Targeting AMPK/eNOS pathway and mitochondria by sonlicromanol protects myocardial cells against ischemia-reperfusion injury

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Abstract. This work evaluated the cardioprotective effects of sonlicromanol, a new mitochondrial-directed drug, on cardiac ischemia/reperfusion (I/R) injury and explored the involvement of inflammatory and oxidative responses *via* activation of AMPK-eNOS-mitochondrial pathway. Male Sprague-Dawley rats underwent regional I/R injury through *in vivo* left anterior descending (LAD) coronary artery ligation for 40 minutes followed by 24 hours of reperfusion. Pretreatment of rats with sonlicromanol considerably reduced cardiac I/R injury in a dose-dependent manner, as indicated by lower infarct size and serum creatine-kinase levels, and improved cardiac function after reperfusion. Sonlicromanol (50 mg/kg) significantly reduced TNF-α, interleukin-1β, NF-κB-p65, and 8-isoprostane levels while increased manganese-superoxide dismutase and nitric-oxide levels and expression of eNOS and AMPK protein. It significantly reduced mitochondrial membrane depolarization and reactive oxygen species (ROS) levels. However, AMPK inhibition significantly reduced sonlicromanol protective actions. Cardioprotection by sonlicromanol was achieved by moderating inflammatory and oxidative responses, and AMPK/eNOS/mitochondrial signaling is a crucial regulator of these actions.

Key words: Mitochondria — Cardioprotection — Ischemia-reperfusion — Oxidative stress — Inflammation — AMPK

Introduction

Cardiac ischemia/reperfusion (I/R) injuries occur when blood returns to the previously ischemic heart tissues. Cardiac ischemia occurs due to temporary or permanent blockage of any of the coronary arteries, during which the amount of blood supplying cardiomyocytes is less than the amount of demand for their normal function; thus, the tissue faces a lack of oxygen, glucose and other substances needed for metabolism. Restoration of blood supply is necessary to save ischemic heart. But, this reperfusion also induces additional damages, exacerbating cardiac function and survival (Heusch 2020). In hospitalized patients, myocardial I/R damage is a primary cause of heart failure and post-operative decreased cardiac function, both of which have unfavorable repercussions (Frank et al. 2012). Increased lipid peroxidation, endoplasmic reticulum stress, mitochondrial dysfunction, apoptosis, and inflammation are among pathophysiological events that contribute to I/R damage etiology (Frank et al. 2012; Kalogeris et al. 2012). Oxidative stress and inflammation share significant contribution to the development of I/R injury and cardiovascular diseases (Kalogeris et al. 2012). Because of the quick recovery of oxygen during reperfusion, considerable reactive oxygen species (ROS) are produced, causing significant tissue damage. Increased ROS levels after cardiac I/R injury activates a number of signaling kinases and transcription factors, damaging DNA and

intracellular organelles such as mitochondria and causing necrosis and apoptosis (Moris et al. 2017). Death of cardiomyocytes affects the extracellular matrix composition of cardiac tissues, resulting in inflammation and, eventually, heart failure (Frank et al. 2012; Moris et al. 2017). There are currently no effective treatments available for lowering oxidative stress and inflammation in cardiac cells after I/R injury and heart failure.

Activating 5' adenosine monophosphate-activated protein kinase (AMPK) lessens the outcomes of myocardial I/R damage (Xu et al. 2021). Increased AMPK activity in cells results in anti-inflammatory and anti-oxidative phenotypes *via* targeting the nuclear and mitochondrial effectors (Jeon 2016). Also, it is now well established that both endogenous and exogenous nitric oxide (NO) sources have significant modulatory effects on mitochondrial function in cardiomyocytes (Davidson and Duchen 2006). NO synthases (NOSs) are expressed in cardiac myocytes, and have modulatory influences on the outcomes of cardiac I/R injury (Davidson and Duchen 2006). Under physiological conditions, endothelial NOS-derived NO in heart and coronary microvasculature regulates myocyte's mitochondrial function. On the other hand, the amounts of NO available during I/R setting are insufficient to carry out this action (Tran et al. 2022). Importantly, AMPK activation may contribute to endothelial NOS (eNOS) phosphorylation and sufficient NO production (Li et al. 2016). Thus, the regulation of mitochondrial homeostasis in I/R conditions can be achieved by enhancing the interactions of these cellular effectors.

Sonlicromanol is a novel mitochondrial-acting medication with high antioxidative capabilities that improves the physiological properties of mitochondria in vital tissues (Beyrath et al. 2018; Xiao et al. 2021). This drug has potent redox modulating activity by targeting intracellular and mitochondrial ROS (Beyrath et al. 2018). Its safety and efficacy have recently been proven in phase 1 and 2 clinical trials in individuals with mitochondrial disorders (Koene et al. 2017; Klein-Gunnewiek et al. 2021). It has been documented that sonlicromanol may inhibit oxidative and inflammatory responses and reduce the symptoms of mitochondrial diseases by decreasing the activity of prostaglandin-E2 (Xiao et al. 2021). Furthermore, a recent study reveals that sonlicromanol's antioxidative effects may occur in some cells *via* an AMPK-dependent activation of mitochondrial biogenesis (Almannai et al. 2020). However, it is not known whether this drug can reduce myocardial infarction and cardiac I/R pathophysiology by affecting AMPK pathway. Since mitochondrial abnormalities and the oxidative/inflammatory reactions are of key players in the progression of myocardial I/R injury and considering the relevant protective potentials of sonlicromanol, it seems that this drug has sufficient capability to be a proper candidate for reducing these consequences in ischemic heart damage. Therefore, this study evaluated the beneficial effects of sonlicromanol on the outcomes of myocardial infarction, oxidative stress, pro-inflammatory cytokines status, and mitochondrial function, and explored the role of AMPK/eNOS activation in these actions.

