Original Research

The protective role of carnosic acid in ischemic/reperfusion injury through regulation of autophagy under T2DM

Min Hu[1], Tianyu Li[1], Zixiang Bo[1] and Feixiang Xiang[2,3]

[1]Department of Cardiovascular Surgery, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China; [2]Department of Ultrasound, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, China; [3]Hubei Province Key Laboratory of Molecular Imaging, Wuhan 430022, China
Corresponding author: Feixiang Xiang. Email: xiangfx@hotmail.com

Impact statement

We have provided, for the first time, evidence that carnosic acid (CA) attenuates ischemia–reperfusion injury of diabetic myocardium, i.e. diabetic myocardial ischemia/reperfusion (DMI/R) injury, via enhancement of autophagy. A greater understanding of the target molecule in CA-enhanced autophagy is necessary for the development of potential chemotherapy for DMI/R injury.

Abstract

Ischemic heart disease (IHD) is the most common cardiovascular disease and is the main cause of death and disability worldwide. Myocardial ischemia/reperfusion (MI/R) injury has been linked to IHD-induced cardiomyocytes apoptosis and tissue damage. Recently, it has been reported that carnosic acid (CA) may function as a potent antioxidant in liver ischemia/reperfusion (I/R). However, whether it regulates I/R in the heart remains unclear. Here, we elucidated the emerging role of CA in MI/R under diabetic myocardial conditions. Diabetes mellitus (DM) was induced in mice by consumption of a high-fat diet for 16 weeks. To create the I/R in mice, the left anterior descending coronary artery was occluded for 30 min, and then occlusion was released to reperfuse the heart for 3 or 24 h. In diabetic myocardial ischemia/reperfusion (DMI/R) mice, pretreatment with CA suppressed the overgeneration of reactive oxygen species (ROS) and production of cytokine. Importantly, the activation of autophagy was significantly increased by CA treatment, as assessed by p62 degradation and LC3-II/LC3-I conversion, as well as by phosphorylation of AMPKα, Akt, and mTOR. Interestingly, all of the protective effects of CA were impeded by the treatment with chloroquine, which is an autophagy inhibitor. These studies suggest that CA prevents DMI/R injury via regulation of autophagy. In conclusion, our findings indicate that CA has potential as a novel therapeutic to prevent DMI/R injury.

Keywords: Autophagy, apoptosis, diabetes mellitus, myocardial ischemia/reperfusion, inflammation, carnosic acid

***Experimental Biology and Medicine* 2019; 244: 602–611. DOI: 10.1177/1535370219840987**

Introduction

Diabetes mellitus (DM)[1] is one of the most common chronic diseases, and affects around 10% of all adults older than 25 years worldwide.[2] Moreover, the prevalence of patients has been increasing substantially during recent decades.[3] DM patients have a high morbidity and mortality caused by ischemic heart injury, including myocardial infarction (MI) and ischemia/reperfusion (I/R) injury. In patients with type 2 diabetes mellitus (T2DM), the incidence of ischemic injury is about fivefold greater than in nondiabetic individuals.[4–6] Clinical studies have indicated that DM-induced hyperglycemia is linked to endothelial dysfunction, development of microvascular injury and alteration of hemodynamic response, as well as tissue damage.[7,8] DM-induced hyperglycemia is also closely associated with susceptibility and severity of myocardial ischemia/reperfusion (MI/R) injury. Thus, recent studies have focused on understanding the myocardial response, aiming to discover the mechanisms of myocardial protection to achieve cardioprotective strategies.[9] It has been reported that hyperglycemia strongly inhibits the formation of autolysosomes via regulation of the mechanistic target of rapamycin (mTOR) signaling[10,11] and largely aggravates I/R injury by the enhancement of oxidative stress.[12–14] These findings support the suggestion that oxidative stress and mTOR signaling are pivotal elements of MI/R injury mechanisms.[15,16] However, the detailed mechanism remains unclear.

Carnosic acid (CA) is a well-known preservative or antioxidant used in food and other products.[17] Currently, CA has been shown to exhibit anti-tumor activity through modulating cell survival via regulation of the AKT/mTOR pathway. Furthermore, CA afforded hepatoprotection in a mouse model of liver I/R injury,[18] suggesting that CA might have a similar protective effect in myocardium against DM-associated I/R injury. Therefore, this study evaluated the protective effect of CA on diabetic myocardial ischemia/reperfusion (DMI/R) injury in mice subjected to an experimental model of DM-induced myocardial injury.

Material and methods

Reagents

6-diamino-2-phenylindole (DAPI), Evans blue (EB), and triphenyltetrazolium chloride (TTC) were obtained from Solarbio Technology (Beijing, China). The primary antibodies against $gp91^{phox}$ (SC-130543), caspase-3 (sc-56053), GAPDH (sc-365062), and β-actin (sc-8432) were purchased from Santa Cruz Biotechnology (Paso Robles, CA, USA). The primary antibodies against p-AMPKα (Thr172, #2531), AMPKα (#5831), p-Akt (Ser473, #4060), Akt (#4691), p-mTOR (Ser2448, #5536), mTOR (#2983), LC3-I (#4599), LC3-II (#3868), and p-62 (39749) were ordered from Cell Signaling Technology (Danvers, MA, USA). Rabbit anti-goat, goat anti-mouse, and goat anti-rabbit secondary antibodies were purchased from the Zhongshan Company (Beijing, China).

Experimental animals

Adult male C57BL/6 mice were purchased from the experimental animal center of the Fourth Military Medical University. All procedures were carried out according to the Center for Animal Resources and Development regulations for animal care and this study was approved by the Ethics Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology. Mice ($n = 180$) were housed at room temperature (22–26°C) with a 12-h light/12-h dark cycle. Food and water were provided *ad libitum* and the body weight of each mouse was recorded every week. Four groups of 10 mice were studied: normal control diet, high-fat diet without I/R, high-fat diet + 3 h reperfusion, and high-fat diet + 24 h reperfusion. First, the mice were fed either a normal or high-fat diet for 16 weeks. After this, the mice with a blood glucose level higher than 300 mg/dL were considered as modeling DM. Before imposition of DMI/R, the DM mice were treated with CA dissolved in 1% tween 80 at a dose of 50 mg/kg via oral gavage for five consecutive days. Five days later, the DM mice were anesthetized with 2% isoflurane, and the heart was exteriorized via a left thoracic incision. A 6–0 silk suture slipknot was tied around the left anterior descending coronary artery for 30 min, producing myocardial ischemia. Reperfusion was initiated by release of the slipknot. The mice were sacrificed by using isoflurane at 3 h (for all assays except for the cardiac function and measurement of infarction size) or 24 h (for collection of blood and tissue) reperfusion. To determine the autophagic flux, 10 mg/kg chloroquine (CQ) was administered by intraperitoneal injection 5 min before coronary artery occlusion. The number of animals per study group was 45. Among that, determination of infarction size was used 10 mice of each group. Ten mice of each group were used for superoxide and MAD measurement. GSH/GSSG analyses were also used 10 mice of each group, 10 mice of each group were used as apoptosis analysis, and the other mice were used for isolation of ventricular myocytes.

