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Tetramethylprazine attenuates myocardial ischemia/reperfusion injury through modulation of autophagy

Zhi Zuo, Peng-fei Zuo, Zu-long Sheng, Xin Wang, Jian-dong Ding**, Gen-shan Ma*

Department of Cardiology, Zhongda Hospital, Medical School of Southeast University, Nanjing City, JiangSu province, China

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ABSTRACT

The current study aimed to investigate the effects of tetramethylprazine (TMP) on myocardial ischemia/reperfusion (MI/R) injury and its underlying mechanisms. MI/R rat model and hypoxia/reoxygenation (H/R) cardiomyocytes model were established. CK level and LDH activity were detected to evaluate MI/R and H/R injury. Cell viability was determined by cell counting kit-8 (CCK-8) assay. Cell apoptosis were identified by flow cytometry and autophagy were detected by western blot. Treatment with TMP significantly reduced CK level and LDH activity and decreased myocardial infarct size in MI/R rats. TMP reduced autophagy dysfunction induced by MI/R. Moreover, TMP treatment decreased H/R-induced injury and attenuated autophagy dysfunction in cardiomyocytes. Inhibiting autophagic flux with chloroquine (CQ) decreased the cardioprotection exerted by TMP *in vivo* and *in vitro*. Additionally, the effects of TMP on the modulation of autophagy were inhibited by LY294002 (a PI3K inhibitor) in H/R cardiomyocytes. Our findings suggested TMP exerted cardioprotection against MI/R injury by decreasing Beclin-1 associated autophagy dysfunction through PI3K pathway.

1. Introduction

Myocardial ischemia is a common pathological condition that is characterized by the decrease of oxygen supply to the heart and abnormal energy metabolism of the myocardium, which result in significant morbidity and mortality [1]. Establishing the reperfusion early for the ischemic myocardium is vital for the rescue of ischemic myocardium. However, reperfusion triggers a complex cascade of events involved in the development of multiple diseases such as irreversible shock, myocardial infarction, and heart failure. Therefore, attenuation of myocardial ischemia/reperfusion (MI/R) injury is vital for the prevention and treatment of ischemic heart diseases.

Tetramethylprazine (TMP) is an alkaloid monomer isolated and purified from *Ligusticum wallichii* (Chuanxiong), a traditional herb in China. It has been well documented that TMP treated lots of diseases such as pulmonary hypertension [2,3], cardiovascular and cerebrovascular diseases [4–6], chronic renal failure [7] and cirrhosis [8,9]. Recently, studies have shown that TMP is protective against MI/R injury through multiple pathways [10]. However, the molecular mechanisms underlying TMP-mediated protective effects against MI/R injury has not been fully clarified.

Autophagy is a conserved lysosome-associated degradation process which is vital for cell survival [11]. Previous studies have shown that autophagy dysfunction is commonly appears in the myocardial ischemia, heart failure, and aging [12,13]. During myocardial ischemia, autophagy is induced by hypoxia or mitochondrial injury through AMP-induced protein kinase (AMPK) pathway, whereas the mechanism of autophagy during reperfusion is related to the increased expression of Beclin-1, a key protein of autophagy [14–16]. Therefore, regulating autophagy is an important approach to the prevention and treatment of MI/R injury [16]. Recently, there is evidence that TMP promotes autophagy in mesenchymal stem cells and improves bone mass in glucocorticoid-induced osteoporosis rats [17]. Therefore, whether autophagy participates in TMP-mediated cardioprotective effects against MI/R injury requires further investigation.

The present study aimed to investigate the upregulation of autophagy flux in MI/R injury treated by TMP and the underlying mechanisms involved.

2. Methods and methods

2.1. Reagents

TMP (purit ≥ 98%, #W323705) were purchased from Sigma-Aldrich Company (St. Louis, MO, USA). Creatine kinase (CK), lactate dehydrogenase (LDH) assay kits were obtained from Jiancheng Reagent

* Corresponding author. Department of Cardiology, Zhongda Hospital, Medical School of Southeast University, No.87 Dingjiaqiao, Nanjing, 210009, China.
** Corresponding author. Department of Cardiology, Zhongda Hospital, Medical School of Southeast University, No.87 Dingjiaqiao, Nanjing, 210009, China.
E-mail addresses: dingjiandong@163.com (J.-d. Ding), genshan_ma@163.com (G.-s. Ma).

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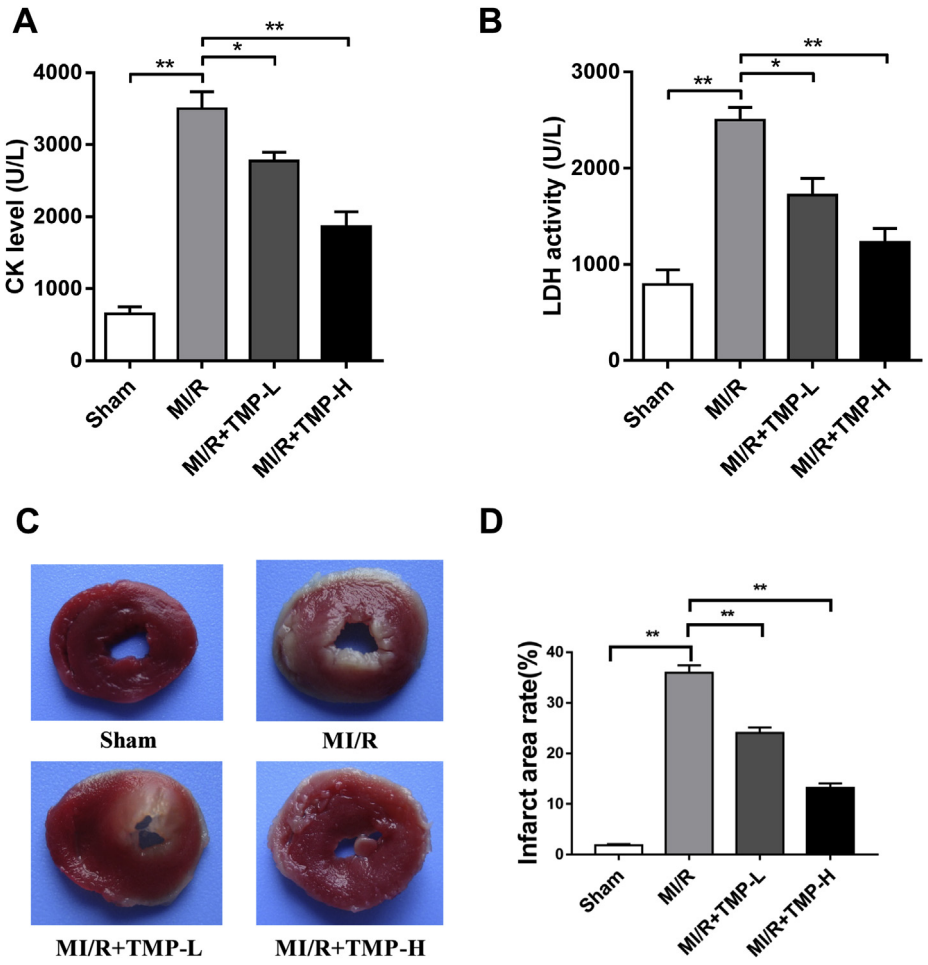

Fig. 1. TMP protected the heart against ischemia/reperfusion injury. (A) Effect of TMP on serum CK level in rats exposed to MI/R. (B) Effect of TMP on LDH activity in rats exposed to MI/R. (C) Myocardial TTC staining images. (D) Analysis of infarct size. TMP-L:TMP at a low dose (10 mg/kg), TMP-H:TMP at a high dose (50 mg/kg). Data are represented as the mean ± SEM, n = 8 per group. $^{*}P < 0.05$ and $^{**}P < 0.01$.

