THE ANATOLIAN JOURNAL OF CARDIOLOGY

Official journal of the TURKISH SOCIETY OF CARDIOLOGY

Effects of Mitochondrial ATP-Sensitive Potassium Channel in Rats with Acute Myocardial Infarction and Its Association with the AKT/mTOR Pathway

ABSTRACT

Background: Myocardial infarction is associated with the autophagy and apoptosis of cardiomyocytes, and the protein kinase B/mammalian target of rapamycin (AKT/mTOR) pathway plays a crucial role in this mechanism.

Methods: Acute myocardial infarction rat models were assessed 0.5, 2, 4, and 6 hours after the induction of the myocardial infarction using hematoxylin and eosin staining, triphenyl tetrazolium chloride staining, myocardial enzyme measurements, and levels of autophagic activity. Additionally, diazoxide, 5-hydroxydecanoate, and LY294002 were intraperitoneally administered to rat models at peak myocardial injury to assess their effects on cardiac injury. The expression levels of autophagy-related and apoptosis-related proteins, as well as p-AKT and p-mTOR, were measured. Electron microscopy was used to assess the ultrastructure and the number of autophagosomes in the cardiac tissue.

Results: We demonstrated that the degree of myocardial injury and the level of autophagy were significantly elevated in the experimental cohort compared with the control cohort. In addition, the myocardial infarct size was significantly smaller in diazoxide-treated acute myocardial infarction rats compared with untreated rats. Diazoxide also decreased the levels of myocardial injury markers, autophagy, and apoptosis, while it also induced the levels of AKT and mTOR phosphorylation, decreased the number of autophagosomes, and improved the myocardial ultrastructure of the acute myocardial infarction rats. 5-Hydroxydecanoate treatment resulted in an opposite effect to those observed upon diazoxide treatment. LY294002 was also able to reverse diazoxide treatment effects.

Conclusion: Peak levels of myocardial tissue injury and autophagy were observed 2 hours post-acute myocardial infarction induction in rats. Diazoxide treatment inhibited myocardial autophagy and apoptosis while protecting cardiac tissue from ischemic injury, which is likely to have proceeded through activation of the AKT/mTOR pathway.

Keywords: AMI, mitochondrial ATP-sensitive potassium channel, PI3K/AKT/mTOR pathway, autophagy, apoptosis

ORIGINAL INVESTIGATION

Qian Zeng [1]
Long-Dan Zhang [1]
Quan-Fang Chen [2]
Wei Wang [1]
Zhou Huang [1]
Dong-Ling Huang [1]
Fan Wang [1]
Feng Yang [1]
Ji-Fei Nong [1]
Jie Yang [1]
Jin-Cheng Li [1]

[1]*Department of Emergency, the First Affiliated Hospital, Guangxi Medical University, Nanning, Guangxi, People's Republic of China*
[2]*Institute of Respiratory Disease, the First Affiliated Hospital, Guangxi Medical University, Nanning, Guangxi, People's Republic of China*

Corresponding author:
Wei Wang
✉ weiwanggx@163.com

Received: August 10, 2022
Accepted: September 29, 2022
Available Online Date: January 1, 2023

Cite this article as: Zeng Q, Zhang L, Chen Q, et al. Effects of mitochondrial ATP-sensitive potassium channel in rats with acute myocardial infarction and its association with the AKT/mTOR pathway. *Anatol J Cardiol.* 2023;27(2):88-99.

DOI:10.14744/AnatolJCardiol.2022.2406

INTRODUCTION

Acute myocardial infarction (AMI) is found at the severe end of the coronary artery disease (CAD) spectrum and is the main cause of global mortality. Currently, myocardial reperfusion through early percutaneous coronary intervention (PCI) remains the most effective means of reducing ischemic damage and preserving cardiac function post-AMI.[1] However, PCI is often not immediately available to all AMI patients due to various socioeconomic factors, leaving a significant proportion of patients subject to AMI complications, such as ventricular fibrillation, cardiogenic shock, or heart failure, while awaiting emergency PCI. Therefore, stabilizing myocardial function is important for reducing the risk of complications during this critical waiting period.[2] The mechanism of myocardial ischemic injury is complicated and involves inflammatory responses, calcium overload, oxidative stress, mitochondrial membrane dysfunction, as well as cardiomyocyte apoptosis and autophagy.[3,4]