Quantification of plasma glucose and insulin

After the mice were sacrificed, the plasma was collected by centrifugation of their blood at 1000*g* for 10 min at 4°C. The plasma glucose was assayed by using commercially available colorimetric assay kits (Cayman Chemicals, Ann Arbor, MI, USA). Briefly, the samples and standard were incubated with an enzyme mixture for 10 min. In this reaction, glucose was oxidized to δ-gluconolactone with concomitant reduction of the glucose oxidase. The reduced form of glucose oxidase was regenerated to its oxidized form to produce hydrogen peroxide. Finally, horseradish peroxidase was added as a catalyst, hydrogen peroxide reacted with 3,5-dichloro-2-hydroxybenzenesulfonic acid, and 4-aminoantipyrine to generate a pink dye with an optical absorbance of 514 nm. The plasma insulin was determined using an ELISA kit (Crystal Chem, Downers Grove, IL, USA). In brief, the plasma was incubated for 2 h at 4°C in 96-well plates coated with an insulin antibody. After washing, the capture antibody was added and incubated for 30 min at room temperature. The reaction was measured by modulation of the absorbance at 450 nm.

Analysis of cardiac function

Cardiac function was evaluated by motion-mode echocardiography by using a VEVO 770 high-resolution *in vivo* imaging system (Visual Sonics, Toronto, ON, Canada). The left ventricular ejection fraction (LVEF) and left ventricular fractional shortening (LVFS) were calculated with computerized algorithms. All measurements represented the mean of five consecutive cardiac cycles.

Determination of myocardial infarction

The infarction area was evaluated by EB/TTC double-staining. The slipknot was retied and 1 mL of 2% EB was then injected into the aorta. The heart was removed and immediately frozen at −20°C, and then divided into 1 mm-thick sections which were incubated in 1% TTC for 10 min at 37°C. The EB-stained area (blue staining, area not at risk (ANAR)), TTC-stained area (red staining, ischemic but viable tissue), and TTC negatively stained area (infarct myocardium) were evaluated using Image Pro Plus software (Media Cybernetics, Rockville, MD, USA). The myocardial infarct area (INF) size was expressed as a percentage of the INF over total area at risk (AAR) (INF/AAR × 100%).

Measurement of myocardial apoptosis

Myocardial apoptosis was detected using a Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay kit (Roche Molecular Biochemicals, Mannheim, Germany). In brief, the myocardium was fixed using 4% formalin for 24 h, and then TUNEL staining was performed according to the manufacturer's instructions. The index of apoptosis was expressed by the number of TUNEL-positively stained apoptotic cardiomyocytes/the total number of cardiomyocytes counted $\times 100\%$.

Quantification of superoxide production and malondialdehyde

Superoxide assay kits were purchased from Jiancheng Bioengineering Institute (Nanjing, China). In brief, the myocardium was incubated with lucigenin suspension in HEPEs buffer for 30 min at 37°C. Lucigenin chemiluminescence was then analyzed using a luminometer (AutoLumat LB953, EG&G Berthold, Gaithersburg, MD, USA). Superoxide production was expressed in relative light units (RLU) per second per milligram of heart weight (RLU/mg/s). The malondialdehyde (MDA) level in the heart homogenates was analyzed using a commercial colorimetric assay kit (Jiancheng Bioengineering Institute, Nanjing, China). The protein fraction from the tissue or standards with known concentrations was incubated with N-methyl-2-phenylindole for 1 h at 45°C, and the generated chromophore was determined spectrophotometrically at 586 nm. The MDA concentration of the samples was calculated according to the standard curve generated under the same conditions.

Measurement of cytokine

Cytokine-6 and cytokine TNF-α were measured using an ELISA kit (R&D Systems; Minneapolis MN, USA) according to the manufacturer's instructions. In brief, the plasma was incubated for 2 h at room temperature in 96-well plates coated with anti-IL-6 or anti-TNF-α antibodies. After washing, the capture antibody was added and incubated for 2 h at room temperature. The reaction was measured by modulation of the absorbance at 450 nm.

Cell culture and treatment

We established the cardiomyocytes model of I/R as described by Wang *et al.*[19] In brief, the mice hearts were removed and perfused by using Ca^{2+}-free bicarbonate-based buffer at 37°C for 3 min post 2% isoflurane anesthetic, followed by enzymatic digestion in collagenase type B/D containing perfusion buffer. Fifty millimoles per liter of Ca^{2+} was added into digestion buffer when the hearts became hard. After 10 min, the sections of left ventricle were further digested by same enzyme containing buffer at 37°C for 10 min. The myocytes in supernatant were collected by filtration and two times centrifugation, further the pellet was resuspended in 250 μM Ca^{2+}-containing bicarbonate-based buffer. Next, the myocytes were plated at 1×10^4 cells/mL in mouse laminin-precoated culture dishes for 1 h, and then the cells were stimulated with I/R. To establish the cardiomyocytes model of I/R, the glucose-free medium supplemented with either a vehicle or globular domain of adiponectin (gAPN, 2 μg/mL) was gassed by using hypoxic gas mixture (95% N_2–5% CO_2) for 5 min, and then the cardiomyocytes were cultured with this hypoxia-hypoglycemic medium in a Napco 8000WJ hypoxia (1% O_2–5% CO_2–94% N_2) incubator (Thermo Scientific, Waltham, MA, USA). After 3 h incubation, the culture medium was changed to normal medium containing vehicle or gAPN. Finally, additional cell culture for 6 h was performed under normoxic conditions.

Measurement of glutathione

The glutathione was assayed using a glutathione detection kit (BioVision, Mountain View, CA, USA). The myocardium lysate was centrifuged at 13,000*g* at 4°C for 2 min. The supernatants were collected and processed as indicated in the manufacturer's protocol. Finally, the samples were incubated with o-phthalaldehyde (OPA) at room temperature for 40 min. In the assay, the OPA reacts with the reduced form of glutathione (GSH) and generates a fluorescence signal. The total glutathione was determined by addition of a reducing agent that converts the oxidized form of glutathione (GSSG) to GSH and the total GSH + GSSG was evaluated.

Myeloperoxidase activity analysis

The enzyme activity was determined using a myeloperoxidase (MPO) assay kit (Abcam, Cambridge, MA, USA). In brief, the myocardium was homogenized with MPO assay buffer which was supported by the assay kit and was washed twice with PBS, followed by centrifugation for 10 min at 10,000*g*. The supernatants were collected and reacted with reaction buffer for 2 h and the reaction was then stopped using stop buffer. The absorbance was measured at 412 nm as a reference wavelength on a microplate reader (Bio-Rad, Hercules, CA, USA).

Flow cytometric analysis

The cells were resuspended in 50 μl of 10 mM HEPES (pH 7.4), 140 mM NaCl and 2.5 mM $CaCl_2$, and placed on ice for 5 min. A further 50 μl of buffer containing 5 μl of Annexin V-Alexa Fluor-488 was added, mixed, and incubated on ice in the dark for 15 min. A 400-μl volume of buffer containing 0.6 μg/mL propidium iodide (PI) was added and the samples were analyzed using a FACS Calibur (BD Biosciences) and FlowJo software (Version X; Tree Star, Ashland, OR, USA).

Immunofluorescence staining

Tissues were sectioned at 8 μm in thickness, and the sections were fixed with cold methanol for 5 min, and then permeabilized with 0.2% Triton X-100 for 10 min. After blocking with BlockAce (DS Pharma Biomedical) for 30 min, the sections were incubated with anti-LC3 antibody for 1 h at room temperature, and then stained with Alexa Fluor 594-conjugated goat anti-rabbit IgG for 1 h at room

temperature. The images were recorded using a BioZero fluorescent microscope BZ-9000 (Keyence).

Measurement of LDH release

The cells were pelleted by centrifugation at 12,000 r/min for 15 s, followed by removal of the supernatants, which were placed on ice. The LDH level was indirectly determined using an LDH assay kit (Sigma-Aldrich, St. Louis, MO, USA), which was used according to the manufacturer's instructions. The values were expressed as the percentage of LDH released relative to the value obtained following permeabilization of the cells with digitonin.

