# Roflumilast reduces myocardial ischemia reperfusion injury *in vivo* and *in vitro* by activating the AMPK signaling pathway

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**Abstract.** Myocardial tissue cell damage induced by myocardial ischemia/reperfusion (MI/R) notably elevates the mortality rate, increases the complications of patients with myocardial infarction and decreases reperfusion benefit in patients suffering from acute myocardial infarction. Roflumilast protect against cardiotoxicity. Therefore, the present study aimed to investigate the effect of roflumilast on MI/R injury and the underlying mechanisms. To simulate MI/R *in vivo* and *in vitro*, the rat model of MI/R was established and H9C2 cells were subjected to hypoxia/reoxygenation (H/R) induction, respectively. The myocardial infarction areas were observed by 2,3,5-triphenyltetrazolium chloride staining. The myocardial enzyme levels in serum and levels of inflammatory cytokines and oxidative stress markers in cardiac tissue were assessed by corresponding assay kits. The cardiac damage was observed by hematoxylin and eosin staining. The mitochondrial membrane potential in cardiac tissue and H9C2 cells was detected using the JC-1 staining kit. The viability and apoptosis of H9C2 cells were detected by Cell Counting Kit-8 and TUNEL assay, respectively. The levels of inflammatory cytokines, oxidative stress markers and ATP in H/R-induced H9C2 cells were analyzed by corresponding assay kits. Western blotting was used for the estimation of AMP-activated protein kinase (AMPK) signaling pathway-, apoptosis- and mitochondrial regulation-associated protein levels. The mPTP opening was detected using a calcein-loading/cobalt chloride-quenching system. The results indicated that roflumilast decreased MI/R-induced myocardial infarction by alleviating myocardial injury and mitochondrial damage through the activation of the AMPK signaling pathway. In addition, roflumilast mitigated viability damage, alleviated oxidative stress, attenuated the inflammatory response and decreased mitochondrial damage in H/R-induced H9C2 cells by activating the AMPK signaling pathway. However, compound C, an inhibitor of the AMPK signaling pathway, reversed the effect of roflumilast on H/R-induced H9C2 cells. In conclusion, roflumilast alleviated myocardial infarction in MI/R rats and attenuated H/R-induced oxidative stress, inflammatory response and mitochondrial damage in H9C2 cells by activating the AMPK signaling pathway.

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*Key words:* roflumilast, myocardial ischemia reperfusion injury, mitochondrial damage, AMP-activated protein kinase signaling pathway

## Introduction

Acute myocardial infarction (AMI) is acute ischemic necrosis of myocardium occurring due to coronary artery disease, which can lead to fatal complications, and even mortality in severe cases (1). Since the beginning of the 21st century, with the changes of the lifestyle and diet of the public, the incidence of AMI has been increasing sharply (2). There are >3 million cases of MI in China (3). The mortality rate of cardiovascular diseases continues to increase. Annual number of deaths owing to cardiovascular diseases increased from 2.51 million to 3.97 million between 1990 and 2016 in China (4). Although the timely implementation of thrombolytic drugs and interventional surgery permits coronary artery recanalization, which is the only effective clinical treatment method at present (5,6), coronary artery recanalization itself can lead to a severe inflammatory response, and continue to expand infarction lesions by >50% (compared with original infarct size), leading to irreversible injury, namely myocardial ischemia/reperfusion injury (MIRI) (7,8). Therefore, it is of importance to find a solution to alleviate heart injury and dysfunction caused by I/R.

Roflumilast is a phosphodiesterase-4 (PDE-4) inhibitor. The US Food and Drug Administration has approved roflumilast for the treatment of severe chronic obstructive pulmonary disease because of its strong anti-inflammatory and immunomodulatory properties (9). Studies have shown that roflumilast prevents ischemic stroke-induced neuronal damage (10) and alleviates sepsis-induced acute kidney injury (11). In addition, roflumilast can protect myocardial cells from nitric oxide-induced apoptosis (12) and decrease cadmium-induced cardiotoxicity by inhibiting oxidative stress (13) and doxorubicin-induced cardiotoxicity by decreasing inflammation (14). These results indicate that roflumilast has cardioprotective

functions; to the best of our knowledge, however, its role in MIRI has not been reported.

Mitochondrial dysfunction is a key cause of MIRI and the main mechanisms include decreased mitochondrial ATP production (15), excessive reactive oxygen species (ROS) production and continuous mitochondrial permeability transition pore (mPTP) opening (16). Roflumilast can reduce mitochondrial dysfunction in smog-induced pulmonary bronchial epithelial cells by downregulating phosphorylated (p)-dynamin-related protein 1 (DRP1) and PTEN-induced kinase 1 (PINK1) (17). Therefore, it may be hypothesized that roflumilast may also alleviate mitochondrial dysfunction caused by MIRI. In addition, AMP-activated protein kinase (AMPK) signaling is an intracellular energy sensor and the activation of the AMPK signaling pathway can decrease mitochondrial damage. Activation of AMPK inactivates DRP1 to inhibit mitochondrial fission, thus preventing the opening of mPTP and contributing to cell survival (18). Activation of AMPK decreases oxidative stress induced by I/R (19) and roflumilast prevents diabetic nephropathy by activating AMPK/sirtuin 1 (SIRT1) (20).

Therefore, the aim of the present study was to investigate the effect of roflumilast on MIRI as well as to discuss the underlying mechanisms. It was hypothesized that roflumilast could alleviate MIRI by improving mitochondrial dysfunction by activating the AMPK signaling pathway.

## Materials and methods

*PubChem*. The chemical structure of roflumilast was determined by PubChem (pubchem.ncbi.nlm.nih.gov/; Fig. 1A).

*MI/R rat model*. The experimental protocol for animal studies was reviewed and approved by the Committee for the Ethics of Animal Experiments, Shenzhen Peking University-The Hong Kong University of Science and Technology Medical Center (approval no. 2021-807; Shenzhen, China). The MI/R rat model was established as previously described (21). A total of 20 male Sprague-Dawley rats (age, 12 weeks; weight, 180-250 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. The rats were raised in an environmentally controlled room (22±2˚C, humidity of 55±5%, 12/12-h light/dark cycle) with free access to standard animal feed and filtered tap water for 7 days. Rats were anesthetized by intraperitoneal injection of 10% chloral hydrate (0.3 ml/100 g) and the intercostal space was opened under mechanical ventilation. The animals exhibited no signs of peritonitis, pain or discomfort. To establish the MI/R rat model, the left anterior descending coronary artery was ligated for 45 min, followed by reperfusion for 2 h. Rats in the control group were not ligated. The rats were randomly divided into four groups (n=5/group): Control; MI/R, MI/R + roflumilast (1 mg/kg; Adooq Bioscience) and MI/R + roflumilast (3 mg/kg) group. Prior to MI/R operation, roflumilast was administered to rats by gavage at 1 or 3 mg/kg once daily for 7 consecutive days. Rats were humanely sacrificed under anesthesia by intraperitoneal injection of 1% pentobarbital sodium (150 mg/kg). The blood samples (~6 ml) collected from the hearts and cardiac tissues were stored at -80˚C until further analysis.

