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Cannabinoid receptor-2 agonist AM1241 attenuates myocardial ischemia/reperfusion- induced oxidative stress in rats via Nrf2/HO-1 pathway

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Research Article

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Abstract

Cannabinoid type II receptor, also known as CB2 receptor, has been reported to have a protective effect on myocardial infarction. However, CB2 receptor's role in oxidative stress involved in myocardial ischemia-reperfusion injury (MIRI) and the underlying mechanism has not been fully elucidated. This study aimed to investigate the protective role of cannabinoid receptor-2 agonist AM1241 against MIRI-induced oxidative stress in rats. In addition, the protective effect of AM1241 on oxidative damage of cardiomyocytes injured by hypoxia-reoxygenation (HR) in vitro and its possible mechanism were investigated by establishing a model of HR of H9c2 cardiomyocytes. Clearly, large infarct size and increased number of inflammatory cells were detected in the heart of rats with MIRI. In the meantime, rats in IR group displayed impaired cardiac function as well as promoted myocardial fibrosis and apoptosis rate. Notably, we observed that ROS production was enhanced in the HR-injured cells, while administration of AM1241 caused a reversal effect, leading to improved cell viability, reduced ROS production and elevated expression of Nrf2 and HO-1 in the cardiomyocytes. In Conclusion, AM1241 exerts beneficial effects on relieving MIRI and attenuating oxidative stress possibly by activating Nrf2/HO-1 signaling pathway.

1. Introduction

Ischemic heart disease is one of the most common cardiovascular diseases and poses a serious threat to human health.[1] The main therapeutic strategy is to effectively restore the blood supply of ischemic myocardium. As such, the mortality of patients with ischemic heart disease can be reduced to a certain extent. Delayed reperfusion may aggravate structural and functional damage to myocardium, causing a series of adverse consequences, such as arrhythmia, cardiac systolic dysfunction and cell death, and eventually myocardial ischemia-reperfusion injury (MIRI).[2,3] The pathogenesis of MIRI is extremely complex, and MIRI has been found to be related to several factors including oxidative stress, endoplasmic reticulum stress and inflammatory responses.[4] Multiple studies have shown that the excessive production of reactive oxygen species (ROS) at the beginning of myocardial reperfusion contributes to enhanced oxidative stress that can subsequently cause harmful damage to the patients.[5,6] Increased expression of HO-1, a stress protein involved in cell defense, can alleviate MIRI-induced cell damage.[7] As a redox-sensitive transcription factor, Nrf2 interacts with antioxidant response elements and plays a key role in transcriptional activation of HO-1. Recently, it has been suggested that upregulation of HO-1 expression induced by Nrf2 activation could protect the heart from oxidative stress.[8,9]

Cannabinoid type II receptor also called CB2 receptor is a G protein coupled receptor with seven transmembrane domains, which is widely distributed in liver, skin, heart, and immune cells.[10] It has recently been demonstrated that endogenous cannabinoid system, especially CB2 receptor, has a protective effect against myocardial infarction.[11] In the meantime, activated CB2 receptor has been shown to possess a great potential in antioxidant stress and anti-inflammatory responses in various disease models.[12,13] Wang et al.[14] found that CB2 receptor agonist AM1241 activates PI3K/Akt/Nrf2

signaling pathway to reduce excessive oxidative stress and inflammation in ischemic heart, eliciting endogenous myocardial regeneration. Zhang et al.[15] showed that AM1241 protects skeletal muscle against ischemia-reperfusion injury via Nrf2 signal transduction pathway. To date, the role of CB2 receptor in MIRI-related oxidative stress and its relevant mechanism have not been fully elucidated.

The present study aimed to investigate the role of CB2 receptor agonist AM1241 in MIRI-induced oxidative stress as well as the underlying molecular basis. The findings of this study suggest that AM1241 appears as a potential therapeutic alternative for the treatment of MIRI.

2. Materials And Methods

2.1. Animals and drugs.

Male Sprague-Dawley (SD) rats weighing 200–250 g were obtained from the Experimental Animal Center of Wenzhou Medical University. All rats were kept in plastic cages that had been irradiated with a fluorescent lamp for 12 h and maintained under controlled environmental conditions (23–25°C, 40–50% humidity), with free access to standard chow and fresh water. AM1241 (Target Mol, USA) was dissolved in 10% DMSO and distilled water for administration, and Nrf2 inhibitor ML385 was purchased from Medchem Express (MCE Co. Ltd., Shanghai, China). All animal experiments were conducted in accordance with the National Institute of Health guidelines (NIH Publications No. 8023, revised 1978), and were approved by the Institutional Animal care and Use Committee of the First Affiliated Hospital of Wenzhou Medical University (Wenzhou, Zhejiang, China).