Western blotting

The proteins from the tissue or cells were washed with PBS and then lysed with Laemmli sample buffer. The protein concentration was measured using a BCA protein assay kit (Merck Millipore Technology, Darmstadt, Germany). The 15 μg protein samples were subjected to SDS-PAGE electrophoresis and the separated proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon-P; EMD Millipore, Darmstadt, Germany). The membrane was blocked with 5% skimmed milk in TBS-T for 2 h and then incubated with the primary antibodies against $gp91^{phox}$, caspase-3, poly ADP-ribose polymerase (PARP), LC3II, LC3I, p62, p-AMPKα, AMPKα, p-mTOR, mTOR, Akt and pAkt, overnight at 4°C. After washing three times in TBS-T, the membrane was incubated with a secondary antibody at room temperature for 1 h. As an internal control, the expression of β-actin was measured by using the anti-β-actin antibody as the primary antibody and the HRP-conjugated anti-rabbit antibody as the secondary antibody. The protein bands were detected by using Luminata Forte Western HRP Substrate (Millipore) with a Bio-Rad ChemiDox XRS+ imaging system (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis

All statistical analyses were performed using GraphPad Prism software version 5.0 (GraphPad Software, San Diego, CA, USA). All data were presented as means ± standard error of the mean (S.E.M). Differences were compared using analysis of variance (ANOVA) followed by Bonferroni correction for *post hoc* t test, where appropriate. Probabilities less than 0.05 were considered to be statistically significant.

Results

CA attenuates DMI/R injury

Firstly, we have established mice that exhibit T2DM and, furthermore, we have successfully induced MI/R as mentioned in the method. As shown in Figure 1(a), the body weight, fasting plasma glucose, and fasting plasma insulin were remarkably increased in the DM mice compared to the healthy mice. We further tested the effect of CA on DMI/R injury in mice. We did not observe any differences in the LVEF and LVFS between the healthy mice and the DM mice. However, the percentages of LVEF and LVFS were reduced by MI/R (Figure 1(b)). Interestingly, treating DMI/R mice with CA caused the recovery of myocardial function (Figure 1(b)) and improvement of myocardial

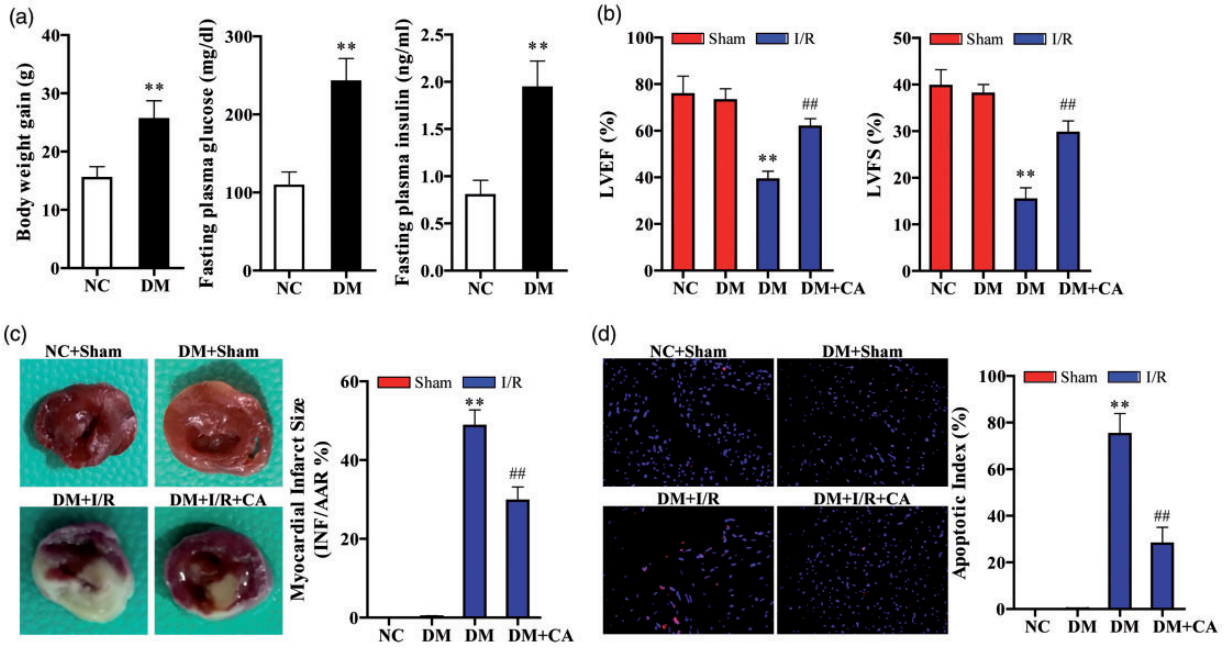

Figure 1. CA attenuated DMI/R-induced heart failure and cardiomyocytes apoptosis. (a) The body weight, fasting plasma glucose, and fasting plasma insulin of mice in each group were measured. (b) The cardiac functions of mice in each group were evaluated. (c) The myocardial infarction sizes of the mice in each group were analyzed using a TTC assay. (d) Cardiomyocytes apoptosis was measured using TUNEL staining. The data were analyzed by one-way ANOVA followed by the Tukey *post hoc* test for pairwise comparison ($n = 10$). Sham NC vs. I/R DM, **, $P < 0.01$; Sham DM vs. I/R DM+CA ##, $P < 0.05$. Each mice group was separated by different color as indicated in the figure. (A color version of this figure is available in the online journal.)

infarction (MI) (Figure 1(c)). We also confirmed cardiomyocytes apoptosis by TUNEL staining, and in contrast to the DMI/R mice, the apoptotic cells in the heart were significantly decreased when DMI/R mice had undergone pre-treatment with CA (Figure 1(d)). These data suggested that CA might prevent mice from experiencing DMI/R injury.

CA diminishes DMI/R-induced oxidative stress and the inflammatory response

Previous studies have shown that reactive oxygen species (ROS) trigger oxidative stress and induce an inflammatory response, which plays an important role in the pathogenesis of DMI/R.[20–22] Thus, we studied the effect of CA on oxidative stress and the inflammatory response in DMI/R injury mice. We found that either superoxide generation or gp91^{phox} was greatly increased in the I/R-induced DM mice (Figure 2(a) and (b)). MDA, a marker of oxidative stress, was produced in greater quantity in the DMI/R mice (Figure 2(c)). GSH, an antioxidant peptide abundantly present in cells, plays an important role in intracellular redox balance. In DMI/R mice, the ratio of GSH to the oxidized form of GSH (GSSG) has been found to be decreased (Figure 2(d)), which suggests that oxidative stress was induced by I/R under DM conditions. The generation of superoxide and MDA was inhibited by CA treatment; moreover, CA also suppressed DMI/R-induced gp91^{phox} expression and reversed GSSG to GSH (Figure 2(a) to (d)). These findings suggest that CA regulates the anti-oxidation response during DMI/R injury.

On the other hand, we investigated the effect of CA on the DMI/R-induced inflammatory response, and we found that CA had a strong inhibitory behavior on DMI/R-induced cytokine production (Figure 2(e) to (g)). MPO is abundantly expressed in granulocytes and monocytes,[23] which plays an important role in MI/R injury.[24,25] A high level of MPO activity was found to be related to DMI/R mice but not to DMI/R mice treated with CA (Figure 2(h)). These results also suggest that CA is involved in DMI/R injury as an anti-inflammatory agent.