Company (Nanjing, China). LY294002 (#9901), chloroquine (CQ) (#14774), primary antibodies directed against Beclin-1 (1:1000, #3738), LC3 (1:1000 , #4108), p62 (1:1000, #5114) and β-actin (1:1000, #3700) were purchased from Cell Signaling Technology (Beverly, MA, USA). Secondary antibodies such as horseradish peroxidase (HRP)–conjugated goat anti-mouse IgG and goat anti-rabbit IgG antibodies were obtained from GeneTex (San Antonio, TX, USA). The Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Laboratories (Kumamoto, Japan). Sodium pentobarbital were purchased from Guoan Institute of Biotechnology (Xi'an, China).

2.2. Animals

Six-week old male Sprague-Dawley (SD) rats (250 ± 20 g) were obtained from Shanghai Silaike Laboratory Animal Co., Ltd. (Shanghai, China).All animal protocols performed in this study were approved by the Institutional Animal Care and Use Committee of Southeast University (Nanjing, China).

2.3. Myocardial ischemia/reperfusion (MI/R) experimental protocols and animal groups

MI/R was induced as described in previous studies [18–20]. SD rats were anesthetized with pentobarbital sodium (60 mg/kg, i.p.) and ventilated on a Harvard rodent respirator via a tracheostomy. The chest was opened through a left intercostals thoracotomy. The heart was exteriorized by a left thoracic incision followed by a slipknot (6-0 silk) around the left anterior descending coronary artery (LAD). Regional ischemia was confirmed by significant ECG changes including widening of the QRS complex and elevation of ST segment. The slipknot was released after 30 min of ischemia after which the animal received 120 min of reperfusion.

Animals were randomly divided into the following groups: (1) sham group: silk was drilled underneath the LAD but the LAD was not ligated; (2) MI/R group: LAD was ligated for 30 min and then allowed 120 min of reperfusion; (3) MI/R + TMP-L group: TMP at a dose of 10 mg/kg was intraperitoneally administered 10 min before ischemia; (4) MI/R + TMP-H group: TMP at a dose of 50 mg/kg was intraperitoneally administered 10 min before ischemia.

2.4. Primary culture of neonatal rat ventricular cardiomyocytes

Neonatal rat ventricular cardiomyocytes were isolated from 1-2-day-old SD rats, as described previously [21,22]. SD rat pups were euthanized by cervical dislocation. Hearts were excised and digested in a mixture of trypsin (0.25%) and collagenase I (10 mg in 9 mL PBS) (1:1 mixed), and cardiomyocytes were collected after 2 h differential adhension. The isolated cardiomyocytes were seeded in 6-well plate at 5×10^5 cells/cm^2 and cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% Fetal Bovine Serum and 0.1 mM 5-bromo-2′-deoxyuridine (BrdU) for 72 h before other treatments.

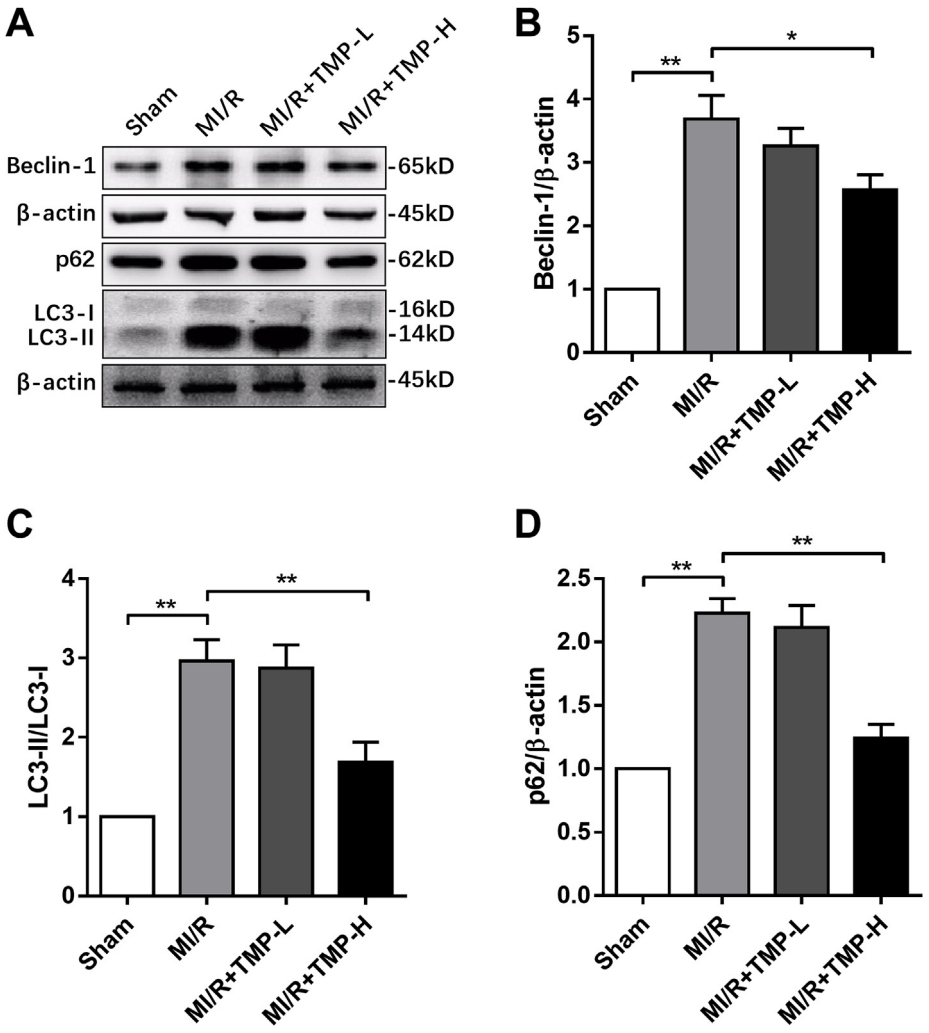

Fig. 2. TMP alleviated cardiac autophagy dysfunction induced by ischemia/reperfusion. (A) Beclin-1, LC3 and p62 expression levels were analyzed by Western blotting. (B) Beclin-1 expression was quantified by densitometry. (C) LC3-I/II expressions were quantified by densitometry. (D) p62 expression was quantified by densitometry. TMP-L:TMP at a low dose (10 mg/kg), TMP-H:TMP at a high dose (50 mg/kg). Data are represented as the mean ± SEM, n = 8 per group. $^{**}P < 0.01$.