2.2. Establishment of a MIRI model and animal grouping.

Rats were anesthetized with thiopental sodium (65 mg/kg, i.p.), fixed in supine position, intubated via mouth and ventilated with an animal ventilator (HX-300S, Chengdu Taimeng Software Co., Ltd., Chengdu, China). Clinical parameters used in the experiments were as follows: respiratory time ratio, 1.5:1; respiratory rate, 60 times/min; and tidal volume, 70 mL/kg. The thoracic cavity was opened at the 3rd and 4th left intercostal space to reveal the heart. Thereafter, the needle with 6 − 0 non-invasive sutures were then inserted into the left edge of pulmonary conus and the lower edge of left atrial appendage. After 10 min of stabilization, a double-layer plastic cannula was inserted and ligation was then performed. The presence of myocardial infarction as evidenced by either ST segment elevation or T-wave peak, as well as local cyanosis in the heart on electrocardiogram (ECG) indicated successful modeling of the MIRI. After 30 min of ischemia, the inner cannula was drawn out for reperfusion for 2 h.

All SD rats (n = 48) were randomly divided into sham group (only threading without ligation); model group (IR group, IR with saline injection); IR + AM1241 group (IR with intraperitoneal injection of AM1241 6 mg/kg); and IR + AM1241 + ML385 group (IR with intraperitoneal injection of AM1241 6 mg/kg and ML385 30 mg/kg). AM1241 and/or ML385 were administered 1 h prior to the modeling.

2.3. Cardiac function test.

Echocardiography was carried out to determine cardiac pump function, including left ventricular fraction (LVEF) and left ventricular fractional shortening (LVFS). The blood sample was taken at the end of reperfusion and centrifuged at 1000 rpm for 15 min. Afterwards, the serum was collected and then subjected to enzyme linked immunosorbent assay (ELISA) for testing cardiac function, including the serum levels of cardiac troponin I (cTnI), aspartate transaminase (AST), lactate dehydrogenase (LDH), and creatine kinase isoenzyme (CK-MB). Results were expressed in U/L. Myocardial cell injury was evaluated and analyzed by using a spectrophotometer (Beijing Shiji Kexin Scientific Instrument Co., Ltd., Beijing, China).

2.4. 2,3,5-triphenyltetrazolium chloride (TTC) staining.

After successful modeling, four rats in each group were killed and their hearts were collected for subsequent analysis. The heart was frozen at -80°C for 20 min, cut into 7–9 transverse slices and then incubated with 1% TTC phosphate buffer (pH = 7.4) in the dark at 37°C for 30 min. The infarct areas were captured and quantified by using a camera and Image J software. Non-TTC-stained area (white) was identified as the infarct area, and the living tissue was stained red.

2.5. Myocardial tissue sectioning and staining.

After reperfusion, the hearts from another 4 rats in each group were fixed in 4% paraformaldehyde for 24 h and cut into slices of 4 μm thickness (Cleica, Nussloch, Germany). Haematoxylin and eosin (H&E) staining and Masson staining were performed to examine histopathological changes under a microscope at 100× (Olympus, Tokyo, Japan). The collagen rich area with fibrous degeneration was stained blue while the cell matrix was stained red. DNA fragmentation was detected in situ on frozen sections with TUNEL apoptosis detection kit (Solarbio, USA). TUNEL positive cells were observed and the apoptosis rate was calculated as a ratio of the positive cells to total cells (the number of green nuclei to the number of blue nuclei).

2.6. Establishment of a hypoxia-reoxygenation (HR) model of cardiomyocytes and cell grouping.

H9c2 cardiomyocytes were obtained from Nanjing Kaiji Biotechnology Co., Ltd. (Nanjing, China) and randomly divided into control, model (HR), HR + AM1241 and HR + AM1241 + ML385 groups. Cells in control group were grown in an incubator with 21% O_2 and 5% CO_2 (Herocell 180, Shanghai Rundu Biotechnology Co., Ltd., Shanghai, China). Cells in HR group were cultured in KRB buffer (0.90 mM NaH_2PO_4, 98.51 mM NaCl, 6.0 mM $NaHCO_3$, 10.0 mM KCl, 2.38 mM $CaCl_2{\cdot}2H_2O$, 2.46 mM $MgSO_4{\cdot}7H_2O$, 20.0 mM HEPES, 0.01% BSA, pH, 7.2–7.4) under hypoxia (1% O_2 and 5% CO_2) for 4 h, followed by incubation in a 5% CO_2 normoxic incubator for another 10 h. Cells in HR + AM1241 group were incubated with 10 μM AM1241 for 6 h and then subjected to hypoxia-reoxygenation. Cells in HR + AM1241 + ML385 group were soaked in 5 μM of ML385 for 1 h after the same treatment as HR + AM1241 group.

2.7. Cell viability assay.

HR-injured cardiomyocytes were seeded in a 96-well plate at a density of 1×10^4 cells per well. 100 μL DMEM (GIBCO, USA) and 10 μL CCK-8 reagent were added to each well. The absorbance was measured at 450 nm using a microplate reader (Multiskan Spectrum; Thermo Fisher Scientific, Waltham, MA, USA).