Materials and Methods

Animals

Sixty male Sprague Dawley rats (220 ± 25 g) were housed in a 23°C temperature and 45% humidity environment with a 12-h light period followed by a 12-h dark period. The animals had unrestricted access to food and water. Before beginning the experiments, the animals were acclimated for two weeks. All animal experiments in this work were conducted in accordance with University's Laboratory Animal Care guidelines and were approved by the local Committee for the Use of Animals in Research (Approved ethical No. K2021-009-01).

Experimental animal groups

The rats were divided into six groups (*n* = 10) at random: Sham; IR; IR+S10; IR+S50; IR+CC; and IR+CC+S50. Sonlicromanol was delivered intraperitoneally (i.p.) to rats in the sonlicromanol-receiving groups at doses of 10 mg/kg (S10) and 50 mg/kg (S50) daily for one week prior to the commencement of left anterior descending (LAD) ligature. Based on our pilot dose-response study (Supplementary materials, Fig. S1), two doses of 10 and 50 mg/kg was selected to use in this study, which were consistent with the effective doses in previous reports (Xiao et al. 2021). Medications were prepared in 1% DMSO, and the untreated rats were i.p. given the similar amount of DMSO. Compound C (CC) is a selective AMPK inhibitor that was given i.p. at 5 mg/kg daily five minutes before the injection of sonlicromanol at 50 mg/kg. Every day, the injectable solutions were freshly provided. Sample size was calculated by relevant software using the power of 80% and alpha level at 0.05 with mean difference of at least 15%. Four rats in each group were allocated to determine the infarct size and the remaining was used for other measurements.

Modeling of myocardial I/R injury

The animals were intubated and ventilated using an animal ventilator after being sedated with an i.p. dose of sodium pentobarbital (50 mg/kg) (Harvard Apparatus VentElit, USA). The chest was then opened on the left side *via* a lateral incision, a 6/0 silk thread was placed around the LAD coronary artery and thread was tied to impede the artery blood flow for 40 min. Following the release of the LAD ligature,

reperfusion was established and lasted for 24 h. The reperfusion duration was extended for two weeks in four animals in each group to examine echocardiographic findings in rats. The rats were placed on a heating pad until they awoke following the surgery. Blood samples were collected two hours after reperfusion began.

Assessment of heart function

A cardiologist who was blinded to the study design performed echocardiography to assess heart function. The animals were anesthetized, and the Vevo 2100 echocardiography system with M-mode tracing was used to assess the contractile function of their hearts (VisualSonics, Toronto, ON, Canada). Three cardiac cycles were used to measure left ventricular end-systolic inner diameter (LVIDs; in cm), left ventricular end-diastolic inner diameter (LVIDd; in cm), left ventricular ejection fraction (EF; in %), and left ventricular fractional shortening (FS; in %).

Infarct size measurement

The LAD was re-ligated at the end of the experiment, and the hearts were perfused with 2.5% Evans-blue for five minutes through the femoral vein. After isolation and weighting of the hearts, approximately five sections of 2 mm thickness were prepared and stained for 15 min at 37°C with 2,3,5-triphenyl-tetrazolium-chloride and then underwent fixation with 10% formaldehyde overnight. Finally, using ImageJ software (NIH, USA), total volumes of left ventricles, risk zones and infarct areas were quantified and their ratios were obtained.

Assay for creatine kinase-mB

During the reperfusion phase, blood samples were taken from the animals' tail veins. The sample was centrifuged at $4500 \times g$ for 15 min at 4°C, after which the sera were collected and used for the measurement of creatine kinase-myocardial band (CK-mB) using a commercial kit (Bioscience, Germany) according to the kit's instructions. The units of measurement were U/l.

Mitochondrial isolation and tissue sampling

The hearts were removed from the bodies immediately after the experiment, and the ischemic tissue including the risk zones of the left ventricles were isolated and homogenized freshly in a mitochondrial-isolation buffer containing 70 mM sucrose, 210 mM mannitol, 1 mM EDTA in 50 mM Tris HCl at pH 7.4. The samples were successively centrifuged at $1300 \times g$ and $12{,}000 \times g$ for 10 min. The collected pellets containing mitochondrial fractions were then transferred to the storage solution having 70 mM sucrose, 210 mM mannitol in 50 mM

Tris HCl at pH 7.4. The bicinchoninic acid assay method was used to estimate the protein's concentrations of each sample.

Mitochondrial production of ROS

The mitochondrial ROS in samples were measured using the dichlorohydro-fluorescein diacetate (DCFDA) fluorometric method. The fluorescence of mitochondrial pellets was identified at an excitation/emission wavelength of 480 nm and 530 nm after 30 min of incubation in a storage buffer with 2 M of DCFDA dye. The ROS levels were expressed as fluorescence intensities *per* mg of samples' proteins.

Mitochondrial membrane potential estimation

The JC-1 staining technique was utilized to detect the alterations of mitochondrial membrane potentials according to the manufacturer's instructions (Sigma Aldrich, USA). The isolated mitochondrial pellets were incubated in JC-1 assay buffer for 15 min at 25°C in a dark room. This incubation reduced JC-1 loss following consumption of the substrate over time, resulting in mitochondrial membrane potential loss. The fluorescence intensities red and green wavelengths were respectively detected at 525 nm and 485 nm excitations, and 590 nm and 530 nm emissions. The intensities were normalized to the samples' proteins content. Mitochondrial depolarization was defined as a decrease in the red/green ratio, with smaller numbers indicating greater depolarization. But for simplicity and easier understanding of depolarization changes, after obtaining the initial values of membrane potential in different groups, the resulting values were subtracted from 100, and thus the obtained numbers indicate the amount of depolarization.

