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Sevoflurane Preconditioning Attenuates Myocardial Ischemia/Reperfusion Injury via Cav-3-Dependent COX-2 Inhibition

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Abstract

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-mediated cardioprotection, and their precise underlying mechanisms of action, remain incompletely understood.

Methods and Results—Sevoflurane preconditioning (SF-PreCon, consisting of 3 cycles of 15 minute-exposures to 2% sevoflurane prior to 30 minutes of MI) decreased MI/R injury in WT mice (caspase-3 activity −29.1%, infarct size −20.2%, and LVEDP −33.8%). In cardiac-specific AMPKα2 dominant negative overexpression (AMPK-DN) mice, the cardioprotective effect of SF-PreCon was largely retained (caspase-3 activity −26.7%, infarct size −16.7%, and LVEDP −25.9%, $P<0.01$). In contrast, SF-PreCon failed to significantly protect Cav3-knockout (Cav3-KO) mice against MI/R injury ($P>0.05$). SF-PreCon significantly decreased MI/R-induced superoxide generation in WT (−43.6%) and AMPK-DN mice (−35.5%, $P<0.01$), but not in Cav3-KO mice. SF-PreCon did not affect NADPH oxidase expression, but significantly inhibited COX-2 expression in WT (−38.7%) and AMPK-DN mice (−35.8%), but not in Cav-3KO mice.

Conclusions—We demonstrate for the first time sevoflurane preconditioning mediates cardioprotection against MI/R injury via Cav-3 dependent-COX-2 inhibition and anti-oxidative effects.

Keywords

preconditioning; reperfusion injury; signal transduction; Caveolin

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Conflict of Interest Disclosures: None

Open-heart surgery patients are subjected to myocardial ischemia/reperfusion (MI/R) injury during the operative and perioperative period[1], a significant challenge faced by modern anesthetic practices. The volatile anesthetic sevoflurane has been demonstrated to be cardioprotective[2]. However, the underlying mechanisms responsible for sevoflurane-mediated cardioprotection remain poorly understood.

The heterotrimeric protein AMP-activated protein kinase (AMPK) plays an essential role in regulating cellular energy metabolism. The cardioprotective effects of AMPK during ischemia are well accepted. However, whether AMPK activation is beneficial or detrimental during reperfusion remains actively debated, due largely to its putative effect upon cardiometabolic regulation, acidosis, and calcium overload. A recent study demonstrated that sevoflurane activates AMPK[3]. Whether AMPK activation is responsible for sevoflurane-mediated cardioprotection remains unknown.

Caveolae are flask-shaped plasma membrane invaginations rich in proteins and lipids processing important signal transduction functions. Caveolins are the structural proteins associated with caveolae morphology and function. Caveolin-3 (Cav-3), specifically expressed in muscular cells, has been recognized as a signaling inhibitor and potent growth suppressor[4]. Recently, it has been demonstrated that cardiac-specific overexpression of Cav-3 induces endogenous cardiac protection by mimicking ischemic preconditioning[5]. However, the mechanisms underlying Cav-3's involvement in sevoflurane-mediated cardioprotection remain largely unknown. We previously demonstrated that the anti-oxidant/anti-nitrative stress effect of adiponectin is not mediated by AMPK, but is dependent upon Cav-3[4, 6]. However, whether the anti-oxidant effect of inhaled anesthetics such as sevoflurane may be 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 following 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 molecule(s) and mechanism(s) responsible for sevoflurane-mediated cardioprotection.

Materials and Method

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* (NIH 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 utilized in this study to avoid the pre-diabetic phenotype associated with Cav-3KO mice of 2 months or older[7]. Prior to MI, animals were individually placed in a gas-tight Plexiglas 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-period 2% sevoflurane interspersed with 15 minutes washout. Animals in the control group were exposed to 3 cycles of 10 minute-period 0% sevoflurane interspersed with 15 minutes washout (Figure 1A)[8]. Subsequently, all mice were anesthetized with 2%

isoflurane, and myocardial ischemia (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 very brief period (<2 minutes)[9]. Slipknot release occurred after 30 minutes MI, and myocardial reperfusion (R) 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 utilized tissue from ischemic/reperfused regions, or areas at risk (identified by Evans blue-negative staining)".