2.5. Hypoxia/reoxygenation (H/R) model and cell groups

To mimic MI/R injury *in vitro*, the cells were cultured under hypoxia/reoxygenation (H/R) conditions. In Brief, cardiomyocytes cultured in glucose-free DMEM were placed in a hypoxic chamber (95% N_2, 5% CO_2, 37 °C) for 2 h, followed by reoxygenation in a standard incubator (5% CO_2, 37 °C) in normal medium for 3 h. Cells in the control group were placed in a standard incubator (5% CO_2, 37 °C) in normal medium.

Cells were randomly divided into the following groups: (1) control group (5%CO_2, 37 °C); (2) H/R group (95% N_2, 5% CO_2, 37 °C); (3) H/R + TMP-L group: TMP (10 μM) was added into cells at the onset of reoxygenation; (4) H/R + TMP-H group: TMP (50 μM) was added into cells at the onset of reoxygenation; (5) H/R + TMP + LY group: LY294002,a specific PI3K inhibitor, was added into cells at the onset of reoxygenation, and 10 min after the administration of LY294002 (10 μM), TMP (50 μM) was added.

2.6. Flow cytometry

After H/R and drug administration, cells were harvested. Fluorescein isothiocyanate–annexin V and propidium iodide were added to the cells and incubated in the dark for 15 min. The fluorescence signals were measured using a flow cytometer (Beckman Coulter, Miami, USA). Data were analyzed using the Cell Quest software (BD Biosciences, CA, USA).

2.7. Cell viability measurement

Cell viability was detected by counting the number of cells with CCK-8 according to the manufacture's protocol. Cells were seeded in 96-well plates before H/R. After H/R and drug administration, CCK-8 was added to the wells (10 μl/well). The absorbance at 450 nm of each well was measured to determine the number of viable cells.

2.8. Western blot

Total proteins were extracted from rat hearts or cardiomyocytes and then lysed with lysis buffer according to the manufacturer's guidelines. Protein concentration was determined using a BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Equal quantities of proteins (30–40 μg/lane) were subjected to SDS–PAGE and electrophoretically transferred to PVDF membranes (Millipore, MA , USA). Membranes were blocked with 10% non-fat dry milk for 1 h at room temperature and incubated overnight with appropriate primary antibodies at 4 °C, followed by incubation with the corresponding secondary antibodies at room

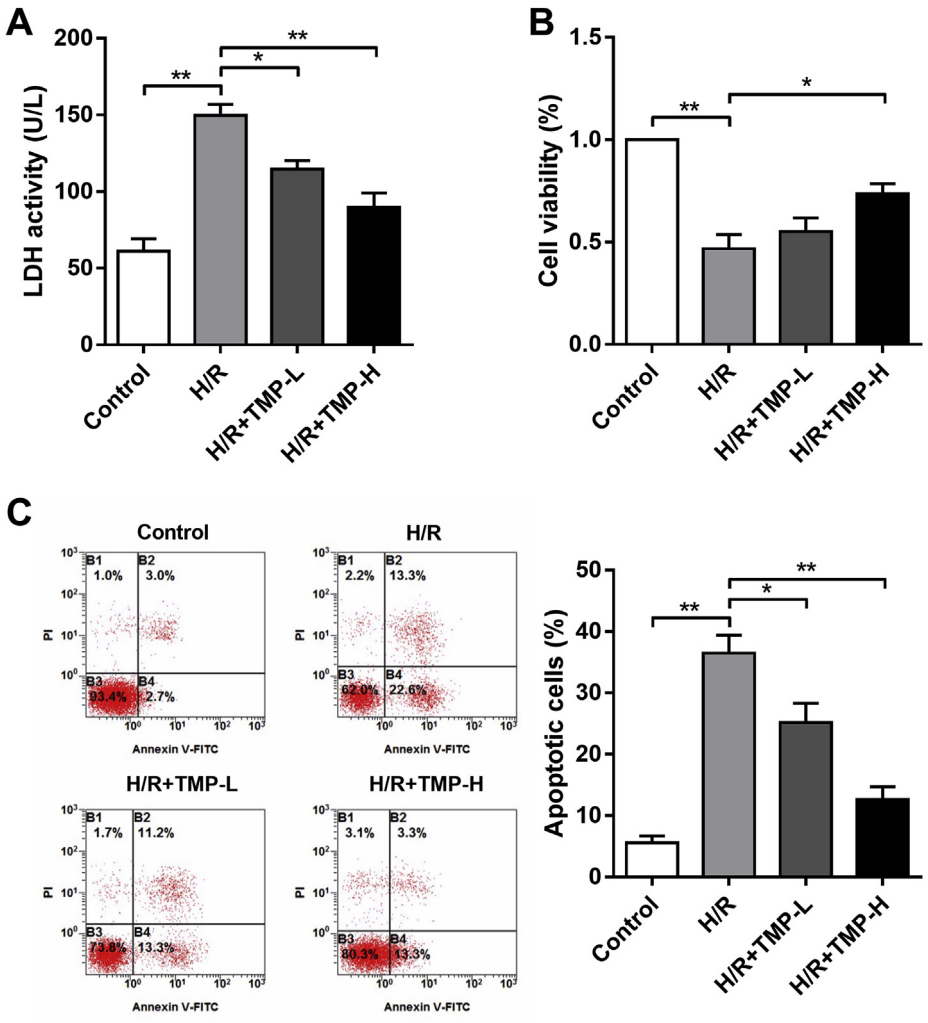

Fig. 3. TMP protected cardiomyocytes against H/R injury. (A) Effects of TMP on LDH activity in cardiomyocytes subjected to H/R. (B) Effects of TMP on cell viability of cardiomyocytes subjected to H/R. (C) Effects of anti-apoptotic effects with TMP in cardiomyocytes subjected to H/R by flow cytometric analysis. TMP-L:TMP at a low dose (10 μM), TMP-H:TMP at a high dose (50 μM). Data are represented as the mean ± SEM, n = 8 per group. *$P < 0.05$ and **$P < 0.01$.

temperature for 1 h. Blot imaging was conducted by an enhanced chemiluminescence detection kit (Millipore, MA, USA).

2.9. Measurement of CK level and LDH activity

After MI/R and H/R, serum and culture supernatant were collected. After the above protocol, the CK level and LDH activity were measured by ELISA kits, respectively. Determination were carried on strictly according to the manufacturer's instructions.

2.10. Detection of myocardial infarct size

The heart was harvested after reperfusion and frozen at −70 °C. Slices were cut along the long axis of the heart. Sections were incubated with 1% 2,3,5-triphenyltetrazolium chloride (TTC) solution for 15 min at 37 °C and photographed. TTC is a dye that stains viable myocardium red due to a formazan reaction with NADH and NADPH, which are washed out from irreversibly injured myocardium, whereas necrotic tissue remains unstained and thus appears white [42].

2.11. Statistical analysis

All values are presented as mean ± SEM. Statistical significance was determined by ANOVA followed by Turkey's correction where appropriate. A probability value of $P < 0.05$ was statistically significant. All data were performed using GraphPad Prism sofware version 5.0 (GraphPad Sofware, Inc., San Diego, CA, USA).

