Activation of AMPK/p38/Nrf2 is involved in resveratrol alleviating myocardial ischemia-reperfusion injury in diabetic rats as an endogenous antioxidant stress feedback

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Background: Myocardial necrosis caused by myocardial ischemia-reperfusion (MI/R) in diabetic patients is prominently aggravated and can cause oxidative stress. Nuclear factor erythroid 2-related factor 2 (Nrf2) is a redox-sensing transcription factor that protects against myocardial ischemia/reperfusion injury (MIRI). However, the mechanism of action of Nrf2 in resveratrol-pretreated cardiomyocytes is complex. We assumed that adenosine 5'-monophosphate (AMP)-activated protein kinase (AMPK)/p38 mitogen-activated protein kinases (p38)/Nrf2 might be involved in resveratrol alleviating MIRI in diabetic rats as an endogenous protective mechanism.

Methods: A total of 50 type 2 diabetes mellitus (T2DM) rat models were randomly divided into 5 groups (n=10 in each group): sham group; MI/R group; AMPK inhibitor compound C + myocardial ischemia-reperfusion (C + MI/R) group; resveratrol + myocardial ischemia-reperfusion (RSV + MI/R) group; and resveratrol + AMPK inhibitor compound C + myocardium ischemia-reperfusion group (RSV + C + MI/R) group. Rats were fed a high fat diet, and the T2DM models were established by intraperitoneal injection of 1% streptozotocin (STZ). The MIRI models were established by ligating the left anterior descending coronary artery for 30 minutes followed by reperfusion for 120 minutes. The size of myocardial infarction was measured. Serum samples were collected to measure the concentrations of creatine kinase-MB (CK-MB). The levels of lactate dehydrogenase (LDH), glutathione (GSH), and superoxide dismutase (SOD) in myocardial tissues were determined. Immunofluorescence analysis of translocase of outer mitochondrial membrane 20 (TOMM20) was performed to observe the pathological changes in myocardial tissues. The protein expressions of AMPK, p-AMPK, p38, p-p38, Nrf2, and heme oxygenase 1 (HO-1) were determined by western blotting.

Results: Compared with the sham group, the expressions of AMPK, p38, Nrf2, and HO-1 in the myocardium were significantly increased in the MI/R group. Compared with the MI/R group, the RSV + MI/R group had a significantly lower oxidative stress level, milder myocardial injury, increased expressions of AMPK, Nrf2, and HO-1, and lower expression of p38. The protein expressions of Nrf2 and HO-1 were partially inhibited in the RSV + C + MI/R group.

Conclusions: Resveratrol can inhibit oxidative stress and alleviate MIRI by activating the AMPK/p38/Nrf2 signaling pathway. Meanwhile, AMPK/p38/Nrf2 is also an endogenous antioxidant stress pathway that protects against stress.

Keywords: Diabetes; resveratrol; myocardial ischemia/reperfusion injury (MIRI); adenosine monophosphate-activated protein kinase (AMPK); nuclear factor erythroid 2-related factor 2 (Nrf2)

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Introduction

The past few years have witnessed a constant rise in the prevalence of diabetes in China (1). Despite the effective control of blood sugar in diabetic patients thanks to the advances in medical science, the long-term complications of hyperglycemia remain a major concern. Since many myocardial protective drugs have been found to be ineffective in diabetic patients, diabetic patients are more susceptible to myocardial ischemia injury than non-diabetics, with poorer prognoses (2,3). Myocardial necrosis caused by myocardial ischemia-reperfusion (MI/R) in diabetic patients is prominently aggravated and is often irreversible. The high blood glucose level increases the production of reactive oxygen species (ROS), which continuously accumulates and aggravates the inflammatory response, eventually leading to redox imbalance and an inflammatory response (4). Therefore, a deeper insight into the pathogenic mechanisms, especially the oxidative stress-related signaling pathways, will assist efforts to extend the lives of diabetic patients.

Resveratrol, a non-flavonoid polyphenolic compound, is an antitoxin produced by many plants when stimulated (5). It can alleviate ischemic injury by suppressing the release of inflammatory cytokines, reducing intracellular oxidative stress, and directly or indirectly mediating a series of molecular mechanisms (6). Research has shown that resveratrol has anti-cancer, antioxidant, anti-inflammatory, and, in particular, cardioprotective activities; in addition, it can prevent ventricular dysfunction from occurring under a chronic high-glucose state (7). It can regulate glucose homeostasis by reducing insulin levels, prevent the overproduction of intracellular ROS, inhibit pro-inflammatory signaling, improve the body's long-term inflammatory status, and alleviate diabetes-related complications (8,9). Inflammation can regulate apoptosis and necrosis of cardiomyocytes, which is the main initiating factor leading to myocardial ischemia/reperfusion injury (MIRI) of cardiomyocytes. Resveratrol has a strong myocardial protective effect, which can significantly reduce apoptosis. Therefore, the application of resveratrol in clinical practice can prevent myocardial injury and reduce oxidative stress in diabetic patients to a certain extent. However, the relevant mechanisms are complex and diverse, and the specific signaling molecules involved require elucidation.

Restoring the redox balance is an important way to reduce MIRI. Adenosine monophosphate-activated protein kinase (AMPK) is a bio-energy metabolism sensor and regulator (10). When the body is exposed to external stimuli, AMPK can quickly respond to decrease cellular oxidative stress by mediating cellular antioxidant pathways and thus plays an anti-inflammatory role. Also, AMPK is a key molecular regulator of the metabolic pathway in diabetic patients (11). By increasing its *in vivo* activity, reducing cellular energy consumption, and decreasing energy loss during ischemia, AMPK can dynamically respond to resveratrol pretreatment (12). A study has shown that the activation of AMPK can regulate the p38 mitogen-activated protein kinase (p38MAPK) signaling pathway, and the p38 cascade can mediate the inflammatory response and mitochondrial apoptosis during ischemia (13). A recent study (14) suggested that the AMPK/p38 pathway might regulate the activation of nuclear factor E2-related factor 2 (Nrf2) to mediate cellular defense functions. As a potential endogenous protective pathway in the body, Nrf2 can bind to Kelch-like ECH-associated protein 1 (Keap1) (15) and exist stably in cells. When the body is under oxidative stress, the expression of Nrf2 increases and the antioxidant effect is activated, which quickly prevents the body from producing excessive ROS, enhances the body's ability to regulate oxidative stress, and up-regulates the downstream antioxidant factor heme oxygenase 1 (HO-1), thereby alleviating myocardial injury (16). Loss of Nrf2 expression in in diabetes (17) weakens antioxidant protection mechanisms, and the body can spontaneously initiate autoimmune defense and enhance its expression under stress. Resveratrol pretreatment can indirectly activate Nrf2 (18) and protect cells from damage caused by external stimuli by triggering the AMPK/p38 pathway. However, it is unclear whether resveratrol can alleviate MIRI in diabetic rats through this signaling pathway. Therefore, we used resveratrol pretreatment to alter the expressions of AMPK/p38/Nrf2 to observe whether it can improve MIRI in the context of diabetes.