CA enhances cardiomyocytes autophagy via regulation of the AMPK and mTOR signal pathway

We attempted to clarify the mechanism of the protective effect of CA on DMI/R by focusing on autophagy. The analysis of autophagy-associated proteins was performed by Western blotting. mTOR is a known critical negative regulator for autophagy, which is activated by Akt phosphorylation and inactivated by AMPKα phosphorylation.[26] In contrast to the healthy mice, the DM mice showed greater expression of Akt phosphorylation and mTOR phosphorylation, and a lower expression of phosphorylated AMPKα (Figure 3(a)). These findings were consistent with a previous report that autophagy is inhibited in DM.[27] Interestingly, pre-treatment of mice with CA reverses the DM-induced inhibition of autophagy, as determined by the expression of autophagy marker proteins (Figure 3(a)). In addition to the three regulators mentioned above, a decreased sequestosome 1 (p62) level and a higher ratio

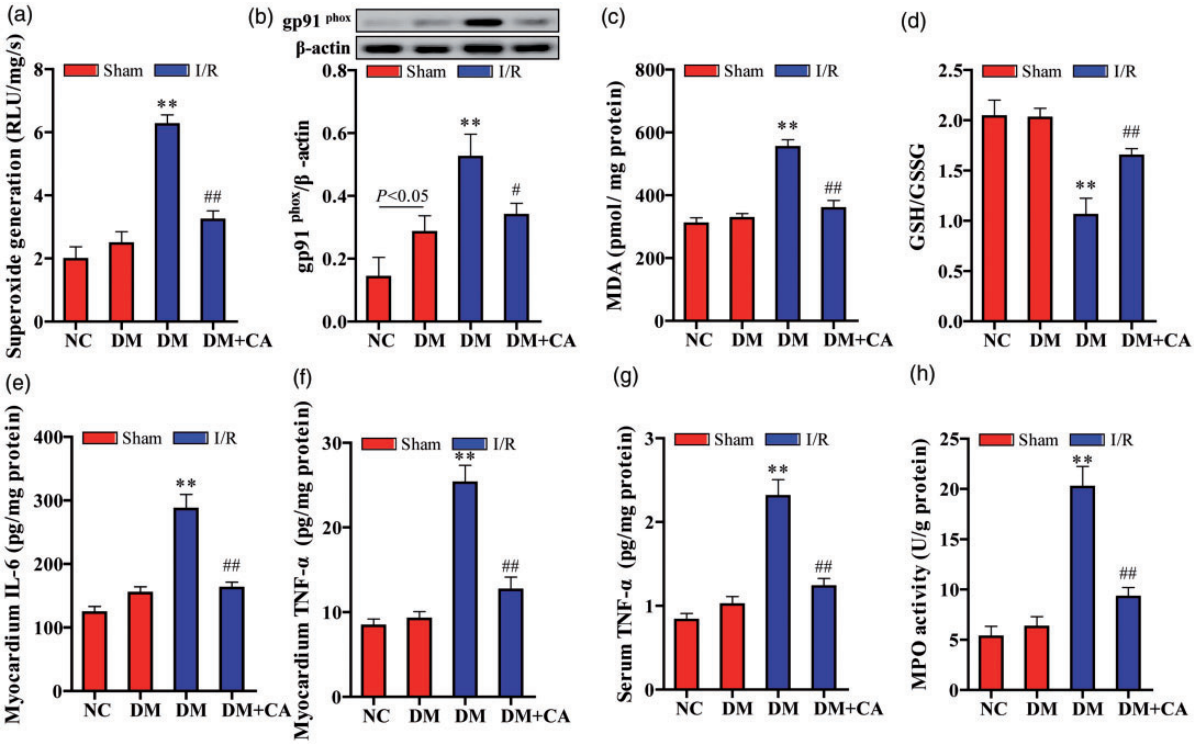

Figure 2. CA promoted antioxidant response and anti-inflammatory response during DMI/R. The oxidative stress was evaluated, as determined by superoxide generation (a), gp91^{phox} expression (b), MDA production (c) and ratio of GSH and GSSG (d). Cytokine IL-6 (e), TNF-α (f, g) as well as MPO activity (h) were quantified using ELISA. The data were analyzed by one-way ANOVA followed by the Tukey *post hoc* test for pairwise comparison ($n = 10$). Sham NC vs. I/R DM, **, $P < 0.01$; Sham DM vs. I/R DM+CA $^{\#}$, $P < 0.05$; $^{\#\#}$, $P < 0.01$. Each mice group was separated by different color as indicated in the figure. (A color version of this figure is available in the online journal.)

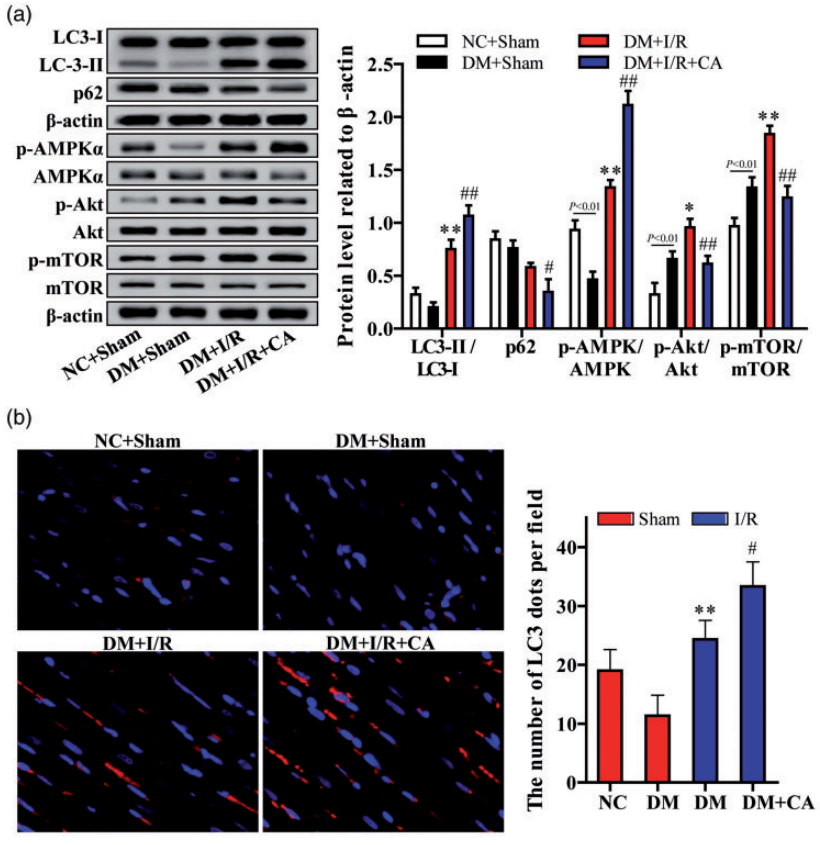

Figure 3. Cardiomyocytes autophagy was regulated by CA. (a) Autophagy-associated protein analysis was performed by Western blotting. (b) The LC3 expression was observed by using immunofluorescence staining. The data were analyzed by one-way ANOVA followed by the Tukey post hoc test for pairwise comparison ($n = 10$). Sham + NC vs. DM + I/R, *, $P < 0.05$; **, $P < 0.01$; Sham + DM vs. DM + I/R + CA $^{\#}$, $P < 0.05$; $^{\#\#}$, $P < 0.01$. Each mice group was separated by different color as indicated in the figure. (A color version of this figure is available in the online journal.)

of LC3-II/LC3-I were observed in CA-treated DMI/R mice. The positive effect of CA on autophagy was also confirmed by immunofluorescence staining (Figure 3(b)). In other words, CA protected against DMI/R injury by eliciting autophagy, probably via regulation of the mTOR-related signaling pathway.

