods
This study was performed in adherence with the guidelines of the Institutional Animal Care and Use Committee (Shanxi Medical University and Thomas Jefferson University) in accordance with The Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No. 85-23, revised 1996).

Animals and Experimental Setup
Male cardiomyocyte-specific AMPKα2 dominant-negative overexpressing (AMPK-DN) and Cav-3 knockout (Cav-3KO) mice, along with each group’s respective wild-type (WT) littermates, were the subject of investigation. Generation, breeding, phenotype characteristics, and genotyping of these mice have been previously described.6,7 Young adult mice (6–7 weeks of age) were used in this study to avoid the prediabetic phenotype associated with Cav-3KO mice of ≥2 months.7 Before MI, animals were individually placed in a gas-tight Plexiglass anesthesia chamber. A calibrated vaporizer was connected to the chamber; either 0% (control group) or 2% sevoflurane (SF-PreCon) gas mixture was delivered. Animals randomized to SF-PreCon treatment were exposed to 3 cycles of 10-minute exposure to 2% sevoflurane interspersed with 15 minutes of washout. Animals in the control group were exposed to 3 cycles of 10-minute exposure to 0% sevoflurane interspersed with 15 minutes of washout (Figure 1A).8 Subsequently, all mice were anesthetized with 2% isoflurane, and MI was induced by temporarily exteriorizing the heart via a left thoracic incision, and a 6-0 silk suture slipknot was tied around the left anterior descending coronary artery. This is a novel surgical-induced MI procedure created by our group that is completed within 2 minutes; animals are only exposed to isoflurane for a brief period (<2 minutes).7 Slipknot release occurred after 30 minutes of MI, and myocardial reperfusion commenced for 3 hours (for all assays excluding cardiac function and infarct size) or 24 hours (for cardiac function, circulating troponin I, and infarct size assays). All assays used tissue from ischemic/reperfused regions or areas at risk (identified by Evans blue–negative staining).

Quantification of Superoxide Production and Determination of Myocardial Apoptosis, Cardiac Function, Circulating Troponin I, and Myocardial Infarct Size
Three hours after reperfusion, mice were anesthetized again with 2% isoflurane. Cardiectomy was performed. Superoxide production was determined using the lucigenin chemiluminescence method. Cardiac function and infarct size were determined using an ex vivo Langendorff perfusion system. Circulating troponin I was determined using ELISA.}

Figure 1. A, Schematic illustration of protocol used in this experiment. Sevoflurane preconditioning (SF-PreCon) improved cardiac function in wild-type (WT; B and C) and AMP kinase dominant-negative (AMPK-DN; B and D) mice subjected to myocardial ischemia/reperfusion (MI/R). Numbers in bars represent the sample size, and the bars above histograms represent SEM. **P<0.01 vs sham MI; #P<0.05 vs MI/R group. LVEDP indicates left ventricular end-diastolic pressure; and LVEF, left ventricular ejection fraction.
production was quantified via lucigenin-enhanced luminescence, and the cellular origin of reactive oxygen species was determined by dihydroethidium staining per manufacturer’s protocol (Molecular Probes, Carlsbad, CA). Myocardial apoptosis was determined by caspase-3 activity assay, as described previously.10 In animals observed for 24 hours after reperfusion, the following outcomes were measured as described previously.10 Cardiac function was determined by echocardiography (VisualSonic VeVo 770 under 2% isoflurane anesthesia) and left ventricular (LV) catheterization (via Millar 1.2F micromanometer). Myocardial infarct size was determined by Evans blue-2,3,5-triphenyl tetrazolium chloride double staining. Blood was collected for serum troponin I measurement per manufacturer’s protocol (Life Diagnostic Inc, West Chester, PA).