Noma was the first to characterize mitochondrial ATP-sensitive potassium channels (mitoK$_{ATP}$) in 1983.[5] mitoK$_{ATP}$ confers myocardial protection under ischemic conditions and can be activated by diazoxide (DZ) and is selectively antagonized by 5-hydroxydecanoate (5-HD) or antioxidants. mitoK$_{ATP}$ activation stimulates transient K+ influx, while increasing electron flux, through the Q cycle, which ultimately stimulates the production of reactive oxygen species (ROS).[6-8] The protective effects exerted by mitoK$_{ATP}$ on the ROS pathway in MI has attracted significant attention. Recent evidence has demonstrated that mitoK$_{ATP}$ is involved in protecting ischemic myocardial tissue against arrhythmias.[9]

The phosphatidylinositol 3-kinase (PI3K)/AKT signaling pathway exerts a significant level of influence on cellular survival, autophagy, and differentiation.[10] It mediates a variety of cytoprotective effects and controls the functions and fate of the cardiomyocytes.[11] Phosphatidylinositol 3-kinase/AKT protects the heart by inhibiting cardiomyocyte apoptosis through several different mechanisms, including suppressing caspase-3 activation, protection against DNA damage, modulation of glucose metabolism, enhancement of Bcl-2-associated molecule functions, and the inhibition of the expression of apoptosis-regulating genes.[12,13] Glycogen synthase kinase 3β (GSK-3β) and mTOR are primary downstream modulators of the PI3K/AKT pathway. Recently, increasing evidence has associated the activation of both the PI3K/AKT/mTOR and PI3K/AKT/GSK-3β signaling pathways in exerting protective effects against ischemia/reperfusion (I/R) injury in several different organs. This effect was blocked upon exposure to a known inhibitor, LY294002.[14,15] There is considerable evidence indicating that activation of PI3K/AKT/mTOR signaling is essential to prevent ischemic cardiomyocyte death.[16] However, the potential mechanism by which mitoK$_{ATP}$ and the AKT/mTOR pathway are associated in ischemic injury induced by AMI in rats has not been clarified as yet. Therefore, in this study, we compared myocardial injury and autophagy levels at various time points within 6 hours of AMI onset and examined the impact and interactions between mitoK$_{ATP}$ and the AKT/mTOR pathway on ischemic injury in AMI rat models.

METHODS

Experimental Animals and Model Establishment

The experimental protocol was approved by the Ethics Committee on May 3, 2021 (no. 202004022). A total of 110 male Sprague–Dawley rats weighing between 200 and 250 g were procured from the Laboratory Animal Center. The models were subjected to controlled standard conditions (50%-60% humidity, 20°C-25°C, 12 hours of alternating light/dark cycles, and ad libitum access to water and chow).

The rats were anesthetized using an intraperitoneal inoculation of 2% sodium pentobarbital (40 mg/kg body weight) and positioned in the supine position. Ventilation was maintained using a rodent ventilator (ALC-V8S, Beijing, China). The rat hearts were exposed by making an incision 2-3 cm above the xiphoid process over the fourth intercostal space. The left anterior descending coronary artery was exposed and ligated using a suture (6-0 silk). The rats in the sham operation group were threaded but were not ligated. Myocardial ischemia was confirmed based on the direct visualization of cardiac tissue distal to the ligated artery turning white and electrocardiograph (ECG) evidence of ST-segment (ST) elevation. An echocardiogram was used to measure the cardiac function of the rats before they were sacrificed. Left ventricular cardiac tissue and serum samples were collected for the analysis.

Experimental Design

Diazoxide (mitoK$_{ATP}$ channel opener), 5-HD (mitoK$_{ATP}$ channels blocker), and LY294002 (an inhibitor of PI3K) were purchased from MCE (Monmouth Junction, NJ, USA). Diazoxide, 5-HD, and LY294002 were dissolved in DMSO (dimethyl sulfoxide) and intraperitoneally injected upon coronary artery ligation.

All rats were divided into 5 groups based on the time of evaluation post-AMI: sham-operated control group (sham), 0.5 hours (MI 0.5 hours), 2 hours (MI 2 hours), 4 hours (MI 4 hours), and 6 hours (MI 6 hours).

Another 60 rats were used to determine the potential mechanism of mitoK$_{ATP}$ during MI. The following 6 cohorts were formed (n=10 for each group): (1) sham-operated control (sham); (2) acute myocardial infarct group (MI group); (3) DZ group (MI + DZ): 10 mg/kg DZ was intraperitoneally injected after surgery; (4) 5-HD group (MI + 5-HD): 10 mg/kg 5-HD was intraperitoneally injected after surgery; (5) DZ plus LY294002 group (MI + DZ + LY): 10 mg/kg DZ and LY294002 were intraperitoneally injected after surgery; (6) 5-HD plus LY294002 group (MI + 5-HD + LY): 10 mg/kg 5-HD and LY294002 were intraperitoneally injected after surgery.

