Effects of maslinic acid on cardiac function in ischemia–reperfusion injury rats

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ABSTRACT

Maslinic acid (MA), a pentacyclic triterpenoid, has been reported to exert broad pharmacological properties. However, it is still unclear whether MA exhibits protective effects against ischemia/reperfusion (I/R) injury. Herein, we aimed to investigate the effects of MA on I/R injury and its underlying mechanisms. A rat model of I/R injury was established and administrated with MA by intraperitoneal injection. Cardiac function was assessed with a color ultrasound diagnosis system and PowerLab system. The levels of oxidative stress-related and I/R-related biomarkers were evaluated by using commercial kits. Apoptosis-related biomarkers and sirtuin (SIRT)1/AMP-activated protein kinase (AMPK) signaling proteins were determined by using quantitative reverse transcription PCR and western blotting, respectively. Treatment with MA improved cardiac performance and cardiac hemodynamic parameters in the I/R injury rat model. Besides, treatment with MA (20 mg/kg) ameliorated I/R injury-related biomarkers in serum. Interestingly, treatment with MA (20 mg/kg) also regulated myocardial apoptosis and inhibited oxidative-stress in left ventricular tissue. Mechanistic studies demonstrated that MA upregulated SIRT1 and AMPK phosphorylation in the left ventricular tissue. In summary, MA exerted protective effects against the impairments of cardiac function in I/R injury rats by the regulation of SIRT1/AMPK signaling pathways.

Significance of this study

What is already known about this subject?

- Maslinic acid (MA), a pentacyclic triterpenoid, has been reported to exert broad pharmacological properties.
- I/R injury can occur in several organs including the heart, kidney, brain, and lungs.
- Acute myocardial ischemia/reperfusion (AMI) injury and its consequences are significant health problems worldwide.

What are the new findings?

- Treatment with MA improved cardiac performance and cardiac hemodynamic parameters in the ischemia/reperfusion (I/R) injury rat model.
- Treatment with MA (20 mg/kg) ameliorated I/R injury-related biomarkers in serum.
- Regulation of SIRT1/AMPK signaling pathways was involved in the protective effects of MA.

How might might these results change the focus of research or clinical practice?

- MA exerted protective effects against cardiac function in I/R injury rats by the regulation of sirtuin 1/AMP-activated protein kinase signaling pathways.

INTRODUCTION

Ischemia–reperfusion (I/R) injury is defined as tissue damage caused by perfusion after a period of ischemia.[1 2] The pathophysiological mechanisms of I/R injury are involved with two stages. Hypoxic injury attributed to cell energy depletion occurs in the ischemic period. Second, the interplay of oxidative disorder, along with inflammatory response and apoptosis, causes superlative damage in the reperfusion period.[2–5] I/R injury can occur in several organs including the heart, kidney, brain, and lungs.[6]

Acute myocardial ischemia/reperfusion (AMI) injury and its consequences are significant health problems worldwide.[7] When AMI occurs, overproduction of reactive oxygen species (ROS) by mitochondrial ROS leads to redox-homeostasis and ion-homeostasis disorders and induces the subsequent inflammatory responses and cell apoptosis.[3 8 9] Mitochondrial ROS plays an important role in the development of AMI and its dysfunction is observed in the pathogenesis of AMI.[10 11] In addition, AMI injury is a complicated process involved with several events, including inflammation, oxidative stress disorder, and cell apoptosis.[2] Therefore, strategies on targeting those events are worthwhile for the treatment of AMI.

Maslinic acid (MA), a pentacyclic triterpenoid isolated from olive–pomace oil, exhibits a series of pharmacological properties including anti-inflammatory, antitumor, antiviral, and antidiabetes.[12] In recent years, MA has also been reported to exert protective effects on cardiac hypertrophy and I/R injury-induced inflammation.[13 14] For instance, in 2018, Liu and colleagues reported that treatment with MA ameliorates cardiac hypertrophy by the regulation of the serine/threonine kinase and extracellular signal-regulated kinase signaling pathways.[15] In 2019, another study performed

by Ampofo and colleagues further demonstrated the protective effects of MA on inflammation caused by I/R injury by the regulation of nuclear factor-κB.[16] In addition, this study also found that treatment with MA ameliorates leukocyte accumulation by the regulation of intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 expression.[16] However, the effects of MA on the AMI injury are still unknown. Therefore, this study was designed to investigate the roles of MA in AMI injury and to elucidate the underlying mechanisms.

AMP-activated protein kinase (AMPK) plays a crucial role in cellular energy homeostasis and has been reported to be associated with cardiac function.[17 18] In 2018, Gélinas and colleagues reported that AMPK activation inhibits cardiac hypertrophy by regulating O-GlcNAcylation.[19] Sirtuin (SIRT)1 is known as a fuel-sensing molecule widely expressed in mammalian cells, which is responsible for the activation of AMPK.[20] For instance, Lan and colleagues reported that SIRT1 overexpressing leads to the activation of AMPK in embryonic kidney cells. However, silencing SIRT1 diminishes the expressions of AMPK.[21] In 2019, Potenza and colleagues, for the first time, reported SIRT1/AMPK signaling pathways involved in I/R injury.[22] Interestingly, MA has been reported to protect against nonalcoholic fatty liver disease associated with its modulatory effects on SIRT1/AMPK signaling pathways.[23] Herein, the present study was designed to investigate whether the protective effects of MA against I/R injury are associated with SIRT1/AMPK signaling pathways.