In the present study, we established models of MIRI in diabetic rats to evaluate the endogenous activation-related pathways of Nrf2 signaling molecules and reveal the exact signaling protection mechanisms; furthermore, we

explored whether resveratrol acts through the AMPK/p38/Nrf2 signaling pathways to alleviate MIRI in diabetic rats, investigated the upstream and downstream relationships between its signaling molecules, and thus validated the cardioprotective effect of resveratrol. We present the following article in accordance with the ARRIVE reporting checklist (available at https://atm.amegroups.com/article/view/10.21037/atm-22-3789/rc).

Methods

Animals

A total of 50 healthy specific-pathogen-free (SPF) Sprague Dawley (SD) male rats aged 2–3 months, weighing 220–280 g were provided by the Animal Experiment Center of Xinjiang Medical University. All rats were kept at room temperature (23–25 ℃) and a suitable humidity (60%). A protocol was prepared before the study without registration. All the animal experimental procedures were approved by the Experimental Animal Ethics Committee of Xinjiang Medical University (No. IACUC20170420-02) and strictly followed the principles of the Institutional Guidelines for the Care and Use of Animals.

Establishment of type 2 diabetes mellitus (T2DM) models

All rats were fed with a standard diet for 1 week and then adaptively with a high-fat diet (HFD; composed of 60% basic feed, 10% lard, 10% sugar, 19% egg yolk powder, 0.5% salt, and 0.5% sesame oil) for 6 weeks, during which a comfortable temperature and humidity were maintained, along with a 12-hour light and dark cycle. After the rats were weighed, 1% streptozotocin (STZ) (Sigma Aldrich, St. Louis, MO, USA) 50 mg/kg was intraperitoneally injected. The modeling was considered successful when the blood glucose remained consistently higher than 16.7 mmol/L and the rats had typical diabetes symptoms including polydipsia, polyuria, and polyphagia (19).

Establishment of rat models of MI/R

All rats were deprived of water for 12 hours before surgery. After anesthesia, the thoracic cavity was separated layer by layer to completely expose the heart, followed by the ligation of the left anterior descending coronary artery. The thoracic cavity was closed after the elevation of the ST segment became visible in the electrocardiogram (ECG), which suggested acute myocardial ischemia. After 30 minutes of ischemia, the ligature was removed to restore coronary blood flow. The establishment of successful reperfusion was indicated when the left ventricle returned ruddy, the heart rate gradually returned normal, and the T wave fell rapidly. The total reperfusion time was 120 minutes. In the sham group, a silk suture was sewn, but no ligation was performed. This modeling method has been well established (20).

Grouping

A total of 50 T2DM rat models were randomly divided into 5 groups (n=10 in each group): a sham group; MI/R group; AMPK inhibitor compound C [MedChemExpress (MCE), Monmouth Junction, NJ, USA] + myocardial ischemia-reperfusion (C + MI/R) group; resveratrol (Sigma Aldrich, USA) + myocardial ischemia-reperfusion (RSV + MI/R) group; and resveratrol + AMPK inhibitor compound C + myocardium ischemia-reperfusion (RSV + C + MI/R) group. A week before surgery, rats in the resveratrol groups were intraperitoneally injected with resveratrol 20 mg/kg on a daily basis (21); rats in the inhibitor groups were given the AMPK inhibitor compound C 0.5 mg/kg via the tail vein 30 minutes before the establishment of MI/R models (22); and the other groups were injected with the same amount of normal saline.

Immunofluorescence staining of myocardial tissues

After 120 minutes of reperfusion, rat myocardial tissue was fixed with neutral formaldehyde (Solarbio, Beijing, China). Sections were baked and then embedded in paraffin (Sinopharm, Shanghai, China). The primary antibody translocase of outer mitochondrial membrane 20 (TOMM20) (Abcam, Cambridge, UK) was added. After the sections were rinsed with phosphate-buffered saline/Tween (PBST), the diluted fluorescent secondary antibody fluorescent CY3-conjugated goat anti-mouse IgG (Boster, Wuhan, China) was added dropwise to stain cell nuclei. The water stains were wiped dry, and the sections were mounted and then observed under a microscope (Olympus BX53 biological microscope) (×400).

Determination of myocardial infarction size

After 120 minutes of reperfusion, myocardial tissue was quickly harvested, and the myocardial infarction size was measured by triphenyltetrazolium chloride (TTC; Sigma,

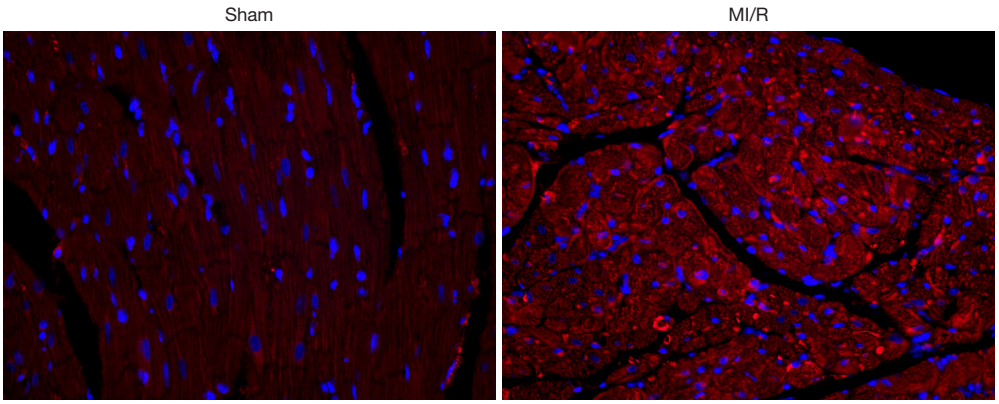

Figure 1 Immunofluorescence staining of TOMM20 in myocardial tissues under a microscope (×400). MI/R, myocardial ischemia/reperfusion; TOMM20, translocase of outer mitochondrial membrane 20.

USA) staining in each group. The following formula was applied: myocardial infarction size (%) = (infarcted area/area of myocardium at the ischemic area) × 100%.

Determination of creatine kinase-MB (CK-MB) and lactate dehydrogenase (LDH) levels

After 120 minutes of reperfusion, arterial blood was collected from the right ventricle of the rats. The supernatant was collected after centrifugation using a C2500-R-230V centrifuge (Labnet, Edison, NJ, USA). Serum CK-MB and LDH levels were determined by enzyme-linked immunosorbent assay (ELISA) using CK-MB assay kits (Elabscience Biotechnology, Wuhan, China) and LDH assay kits (Nanjing Jiancheng, Nanjing, China).

Determination of glutathione, superoxide dismutase (SOD), and ROS activities

After 120 minutes of reperfusion, 120 mg of ischemic left ventricular myocardium was collected and stored. The levels of glutathione (GSH), SOD, and ROS were determined according to manufacturer's instructions (Nanjing Jiancheng, China).