Quantification of superoxide production, determination of myocardial apoptosis, cardiac function, circulating troponin-I, and myocardial infarct size

3 hours after reperfusion, mice were anesthetized again with 2% isoflurane. Cardiectomy was performed. Superoxide 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, Carlsbas, 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 1.2-Fr micromanometer). Myocardial infarct size was determined by Evans blue-2,3,5-triphenyl tetrazolium chloride (TTC) double staining. Blood was collected for serum troponin-I (TnI) 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 upon SI/R-induced cell death was determined by LDH release after 6 hours reoxygenation. Cellular survival rate was determined at 0, 3, and 6 hours (different plates were used) after reoxygenation as previously reported[12]. Sevoflurane preconditioning was performed with 1.5 mM sevoflurane[13], administered in 3 cycles of 15 minute-period exposures prior to simulated ischemia.

Western blot

Proteins were separated on SDS-PAGE gels, transferred to nitrocellulose membranes, and incubated with primary antibodies against AMPK, phosphorylated acetyl-CoA carboxylase (ACC), cyclooxygenase-2 (COX-2), $gp91^{phox}$ (all preceding antibodies from Transduction Laboratories, San Jose, CA), and glyceraldehyde 3-phosphate dehydrogenase 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 three 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 Barlett's test, and sample distribution was determined by the D'Agostino-Pearson omnibus normality test. Probabilities less than or equal to 0.05

(with Bonferroni-corrected multiple pairwise comparisons) were considered statistically significant.

Results

Sevoflurane preconditioning augments cardiac function, reduces infarct size, and decreases cell death in WT mice both in vivo and in vitro

Compared to control, SF-PreCon significantly improved cardiac function, as evidenced by increased left ventricular ejection fraction (LVEF) and reduced LVEDP (Figures 1B and 1C), as well as reduced infarct size, decreased serum TnI, and decreased apoptotic cell death determined by caspase-3 activation (Figures 2A and 2B). To further investigate whether SF-PreCon directly protects cardiomyocytes from ischemia/reperfusion injury or indirectly via in vivo neuronal-humeral factors, the effect of SF-PreCon upon 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 (Figures 3A and 3B), suggesting SF-PreCon directly protects cardiomyocytes from I/R injury.

Pro-survival effect of SF-PreCon is largely preserved in AMPK-DN mice

AMPK is a pro-survival 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 dominant negative (AMPK-DN) mice. SF-PreCon increased LVEF, reduced LVEDP (Figures 1B and 1D), decreased infarct size, and reduced circulating cTnI and apoptosis (Figures 2A and 2C) in AMPK-DN mice in vivo. SF-PreCon significantly improved cell survival and reduced LDH release (Figures 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 utilized 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 upon ACC phosphorylation, the primary downstream molecule responsible for AMPK's metabolic regulation, was determined. SF-PreCon significantly increased AMPK (Figure 4A) and ACC phosphorylation (Figure 4B), which was completely abolished in AMPK-DN mice (Figure 4C). Together, these results indicate 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 Cav3KO 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. Moreover, the beneficial effect of SF-PreCon upon infarct size, cTnI, and apoptosis was virtually abolished in Cav-3KO mice (Figures 5B, C, D). Together, these results demonstrate that sevoflurane-mediated cardioprotection is Cav-3 dependent.