MATERIALS AND METHODS

Animal experimental design

Male Sprague Dawley rats (230±15 g, 7–8 weeks old) were purchased from Cyagen Biosciences Inc (Suzhou, China). MA (purity ≥98%) was purchased from Sigma Aldrich (St. Louis, Missouri, USA).

After 1 week of acclimation, the rat model of I/R injury was established according to a previously reported method.[24] In brief, the rat was intraperitoneally injected with sodium pentobarbital. Heart reperfusion was allowed for 48 hours by releasing ligation after 30 min of ischemia. No permanent ligated animals were observed in this study. The animals were divided into six groups including sham, I/R, I/R+MA (5 mg/kg), I/R+MA (10 mg/kg), I/R+MA (20 mg/kg), and I/R+MA (20 mg/kg)+EX527 (75 mg/kg). To investigate whether MA therapy is associated with activation of the SIRT1, SIRT1 inhibitor (EX527) was applied. The animals were intraperitoneally injected with MA or EX527 at 30 min after ischemia and at 12, 24, and 48 hours after reperfusion. At the end of the experimental period, the animals were sacrificed and the tissues were collected for further biochemical assays.

Evaluation of cardiac function

Color ultrasound diagnosis systems equipped with 1–5 MHz probe were applied for the evaluation of cardiac function in rats 2 hours after the final treatment. Rats were anesthetized with 2% isoflurane gas followed by transthoracic echocardiography examination. M-mode tracing of the left ventricle was obtained from the parasternal long-axis view. Parameters including the left ventricular end diastolic dimension (LVEDD), left ventricular end systolic diameter (LVESD), left ventricular ejection fraction (LVEF), and left ventricular fractional shortening were determined using computer algorithms. All measurements represent a mean of five consecutive cardiac cycles.

Assessment of hemodynamic parameters

Following evaluation of cardiac function described earlier, cardiac hemodynamic parameters were evaluated by the PowerLab system (ADInstruments, Shanghai, China). All rats were anesthetized by intraperitoneal injection of the mixtures of ketamine (50 mg/kg) and diazepam (5 mg/kg) and were fixed on a plank with supine position. Parameters including the left ventricular systolic pressure (LVSP), left ventricular end diastolic pressure (LVEDP), left indoor systolic pressure increment (+dp/dt), and diastolic decrement (−dP/dt) were recorded by using an multichannel physiological recorder.

Measurement of the infarct size

Infarct size of the heart was determined by using triphenyl–tetrazolium chloride staining according to a previously reported method.[25] After the heart was collected, it was frozen at −80°C for 30 min followed by sectioning from apex to base as 2–4 mm thickness sections. The slices were then incubated in 1% 2, 3, 5-triphenyltetrazolium chloride solution at 37°C for 5 min prior to fixing in 4% paraformaldehyde overnight at room temperature. The slides were photographed and analyzed using ImageJ software (Wayne Rasband, National Institutes of Health). The infarct size was calculated as the percent of unstained tissue over the whole left ventricular area.

Biochemical analysis

Biomarkers including lactate dehydrogenase (LDH), creatine kinase-MB (CK-MB), troponin I, and alanine aminotransferase (ALT) were determined by using commercial reagents according to the instructions of the manufacturers. LDH and CK-MB were measured by using commercialized colorimetric kits (R&D Systems, Minneapolis, Minnesota, USA). Troponin I and ALT were measured by using specific ELISA kits (R&D Systems). Besides, the left ventricular tissue was collected and tissue homogenates were prepared. Biomarkers including malondialdehyde (MDA), ROS, reduced glutathione (GSH), and manganese-dependent superoxide dismutase (MnSOD) activity were determined by using commercial reagents according to the instructions of the manufacturers (R&D Systems).

Quantitative reverse transcription PCR (RT-qPCR)

The primers used in the present study were synthesized by GeneScript (Nanjing, China). The sequences of primers were shown as follows: *SIRT1* forward: 5′-GGC ACC GAT CCT CGA ACA AT-3′, and reverse: 3′-CGC TTT GGT GGT TCT GAA AGG-3′; *Bax* forward: 5′-CCA AGA AGC TGA GCG AGT GTC TC-3′, and reverse: 5′-AGT TGC CAT CAG CAA ACA TGT CA-3′; *Bcl-2* forward: 5′-GGA GCG TCA ACA GGG AGA TG-3′, and reverse: 5′-GAT GCC GGT TCA GGT ACT CAG-3′; *β-actin* forward: 5′-CAG GGT GTG ATG GTG GGT ATG G-3′, and reverse: 5′-AGT TGG TGA CAA TGCC GTG TTC-3′. Total RNA