Western blotting

Myocardial tissue samples were harvested, and total protein was extracted for electrophoresis and transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Burlington, MA, USA). The specific antibodies including anti-AMPK (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-p-AMPK (1:200, Abcam, UK), anti-p38, anti-p-p38 (1:1,000, Affinity Company, San Francisco, CA, USA), anti-Nrf2 (1:1,000, Proteintech, Wuhan, China), and anti-HO-1 (1:1,000, Novus, Centennial, CO, USA) were added, and the mixture was incubated at 4 ℃ overnight. After washing 3 times with TBST, the secondary antibody (1:10,000, Santa Cruz Company, USA) was added. Blots were developed with electrochemiluminescence (ECL) substrate (Thermo Fisher, Waltham, MA, USA). Gray values were calculated using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 8.0 software (GraphPad Software Inc., San Diego, CA, USA). The normally distributed measurement data were presented as mean ± standard deviations ($\bar{x}$±SD). Inter-group comparison was based on one-way analysis of variance (ANOVA). The Mann-Whitney test or Student's *t*-test was applied for the parameters without normal distribution. A P value of less than 0.05 was considered statistically significant.

Results

MI/R significantly aggravated myocardial injury in diabetic rats

It was revealed that TOMM20 was mainly expressed in the cytoplasm of cardiomyocytes, and its expression level was relatively lower in the sham group (*Figure 1*); mitochondria were damaged during MI/R, and the

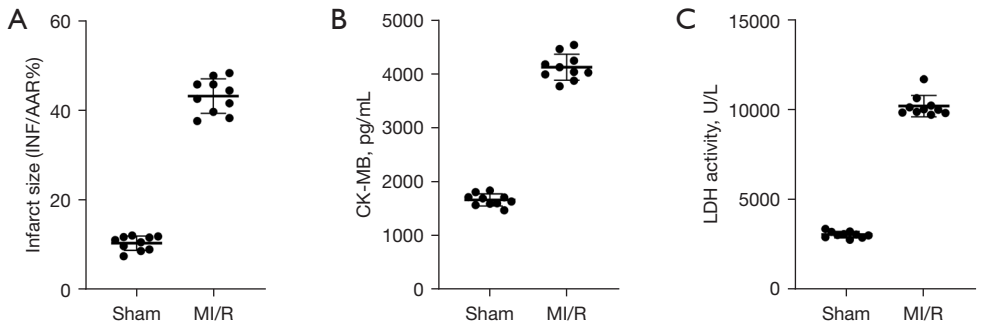

Figure 2 Comparison of myocardial infarction size and myocardial injury indicators between the sham group and MI/R group. (A) myocardial infarct size; (B) CK-MB; (C) LDH. CK-MB, creatine kinase-MB; LDH, lactate dehydrogenase; MI/R, myocardial ischemia/reperfusion; INF, myocardial infarct size; AAR, percentage of total ischemia-reperfusion risk area.

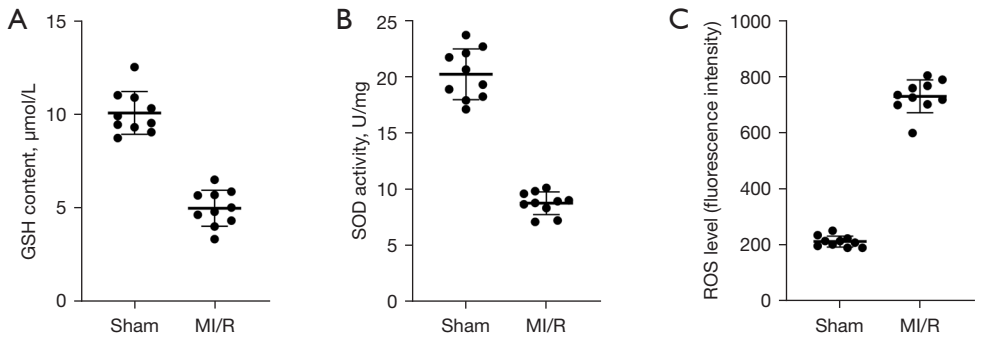

Figure 3 Comparison of oxidative stress markers between the sham group and MI/R group. (A) GSH; (B) SOD; (C) ROS. GSH, glutathione; SOD, superoxide dismutase; ROS, reactive oxygen species; MI/R, myocardial ischemia/reperfusion.

expression of TOMM20 was relatively increased, which induced mitophagy. In addition, compared with the sham group, the MI/R group had an increased percentage of myocardial infarct size, increased serum CK-MB and LDH concentrations (P<0.05) (*Figure 2A-2C*), and decreased GSH and SOD levels and increased ROS level in myocardial tissues (P<0.05) (*Figure 3A-3C*), indicating that the oxidative stress and myocardial damage were further aggravated during MI/R in diabetic rats, which verified the successful modeling of MI/R in our experiment.

AMPK/p38/Nrf2 was activated as an endogenous anti-oxidative stress mechanism during myocardial injury

After the use of AMPK inhibitor compound C, the percentage of myocardial infarction area increased, serum CK-MB and LDH concentrations increased (both P<0.05), GSH and SOD levels decreased, and ROS levels increased in the C + MI/R group (P<0.05), suggesting that the endogenous anti-oxidative stress mechanism was inhibited (*Figure 4A-4F*). Compared with the sham group, the expressions of AMPK, p38, Nrf2, and HO-1 in the MI/R group were significantly up-regulated (all P<0.05), suggesting the body had initiated an active anti-oxidative stress mechanism to protect against myocardial ischemic injury (*Figure 5A-5F*).

The AMPK/p38/Nrf2 pathway was involved in resveratrol alleviating MIRI in diabetic rats

We further evaluated the effect of resveratrol on MIRI in T2DM rats. In the RSV + C + MI/R group, AMPK inhibitor compound C was also used along with resveratrol pretreatment to observe whether compound C can block the myocardioprotective effect of resveratrol. Compared with the sham group, the MI/R group had significantly higher expression of TOMM20, which induced mitophagy; compared with the MI/R group, the RSV + MI/R group had