Measurement of inflammatory and oxidative stress markers

The cardiac samples from ischemic left ventricles were isolated, homogenized, and centrifuged at 10,000 rpm in lysing buffer (Beyotime, Jiangsu, China). Following the collection of sample supernatants, the levels of pro-inflammatory cytokines and mediators including interleukin-1β (IL-1β), tumor necrosis factor α (TNF-α), and nuclear factor-κB (NF-κB), as well as oxidative stress markers such as 8-isoprostane and manganese superoxide dismutase (MnSOD) were measured using appropriate ELISA kits (MyBioSource, Inc., USA). The levels of inflammatory mediators and oxidative stress markers were normalized using the Bradford method to determine the sample's protein content, and the results were expressed in mg of protein.

NO levels measurement

NO levels were measured using the Griess colorimetric method in accordance with the protocol of relevant kit (Ther-

moFisher Scientific, Germany). The principle of this method is color development in an acidic medium using Griess reagent (N-naphthyl ethylene diamine and sulfanilamide) after nitrate conversion to nitrite by copperized cadmium granules. This assay's calibration was done with sodium nitrite standard solutions. The levels of NO metabolite were calculated after reading the optical density of samples at 540 nm using a 96-well microplate, and normalized with the protein content of samples.

Western blot analysis

The protein expression of phosphorylated form of AMPK, eNOS, and GAPDH in cardiac samples was detected using the Western blotting method. Each sample had approximately 20 g of proteins was electrophoresed on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred on a PVDF membrane. The membranes were then blocked for an hour in five percent skim milk with 0.1 percent Tween-20. Then the membranes were exposed overnight at 4°C to the primary antibodies to target proteins (1:1000, Cell Signaling Technology, USA), followed by three washes with Tris buffer saline (TBS). After that, an hour was spent using a horseradish-peroxidase conjugated secondary antibody (1:2500, Cell Signaling Technology, USA) to develop specific binding. Before being exposed to the X-ray film, the membrane was rinsed in TBS and incubated in the darkroom with the enhanced chemiluminescence reagents. The protein bands were identified, as well as their corresponding intensities. The protein bands of AMPK and eNOS were detected using ImageJ software (NIH, USA), and their intensities were then normalized to the GAPDH intensity of the samples.

Statistical analysis

The information was presented as a mean ± standard deviation. Once the normal distribution of the data was confirmed, one-way analysis of variance and Tukey tests were selected to detect the between-group differences. The p value of 0.05 was thought to be the bare minimum of statistical significance.

Results

Cardiac function

In comparison to the Sham group, induction of 40 min ischemia followed by 24 h reperfusion significantly reduced EF and FS ($p < 0.01$). When compared to the IR group, sonlicromanol at 10 mg/kg had no significant effect on EF and FS. Nonetheless, sonlicromanol at 50 mg/kg significantly increased these parameters (Table 1). Similarly, the IR group had significantly greater LVIDs and LVIDd than those of the Sham group ($p < 0.01$). Furthermore, when compared to the IR group, administration of sonlicromanol at 50 mg/kg resulted in a significant reduction in LVIDs and LVIDd. Inhibition of AMPK activity using CC considerably attenuated the beneficial effects of sonlicromanol on cardiac function following I/R injury (Table 1).

Myocardial tissue injury

After LAD ligation for 40 min, the hearts of all groups developed comparable area at risk indicated a similar degree of ischemic injury (area at risk) in all groups (Fig. 1A). Inducing myocardial I/R injury resulted in a 54% increase in infarct size compared to the Sham group ($p < 0.001$) (Fig. 1B). Similarly, the IR group's CK-mB release was significantly higher than that of the Sham group (Fig. 1C). CK-mB release or infarct size did not show significant reduction after administering sonlicromanol at 10 mg/kg. However, sonlicromanol at 50 mg/kg significantly reduced infarct size (to 28%) and CK-mB levels when compared to the IR group ($p < 0.01$ and $p < 0.05$, respectively). Finally, concomitant application of

Table 1. Hemodynamic function of the heart

Group	EF (%)	LVIDd (cm)	LVIDs (cm)	FS (%)
Sham	75.6 ± 5.9	0.55 ± 0.05	0.31 ± 0.05	44.7 ± 4.9
IR	48.9 ± 5.4[**]	0.77 ± 0.04[**]	0.53 ± 0.05[**]	25.3 ± 3.0[**]
IR+S10	61.2 ± 6.0	0.68 ± 0.04	0.45 ± 0.04	34.7 ± 5.5
IR+S50	74.7 ± 5.3[#]	0.51 ± 0.05[#]	0.34 ± 0.04[#]	41.5 ± 3.6[#]
IR+CC	56.3 ± 4.8	0.72 ± 0.04	0.47 ± 0.02	27.8 ± 3.0
IR+CC+S50	62.7 ± 3.7[+]	0.65 ± 0.05[+]	0.44 ± 0.05[+]	33.3 ± 7.1[+]