was isolated from left ventricular tissue by using TRIzol reagent. The concentrations and purities of RNA were then determined by using Nanodrop spectrophotometer. A reverse transcriptional kit was applied to synthesize cDNA before the advanced master mix was applied to the quantitative analysis. qPCR was carried out in a CFX96 real-time PCR system (Bio-Rad, California, USA). In brief, each individual reaction that contained diluted cDNA (2 µL), 10 µM forward and reverse primers (0.15 µL of each), SYBR green master mix (10 µL), and nuclease-free water (7.7 µL). The qPCR run is composed of four steps. Step 1 is enzyme inactivation for 30 s at 95°C. Step 2 is denaturation at 95°C for 5 s. Step 3 is annealing/extension at 60°C for 30 s. Step 4 is melting at 95°C for 1 s. Steps 2 and 3 were carried out for 35 cycles and steps 1 and 4 were performed as 1 cycle. To analyze the accuracy of the PCR reaction, the melt curves were used. GAPDH was selected as an internal control. To evaluate the expressions of genes including SIRT1, bax, and bcl-2, $2^{-\triangle\triangle}$Ct values were calculated.

Western blotting

The primary antibodies are anti-Bax (1:2000, ab182733, rabbit monoclonal to Bax), anti-bcl-2 (1:1000, ab194583, rabbit polyclonal to Bcl-2), anti-SIRT1 (1:1000, ab189494, rabbit monoclonal to SIRT1), anti-AMPK (1:1000, ab207442, rabbit monoclonal to AMPK), anti-phosphorylated AMPK (1:1000, ab240058, rabbit monoclonal), and anti-β-actin (1:1000, ab8227, rabbit polyclonal). The secondary horseradish peroxidase (HRP)-conjugated antibody is anti-rabbit IgG (1:2000, ab6721). All antibodies were purchased from Abcam (Cambridge, Massachusetts, USA).

Cardiac tissues were taken from each group. Protein samples were prepared from the left ventricular tissue. After being washed with phosphate-buffered saline (PBS) three times, the tissue homogenates of the left ventricular were prepared by using immunoprecipitation buffer supplemented with protease inhibitor. The lysate concentration was then centrifuged at a rate of 12 000 rpm for 20 min, followed by collection of the supernatant. The protein concentrations of each sample were qualified by the bicinchoninic acid protein kit. The equal amount of proteins was loaded into the sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel followed by transferring the proteins into the polyvinylidene fluoride membrane. The membrane was then blocked in 5% bovine serum albumin solution at room temperature for 1 hour, washed with PBS for 2 min and finally incubated with the primary antibodies at a temperature of 4°C overnight. After being washed with Tris-buffered saline with Tween 20 three times, the membrane was incubated HRP-conjugated secondary antibodies at room temperature for 1 hour. An electrochemiluminescence detection kit was applied for chemiluminescence development. β-actin was selected as an internal control.

Statistical analysis

Statistical analysis was performed with GraphPad Prism V.8 (San Diego, California, USA). Data were presented as mean±SD. One-way analysis of variance and a Tukey's post hoc test were employed. A p value of less than 0.05 was considered as a statistical significance.

RESULTS

Treatment with MA improved cardiac performance in the I/R injury rat model

We assessed the cardiac performance in the presence or absence of MA by using echocardiography examination. The results demonstrated the significant increase of LVEDD and LVESD in the I/R injury rat model as compared with the sham group. Treatment with MA dramatically decreased the LVEDD and LVESD in a dose-dependent manner (figure 1A,B). However, the presence of EX527 significantly increased the LVEDD and LVESD as compared with the I/R+MA (20 mg/kg) group (online supplemental figure S1A,B). In addition, a significant decrease of LVEF and left ventricular fraction shortening (LVFS) was observed in the I/R group. Treatment with MA (10 and 20 mg/kg) significantly increased the LVEF and LVFS in a dose-dependent manner (figure 1C,D). However, the presence of EX527 significantly decreased the LVEF and LVFS as compared with the I/R+MA (20 mg/kg) group (online supplemental figure S1C,D). We observed that treatment with MA (5 mg/kg) did not significantly affect LVEDD, LVESD, and LVEF.

In addition to echocardiography examination, we also measured cardiac hemodynamic parameters. The results showed that the hemodynamic parameters including LVSP, +dp/dt, and −dp/dt significantly decreased in the I/R group as compared with the sham group. Treatment with MA significantly increased the LVSP, +dp/dt, and −dp/dt in a dose-dependent manner (figure 2A,C,D). However, the presence of EX527 significantly decreased the LVSP, +dp/dt, and −dp/dt as compared with the I/R+MA (20 mg/kg) group (online supplemental figure S2A,C,D). Additionally, an increase of LVEDP was observed in the I/R group as compared with the sham group, whereas treatment with MA (10 and 20 mg/kg) significantly decreased the LVEDP (figure 2B). However, the presence of EX527 significantly increased the LVEDP as compared with the I/R+MA (20 mg/kg) group (online supplemental figure S2B). Interestingly, treatment with MA (5 mg/kg) did not significantly affect these hemodynamic parameters. Taken together, these results supported that treatment with MA (10 and 20 mg/kg) improved cardiac function in a rat model of I/R injury, while MA at a lower dose (5 mg/kg) did not exert cardiac protective effects.