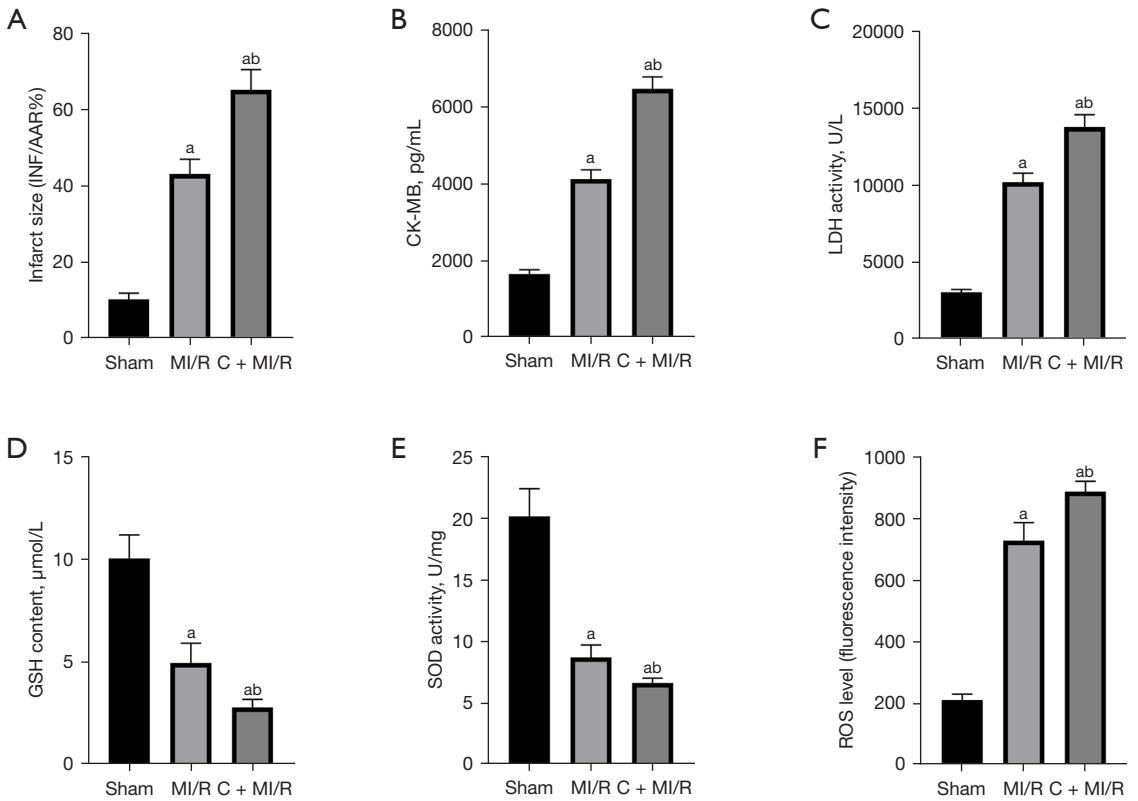

Figure 4 Effect of the inhibitor compound C on the endogenous protective mechanism of the body. (A) Myocardial infarct size; (B) CK-MB; (C) LDH; (D) GSH; (E) SOD; (F) ROS. [a], P<0.05, compared with the Sham group; [b], P<0.05. compared with the MI/R group. GSH, glutathione; SOD, superoxide dismutase; ROS, reactive oxygen species; MI/R, myocardial ischemia/reperfusion; C, compound C; CK-MB, creatine kinase-MB; LDH, lactate dehydrogenase; INF, myocardial infarct size; AAR, percentage of total ischemia-reperfusion risk area.

the significantly lower expression of TOMM20, suggesting the myocardial mitochondrial damage was alleviated; in contrast, TOMM20 expression was slightly elevated in the RSV + C + MI/R group compared with RSV + MI/R group (*Figure 6*). In addition, compared with the MI/R group, the myocardial infarction area and oxidative stress indicators significantly decreased in the RSV + MI/R group (all P<0.05); compared with the RSV + MI/R group, the myocardial infarction area increased in the RSV + C + MI/R group (the infarct area was massively gray-white in color), and the oxidative stress indicators rebounded (all P<0.05), indicating that the use of the inhibitor compound C partially blocked the antioxidant effect of resveratrol (*Figures 7,8*). Meanwhile, the expressions of AMPK, p-AMPK, Nrf2, and HO-1 were increased and the expressions of pro-inflammatory factors p38 and p-p38 were decreased in the RSV + MI/R group, indicating that resveratrol further activated the functions of cardioprotective signaling molecules and relieved the oxidative stress in the body. In order to further confirm the upstream and downstream relationship between the signaling molecules of this pathway, we synergistically applied the inhibitor compound C on top of resveratrol pretreatment. It was found that in the RSV + C + MI/R group, the expressions of AMPK, p-AMPK, Nrf2, and HO-1 were partially inhibited, the expression of p38 was somehow up-regulated, and expression of p-p38 showed no significant difference (*Figure 5A-5F*). Therefore, the AMPK/p38/Nrf2 signaling pathway synergistically regulated MIRI in diabetic rats and participated in the myocardioprotective effect of resveratrol.

Discussion

Myocardial ischemia-reperfusion injury is a complex pathological process involving multiple factors, including oxidative stress (free radical damage), inflammatory response, calcium overload and cardiomyocyte apoptosis. At present, there is no effective treatment for this disease.