Data was presented as mean ± SD; n = 6/each group. IR, ischemia/reperfusion; S10 and S50, sonlicromanol at 10 and 50 mg/kg respectively; CC, compound C; EF, ejection fraction; LVIDd, left ventricular end-diastolic inner diameters; LVIDs, left ventricular end-systolic inner diameters; FS, fractional shortening. [**] $p < 0.01$ *vs.* Sham group; [#] $p < 0.05$ *vs.* IR group; [+] $p < 0.05$ *vs.* IR+S50 group.
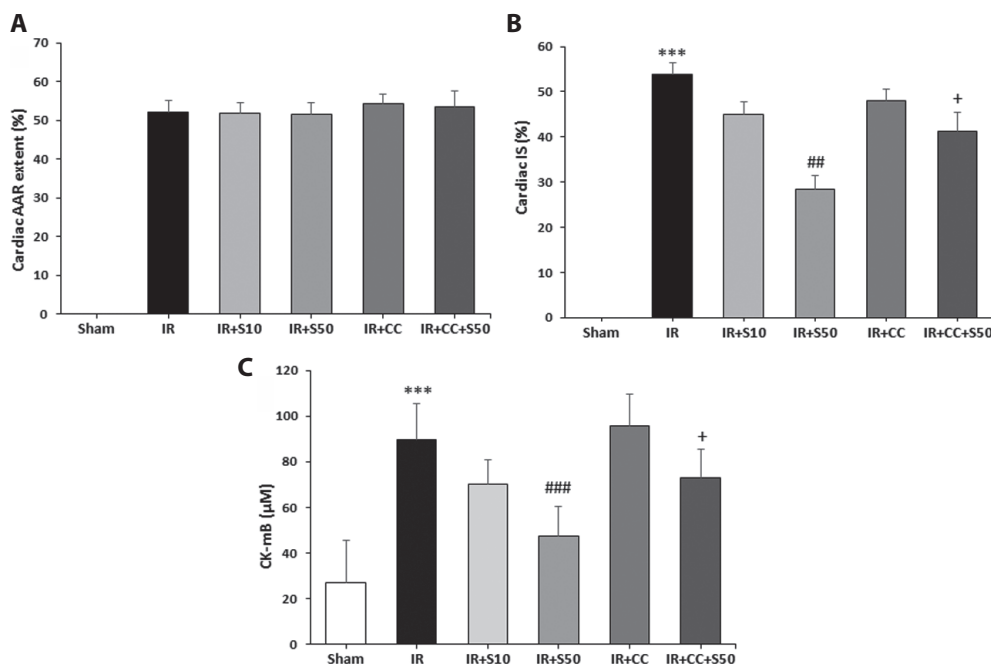
Figure 1. Cardiac injury markers: area at risk (AAR; **A**), infarct size (IS; **B**), and creatine kinase myocardial band (CK-mB; **C**) in differential rat groups. Data was presented as mean ± SD; $n = 4$/each group. *** $p < 0.001$ *vs.* Sham group; ## $p < 0.01$, ### $p < 0.001$ *vs.* IR group; + $p < 0.05$ *vs.* IR+S50 group. IR, ischemia/reperfusion; S10 and S50, sonlicromanol at 10 and 50 mg/kg, respectively; CC, compound C (5 mg/kg).

CC, an AMPK blocker, significantly abolished the impact of sonlicromanol on infarct size and CK-mB release ($p < 0.05$) (Fig. 1). The findings of IR+CC group were not significantly different from the IR group.

Cardiac mitochondrial function

Following I/R injury, mitochondrial ROS production significantly augmented and mitochondrial membrane showed significant depolarization ($p < 0.001$) (Fig. 2A,B). Administration of sonlicromanol at 50 mg/kg into I/R rats significantly lessened mitochondrial ROS levels ($p < 0.001$) and increased its membrane potential ($p < 0.01$), while sonlicromanol at 10 mg/kg had no significant effects. Furthermore, CC significantly suppressed the beneficial impacts of sonlicromanol on mitochondrial parameters ($p < 0.05$).

Cardiac inflammatory mediators

After inducing I/R injury, we discovered a significant rise in proinflammatory mediators levels TNF-α, IL-1β, and NF-κB-p65 ($p < 0.001$) (Fig. 3A–C). In comparison to I/R rats, sonlicromanol at 50 mg/kg, but not 10 mg/kg, treatment significantly reduced TNF-α ($p < 0.01$), IL-1β, and NF-κB-p65 ($p < 0.001$) levels. In these rats, however, co-administration of CC significantly reversed the anti-inflammatory effects of sonlicromanol ($p < 0.05$).

Cardiac oxidative stress mediators

Figure 4 shows that after inducing I/R injury in rats, cardiac levels of 8-isoprostane increased while MnSOD levels decreased ($p < 0.001$) (Fig. 4A,B). Furthermore, sonlicromanol at 50 mg/kg
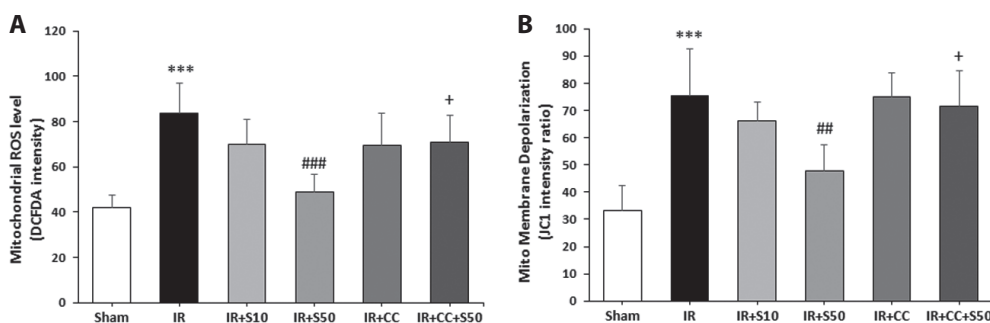