Treatment with MA attenuated infarct size and regulated cardiac ischemia-related markers in the I/R injury rat model

We first evaluated the effects of MA on infarct size. The results demonstrated that infarct size was significantly increased in the rat model of I/R injury as compared with the sham group (online supplemental figure S3A,B). Treatment with MA (20 mg/kg) significantly reduced infarct size as compared with the I/R group (online supplemental figure S3A,B).

We next evaluated the effects of MA on cardiac ischemia-related markers in a rat model of I/R injury. It is known that I/R injury is accompanied by an elevation of cardiac ischemia-related markers. As expected, the levels of LDH, CK-MB, troponin I, and ALT significantly increased in the I/R group as compared with the sham group (figure 3A–D). These results indicate that cardiac ischemia-related markers

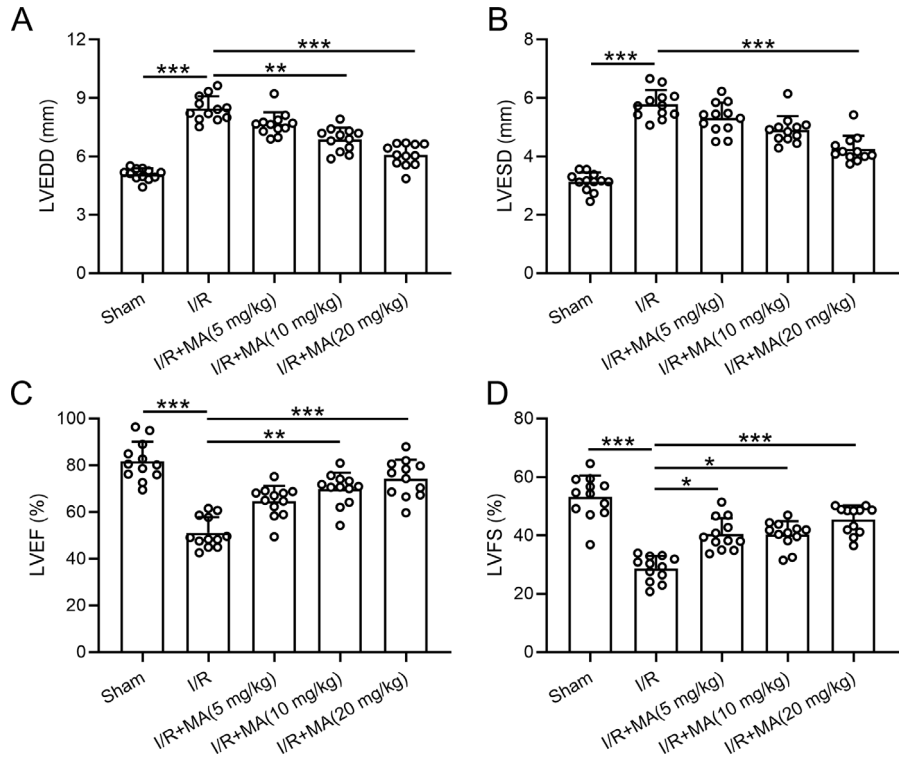

Figure 1 Assessment of cardiac performance in the presence or absence of MA. Cardiac performance-related parameters including LVEDD (A), LVESD (B), LVEF (C), and LVFS (D) were determined using echocardiography. The data were expressed as means±SD, n=12 for each group. *P<0.05, **P<0.01, and ***P<0.001 indicate significant difference. I/R, ischemia/reperfusion; LVEDD, left ventricular end diastolic dimension; LVEF, left ventricular ejection fraction, LVFS, left ventricular fraction shortening, LVESD, left ventricular end systolic diameter; MA, maslinic acid.

significantly increased due to the I/R injury. Treatment with MA (10 and 20 mg/kg) significantly reduced the levels of cardiac ischemia-related markers including LDH, CK-MB, troponin I, and ALT as compared with the I/R group (figure 3A–D). Interestingly, we also observed that MA at a lower dose (5 mg/kg) did not significantly affect the levels of these biomarkers. Thus, only MA (20 mg/kg) was selected for further mechanistic studies.

Treatment with MA (20 mg/kg) regulated myocardial apoptosis in the I/R injury rat model

Next, we sought to explore the effects of MA on myocardial apoptosis in a rat model of I/R injury. Apoptosis-related biomarkers were determined in the left ventricular tissue using RT-qPCR and western blotting, respectively. The results showed that the mRNA levels of Bax were significantly increased, while the mRNA levels of Bcl-2 were significantly decreased in the I/R group as compared with the sham group (figure 4A,B). Consistently, the protein expressions of Bax were increased and the mRNA levels of Bcl-2 were decreased in the I/R group as compared with the sham group (figure 4E,F). MA (20 mg/kg) exerted significantly inhibitory effects on the levels of Bax and improved effects on the levels of Bcl-2. In addition, we also evaluated the mRNA and protein levels of cleaved caspase-3. The results demonstrated that treatment with MA (20 mg/kg) significantly suppressed the levels of cleaved caspase-3 as compared with the I/R group (figure 4C,D). Interestingly, the regulatory effects of MA on these apoptosis-related biomarkers (Bax, Bcl-2, and cleaved caspase-3) were in transcriptional and post-transcriptional manners.