Adult Mouse Cardiomyocyte Culture
Adult mouse cardiomyocytes were isolated from WT and AMPK-DN mice and subjected to 3 hours of simulated ischemia and 6 hours of reoxygenation (SI/R), as described previously.11 The effect of SF-PreCon on SI/R-induced cell death was determined by lactate dehydrogenase (LDH) release after 6 hours of reoxygenation. Cellular survival rate was determined at 0, 3, and 6 hours (different plates were used) after reoxygenation as previously reported.12 SF-PreCon was performed with 1.5 mmol/L sevoflurane,13 administered in 3 cycles of 15-minute exposure before simulated ischemia.

Western Blot
Proteins were separated on SDS-PAGE gels, transferred to nitrocellulose membranes, and incubated with primary antibodies against AMPK, phosphorylated acetyl-coenzyme A carboxylase, cyclo-oxygenase-2 (COX-2), pp91 Akt (all preceding antibodies from Transduction Laboratories, San Jose, CA), and GAPDH (Cell Signaling, Danvers, MA), followed by a horseradish peroxidase–conjugated secondary antibody. Blots were developed by a Supersignal Chemiluminescence detection kit (Pierce, Rockford, IL) and visualized with a Kodak Image Station 400 (Rochester, NY). Blot densities were analyzed by Kodak 1D software.

Statistical Analysis
All values in the text and figures are presented as mean±SEM of n independent experiments. Using ANOVA, we compared the 3 groups: sham MI/R, MI/R, and SF-PreCon MI/R. Post hoc pairwise tests for certain group pairs with assessment of statistical significance were performed after Bonferroni correction of the overall significance level. Homoscedasticity was determined by the Bartlett test, and sample distribution was determined by the D’Agostino-Pearson omnibus normality test. Ps<0.05 (with Bonferroni-corrected multiple pairwise comparisons) were considered statistically significant.

Results
SF-PreCon Augments Cardiac Function, Reduces Infarct Size, and Decreases Cell Death in WT Mice Both In Vivo and In Vitro
Compared with control, SF-PreCon significantly improved cardiac function, as evidenced by increased LV ejection fraction and reduced LV end-diastolic pressure (Figure 1B and 1C), as well as reduced infarct size, decreased serum troponin I, and decreased apoptotic cell death determined by caspase-3 activation (Figure 2A and 2B). To further investigate whether SF-PreCon directly protects cardiomyocytes from ischemia/reperfusion injury or indirectly via in vivo neural-humoral factors, the effect of SF-PreCon on isolated adult mouse cardiomyocytes subjected to simulated MI/R (SI/R) was determined. SF-PreCon significantly improved cell survival and reduced LDH release after SI/R (Figure 3A and 3B), suggesting SF-PreCon directly protects cardiomyocytes from I/R injury.

Prosurvival Effect of SF-PreCon Is Largely Preserved in AMPK-DN Mice
AMPK is a prosurvival kinase. SF-PreCon has been previously demonstrated to activate AMPK, suggesting SF-PreCon may protect the heart via AMPK activation. Surprisingly, the cardioprotective effect of SF-PreCon is largely preserved in AMPK-DN mice. SF-PreCon increased LV ejection fraction, reduced LV end-diastolic pressure (Figure 1B and 1D), decreased infarct size, and reduced circulating cardiac troponin I and apoptosis (Figure 2A and 2C) in AMPK-DN mice in vivo. SF-PreCon significantly improved cell survival and reduced LDH release (Figure 3C and 3D) in adult cardiomyocytes isolated from AMPK-DN mice subjected to SI/R in vitro.

Because a cardiomyocyte-specific dominant-negative overexpression animal model was used in this study, the concern exists that AMPK signaling is not completely blocked, and residual AMPK signaling might be responsible for SF-PreCon–mediated cardioprotection in these animals. To directly address this concern, the effect of SF-PreCon on acetyl-coenzyme A carboxylase phosphorylation, the primary downstream molecule responsible for the metabolic regulation of AMPK, was determined. SF-PreCon significantly increased AMPK (Figure 4A) and acetyl-coenzyme A carboxylase phosphorylation (Figure 4B), which was completely abolished in AMPK-DN mice (Figure 4C). Together, these results indicate that a significant portion of sevoflurane-mediated cardioprotection is AMPK independent, suggesting the existence of other signaling mechanisms mediating SF-PreCon cardioprotection.