Figure 2. Mitochondrial function. **A.** Mitochondrial reactive oxygen species (ROS) production. **B.** Mitochondrial membrane potential depolarization. Data was presented as mean ± SD; $n = 6$/each group. *** $p < 0.001$ *vs.* Sham group; ## $p < 0.01$, ### $p < 0.001$ *vs.* IR group; + $p < 0.05$ *vs.* IR+S50 group. DCFDA, dichlorohydro-fluorescein diacetate; JC-1, a fluorescent mitochondrial dye; Mito, mitochondrial. For other abbreviations, see Figure 1.
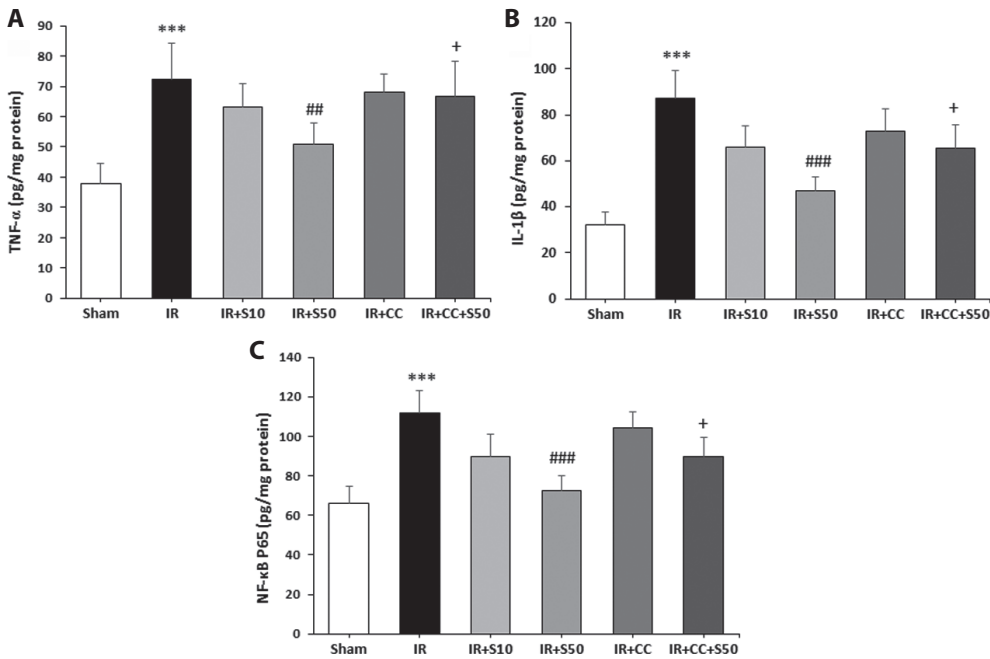
Figure 3. Pro-inflammatory mediators TNF-α (**A**), IL-1β (**B**), and NF-κB-p65 (**C**). Data was presented as mean ± SD; n = 6/each group. *** $p < 0.001$ *vs.* Sham group; ## $p < 0.01$, ### $p < 0.001$ *vs.* IR group; + $p < 0.05$ *vs.* IR+S50 group. TNF-α: tumor necrosis factor-alpha; IL-1β: interleukin-1 beta; and NF-κB: nuclear factor-kappa B. For other abbreviations, see Figure 1.

significantly reduced 8-isoprostane level ($p < 0.05$) and augmented MnSOD level ($p < 0.001$) when compared to the IR group. In comparison to the IR+S50 group, blocking AMPK activity significantly reduced sonlicromanol's protective effects on cardiac oxidative stress markers ($p < 0.05$) (Fig. 4).

Cardiac NO production

Following myocardial I/R injury, NO production significantly reduced ($p < 0.01$ *vs.* Sham group) (Fig. 5). sonlicromanol at 10 mg/kg had no statistically significant effects on NO levels in rats. In contrast, sonlicromanol at 50 mg/kg significantly increased myocardial NO metabolites levels when compared to the IR group ($p < 0.01$). Also, CC administration significantly reversed the effect of sonlicromanol on NO production ($p < 0.05$).

Cardiac expression of phosphorylated-AMPK and eNOS

Following myocardial I/R injury, the expression of phosphorylated form of AMPK (p-AMPK) and eNOS was

significantly downregulated in I/R hearts in comparison to the Sham hearts ($p < 0.001$) (Fig. 6A,B). Treatment of rats with sonlicromanol at 10 mg/kg had no statistically significant effects on eNOS expression, while increased p-AMPK expression ($p < 0.05$). Sonlicromanol at 50 mg/kg, on the other hand, significantly upregulated the expression of both p-AMPK and eNOS in comparison to the IR group ($p < 0.001$). The sonlicromanol's influence on the regulation of p-AMPK and eNOS expression was significantly reversed by administration of CC ($p < 0.01$), confirming the AMPK-dependence of sonlicromanol effects in I/R hearts.