2.8. Dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay.

After modeling, cells were washed with PBS buffer and then incubated with 500 μL of DCFH-DA staining solution (Jiangsu biyuntian Biotechnology Co., Ltd., Nantong, China) at 37°C for 20 min. Afterwards, the staining solution was removed and the cells were washed with serum-free medium for three times. The fluorescence intensity was detected by Accuri C6 flow cytometry (BD, biosciences, CA, USA). The excitation wavelength was 488 nm, and the emission wavelength was 525 nm.

2.9. Immunofluorescence.

Myocardial tissues were embedded in Polylysine, fixed with precooled 4% paraformaldehyde at room temperature and then sectioned at a thickness of 4 μm. The slices were washed with PBS and subjected to antigen retrieval. After being blocked with PBS containing 2% BSA at room temperature for 1 h, the sections were incubated with Nrf2 polyclonal antibody (1:200, Santa Cruz, USA) and FITC-labeled anti-rabbit secondary antibody (Jackson ImmunoResearc, USA) at 37°C for 1 h. Fluorescence images were captured under laser confocal fluorescence microscopy (Leica TCS-SP2-AOBS-MP, Germany), and 5 different fields of view were randomly taken from each section.

2.10. Western blot.

The concentration of total protein extracted from myocardial tissues or H9c2 cardiomyocytes was detected with a BCA protein assay kit. The protein samples were separated by 12% SDS-PAGE and transferred to a PVDF membrane (Millipore, Bedford, MA). The membrane was blocked in TBST (Tris HCl and Tween 20) solution containing 5% skimmed milk powder at 25°C for 1.5 h and then incubated overnight at 4°C with primary antibodies against Nrf2 (1:1000, Abcam, Cambridge, UK) or HO-1 (1:1000, Abcam, Cambridge, UK). On the next day, the membrane was washed with TBST buffer and incubated with alkaline phosphatase labeled IgG (Promega, USA) for 1 h at room temperature. β-actin was included as an internal reference. Immunoreactive proteins were visualized using a chemiluminescence imaging system (EI600, Beyotime, Shanghai, China), and Image J software was used to determine the gray value of protein bands.

2.11. Statistical analysis.

Statistical analyses were performed using GraphPad Prism 8.0 software. The experimental data were expressed as mean ± standard deviation (SD). Differences between multiple groups were analyzed with one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Statistical significance was defined as $p < 0.05$.

3. Results

3.1. AM1241 Inhibits MIRI in vivo.

To investigate the role of AM1241 in MIRI, we performed TTC staining and H&E staining to determine the infarct size as well as characteristics of myocardial histomorphology. As shown by TTC staining, almost no infarct area (white) was identified from rat heart slices in sham group, heart slices in IR group contained large white areas (Fig. 1A). Moreover, the infract sizes in IR + AM1241 + ML385 group were significantly larger than those in IR + AM1241 group, while the sizes in IR + AM1241 or IR + AM1241 + ML385 group remained considerably smaller than those in IR group (Fig. 1B). In the meantime, H&E staining revealed myocardial rupture, necrosis, congestion, and inflammatory response in IR group, but normal myocardial structure in IR + AM1241 group (Fig. 1C). Notably, administration of AM1241 (6 mg/kg) significantly reduced the number of inflammatory cells in the IR injured tissues ($p < 0.001$), while this inhibitory effect of AM1241 was relieved by Nrf2 inhibitor ML385 ($p < 0.01$) (Fig. 1D).

We next performed echocardiography on rats for cardiac function test. As illustrated in Fig. 2A-C, a significant reduction in LVEF and LVFS values was observed in IR group as compared to sham group. Moreover, the serum levels of cTnI, AST, LDH, and CK-MB in IR rats were significantly higher than those in the controls, indicating an IR-induced injury in cardiomyocytes (Fig. 2D-G). As expected, we found that while AM1241 treatment markedly attenuated the IR injury in rats ($p < 0.001$), co-treatment with ML385 (30 mg/kg) reversed cardiac function improvement caused by AM1241.

3.2 AM1241 alleviates myocardial fibrosis in rats with MIRI and reduces cardiomyocyte apoptosis following IR injury.

Masson staining was performed to detect the degree of myocardial fibrosis in the rats. In the experiments, myocardial fibers were stained red and collagen fibers were stained blue. As depicted in Fig. 3A, obvious myocardial fibrosis (shown in blue) was observed in IR group compared with sham group. Moreover, we showed that the degree of myocardial fibrosis was significantly reduced in IR + AM1241 group ($p < 0.001$), while administration of ML385 relieved the inhibitory effect of AM1241 on myocardial fibrosis in IR rats ($p < 0.05$) (Fig. 3B).