Anti-oxidative effect of SF-PreCon is Cav-3, but not AMPK, dependent

Because oxidative stress plays a critical role in reperfusion injury, we next determined if SF-PreCon exerted anti-oxidative effect in MI/R injury, and whether such effect was Cav-3 dependent. In WT mice, SF-PreCon decreased superoxide generation by 43.6% (Figures 6A and 6B). This anti-oxidant property is largely retained in AMPK-DN mice (−35.5%, Figures 6A and 6B). In contrast, SF-PreCon did not reduce superoxide generation after MI/R in

Cav-3KO mice (Figures 6A and 6B). Together, these results demonstrate SF-PreCon-mediated attenuation of superoxide generation is highly dependent upon Cav-3, and only partially dependent upon 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 NADPH oxidase, mitochondria, xanthine/xanthine oxidase, and COX-2, contribute to superoxide overproduction following 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 (Figures 7B). However, SF-PreCon significantly inhibited COX-2, but not NADPH oxidase expression (Figures 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 Cav3KO heart (Figure 7B, right). These 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. 10 minutes before reperfusion, mice were randomized to receive either vehicle or parecoxib[14] (a soluble COX-2 inhibitor, 0.75mg/kg intraperitoneal). 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 myocardial ischemia 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 SF-PreCon protects against MI/R injury via AMPK signaling[15]. However, our current study argues against the integral role of AMPK in SF-PreCon cardioprotection. Consistent with previously reported results, we demonstrate that sevoflurane preconditioning 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 indicates that SF-PreCon-induced AMPK activation is associated, rather than causatively related, with SF-PreCon cardioprotection. At least two possibilities may explain the discrepancy between our current experimental findings and previously reported results. Firstly, a cardiomyocyte-specific APMKα2 dominant negative transgenic mouse model was employed in the current study. This approach not only avoided potential non-specific effects of chemical AMPK inhibitors, but also minimized any secondary effects due to systemic AMPK knockout[6]. More importantly, the previous study demonstrating the important role of AMPK utilized 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 insulin signaling may be an exception, in which caveolin is requisite for transmembrane signaling[4]. More importantly, Horikawa et al originally reported that Cav-3 expression and caveolae are required for isoflurane-induced cardiac protection from hypoxia and ischemia/reperfusion injury[16]. A more recent study by Tsutsumi et al demonstrated that the cardioprotective effects of isoflurane bolus administration pre-ischemia is abolished when caveolae formation is disrupted or Cav-3 is knocked out[17]. 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.

It is well-recognized that modestly increased superoxide production during preconditioning prevents massive superoxide production following reperfusion, thereby protecting the heart against reperfusion injury. Our current study makes two 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, PI3 kinase, GSK-3β, and protein kinase C (PKC)[18], as well as modulating ATP-sensitive potassium channel activity and mitochondrial permeability transition pore opening[19]. Importantly, these signaling molecules and effector systems either interact directly with the scaffolding domain of caveolin or are known to localize to caveolae[20]. Therefore, it is conceivable that loss of Cav-3 may interrupt multiple anti-oxidant signaling systems through which preconditioning protects the heart against reperfusion injury. Moreover, we demonstrate for the first time that although MI/R stimulates superoxide production through multiple pathways, COX-2 is the molecule upregulated by MI/R that is significantly inhibited by SF-PreCon in a Cav-3-dependent fashion. Although SF-PreCon no longer exhibits anti-oxidative/anti-apoptotic effects in Cav-3KO mice, selective COX-2 inhibition remains effective in reducing post-MI superoxide production and cardiomyocyte apoptosis in these animals, reinforcing the involvement of Cav-3-dependent inhibition of COX-2-derived superoxide[21] with SF-PreCon-mediated-cardioprotection. The molecular mechanisms responsible for the selective COX-2 inhibition by SF-PreCon are currently unknown, and are likely complex. This important question will be addressed in our future studies.

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 anti-oxidant signaling and cardioprotection. Although caution must always be taken when extrapolating experimental findings to clinical practice, our results suggest bolstering Cav-3 signaling may be a novel approach reducing perioperative cardiac injury in patients subjected to limited volatile anesthetics. Conversely, our results suggest that impaired Cav-3 expression/ localization, as seen in diabetes and aging, might be responsible for attenuated response to volatile anesthetic-mediated perioperative cardioprotection.