Determination of Infarct Size

The rat hearts were harvested and snap frozen for 5 minutes at −80°C before being sectioned into 2- to 3-mm transverse slices. The sections were treated for 10 minutes at 37°C with 1% triphenyl tetrazolium chloride (TTC) in a pH 7.4 buffer. Triphenyl tetrazolium chloride-stained viable cardiac tissues turned brick red through its interaction with dehydrogenase enzymes, while necrotic tissues remained white. The infarct size was assessed based on the ischemic area as a percentage of the entire area of the left ventricle.

CK-MB and cTnI Release Evaluation

An enzyme-linked immunosorbent assay kit (Cusabio Biotech Co., Ltd., Wuhan, China) was used to assess serum cardiac troponin I (cTnI) and creatine kinase-MB (CK-MB) levels based on the instructions provided by the manufacturer. After the

HIGHLIGHTS

- Myocardial tissue injury and autophagic activity are the highest at 2 hours post-ischemic injury in rat models of acute myocardial infarction.
- MitoK$_{ATP}$ activator diazoxide reduces autophagy and apoptosis by activating AKT/mTOR pathway.
- Diazoxide ameliorates myocardial ischemia injury.

rats were sacrificed, blood was drawn from the abdominal aorta and centrifuged for 15 minutes at 3000 rpm to obtain serum samples, which were frozen until further analysis.

Hematoxylin and Eosin and Immunohistochemistry Staining

Four percent paraformaldehyde was used for 24 hours to fix the rat hearts before they were paraffin embedded. The tissue blocks were processed into 4-µm-thick serial sections, deparaffinized, and rehydrated. Hematoxylin and eosin staining was performed for further immunohistochemistry (IHC) analysis. After antigen retrieval, 0.3% hydrogen peroxide was used to block the endogenous peroxidase activity for 10 minutes at room temperature. The sections were further exposed to 2% bovine serum albumin in phosphate-buffered saline before being incubated overnight with rabbit antibodies specific for LC3B (1:500; Cell Signaling Technology, Beverly, Mass, USA) and Beclin1 (1:200; Proteintech, Chicago, Ill, USA) in a moist chamber at 4°C. The next day, the samples were incubated for 60 minutes with the horseradish peroxidase (HRP)-labeled anti-rabbit secondary antibody at room temperature. Negative controls were not exposed to the primary antibody. Diaminobenzidine was used to develop colors before the sections were counterstained with hematoxylin. A light microscope attached to a camera (Axio Imager A2, Zeiss, Oberkochen, Germany) was used for image analysis. Subsequently, previously described methods for assessing MI severity using an optical microscope were followed.[17] A score of 0 indicates the complete absence of injury, 1 indicates the presence of areas of focal necrosis and interstitial edema (mild), 2 indicates the presence of larger areas of necrosis and cardiomyocyte swelling (moderate), 3 indicates inflammatory cell infiltration and necrosis with contraction bands (severe), while 4 indicates hemorrhage, inflammatory cell infiltration, and expanded necrosis with contraction bands (highly severe).

Transmission Electron Microscopy

Fresh left ventricular cardiac tissues (1 mm^3) were fixed in 4% glutaraldehyde, after serial alcohol dehydration and embedding in epoxy resin. Then, 1-µm-thick sections were cut and stained with 2% uranyl acetate and lead citrate. A Hitachi transmission electron microscope (TEM) (Hitachi, H-7500, IBARAKI, Japan) was used to observe the tissue sections.

Western Blotting Analysis

The protein samples were analyzed to determine the concentration and subsequently homogenize the proteins. Then, 100 µg of the sample was separated using 6%-12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis before the sample was blotted onto polyvinylidene difluoride membranes. Thereafter, 5% nonfat milk in Tris-buffered saline with 0.1% Tween 20 (TBST) was used to block the membranes. The samples were subjected to overnight incubation at 4°C using the following primary antibodies: LC3B, Beclin1, Bcl-2, GAPDH (1:1000; Cell Signaling Technology), Bax, p-AKT, AKT, p-mTOR, and mTOR (1:5000; Abcam, Cambridge, UK). Then, the membranes were rinsed thrice (5 minutes each time) with TBST before an additional 2-hour period of incubation with HRP-conjugated secondary antibodies. The samples were again washed thrice with TBST. The protein bands were evaluated and assessed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction

Total RNA was extracted from the rat hearts post-AMI induction and was purified using a TRIzol reagent kit (Invitrogen, California, USA). cDNA was reverse transcribed from 1 mg of the isolated RNA using a cDNA synthesis kit (Vazyme Biotech Co. Ltd., Nanjing, China). Quantitative real-time polymerase chain reaction was performed with the aid of the SYBR Green Supermix (Bio-Rad) using an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Carlsbad, USA). The relative mRNA expression levels were analyzed using the 2−ΔΔCT method. The primer sequences used for RT-PCR are presented in Table 1.