Inhibition of autophagy partially abolishes the protective effects of CA on the myocardium

To further verify the myocardial protective function of autophagy, CQ, a specific inhibitor of autophagy, was used in the following experiments. It was found that CQ blocked CA-induced p62 degradation, although the CQ ameliorated ratio of LC3-II/LC3-I was not observed (Figure 4(a)). Importantly, CQ treatment effectively inhibited the protective effect of CA on I/R mice, as evidenced by the reduced myocardial function (Figure 4(b)), increased cardiomyocytes apoptosis (Figure 4(c)) and MI (Figure 4 (d)). Both oxidative stress (Figure 4(e) and (f)) and cytokine production (Figure 4(g)) showed significantly higher levels in CQ-treated DMI/R mice compared to DMI/R mice. These results provide further evidence that CA exerts a cardioprotective effect against DMI/R injury via inducement of autophagy.

CA recovers the DMI/R-induced cardiomyocytes apoptosis via regulation of autophagy *in vitro*

A parallel study was performed using the cardiomyocytes isolated from mice. Levels of PI^{+} Annexin-V^{+} cells, an index of apoptotic cells, increased after the cells were stimulated with high glucose and high fat in both untreated cells and I/R-induced cells (Figure 5(a)). In contrast, the apoptosis of cells, which was obviously lower in CA-treated I/R cells compared to untreated I/R cells, was nullified by treatment of the CA-treated I/R cells with CQ (Figure 5(a)). A similar tendency was observed with the LDH assay: CQ cancelled the protective effect of CA against DMI/R-induced cell death (Figure 5(b)). The expression of cleaved caspase-3 and its substrate, PARP, were then analyzed using Western blotting. These two molecules were the main cell apoptosis markers.[28] Our data revealed that cleaved caspase-3 was highly expressed in DMI/R cells. CA treatment improved caspase-3 cleavage, which was inhibited by CQ treatment (Figure 5(c)). A similar tendency was observed in the expression of cleaved PARP. No significant level of LC3-II/LC3-I was detected in the presence of CQ; however, CA-induced p62 degradation in DMI/R cells was suppressed by CQ treatment (Figure 5(c)). All of these findings suggest that CA induces autophagy against DMI/R-induced apoptosis *in vitro*.

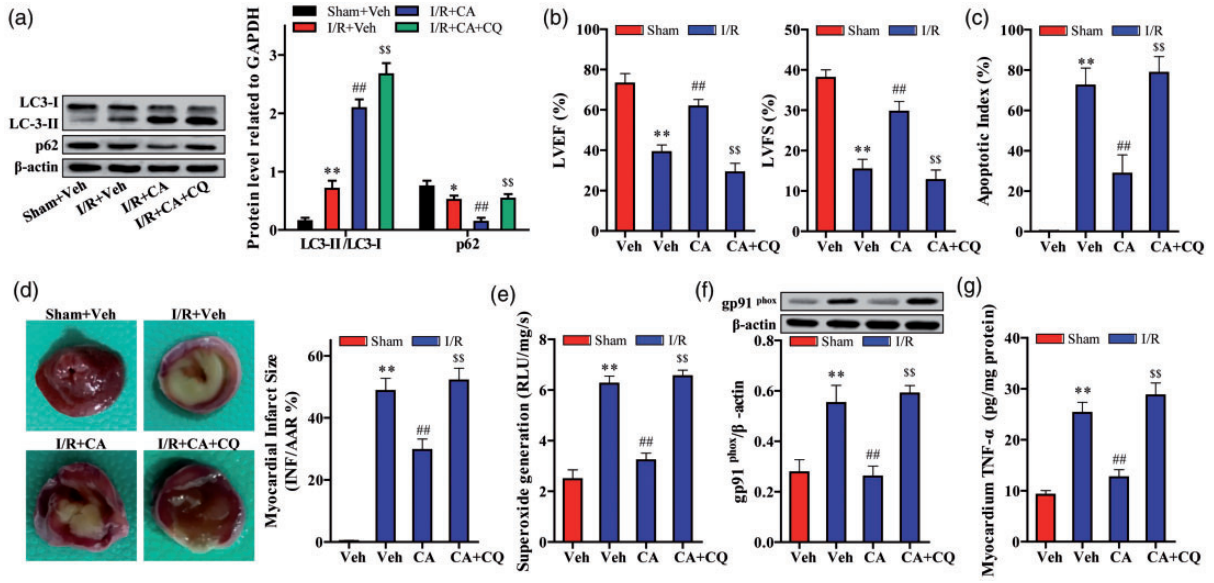

Figure 4. Chloroquine inhibited the protective effects of CA on the myocardium. (a) Autophagy-associated proteins were analyzed by Western blotting. (b) The cardiac functions were evaluated, as determined by the percentage of LVEF and LVFS. (c) Cardiomyocytes apoptosis of mice in each group was measured using TUNEL staining. (d) The myocardial infarction sizes of mice in each group were analyzed using a TTC assay. (e) Superoxide production was measured by assay kit. (f) The expression of gp91^{phox} was detected by Western blotting. (g) Cytokine TNF-α in myocardium was measured using ELISA. The data were analyzed by one-way ANOVA followed by the Tukey *post hoc* test for pairwise comparison ($n = 10$). Sham + Veh vs. I/R + Veh, *, $P < 0.05$, **, $P < 0.01$; I/R + Veh vs. I/R + CA, $^{\#\#}$, $P < 0.01$; I/R + Veh vs. I/R + CA + CQ, $^{\$\$}$, $P < 0.01$. Each mice group was separated by different color as indicated in the figure. (A color version of this figure is available in the online journal.)

Discussion

It has been reported that hyperglycemia induces the activation of NADPH oxidase and depletion of endogenous antioxidants,[29] which further results in enhancement of the assembly of ROS, which promotes tissue damage such as MI/R.[30] Our data showed that CA downregulates the gp91^{phox} expression and upregulates the synthesis of the main intracellular antioxidants (Figure 2(a) to (d)), and then attenuated I/R-produced oxidative stress occurs in DMI/R mice. Mitochondria damage has been considered to be the main source of intracellular ROS generation, which is induced by cell apoptosis during DMI/R injury.[31] CA may be a critical regulator of DMI/R-induced mitochondria damage. On the other hand, persistent hyperglycemia alters some genes promoters such as Suv39h1, a histone methyltransferase,[32] which is associated with transcription of cytokine genes, including IL-6, TNF-α, and MCP-1.[33,34] We found that CA suppresses the production of DMI/R-induced cytokine IL-6 and cytokine TNF-α (Figure 2(e) to (g)), which suggests that CA plays a key role in the methylation of histone during DMI/R injury.