Discussion

We discovered that sonlicromanol protected the rats' cardiac tissue against I/R injury-induced oxidative stress and inflammatory responses *via* an AMPK-dependent mechanism. sonlicromanol treatment reduced infarct size and
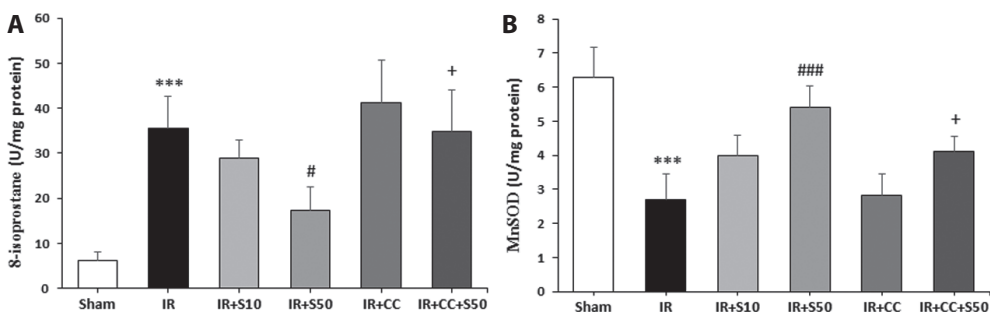

Figure 4. Oxidative stress markers 8 isoprostane (**A**), and manganese superoxide-dismutase (MnSOD; **B**). Data was presented as mean ± SD; n = 6/each group. *** $p < 0.001$ *vs.* Sham group; # $p < 0.05$, ### $p < 0.001$ *vs.* IR group; + $p < 0.05$ *vs.* IR+S50 group. For abbreviations, see Figure 1.
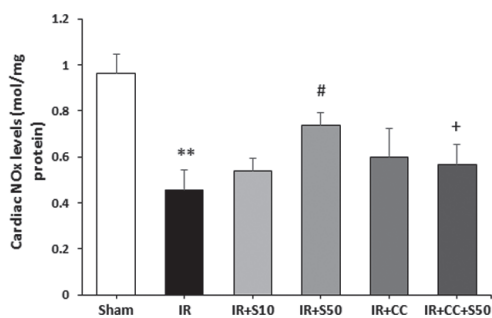
Figure 5. Nitric oxide metabolites (NOx). Data was presented as mean ± SD; n = 6/each group. ** $p < 0.01$ *vs.* Sham group; [#] $p < 0.05$ *vs.* IR group; [+] $p < 0.05$ *vs.* IR+S50 group. For abbreviations, see Figure 1.

serum CK-mB levels while increasing cardiac hemodynamic function. Also, sonlicromanol resulted in an improved mitochondrial function, increased antioxidant capacity, eNOS expression and NO levels, and lowered production of pro-inflammatory cytokines. The protective effects of sonlicromanol on cardiac tissue were significantly reversed when AMPK activity was inhibited, suggesting that AMPK signaling is involved in this protection.

Sonlicromanol is a newly developed drug having anti-oxidative and anti-inflammatory properties that targets mitochondria and improves mitochondrial-related diseases (de Haas et al. 2017; Koene et al. 2017; Beyrath et al. 2018; Klein-Gunnewiek et al. 2021; Xiao et al. 2021). Our findings confirm the presumption that this compound shows promising anti-oxidative and anti-inflammatory potentials in rats hears with I/R damage. During I/R circumstances, the overproduction of ROS and free radicals contributes significantly to lipid peroxidation, which causes oxidative

destruction of membrane lipids, proteins, and other target macromolecules (Moris et al. 2017; Zhou et al. 2018). In the present work, the main lipid peroxidation marker, 8-iso-prostane, as well as mitochondrial ROS levels were reduced in ventricular samples after sonlicromanol administration, whereas endogenous antioxidant MnSOD was increased, demonstrating the attenuation of I/R-induced oxidative damage in rats.

In addition, inflammatory responses and oxidative stress unite in myocardial I/R injury and play an important role in injury progression (González-Montero et al. 2018). Under excessive inflammatory responses, inflammatory cells were recruited from the bloodstream into the injured tissue (Chen et al. 2017). Cytokines TNF-α and IL-1β are typical pro-inflammatory mediators which are released in response to this condition. Furthermore, excessive ROS produced during reperfusion could trigger this inflammatory response, exacerbating I/R injury (González-Montero et al. 2018). We revealed that sonlicromanol administration reduced the production of pro-inflammatory cytokines as well as NF-κB-p65, which were all elevated in rat's hearts after I/R damage. NF-κB is a transcription factor that regulates central physiological processes such as inflammation, oxidative stress, and apoptosis (Chen et al. 2016). It is activated by a variety of death signals, resulting in increased cytokine release, inflammatory reactions, and oxidative stress, augmenting cardiomyocytes sensitivity to I/R insult. Normal mitochondrial function is essential in repairing oxidative and inflammatory damage in cardiomyocytes (Chang et al. 2020). However, myocardial I/R injuries severely impair mitochondrial function and makes cells prone to associated cellular damages. On the other hand, sonlicromanol administration has been able to prevent mitochondrial dysfunction following I/R injury, and therefore the benefits of the drug
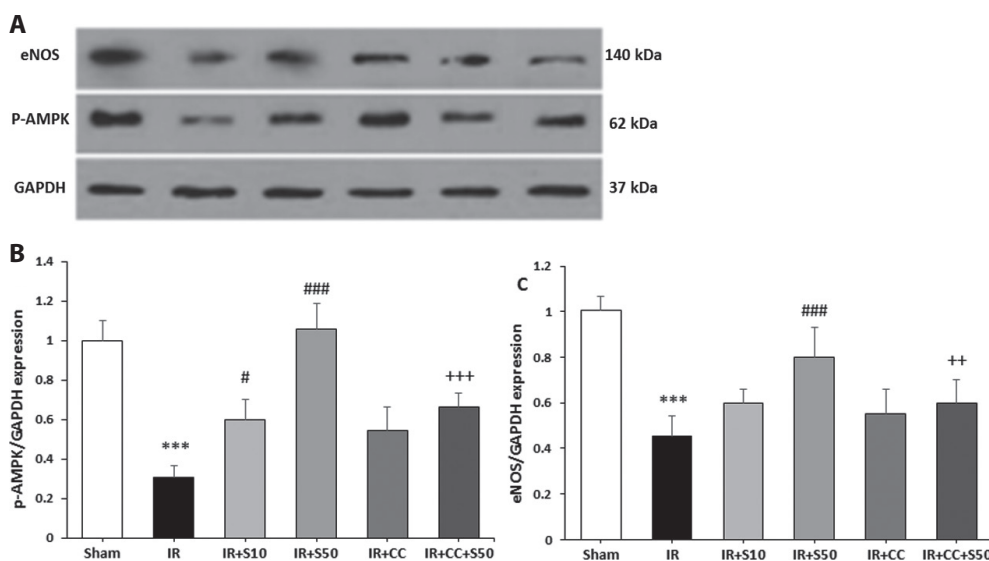