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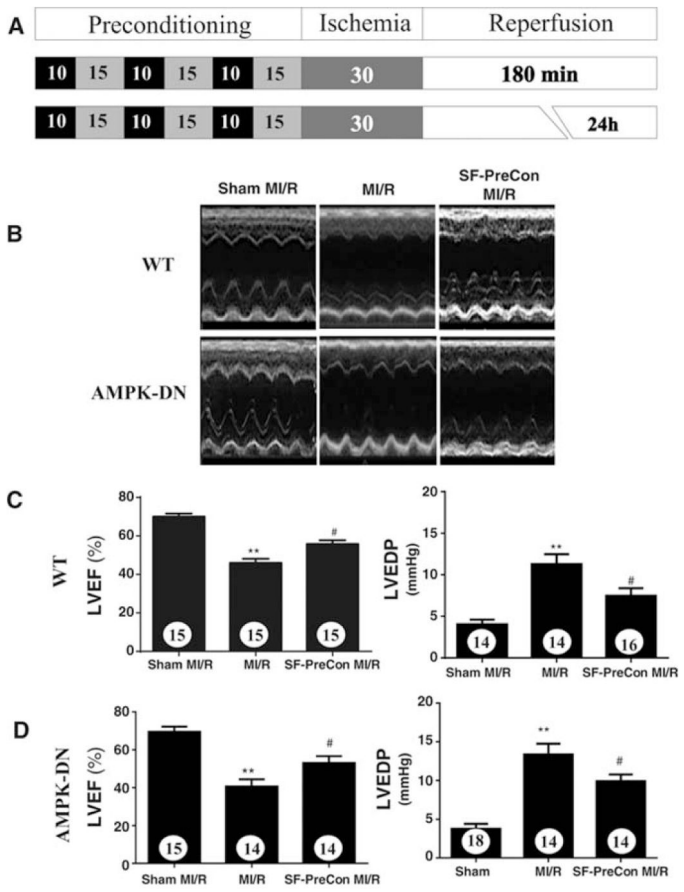

Figure 1.

A: Schematic illustration of protocol used in this experiment. Sevoflurane preconditioning improved cardiac function in WT (B, C), as well as in AMPK-DN (B, D) mice subjected to MI/R. Numbers in bars represent the sample size, the bars above histograms represent SEM. **p<0.01 vs. Sham MI; #P<0.05 vs. MI/R group. Abbreviations: SF-PreCon, sevoflurane preconditioning; MI/R, myocardial ischemia/reperfusion; WT, wild type; AMPK-DN, AMP kinase dominant negative; LVEF, left ventricular ejection fraction; LVEDP; left ventricular end diastolic pressure.