*Hypoxia/reoxygenation (H/R) injury induction in vitro and roflumilast treatment*. H9C2 cells were obtained from the American Tissue Culture Collection (ATCC; cat. no. CRL-1446). H9C2 cells were cultured in ATCC-formulated Dulbecco's Modified Eagle's Medium (cat. no. ATCC 30-2002) supplemented with 10% fetal bovine serum (cat. no. ATCC 30-2020) in 95% air and 5% $CO_2$ at 37˚C.

For H/R stimulation, H9C2 cells were grown in an anoxic chamber with 5% $CO_2$ and 95% $N_2$ for 6 h and then in a normal chamber with 95% air and 5% $CO_2$ for 12 h at 37˚C.

For roflumilast treatment, cultured cells were pre-incubated with roflumilast (1.0, 2.5 and 5.0 $\mu$M; Adooq Bioscience) or compound C (10 $\mu$M; an inhibitor of the AMPK signaling pathway; MedChemExpress, United States) for 30 min at 37˚C before H/R treatment.

*2,3,5-triphenyltetrazolium chloride (TTC) staining*. The MI area in each group was observed by TTC staining. Following storage at -18˚C for 15 min, the heart tissue perpendicular to the coronary sulcus was cut into five equal pieces. Following incubation with 1% TTC solution (Sigma-Aldrich; Merck KGaA) for 10 min at 37˚C and 10% neutral buffered formalin (Thermo Fisher Scientific, Waltham, MA) for 90 min at 37˚C in the dark, all slices (1 mm) were imaged using a digital camera. The MI areas were determined by Image-Pro Plus image analysis software (version 4.1; Media Cybernetics, Inc.). Finally, calculation of MI area was conducted according to the following formula: Myocardial infarct size (%)=(infarct area/whole heart area) x100%.

*Hematoxylin and eosin (H&E) staining*. The cardiac tissue samples collected from the left ventricle were fixed in 4% paraformaldehyde overnight at 4˚C, dehydrated in ascending ethanol gradient and embedded in paraffin for 50-60 min at room temperature. Subsequently, the embedded cardiac tissues were cut into 4 $\mu$m slices, followed by staining with 0.5% hematoxylin for 5 min and eosin for 2 min at room temperature. Then, the sections were mounted and observed under a light microscope (magnification, x100/400; Olympus Corporation BX53).

*Mitochondrial membrane potential detection*. The mitochondrial membrane potential in cardiac tissue and H9C2 cells was detected using the JC-1 staining kit (cat. no. C2006; Beyotime Institute of Biotechnology) according to the manufacturer's instructions. In brief, the isolated cardiomyocyte suspension and H9C2 cells were stained with 2.5 mg/ml JC-1 solution for 20 min at 37˚C. Subsequently, the cells were washed with JC-1 staining buffer twice and observed using a fluorescence microscope (magnification, x200; Olympus Corporation BX63).

*Detection of myocardial enzyme levels in serum*. The collected peripheral blood (15 ml) was centrifuged at 2,072 x g at 4˚C for 10 min to separate the serum. Heart muscle damage indicators, including aspartate transaminase (AST), creatine kinase-myocardial band (CK-MB) and lactate dehydrogenase (LDH) in serum were detected by AST (cat. no. C010-2-1), CK-MB (cat. no. A032-1-1) and LDH (cat. no. A020-2-2) assay kits (all Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's instructions, respectively.

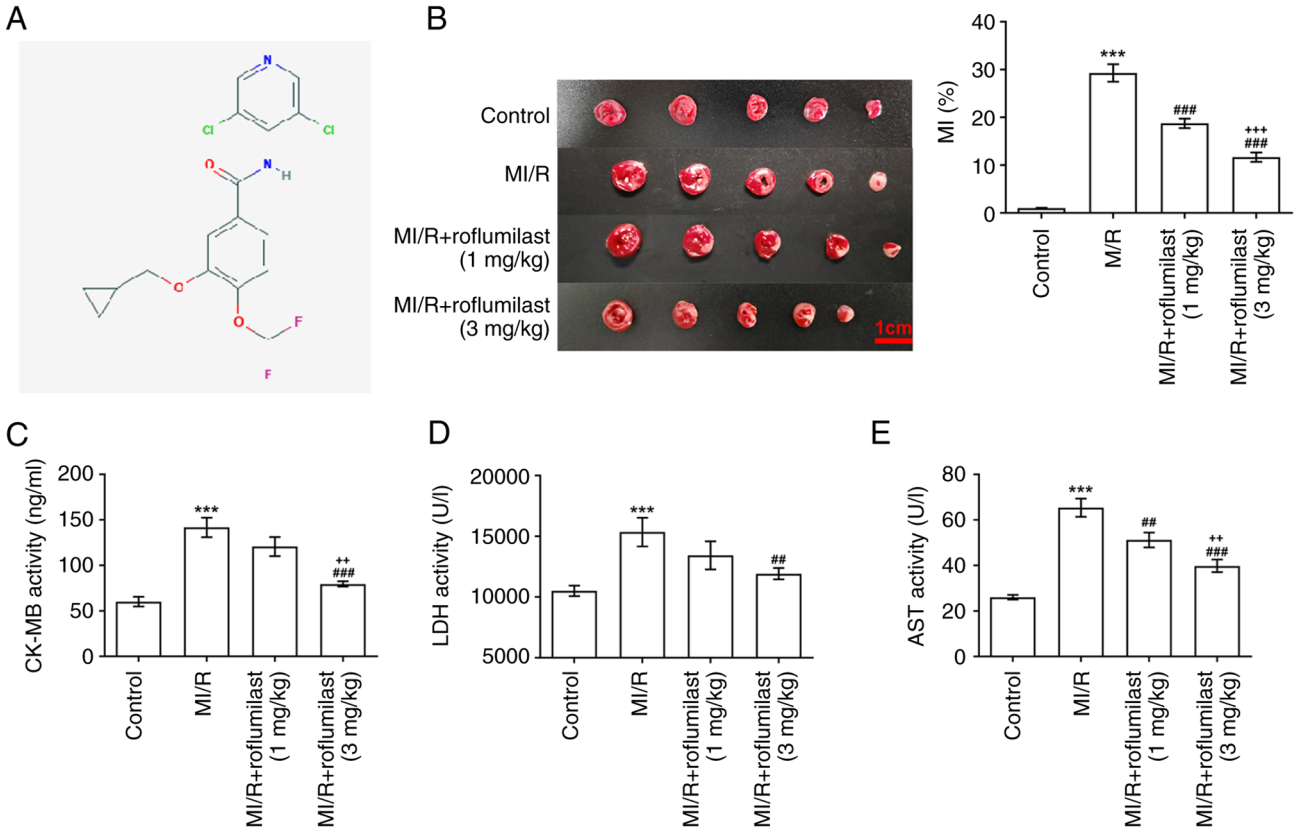


Figure 1. Roflumilast decreases MI/R-induced myocardial infarction. (A) Chemical structure of roflumilast was obtained from PubChem. (B) Representative images of myocardial infarct size demonstrated by TTC staining. The non-stained areas (white or pale) indicate infarct areas; TTC-stained areas (red) indicate non-infarct areas. The activities of (C) CK-MB, (D) LDH and (E) AST in rat serum were detected by corresponding assay kits. $^{***}$P<0.001 vs. Control. $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. MI/R. $^{++}$P<0.01 and $^{+++}$P<0.001 vs. MI/R + roflumilast (1 mg/kg). MI/R, myocardial ischemia/reperfusion; TTC, 2,3,5-triphenyltetrazolium chloride; CK-MB, creatine kinase-myocardial band; LDH, lactate dehydrogenase; AST, aspartate transaminase; MI, myocardial infarction.