Treatment with MA (20 mg/kg) ameliorated oxidative stress-related biomarkers in a rat model of I/R injury

To evaluate the effects of MA on oxidative stress in a rat model of I/R injury, we measured the levels of oxidative-stress related biomarkers in the left ventricular tissue. As expected, the levels of MDA and ROS were significantly increased in the I/R group as compared with the sham group (figure 5A,B). However, treatment with MA (20 mg/kg) significantly suppressed the levels of MDA and ROS as compared with the I/R group (figure 5A,B). Additionally, the results showed that the levels of GSH and MnSOD were significantly decreased in the I/R group as compared with the sham group, whereas treatment with MA (20 mg/kg) significantly ameliorated the levels of GSH and MnSOD as compared with the I/R group (figure 5C,D). Taken together, these results suggested that treatment with MA (20 mg/kg) ameliorated the I/R injury-induced oxidative stress in the left ventricular tissue.

Treatment with MA (20 mg/kg) upregulated SIRT1 and AMPK phosphorylation in a rat model of I/R injury

Finally, we explored the underlying mechanisms of MA on the I/R injury. Previous studies have reported that MA regulated SIRT1 and AMPK phosphorylation in a mouse model of non-alcoholic fatty liver disease. Therefore, we speculated

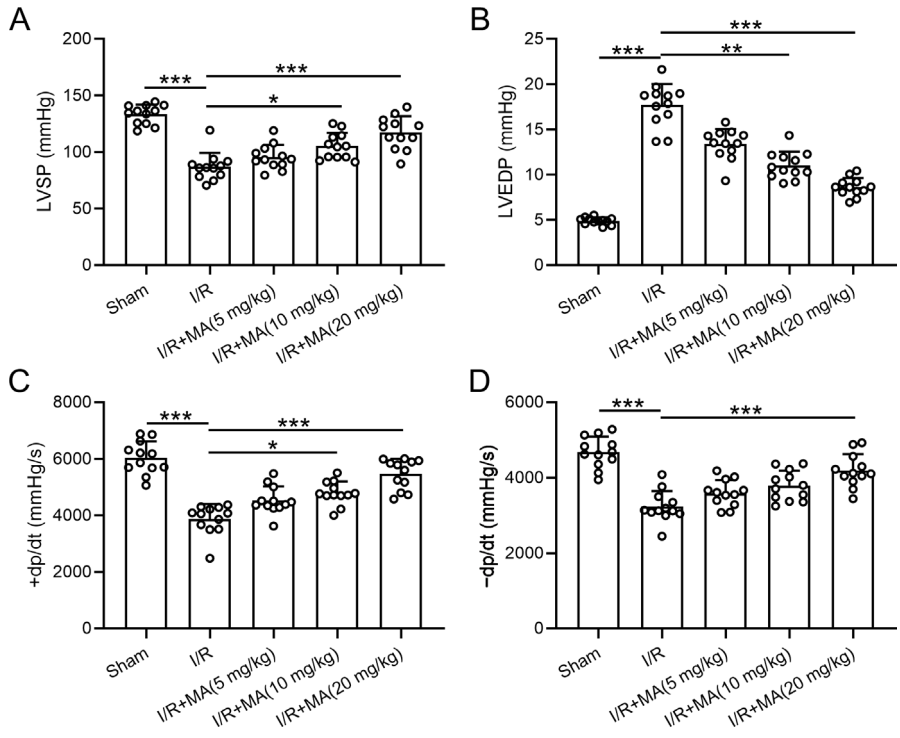

Figure 2 Assessment of left ventricle hemodynamic parameters in the presence or absence of MA. Left ventricle hemodynamic parameters including LVSP (A), LVEDP (B), +dp/dt (C), and −dp/dt (D) from the left ventricle were determined using hemodynamic measurement. The data were expressed as means±SD (n=12) for each group. *P<0.05, **P<0.01, and ***P<0.001 indicate significant difference. +dp/dt, left indoor systolic pressure increment; −dp/dt, diastolic decrement; I/R, ischemia/reperfusion; LVEDP, left ventricular end diastolic pressure; LVSP, left ventricular systolic pressure; MA, maslinic acid.

that MA might also target SIRT1 and AMPK in a rat model of I/R injury. The results revealed that the mRNA and protein levels of SIRT1 were significantly decreased in the I/R group, whereas treatment with MA (20 mg/kg) significantly ameliorated the mRNA and protein levels of SIRT1 (figure 6A–C). Moreover, we also observed a reduction of AMPK phosphorylation in the I/R group as compared with the sham group, whereas treatment with MA (20 mg/kg) significantly reduced AMPK phosphorylation but did not affect the protein levels of AMPK (figure 6D–F). It is worth noting that MA treatment did not affect SIRT1 expression and AMPK phosphorylation in sham mouse (data not shown). In summary, these results supported that treatment with MA (20 mg/kg) upregulated SIRT1 and AMPK phosphorylation in a rat model of I/R injury.

DISCUSSION

We first investigated the effects of MA on cardiac function by using echocardiography and the PowerLab system, which are commonly used tools for assessing cardiac function.[26 27] Treatment with MA (10 and 20 mg/kg) was able to decrease the LVEDD and LVESD and to increase the LVEF and LVFS in a dose-dependent manner. Besides, treatment with MA was also able to increase the LVSP, +dp/dt, and −dp/dt and to decrease the LVEDP in a dose-dependent manner. These results are consistent with a previous study in which MA altered +dp/dt and −dp/dt in mice with aortic banding surgery.