SF-PreCon–Mediated Cardioprotection Is Lost in Cav-3KO Mice
Having demonstrated that SF-PreCon–mediated cardioprotection is largely preserved in AMPK-DN mice, we further determined whether such effects are mediated by Cav-3. SF-PreCon failed to rescue cardiac function (Figures 5A) in Cav-3KO mice subjected to MI/R. Furthermore, the beneficial effect of SF-PreCon on infarct size, cardiac troponin I, and apoptosis was virtually abolished in Cav-3KO mice (Figures 5B–5D). Together, these results demonstrate that sevoflurane-mediated cardioprotection is Cav-3 dependent.

Antioxidative Effect of SF-PreCon Is Cav-3, But Not AMPK, Dependent
Because oxidative stress plays a critical role in reperfusion injury, we next determined whether SF-PreCon exerted antioxidative effect in MI/R injury and whether such effect was Cav-3 dependent. In WT mice, SF-PreCon decreased superoxide generation by 43.6% (Figure 6A and 6B). This antioxidant property is largely retained in AMPK-DN mice (~35.5%; Figure 6A and 6B). In contrast, SF-PreCon did not reduce superoxide generation after MI/R in Cav-3KO mice (Figure 6A and 6B). Together, these results demonstrate that SF-PreCon–mediated attenuation of superoxide generation is
highly dependent on Cav-3 and only partially dependent on the AMPK signaling axis.

SF-PreCon Attenuates COX-2 Production in a Cav-3–Dependent Manner

Having demonstrated that SF-PreCon reduced oxidative stress in an AMPK-independent and Cav3-dependent manner, we further attempted to identify the sources of superoxide inhibited by SF-PreCon. Our initial experimental results demonstrated that several superoxide-generating systems, including nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, mitochondria, xanthine/xanthine oxidase, and COX-2, contribute to superoxide overproduction after MI/R. However, among these superoxide-generating systems, COX-2 is the molecule most significantly inhibited by SF-PreCon. MI/R significantly increased both NADPH oxidase (Figure 7A) and COX-2 expression (Figure 7B). However, SF-PreCon significantly inhibited COX-2 but not NADPH oxidase expression (Figure 7A and 7B). Finally, SF-PreCon significantly inhibited COX-2 expression in the AMPK-DN heart (Figure 7B, middle), an effect virtually abolished in the Cav-3KO heart (Figure 7B, right). These

Figure 2. Sevoflurane preconditioning (SF-PreCon) reduced infarct size, decreased circulating troponin I, and reduced apoptotic cell death in wild-type (WT; A and B) and AMP kinase dominant-negative (AMPK-DN; A and C) mice subjected to myocardial ischemia/reperfusion (MI/R). n=13 to 15 mice/group. **P<0.01 vs sham MI; #P<0.05 vs MI/R group.
data demonstrate that SF-PreCon inhibits COX-2 production in a Cav-3–dependent, AMPK-independent manner.

Parecoxib Preferentially Inhibits Superoxide Production and Attenuates Apoptosis in Cav3−/− Mice Subjected to MI/R

The results presented above strongly suggest that inhibition of COX-2 expression and subsequent superoxide production are involved in Cav-3–dependent SF-PreCon–mediated cardioprotection. To obtain more evidence supporting this notion, an additional experiment was performed. Cav-3KO and their WT littermates were subjected to MI/R as described above. Ten minutes before reperfusion, mice were randomized to receive either vehicle or parecoxib14 (a soluble COX-2 inhibitor, 0.75 mg/kg IP). Parecoxib inhibited superoxide production and attenuated cardiac apoptosis in WT mice (Figure 8A). More importantly, although SF-PreCon failed to inhibit superoxide production and cardiac apoptosis in Cav-3KO mice (Figures 5 and 6), parecoxib effectively inhibited superoxide production and attenuated cardiomyocyte apoptosis in these animals (Figure 8B).