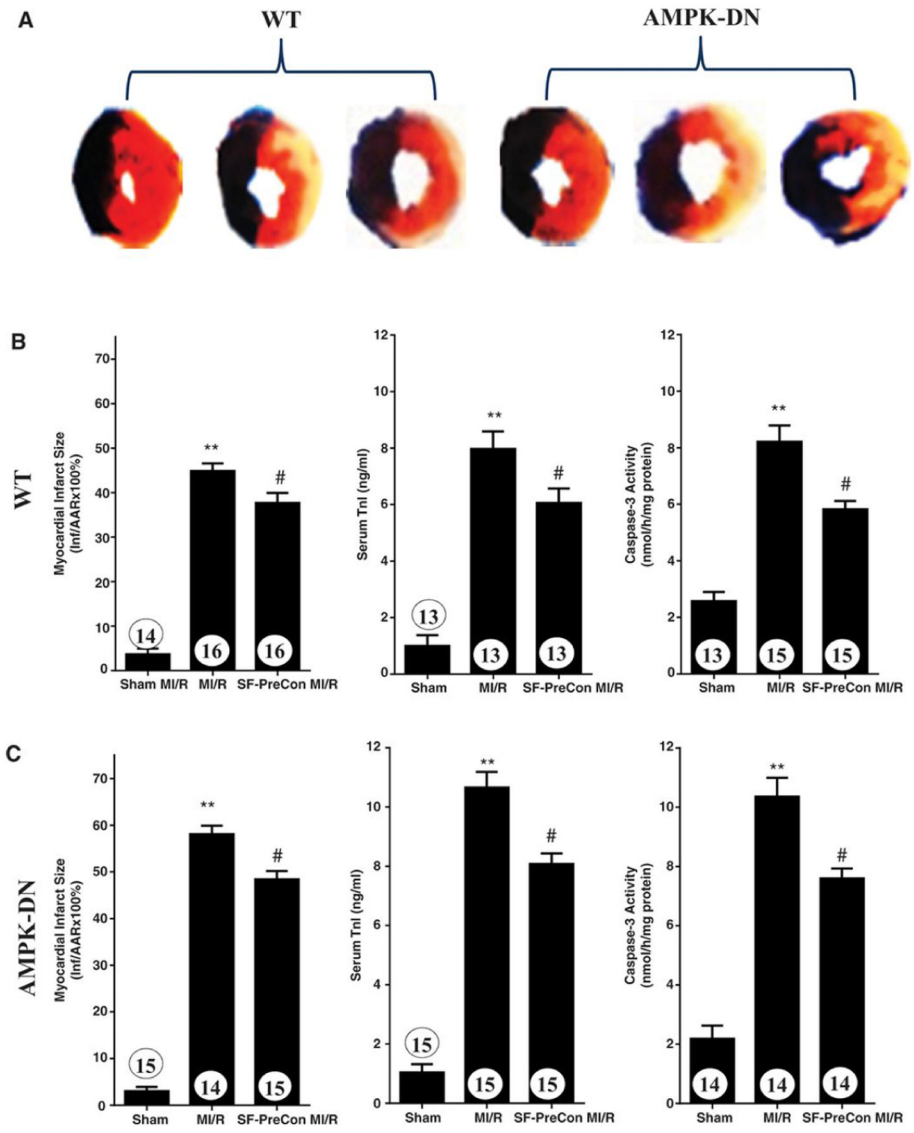

Figure 2.

Sevoflurane preconditioning reduced infarct size, decreased circulating troponin I, reduced apoptotic cell death in WT (A, B) as well as in AMPK-DN (A, C) mice subjected to MI/R. N=13–15 mice/group. **p<0.01 vs. Sham MI; #P<0.05 vs. MI/R group.

