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Autophagy assuages myocardial infarction through Nrf2 signaling activation-mediated reactive oxygen species clear

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Abstract. – OBJECTIVE: The activation of autophagy was shown to shrink infarct size and mitigate cardiac dysfunction caused by myocardial infarction (MI). However, the underlying mechanisms remain largely unknown. As excessive generation of reactive oxygen species (ROS) deteriorates MI process and Nrf2 signaling exerts an antioxidant role, we explored whether autophagy assuaged MI through Nrf2 signaling activation-mediated ROS clear.

MATERIALS AND METHODS: MI models were induced by ligation of the left descending coronary artery (LAD) in C57BL/6J mice or Nrf2 knockout mice (Nrf2-KO). Rapamycin and 3-methyladenine (3-MA) were used to activate and repress autophagy in MI mice, respectively. Aspirin, a cardioprotective drug was given to MI mice to evaluate its effects on autophagy.

RESULTS: Compared with the MI group, rapamycin treatment remarkably decreased the infarct size, cell apoptosis and blood troponin I level, accompanied by the reduced redox potential (Eh), ROS, malondialdehyde (MDA) and cytochrome C levels, and the increased reduced glutathione (GSH) level. Also, rapamycin treatment increased the expressions of bcl-2, bcl-xL, HSP70, and HSP90. In addition, rapamycin treatment promoted the nuclear accumulation of Nrf2 protein. However, Nrf2 downregulation significantly impaired the effects of rapamycin on the reductions of infarct size, cell apoptosis, troponin I and ROS levels. Similarly, to rapamycin roles, aspirin treatment also remarkably reduced infarct size, cell apoptosis and troponin I in mice with MI surgery, as well as increased the expression level of LC3II/LC3I.

CONCLUSIONS: This study demonstrated that autophagy enhancement contributed to the improvement of MI through Nrf2 signaling activation-mediated ROS clear.

Key Words:
Autophagy, Nrf2, Apoptosis, CytC, ROS, Myocardial infarction.

Introduction

As one of the ischemic heart diseases (IHD), myocardial infarction (MI) caused by prolonged ischemia[1], is one of the main causes of morbidity and mortality in the globe[2]. The myocardial necrosis induced by MI is severe and enduring[3], bringing a huge threat to human death. Therefore, it is necessary to find new potent methods to cure MI.

It is well documented that the excessive generation of reactive oxygen species (ROS) plays an important role in the development of ventricular remodeling induced by MI[4,5]. MI is mostly initiated from myocardial ischemia due to coronary artery obstruction. ROS was reported to be generated in the ischemic myocardium, especially after reperfusion[6], in which phagocyte nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, xanthine oxidase and mitochondria are the main sources of ROS[7]. The excessive generation of ROS directly injures tissue as a key inducer for cell death, as well as aggravates inflammation to cause further injury[8]. Some studies[9,10] have demonstrated that the reduced ROS level is intimately linked to the small infarct size in MI animal or cell models.

Autophagy is an evolutionarily conserved process which delivers cytoplasmic cargo to the lysosome and is characterized by the appearance of double membrane cytoplasmic vesicles[11,12]. Autophagy

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process is rapidly accelerated when cells need more intracellular nutrients or energy[11,12]. It is reported that autophagy process is strongly implicated in MI. For instance, McCormick et al[13] demonstrated that the enhanced autophagy induced by the repression of signal transducers and activators of transcription 1 (STAT1) contributed to the reduction of infarcts in MI mice. Pre-treatment with 3-methyladenine (3-MA), an inhibitor of autophagy, impaired the cardio-protective roles of STAT1 repression[13]. Aisa et al[14] found that autophagy enhancement with rapamycin significantly reduced the ventricular infarction area in MI rats with left anterior descending (LAD) ligation. All of these findings suggest that autophagy benefit to MI relaxation. However, the mechanisms underlying the cardio-protective roles of autophagy remain largely unknown.

Nuclear-factor-E2-related factor (Nrf2) is a key transcription factor that regulates the expression of antioxidant genes that are used to detoxify pro-oxidative stressors[15]. By inhibiting the excessive generation of ROS, Nrf2 signaling is considered to alleviate MI[16]. However, whether Nrf2 signaling-induced ROS reduction is involved in autophagy-mediated MI improvement remains unknown.

In the current study, we aimed to reveal whether Nrf2 signaling is involved in autophagy-mediated alleviation of MI. MI models were induced by the permanent ligation of LAD in C57BL/6J mice or Nrf2 knockout mice (Nrf2-KO). Rapamycin and 3-MA were used to activate and repress autophagy in MI mice, respectively.