Discussion

AMPK controls energy metabolism. Its protective role during MI is well accepted, although its role in reperfusion injury remains debated. Previous studies have demonstrated that SF-PreCon activates AMPK, improves myocardial recovery, and ameliorates cardiac injury.15 A recent study reported that compound C, an AMPK inhibitor, abolished SF-PreCon cardioprotection in the isolated perfused heart, suggesting...

Figure 3. Sevoflurane preconditioning (SF-PreCon) reduced simulated ischemia/reperfusion-induced cell death as determined by LDH release (A) and cell survival (B). Representative cellular images presented in B and D were taken after 6 hours of reoxygenation. n=12 to 15 dishes/experimental condition using cells isolated from ≥8 different animals. **P<0.01 vs sham simulated ischemia/reoxygenation (SI/R); #P<0.05 vs SI/R group. AMPK-DN indicates AMPK kinase dominant-negative mice; LDH, lactate dehydrogenase; and WT, wild type.

Figure 4. Sevoflurane preconditioning (SF-PreCon) significantly activated AMP kinase (AMPK; A) and acetyl-coenzyme A carboxylase (ACC; B) in wild-type (WT) but not in AMPK dominant-negative (AMPK-DN; C) mice. n=13 to 15 mice/group. *P<0.05 vs sham myocardial ischemia (MI); #P<0.05 vs MI reperfusion (MI/R) group.
SF-PreCon protects against MI/R injury via AMPK signaling. However, our current study argues against the integral role of AMPK in SF-PreCon cardioprotection. Consistent with previously reported results, we demonstrate that SF-PreCon significantly activates AMPK signaling in the WT heart subjected to MI/R. However, SF-PreCon–mediated cardioprotection remains largely preserved in cardiomyocyte-specific AMPKα2 dominant-negative transgenic mice. This result

**Figure 5.** Sevoflurane preconditioning (SF-PreCon) failed to improve cardiac function (A) and did not reduce infarct size (B), circulating troponin I (TnI; C), and apoptosis (D) in caveolin-3 knockout (Cav-3KO) mice. n=15 mice/group. *P<0.05, **P<0.01 vs myocardial ischemia/reperfusion (MI/R). LVEF indicates left ventricular ejection fraction; and WT, wild type.

**Figure 6.** A and B, Antioxidant effect of sevoflurane preconditioning (SF-PreCon) is blocked in caveolin-3 knockout (Cav-3KO) mice but not in AMPK dominant-negative (AMPK-DN) mice. n=16 mice/group. *P<0.05 vs myocardial ischemia/reperfusion (MI/R). WT indicates wild type.
indicates that SF-PreCon–induced AMPK activation is associated, rather than causatively related, with SF-PreCon cardioprotection. At least 2 possibilities may explain the discrepancy between our current experimental findings and previously reported results. First, a cardiomyocyte-specific AMPKα2 dominant-negative transgenic mouse model was used in the current study. This approach not only avoided potential nonspecific effects of chemical AMPK inhibitors but also minimized any secondary effects caused by systemic AMPK KO.6 More importantly, the previous study demonstrating the important role of AMPK used an isolated perfused heart,15 an experimental model in which cardiac energy production is completely glucose dependent. The role of AMPK (a critical regulator of metabolism) in cardiac metabolism and cardiomyocyte function differs from an isolated perfused heart system and an in vivo model.