Autophagy is an essential cell event that occurs in response to various cellular stresses, such as nutrient starvation, oxidative stress, and infection.[20] The molecular mechanisms of autophagy are not completely understood, but studies have demonstrated that the formation of double-membrane vesicles initiated by autophagy is called autophagosomes. The outer membrane of the autophagosomes subsequently fuses with the endosome and then lysosomes to form autolysosomes containing enzymes that degrade the molecules enclosed within.[35] These processes are precisely controlled by several regulators including mTOR, a serine/threonine protein kinase of the PI3K-related kinase family which is a central negative regulator of autophagy.[36] mTOR forms two distinct protein complexes, mTOR complex 1 (mTORC1) and 2 (mTORC2), which are generated by the stimulation of autophagy initiators, including growth factors, energy, oxygen, amino acids, and many metabolic signals.[36] Inhibition of mTORC1 causes autophagy activation, accompanied by several phenomena. For instance, the microtubule-associated protein 1 light chain 3 (LC3-I) binds with phosphatidylethanolamine to form LC3-II, which then is incorporated into the inner and the outer membranes of the autophagosomes until it is either degraded or recycled into the cytosolic pool.[37] This conversion of LC3-I/LC3-II is taken as powerful evidence for autophagy promotion. Additionally, p62 is an autophagy substrate which reports autophagy activity.[38] Autophagy is associated with several kinds of human pathologies including tumorigenesis, renal I/R and cardiac disease, in particular MI/R.[27,39–41]

In the present study, we provide evidence for the protective effect of CA against DMI/R injury via CQ activated up-regulation of autophagy. As shown in Figure 5(c), the ratio of LC3-II/LC3-I was increased even in the presence of CQ, which can be explained by the fact that the LC3-II cannot return to LC3-I when CQ inhibits the LC3-I/LC3-II conversion. To the best of our knowledge, this is the first report on the cytoprotective effect of CA against DMI/R injury via enhanced autophagy. In contrast, Zhang's group reported that N-acety-l-cysteine (NAC) attenuates DMI/R injury through inhibition of autophagy.[42]

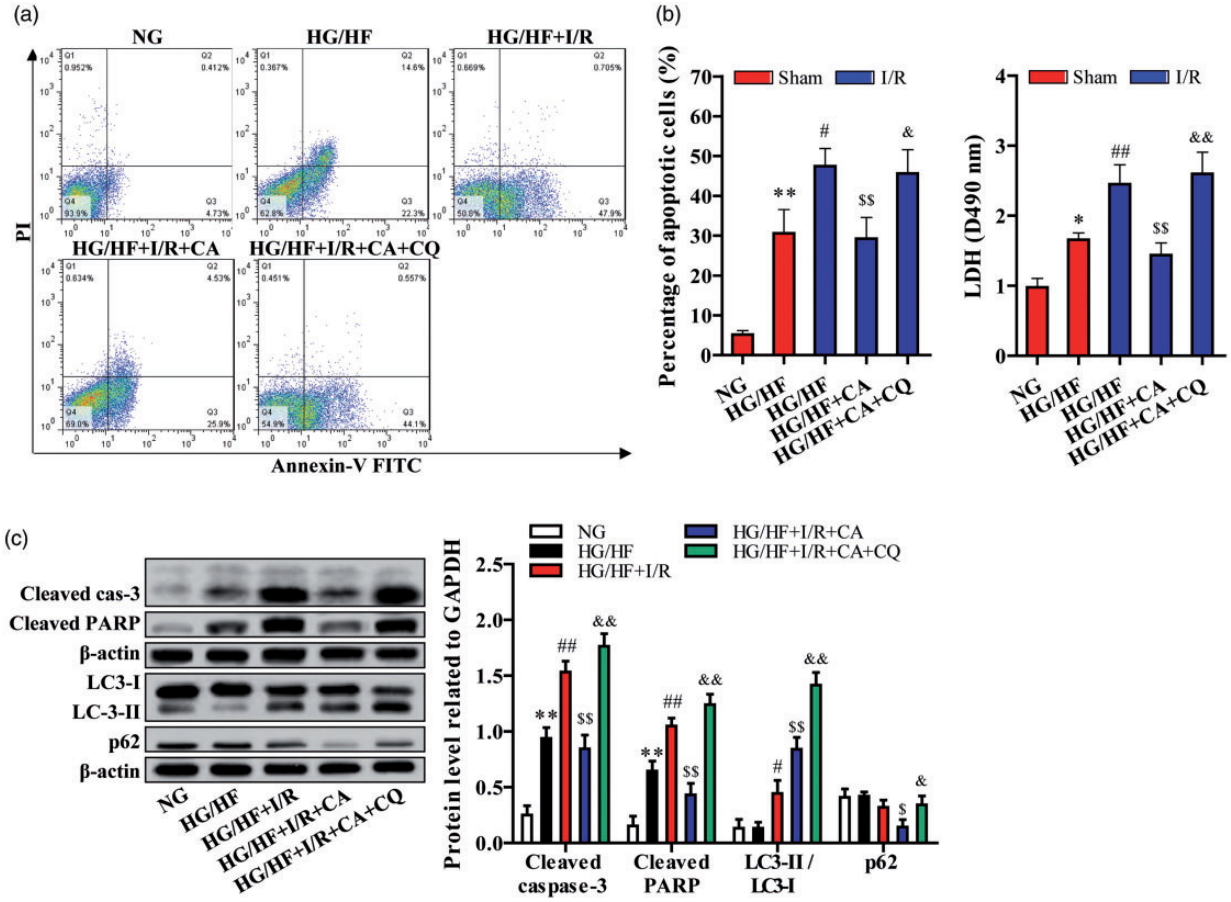

Figure 5. CA protected cardiomyocytes from DMI/R-induced apoptosis *in vitro*. Cardiomyocytes apoptosis was analyzed by FACs (a), and LDH assays (b). (c) Cleaved caspase-3, cleaved PARP, and autophagy-associated proteins were detected by Western blotting. The data were analyzed by one-way ANOVA followed by the Tukey *post hoc* test for pairwise comparison ($n = 10$). NG vs. HG/HF, *, $P < 0.05$, **, $P < 0.01$; I/R + HG/HF vs. HG/HF, #, $P < 0.05$, ##, $P < 0.01$; I/R + HG/HF vs. I/R + HG/HF + CA, $, $P < 0.05$, $$, $P < 0.01$; I/R + HG/HF vs. I/R + HG/HF + CA + CQ, &, $P < 0.05$, &&, $P < 0.01$. Each mice group was separated by different color as indicated in the figure. (A color version of this figure is available in the online journal.)

The expression of phosphorylated AMPKα (p-AMPKα) and total AMPKα was significantly increased in diabetic rats compared to healthy rats; I/R further enhanced the phosphorylation of AMPKα but did not have a remarkable effect on the total AMPKα. Autophagy markers, including the ratio of LC3-II/LC3-I or expression of p62, were obviously increased in DMI/R rats. However, the results showed that the inhibitory effect of NAC on DMI/R rats was via inhibition of autophagy, as evidenced by a decrease in the ratio of LC3-II/LC3-I and protein expression of p62, AMPKα, and mTOR. mTOR negatively regulates the autophagy by phosphorylation at Ser2448 or at Ser2481,[43,44] rather than the phosphorylation of mTOR. Furthermore, the effects of NAC on the upstream signals of mTOR including p-AMPKα and AMPKα were unclear. Finally, p62, a terminal protein of autophagy, is degraded by autophagy.[38] In contrast, the p62 level in NAC-treated DMI/R rats was lower than the untreated DMI/R rats, which suggested that NAC treatment enhances the autophagy process. Therefore, it is hard to say whether NAC inhibits excessive autophagy.

mTOR belongs to the phosphoinositide 3-kinase-related kinase family and interacts with several proteins to form two complexes named mTORC1 and mTORC2. mTOR signaling is much more complex than was originally anticipated. mTORC1 has six known protein components and mTORC2 has seven.[45] mTORC1 is responsible for autophagy induction in response to starvation. On the other hand, mTORC2 is involved in the regulation of phosphorylation and activation of Akt, protein kinase C, serum- and glucocorticoid-induced protein kinase 1. As Akt positively regulates mTORC1, it would be reasonable to speculate that mTORC2 acts as a negative regulator of autophagy, and, indeed, it has been found that mTORC2 inhibition induces autophagy.[26] Ma *et al.*[46] proposed that mTORC1 overexpression would suppress the pro-survival process of autophagy. In contrast, Aoyagi *et al.*[47] reported that overexpression of mTORC1 protected the heart from I/R injury. Actually, the beneficial effects of mTORC1 overexpression are mainly attributed to the suppression of the inflammatory response during reperfusion, and the benefits of a decreased inflammatory response exceed the functional loss of autophagy.[46] mTORC2 is also involved in the induction of autophagy and associated with enhanced cardiac cell survival after I/R.[48] However, Volkers *et al.*[49] proposed a new approach for cardiac ischemia by selectively increasing mTORC2 while inhibiting mTOC1 signaling, which indicated that autophagy is regulated by either the PI3K-Akt-mTORC1 pathway or by mTORC1/mTORC2 balancing.