3. Results

3.1. TMP protected against MI/R injury

To investigate the cardioprotective effects of TMP on MI/R injury, we measured the CK level and LDH activity, which represent markers of myocardial injury in serum. As shown in Fig. 1A and B, the CK level and LDH activity in the MI/R group were significantly elevated compared with the sham group. Treatment with TMP at a low (10 mg/kg) and high (50 mg/kg) dose reduced serum CK level and LDH activity in MI/R rats (Fig. 1A and B). The myocardial infarct size were measured by TTC staining (Fig. 1C and D), Treatment with TMP at a low (10 mg/kg) and high (50 mg/kg) dose reduced myocardial infarct size. These results suggested that TMP protected against MI/R injury.

3.2. TMP suppressed MI/R-induced autophagy dysfunction

MI/R injury induces cardiomyocyte death through blocking autophagy flux [23,24]. To identify the effects of TMP on autophagy in MI/R cardiomyocyte, we used immunoblotting to measure the expression of

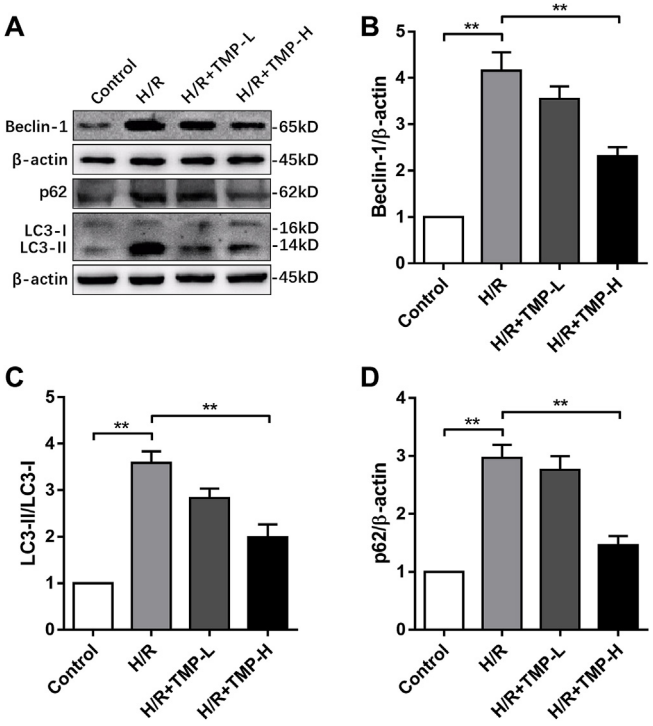

Fig. 4. TMP improved H/R-induced autophagy dysfunction in cardiomyocytes. (A) Beclin-1, LC3 and p62 expression levels were analyzed by Western blotting. (B) Beclin-1 expression was quantified by densitometry. (C) LC3 expression were quantified by densitometry. (D) p62 expression was quantified by densitometry. TMP-L:TMP at a low dose (10 μM), TMP-H:TMP at a high dose (50 μM). Data are represented as the mean ± SEM, n = 8 per group. $^{**}P < 0.01$.

autophagy markers such as Beclin-1, LC3, and p62, in MI/R cardiomyocyte obtained from each experimental group. As shown in Fig. 2, compared with the sham group, the expression of Beclin-1 and p62 and ratio of LC3-II/LC3-I were significantly increased in the MI/R group, suggesting that MI/R contributed to the impairment of autophagosome clearance. Treatment with TMP at 10 mg/kg did not change the expression of Beclin-1 and p62 and LC3-II/LC3-I ratio in MI/R rats. But administration of TMP (50 mg/kg) significantly reduced the ratio of LC3-II/LC3-I and decreased the expression of Beclin-1 and p62. These data indicated that TMP improved autophagy dysfunction in MI/R.

3.3. TMP protected cardiomyocytes against H/R injury

To demonstrate the effects of TMP on cell survival subjected to H/R, primary cultures of neonatal rat ventricular cardiomyocytes were collected. As shown in Fig. 3A, LDH activity in H/R group was increased compared with the control group. Treatment with TMP (10 and 50 μM) remarkably reversed H/R-induced increase of LDH activity in a dose-dependent manner. Fig. 3B illustrates that, compared with the control group, the cell viability was decreased in H/R group. Treatment with TMP (50 μM) significantly increased the cell viability under H/R conditions. Next, we further determined cell apoptosis by flow cytometry (Fig. 3C). Flow cytometry experiments indicated that TMP treatment induced a dose-dependent decrease in cell apoptosis when compared with the control group. Our results showed that TMP significantly attenuated cellular apoptosis and elevated cell survival in cells subjected to H/R.

3.4. TMP attenuated autophagy dysfunction in cardiomyocytes exposed to H/R

To confirm the effects of TMP on autophagy in cardiomyocytes subjected to H/R, cells were treated with TMP under standard or H/R