We further examined apoptosis in the cardiomyocytes by TUNEL assay. As illustrated in Fig. 3C and 3D, the number of apoptotic cardiomyocytes stained green was noticeably increased in IR group compared with sham group ($p < 0.0001$). Meanwhile, treatment of IR rats with AM1241 (6 mg/kg) led to a marked decrease in the apoptosis rate ($p < 0.01$). Notably, co-treatment with ML385 partially reversed AM1241-induced inhibition of apoptosis in IR rats, albeit the apoptosis rate in IR + AM1241 + ML385 group remained lower than that in IR group ($p < 0.05$). Together, these observations indicated that AM1241 inhibited apoptosis in the cardiomyocytes of IR rats.

We then performed cell viability assay to determine the effect of AM1241 on cell survival of H9c2 cardiomyocytes. As shown in Fig. 3E, AM1241 treatment caused distinctive changes in cell viability of

H9c2 cells. In this case, a significant increase in the cell viability was observed in the cardiomyocytes treated with 10 μM AM1241 as compared to the control cells ($p < 0.01$). We, therefore, chose 10 μM AM1241 for subsequent experiments.

3.3 Effects of AM1241 on oxidative stress and myocardial protection in HR-injured cardiomyocytes.

We conducted DCFH-DA assay to evaluate the production of ROS in rat cardiomyocytes. As depicted in Fig. 4A-B, a dramatic accumulation of ROS was observed in HR group compared with control group. In the meantime, ROS fluorescence intensity in HR + AM1241 group was significantly lower than that in HR group ($p < 0.001$). Notably, co-treatment with ML385 relieved AM1241-induced decrease of ROS fluorescence intensity in HR-injured cells ($p < 0.05$). These data demonstrated that 10 μM AM1241 could effectively inhibit HR-induced production of ROS.

We further examined the expression levels of Nrf2 and HO-1 in the cardiomyocytes. Immunofluorescence assay showed that AM1241 treatment led to an increase in the intensity of green fluorescence in HR-injured cells, suggesting an elevated expression of Nrf2 in myocardium (Fig. 4C). Moreover, western blot analysis revealed that while the expression of Nrf2 and HO-1 was upregulated in HR cells treated with AM1241, co-administration of ML385 partially reversed AM1241-induced increase of Nrf2 and HO-1 expression in HR-injured cells (Fig. 4D). Taken together, these results suggested that the protective effect of AM1241 on MIRI rats may involve Nrf2/HO-1 signaling pathway.

4. Discussion

IR injury in the heart could lead to changes in myocardial morphology, metabolism and function. Previous studies have suggested that IR injury may be linked to calcium overload, inflammatory responses, dysfunction of energy metabolism, and increased ROS production.[16,17] In this study, we showed that rats with MIRI display inflammatory cell infiltration and necrosis followed by a marked increase in myocardial apoptosis and fibrosis, providing consistent data with the previous reports.[18,19] AM1241, a selective agonist of cannabinoid CB2 receptor, has been shown to improve cardiac function and alleviate myocardial fibrosis through Nrf2-mediated TGF-β1/Smad3 signaling pathway, while preventing ethanol-induced cardiotoxicity.[20,21] Currently, little is known about the therapeutic effects of AM1241 on ischemic heart disease as well as its cellular regulation. The present study aimed to investigate the role of AM1241 in rats with MIRI and the underlying molecular mechanism. Strikingly, we found that AM1241 treatment significantly reduced the infraction size of the heart and down-regulated the serum activity levels of cTnI, AST, LDH, and CK-MB, suggesting a protective role of AM1241 in ischemia-induced myocardial damage in rats.

Herein, we provided more evidence that oxidative stress is closely related to the occurrence and development of IR injury. Given that primary cardiomyocytes are terminally differentiated cells without proliferating activities, the present study generated a HR injury model using H9c2 cardiomyocytes for

mimicking MIRI and further unveiled the potential mechanism underlying the antioxidant effect of AM1241 on myocardial HR injury. Under pathological conditions such as HR injury, the myocardial infarction and cardiac function damage could lead to overproduction of ROS, resulting in oxidative stress.[22] It has been showed that HR injury up-regulates the expression of downstream genes related to antioxidation. Upon reperfusion of ischemic cardiomyocytes, excessive oxygen molecules in the bloodstream enter the cardiomyocytes, allowing the production of a large amount of hydrogen peroxide and superoxide anions.[23,24] In this study, we employed the DCFH-DA method to further investigate the protective effects of AM1241 on HR injury-induced overproduction of ROS. The DCFH-DA assay revealed that AM1241 inhibited ROS production in HR-injured cardiomyocytes, suggesting an antioxidant effect of AM1241 on the IR injury. This finding was in accordance with the previous observation showing an antioxidant effect of AM1241 in hepatic stellate cell line.[25]