*Detection of inflammatory cytokines*. The concentrations of IL-1β and IFN-γ in myocardial tissue and in cell supernatant were measured using ELISA kits for IL-1β (cat. no. E-EL-R0012c; Elabscience Biotechnology, Inc.) and IFN-γ (cat. no. E-EL-R0009c; Elabscience Biotechnology, Inc.) according to the manufacturer's instructions.

*Detection of oxidative stress*. The activity of malondialdehyde (MDA) and superoxide dismutase (SOD) in myocardial tissue and H9C2 cells were measured using MDA (cat. no. S0131S; Beyotime Institute of Biotechnology) and SOD assay kits (cat. no. S0109; Beyotime Institute of Biotechnology) according to the manufacturer's instructions.

*Detection of ATP levels*. The detection of ATP concentration in H9C2 cells was conducted using ATP assay kit (cat. no. S0026; Beyotime Institute of Biotechnology) according to the manufacturer's instructions. Briefly, H9C2 cells were collected and mixed with cell lysis buffer for 10 min at 4˚C, followed by centrifugation at 12,000 x g at 4˚C for 5 min. Subsequently, cell supernatant was incubated with 100 $\mu$l kit solution at room temperature for 5 min and the ATP levels in the cell supernatant were detected using a LuminMax-C luminometer (Maxwell Sensors Inc.).

*Western blotting*. Protein from cardiac tissues and H9C2 cells was obtained using RIPA lysis buffer (Beyotime Institute of Biotechnology) and qualified with a BCA detection kit (Beyotime Institute of Biotechnology). A total of 25 $\mu$g/lane protein was separated by 10% SDS-PAGE and transferred to a PVDF membrane. Following blocking with 5% BSA (Beyotime Institute of Biotechnology) at room temperature for 2 h, the membrane was incubated with primary antibodies targeting AMP-activated protein kinase alpha (AMPKα; cat. no. 5831; 1:1,000; Cell Signaling Technology, Inc.), phosphorylated (p-) AMPKα (cat. no. 50081; 1:1,000; Cell Signaling Technology, Inc.), SIRT1 (cat. no. ab189494; 1:1,000; Abcam), Bcl-2 (cat. no. ab196495; 1:1,000; Abcam), Bax (cat. no. ab32503; 1:1,000; Abcam), PINK1 (cat. no. ab186303; 1:1,000; Abcam), DRP1 (cat. no. 8570; 1:1,000; Cell Signaling Technology, Inc.), p-DRP1 (cat. no. 4867; 1:1,000; Cell Signaling Technology, Inc.) and β-actin (cat. no. 93473; 1:1,000; Cell Signaling Technology, Inc.) overnight at 4˚C. Then, membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (cat. no. ab6721; 1:2,000; Abcam) or goat anti-mouse IgG (cat. no. ab6789; 1:2,000; Abcam) for 1 h at room temperature. Finally, the bands were examined with ECL reagent (Beyotime Institute of Biotechnology) and band density was quantified using ImageJ Software (version 1.46; National Institutes of Health).

*Cell Counting Kit-8 (CCK-8) assay*. H9C2 cells were inoculated into a 96-well plate at a density of $1x10^3$ cells/well. H9C2 cells were treated with roflumilast (1.0, 2.5 and 5.0 $\mu$M) for

24 h at 37˚C and induced by H/R. H9C2 cells in each well were mixed with 10 $\mu$l CCK-8 solution (Beyotime Institute of Biotechnology) and incubated at 37˚C for 1 h. The optical density at 450 nm was detected by a spectrophotometer (Bio-Rad Laboratories, Inc.).

*TUNEL assay*. The apoptosis of H9C2 cells was determined by *In Situ* Cell Death Detection kit (cat. no. 11684795910; Roche) for the TUNEL assay. Briefly, H9C2 cells in a 24-well plate ($1x10^5$ cells/well) were fixed in 4% paraformaldehyde at room temperature for 15 min, then 0.1% Triton-X-100 at room temperature for 10 min. Then, cells on the slides were stained with TUNEL reaction mixture (50 $\mu$l terminal deoxynucleotidyl-transferase and 450 $\mu$l fluorescein-labeled deoxyuridine triphosphate) at 37˚C for 1 h in the dark and nuclei were stained with 10 $\mu$g/ml DAPI at room temperature for 5 min. The cells were mounted with PBS and glycerol (ratio, 1:2) and observed by fluorescence microscopy in five randomly selected high-power microscope fields (magnification, x200). The formula used to calculate the percentage of cell apoptosis was as follows: Cell apoptosis (%)=(number of apoptotic H9C2 cells/total number of H9c2 cells) x100%.

*mPTP opening assay*. The opening of the mPTP was detected using a calcein-loading/cobalt chloride ($CoCl_2$)-quenching system. Briefly, $2x10^5$ H9C2 cells seeded in a 6-well plate were treated with 1 $\mu$M calcein and 2 mM $CoCl_2$ for 20 min at 37˚C in the dark. After washing with PBS, H9C2 cells were observed and imaged using a confocal laser scanning microscope (model no. LSM 880; Carl Zeiss AG; magnification, x100). The mean green fluorescence intensities in the mitochondria were quantified using ImageJ Software (version 1.46; National Institutes of Health). The changes of green fluorescence intensity in the mitochondria were the index of mPTP opening.

*Statistical analysis*. GraphPad (version 8.0.1; GraphPad Software, Inc.; Dotmatics) was used to analyze the experimental data. Data are shown as the mean ± the standard deviation from three independent experiments. The comparisons between multiple groups were conducted by one-way ANOVA followed by Tukey's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

## Results

*Roflumilast decreases MI/R-induced MI*. The chemical structure of roflumilast is shown in Fig. 1A. TTC staining results showed that the infarct area in the MI/R group was significantly increased compared with that in the control group, indicating that the rat model of MI/R was successfully constructed. Moreover, roflumilast (1 and 3 mg/kg) significantly decreased the increased infarct size in rats subjected to MIRI in a dose-dependent manner (Fig. 1B). Furthermore, the increased levels of CK-MB (Fig. 1C), LDH (Fig. 1D) and AST (Fig. 1E) in the MI/R group were decreased by treatment of roflumilast.