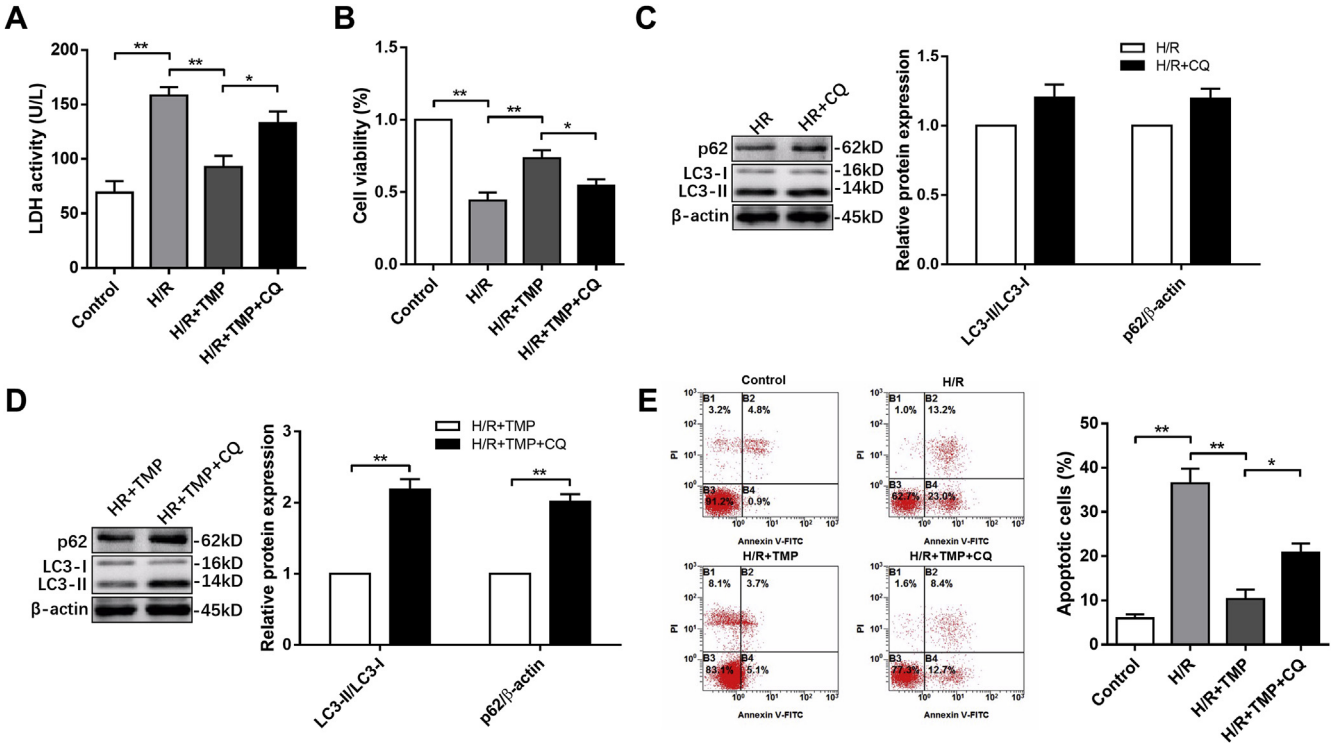

Fig. 5. Inhibiting autophagic flux with CQ suppressed TMP-exerted cardioprotection *in vivo*. (A) CK level in rats subjected to MI/R with TMP (50 mg/kg) treatment, in the absence or presence of CQ (10 mg/kg). (B) LDH activity in rats subjected to MI/R with TMP (50 mg/kg) treatment, in the absence or presence of CQ (10 mg/kg). (C) Representative blots and statistical data showing the expressions of LC3 and p62 in MI/R rats with or without CQ (10 mg/kg). (F) Representative blots and statistical data showing the expressions of LC3 and p62 in MI/R rats with TMP (50 mg/kg) treatment, in the absence or presence of CQ (10 mg/kg). Data are represented as the mean ± SEM, n = 8 per group. *P < 0.05 and **P < 0.01.

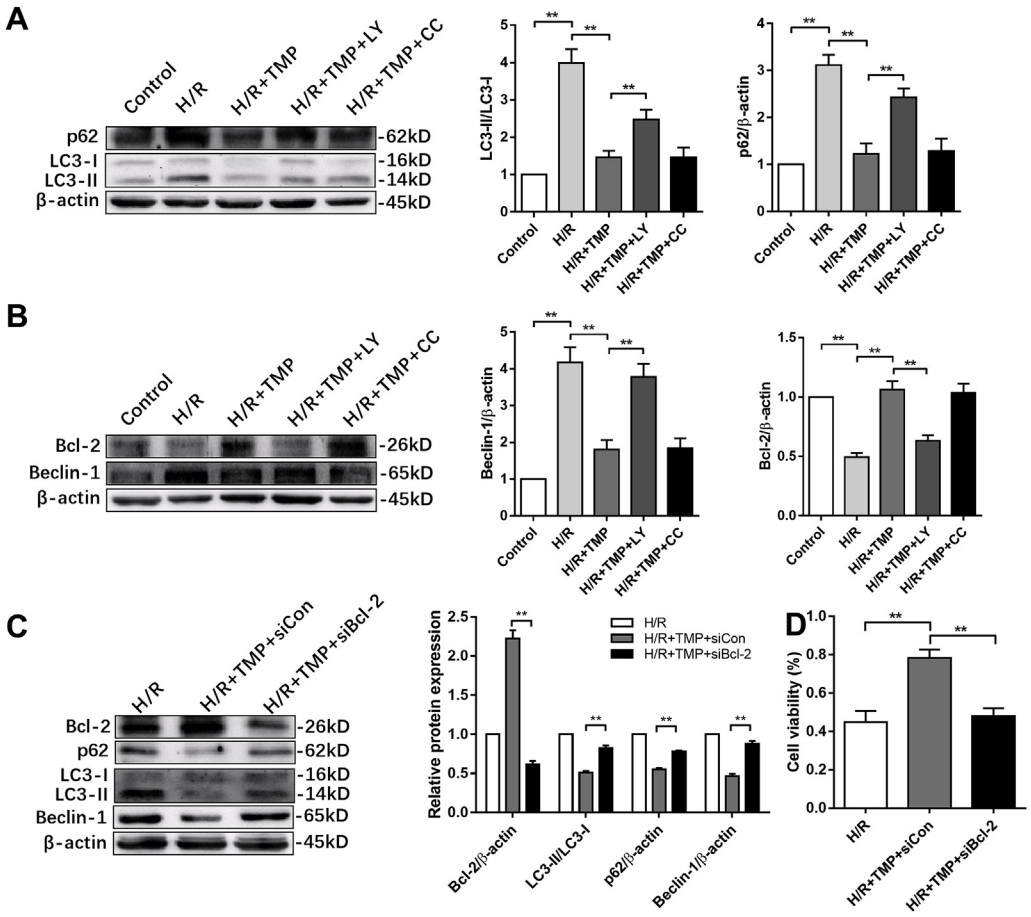

Fig. 6. Inhibiting autophagic flux with CQ suppressed TMP-exerted cardioprotection *in vitro*. (A) LDH activity in cardiomyocytes subjected to H/R with TMP (50 μM), in the absence or presence of CQ (10 μM). (B) Cell viability of cardiomyocytes subjected to H/R with TMP (50 μM), in the absence or presence of CQ (10 μM). (C) Representative blots and statistical data showing the expressions of LC3 and p62 in cultured cardiomyocytes subjected to H/R with or without CQ (10 μM). (F) Representative blots and statistical data showing the expressions of LC3 and p62 in cultured cardiomyocytes subjected to H/R with TMP (50 μM), in the absence or presence of CQ (10 μM). (E) Flow cytometric analysis of apoptosis in cardiomyocytes subjected to H/R with TMP (50 μM), in the absence or presence of CQ (10 μM). Data are represented as the mean ± SEM, n = 8 per group. $^{*}P < 0.05$ and $^{**}P < 0.01$.

conditions. The expression of the autophagy markers Beclin-1, LC3, and p62 was analyzed by immunoblotting. As shown in Fig. 4, Beclin-1 and p62 expression and the LC3B-II/-I ratio were increased in the H/R group, but TMP at 50 μM significantly counteracted these elevations. Our data indicated that TMP prevented excessive autophagy to a normal level in cardiomyocytes under H/R conditions.

3.5. Inhibiting autophagic flux with chloroquine decreased TMP-exerted cardioprotection

Chloroquine (CQ), an autophagic inhibitor, prevent autophagosome-lysosome fusion, thereby interfering with the processing of autophagosomes and inhibiting autophagic flux. To clarify the role of autophagic flux in TMP-induced cardioprotection, we used CQ to block autophagic flux both *in vivo* and *in vitro*. As shown in Fig. 5A and B, treatment with CQ profoundly inhibited the CK level and LDH activity in MI/R rats, indicating that CQ suppressed the cardioprotective effects of TMP. In addition, preventing autophagosome-lysosome fusion with CQ did not increase LC3-II/LC3-I ratio and p62 expression after MI/R (Fig. 5C), indicating that MI/R induced impairment of autophagosome clearance. However, CQ elevated the LC3-II/LC3-I ratio and p62 expression in TMP-treated MI/R rats (Fig. 3D), indicating preserved "intact" autophagic flux by TMP.

We further found that the reduction of LDH activity and the enhancement of cell viability induced by TMP (50 μM) in cardiomyocytes subjected to H/R were opposed by co-administration with CQ (Fig. 6A and B). Our experiments also showed that CQ counteracted the suppression of cellular apoptosis induced by TMP (Fig. 6E). Consistently with animal experiments, CQ did not influence the LC3-II/LC3-I ratio and p62 expression after H/R in vehicle-treated cardiomyocytes (Fig. 6C), but significantly increased the LC3-II/LC3-I ratio and p62 expression in TMP-treated cardiomyocytes (Fig. 6D). Our data demonstrated that TMP-exerted cardioprotection were partly contributed to the improvement of autophagic flux.