Materials and Methods

Ethic Statement

All protocols involving animals were approved by the Animal Ethics Committee of The First Affiliated Hospital of Harbin Medical University and were performed in accordance to the national guidelines.

Animal Grouping

Male C57BL/6J mice (12-14-week-old, weight 25-30 g) or Nrf2 knockout mice (Nrf2-KO) at C57BL/6J background were all obtained from Charles River Laboratories (Beijing, China). C57BL/6J mice were divided into 5 groups, sham, MI, MI+rapamycin, MI+3-MA and MI+aspirin groups, with 5 mice in each group.

MI Induction and Mice Administration

MI was induced *via* permanent ligation of the LAD as previously reported[17]. In detail, mice were anesthetized *via* intraperitoneal injection of pentobarbital sodium salt anesthesia (50 mg/kg) and the anesthetic effect was assessed by the pedal reflex. Then, thoracotomy in the left side was carried out and the LAD was ligated 2 mm below the left auricular appendage. A pallor region was used to evaluate whether occlusion was successfully built. The mice in sham group were give the same procedure except for LAD ligation. The chest, musculature and cutaneous were closed. After the surgery, mice were immediately intramuscularly injected lidocaine (6 mg/kg) and atropine (0.04-0.10 mg/kg), followed by lidocaine and atropine administration every 4 h for the first 24 h to prevent arrhythmias. 24 h post-surgery, rapamycin (1 mg/kg/day; MedChemExpress LLC, Shanghai, China), 3-MA (20 mg/kg/day; MedChemExpress LLC, Shanghai, China) or aspirin (80 mg/kg/day; MedChemExpress LLC, Shanghai, China) was intraperitoneally injected to mice for a total of 21 days, with DMSO (7.5%, Solarbio, Beijing, China) as a negative control.

Sample Collection

On the 21-day post-MI surgery, mouse was sacrificed by CO_2 inhalation followed by spinal dislocation. Then, the apical blood sample was taken from each mouse and kept at room temperature for 30 min. Next, the blood samples were centrifuged at 1,000 ´g for 10 min at 4˚C and stored at -80°C until assayed. Meanwhile, the heart tissues were extracted from each mouse and submitted to ROS and infarct size evaluation.

Blood MDA, GSH and Troponin I Detection

The blood level of malondialdehyde (MDA) in mice were detected by thiobarbital colorimetric assay (No. A003-1, Nanjing Jiancheng Bio-Engineering Institute Co., Ltd., Nanjing, China). The blood level of glutathione (GSH) was determined by using the commercial kit (No. A006-1-1, Nanjing Jiancheng Bio-Engineering Institute Co., Ltd.) based on the manufacturer's instructions. The serum concentrations of troponin-I were detected by using a Human Cardiac Troponin I ELISA Kit (No. ab200016, Abcam, Cambridge, MA, USA) according to the manufacturer's instructions.

Tissue ROS Detection

The levels of ROS in the heart tissue homogenates were measured by an active oxygen detection kit (No. CA1410-100T; Solarbio, Beijing,

China) according to the manufactory's instructions, with distilled water as a negative control. The optical density at 550 nm for each sample was measured by using Fisherbrand™ accuSkan™ GO UV/Vis Microplate Spectrophotometer (Thermo Fisher scientific, Waltham, MA, USA).

Evaluation of Infarct Size

On the 21-day post-MI surgery, mice were killed, and the hearts were rapidly removed. After blood removal, hearts were fixed in OCT compound for 15 min at -20°C and cut into 6-8 slices with 1 mm thickness. Then, the slices were thawed and incubated in 1% of 2, 3, 5-triphenyltetrazolium chloride (TTC; No. G3005, Solarbio, Beijing, China) diluted in phosphate buffer solution (PBS) at 37°C for 20 min. Then, the slices were photographed with a digital camera. The quantification of infarct size was determined by Image J software as a percentage of LV mass.

TUNEL Staining

The terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) staining was used for tissue cell apoptosis detection. In brief, the cardiac tissues were embedded and sliced into 4 μm in thickness and placed in an oven at 45°C overnight. Then, the sections were dewaxed, and antigen repaired with 20 mg/ml of proteinase K diluted with 10 mM Tris-HCl (pH 7.4-7.8) and incubated at 37°C for 20 min. The sections were then washed twice with PBS for 5 min, followed by incubation with TdT buffer for 10 min. After that, the sections were probed with 100 μl TUNEL mixture at 37˚C for 90 min. Then, the sections were placed in Tris-HCl buffer and incubated for 15 min at room temperature. Cell nucleus were stained with 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, St. Louis, MO, USA). The staining was assessed under a fluorescence microscope.