Figure 6. Proteins expression. **A.** Representative immunoblots. **B.** Phosphorylated form of adenosine monophosphate-activated protein kinase (p-AMPK). **C.** Endothelial nitric oxide synthase (eNOS). Data was presented as mean ± SD; n = 3/each group. *** $p < 0.001$ *vs.* Sham group; [#] $p < 0.05$, [###] $p < 0.001$ *vs.* IR group; [+] $p < 0.05$ *vs.* IR+S50 group. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. For other abbreviations, see Figure 1.
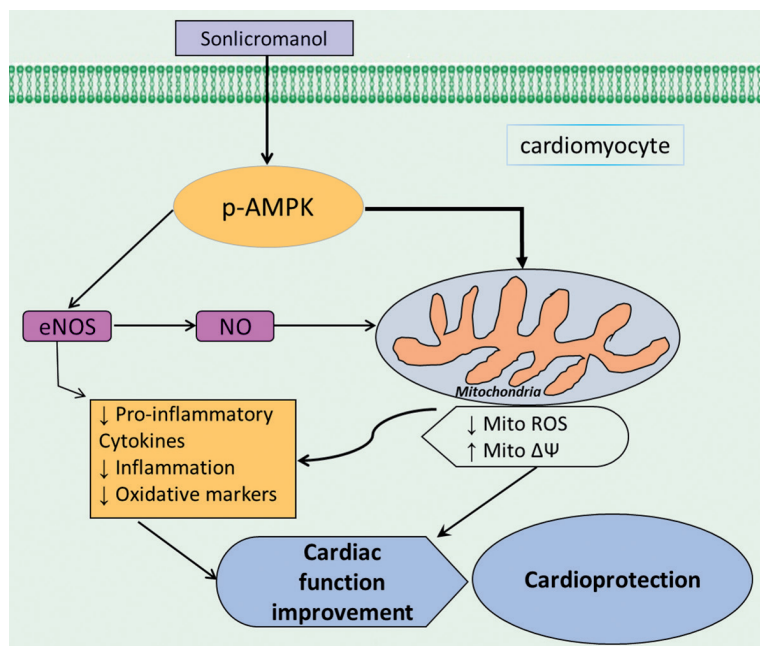
Figure 7. Schematic diagram of the study. Sonlicromanol has the potential to enhance mitochondrial function in rat hearts suffering from ischemia-reperfusion injury by activating AMPK. Furthermore, the activation of eNOS and subsequent production of NO are involved in this beneficial effect. These processes are then followed by a decrease in inflammatory responses and oxidative stress, ultimately leading to cardioprotection. AMPK, adenosine monophosphate-activated protein kinase; eNOS, endothelial nitric oxide synthase; NO, nitric oxide; Mito ROS, mitochondrial reactive oxygen species, Mito ΔΨ, mitochondrial membrane potential.

in reducing oxidative and inflammatory damages can be attributed to this mitochondrial action.

The interesting finding from this study is that sonlicromanol's cardioprotective effects were AMPK-dependent. In cardiac cells, AMPK serves as the primary energy sensor, and its dysregulation contributes to the development and progression of heart failure and I/R injury (Qi and Young 2015). By promoting SIRT1 activity, AMPK suppresses inflammatory responses *via* regulating numerous downstream signaling pathways such as NF-κB/TNF-α/interleukins (Yang et al. 2022). AMPK can also improve endothelial dysfunction by reducing TNF-dependent leukocyte-endothelial cell interactions and endoplasmic reticulum stress-induced lipid peroxidation (Qi et al. 2015; Yang et al. 2022). sonlicromanol phosphorylated the AMPK protein in rat cardiomyocytes. Proper activation of the AMPK signaling pathway components is required for the anti-oxidative and anti-inflammatory effects of this compound in rats subjected to cardiac I/R injury. Importantly, sonlicromanol treatment increased the expression of eNOS and NO levels. eNOS is also activated after AMPK phosphorylation and is primarily responsible for sufficient NO production (Li et al. 2016). By regulating the opening of ATP-sensitive potassium channels in mitochondrial membrane, NO significantly contributes to the better regulation of mitochondrial homeostasis (Doul et al. 2019; Tran et al. 2022). The contribution of these channels in protective impacts of sonlicromanol under AMPK activation needs to be addressed. It is worth noting that other AMPK activators can have effects similar to sonlicromanol in reducing oxidative stress and inflammation leading to

cardioprotection. For example, metformin, resveratrol, and aerobic exercise all reduce NF-κB activity and activate eNOS in cardiac cells *via* AMPK phosphorylation, thus mitigating the pathophysiology of I/R injury and subsequent heart failure (Dolinsky and Dyck 2014; Salvatore et al. 2021).

