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Metformin Attenuates Myocardial Ischemia-Reperfusion Injury through the AMPK-HMGCR-ROS Signaling Axis

Objective	To explore the role and mechanism of metformin (MET) in regulating myocardial injury caused by cardiac ischemia-reperfusion.
Material and methods	A rat model of myocardial ischemia-reperfusion injury was established by ligation of the anterior descending branch of the left coronary artery. The myocardial area at risk and the infarction size were measured by Evans blue and 2,3,5-triphenyltetrazole chloride (TTC) staining, respectively. Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End Labeling (TUNEL) staining was used to detect apoptosis of cardiomyocytes. The expression of 4-hydroxynonenal (4-HNE) was detected by immunohistochemical staining. Real-time quantitative polymerase chain reaction (RT-PCR) and Western blot were used to detect mRNA and expression of the Adenosine 5'-monophosphate-activated protein kinase (AMPK) – 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) signaling pathway, respectively.
Results	MET treatment decreased the infarct size and the activity of the myocardial enzyme profile, thus demonstrating protection of ischemic myocardium. The number of TUNEL positive cells significantly decreased. Immunohistochemical results showed that MET decreased the expression of 4-HNE in myocardial tissue and the content of malondialdehyde (MDA) in myocardial cells. Further experimental results showed that MET decreased HMGCR transcription and protein expression, and increased AMPK phosphorylation. In the model of hypoxia and reoxygenation injury of cardiomyocytes, MET increased the viability of cardiomyocytes, decreased the activity of lactic dehydrogenase (LDH), decreased malondialdehyde content and intracellular reactive oxygen species (ROS) concentrations, and regulate the AMPK-HMGCR signaling pathway through coenzyme C (ComC).
Conclusion	MET inhibits the expression of HMGCR by activating AMPK, reduces oxidative damage and apoptosis of cardiomyocytes, and alleviates myocardial ischemia-reperfusion injury.

Keywords	Metformin; AMPK; HMGCR; ischemia-reperfusion; oxidative stress
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Introduction

Coronary artery disease is one of the most common chronic cardiovascular diseases, with globally increasing incidence and mortality [1]. Early thrombolytic reperfusion and percutaneous coronary intervention are effective strategies for treating acute myocardial infarction by helping to restore coronary blood flow and reduce the size of the infarct. However, the process of coronary artery blood flow restoration may lead to further myocardial damage, known as myocardial ischemia-reperfusion injury (MIRI). Mechanisms involved in MIRI include oxidative stress, myocardial cell apoptosis, inflammatory response, calcium overload, and mitochondrial dysfunction [2, 3].

Oxidative stress refers to an excessive accumulation of intracellular reactive oxygen species (ROS) that surpasses the body's antioxidant clearing capacity and results in oxidative damage [4]. Previous studies have indicated that 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) is a significant source of ROS in myocardium, and that it plays a crucial regulatory role in myocardial cell proliferation and death [5]. Inhibiting HMGCR helps to reduce ROS and to alleviate MIRI. Research on 5'-monophosphate-activated protein kinase (AMPK) in knockout mice has confirmed the vital role of AMPK activation in cardiac protection during MIRI [6], although the specific mechanism remains unclear.

Metformin (MET) is a first-line medication for treating type 2 diabetes [7]. In addition, MET can significantly reduce

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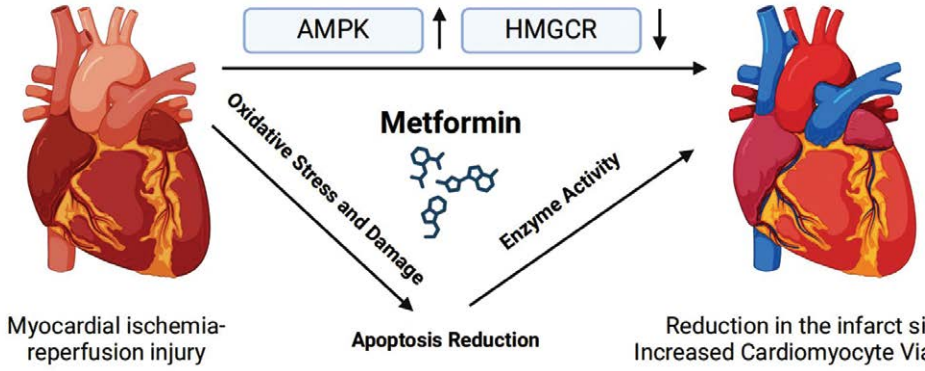
Central illustration. Metformin Attenuates Myocardial Ischemia-Reperfusion Injury through the AMPK-HMGCR-ROS Signaling Axis



the incidence of myocardial infarction and mortality in patients with type 2 diabetes [8]. Therefore, besides controlling blood sugar, MET also has protective effects in various cardiovascular diseases, including myocardial ischemia [8, 9].