Redox State Evaluation

The redox state of each mouse was evaluated according to a previous study[18]. In brief, blood samples are thawed on ice and prepared for high performance liquid chromatography (HPLC) using dansyl chloride as described previously[19,20]. GSH and glutathione disulfide (GSSG) were quantified by reverse-phase HPLC analysis on a Waters 2695 Alliance Separations Module fitted with a Supelcosil LC-NH2 column (Sigma-Aldrich, St. Louis, MO, USA). Mobile phases consisted of 80% HPLC grade methanol and 20% ddiH_2O, and 62.5% methanol, 12.5% glacial acetic acid, 214 mg/ml CH_3COONa-$3H_2O$ in H_2O with gradient flow at a speed of 1.0 ml per min. The peaks were visualized by fluorescence detection with excitation at 335 nm and emission at 518 nm. Then, the calculation of redox potential (Eh) of the GSH/GSSG in blood samples was obtained using the Nernst equation for pH 7.4: GSH/GSSG, Eh (mV) = -264+30 log ([GSSG]/[GSH]^2).

Western Blotting Assay (WB)

Total proteins were extracted from heart tissues by using RIPA lysis buffer (Solarbio, Beijing, China) containing 1% protease inhibitor (Solarbio, Beijing, China). Nuclear and cytoplasmic proteins were obtained with the help of the Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime, Jiangsu, China). Mitochondrial protein was extracted from tissues using the Tissue mitochondrial protein extraction kit (No. HR0143, Baiaolaibo Co., LTD., Beijing, China). The supernatants were collected and submitted to protein quantification measurement with standard Bicinchoninic acid kit (BCA, Thermo Fisher Scientific, Waltham, MA, USA). Then, 30 mg of protein from each sample was loaded into lanes and the proteins were separated by electrophoresis on 10% polyacrylamide gels. After that, the proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Thermo Fisher Scientific, Waltham, MA, USA). Subsequently, the membranes were blocked with 5% fat-free milk and incubated overnight at 4°C with the indicated primary antibodies: anti-cytochrome C (No. 4272, Cell Signaling Technology, Danvers, MA, USA), anti-bcl-2 (No. 2872, Cell Signaling Technology, Danvers, MA, USA), anti-bcl-xL (No. 2762, Cell Signaling Technology, Danvers, MA, USA), anti-HSP70 (No. 4872, Cell Signaling Technology, Danvers, MA, USA), anti-HSP90 (No. 4874, Cell Signaling Technology, Danvers, MA, USA), anti-Nrf2 (No. ab137550, Abcam, Cambridge, MA, USA), anti-Tubulin (No. ab6046, Abcam, Cambridge, MA, USA), anti-Histone (No. ab1791, Abcam, Cambridge, MA, USA), anti-COX IV (No. 4844, Cell Signaling Technology, Danvers, MA, USA) and anti-GAPDH (Proteintech, Wuhan, China). Tubulin, Histone, GAPDH and COX IV were used as internal references for cytoplasmic protein, nuclear protein, mitochondrial protein and total protein, respectively. After washing for three times, the membranes were exposed to horse radish peroxidase (HRP)-conjugated secondary antibodies (Proteintech, Wuhan, China). Protein