During the process of cardiac I/R, mitochondrial damage, including mitochondrial fission and fragmentation were observed in cardiomyocytes.[50] Excessive damage to mitochondria caused ROS over-production and resulted in oxidative stress and further myocardial cell death. Under these conditions, autophagy is induced to remove dysfunctional mitochondria, which is known as mitophagy.[51] Undoubtedly, mitophagy is a protective form of autophagy which occurs during I/R injury. CA showed an inhibitory effect on DMI/R-induced oxidative stress (Figures 2 and 4), which could be caused by inhibition of mitochondrial damage. Additionally, CA also regulates the PI3K-Akt-mTOR signaling pathway. Taken together, the protective effect of CA on DMI/R is probably associated with both mitochondrial protection and PI3K-Akt-mTOR pathway regulation.

In conclusion, we have provided, for the first time, evidence that CA attenuates DMI/R injury via enhancement of autophagy. A greater understanding of the target molecule in CA-enhanced autophagy is necessary for the development of potential chemotherapy for DMI/R injury.

Authors' contributions: MH and TYL performed the study and drafted the manuscript. TYL and ZXB analyzed the data, and performed the literature search and statistical analysis. FXX conceived and designed the study, and revised the manuscript accordingly prior to submission. All authors have read and agreed with the final content of this manuscript.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

FUNDING

The author(s) received no financial support for the research, authorship, and/or publication of this article.

REFERENCES

1. Baldus S, Heitzer T, Eiserich JP, Lau D, Mollnau H, Ortak M, Petri S, Goldmann B, Duchstein HJ, Berger J, Helmchen U, Freeman BA, Meinertz T, Munzel T. Myeloperoxidase enhances nitric oxide catabolism during myocardial ischemia and reperfusion. *Free Radical Biol Med* 2004;**37**:902–11
2. Hyder AA, Paichadze N, Toroyan T, Peden MM. Monitoring the decade of action for global road safety 2011-2020: an update. *Glob Public Health* 2017;**12**:1492–505
3. Rathmann W, Giani G. Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. *Diabetes Care* 2004;**27**:2568–9
4. Thom T, Haase N, Rosamond W, Howard VJ, Rumsfeld J, Manolio T, Zheng ZJ, Flegal K, O'Donnell C, Kittner S, Lloyd-Jones D, Goff DC, Jr., Hong Y, Adams R, Friday G, Furie K, Gorelick P, Kissela B, Marler J, Meigs J, Roger V, Sidney S, Sorlie P, Steinberger J, Wasserthiel-Smoller S, Wilson M, Wolf P. Heart disease and stroke statistics – 2006 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. *Circulation* 2006;**113**:e85–151
5. Iris JM, Villasis-Keever MA. Angiographic findings and outcome in diabetic patients with myocardial infarction – the GUSTO-I experience. *J Am Coll Cardiol* 1998;**31**:1699–701
6. Yu Q, Gao F, Ma XL. Insulin says NO to cardiovascular disease. *Cardiovasc Res* 2011;**89**:516–24
7. Gilbert RE. Endothelial loss and repair in the vascular complications of diabetes: pathogenetic mechanisms and therapeutic implications. *Circ J* 2013;**77**:849–56
8. Everett BM, Brooks MM, Vlachos HE, Chaitman BR, Frye RL, Bhatt DL. Troponin and cardiac events in stable ischemic heart disease and diabetes. *N Engl J Med* 2015;**373**:610–20
9. Yellon DM, Hausenloy DJ. Myocardial reperfusion injury. *N Engl J Med* 2007;**357**:1121–35
10. Kobayashi S, Xu X, Chen K, Liang Q. Suppression of autophagy is protective in high glucose-induced cardiomyocyte injury. *Autophagy* 2012;**8**:577–92
11. Baranyai T, Nagy CT, Koncsos G, Onodi Z, Karolyi-Szabo M, Makkos A, Varga ZV, Ferdinandy P, Giricz Z. Acute hyperglycemia abolishes cardioprotection by remote ischemic perconditioning. *Cardiovasc Diabetol* 2015;**14**:151
12. Aragno M, Mastrocola R, Alloatti G, Vercellinatto I, Bardini P, Geuna S, Catalano MG, Danni O, Boccuzzi G. Oxidative stress triggers cardiac fibrosis in the heart of diabetic rats. *Endocrinology* 2008;**149**:380–8
13. Su H, Ji L, Xing W, Zhang W, Zhou H, Qian X, Wang X, Gao F, Sun X, Zhang H. Acute hyperglycaemia enhances oxidative stress and aggravates myocardial ischaemia/reperfusion injury: role of thioredoxin-interacting protein. *J Cell Mol Med* 2013;**17**:181–91
14. Ghattas MH, Abo-Elmatty DM. Association of polymorphic markers of the catalase and superoxide dismutase genes with type 2 diabetes mellitus. *DNA Cell Biol* 2012;**31**:1598–603
15. Anderson EJ, Kypson AP, Rodriguez E, Anderson CA, Lehr EJ, Neufer PD. Substrate-specific derangements in mitochondrial metabolism and redox balance in the atrium of the type 2 diabetic human heart. *J Am Coll Cardiol* 2009;**54**:1891–8
16. Braunwald E. Biomarkers in heart failure. *N Engl J Med* 2008;**358**:2148–59
17. Steiner M, Priel I, Giat J, Levy J, Sharoni Y, Danilenko M. Carnosic acid inhibits proliferation and augments differentiation of human leukemic cells induced by 1,25-dihydroxyvitamin D3 and retinoic acid. *Nutr Cancer* 2001;**41**:135–44
18. Kim HS, Lee JY, Lim SH, Park K, Sun JM, Ko YH, Baek CH, Son YI, Jeong HS, Ahn YC, Lee MY, Hong M, Ahn MJ. Association between PD-L1 and HPV status and the prognostic value of PD-L1 in oropharyngeal squamous cell carcinoma. *Cancer Res Treat* 2016;**48**:527–36
19. Wang Y, Tao L, Yuan Y, Lau WB, Li R, Lopez BL, Christopher TA, Tian R, Ma XL. Cardioprotective effect of adiponectin is partially mediated by its AMPK-independent antinitrative action. *Am J Physiol Endocrinol Metab* 2009;**297**:E384–91
20. Zweier JL, Talukder MA. The role of oxidants and free radicals in reperfusion injury. *Cardiovasc Res* 2006;**70**:181–90
21. Maxwell SR, Lip GY. Reperfusion injury: a review of the pathophysiology, clinical manifestations and therapeutic options. *Int J Cardiol* 1997;**58**:95–117
22. Petrosillo G, Ruggiero FM, Di Venosa N, Paradies G. Decreased complex III activity in mitochondria isolated from rat heart subjected to ischemia and reperfusion: role of reactive oxygen species and cardiolipin. *FASEB J* 2003;**17**:714–6
23. Hristova M, Rocha-Ferreira E, Fontana X, Thei L, Buckle R, Christou M, Hompoonsup S, Gostelow N, Raivich G, Peebles D. Inhibition of signal transducer and activator of transcription 3 (STAT3) reduces neonatal hypoxic-ischaemic brain damage. *J Neurochem* 2016;**136**:981–94
24. Austin GE, Chan WC, Zhao W, Racine M. Myeloperoxidase gene expression in normal granulopoiesis and acute leukemias. *Leuk Lymphoma* 1994;**15**:209–26
25. Kalasz J, Pasztor ET, Fagyas M, Balogh A, Toth A, Csato V, Edes I, Papp Z, Borbely A. Myeloperoxidase impairs the contractile function in isolated human cardiomyocytes. *Free Radic Biol Med* 2015;**84**:116–27
26. Jung CH, Ro SH, Cao J, Otto NM, Kim DH. mTOR regulation of autophagy. *FEBS Lett* 2010;**584**:1287–95
27. Han Z, Cao J, Song D, Tian L, Chen K, Wang Y, Gao L, Yin Z, Fan Y, Wang C. Autophagy is involved in the cardioprotection effect of remote