Nrf2 is a transcription factor involved in various intracellular signal transduction pathways which protect organs against oxidative stress. Diao et al.[26] found that Nrf2 could inhibit oxidative damage and reduce ROS production in a mouse model of renal IR injury. Nrf2 upregulation can promote antioxidant signal transduction in the diabetic MIRI model. Increased expression of HO-1, a downstream protein of Nrf2, has been identified as an indicator for activation of Nrf2 signaling pathway. Here, we hypothesized that the potential mechanism of myocardial HR injury may involve Nrf2/HO-1 pathway. To test this scenario, we performed assays using Nrf2 inhibitor ML385. As expected, we showed that the administration of ML385 led to a downregulation of the expression of Nrf2 and HO-1. Notably, accumulating evidences have demonstrated that HO-1 possesses the multiple effects, including antioxidant, anti-inflammatory activities, signal transduction and immune regulation, as well as inhibition of adhesion molecule expression. A recent research has found that HO-1 can protect the intestine against IR injury and inflammation, while slowing the progression of many diseases such as cerebral IR injury.[27] In this study, AM1241 upregulated the expression of HO-1 and Nrf2, providing consistent data with the previous research.[14] Moreover, treatment with Nrf2 inhibitor partially reversed AM1241-induced increase in the expression of HO-1 and Nrf2, suggesting that AM1241 protects against MIRI presumably by activating Nrf2/HO-1 pathway. At present, artificial intelligence has been applied to various fields including the diagnosis and treatment of cardiovascular diseases.[28] Hence, prediction models that have been used to identify potential targets for Nrf2/HO-1 pathway may have significant implications for the treatment of ischemic heart diseases.

5. Conclusion

This study provides evidence that Cannabinoid receptor-2 agonist AM1241 improves the heart function and alleviates myocardial infarction and inflammatory response in rats with MIRI. Further studies show that it suppresses HR-induced oxidative stress possibly through activating Nrf2/HO-1 signaling pathway. Therefore, AM1241 may serve as a promising agent for the treatment of MIRI.

Declarations

Data Availability

The data were available from the corresponding author on reasonable request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Authors' Contributions

Mingxiao Zhang proposed the concept, designed the methodology; Formal analysis, data investigations and management were completed by Qingxin Tian; Jianlong Liu made critical revisions to the manuscript. All authors wrote, read and approved the final manuscript.