Statistical Analysis

Data are presented as mean ± standard deviation (SD), while all statistical analyses were performed using Statistical Package for Social Sciences SPSS 25.0 software (SPSS Inc., Chicago, Ill, USA). Intergroup differences were analyzed using a one-way analysis of variance and the Student–Newman–Keuls post-hoc test. A *P*-value of <.05 was interpreted to be of statistical significance.

RESULTS

The Representative Changes in the ECG Were Used to Verify the Success of the Myocardial Infarction Model

To confirm the success of the MI model, we evaluated changes in the ECG. As shown in Figure 1, the ECG pattern was not found to vary in the sham group before and after threading. In contrast, remarkable elevation of the ST segment was found in the MI group after ligation.

Myocardial Ischemic Injury Peaked 2 Hours After Acute Myocardial Infarction Induction

To determine the point of maximal myocardial injury, we determined changes at 4 time points. The extent of myocardial injury caused by AMI was evaluated based on myocardial infarct size (TTC), myocardial biomarkers (CK-MB and cTnI), and morphological changes [hematoxylin and eosin (HE) staining). As shown in Figure 2, the sham group possessed

Table 1. The Primer Sequences Used for the RT-PCR

LC3	Forward	5′-TTCACTGCCGCCCTAAAG-3′
	Reverse	5′-TCCAACCCACAAAGACGC-3′
Beclin1	Forward	5′-GGAGCAAATGAATGAGGGC-3′
	Reverse	5′-CAGAACAGTACAACGGCAACTCCTT-3′
Bax	Forward	5′-GCTGGACACTGGACTTCCTC-3′
	Reverse	5′-ACTCCAGCCACAAAGATGGT-3′
Bcl-2	Forward	5′-AGGATTGTGGCCTTCTTTGA-3′
	Reverse	5′-CAGATGCCGGTTCAGGTACT-3′
GAPDH	Forward	5′-GCCAAGGTCATCCATGACAAC-3′
	Reverse	5′-GTGGATGCAGGGATGATGTTC-3′

RT-PCR, real-time polymerase chain reaction.

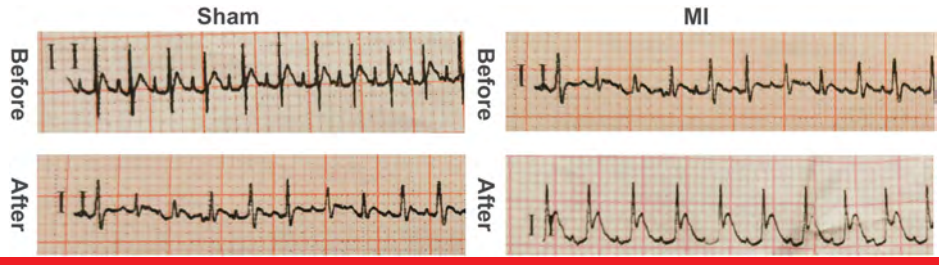

Figure 1. Lead II of ECG in the sham group and model group.

intact and regular cardiomyocytes. The myocardial cells of the MI groups were disordered and were partially ruptured. There was evidence of nuclear dissolution, cardiomyocyte necrosis, and interstitial edema. The results showed that the ratio of the myocardial infarction area and damage score were the highest in the MI 2-hour group, and the differences were statistically significant compared with the other groups ($P < .05$). Figure 3E and F demonstrate that serum CK-MB and cTnI levels in all MI groups were significantly elevated compared with the sham group ($P < .05$). Serum values of the MI 2-hour group were significantly higher than all the other groups ($P < .05$). We concluded that 2-hour post-AMI represents the peak time of myocardial injury.

Levels of Myocardial Autophagy Peaked 2 hours After Acute Myocardial Infarction Induction

The expression levels of LC3 and Beclin1, which are markers of autophagy, were evaluated using IHC, western blotting, and quantitative real-time polymerase chain reaction (qRT-PCR). As shown in Figures 3, 4, and 5, both these molecules were highly expressed in the MI group, in contrast to the sham group ($P < .05$). Beclin1 and LC3 expression levels were the highest in the MI 2-hour group ($P < .05$). Therefore, we