*Roflumilast attenuates MI/R-induced myocardial injury*. The H&E staining results revealed that the myocardial fiber structure was damaged, vascular walls were broken and hemocyte infiltration was apparent in the MI/R group. However, MI/R rats pre-injected with roflumilast exhibited mild tissue damage (Fig. 2A). The inflammatory factors (IL-1β and IFN-γ) in cardiac tissues were significantly increased in the MI/R group but significantly reduced by roflumilast treatment (Fig. 2B).

*Roflumilast attenuates myocardial mitochondrial damage induced by MI/R*. The ratio of green and red fluorescence intensity was significantly increased in MI/R rats compared with control rats; this was reversed by roflumilast treatment, indicating that roflumilast decreased depolarization of the mitochondrial membrane (Fig. 3A). In addition, MDA was significantly increased and SOD was significantly decreased in the cardiac tissues of rats subjected to MI/R; these effects were subsequently reversed by roflumilast (Fig. 3B).

*Roflumilast activates the AMPK signaling pathway in MIRI*. The expression of p-AMPK and SIRT1 in the cardiac tissue of rats subjected to MI/R were significantly decreased compared with the control group, while roflumilast treatment promoted the expression of p-AMPK and SIRT1 in a dose-dependent manner (Fig. 4).

*Roflumilast mitigates damage of H/R to H9C2 cell viability by activating the AMPK signaling pathway*. After H9C2 cells were treated with roflumilast, their viability was unchanged, indicating that roflumilast has no significant effect on H9C2 cells at these concentrations (Fig. 5A). H/R-induced H9C2 cells showed significantly decreased viability, which was reversed by roflumilast in a dose-dependent manner (Fig. 5B). H/R induction significantly decreased the expression of p-AMPK and SIRT1, while roflumilast promoted the expression of p-AMPK and SIRT1 in H/R-induced H9C2 cells (Fig. 5C). H9C2 cells subjected to H/R induction demonstrated a significant increase in the number of TUNEL-positive/apoptotic cells; this was significantly decreased following treatment with roflumilast, while compound C weakened the effect of roflumilast (Fig. 5D). Roflumilast resulted in the increased expression of Bcl-2 and decreased expression of Bax in H/R induced H9C2 cells, which was then reversed by compound C (Fig. 5E).

*Roflumilast activates the AMPK signaling pathway to alleviate oxidative stress and the inflammatory response of H9C2 cells induced by H/R*. Roflumilast significantly suppressed MDA and significantly upregulated SOD in H/R-induced H9C2 cells, while compound C significantly impaired the function of roflumilast (Fig. 6A). ELISA revealed a significant increase in levels of IL-1β and IFN-γ in the H/R group compared with the control group, which were significantly decreased by roflumilast treatment. However, the decreased levels of IL-1β and IFN-γ in the H/R + roflumilast (5 $\mu$M) group were partially elevated by compound C (Fig. 6B).

*Roflumilast activates the AMPK signaling pathway to decrease mitochondrial damage of H9C2 cells induced by H/R*. Roflumilast treatment significantly decreased the ratio of green and red fluorescence intensity in H/R-induced H9C2 cells compared with that in the H/R group; this was increased following the administration of compound C (Fig. 7A). H/R induction significantly decreased the ATP levels, which were

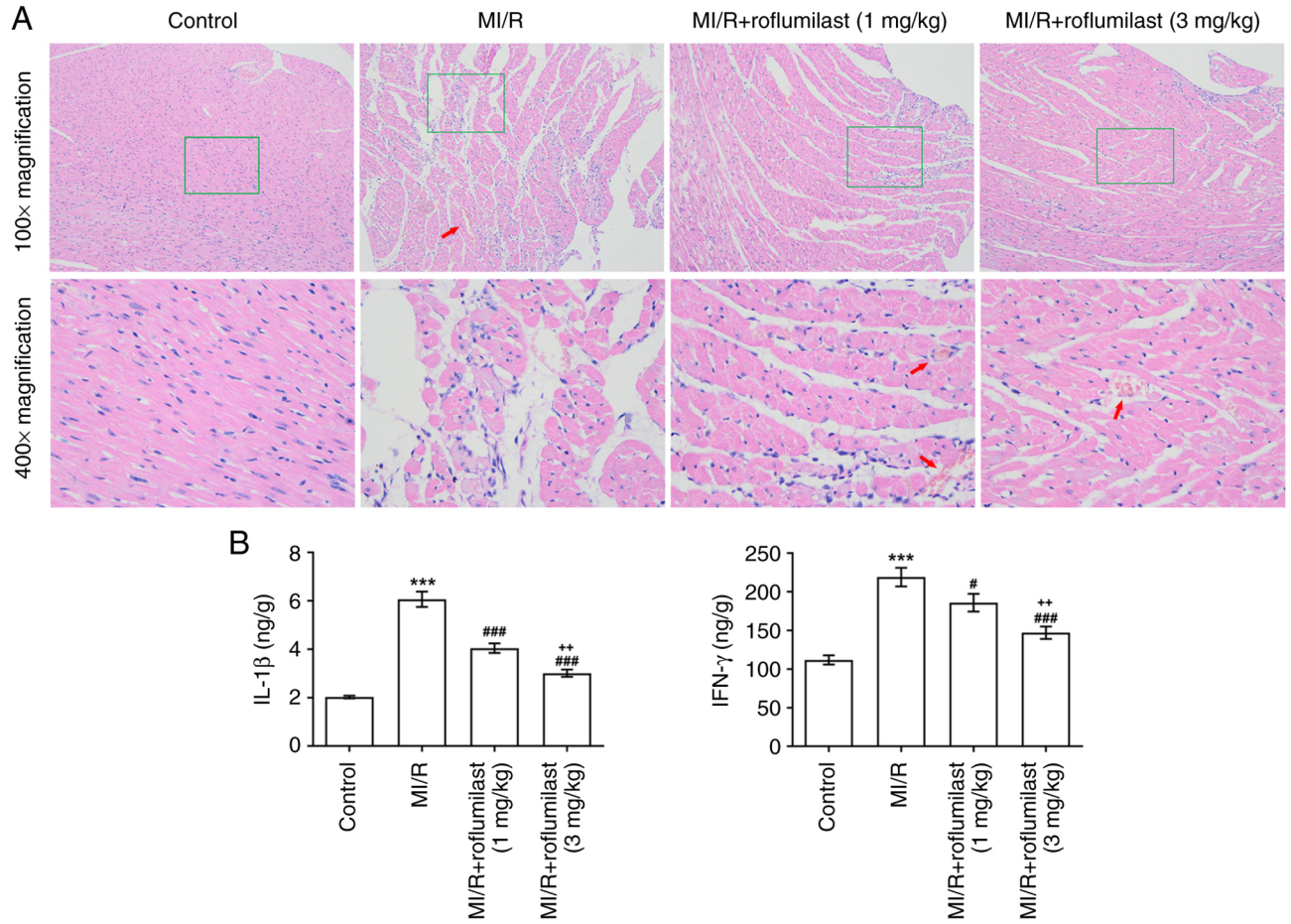


Figure 2. Roflumilast attenuates MI/R-induced myocardial injury. (A) Changes of pathological injury of myocardial tissue were detected by H&E staining. The arrows indicated hemocyte infiltration. (B) Inflammatory factors (IL-1β and IFN-γ) in myocardial tissue were detected by corresponding assay kits. $^{***}$P<0.001 vs. Control. $^{\#}$P<0.05 and $^{\#\#\#}$P<0.001 vs. MI/R. $^{++}$P<0.01 vs. MI/R + roflumilast (1 mg/kg). MI/R, myocardial ischemia/reperfusion.