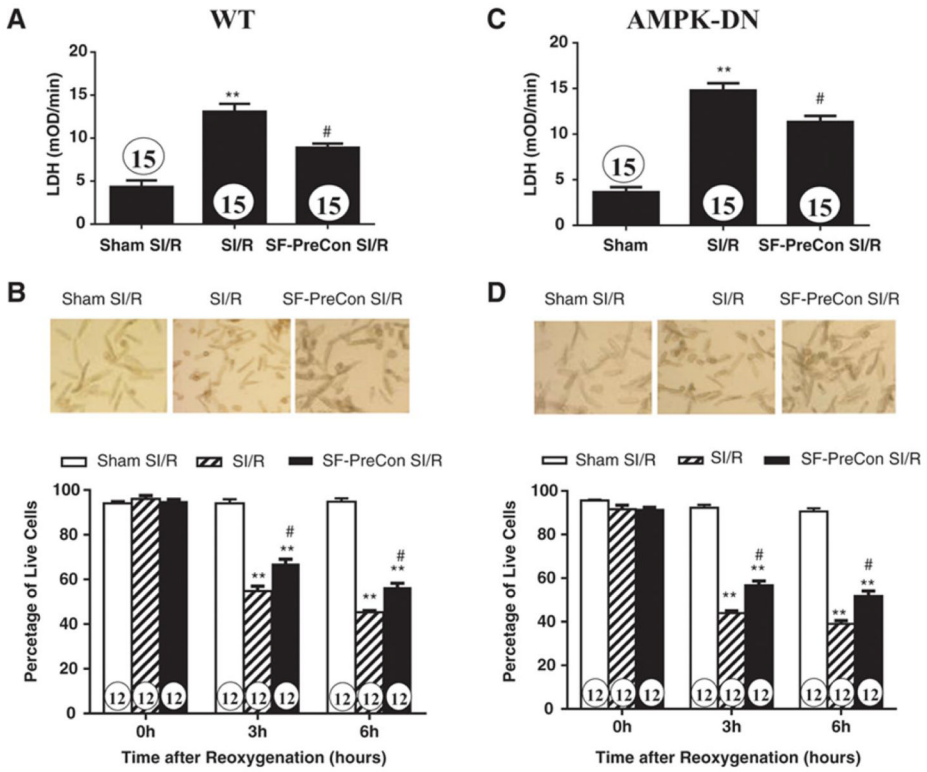

Figure 3.

Sevoflurane preconditioning 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=12–15 dishes/experimental condition utilizing cells isolated from at least 8 different animals. **p<0.01 vs. Sham SI/R; #P<0.05 vs. SI/R group.

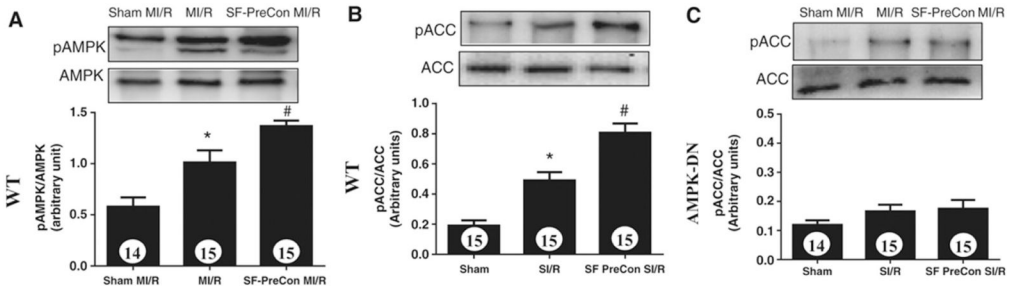

Figure 4.

Sevoflurane preconditioning significantly activated AMPK (A) and ACC (B) in WT but not AMPK-DN (C) mice. N=13–15 mice/group. *p<0.05 vs. Sham MI; #P<0.05 vs. MI/R group.