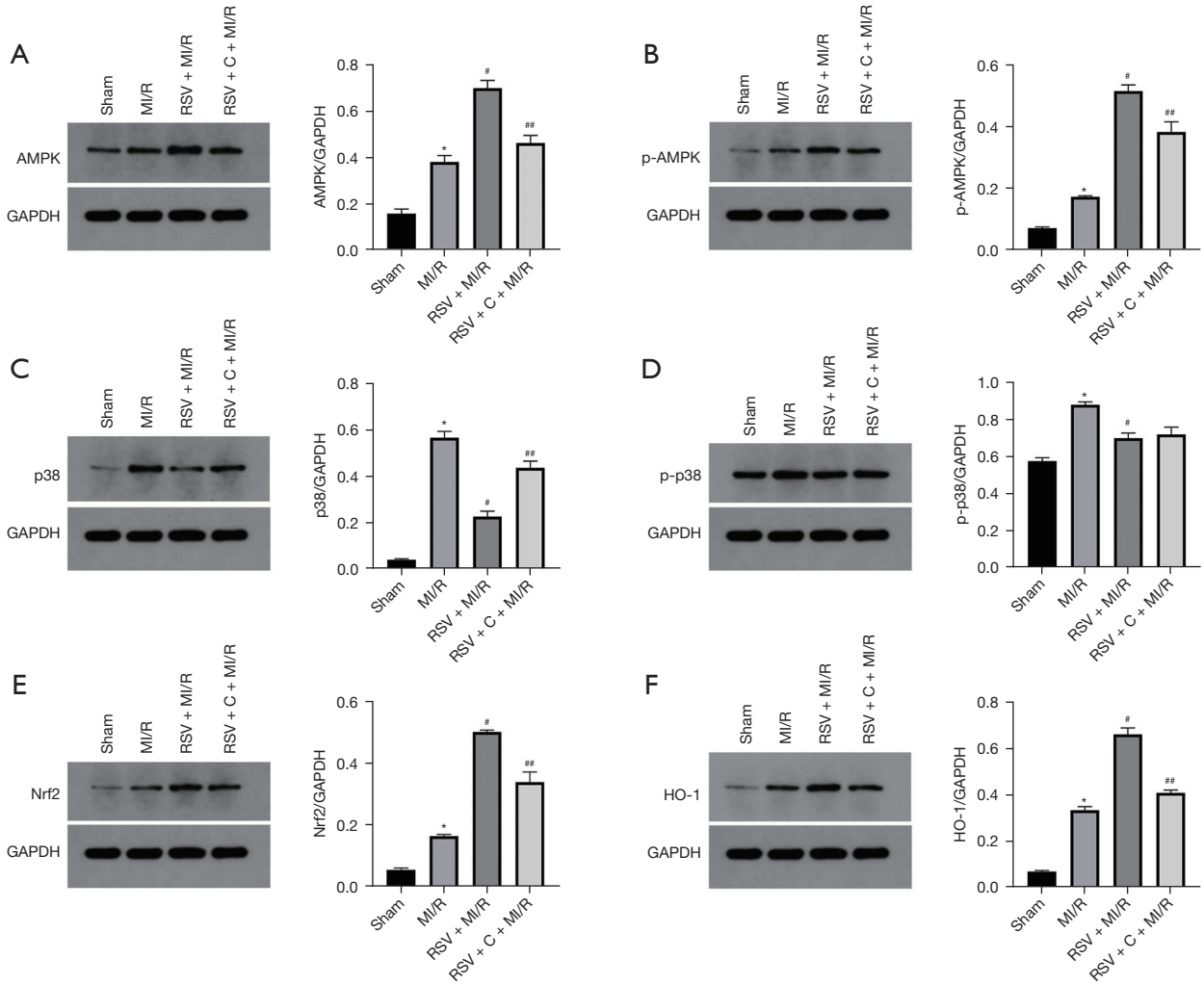

Figure 5 Roles of AMPK, p-AMPK, p38, p-p38, Nrf2, and HO-1 proteins in MIRI in resveratrol-pretreated diabetic rats. (A) AMPK; (B) p-AMPK; (C) p38; (D) p-p38; (E) Nrf2; (F) HO-1. *, P<0.05, compared with Sham group; #, P<0.05, compared with MI/R group; ##, P<0.05, compared with RSV + MI/R group. MI/R, myocardial ischemia/reperfusion; RSV, resveratrol; C, compound C; MIRI, myocardial ischemia/reperfusion injury; AMPK, adenosine monophosphate-activated protein kinase; Nrf2, nuclear factor erythroid 2-related factor 2; HO-1, heme oxygenase 1.

In order to improve the prognosis of patients, more and more studies have shown that a variety of intervention drugs can regulate cell signaling pathway and effectively reduce myocardial injury in diabetic patients. Compared with non-diabetic patients, those with diabetes have more damage in the myocardial signaling pathway, which further aggravates their MIRI. Based on the findings of a previous study (23), we further explored the myocardial protection in diabetic rats in our current study. At the same time, we further explored the endogenous protective pathway of the body. First, we demonstrated the cardioprotective effect of resveratrol in T2DM rats. Second, our data showed that the AMPK/p38/Nrf2 signaling pathway, as an endogenous antioxidant response mechanism, was activated upon myocardial injury; the protein expression of this pathway was further enhanced after pretreatment with resveratrol, thereby alleviating MIRI in diabetic rats. Finally, we confirmed the cardioprotective effect mediated by the AMPK/p38/Nrf2 signaling pathway was involved in inhibiting oxidative stress.

Diabetes has become one of the major health threats, with a constantly rising prevalence (24). Due to the chronic

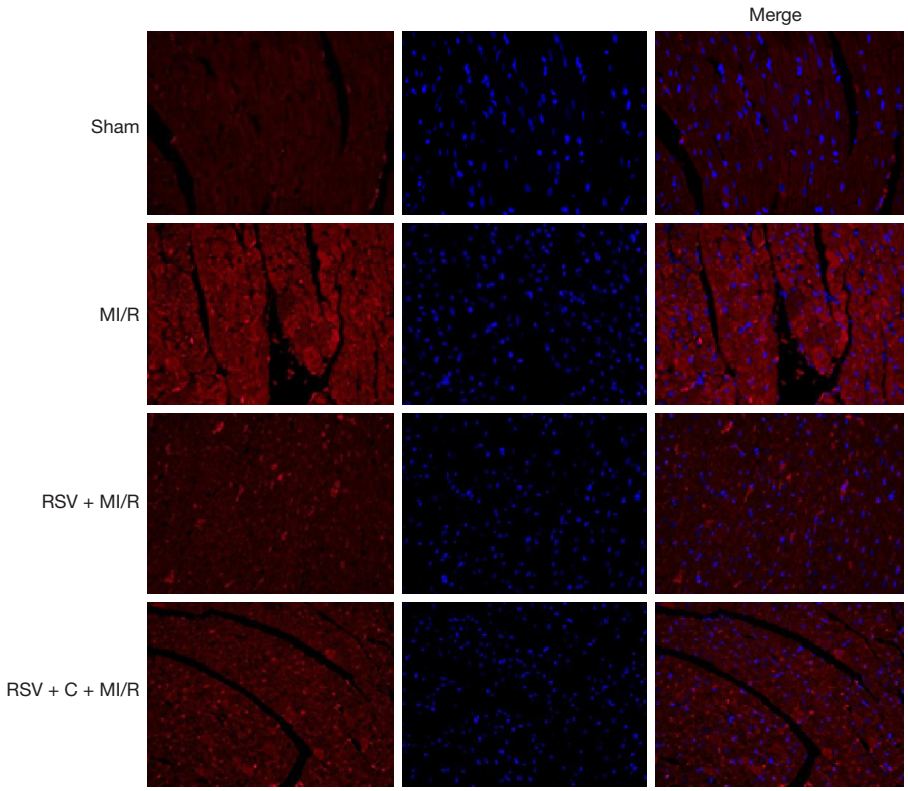

Figure 6 Immunofluorescence staining of TOMM20 in myocardial tissues under a microscope in 4 groups (×400). MI/R, myocardial ischemia/reperfusion; RSV, resveratrol; C, compound C; TOMM20, translocase of outer mitochondrial membrane 20.

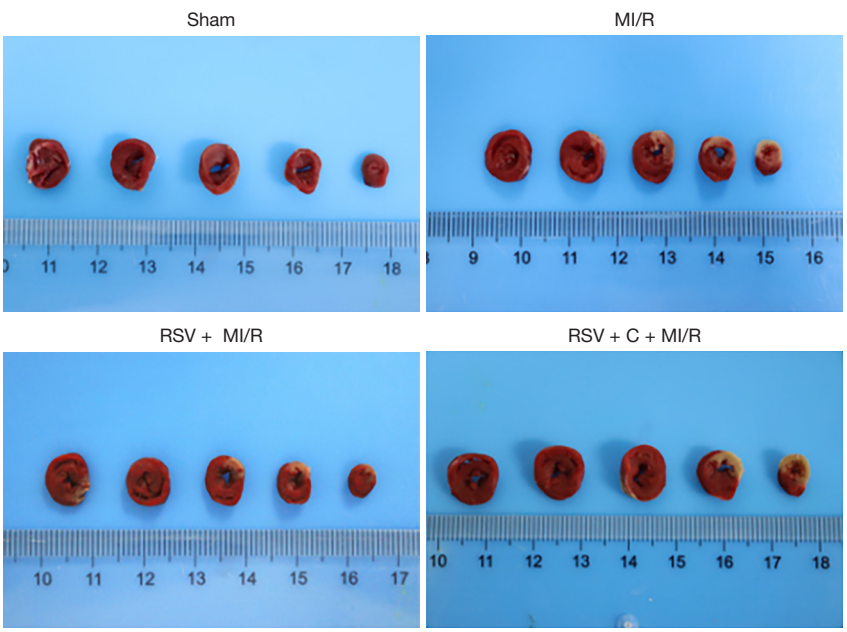

Figure 7 Comparison of myocardial infarction size among 4 groups. MI/R, myocardial ischemia/reperfusion; RSV, resveratrol; C, compound C.