promoted by roflumilast. Compound C decreased the ATP levels in H/R + roflumilast + compound C group compared with the H/R + roflumilast group (Fig. 7B). Compared with the control, the fluorescence intensity of mitochondrial calcein was significantly decreased in the H/R group, indicating that the extent of mPTP opening was enhanced following H/R. Moreover, roflumilast significantly increased fluorescence intensity compared with the H/R group, indicating that roflumilast inhibited H/R-induced mPTP opening in H9C2 cardiomyocytes, which was reversed by compound C (Fig. 7C and D). The enhanced expression of PINK1 and p-DRP1 in H9C2 cells due to H/R induction were suppressed by roflumilast treatment; these effects were then reversed by compound C (Fig. 7E).

## Discussion

The present study demonstrated that roflumilast treatment decreased MIRI *in vivo* and *in vitro* by activating the AMPK signaling pathway. Furthermore, mechanistic investigations demonstrated that compound C, an inhibitor of the AMPK signaling pathway, reversed the protective effects of roflumilast on MIRI *in vivo* and *in vitro*.

The rhythmic contraction of cardiomyocytes consumes a lot of energy, and 90% of ATP is produced by mitochondria. Therefore, maintaining good mitochondrial morphology and function is crucial for the survival and normal function of cardiomyocytes (22). Mitochondria are also involved in calcium homeostasis, which regulates cell division and initiates signal transduction pathways (23-25). Mitochondrial dysfunction plays a key role in H/R injury, including decreased ATP synthesis, excessive production of ROS, $Ca^{2+}$ overload and continuous opening of mPTP. Abnormal mitochondrial function can lead to systolic-diastolic dysfunction of cardiac myocytes, and apoptosis (22,26-28). mPTP opening reduces mitochondrial membrane potential, which is an important factor for the decrease of ATP production, resulting in the death of myocardial cells (29). The present study indicated that abnormal mitochondrial function occurred in the MI/R rat model. The enhanced mPTP opening occurred in H/R injury, which resulted in a decreased mitochondrial membrane potential and ATP levels and increased apoptosis.

When the energy crisis of the body is caused by stress conditions (ischemia, hypoxia, oxidative stress and other factors), the ATP levels in the body decrease or the newly generated ATP cannot rapidly replace its consumption by tissues and organs, resulting in the insufficient energy supply in cells and the activation of AMPK (30). Notably, previous study found that mitochondrial dysfunction could activate AMPK signaling to

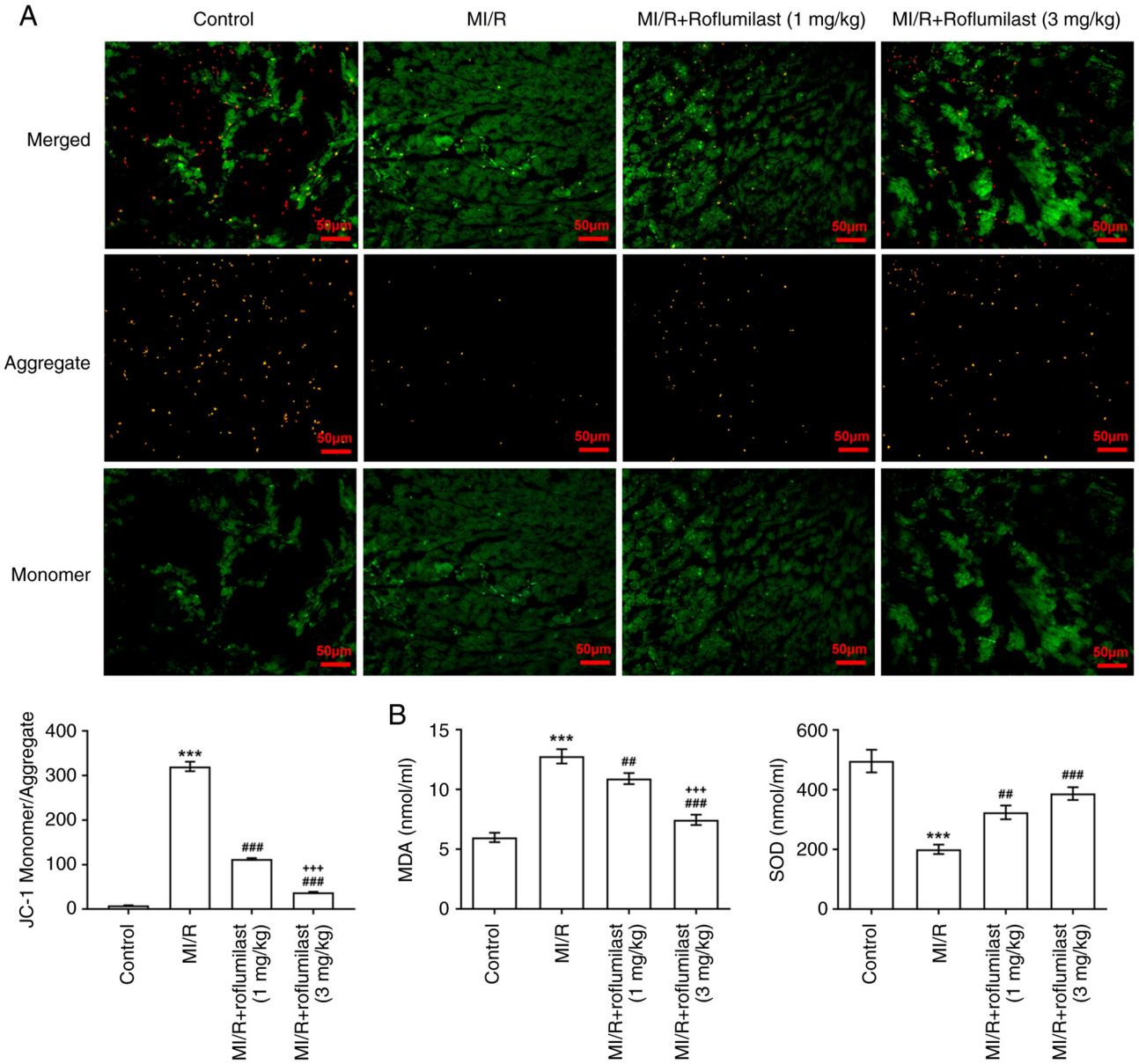


Figure 3. Roflumilast attenuates myocardial mitochondrial damage induced by MI/R. (A) Changes in mitochondrial membrane potential were detected by JC-1 staining. (B) Levels of oxidative stress markers in myocardial tissue were detected by assay kits. $^{***}$P<0.001 vs. Control. $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. MI/R. $^{+++}$P<0.001 vs. MI/R + roflumilast (1 mg/kg). MI/R, myocardial ischemia/reperfusion; MDA, malondialdehyde; SOD, superoxide dismutase.