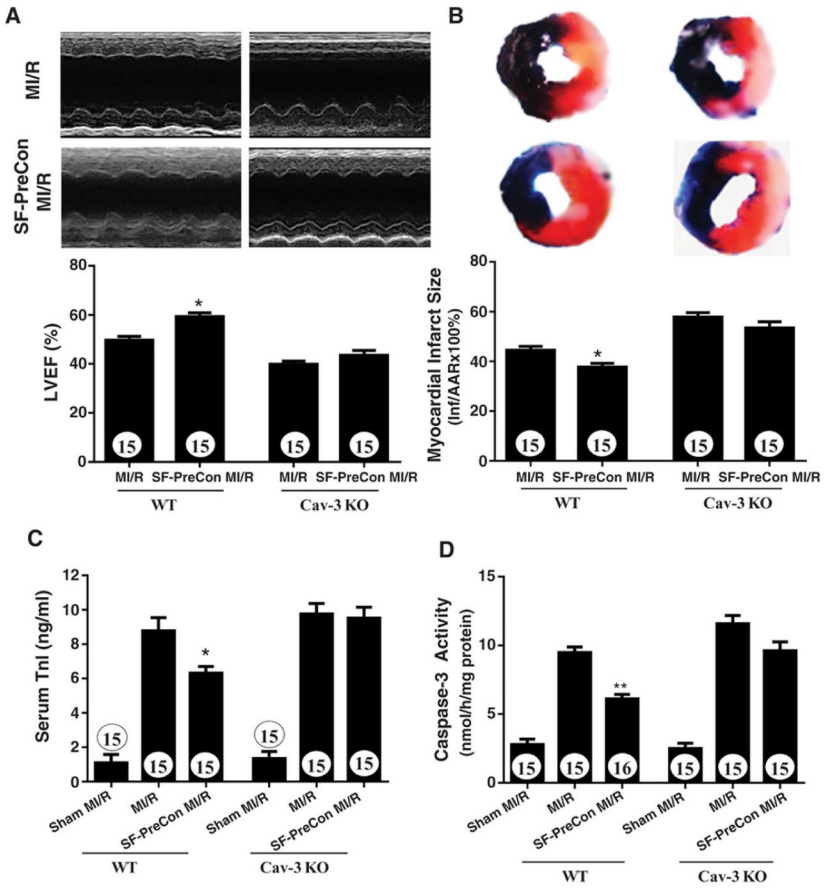

Figure 5.

Sevoflurane preconditioning failed to improve cardiac function (A), and did not reduce infarct size (B), circulating troponin I (C) and apoptosis (D) in Cav-3KO mice. N=15 mice/group. *P<0.05, **p<0.01 vs. MI/R.

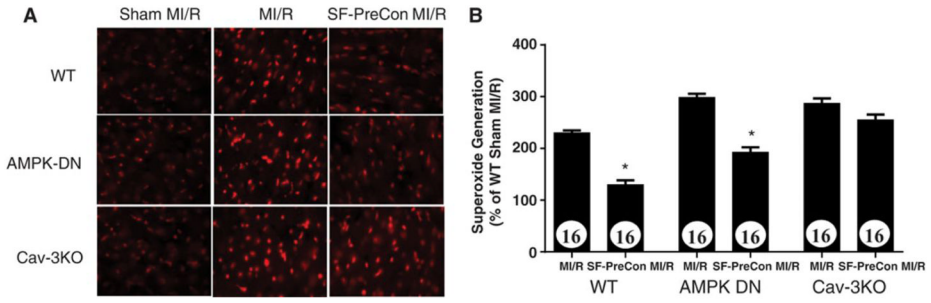

Figure 6.

Anti-oxidant effect of sevoflurane preconditioning is blocked in Cav-3KO mice but not in AMPK-DN mice. N=16 mice/group. *p<0.05 vs. MI/R.