In addition to the assessment of cardiac function, we next sought to explore the effects of MA on I/R injury-related enzymes including LDH, CK-MB, troponin I, and ALT. LDH is a biomarker for evaluating heart injury.[28] The levels of CK-MB can be used to reflect cardiotoxicity and membrane integrity.[29 30] Treatment with MA (10 and 20 mg/kg) significantly reduced the levels of LDH and CK-MB in I/R injury rats. We also investigated the effects of MA on the levels of troponin I and ALT. Troponin I serves as a classic marker for cardiac ischemic injury.[31] Besides, an increase in ALT is one of the characteristics of I/R injury.[28] Our results revealed that MA (10 and 20 mg/kg) significantly reduced the levels of Troponin I and ALT in the I/R injury rat model.

Furthermore, we evaluated the effects of MA on apoptosis by assessing apoptosis-related proteins including Bax, Bcl-2, and Caspase-3.[32] The roles of apoptosis in I/R injury have been well documented in many studies.[33] Myocardial ischemia causes cell necrosis and reperfusion promotes cell apoptosis.[34] When the I/R injury occurs, death signals trigger cell shrinkage and subsequent formation of apoptotic bodies.[34] Interestingly, some studies revealed that targeting apoptosis is an effective strategy for the treatment of I/R injury.[34 35] In this study, treatment with MA (20 mg/kg) was able to decrease the levels of Bax and caspase-3 and to increase the levels of Bcl-2. Moreover, our results also demonstrated that treatment with MA (20 mg/kg) exerted regulatory effects on these apoptosis-related biomarkers (Bax, Bcl-2, and cleaved caspase-3) in the transcriptional and post-transcriptional manners. Interestingly, our results are different from previously reported studies. Reyes-Zurita and colleagues demonstrated that MA induces cell apoptosis by increasing the caspase-3 and caspase-8 in

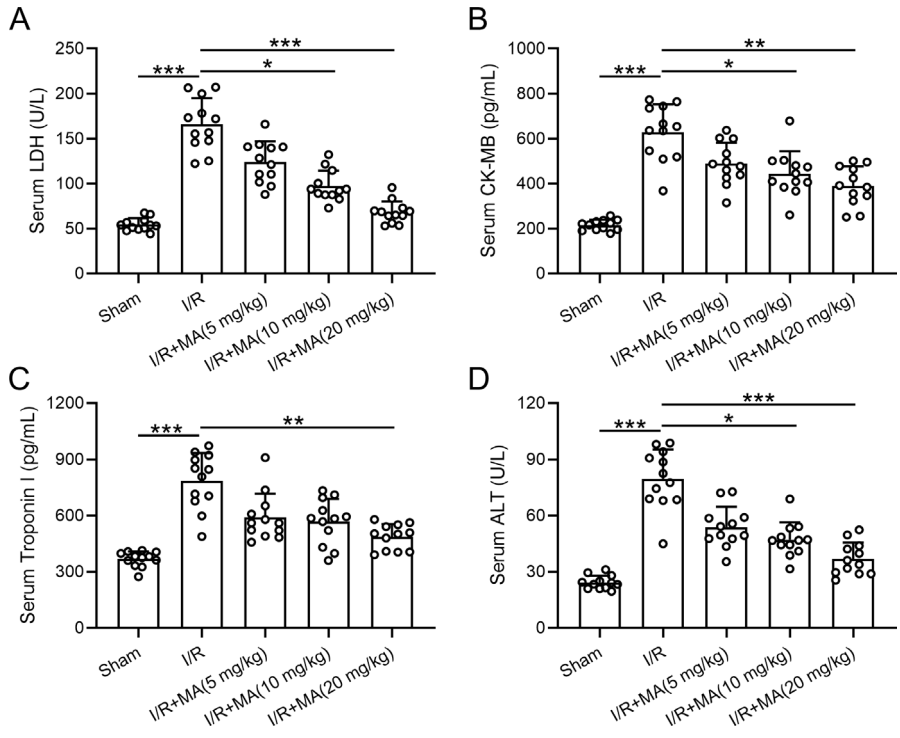

Figure 3 Treatment of MA-regulated cardiac ischemia-related markers. Serum LDH (A), CK-MB (B), troponin I (C), and ALT (D) were determined by using biochemical analysis. The data were expressed as means±SD (n=12) for each group. *P<0.05, **P<0.01, and ***P<0.001 indicate significant difference. ALT, alanine aminotransferase; CK-MB, creatine kinase-MB; +dp/dt, left indoor systolic pressure increment; I/R, ischemia/reperfusion; LDH; lactate dehydrogenase; MA, maslinic acid.

colon cancer cells.[36] Similarly, another study showed that MA increases the expressions of caspase-3, caspase-8, and caspase-9 in A549 cells. These studies demonstrated that MA exerts promoting effects on the activity of caspase-3 in cancer cells. However, cancer cells differ from normal cells in many ways, including the way their cells repair and cell death. Two previous studies also reported that treatment with MA suppresses caspase-3 activity in cortical neurons and the hippocampus of mice.[37 38] Our results are consistent with these studies.