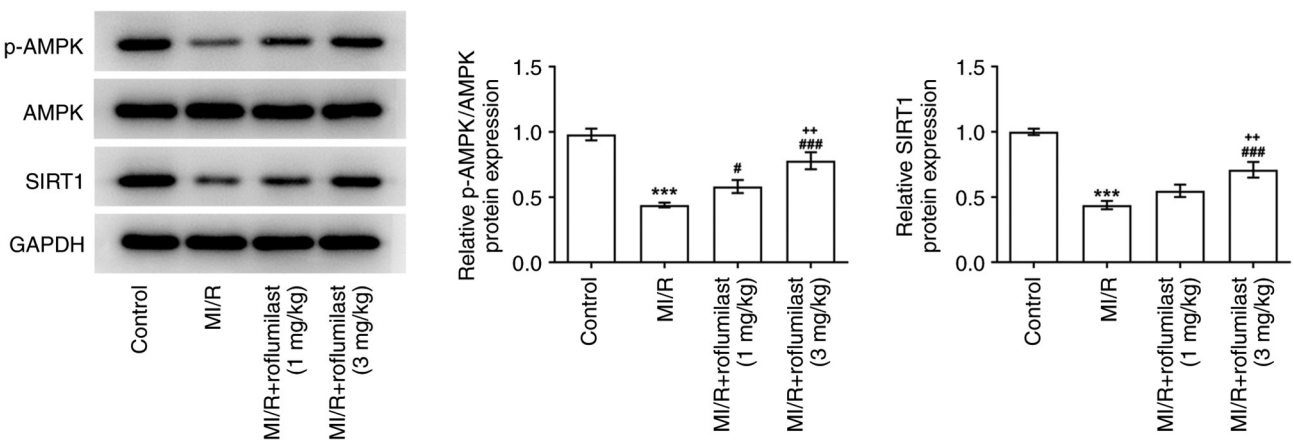


Figure 4. Roflumilast activates the AMPK signaling pathway in MI/R injury. The expression of AMPK signaling pathway-associated proteins in myocardial tissue was detected by western blotting. $^{***}$P<0.001 vs. Control. $^{\#}$P<0.05 and $^{\#\#\#}$P<0.001 vs. MI/R. $^{++}$P<0.01 vs. MI/R + roflumilast (1 mg/kg). AMPK, AMP-activated protein kinase; MI/R, myocardial ischemia/reperfusion; p, phosphorylated; SIRT1, sirtuin 1.

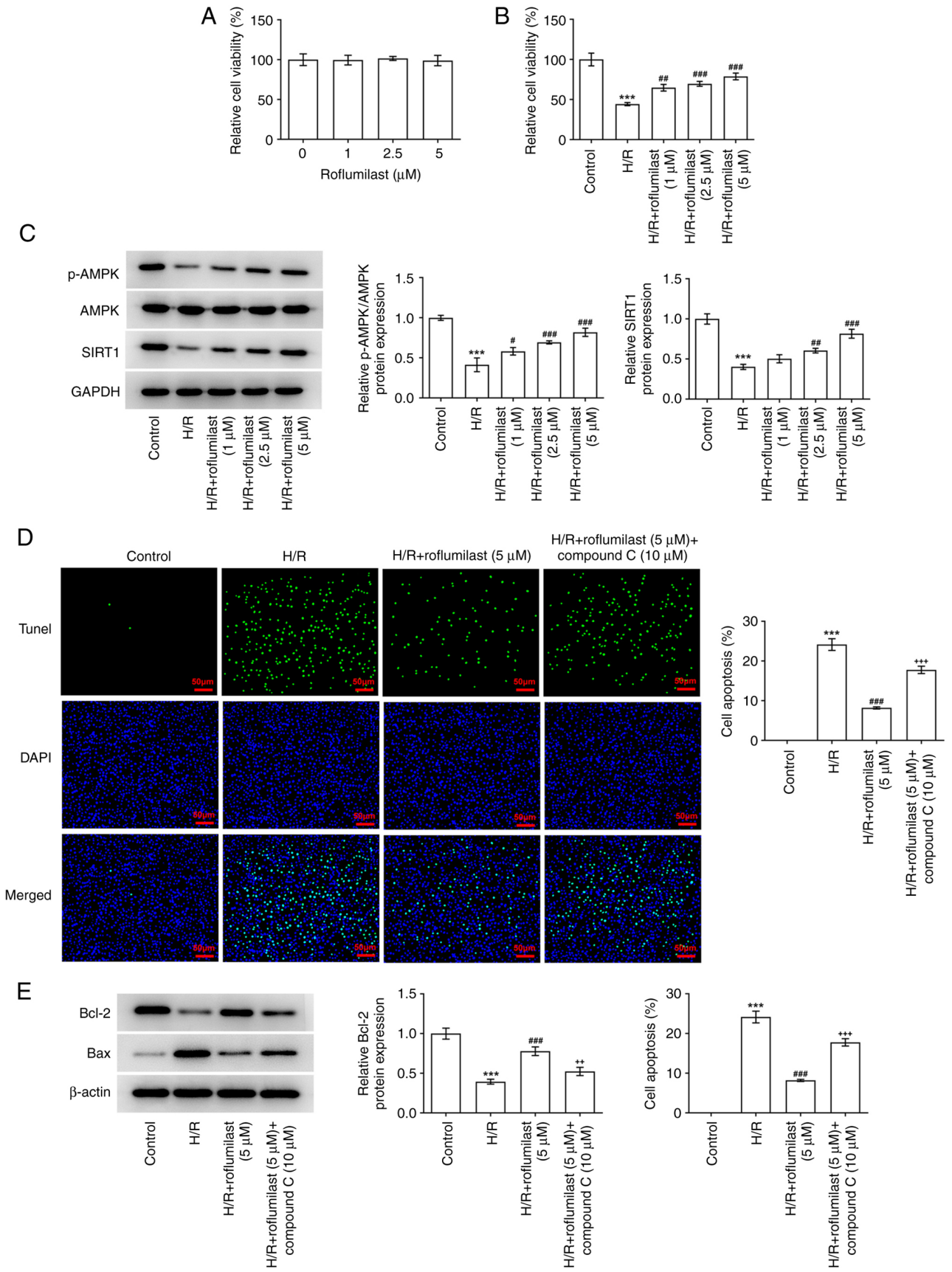


Figure 5. Roflumilast mitigates damage of H/R to H9C2 cell viability by activating the AMPK signaling pathway. (A) Effect roflumilast on H9C2 cell viability was analyzed using CCK-8 assay. (B) Viability of H/R-induced H9C2 cells treated with roflumilast was analyzed by CCK-8 assay. (C) Expression AMPK signaling pathway-related proteins in H/R-induced H9C2 cells with roflumilast treatment was detected by western blotting. (D) Apoptosis of H/R-induced H9C2 cells with roflumilast and compound C treatment was analyzed by TUNEL assay. (E) Expression of apoptosis-related proteins in H/R-induced H9C2 cells with roflumilast and compound C treatment was detected by western blotting. $^{***}$P<0.001 vs. Control. $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. H/R. $^{++}$P<0.01 and $^{+++}$P<0.001 vs. H/R + roflumilast (5 $\mu$M). H/R, hypoxia/reoxygenation; AMPK, AMP-activated protein kinase; CCK-8, Cell Counting Kit-8; p, phosphorylated; SIRT1, sirtuin 1.