In a rat model, intracoronary infusion of MET before reperfusion reduced infarct size by 40–50% [10]. In a mouse model, treatment with MET for 14 days following ischemia/reperfusion provided significant protection against myocardial hypertrophic and apoptotic remodeling [11]. Several studies have shown that MET's cardioprotective effects partially depend on the activation of AMPK [12, 13]. Although the effects of MET on MIRI are somewhat established, its mechanisms have not been fully elucidated. Research on MET's antioxidant effects has primarily focused on whether it can reduce ROS synthesis sufficiently to decrease tissue fibrosis [14]. In fact, MET has been reported to inhibit heart fibrosis by suppressing HMGCR expression and reducing ROS production [15]. Studies in a model of high-sugar-induced cell damage indicated that AMPK inhibition of HMGCR expression can reduce ROS production [16, 17].

The relationship between AMPK and HMGCR during development of MIRI remains unclear. Therefore, this study aimed to explore whether the protective effects of MET are related to AMPK activation, to inhibition of HMGCR expression, and to reduction of excessive oxidative stress damage through in vivo and in vitro applications of models of MIRI.

Material and methods

Animal source and other details

Sprague-Dawley (SD) male rats (200–250 g; 6–8 wk old; n=50) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (animal production license number of 2021–0006). The rats were acclimated at the Experimental Animal Center of Henan University of Science and Technology for 1 wk before the experiment at 20–22° C, 45%–65% relative humidity, and a light-dark cycle. This animal study was conducted after approval by the Medical Ethics Committee of Henan University of Science and Technology. Euthanasia was performed using excessive anesthesia (intraperitoneal injection of pentobarbital sodium at a dose of 200 mg/kg).

Model of MIRI

The rats were anesthetized with a intraperitoneal injection of pentobarbital sodium (50 mg/kg), followed by intubation and ventilation with a rodent ventilator. To create regional myocardial ischemia/reperfusion, the heart was exposed, and the left anterior descending coronary artery (LAD) was ligated with 6–0 silk thread for 45 min. Myocardial whitening and regional motion abnormalities in the ischemic area and elevation of the ST segment in the electrocardiogram confirmed regional ischemia. Subsequently, the ligature was released to allow for 1 h of reperfusion, as evidenced by marked congestion in the reperfused region.

The rats were randomly divided into three groups: Sham group (surgery simulation, n=3), MIRI group (LAD ligation for 45 min followed by 1 h reperfusion, n=3), and MIRI + MET group (LAD ligation for 45 min, intravenous injection of MET (5 mg/kg) via the jugular vein, followed by 1 h reperfusion, n=3. Experimentally, this was best done immediately after MI). The MET dosage was based on the previous report of Apaijai et al. [18].

At the end of reperfusion, the LAD was ligated again, and Evan's blue dye was injected into the aorta to retrogradely perfuse the coronary circulation. The heart was excised and frozen at –20°C for 30 min and then sliced into six 2 mm sections. The sections were incubated in TTC at 37°C for 10 min, followed by immersion in 4% formaldehyde at room temperature for 4 hrs. The tissue distal to the LAD ligature, i.e., the area at-risk (AR) was evident from the absence of blue color. TTC staining differentiated the white infarcted area (IS) from the red non-infarcted at-risk area. The myocardial sections were arranged from top to bottom (distance below the suture) and photographed. ImageJ soft-

ware (version 1.47; National Institutes of Health, USA) was used to analyze the staining of the sections, and the final results are presented in the form of IS/AR ratios.

Hypoxia-reoxygenation injury model of primary cardiomyocytes

SD rats (1–3 days old, n=30) were sacrificed by cervical dislocation, and their hearts were quickly excised, minced, and dissociated in a solution of 0.1% trypsin and 0.03% type II collagen. The dispersed neonatal rat cardiomyocytes (NRCMs) were then plated at a density of 2×10^5 cells/cm^2 in 6-well plates containing 2 ml per well of Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum. After serum starvation (37°C, 95% O_2, 5% CO_2, 24 h), the cardiomyocytes were subjected to hypoxia (95% N_2, 5% CO_2) in a CO_2 incubator at 37°C for 4 h. Subsequently, the medium was replaced with fresh, oxygenated plating medium (68% DMEM, 17% medium 199, 10% horse serum, 5% FCS), and the plates were transferred to a normal oxygen concentration incubator (5% CO_2, 20% O_2) for reoxygenation for 6 h. This model of hypoxic-reoxygenation injury in primary cardiomyocytes was referred to in previous studies [19].