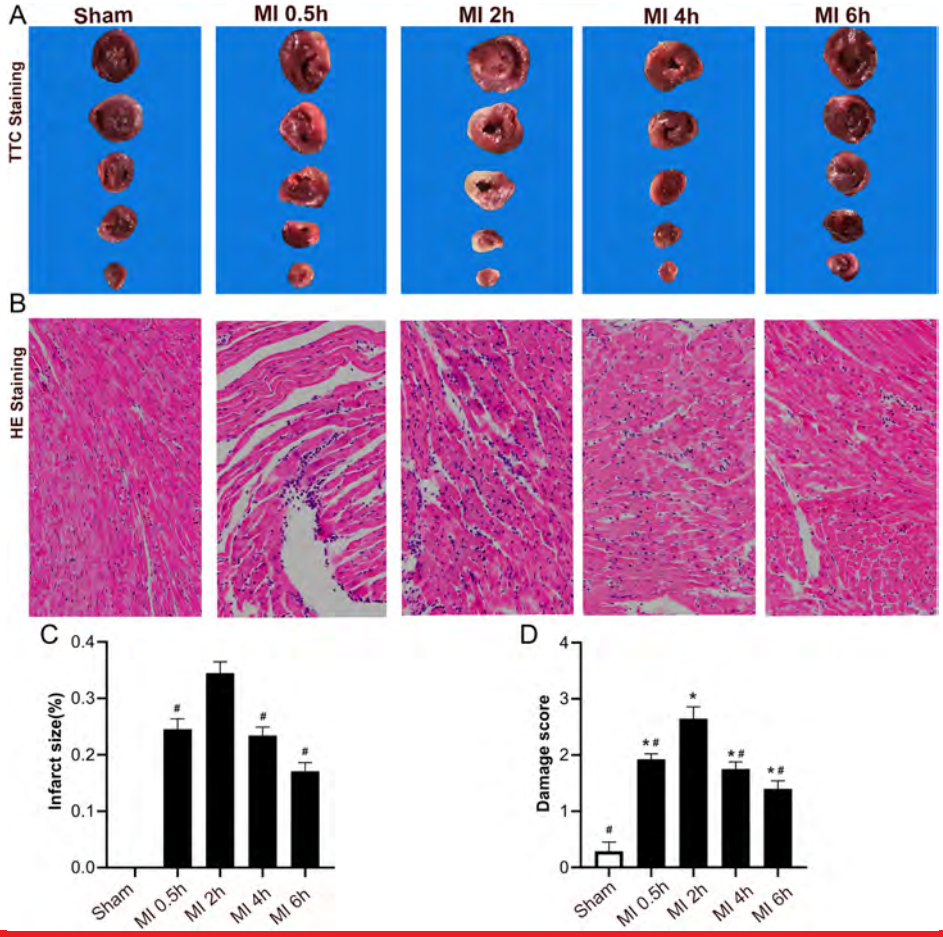

Figure 2. Myocardial infarct areas and myocardial tissue structure in rats at 0, 0.5, 2, 4, and 6 hours after ischemic injury (n = 4); (A) representative cardiomyocytes stained using TTC (n = 4); (B) representative cardiomyocytes stained using HE (×200; n = 6); (C) quantitative analysis of the myocardial infarct area (n = 4); (D) damage score (n = 6). *$P < .05$ vs. sham group; #$P < .05$ vs. MI 2-hour group. HE, hematoxylin and eosin; TTC, triphenyl tetrazolium chloride.