Cav-3 is the caveolin isoform specifically expressed in muscular cells. Many signaling molecules compartmentalize within cardiomyocyte caveolae and interact with the scaffolding domain of Cav-3. Many studies have demonstrated that, similar to Cav-1, the Cav-3 scaffolding domain binding inhibits the function of multiple caveolar proteins involved in cell growth and proliferation.4 Thus, Cav-3 has been generally recognized as a signal inhibitor and a potent growth suppressor. However, recent studies suggest that insulin signaling may be an exception, in which caveolin is a requisite for transmembrane signaling.4 More importantly, Horikawa et al16 originally reported that Cav-3 expression and caveolae are required for isoflurane-induced cardiac protection from hypoxia and ischemia/reperfusion injury. A more recent study by Tsutsumi et al17 demonstrated that the cardioprotective effects of isoflurane bolus administration preischemia are abolished when caveolae formation is disrupted or Cav-3 is knocked out. Our current study provides the first evidence that SF-PreCon–mediated cardioprotection is Cav-3 dependent. We demonstrate that Cav-3–mediated cardioprotection is not unique to isoflurane; rather, it is likely a common signaling property shared by many cardioprotective volatile anesthetics.

Figure 7. Sevoflurane preconditioning failed to attenuate nicotinamide adenine dinucleotide phosphate oxidase (A, determined by gp91phox) expression but reduced cyclooxygenase-2 (COX-2) expression (B) in a manner dependent on caveolin-3 (Cav-3) but independent of AMP kinase (AMPK). Bar graphs represent density analysis of Western blots from ≥3 repeated experiments. n=15 to 16 mice/group. **P<0.01 vs sham myocardial ischemia (MI); #P<0.05 vs MI/reperfusion (MI/R) group. AMPK-DN indicates AMPK dominant-negative mice; KO, knockout; and WT, wild type.
superoxide production after reperfusion, thereby protecting the heart against reperfusion injury. Our current study makes 2 additional novel observations. To the best of our knowledge, we provide the first direct evidence that SF-PreCon inhibits superoxide production in a Cav-3–dependent fashion. Previous studies have demonstrated that preconditioning is cardioprotective by activating multiple intracellular signaling systems, including Src tyrosine kinase, phosphoinositide 3 kinase, glycogen synthase kinase-3 β, and protein kinase C, as well as modulating ATP-sensitive potassium channel activity and mitochondrial permeability transition pore opening. Importantly, these signaling molecules and effector systems either interact directly with the scaffolding domain of caveolin or are known to localize to caveolae. Therefore, it is conceivable that loss of Cav-3 may interrupt multiple antioxidative/superoxide overproduction (A) and apoptosis (B) in caveolin-3 knockout (Cav-3KO) mice. *P<0.05 vs MI/R group. WT indicates wild type.

In summary, we have demonstrated that although SF-PreCon is capable of activating AMPK and Cav-3, only Cav-3 is causatively related to SF-PreCon–mediated antioxidant signaling and cardioprotection. Although caution must always be taken when extrapolating experimental findings to clinical practice, our results suggest that bolstering Cav-3 signaling may be a novel approach to reduce perioperative cardiac injury in patients subjected to limited volatile anesthetics. Conversely, our results suggest that impaired Cav-3 expression/localization, as seen in diabetes mellitus and aging, might be responsible for attenuated response to volatile anesthetic-mediated perioperative cardioprotection.

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**Disclosures**

None.

**References**

7. Capozza F, Combs TP, Cohen AW, Cho YR, Park SY, Schubert W, Williams TM, Brasaemle DL, Jerkels LA, Scherer PE, Kim JK, Lisanti MP. Caveolin-3 knockout mice show increased adiposity and whole body insulin resistance, with ligand-induced insulin resistance and whole body insulin resistance, with ligand-induced insulin resistance and whole body insulin resistance, with ligand-induced insulin resistance and whole body insulin resistance, with ligand-induced insulin resistance and whole body insulin resistance, with ligand-induced insulin resistance and whole body insulin resistance, with ligand-induced insulin resistance and whole body insulin resistance, with ligand-induced insulin resistance and whole body insulin resistance, with ligand-induced insulin resistance and whole body insulin resistance, with ligand-induced insulin resistance and whole body insulin resistance, with ligand-induced insulin resistance and whole body insulin resistance, with ligand-induced


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