Cell grouping

1) AMPK inhibitor experiment. NRCMs were randomly divided into four groups: control group (CON), cells were incubated under normal culture conditions; hypoxia-reoxygenation (HR) group, cells were incubated under hypoxic conditions for 4 h followed by reoxygenation for 6 h; HR + MET group, MET (0.1 mM) was added to the DMEM at the start of reoxygenation; HR + MET + Compound C (ComC) group, MET and ComC (10 nM; ab120843; Abcam) were simultaneously added to the medium at the start of reoxygenation. ComC is an AMPK activity inhibitor.
2) AMPK silencing experiment. NRCMs were randomly divided into four groups: CON + NS-siRNA group, cells were pretreated with NS-siRNA and then incubated under normoxia; HR + NS-siRNA group, cells were pretreated with NS-siRNA, subjected to 4 h of hypoxia followed by 6 h of reoxygenation; HR + MET + NS-siRNA group, cells were pretreated with NS-siRNA, subjected to 4 h of hypoxia and 6 h of reoxygenation, MET (0.1 mM) was added at the start of reoxygenation; HR + MET + AMPK-siRNA group, cells were pretreated with AMPK-siRNA, subjected to 4 h of hypoxia and 6 h of reoxygenation, MET (0.1 mM) was added to the DMEM at the start of reoxygenation.

Evaluation of cell viability by MTT assay

Cardiomyocytes were seeded in a 96-well culture plate at a density of 104 cells per well. After oxygenation, the cells were incubated with MTT reaction solution (C0009S, Beyotime) for 4 h and then the culture medium was removed. Subsequently, 100 μl of dimethyl sulfoxide (DMSO, HY-Y0320, MedChemExpress) was added to each well and incubated for 10 min, followed by thorough mixing using a mechanical plate shaker. Finally, the absorbance was measured at a wavelength of 530 nm using an enzyme immunoassay reader.

Detection of creatine kinase MB (CK-MB) and lactate dehydrogenase (LDH) activities in plasma and cell culture supernatant

Blood samples and the cell culture supernatant were centrifuged at 1,800 × g for 10 min at 4°C. LDH (A020–1, Nanjing Jiancheng Bioengineering Institute). CK-MB (E006-1-1, Nanjing Jiancheng Bioengineering Institute) concentrations were measured according to the instruction manual from Nanjing Jiancheng Bioengineering.

Malondialdehyde (MDA) detection

MDA concentration in isolated cardiomyocytes was estimated to assess lipid peroxidation using a lipid peroxidation assay kit (60745ES50, YEASEN). In brief, the cells were lysed and mixed with reagent R1 (N-methyl-2-phenylindole in acetonitrile and methanol) at 45°C for 60 min. Subsequently, the samples were centrifuged at 21,130 × g for 10 min at room temperature. The optical density of the supernatant was measured at 586 nm with a spectrophotometer. MDA concentration was expressed as μmol/g.

Detection of ROS in cardiomyocytes

ROS generation in NRCMs was detected using the DCFH-DA fluorescence method. ROS within the cells can oxidize non-fluorescent DCFH to generate fluorescent DCF. Following treatment with HR or MET, NRCMs were incubated in serum-free DMEM medium with 10 mM DCFH-DA solution at 37°C in the dark for 30 min. After incubation, the cells were washed three times with serum-free DMEM medium and fluorescence intensity was measured using a fluorescence microplate reader at an excitation/emission wavelength of 485/535 nm.

The left ventricle of rat hearts was fixed in 4% paraformaldehyde at room temperature for 24 h, embedded in paraffin at 56°C for 1.5 h, and then sliced (thickness, 6 μm). Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was used to detect apoptotic cells in the heart tissue sections. The sections were incubated in the dark at 37°C for 60 min in a mixture for TUNEL reaction. They were then stained with hematoxylin for 2 min at room temperature, washed with PBS, and sealed with a mounting medium (G8590, Solarbio). The nuclei of cells in 10 random fields of view for each section were counted using an optical microscope (magnification, ×400), and the results are

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presented as the percentage of TUNEL-positive nuclei relative to the total number of nuclei.