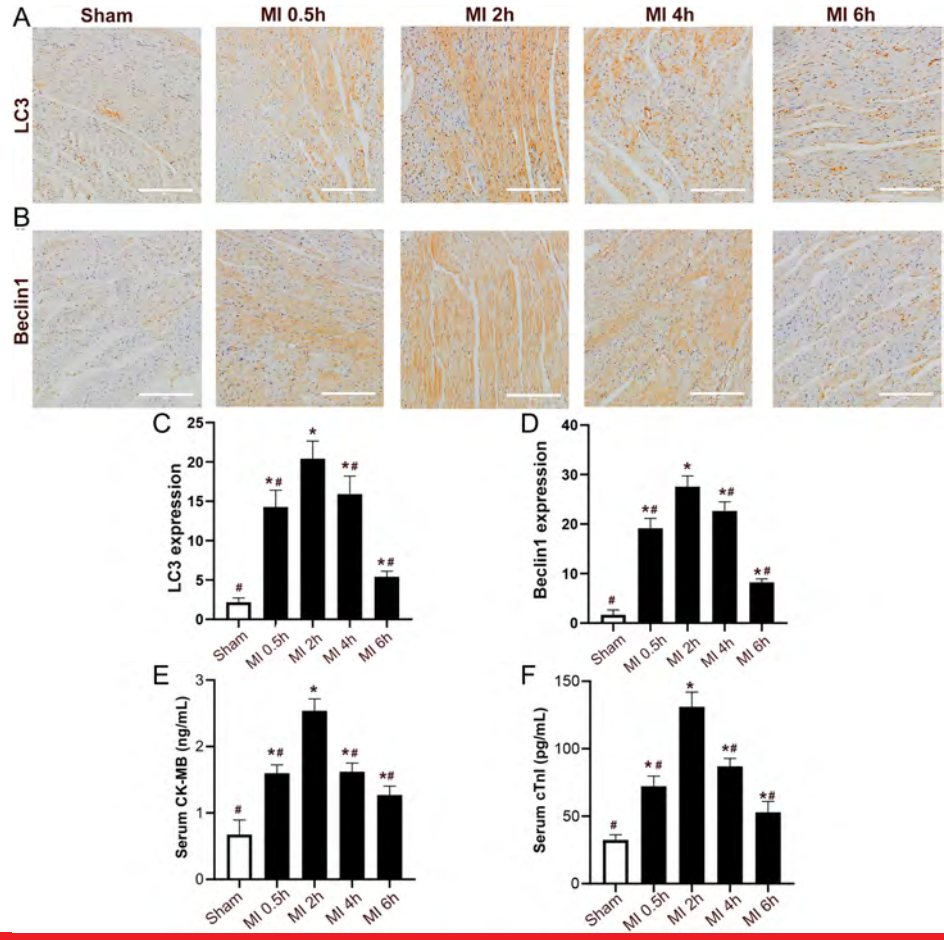

Figure 3. Diazoxide affects the expression levels of LC3B and Beclin1 proteins in the hearts and serum of rats subjected to AMI (A, B). Left ventricular cross-sections showing Beclin1 and LC3B visualized using IHC in rats at 0, 0.5, 2, 4, and 6 hours (×200) after ischemic injury (C, D). Immunohistochemical quantitative analysis of LC3B and Beclin1 (E, F). Levels of CK-MB and cTnI in the serum of rats at 0, 0.5, 2, 4, and 6 hours after ischemic injury (n = 6). *$P < .05$ vs. sham group; #$P < .05$ vs. MI 2-hour group. AMI, acute myocardial infarction; DZ, diazoxide; IHC, immunohistochemistry.

concluded that 2-hour post-AMI represents the peak time for the expression of autophagic markers.

Diazoxide Mitigates the Degree of Acute Myocardial Infarction-Induced Ischemic Injury, While LY294002 Blocks Its Protective Effect

Triphenyl tetrazolium chloride staining was used to evaluate the size of the myocardial infarcts. Rats in the MI + DZ group demonstrated a reduced infarct size, as shown in Figure 6a and c ($P < .05$, compared with the MI group). The MI + DZ + LY group had a larger infarct size than that of the MI + DZ group. We did not note any significant variability in the infarct sizes between the MI + 5-HD and MI + 5-HD + LY groups ($P > .05$).

Hematoxylin and eosin staining was used to observe cardiomyocytes. As described in Figure 6B and D, cardiomyocyte

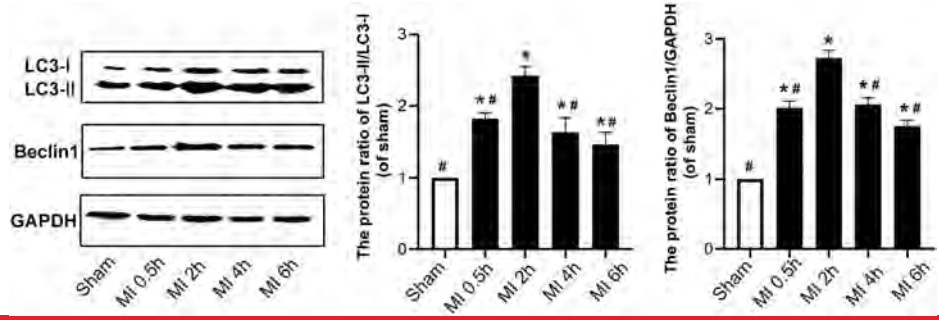

Figure 4. Western blotting bands and LC3-II and Beclin1 analysis of the myocardial tissue of rats at 0, 0.5, 2, 4, and 6 hours after ischemic injury (n = 6). *$P < .05$ vs. sham group; #$P < .05$ vs. MI 2-hour group. MI, myocardial infarction.