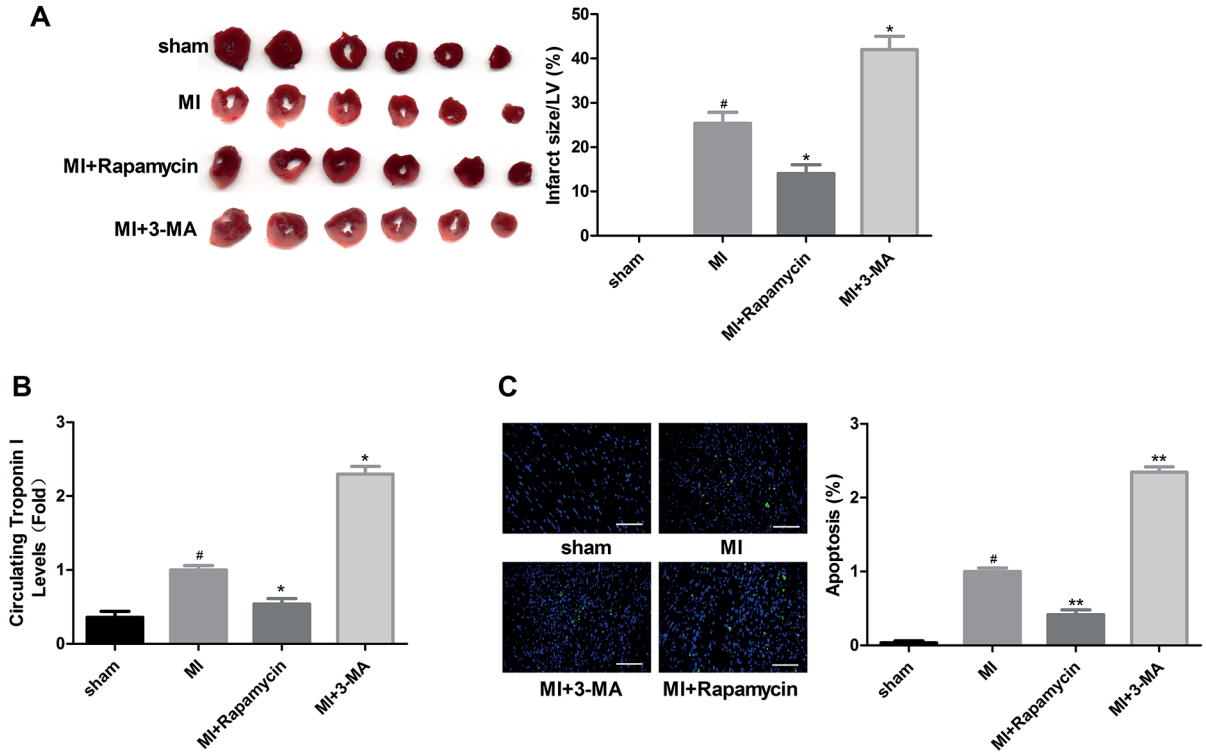

Figure 1. Evaluation of the effects of autophagy on the infarct size, blood Troponin I level and myocardial cell apoptosis in MI mice. C57BL/6J mice were randomly divided into sham, MI, MI+Rapamycin and MI+3-MA groups, and the blood and heart samples were obtained from all mice 21 days post-procedure. **A,** TTC staining was used to assess infarct size of mice heart from different groups. **B,** Blood levels of Troponin I in mice from different groups were detected by ELISA assay. **C,** Cell apoptosis in heart tissues was assessed by TUNEL assay (magnification: 100x). ($^{\#}p<0.05$, MI group compared with sham group; $^{*}p<0.05$, MI+Rapamycin/MI+3-MA group compared with MI group).

signals were enlarged by the enhanced chemiluminescence detection kit (ECL; Millipore, Billerica, MA, USA). Image-analysis software Image J was used to determine the expression of proteins.

Statistical Analysis

Data are presented as mean ± standard deviation (SD). Differences between groups were assessed using unpaired Student's *t*-test for 2 groups and One-way ANOVA with a post-hoc Student-Newmann-Keuls test for multiple comparisons. All analyses were performed by using GraphPad Prism 6.0 software (La Jolla, CA, USA) and statistical significance was set at $p<0.05$.

Results

Autophagy Enhancement Reduces the Myocardial Infarct Size and Cell Apoptosis in MI Mice

To explore the mechanism of autophagy-mediated cardioprotection in MI, we first explored its function in myocardial infarct size and cell apoptosis of mice with LAD ligation. Compared with the sham group, the infarct size (Figure 1A) was apparently increased in MI group, along with increased blood level of Troponin I (Figure 1B) and cell apoptosis (Figure 1C), suggesting that the MI models were successfully built. To probe the effects of autophagy on MI, MI mice were given rapamycin or 3-MA to activate or repress autophagy. The results showed that rapamycin treatment significantly reduced the infarct size, blood level of Troponin I, and myocardial cell apoptosis, whereas 3-MA treatment showed the opposite results with increased infarct size, blood level of Troponin I and myocardial cell apoptosis (Figure 1A-1C). These results suggest that autophagy exerts a protective role in MI.