Western blot analyses

The RIPA solution lysis (89900, ThermoFisher) was used to extract proteins from frozen tissue samples and cultured myocardial cells. The BCA assay (A55861, ThermoFisher) was used to measure the concentration of protein in the samples. Subsequently, proteins extracted at 50 μg/lane are separated by 10% SDS-PAGE and transferred to a PVDF membrane (FFP39, Beyotime). The membrane was blocked with 5% milk at room temperature for 2 h. Then the membrane is incubated overnight with the primary antibody in buffer (10 mM Tris-HCl; pH 7.5; 150 mM NaCl, 2% Tween-20) at 4°C, followed by a 1.5-h incubation with the secondary antibody at room temperature. Detection was performed using ECL chemiluminescence (P0018S, Beyotime). Antibodies: phosphorylated-AMPK (Thr172; 1:1,000; 2535T; Cell Signaling Technology), AMPK (1:1,000; 5831T; Cell Signaling Technology), and HMGCR (1:1,000; ab109225; Abcam); 4-HNE (1:200; ab48506; Abcam); GAPDH (1:10,000; BM3896; BOSTER) and the corresponding horseradish peroxidase (HRP) – conjugated secondary antibody (C31430100, 1:500, Thermofisher).

Immunohistochemistry

Tissue sections (6 um thickness) were deparaffinized and then blocked at 37°C for 1 h. The sections were incubated with anti-4HNE primary antibody (ab46545, Abcam) at 37°C for 2 h, followed by incubation with an HRP-conjugated secondary antibody for 1 h. The sections were stained in 3,3' – diaminobenzidine solution for 3 min at room temperature, counterstained with hematoxylin for 2 min at room temperature, washed with PBS, and then mounted with a mounting medium.

RNA Extraction and quantitative PCR (RT-qPCR)

Total RNA from cardiac tissue and NRCMs was extracted using TRIzol® reagent (Invitrogen; Thermo Fisher) for PCR. TaqMan® reverse transcription reagent (catalog number N8080234; Thermo Fisher) was used. All samples were quantified using the comparative Cq method for relative gene expression quantification and normalized to GAPDH.

The primers used in this study were as follows: HMGCR, forward primer, 5'-TTGCACGTCTAC AGAAACTTCATAC-3', reverse primer, 5'-CCTGAC CTGGACTGGAAACG-3'; AMPK, forward primer, 5'-GATCGGACACTACGTGCTGG-3', reverse primer, 5'-TAGTTGCTCGCTTCAAGGGG-3'; and GAPDH, forward primer, 5'-TGATGACATCA AGAAGGTGGTGAAG-3' and reverse primer, 5'-TCCTTGGAGGCCATGTAGGCCAT-3'.

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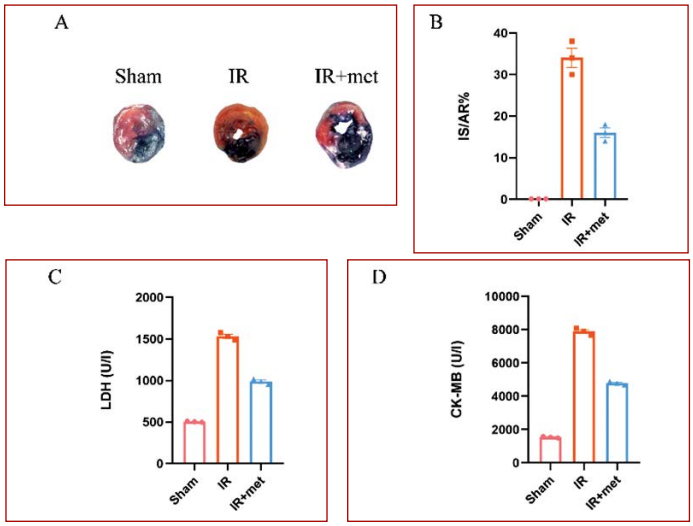
Figure 1. Protective effect of MET on myocardial ischemia-reperfusion injury



(A) Evan's Blue and triphenyltetrazolium chloride staining observed in heart tissue slices. The distribution of blue, non-blue, and white areas represents non-ischemic, AR, and IS areas. (B) IS/AR ratio in different groups. (C) Plasma LDH activity. (D) Plasma CK-MB activity. Sham, sham surgery control group; IR, ischemia-reperfusion group; LDH, lactate dehydrogenase; CK-MB, creatine kinase-MB; MET metformin. (n=3/group).

group, HMGCR mRNA significantly increased in the IR group ($p<0.05$; Figure 3A); this increase was markedly reduced after MET treatment ($p<0.05$; Figure 3A). Similarly, the HMGCR protein concentrations in the IR group were significantly higher than those in the Sham group ($p<0.05$; Figure 3B); after MET treatment, the elevated concentrations of HMGCR were significantly reversed ($p<0.05$; Figure 3B and C). This study further investigated the activation of the downstream target gene AMPK by MET. The phosphorylation concentration of AMPK in the IR group was higher compared to the Sham group ($p<0.05$; Figure 3B and D). MET significantly promoted the expression of phosphorylated AMPK compared to the IR group ($p<0.01$; Figure 3B and D).