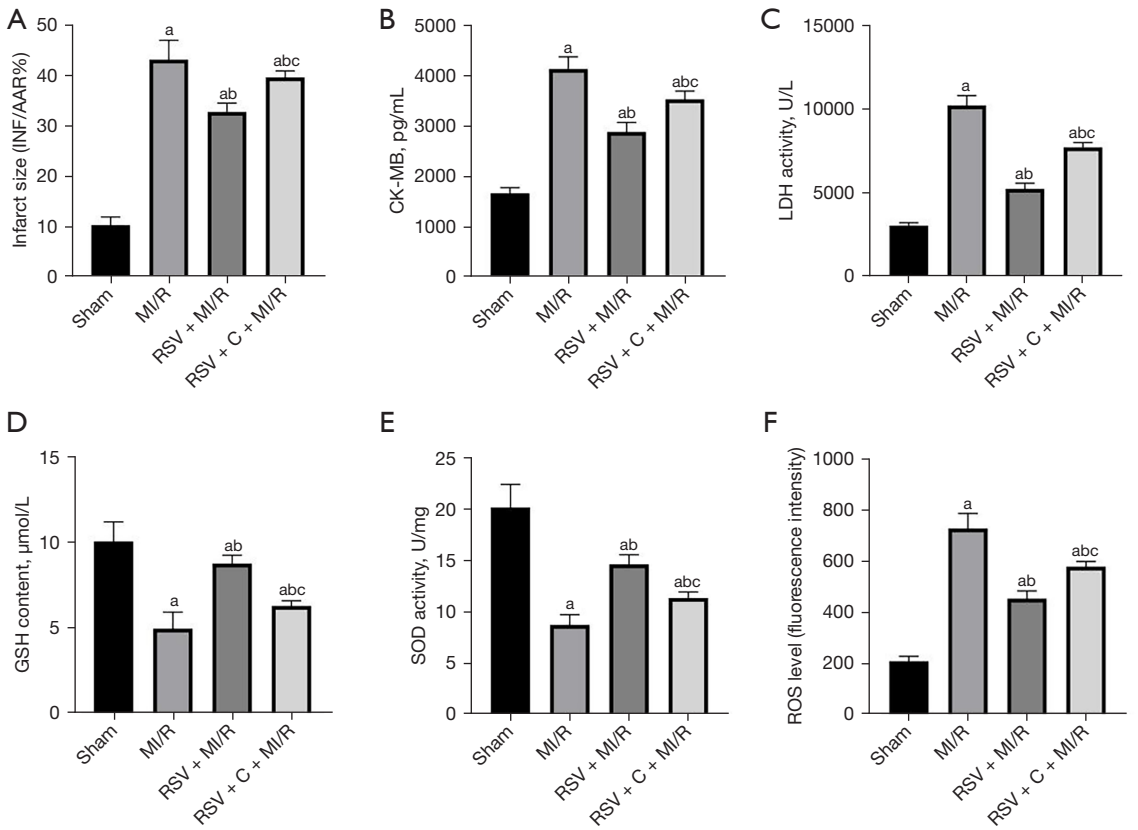

Figure 8 The protective effect of resveratrol and the effect of synergistic use of the inhibitor compound C on the antioxidant mechanism. (A) Myocardial infarct size; (B) CK-MB; (C) LDH; (D) GSH; (E) SOD; (F) ROS. [a], P<0.05, compared with Sham group; [b], P<0.05, compared with MI/R group; [c], P<0.05, compared with RSV + MI/R group. CK-MB, creatine kinase-MB; LDH, lactate dehydrogenase; GSH, glutathione; SOD, superoxide dismutase; ROS, reactive oxygen species; MI/R, myocardial ischemia/reperfusion; RSV, resveratrol; C, compound C; INF, myocardial infarct size; AAR, percentage of total ischemia-reperfusion risk area.

state of hyperglycemia, diabetic patients have higher levels of oxidative stress and inflammatory factors than non-diabetic patients, especially in the context of ischemic injury (25). As a potent biological antioxidant, resveratrol has received much clinical interest (26). Resveratrol has multiple antioxidant pathways. It can regulate the oxidative phosphorylation system, improve the content of high-energy compounds in the heart muscle (27), and prevent myocardial injury. Meanwhile, it can effectively stimulate the SIRT1 deacetylation of PGC-1α (28), inhibit the production of ROS, and alleviate mitochondrial damage during myocardial ischemia. In addition, resveratrol has been shown to act on the Sirt1/Nrf2 signaling pathway to regulate vascular functions and improve acute lung injury after intestinal ischemia (29); it also increases Nrf2 activity through Sirt1 overexpression, thus alleviating MIRI and the increased oxidative stress in diabetes (30). Although the cardioprotective effect of resveratrol has been demonstrated, it was unclear whether resveratrol can increase Nrf2 expression through the AMPK/p38 pathway. In our present study, we once again confirmed the cardioprotective effect of resveratrol and found that MI/R significantly aggravated myocardial injury in diabetic rats, along with remarkably increased oxidative stress markers. Meanwhile, we found that resveratrol pretreatment up-regulated the protein expressions of AMPK, Nrf2, and HO-1 and down-regulated the protein expression of the inflammatory signaling molecule p38 in myocardial tissue, suggesting the antioxidant and myocardioprotective effects of resveratrol may be related to the AMPK/p38/Nrf2 signaling pathway.

Nrf2 is an anti-oxidative stress transcription factor. Enhancing the activation of Nrf2 can protect the heart from MIRI. Overexpression of Nrf2 can stabilize ROS

homeostasis in cardiomyocytes (31), maintain vascular stability, and suppress oxidative stress, whereas inhibition of Nrf2 significantly blocked these effects (32,33). These findings further clarified the beneficial roles of Nrf2 in MIRI. In a study showing that melatonin significantly improved cardiac function after ischemia, it was found that melatonin inhibited oxidative stress by increasing AMPK activation and Nrf2 nuclear translocation, thereby alleviating myocardial ischemia injury (34). In our current study, we demonstrated that Nrf2 functioned as an endogenous antioxidant defense system in the body and confirmed the upstream-downstream relationship between AMPK/p38 and Nrf2. Compared with the sham group, the MI/R group had significantly higher Nrf2 expression, indicating that Nrf2 is activated as an endogenous antioxidant defense system when the body is stimulated and exerts its defensive role to protect cardiomyocytes.