Enhanced Autophagy Inhibits the Excessive Generation of ROS in MI Mice

Next, we explored the effects of autophagy in the oxidative stress-induced by MI *via* recruiting

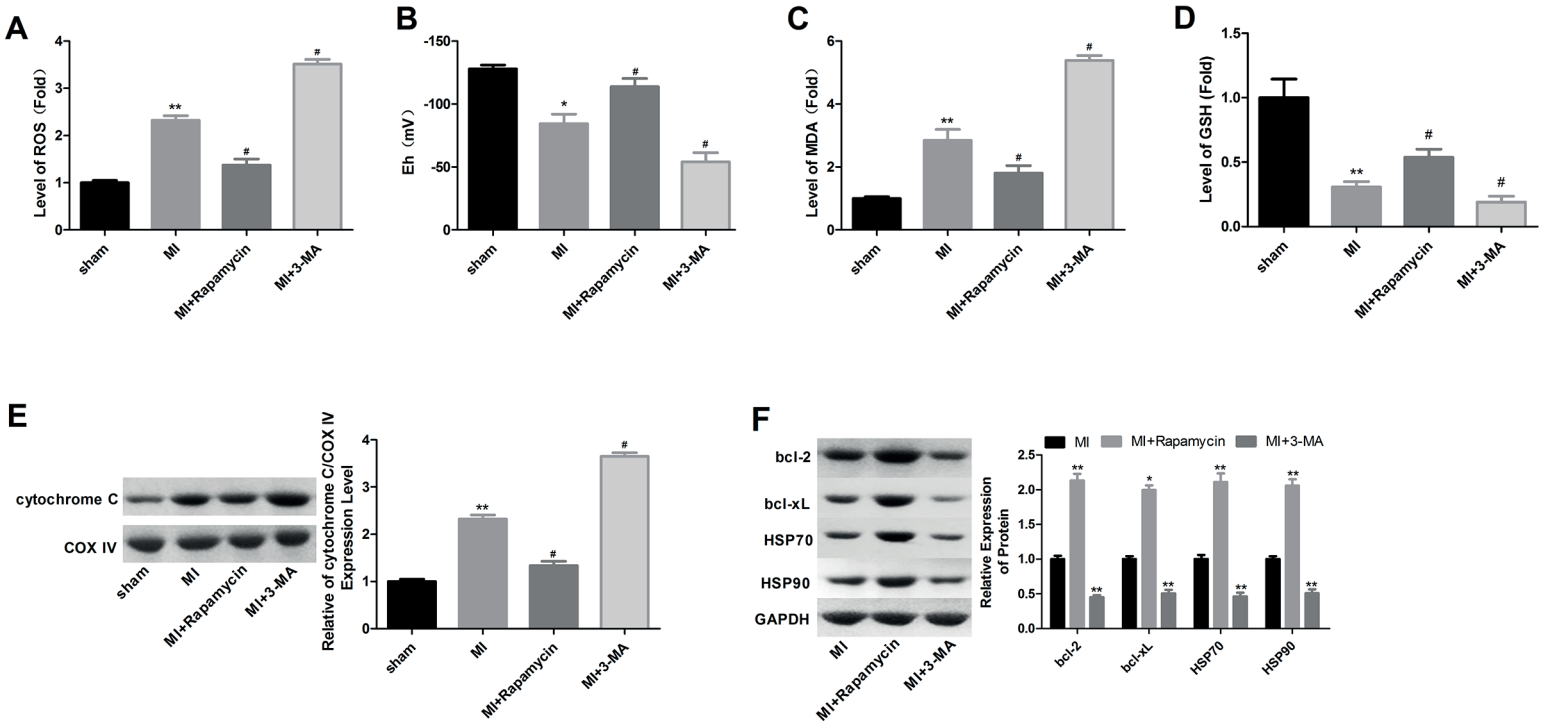

Figure 2. Evaluation of the effects of autophagy on ROS. **A,** The ROS levels in mice heart tissues were detected by using an active oxygen detection kit (No. CA1410-100T; Solarbio, Beijing, China). **B,** Blood Eh levels in mice with different treatments were calculate by Nernst equation for pH 7.4: GSH/GSSG, Eh (mV)=-264+30 log ([GSSG]/[GSH]2). **C,** Blood levels of MDA in mice from different groups were detected by ELISA assay. **D,** Blood levels of GSH in mice from different groups were detected by using a commercial kit. **E,** The expression of cytochrome C in the heart tissues were detected by WB assay, COX IV was used as an internal reference. **F,** The expression of bcl-2, bcl-xL, HSP70 and HSP90 in mice heart tissues were tested by WB assay, with GAPDH as an internal reference. ($^{\#}p<0.05$, MI group compared with sham group; $^{*}p<0.05$, $^{**}p<0.01$, MI+Rapamycin/MI+3-MA group compared with MI group).