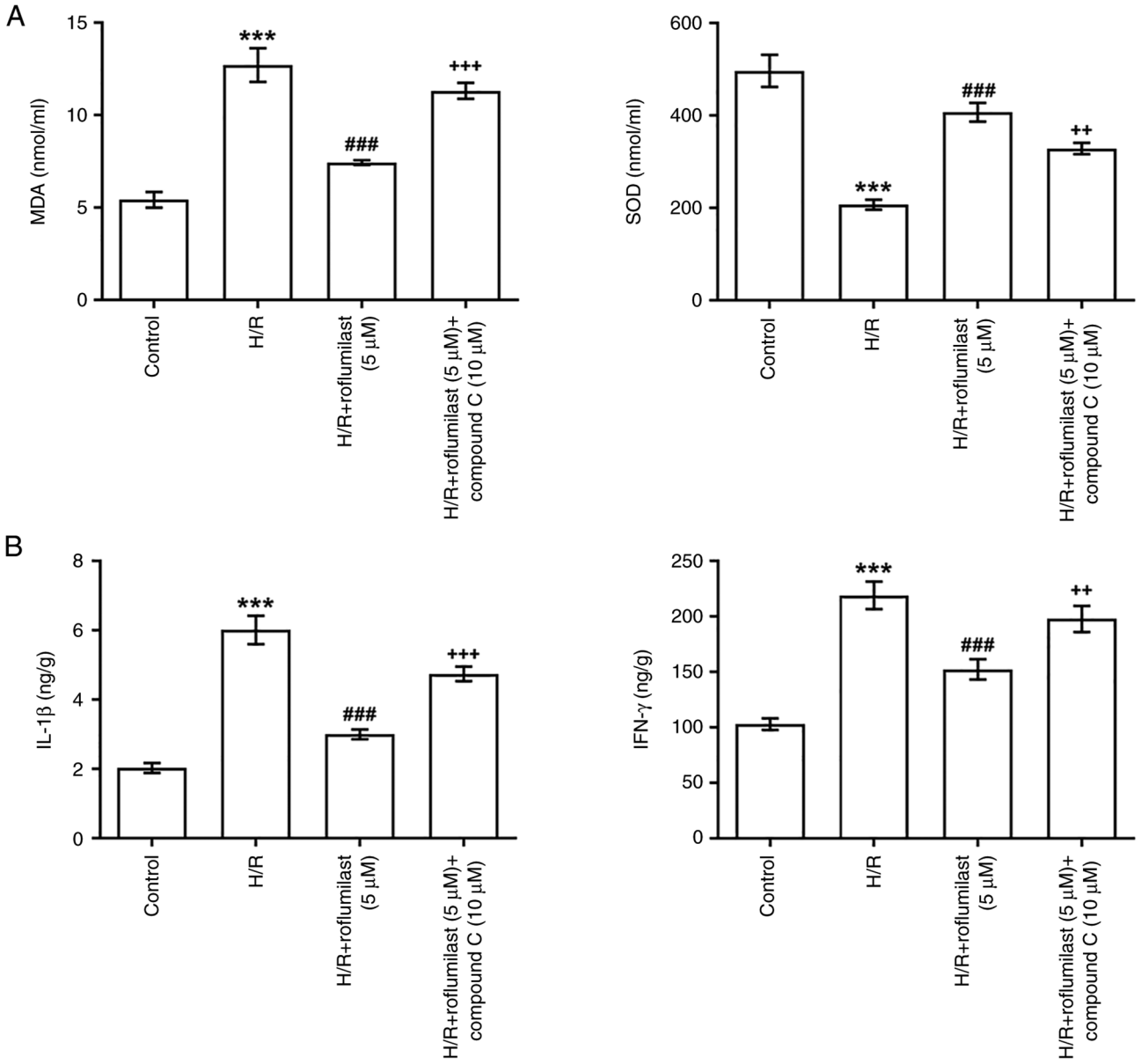


Figure 6. Roflumilast activates the AMP-activated protein kinase signaling pathway to alleviate oxidative stress and the inflammatory response of H9C2 cells induced by H/R. (A) Oxidative stress in H/R-induced H9C2 cells with roflumilast and compound C treatment were detected by assay kits. (B) Inflammatory factors in H/R-induced H9C2 cells with roflumilast and compound C treatment were detected by assay kits. $^{***}$P<0.001 vs. Control. $^{###}$P<0.001 vs. H/R. $^{++}$P<0.01 and $^{+++}$P<0.001 vs. H/R + roflumilast (5 $\mu$M). H/R, hypoxia/reoxygenation; MDA, malondialdehyde; SOD, superoxide dismutase.

promote cell survival (31). At the initial stage of reperfusion, oxidative stress injury caused by oxygen free radical explosion is one of the main pathogenic mechanisms of IRI. Activation of the AMPK signaling pathway increases cell viability and alleviates cardiomyocyte apoptosis induced by oxidative stress (32-34). Activated AMPK can inhibit myocardial cell apoptosis (35,36). Numerous studies suggested that pharmacological agonists of AMPK had anti-apoptotic activity in MI/R by activating the AMPK signaling pathway (37,38). In addition, AMPK decreases production of proinflammatory factors IL-1β and TNF-α, increases the content of anti-inflammatory factor IL-10 and alleviates myocardial injury from MI in rats (39). To confirm the effects of AMPK on inflammation, oxidative stress, apoptosis and mitochondrial function in I/R injury, compound C was administered to cells. As expected, compound C promoted inflammation, oxidative stress and apoptosis and aggravated mitochondrial injury, in H/R-induced H9C2 cells.