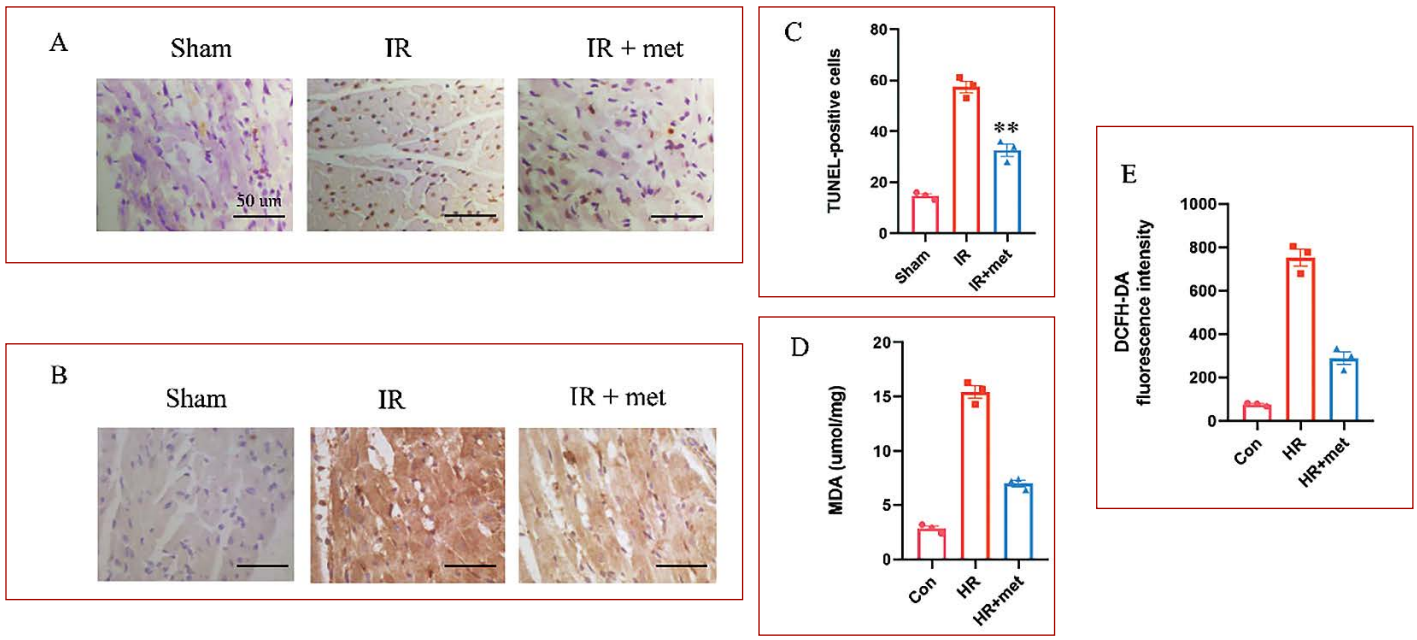
Reversal of MET-induced AMPK inhibition by extracellular signals in regulating the AMPK-HMGCR signaling pathway

In the NRCMs injury model induced by HR, treatment with MET significantly increased the p-AMPK concentration ($p<0.01$; Figure 4A and B) and significantly reduced HMGCR expression ($p<0.01$; Figure 4A and C). This study also used the AMPK inhibitor ComC to inhibit AMPK activation and explore the role of HMGCR in this process. Pre-treatment of NRCMs in the HR model with ComC completely reversed the effect of MET on regulating the AMPK-HMGCR signaling pathway ($p<0.05$; Figure 4A-4C).

siAMPK abolished the regulation of MET on the AMPK-HMGCR signaling pathway

This study further investigated the relationship between AMPK and HMGCR using siAMPK. Transfection with siAMPK led to significantly downregulated expression of AMPK in the Con + siAMPK group and HR + siAMPK group compared to the siNS group ($p<0.05$; Figure 5A), indicating successful silencing. Western blot results

Figure 2. MET reduces oxidative damage and cell apoptosis



(A) Apoptosis of myocardial cells as measured by TUNEL staining; brown cells showed TUNEL positivity. (B) Immunohistochemical staining for 4-hydroxynonenal (4-HNE) (brown). (C) Percentage of TUNEL-positive cells (n=3). (D) MDA content in myocardial cells (n=3). (E) ROS content in myocardial cells.(n=20, neonatal rats).