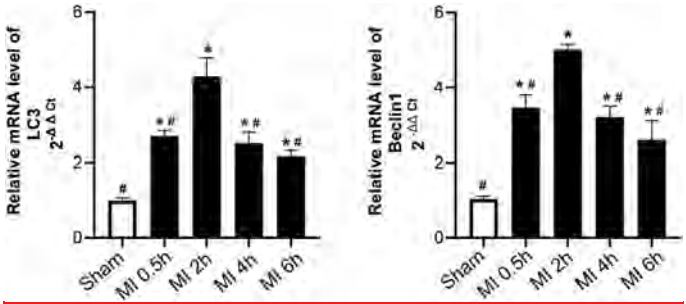

Figure 5. The mRNA expression of LC3 and Beclin1 in the myocardial tissues of rats at 0, 0.5, 2, 4, and 6 hours after ischemic injury (n = 6). *$P < .05$ vs. sham group; #$P < .05$ vs. MI 2-hour group. MI, myocardial infarction.

integrity was worse in the MI group compared with the sham group. Diazoxide treatment appeared to reduce the severity of cardiac injury. Interestingly, the co-administration of DZ and LY294002 in the MI + DZ + LY group resulted in worse injury scores than those observed in the MI + DZ group ($P < .05$). We did not observe any differences between the MI + 5-HD and MI + 5-HD + LY in terms of myocardial tissue structure ($P > .05$).

As shown in Figure 7C and D, MI significantly elevated serum CK-MB and cTnI levels. The MI + DZ cohort showed a reduction in cardiac marker levels compared with the MI group. Similar to our findings on the cardiomyocytes, serum cardiac marker expression levels in the MI + DZ + LY were higher than that of the MI + DZ group ($P < .05$). 5-Hydroxydecanoate treatment did not appear to influence serum cardiac marker levels compared with that of the MI + 5-HD + LY group ($P > .05$).

Diazoxide Diminished Autophagosomes and Maintained Mitochondrial Integrity Following Acute Myocardial Infarction-Induced Ischemic Injury While LY294002 Counteracts This Protective Effect

As shown in Figure 7A and B, MI-exposed rats showed an increase in the number of autophagosomes ($P < .05$) and demonstrated a distorted mitochondrial ultrastructure, which included swelling, disintegration, and a reduction or absence of cristae. Diazoxide treatment decreased the number of autophagosomes ($P < .05$) and appeared to maintain mitochondrial integrity in the same cohort of MI rats. However, co-exposure of DZ with LY294002 resulted in increased mitochondrial distortion and autophagosomes ($P < .05$). Furthermore, treatment with 5-HD alone appears