Following I/R injury, cardiac cells show low basal expression of eNOS and AMPK (Singhal et al. 2010; Xu and Si 2010). Similarly, we found that eNOS expression, as well as its downstream target, NO, was reduced in rat's heart with I/R injury. sonlicromanol, on the other hand, completely reversed the I/R-induced effects. Furthermore, previous evidence suggest that inhibition of NF-κB transcription factor and activation of eNOS following AMPK phosphorylation has a competitive advantage (Okayasu et al. 2008). Silencing AMPK expression or inhibiting AMPK activity in human umbilical vein endothelial cells have significantly decreased eNOS activity and NO production. Together, NF-κB activity inhibition has also been detected in mouse endothelial cells following AMPK inhibition (Okayasu et al. 2008). There is no study yet indicating the possible direct effect of sonlicromanol on eNOS or NF-κB activity, but since this drug lowers oxidative stress by modulating prostaglandin-E2 pathway (Xiao et al. 2021), it may modulate NF-κB activity and related inflammatory responses in ischemic disorders. Additionally, eNOS activation reduces NF-κB signaling (Caviedes et al. 2021). As a result, we can deduce that the proportion of NF-κB and AMPK/eNOS in a cell determines the status of cellular and tissue inflammatory and oxidative responses. Accordingly, our findings suggest that sonlicromanol's protective properties in rat's heart are due to the modulation of

the activity of AMPK/eNOS/NF-κB pathway. However, it is still not known by what mechanisms (directly or indirectly) sonlicromanol can increase AMPK activity. Two upstream molecules, calcium/calmodulin-dependent protein kinase kinase 2 (CAMKK2) and serine/threonine liver kinase B1 (LKB1), are major kinases involved in activating AMPK in stressful conditions such as exercise or calorie restriction (Willows et al. 2017). Whether sonlicromanol contributes to AMPK activation through modulating these upstream mediators requires further investigation.

Finally, sonlicromanol appears to have other extramitochondrial and intramitochondrial effects, such as regulating protein kinase C and intracellular Ca^{2+} signaling, mitochondrial biogenesis, oxidative-phosphorylation chain, apoptosis, and pyroptosis, which may act independently of AMPK and contribute to the ultimate efficacy of sonlicromanol in regression of I/R injury.

Conclusion

In conclusion, this study revealed that sonlicromanol provided substantial protection against myocardial I/R injury in rats by improving cardiac function and decreasing inflammatory responses and oxidative stress, and preventing mitochondrial dysfunction. sonlicromanol modification of eNOS/NF-κB/mitochondrial pathways components was linked to these promising results, and AMPK activation was a key regulator of this protective signaling (Fig. 7). The concentrations of sonlicromanol used in previous studies for mitochondrial disorders i were ranged from 2 to 100 mg, comparable to the drug doses used in this study. Regardless of this, additional studies are needed to determine the effective dose of the drug in similar settings but in the presence of cardiac risk factors and other comorbidities.

Conflict of interest. The authors declare that they have no conflicts of interest to report regarding the present study.

Availability of data and materials. The data that support the findings of this study are available on request from the corresponding authors.

Author contribution. XX and LL conceptualized and designed the experiments. KL, FH, and XX performed the experiments. KL, XX, and LL analyzed and interpreted the data. KL and FH drafted the initial draft of the manuscript. XX critically revised the draft. All authors reviewed the results and approved the final version of the manuscript.

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Supplementary Material

Targeting AMPK/eNOS pathway and mitochondria by sonlicromanol protects myocardial cells against ischemia-reperfusion injury

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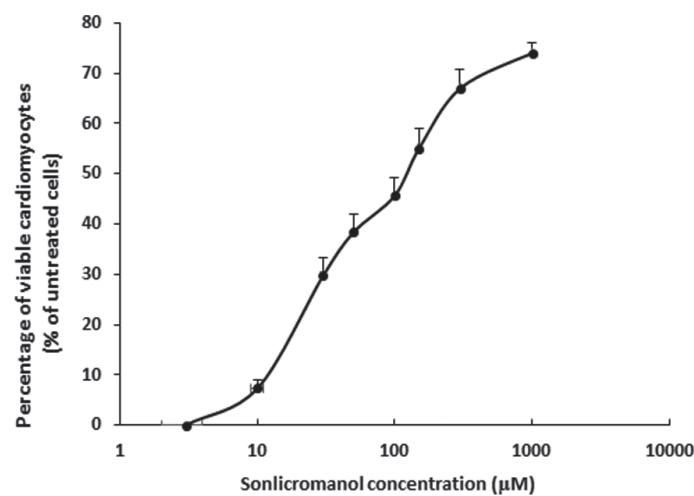
Supplementary Figure

Figure S1. Dose-response tracing of the effect of different concentrations of sonlicromanol in µM on H9C2 cardiomyocytes viability (in percent of untreated H9C2 cells) following hypoxia/reoxygenation injury model. A dose-response pilot study was conducted to determine the effect sonlicromanol on cell viability in H9C2 cardiomyocytes exposed to hypoxia/reoxygenation injury. We selected two concentrations of 30 to 150 µM and then calculated their injectable doses to use in our animal study.