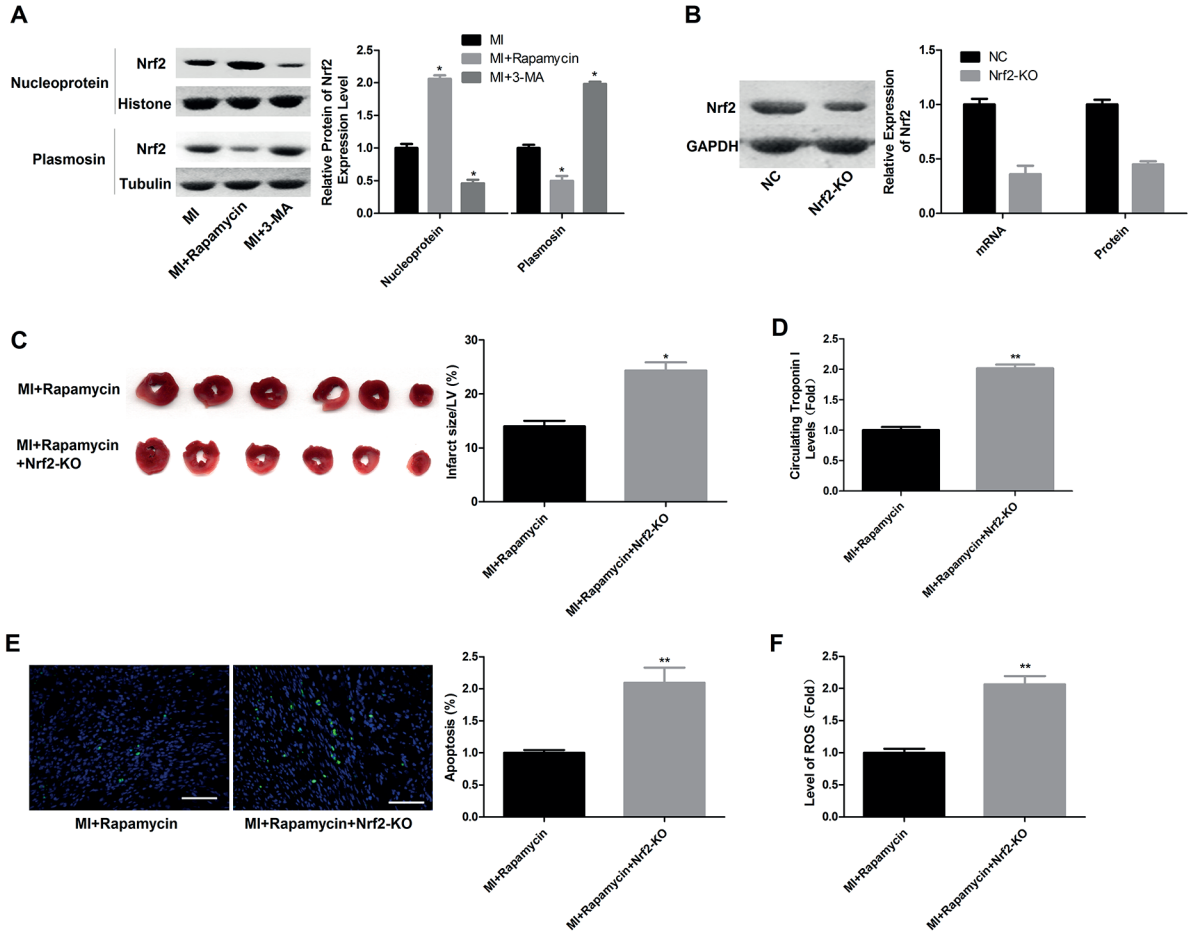

Figure 3. Autophagy-induced MI alleviation depended on the activation of Nrf2 signaling. **A,** The expression of Nrf2 protein in nuclear and cytoplasm of the heart tissues from mice with MI surgery, MI+rapamycin and MI+3-MA treatments were detected by WB assay. The expression levels of Histone and Tublin were used as internal references for nuclear protein and cytoplasm protein, respectively. **B,** The expression levels of Nrf2 in the heart tissues from the Nrf2-KO mice or normal mice were detected by WB. **C,** TTC staining was used to assess the myocardial infarct size of mice from different groups. **D,** Blood levels of Troponin I in mice from different groups were detected by ELISA assay. **E,** Cell apoptosis in heart tissues was assessed by TUNEL assay (magnification: 100x). **F,** The ROS levels in mice heart tissues were detected by using an active oxygen detection kit (No. CA1410-100T; Solarbio, Beijing, China). ($^{*}p<0.05$, $^{**}p<0.01$).