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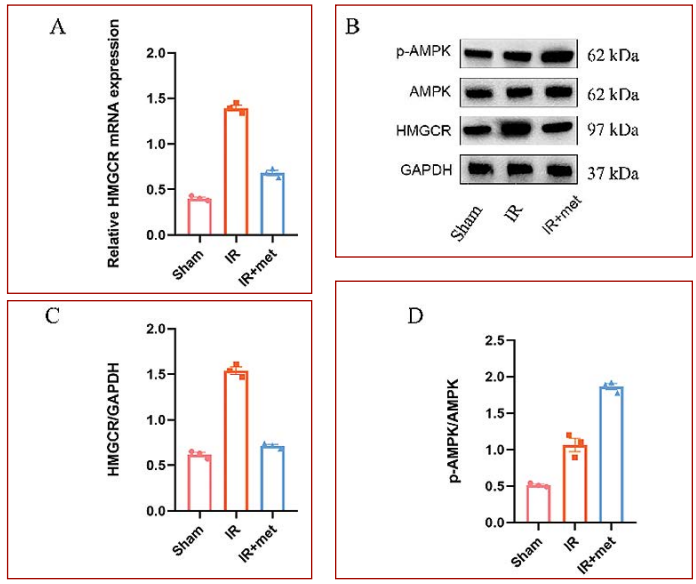
showed that in the HR + siNS NRCMs model, MET treatment significantly enhanced AMPK phosphorylation ($p<0.05$; Figures 5B, C, and F), while simultaneously reducing significantly HMGCR expression ($p<0.01$; Figures 5B and D). In the HR + MET + siAMPK group, the expression of AMPK and phosphorylated AMPK was significantly decreased compared to the HR + MET + siNS group ($p<0.01$; Figures 5B, C, and E), further demonstrating successful AMPK silencing and the reversal of the trend of MET lowering HMGCR expression (Figures 5B and D).

Figure 3. The effect of MET on the expression of HMGCR and phosphorylation of AMPK



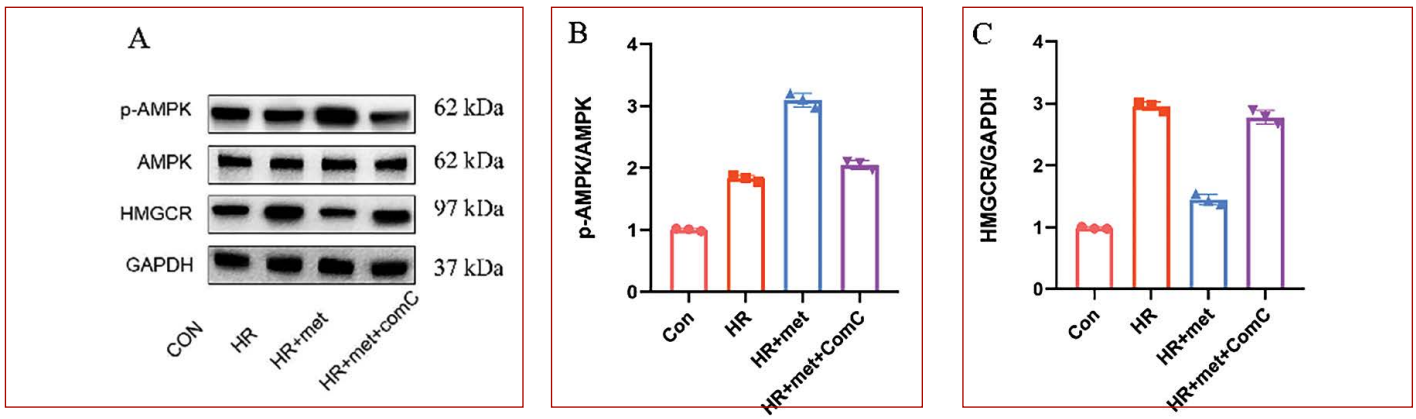
(A) Real-time quantitative PCR was used to measure the expression of HMGCR mRNA.
(B) Western blot method was used to detect the protein expression concentrations of p-AMPK, AMPK, and HMGCR, represented in the figure.
(C) Semi-quantitative analysis of HMGCR protein concentrations.
(D) Semi-quantitative analysis of the p-AMPK/AMPK ratio.

Discussion

This study aimed to explore the protective mechanism(s) of MET on MIRI in the heart, using animal and cell models for an in-depth, mechanistic investigation. While previous studies have shown that MET can reduce MIRI [20], whether the AMPK-HMGCR signaling pathway is involved remained unclear. This study found that MET significantly reduced myocardial infarct size, alleviated myocardial oxidative damage and cell apoptosis, promoted AMPK activation, and simultaneously decreased HMGCR expression. Similarly, in cardiomyocytes of NRCMs subjected to hypoxia-reoxygenation, MET activated AMPK and reduced HMGCR expression. However, this regulatory effect of MET on the AMPK-HMGCR signaling pathway could be reversed or even abolished by the use of an AMPK inhibitor (ComC) or by siAMPK. These results indicate that the protective effect of MET on MIRI in the myocardium is at least partially achieved by activating AMPK and inhibiting HMGCR expression.