Besides being a key regulator of energy balance in eukaryotic cells, AMPK regulates inflammatory response, downregulates p38 and NF-κB signaling (35), and suppresses the expressions of pro-inflammatory mediators. Resveratrol has been reported to inhibit neointimal hyperplasia following arterial injury, especially in rodents fed an HFD, and such effects may be mediated by AMPK (36). As part of its role as a key regulator of proinflammatory cytokine biosynthesis (37), p38 regulates protein transcription and translation. Stress may activate p38. In particular, I/R injury can lead to cell death, which may be due to prolonged p38 activation (38). In our current experiment, the myocardial infarction area increased, the levels of GSH and SOD in myocardial tissue decreased, the level of ROS increased, and the expression of p38 also increased in the MI/R group, indicating that ischemic injury causes the massive generation of pro-inflammatory factors, and p38 is rapidly activated to mediate the expressions of pro-inflammatory factors and the apoptosis, leading to the aggravation of the myocardial injury. Furthermore, the damaged myocardial mitochondria, the accumulated outer membrane translocase in the mitochondrial outer membrane, and the increased TOMM20 level also contribute to mitochondrial autophagy. In addition, AMPK, Nrf2, and HO-1 signaling molecules in cardiomyocytes are also activated, and the body activates its own antioxidant defense mechanism to combat the pro-inflammatory effect of p38; meanwhile, to a certain extent, it down-regulates the expression of p38 and promotes the expressions of Nrf2 and downstream HO-1, which trigger the AMPK/p38 cascade and alleviate the myocardial injury in rats.

In our present study, rats were pretreated with resveratrol to further verify whether the AMPK/p38/Nrf2 signaling pathway is involved in the cardioprotective activity of resveratrol. In the RSV + MI/R group, the infarct area was reduced, the oxidative stress index (OSI) was decreased, and the myocardial injury was alleviated, indicating that resveratrol has a cardioprotective effect in T2DM rats. Meanwhile, TOMM20 expression was significantly reduced, myocardial mitochondrial damage was greatly alleviated, and cardiomyocyte apoptosis was reduced. In addition, the expressions of AMPK, p-AMPK, Nrf2, and HO-1 in myocardial tissue were further increased, the expression of p38 was decreased, and the phosphorylation levels of p38 showed no significant change, indicating that resveratrol pretreatment further activates the expressions of protective signaling molecules and suppresses the generation of pro-inflammatory factor p38. Therefore, the myocardioprotective effect of resveratrol may be mediated through the AMPK/p38/Nrf2 signaling pathway. Finally, we synergistically applied the AMPK inhibitor compound C to further identify the upstream and downstream relationship of this pathway. After the use of compound C, compared with the RSV + MI/R group, the RSV + C + MI/R group had significantly lower expression of AMPK, decreased expressions of Nrf2 and HO-1, and slightly increased expression of p38, suggesting that the signaling pathway had been successfully blocked by compound C; in addition, the increased myocardial infarction area, the release of inflammatory factors, and the relative increase of OSI and TOMM20 expression demonstrated that both Nrf2 and HO-1 are in the downstream of AMPK/p38 signaling pathway. Further, as AMPK was partially inhibited, its ability to down-regulate p38 was also weakened; the increase in pro-inflammatory factors induces autophagy, leading to more severe myocardial injury (also compared with the RSV + MI/R group). Therefore, the AMPK/p38 pathway will affect the expressions of its downstream Nrf2 and HO-1 proteins. This study aims at effectively reducing the oxidative stress response in diabetic patients, and explores the endogenous protective mechanism of the body without external intervention, which provides a new prospect for clinical search for protective methods to reduce diabetic MIRI. In addition, a previous study has shown that resveratrol not only has myocardial protective effect, but also can prevent kidney injury caused by oxidative stress, enhance antioxidant capacity, reduce inflammation and fibrosis response, and has certain neuroprotective effect (39).

To sum up, our data reconfirmed the protective effect of

resveratrol on MIRI in diabetic rats, which was associated with the AMPK/p38/Nrf2 signaling pathway. When the body is under stress due to ischemic injury, the AMPK/p38/Nrf2 signaling pathway, as an endogenous anti-oxidative stress feedback, quickly produces a defensive effect and alleviates the ischemia-reperfusion injury. In addition, resveratrol further increases AMPK expression and suppresses the production of pro-inflammatory cytokine p38, finally activating the downstream Nrf2/HO-1 signaling pathway and reducing oxidative stress in the body. Therefore, resveratrol greatly enhances the endogenous protection against oxidative stress via the AMPK/p38/Nrf2 signaling pathway and improves the expression of the AMPK/p38/Nrf2 pathway, which may effectively mitigate MIRI in diabetic patients. However, only the inhibitor of AMPK protein was used in our current experiment. More convincing evidence may be obtained based on techniques such as gene knockout and lentivirus packaging, which may enable us to explore the molecular regulation mechanisms in a more comprehensive and specific manner.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All the animal experimental procedures were approved by the Experimental Animal Ethics Committee of Xinjiang Medical University (No. IACUC20170420-02) and strictly followed the principles of the Institutional Guidelines for the Care and Use of Animals.

References

1. Sun H, Saeedi P, Karuranga S, et al. IDF Diabetes Atlas: Global, regional and country-level diabetes prevalence estimates for 2021 and projections for 2045. Diabetes Res Clin Pract 2022;183:109119.
2. Bugger H, Bode C. The vulnerable myocardium. Diabetic cardiomyopathy. Hamostaseologie 2015;35:17-24.
3. Evangelista I, Nuti R, Picchioni T, et al. Molecular Dysfunction and Phenotypic Derangement in Diabetic Cardiomyopathy. Int J Mol Sci 2019;20:3264.
4. Takayanagi R, Inoguchi T, Ohnaka K. Clinical and experimental evidence for oxidative stress as an exacerbating factor of diabetes mellitus. J Clin Biochem Nutr 2011;48:72-7.
5. Tang PC, Ng YF, Ho S, et al. Resveratrol and cardiovascular health--promising therapeutic or hopeless illusion? Pharmacol Res 2014;90:88-115.
6. Miao J, Huang Z, Liu S, et al. Hydroxytyrosol protects against myocardial ischemia reperfusion injury by inhibiting mitochondrial permeability transition pore opening. Exp Ther Med 2019;17:671-8.
7. Mao ZJ, Lin H, Hou JW, et al. A Meta-Analysis of Resveratrol Protects against Myocardial Ischemia/Reperfusion Injury: Evidence from Small Animal Studies and Insight into Molecular Mechanisms. Oxid Med Cell Longev 2019;2019:5793867.
8. Mahjabeen W, Khan DA, Mirza SA. Role of resveratrol supplementation in regulation of glucose hemostasis, inflammation and oxidative stress in patients with diabetes mellitus type 2: A randomized, placebo-controlled trial. Complement Ther Med 2022;66:102819.