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Figures

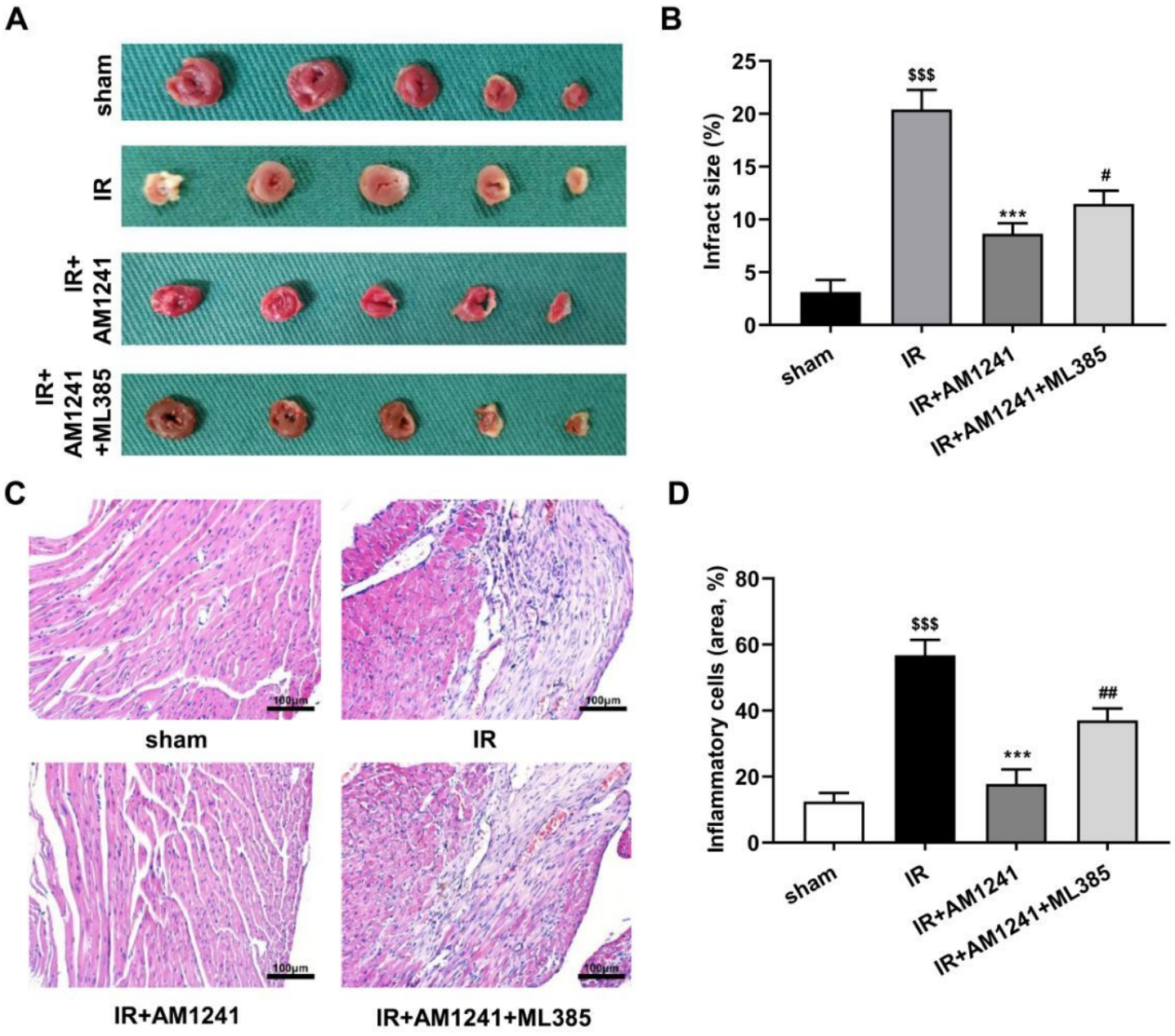

Figure 1

AM1241 alleviated myocardial infarct of rats with MIRI

(A) Representative images of TTC staining. (B) The percentage of infarct volume to left ventricular volume. (C) Myocardial tissues of normal rats and IR rats stained by H&E (×100). (D) Quantitative analysis of the number of inflammatory cells. $^{\$\$\$}$p<0.001 vs. sham group; ***p<0.001 vs. IR group; #p<0.05 and ##p<0.01 vs. IR+AM1241 group.

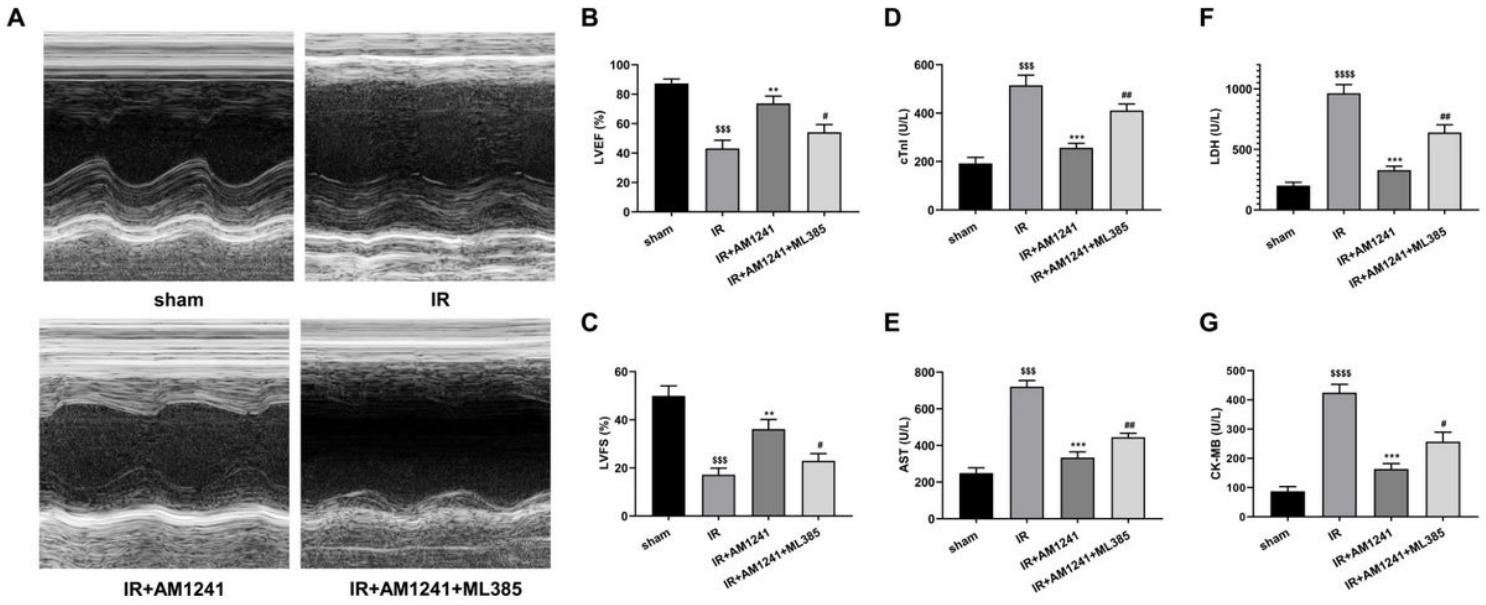

Figure 2

Protective effect of AM1241 on cardiac function of rats with MIRI

(A-C) Echocardiography was carried out to evaluate the cardiac function indexes of ejection fraction (LVEF) and shortening fraction (LVFS) of rats. (D-G) The level of cTnI, AST, LDH and CK-MB in serum of rats in each group. Results were presented as mean ± SD (n=4). $^{\$\$\$}p<0.001 and $^{\$\ \$\$\$}p<0.0001 vs. sham group; $^{**}p<0.01$ and $^{***}p<0.001$ vs. IR group; $^{\#}p<0.05$ and $^{\#\#}p<0.01$ vs. IR+AM1241 group.