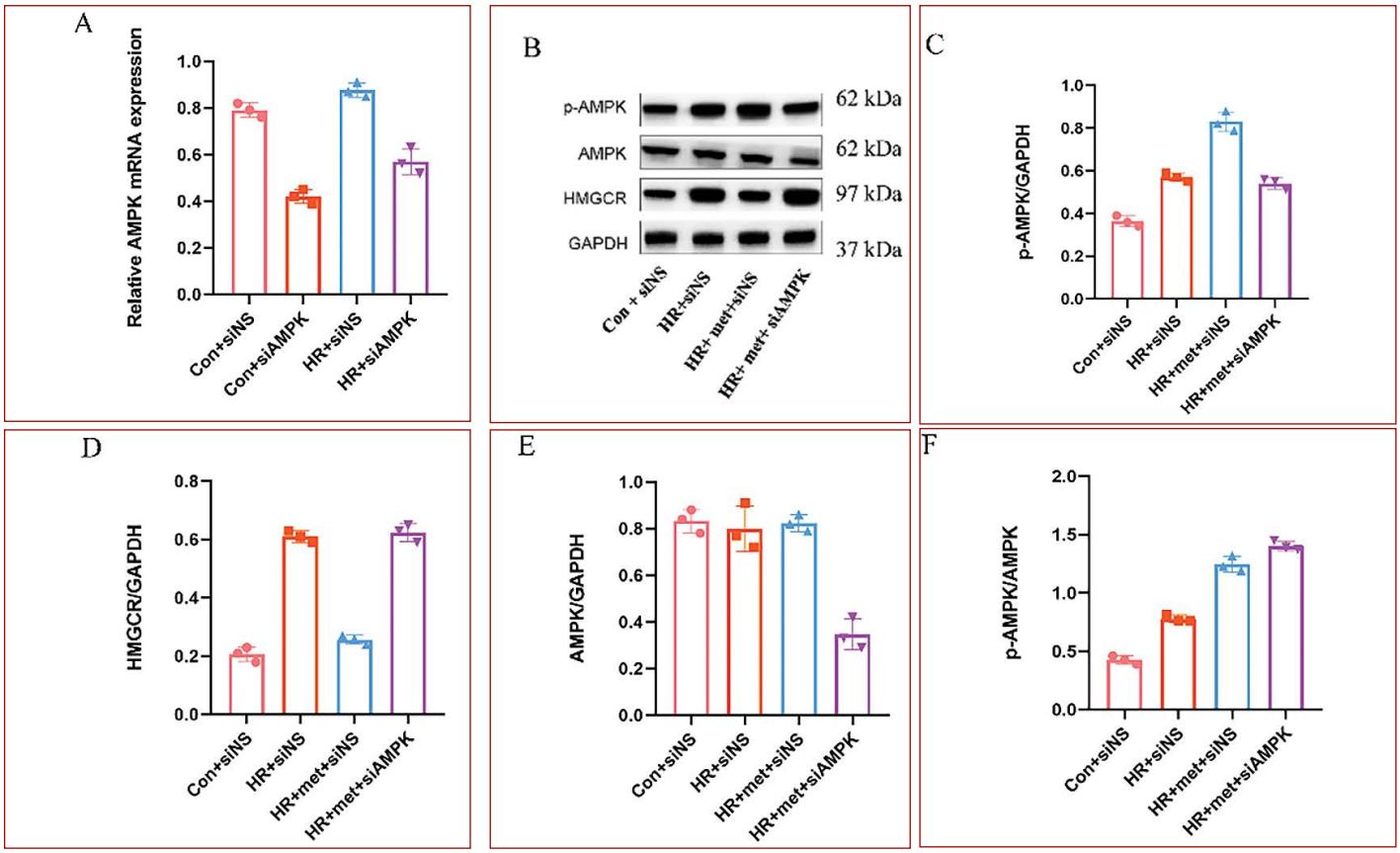
Previous studies have indicated that post-ischemic treatment can effectively reduce myocardial infarct size [21], but its application has certain limitations. Therefore, researchers are actively seeking drugs with potential cardiac protective effects to mimic the effects of post-ischemic treatment in a way that is more readily accepted by clinical patients. Various endogenous substances, such as adenosine, erythropoietin, and growth factors, play crucial roles in alleviating ischemia-reperfusion injury [22]. Another approach is to explore existing drugs with cardiac protective effects by expanding their clinical indications and, thus, to significantly reduce the cost of new drug development. Statins, in addition to lipid regulation, have cardiac protective effects, including antioxidant and anti-apoptotic properties [23]. Previous research has shown that MET also exhibits cardiac protective effects. For example, in a prospective study on diabetes, MET was found to reduce

Figure 4. Reverse effect of AMPK inhibitor on the regulation of the AMPK-HMGCR signaling pathway by MET



(A) Western blot analysis of protein concentrations of p-AMPK, AMPK, and HMGCR.
(B) Semi-quantitative analysis of p-AMPK/AMPK. (C) Semi-quantitative analysis of HMGCR protein concentrations. (n=10, neonatal rats).