MI mice with rapamucin or 3-MA administration. Rapamycin treatment significantly decreased the ROS level in heart tissues (Figure 2A), the blood levels of Eh (Figure 2B), MDA (Figure 2C) and GSH (Figure 2D) and increased the expression of cytochrome C in the chondriosome (Figure 2E) induced by LAD ligation. However, these indicators were all enhanced when the MI mice were given 3-MA (Figure 2A-2E). Furthermore, rapamycin treatment promoted the expressions of anti-apoptosis proteins bcl-2 and bcl-xL, and the antioxidant proteins HSP70 and HSP90 (Figure 2F). These discoveries suggest that autophagy can significantly inhibit the oxidative stress in MI mice.

Autophagy Assuages MI by Activating Nrf2 Signaling In Vivo

Next, we explored whether Nrf2 signaling was involved in autophagy-mediated MI mitigation. Compared with the MI group, rapamycin treatment significantly increased Nrf2 expression in nuclear while decreased its cytoplasm content, and 3-MA treatment resulted in the opposite results (Figure 3A), suggesting that Nrf2 signaling might play a role in autophagy-induced MI improvement. To this end, we recruited the transgenic mice with Nrf2-KO to study the effects of Nrf2 in autophagy-mediated alleviation of MI. The expression of Nrf2 in

Nrf2-KO mice was significantly decreased as compared with the control group (Figure 3B). In addition, compared with MI+rapamycin group, the infarct size (Figure 3C), blood Troponin I level (Figure 3D) and tissue cell apoptosis (Figure 3E), as well as ROS level (Figure 3F) were all increased in Nrf2-KO mice underwent MI surgery and rapamycin treatment. These results suggest that autophagy assuages MI by activating Nrf2 signaling.

Aspirin Treatment Reduces Infarct Size and Cell Apoptosis Induced by MI Surgery by Activating Autophagy

As aspirin had the potential to reduce the incidence of cardiovascular disease[21], we explored whether autophagy could be activated by aspirin in MI mice. Similarly to rapamycin treatment, aspirin administration significantly reduced the infarct size in MI mice (Figure 4A) and reduced blood troponin I content (Figure 4B), as well as inhibited cell apoptosis in mouse heart tissues (Figure 4C). Moreover, the WB results showed that the expression level of LC3II/LC3I was increased when MI mice were given aspirin (Figure 4D). Together, these above results indicate that the activation of autophagy is strongly implicated in aspirin-mediated MI improvement.

Discussion

Cardiac myocytes apoptosis is one of the main etiological agents for MI, which is closely associated with the infarct size and the poor prognosis of patients with MI[22,23]. In addition, autophagy may constitute an alternative pathophysiological response to ischemia during heart ischemia/reperfusion (I/R) injury[24]. Zhu et al[25] showed that the overexpression of Pim1 could inhibited cardiomyocytes apoptosis under hypoxia and oxidative stress *via* activating cell autophagy, suggesting that autophagy plays a protective role in preventing cardiomyocytes against apoptosis. Zhang et al[26] found that chloroquine intervention significantly inhibited cell apoptosis in infarction region induced by LAD ligation by activating autophagy. The heart may uniquely rely on stress-induced autophagy activation to make sure the usability of energy substrates and benefit to the success process of cellular remodeling when exposed to ischemia, which may indeed protect the hearts[27]. Although a number of studies[28-31] demonstrated that autophagy enhancement contributed to the alleviation of MI, the underlying mechanisms remain not entirely clear. Here, we demonstrated, for the first time, that autophagy could assuage myocardial infarction through Nrf2 signaling activation-mediated ROS clear.