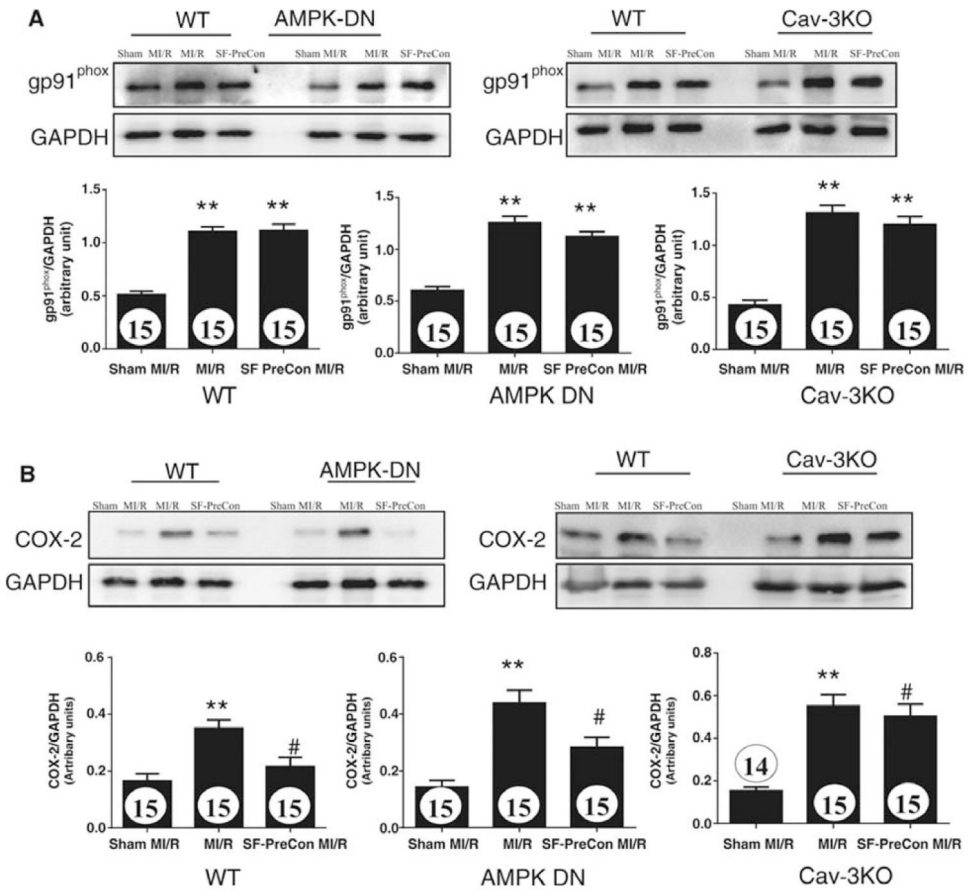

Figure 7.

Sevoflurane preconditioning failed to attenuate NADPH oxidase (A, determined by $gp91^{phox}$) expression but reduced COX-2 expression (B) in a manner dependent upon Cav-3, but independent of AMPK. Bar graphs represent density analysis of Western blots from at least 3 repeated experiments. N=15–16 mice/group. **p<0.01 vs. Sham MI; $^{\#}$P<0.05 vs. MI/R group.

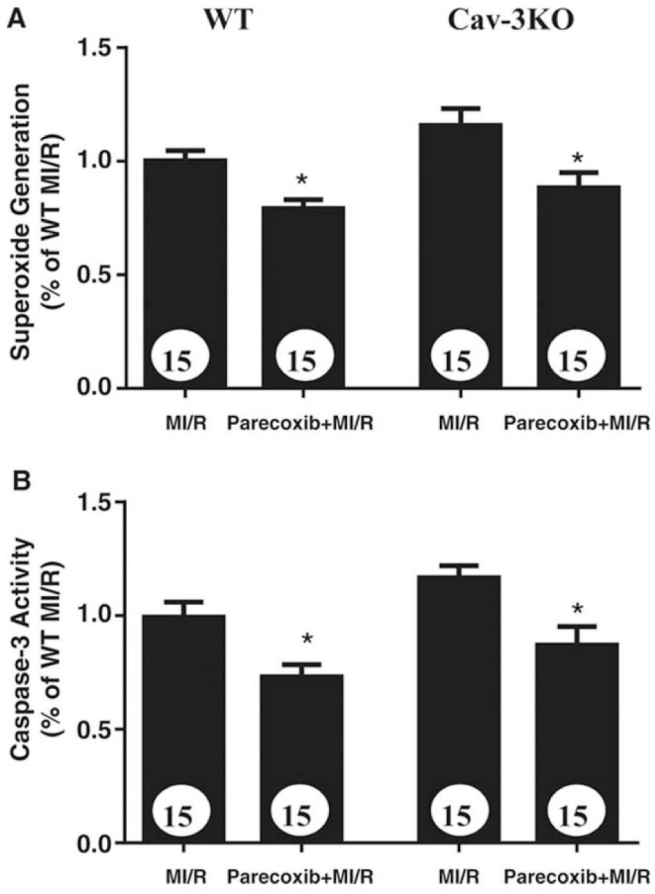

Figure 8.

Paracoxib (a COX-2 inhibitor), but not sevoflurane preconditioning, attenuates MI/R-induced superoxide overproduction (A) and apoptosis (B) in Cav-3KO mice. N=15 mice/group. *p<0.05 vs. MI/R group.