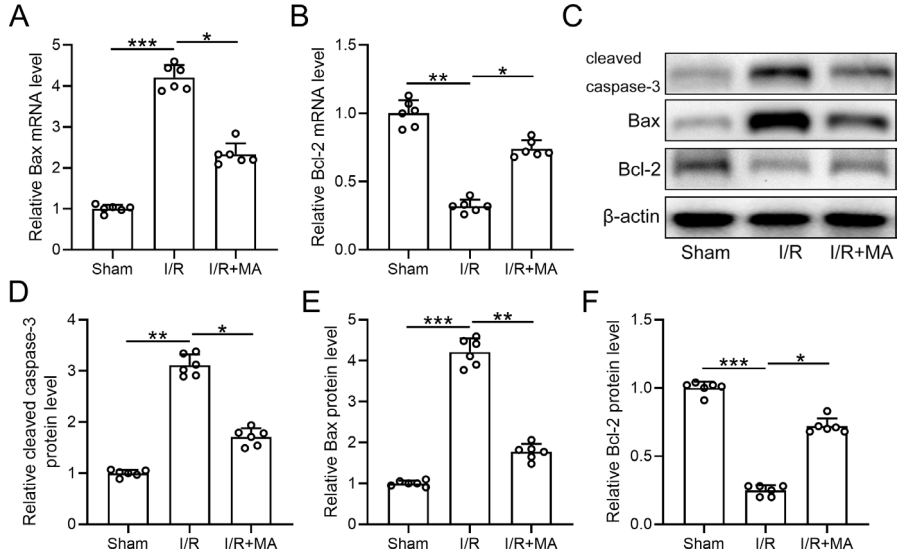

Figure 4 Treatment of MA (20 mg/kg) ameliorated myocardial apoptosis caused by I/R injury. The mRNA levels of bax (A) and bcl-2 (B) in the left ventricular tissue were determined by using quantitative reverse transcription PCR. (C) The protein levels of cleaved caspase-3 (D), bax (E), and bcl-2 (F) in the left ventricular tissue were determined by using western blotting in individual rat. The relative expressions of target proteins were normalized to sham. The data were expressed as means±SD (n=6) for each group. *P<0.05, **P<0.01, and ***P<0.001 indicate significant difference. I/R, ischemia/reperfusion; MA, maslinic acid.

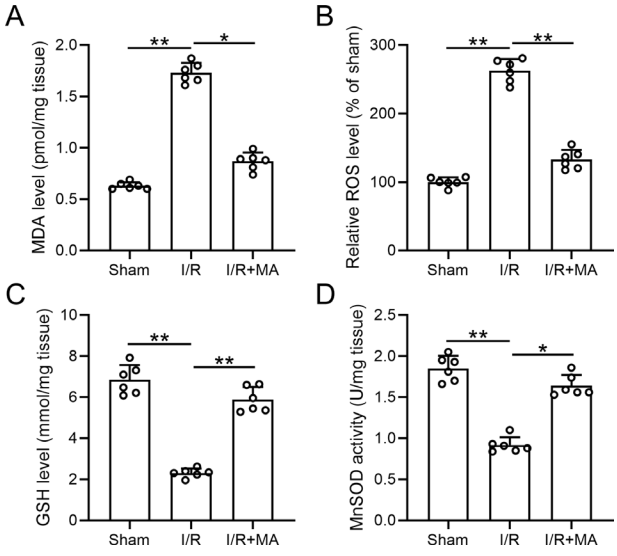

Figure 5 Treatment of MA (20 mg/kg) ameliorated oxidative stress caused by I/R injury. At the end of the experimental period, the animals were used and the left ventricular tissues were collected. The levels of MDA (A), ROS (B), GSH (C), and MnSOD activity (D) in the left ventricle were determined using biochemical assays. The data were expressed as means±SD (n=6) for each group. *P<0.05, **P<0.01 indicate significant difference. GSH, glutathione; I/R, ischemia/reperfusion; MA, maslinic acid; MDA, malondialdehyde; MnSOD, manganese-dependent superoxide dismutase; ROS, reactive oxygen species.

I/R injury is a complex pathological process associated with cell apoptosis and oxidative stress disorder.[2 10] Oxidative stress has been considered as a major contributor to the I/R injury.[8 10] Therefore, we investigated the effects of MA on oxidative stress by assessing the levels of MDA, ROS, and GSH and the activities of MnSOD. In the present study, treatment with MA (20 mg/kg) significantly suppressed the levels of MDA and ROS. Besides, the levels of the endogenous antioxidant GSH and the activities of mitochondrial ROS scavenging enzyme MnSOD were also determined. Treatment with MA (20 mg/kg) significantly ameliorated the levels of GSH and the activities of MnSOD. These results suggested that the ameliorated effects of MA on the I/R injury were associated with its antioxidative properties.