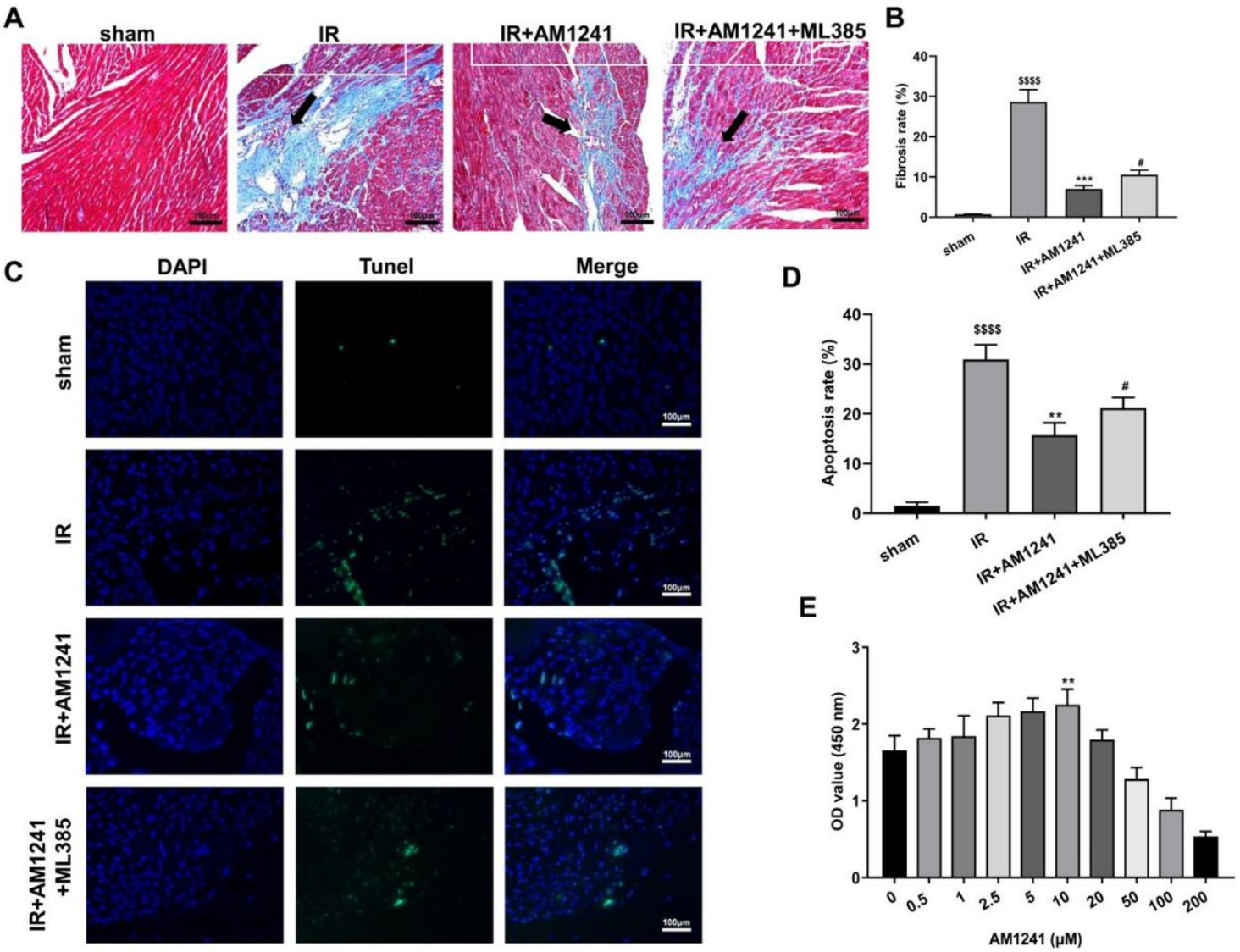

Figure 3

AM1241 suppressed myocardial fibrosis and myocardial cell apoptosis in rats

(A) Masson staining (×100) and (B) quantitative analysis of myocardial necrosis in rats with MIRI. $ $$$p<0.0001 vs. sham group; ***p<0.001 vs. IR group; #p<0.05 vs. IR+AM1241 group. (C) Apoptosis of cardiomyocytes in each group was detected by TUNEL assay. (D) Myocardial cell apoptosis rate in each group. $ $$$p<0.0001 vs. sham group; **p<0.01 vs. IR group; #p<0.05 vs. IR+AM1241 group. (E) CCK-8 assay was performed to assess the viability of H9c2 cells treated with different concentrations of AM1241 for 24 h. **p<0.01 vs. control group (cells without AM1241 treatment).