3.6. PI3K/Bcl-2 cascade was involved in the improvement of autophagy dysfunction by TMP

To investigate whether the PI3K pathway was involved in the TMP-induced modulation of autophagy, LY294002 (a PI3K inhibitor) and Compound C (CC; an AMPK inhibitor) were administered together with TMP (50 μM) to cardiomyocytes subjected to H/R. As shown in Fig. 7A, TMP plus LY294002 respectively decreased the ratio of LC3-II/LC3-I, p62 and Beclin-1 expressions compared with TMP only. CC did not exert these effects induced by TMP. These data indicated that PI3K was involved in the modulation of autophagy in cardiomyocytes afforded by TMP.

Beclin-1 mediated autophagy activation is associated with Bcl-2 down-regulation during reperfusion [25,26]. We further found that TMP inhibited the decrease of Bcl-2 expression in cardiomyocytes in response to H/R (Fig. 7B). In addition, LY294002 attenuated the elevation of Bcl-2 produced by TMP, while CC had no effect. To demonstrate that Bcl-2 was involved in the modulation of autophagy induced by TMP, we used siRNA (siBcl-2) to knock down Bcl-2 in cardiomyocytes. As shown in Fig. 7C, siRNA treatment significantly inhibited Bcl-2 expression. Knocking down Bcl-2 significantly reduced the modulation of autophagy conferred by TMP, as shown by the differences in LC3-II/LC3-I ratio, and p62 and Beclin-1 expressions in cultured cardiomyocytes exposed to H/R in absence and presence of siBcl-2. Moreover, TMP increased cell viability in cultured cardiomyocytes exposed to H/R, and the effect was inhibited by siRNA knocking down (Fig. 7D). These results demonstrated that PI3K/Bcl-2 cascade was

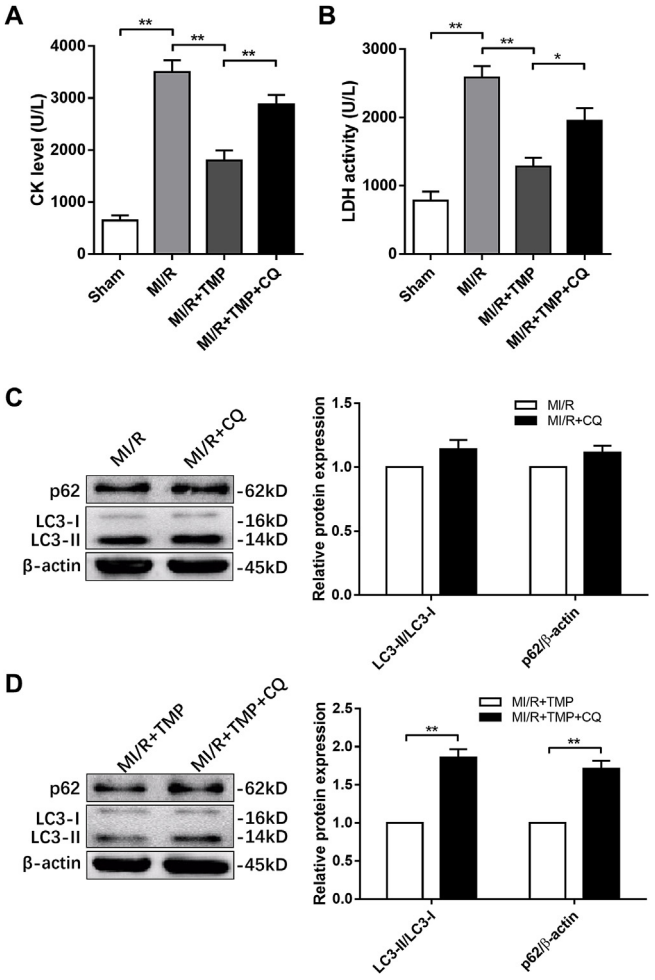

Fig. 7. TMP improved autophagy dysfunction via Bcl-2 induction in cardiomyocytes subjected to H/R. (A) LC3 and p62 expression levels in cardiomyocytes subjected to H/R with TMP (50 μM) treatment were analyzed by Western blotting in the absence or presence of LY294002 (10 μM) and Compound C (10 μM). (B) Representative blots and statistical data showing the expressions of Bcl-2 and Beclin-1 in cultured cardiomyocytes treated as in A. (C) Representative blots and statistical data showing the expressions of Bcl-2, LC3, p62 and Beclin-1 in cultured cardiomyocytes transfected with siRNA of Bcl-2 (siBcl-2) or siRNA negative control (siCon), followed by H/R with TMP (50 μM) treatment. (D) Cell viability of cardiomyocytes treated as in C subjected to H/R. LY: LY294002, CC: Compound C. Data are represented as the mean ± SEM, n = 8 per group. $^{*}P < 0.05$ and $^{**}P < 0.01$.

possibly implicated in the Beclin-1-assosicated modulation of autophagy induced by TMP in cardiomyocytes exposed to H/R.

4. Discussion

In the present study, we established models of animal and cell to investigate the protective effects of TMP against MI/R injury. We demonstrated that TMP reduced serum CK level and LDH activity, decreased MI/R-induced autophagy dysfunction. At the cellular level, TMP attenuated LDH activity and cardiomyocyte apoptosis , improved cell viability and myocardial autophagy dysfunction subjected to H/R. Moreover, autophagy inhibition with chloroquine resulted in the loss of TMP-induced protection including improved cell viability and decreased CK level, LDH activity, and cellular apoptosis. Our data also showed that PI3K/Bcl-2 cascade was linked to the ability of TMP to inhibit Beclin-1 associated autophagy and restore autophagic flux that help to reduce MI/R injury. These results demonstrated a novel mechanism for TMP in MI/R injury.

MI/R is a common pathological and physiological phenomenon and involves myocardial metabolic disorders and structural remodeling [27]. Previous studies indicated that MI/R is caused by inflammatory response, microembolization, and cell death, resulting in serious disorders of the cardiovascular function [28]. Although many therapies such as percutaneous coronary intervention, calcium-channel blockers and anticoagulation are used in the treatment of MI/R, the efficacy is limited due to various complications [29,30]. TMP is the main component of saponins extracted from *Ligusticum wallichii* (Chuanxiong). As a traditional Chinese medicine, TMP inhibit the activation of inflammatory cells, reduce the production of pro-inflammatory cytokines, exert antioxidant, anti-apoptotic effect and has been widely used in the treatment of acute heart-brain injury and spinal cord injury [31,32]. In the present study, we found that TMP significantly attenuated the increase of CK level and LDH activity in MI/R rats. Moreover, our data indicated that TMP increased cell viability, reduced LDH activity, decreased cellular apoptosis in H/R cardiomyocytes. Therefore, our data demonstrated that TMP elicited protective effect against MI/R injury.