Figure 5. siAMPK disrupted the regulation of the AMPK-HMGCR signaling pathway by MET



(A) Quantitative PCR detection of AMPK mRNA expression. (B) Western blot analysis of p-AMPK, AMPK, and HMGCR protein concentrations. (C) Semi-quantitative analysis of p-AMPK concentrations. (D) Semi-quantitative analysis of HMGCR protein concentrations. (E) Semi-quantitative analysis of AMPK protein concentrations. (F) Semi-quantitative analysis of p-AMPK/AMPK ratio. (n=11, neonatal rats).

the risk of acute myocardial infarction (AMI) in type II diabetes patients [24]. Compared to sulfonylureas, MET treatment in AMI patients was found to more significantly reduce mortality [25]. In this study, MET treatment in an IR rat model significantly reduced infarct size, consistent with previous research results. A concentration of 5 mg/kg of MET was sufficient to protect the myocardium in mice without altering blood glucose concentrations.

During the MIRI process, a large amount of ROS is produced intracellularly, causing damage to cardiac tissues through various pathways, including destruction of structural proteins and enzymes, DNA damage, mitochondrial dysfunction, calcium overload, and cardiomyocyte apoptosis [26]. Previous studies have reported that MET can reduce ROS formation in myocardial fibrosis [27]. MDA and 4-HNE are products of lipid peroxidation that indirectly reflect the concentration of ROS generation. Recent studies have found that MET can reduce 4-HNE concentrations in a MIRI rat model and MDA concentrations in an HR cell model, indicating its ability to inhibit excessive oxidative stress in vivo.

Intracellular ROS are mainly produced through the mitochondrial pathway. Previous research reports have indicated that HMGCR is one of the main sources of cardiac reactive oxygen species production, playing an important role in cardiac cell growth and death [28]. The exact role of HMGCR in MIRI is still unclear. Therefore, this study aimed to investigate the role of HMGCR in MET 's anti-MIRI effect. Our results show that after MIRI in mice or HR cell modeling, the expression concentrations of HMGCR significantly increased, consistent with the significant increase in 4-HNE concentrations and the accumulation of ROS and elevated MDA content in cardiomyocytes. Treatment with MET reversed these trends, suggesting that HMGCR may exacerbate MIRI by promoting oxidative stress, and MET 's anti-MIRI effect is related to this. Consistent with previous research results, MET was found to inhibit HMGCR expression [29], indicating a potential regulatory relationship between MET and HMGCR.

MET can inhibit intracellular oxidative stress by activating AMPK, thereby exerting a cardioprotective effect. Previous studies have shown that AMPK reduces cell apoptosis by inhibiting HMGCR expression, indicating a certain relationship between AMPK and HMGCR [30]. As a downstream effector of MET, it has been proven that activating AMPK can inhibit cardiomyocyte apoptosis and improve cardiac function [31]. In this study, we observed an increase in AMPK phosphorylation concentrations in the IR

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group compared to the Sham group. After MET treatment, p-AMPK significantly increased compared to the IR group, consistent with previous research results. Additionally, we observed that the trends of HMGCR in the IR group and the MET-treated group were opposite, suggesting a relationship between AMPK and HMGCR in the MIRI rat model. In the cell model, pre-treatment with the AMPK inhibitor, ComC, significantly reduced the expression of HMGCR protein by inhibiting AMPK activation. To further confirm the relationship between AMPK and HMGCR, we used siRNA transfection technology to knock down AMPK, resulting in a significant decrease in AMPK gene or protein expression concentrations. In the NRCM model with damage caused by HR, pre-treatment with AMPK-siRNA reversed the regulatory effect of MET on the AMPK-HMGCR signaling pathway. These results suggest that MET 's protective effect on MIRI depends on the AMPK-HMGCR signaling pathway.

In conclusion, the results of this study indicate that MET alleviates myocardial oxidative damage, cell apoptosis, and infarct size by activating AMPK and inhibiting HMGCR expression, thereby reducing MIRI.

No conflicts of interest are reported.

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