limb ischemic postconditioning on myocardial ischemia/reperfusion injury in normal mice, but not diabetic mice. *PLoS One* 2014;**9**:e86838
28. Morris G, Walker AJ, Berk M, Maes M, Puri BK. Cell death pathways: a novel therapeutic approach for neuroscientists. *Mol Neurobiol* 2018;**55**:5767–86
29. Huynh K, Kiriazis H, Du XJ, Love JE, Jandeleit-Dahm KA, Forbes JM, McMullen JR, Ritchie RH. Coenzyme Q10 attenuates diastolic dysfunction, cardiomyocyte hypertrophy and cardiac fibrosis in the db/db mouse model of type 2 diabetes. *Diabetologia* 2012;**55**:1544–53
30. Koka S, Das A, Salloum FN, Kukreja RC. Phosphodiesterase-5 inhibitor tadalafil attenuates oxidative stress and protects against myocardial ischemia/reperfusion injury in type 2 diabetic mice. *Free Radic Biol Med* 2013;**60**:80–8
31. Lesnefsky EJ, Chen Q, Tandler B, Hoppel CL. Mitochondrial dysfunction and myocardial ischemia-reperfusion: implications for novel therapies. *Annu Rev Pharmacol Toxicol* 2017;**57**:535–65
32. Martin C, Zhang Y. The diverse functions of histone lysine methylation. *Nat Rev Mol Cell Biol* 2005;**6**:838–49
33. Villeneuve LM, Reddy MA, Lanting LL, Wang M, Meng L, Natarajan R. Epigenetic histone H3 lysine 9 methylation in metabolic memory and inflammatory phenotype of vascular smooth muscle cells in diabetes. *Proc Natl Acad Sci U S A* 2008;**105**:9047–52
34. Chen X, El Gazzar M, Yoza BK, McCall CE. The NF-kappaB factor RelB and histone H3 lysine methyltransferase G9a directly interact to generate epigenetic silencing in endotoxin tolerance. *J Biol Chem* 2009;**284**:27857–65
35. Parzych KR, Klionsky DJ. An overview of autophagy: morphology, mechanism, and regulation. *Antioxidants Redox Signal* 2014;**20**:460–73
36. Saxton RA, Sabatini DM. mTOR signaling in growth, metabolism, and disease. *Cell* 2017;**168**:960–76
37. Kabeya Y, Mizushima N, Yamamoto A, Oshitani-Okamoto S, Ohsumi Y, Yoshimori T. LC3, GABARAP and GATE16 localize to autophagosomal membrane depending on form-II formation. *J Cell Sci* 2004;**117**:2805–12
38. Liu WJ, Ye L, Huang WF, Guo LJ, Xu ZG, Wu HL, Yang C, Liu HF. p62 links the autophagy pathway and the ubiqutin-proteasome system upon ubiquitinated protein degradation. *Cell Mol Biol Lett* 2016;**21**:29
39. Lamming DW, Ye L, Sabatini DM, Baur JA. Rapalogs and mTOR inhibitors as anti-aging therapeutics. *J Clin Invest* 2013;**123**:980–9
40. Liang XH, Jackson S, Seaman M, Brown K, Kempkes B, Hibshoosh H, Levine B. Induction of autophagy and inhibition of tumorigenesis by beclin 1. *Nature* 1999;**402**:672–6
41. Xie Z, Lau K, Eby B, Lozano P, He C, Pennington B, Li H, Rathi S, Dong Y, Tian R, Kem D, Zou MH. Improvement of cardiac functions by chronic metformin treatment is associated with enhanced cardiac autophagy in diabetic OVE26 mice. *Diabetes* 2011;**60**:1770–8
42. Wang S, Wang C, Yan F, Wang T, He Y, Li H, Xia Z, Zhang Z. N-acetylcysteine attenuates diabetic myocardial ischemia reperfusion injury through inhibiting excessive autophagy. *Mediat Inamm* 2017;**2017**:9257291
43. Nave BT, Ouwens M, Withers DJ, Alessi DR, Shepherd PR. Mammalian target of rapamycin is a direct target for protein kinase B: identification of a convergence point for opposing effects of insulin and amino-acid deficiency on protein translation. *Biochem J* 1999;**344**:427–31
44. Peterson RT, Beal PA, Comb MJ, Schreiber SL. FKBP12-rapamycin-associated protein (FRAP) autophosphorylates at serine 2481 under translationally repressive conditions. *J Biol Chem* 2000;**275**:7416–23
45. Laplante M, Sabatini DM. mTOR signaling in growth control and disease. *Cell* 2012;**149**:274–93
46. Ma S, Wang Y, Chen Y, Cao F. The role of the autophagy in myocardial ischemia/reperfusion injury. *Biochim Biophys Acta* 2015;**1852**:271–6
47. Aoyagi T, Kusakari Y, Xiao CY, Inouye BT, Takahashi M, Scherrer-Crosbie M, Rosenzweig A, Hara K, Matsui T. Cardiac mTOR protects the heart against ischemia-reperfusion injury. *Am J Physiol Heart Circ Physiol* 2012;**303**:H75–85
48. Gurusamy N, Lekli I, Mukherjee S, Ray D, Ahsan MK, Gherghiceanu M, Popescu LM, Das DK. Cardioprotection by resveratrol: a novel mechanism via autophagy involving the mTORC2 pathway. *Cardiovasc Res* 2010;**86**:103–12
49. Volkers M, Konstandin MH, Doroudgar S, Toko H, Quijada P, Din S, Joyo A, Ornelas L, Samse K, Thuerauf DJ, Gude N, Glembotski CC, Sussman MA. Mechanistic target of rapamycin complex 2 protects the heart from ischemic damage. *Circulation* 2013;**128**:2132–44
50. Hwang SJ, Kim W. Mitochondrial dynamics in the heart as a novel therapeutic target for cardioprotection. *Chonnam Med J* 2013;**49**:101–7
51. Eltzschig HK, Eckle T. Ischemia and reperfusion – from mechanism to translation. *Nat Med* 2011;**17**:1391–401

(Received August 22, 2018, Accepted March 8, 2019)