Autophagy is an important evolutionarily conserved process in eukaryotic organisms for the turnover of intracellular substances. TMP has been reported to be closely linked to induction of autophagy. Yu et al. reported that TMP phosphate and borneol combination therapy synergistically attenuated ischemia-reperfusion injury of the hypothalamus and striatum via regulation of apoptosis and autophagy in a rat model [33]. He also reported that the synergic effect of TMP phosphate and borneol for protecting against ischemia injury in cortex and hippocampus regions by modulating apoptosis and autophagy [34]. It was reported that TMP protected against sodium arsenite-induced nephrotoxicity by suppressing ROS production, mitochondrial dysfunction, pro-inflammatory signaling pathways and programed cell death [35]. Although autophagy seems to be a necessary process in many diseases, it remains unknown whether autophagy is involved in TMP-mediated protection against MI/R injury. In our study we found that TMP decreased the ratio of LC3-II/LC3-I, reduced expressions of Beclin-1 and p62 in MI/R rats as well as H/R cardiomyocytes. However, the role of autophagy in MI/R injury is still controversial. In order to investigate the role of autophagy in TMP-induced cardioprotection, CQ was used in MI/R and H/R models. Previous studies showed that CQ improved cardiac dysfunction by blocking autophagy flux, but impaired autophagosome clearance by CQ was sufficient to cause cell death in normal cardiomyocytes [23,40]. Our results demonstrated that inhibition of autophagic flux by CQ decreased protective effects of treatment with TMP in MI/R rats as well as in H/R cardiomyocytes. Importantly, inhibition autophagic flux by CQ inhibited TMP-exerted protective effects in MI/R rats as well as in H/R cardiomyocytes. Taken together, these findings suggested that inhibiting autophagy dysfunction played an important role in TMP-exerted protection against MI/R injury.

The mechanisms responsible for cardioprotection of TMP against MI/R injury involve prevention of injury in endothelial cell and proliferation in vascular smooth muscle cell [38]. Recent studies have reported that the autophagic process in cardiomyocytes results in major phases of myocardial injury [39]. Myocardial ischemia induces autophagy through an AMPK-dependent pathway, whereas reperfusion stimulates autophagy via a Beclin-1-dependent pathway [36,37]. We demonstrated that TMP inhibited Beclin-1 associated autophagy overactivation and facilitated autophagic flux in MI/R rats as well as in H/R cardiomyocytes. Moreover, we found that TMP decreased MI/R-induced upregulation of Beclin-1. Using inhibitors and siRNA, we further identified that PI3K/Bcl-2 cascade induced the inhibitory effect of TMP on autophagy dysfunction in H/R cardiomyocytes. This is in line with the previous reports that Beclin-1mediated excessive autophagy activation during reperfusion is associated with Bcl-2 down-regulation and that there was negative regulation of Beclin-1-mediated autophagy by Bcl-2 [41].

5. Conclusion

Our present study demonstrates that the improvement of autophagy dysfunction participates in TMP-induced protection against MI/R injury. TMP alleviates Beclin-1 associated autophagy dysfunction via PI3K/Bcl-2 cascade. These findings provide evidence that TMP play a novel role in regulating autophagy dysfunction against MI/R injury, which ispotential applied to the treatment of ischemic heart disease.

Author contributions

Gen-shan Ma conceived of the study, and participated in its design and coordination and modified the manuscript. All authors read and approved the final manuscript.

Conceptualization: Zhi Zuo, Gen-shan Ma; Data curation: Zhi Zuo, Peng-fei Zuo.

Formal analysis: Zhi Zuo, Peng-fei Zuo.

Funding acquisition: Gen-shan Ma.

Investigation: Jian-dong Ding, Gen-shan Ma;

Methodology: Zhi Zuo, Peng-fei Zuo.

Project administration: Zhi Zuo, Peng-fei Zuo, Zu-long Sheng, Xin Wang.

Resources: Zu-long Sheng, Xin Wang.

Software: Jian-dong Ding.

Supervision: Gen-shan Ma.

Validation: Zhi Zuo, Peng-fei Zuo.

Roles/Writing - original draft: Zhi Zuo.

Writing - review & editing: Gen-shan Ma.

Declaration of competing interest

The authors declare that they have no competing interests.