9. Paradies G, Petrosillo G, Pistolese M, et al. Decrease in mitochondrial complex I activity in ischemic/reperfused rat heart: involvement of reactive oxygen species and cardiolipin. Circ Res 2004;94:53-9.
10. Pelosse M, Cottet-Rousselle C, Bidan CM, et al. Synthetic energy sensor AMPfret deciphers adenylate-dependent AMPK activation mechanism. Nat Commun 2019;10:1038.
11. Yang H, Feng A, Lin S, et al. Fibroblast growth factor-21 prevents diabetic cardiomyopathy via AMPK-mediated antioxidation and lipid-lowering effects in the heart. Cell Death Dis 2018;9:227.
12. Wan D, Zhou Y, Wang K, et al. Resveratrol provides neuroprotection by inhibiting phosphodiesterases and regulating the cAMP/AMPK/SIRT1 pathway after stroke in rats. Brain Res Bull 2016;121:255-62.
13. Liu XM, Chen QH, Hu Q, et al. Dexmedetomidine protects intestinal ischemia-reperfusion injury via inhibiting p38 MAPK cascades. Exp Mol Pathol 2020;115:104444.
14. Song C, Heping H, Shen Y, et al. AMPK/p38/Nrf2 activation as a protective feedback to restrain oxidative stress and inflammation in microglia stimulated with sodium fluoride. Chemosphere 2020;244:125495.
15. Bollong MJ, Lee G, Coukos JS, et al. A metabolite-derived protein modification integrates glycolysis with KEAP1-NRF2 signalling. Nature 2018;562:600-4.
16. Ding YW, Zhao GJ, Li XL, et al. SIRT1 exerts protective effects against paraquat-induced injury in mouse type II alveolar epithelial cells by deacetylating NRF2 in vitro. Int J Mol Med 2016;37:1049-58.
17. Miller WP, Sunilkumar S, Giordano JF, et al. The stress response protein REDD1 promotes diabetes-induced oxidative stress in the retina by Keap1-independent Nrf2 degradation. J Biol Chem 2020;295:7350-61.
18. Li S, Zhao G, Chen L, et al. Resveratrol protects mice from paraquat-induced lung injury: The important role of SIRT1 and NRF2 antioxidant pathways. Mol Med Rep 2016;13:1833-8.
19. King AJ. The use of animal models in diabetes research. Br J Pharmacol 2012;166:877-94.
20. Gao J, Min F, Wang S, et al. Effect of Rho-Kinase and Autophagy on Remote Ischemic Conditioning-Induced Cardioprotection in Rat Myocardial Ischemia/Reperfusion Injury Model. Cardiovasc Ther 2022;2022:6806427.
21. Yan Y, Tong F, Chen J. Endogenous BMP-4/ROS/COX-2 Mediated IPC and Resveratrol Alleviated Brain Damage. Curr Pharm Des 2019;25:1030-9.
22. Handa N, Takagi T, Saijo S, et al. Structural basis for compound C inhibition of the human AMP-activated protein kinase α2 subunit kinase domain. Acta Crystallogr D Biol Crystallogr 2011;67:480-7.
23. Xu G, Zhao X, Fu J, et al. Resveratrol increase myocardial Nrf2 expression in type 2 diabetic rats and alleviate myocardial ischemia/reperfusion injury (MIRI). Ann Palliat Med 2019;8:565-75.
24. Crisafulli A, Pagliaro P, Roberto S, et al. Diabetic Cardiomyopathy and Ischemic Heart Disease: Prevention and Therapy by Exercise and Conditioning. Int J Mol Sci 2020;21:2896.
25. Ding M, Lei J, Han H, et al. SIRT1 protects against myocardial ischemia-reperfusion injury via activating eNOS in diabetic rats. Cardiovasc Diabetol 2015;14:143.
26. Kaur A, Tiwari R, Tiwari G, et al. Resveratrol: A Vital Therapeutic Agent with Multiple Health Benefits. Drug Res (Stuttg) 2022;72:5-17.
27. Fourny N, Lan C, Sérée E, et al. Protective Effect of Resveratrol against Ischemia-Reperfusion Injury via Enhanced High Energy Compounds and eNOS-SIRT1 Expression in Type 2 Diabetic Female Rat Heart. Nutrients 2019;11:105.
28. Fang WJ, Wang CJ, He Y, et al. Resveratrol alleviates diabetic cardiomyopathy in rats by improving mitochondrial function through PGC-1α deacetylation. Acta Pharmacol Sin 2018;39:59-73.
29. Chai D, Zhang L, Xi S, et al. Nrf2 Activation Induced by Sirt1 Ameliorates Acute Lung Injury After Intestinal Ischemia/Reperfusion Through NOX4-Mediated Gene Regulation. Cell Physiol Biochem 2018;46:781-92.
30. Xu JJ, Cui J, Lin Q, et al. Protection of the enhanced Nrf2 deacetylation and its downstream transcriptional activity by SIRT1 in myocardial ischemia/reperfusion injury. Int J Cardiol 2021;342:82-93.
31. Li N, Zhan X. Machine Learning Identifies Pan-Cancer Landscape of Nrf2 Oxidative Stress Response Pathway-Related Genes. Oxid Med Cell Longev 2022;2022:8450087.
32. Katsumata Y, Shinmura K, Sugiura Y, et al. Endogenous prostaglandin D2 and its metabolites protect the heart against ischemia-reperfusion injury by activating Nrf2. Hypertension 2014;63:80-7.
33. Li J, Ichikawa T, Villacorta L, et al. Nrf2 protects against maladaptive cardiac responses to hemodynamic stress. Arterioscler Thromb Vasc Biol 2009;29:1843-50.
34. Xu C, Wang J, Fan Z, et al. Cardioprotective effects of melatonin against myocardial ischaemia/reperfusion

injury: Activation of AMPK/Nrf2 pathway. J Cell Mol Med 2021;25:6455-9.
35. Huang BP, Lin CH, Chen HM, et al. AMPK activation inhibits expression of proinflammatory mediators through downregulation of PI3K/p38 MAPK and NF-κB signaling in murine macrophages. DNA Cell Biol 2015;34:133-41.
36. Guo J, Pereira TJ, Mori Y, et al. Resveratrol Inhibits Neointimal Growth after Arterial Injury in High-Fat-Fed Rodents: The Roles of SIRT1 and AMPK. J Vasc Res 2020;57:325-40.
37. Rajpoot S, Kumar A, Zhang KYJ, et al. TIRAP-mediated activation of p38 MAPK in inflammatory signaling. Sci Rep 2022;12:5601.
38. Mackay K, Mochly-Rosen D. Involvement of a p38 mitogen-activated protein kinase phosphatase in protecting neonatal rat cardiac myocytes from ischemia. J Mol Cell Cardiol 2000;32:1585-8.
39. Salami M, Salami R, Mafi A, et al. Therapeutic Potential of Resveratrol in Diabetic Nephropathy According to Molecular Signaling. Curr Mol Pharmacol 2022;15:716-35.

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