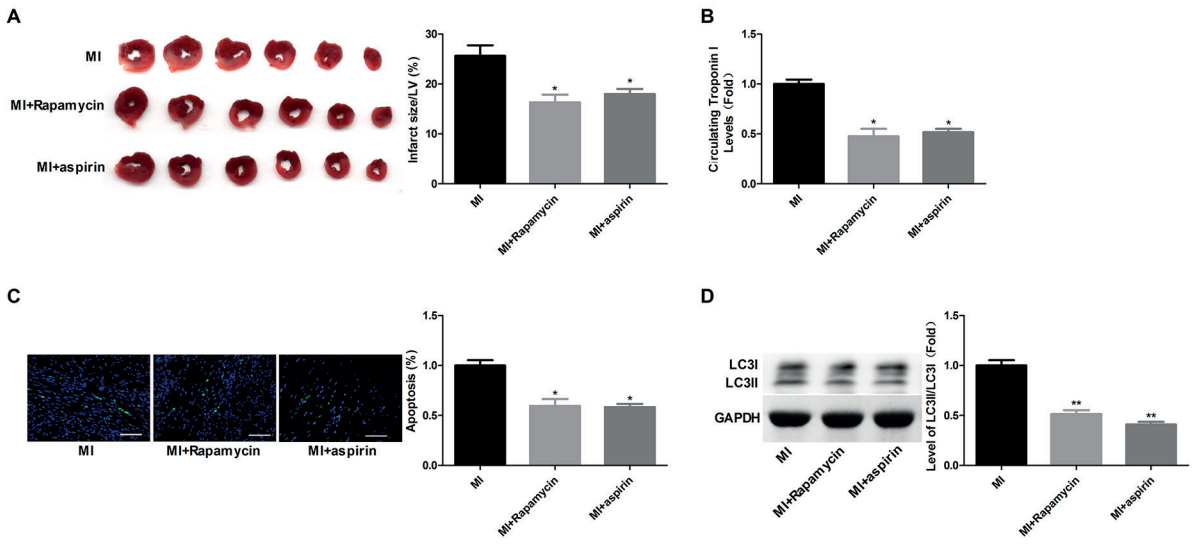

Figure 4. Autophagy activation was strongly implicated in aspirin-mediated MI improvement. **A,** TTC staining was used to assess the myocardial infarct size of mice from different groups. **B,** Blood levels of Troponin I in mice from different groups were detected by ELISA assay. **C,** Cell apoptosis in heart tissues was assessed by TUNEL assay (magnification: 100x). **D,** The expression levels of LC3II/I in heart tissues were measured by WB technology. ($^{*}p<0.05$, $^{**}p<0.01$, compared with MI group).

In the current study, we gave MI mice rapamycin and 3-MA to induce and repress autophagy, respectively. The results showed that autophagy enhancement significantly reduced the infarct size and inhibited cell apoptosis of the MI mice, and reduced the excessive oxidative stress with reduced ROS, MDA, Eh and cytochrome C, and increased GSH, HSP70 and HSP90, antioxidant markers[32]. These findings indicated that ROS reduction was involved in the cardioprotective role of autophagy played in MI. It is reported[33] that oxidative stress with enhanced levels of ROS and reactive nitrogen species (RNS) is the main intracellular signal transducer of the sustaining autophagy. In the current study, we demonstrated that autophagy activation could inhibit the excessive production of ROS, hence, we hypothesized that there is a negative loop between autophagy and ROS in heart. On one hand, excessive ROS can serve as a stimulus to activate autophagy; on the other hand, the activation of autophagy can repress the generation of ROS, and then, protect myocardial cells from apoptosis.

In mechanism, we found that the activation of autophagy with rapamycin in MI mice significantly increased Nrf2 expression in nuclear while decreased its expression in cytoplasm, indicating that autophagy could promote the activation of Nrf2 signaling. Under oxidative stress condition, Nrf2 dissociates with Kelch-like ECH-binding protein 1 (Keap1) and translocates into nucleus, and then, promotes the transcription of anti-oxidation and detoxification enzyme genes, such as glutathione S-transferase (GST), heme oxygenase-1 (HO-1), and NAD(P)H: quinone oxidoreductase 1 (NQO1)[34], thereafter clearing the excessive produced ROS. To explore whether Nrf2 was involved in autophagy-mediated MI improvement, we built MI in Nrf2-KO mice. The results showed that the effects of rapamycin administration on the reduction of infarct size, cell apoptosis inhibition and ROS clear were abolished in Nrf2-KO mice underwent LAD ligation, indicating that autophagy assuaged MI *via* Nrf2 signaling activation-mediated ROS clear.

As aspirin (acetylsalicylic acid) is widely used for preventing and treating cardiovascular and cerebrovascular diseases[35], we also explored whether autophagy activation was involved in the cardioprotective effect of aspirin. The results confirmed that aspirin administration significantly reduced infarct size, cell apoptosis and troponin I in mice with MI surgery, as well as increased the expression level of LC3II/LC3I. Our results were consistent with those of Huang et al[36] who demonstrated that aspirin could activate autophagy in hepatocellular carcinoma cells. Our results suggest that active autophagy was closely related to aspirin-mediated cardioprotection.

Conclusions

Together, the present study indicated that the cardioprotective effects of autophagy were dependent on Nrf2 signaling activation-induced ROS reduction. Furthermore, this study clarified that autophagy activation was involved in aspirin-mediated infarct size and cell apoptosis reduction. In a word, the present study demonstrated that autophagy enhancement contributed to the improvement of MI through Nrf2 signaling activation-mediated ROS clear, which might provide a new method for MI clinical treatment, such as aspirin.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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