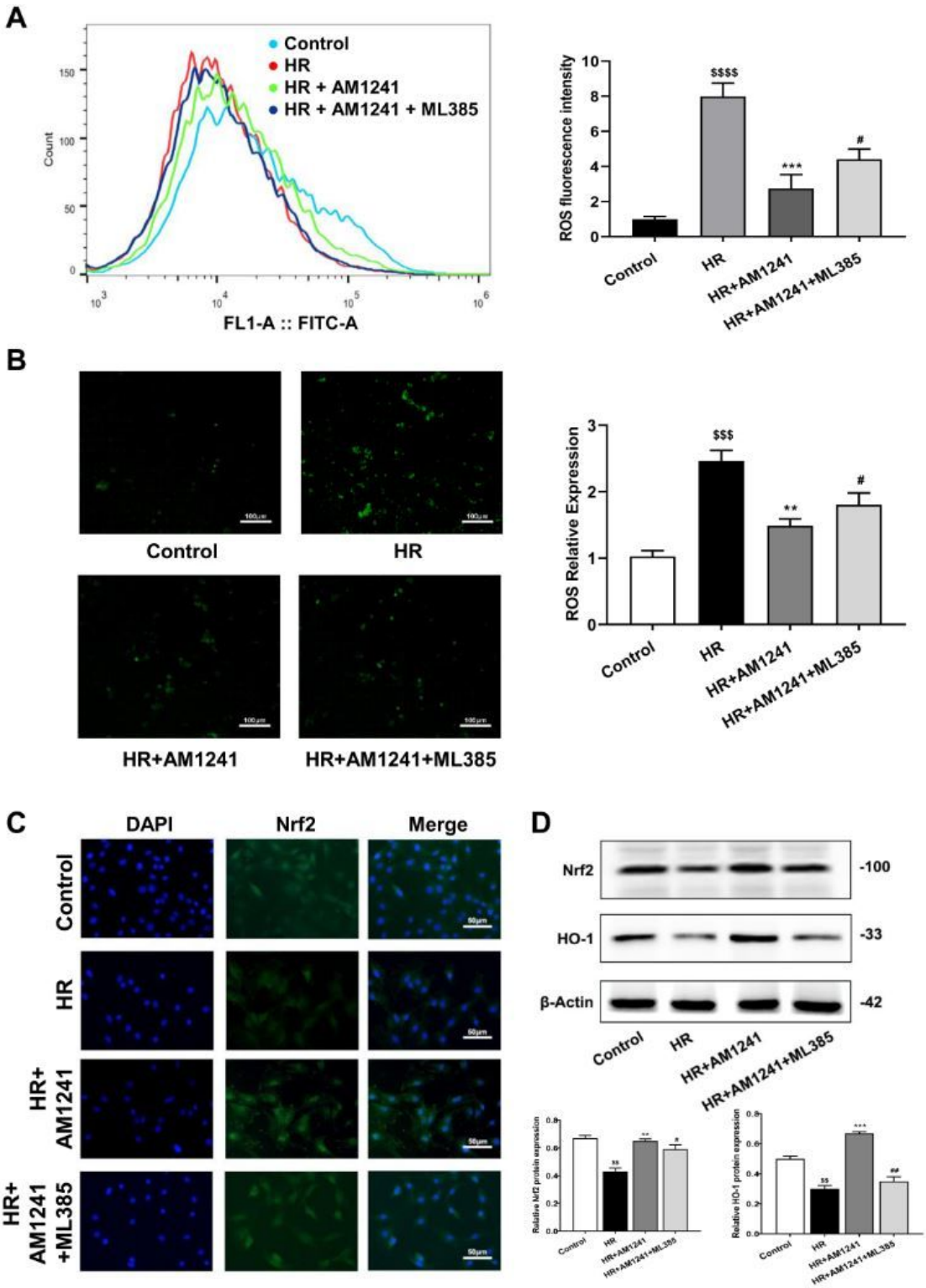

Figure 4

Inhibitory effect of AM1241 on ROS production induced by HR in H9c2 cells and alleviated the damage owing to oxidative stress via the Nrf2/HO-1 signaling pathway

(A) DCFH-DA fluorescence staining was used to evaluate the effect of AM1241 on intracellular ROS production. (B) Effects of AM1241 on intracellular ROS production. $^{\$\$\$}p<0.001$ and $^{\$\ \$\$\$}p<0.0001$ vs.

control group; $^{**}p<0.01$ and $^{***}p<0.001$ vs. HR group; $^{\#}p<0.05$ vs. HR+AM1241 group. (C) The subcellular localization of Nrf2 protein was observed by immunofluorescence staining (green) under confocal microscope LSM (magnification 200×). (D) Expression of Nrf2 and HO-1 measured by Western blotting. β-Actin served as control for equal protein loading. $^{\$\$}p<0.01$ vs. control group; $^{**}p<0.01$ and $^{***}p<0.001$ vs. HR group; $^{\#\#}p<0.01$ vs. HR+AM1241 group.