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References

[1] S.C. Chung, R. Gedeborg, O. Nicholas, et al., Acute myocardial infarction: a comparison of short-term survival in national outcome registries in Sweden and the UK, Lancet 383 (2014) 1305–1312.
[2] Z. Li, D. Li, J. Huang, et al., Preparation of cardiovascular disease-related genes microarray and its application in exploring ligustrazine-induced changes in endothelial gene expression, Pol. J. Pharmacol. 56 (2004) 427–433.
[3] H. Chen, G. Li, P. Zhan, X. Liu, Ligustrazine derivatives. Part 5: design, synthesis and biological evaluation of novel ligustrazinyloxy-cinnamic acid derivatives as potent cardiovascular agents, Eur. J. Med. Chem. 46 (2011) 5609–5615.
[4] Z. Wang, Q. Wang, C. Wang, et al., Tetramethylpyrazine attenuates periorbital allodynia and neuroinflammation in a model of traumatic brain injury, J. Inflamm. 14 (2017) 13.
[5] S.H. Xu, M.S. Yin, B. Liu, et al., Tetramethylpyrazine-2'-O-sodium ferulate attenuates blood-brain barrier disruption and brain oedema after cerebral ischemia/reperfusion, Hum. Exp. Toxicol. 36 (2017) 670–680.
[6] L. Jian-Sheng, W. Hai-Feng, B. Yun-Ping, et al., Ligustrazine injection for chronic pulmonary heart disease: a systematic review and meta-analysis, Evid. Based Compl. Alt. (2012) 792726.
[7] C.F. Liu, M.H. Lin, C.C. Lin, et al., Protective effect of tetramethylpyrazine on absolute ethanol-induced renal toxicity in mice, J. Biomed. Sci. 9 (2002) 299–302.
[8] X. Wu, F. Zhang, X. Xiong, et al., Tetramethylpyrazine reduces inflammation in liver fibrosis and inhibits inflammatory cytokine expression in hepatic stellate cells by modulating NLRP3 inflammasome pathway, IUBMB Life 67 (2015) 312–321.
[9] S. Zhao, Z. Zhang, L. Qian, et al., Tetramethylpyrazine attenuates carbon tetrachloride-caused liver injury and fibrogenesis and reduces hepatic angiogenesis in rats, Biomed. Pharmacother. 86 (2017) 521–530.
[10] L. Lv, Q. Meng, J. Xu, et al., Ligustrazine attenuates myocardial ischemia reperfusion injury in rats by activating the phosphatidylinositol 3-kinase/Akt pathway, Ann. Clin. Lab. Sci. 42 (2012) 198–202.
[11] N. Mizushima, B. Levine, A.M. Cuervo, D.J. Klionsky, Autophagy fights disease through cellular self-digestion, Nature 451 (2008) 1069–1075.
[12] G.G. Schiattarella, J.A. Hill, Therapeutic targeting of autophagy in cardiovascular disease, J. Mol. Cell. Cardiol. 95 (2016) 86–93.
[13] K. Nishida, K. Otsu, Autophagy during cardiac remodeling, J. Mol. Cell. Cardiol. 95 (2016) 11–18.
[14] S. Sciarretta, M. Volpe, J. Sadoshima, Mammalian target of rapamycin signaling in cardiac physiology and disease, Circ. Res. 114 (2014) 549–564.
[15] M. Fukui, N. Yamabe, H.J. Choi, et al., Mechanism of ascorbate-induced cell death in human pancreatic cancer cells: role of bcl-2, Beclin 1 and autophagy, Planta Med. 81 (2015) 838–846.
[16] a Y. Matsui, H. Takagi, X. Qu, M. Abdellatif, H. Sakoda, T. Asano, B. Levine, J. Sadoshima, Distinct roles of autophagy in the heart during ischemia and reperfusion: roles of AMP-activated protein kinase and Beclin 1 in mediating autophagy, Circ. Res. 100 (2007) 914–922;
b L.L. Ma, X. Ma, F.J. Kong, et al., Mammalian target of rapamycin inhibition attenuates myocardial ischaemia-reperfusion injury in hypertrophic heart, J. Cell Mol. Med. 22 (2018) 1708–1719.
[17] L. Wang, H.Y. Zhang, B. Gao, et al., Tetramethylpyrazine protects against glucocorticoid-induced apoptosis by promoting autophagy in mesenchymal stem cells and improves bone mass in glucocorticoid-induced osteoporosis rats, Stem Cells Dev. 26 (2017) 419–430.
[18] J. Gao, W. Fu, Z. Jin, X. Yu, Acupuncture pretreatment protects heart from injury in rats with myocardial ischemia and reperfusion via inhibition of the beta(1)-adrenoceptor signaling pathway, Life Sci. 80 (2007) 1484–1489.
[19] C.C. Lai, P.H. Huang, A.H. Yang, et al., Baicalein attenuates lung injury induced by myocardial ischemia and reperfusion, Am. J. Chin. Med. 45 (2017) 791–811.
[20] S.G. Qin, H.Y. Tian, J. Wei, et al., 3-Bromo-4,5-Dihydroxybenzaldehyde protects against myocardial ischemia and reperfusion injury through the Akt-PGC1alpha-Sirt3 pathway, Front. Pharmacol. 9 (2018) 722.
[21] W.J. Li, S.P. Nie, Y. Yan, S.B. Zhu, M.Y. Xie, The protective effect of Ganoderma atrum polysaccharide against anoxia/reoxygenation injury in neonatal rat cardiomyocytes, Life Sci. 85 (2009) 634–641.
[22] S.R. Frolova, V.S. Gorbunov, N.S. Shubina, et al., Stilbene derivative as a photosensitive compound to control the excitability of neonatal rat cardiomyocytes, Biosci. Rep. 39 (2019).
[23] X. Ma, H. Liu, S.R. Foyil, et al., Impaired autophagosome clearance contributes to cardiomyocyte death in ischemia/reperfusion injury, Circulation 125 (2012) 3170–3181.
[24] S. Ma, Y. Wang, Y. Chen, F. Cao, The role of the autophagy in myocardial ischemia/reperfusion injury, Biochim. Biophys. Acta 1852 (2015) 271–276.
[25] C. Chen-Scarabelli, P.R. Agrawal, L. Saravolatz, C. Abuniat, G. Scarabelli, A. Stephanou, L. Loomba, J. Narula, T.M. Scarabelli, R. Knight, The role and modulation of autophagy in experimental models of myocardial ischemia-reperfusion injury, Journal of geriatric cardiology : JGC. 11 (2014) 338–348.
[26] S. Pattingre, A. Tassa, X. Qu, R. Garuti, X.H. Liang, N. Mizushima, M. Packer, M.D. Schneider, B. Levine, Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy, Cell 122 (2005) 927–939.
[27] L.C. Becker, G. Ambrosio, Myocardial consequences of reperfusion, Prog. Cardiovasc. Dis. 30 (1987) 23–44.
[28] A.T. Turer, J.A. Hill, Pathogenesis of myocardial ischemia-reperfusion injury and rationale for therapy, Am. J. Cardiol. 106 (2010) 360–368.
[29] X. Chen, F. Liu, H. Xu, et al., Left ventricular diastolic dysfunction in patients with ST-elevation myocardial infarction following early and late reperfusion by coronary intervention, Int. J. Cardiol. 228 (2017) 886–889.
[30] J.P. Collet, J. Silvain, O. Barthelemy, et al., Dual-antiplatelet treatment beyond 1 year after drug-eluting stent implantation (ARCTIC-Interruption): a randomised trial, Lancet 384 (2014) 1577–1585.
[31] L. Lv, S.S. Jiang, J. Xu, J.B. Gong, Y. Cheng, Protective effect of ligustrazine against myocardial ischaemia reperfusion in rats: the role of endothelial nitric oxide synthase, Clin. Exp. Pharmacol. Physiol. 39 (2012) 20–27.
[32] X. Xiao, Y. Liu, C. Qi, et al., Neuroprotection and enhanced neurogenesis by tetramethylpyrazine in adult rat brain after focal ischemia, Neurol. Res. 32 (2010) 547–555.
[33] B. Yu, M. Ruan, T. Liang, et al., Tetramethylpyrazine phosphate and borneol combination therapy synergistically attenuated ischemia-reperfusion injury of the hypothalamus and striatum via regulation of apoptosis and autophagy in a rat model, Am. J. Transl. Res. 9 (2017) 4807–4820.
[34] B. Yu, M. Ruan, T. Liang, et al., The synergic effect of tetramethylpyrazine phosphate and borneol for protecting against ischemia injury in cortex and hippocampus regions by modulating apoptosis and autophagy, J. Mol. Neurosci. 63 (2017) 70–83.
[35] X. Gong, V.N. Ivanov, M.M. Davidson, T.K. Hei, Tetramethylpyrazine (TMP) protects against sodium arsenite-induced nephrotoxicity by suppressing ROS production, mitochondrial dysfunction, pro-inflammatory signaling pathways and programed cell death, Arch. Toxicol. 89 (2015) 1057–1070.
[36] I. Tamargo-Gomez, G. Marino, AMPK: regulation of metabolic dynamics in the context of autophagy, Int. J. Mol. Sci. 19 (2018).
[37] Y. Maejima, M. Isobe, J. Sadoshima, Regulation of autophagy by Beclin 1 in the heart, J. Mol. Cell. Cardiol. 95 (2016) 19–25.
[38] K.L. Wong, K.C. Wu, R.S. Wu, Tetramethylpyrazine inhibits angiotensin II-increased NAD(P)H oxidase activity and subsequent proliferation in rat aortic smooth muscle cells, Am. J. Chin. Med. 35 (2007) 1021–1035.
[39] J.A. Riquelme, M.N. Chavez, D. Mondaca-Ruff, et al., Therapeutic targeting of autophagy in myocardial infarction and heart failure, Expert Rev. Cardiovasc Ther. 14 (2016) 1007–1019.
[40] N. Mizushima, T. Yoshimori, B. Levine, Methods in mammalian autophagy research, Cell 140 (2010) 313–326.
[41] S. Pattingre, A. Tassa, X. Qu, R. Garuti, X.H. Liang, N. Mizushima, M. Packer, M.D. Schneider, B. Levine, Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy, Cell 122 (2005) 927–939.
[42] M.L. Lindsey, R. Bolli, J.M. Canty Jr.et al., Guidelines for experimental models of myocardial ischemia and infarction, Am. J. Physiol. Heart Circ. Physiol. 314 (2018) H812–H838.