Finally, the underlying mechanisms of MA on the I/R injury were explored. I/R injury is characterized by cellular energy and ion homeostasis disorder.[2 3] AMPK plays a crucial role in the energy homeostasis and has clinical significance for improving cardiac function.[17] SIRT1 is known as a fuel-sensing molecule which is responsible for the activation of AMPK.[20] Previous studies have demonstrated that SIRT1 overexpression leads to the activation of AMPK, and silencing SIRT1 diminishes the expressions of AMPK.[21] Liou and colleagues reported that treatment with MA ameliorates non-alcoholic fatty liver disease by the regulation of the SIRT1/AMPK signaling pathways.[23] Thus, we speculated that the protective mechanisms of MA against I/R injury might be linked with its regulatory effects on SIRT1/AMPK signaling pathways. Our results demonstrated that treatment with MA (20 mg/kg) ameliorated I/R injury. The presence of the SIRT1 selective inhibitor, EX527, significantly reduced the protective effects of MA on myocardial function. Therefore, we speculated that the protective effects of MA on myocardial function were associated with its regulatory effects on SIRT1 and AMPK phosphorylation. Interestingly, a previous study also reported that MA decreased phosphorylated GSK3β levels in a pressure-overload model of left ventricular

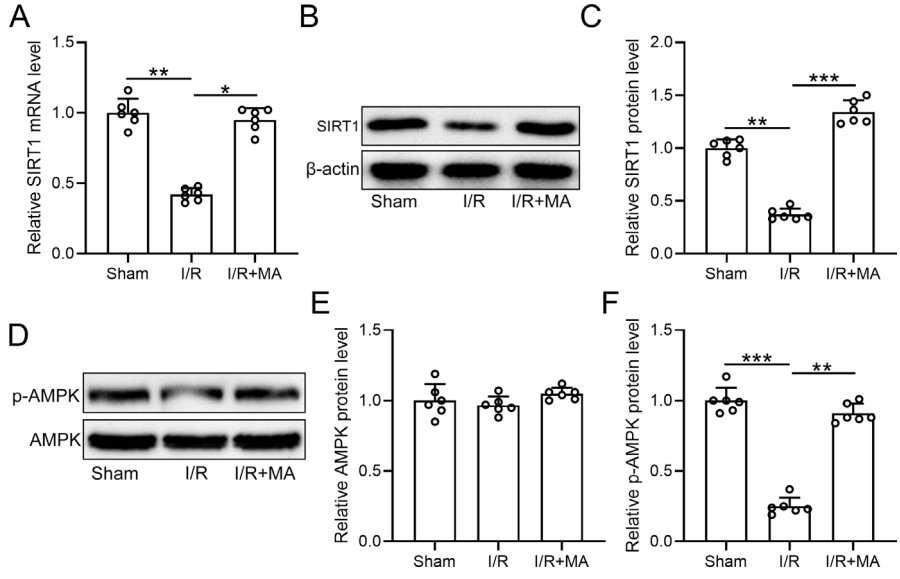

Figure 6 Treatment of MA (20 mg/kg) upregulated SIRT1 and AMPK phosphorylation in the rat model of I/R injury. (A) The mRNA levels of SIRT1 in the left ventricular tissue were determined by using quantitative reverse transcription PCR in the left ventricular tissues. (B–F) Besides, the protein expressions of SIRT1, AMPK, and phosphorylated AMPK in the left ventricular tissue were determined by using western blotting in individual rats. The relative expressions of target proteins were normalized to sham. Data were expressed as means±SD n=6 for each group. *p<0.05, **p<0.01, and ***p<0.001 indicate significant difference. AMP-activated protein kinase; I/R, ischemia/reperfusion; MA, maslinic acid; SIRT, sirtuin.

hypertrophy.[15] GSK3β is also known as an important mediator of I/R.[39 40] These results demonstrated the activities of MA in protecting the heart from I/R injury also associated with other signaling pathways. Instead of investigating broad I/R-related cell signaling pathways, our limitation is focused only on the relationship between MA and SIRT1/AMPK signaling. Therefore, the effects of MA on other I/R--related signaling pathways are warranted in further studies.

It must be noted that there are several limitations. One limitation of this study is performing 48 hours of reperfusion. The construction of the I/R injury model showed different reperfusion times, including 4, 24, and 48 hours.[24 25 41] However, biomarkers including troponin and LDH lose their sensitivity when measured at 48 hours after AMI construction. It is important to investigate the effects of MA on AMI with different reperfusion time in further studies. Another limitation is that only male rats were employed; female rats could be studied if some factors, such as estrogen secretion and menstrual period, can be well controlled. Last, we did not include the sham group treated with MA, although MA has been well documented for its biosafety.

CONCLUSION

Treatment with MA improved cardiac performance and cardiac hemodynamic parameters in I/R injury rats. Besides, treatment with MA was able to reduce infarct size, ameliorate I/R injury-related biomarkers in serum, regulate myocardial apoptosis, and inhibit oxidative-stress in the left ventricular tissues. Mechanistic studies revealed that MA exerted protective effects on cardiac function in I/R injury rats by the regulation of SIRT1/AMPK signaling pathways.

Contributors Collected and analyzed the data: NW, ZM, CC and NX; designed the study and wrote the manuscript: NW and ZM; all authors approved the final submission.

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Patient consent for publication Not required.

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