A previous study indicated that roflumilast could mitigate inflammation, oxidative stress and apoptosis in the acute lung injury of rabbits (40). Xu *et al* (10) demonstrated that roflumilast inhibits oxidative stress caused by ischemic stroke. Additionally, it was also discovered that roflumilast protected against cardiotoxicity by reducing inflammation and oxidative stress (13,14). In the present study, it was also found that roflumilast could suppress inflammation, oxidative stress and apoptosis in H/R-induced H9C2 cells and inhibit inflammation in rats subjected to MI/R. In addition, roflumilast could inhibit weight gain, promote insulin sensitivity and suppress hepatic steatosis in mice by increasing mitochondrial chondrogenesis (41). A PDE-4 inhibitor (rolipram) protected against malathion-induced toxic damage in rat blood and

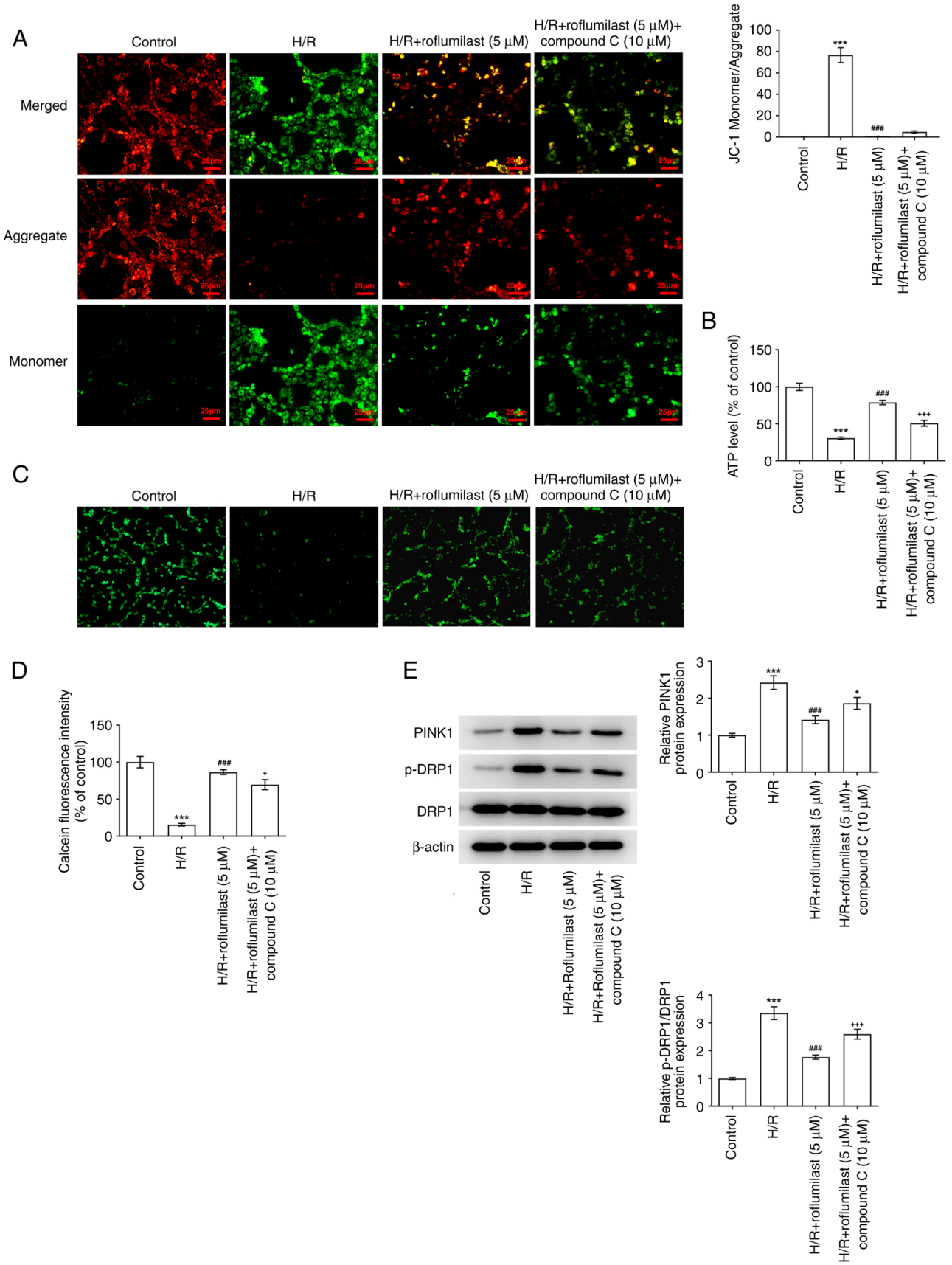


Figure 7. Roflumilast activates the AMP-activated protein kinase signaling pathway to decrease mitochondrial damage of H9C2 induced by H/R. (A) Changes of mitochondrial membrane potential in H/R-induced H9C2 cells with roflumilast and compound C treatment were detected by JC-1 staining. (B) ATP levels in H/R-induced H9C2 cells with roflumilast and compound C treatment were detected by ATP assay kit. (C) mPTP in H/R-induced H9C2 cells with roflumilast and compound C treatment was examined via a calcein-loading/$CoCl_2$-quenching system (magnification, x100). (D) Quantification of mPTP opening level. (E) Expression of mitochondrial regulatory proteins in H/R-induced H9C2 cells with roflumilast and compound C treatment was determined by western blotting. $^{***}$P<0.001 vs. Control. $^{###}$P<0.001 vs. H/R. $^{+}$P<0.05 and $^{+++}$P<0.001 vs. H/R + roflumilast (5 $\mu$M) group. H/R, hypoxia/reoxygenation; mPTP, mitochondrial permeability transition pore; PINK1, PTEN-induced kinase 1; p, phosphorylated; DRP1, dynamin-related protein 1.

brain mitochondria (42). Based on aforementioned findings, it was hypothesized that a PDE-4 inhibitor might be related to the regulation of mitochondrial function. In the present study, roflumilast improved mitochondrial function in MI/R rats and H/R-induced H9C2 cells. Xu *et al* (43) found that inhibition of AMPKα by compound C almost abolished the promotive effects of roflumilast on proliferator-activated Receptor-gamma and CCAAT enhancer-binding protein alpha: When AMPKα was inhibited, roflumilast treatment was almost abated. The protective effect of roflumilast on injury in MI/R rats or H/R-induced H9C2 cells was observed in the present study; this was weakened by compound C via the inhibition of the AMPK signaling pathway.

However, there were certain limitations to the current study. Firstly, cardiac functional studies, such as imaging and cardiac echo, were not performed. Secondly, female rats were not included in the MIRI model. Finally, the target of roflumilast in the MIRI model was not determined. These factors should be considered in further studies.

In conclusion, the present study demonstrated that roflumilast could alleviate MI in rats subjected to MI/R and attenuate H/R-induced oxidative stress, inflammatory response and mitochondrial damage in H9C2 cells by activating the AMPK signaling pathway.

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**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Authors' contributions**

ZH designed and conceived the study. BL conducted the experiments, analyzed the data and drafted the manuscript. ZH and BL confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

**Ethics approval and consent to participate**

The experimental protocol for the animal studies was reviewed and approved by the Committee for the Ethics of Animal Experiments, Shenzhen Peking University-The Hong Kong University of Science and Technology Medical Center (approval no. 2021-807; Shenzhen, China).

**Patient consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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