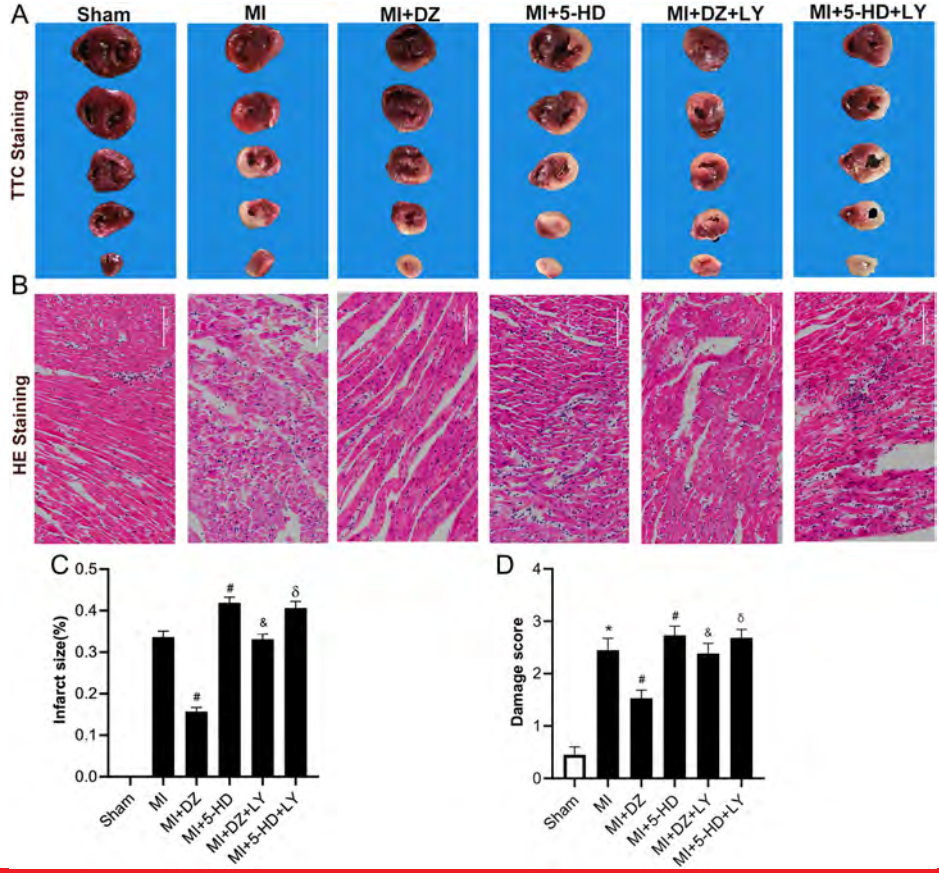

Figure 6. Diazoxide (10 mg/kg) exposure reduced AMI-induced infarct size, damage scores, and myocardial injury. These effects were reversed after exposure to LY294002; (A) cardiomyocytes of various cohorts stained using TTC (n = 4); (B) cardiomyocytes of various cohorts stained using HE (×200; n = 6); (C) quantitative analysis of the myocardial infarct area (n = 4); (D) damage scores of the different groups (n = 6). *$P < .05$ vs. sham group; #$P < .05$ vs. MI group; &$P < .05$ vs. MI + DZ group; δ$P > .05$ vs. MI + 5-HD group. AMI, acute myocardial infarction; DZ, diazoxide; HE, hematoxylin and eosin; TTC, triphenyl tetrazolium chloride; 5-HD, 5-hydroxydecanoate.

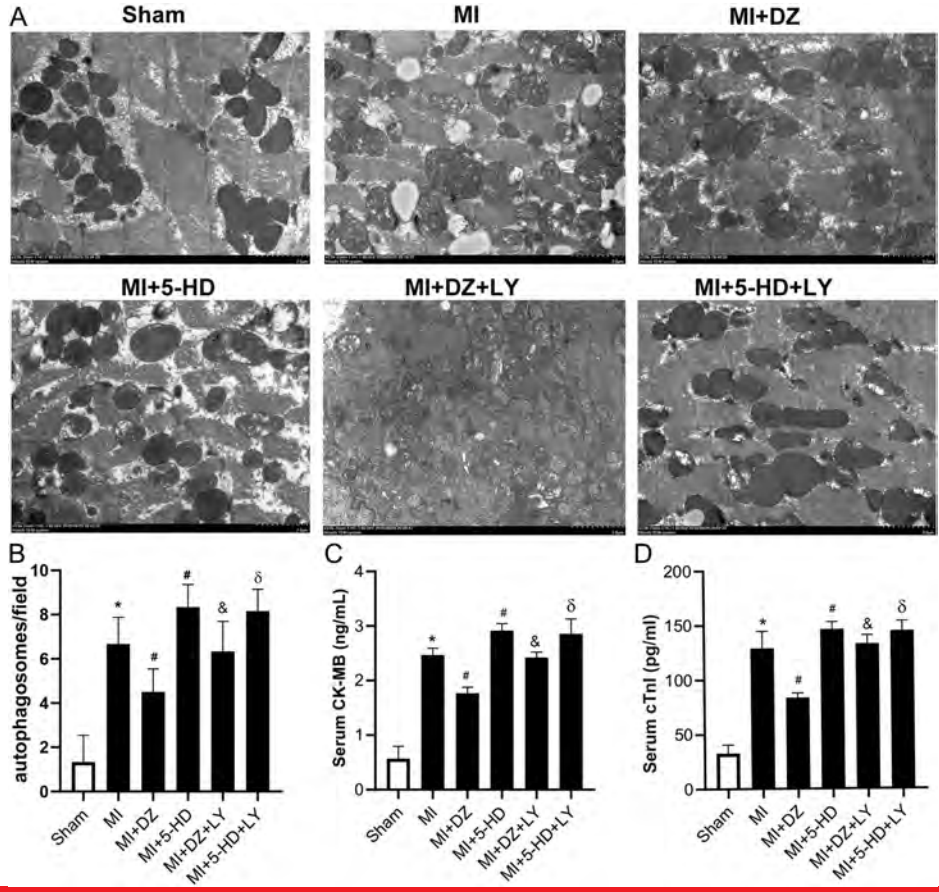

Figure 7. Transmission electron microscope (TEM) images and expression levels of markers of myocardial injury in rats in the different groups; (A) typical TEM (×3000) images of rat cardiac tissues; (B) quantification of the number of autophagosomes; (C) serum level of CK-MB; (D) serum level of cTnI. $^{*}P < .05$ vs. sham group; $^{\#}P < .05$ vs. MI group; $^{\&}P < .05$ vs. MI + DZ group; $^{\delta}P > .05$ vs. MI + 5-HD group. DZ, diazoxide; MI, myocardial infarction; 5-HD, 5-hydroxydecanoate.

to have conferred no significant difference in the MI + 5-HD group compared with the MI + 5-HD + LY group ($P > .05$).

Diazoxide Mitigates the Expression of Autophagy/Apoptosis-Related Proteins Following Acute Myocardial Infarction-Induced Ischemic Injury and LY294002 Abolishes This Effect

The expression of autophagy indicators, LC3B and Beclin1, and apoptotic indicators, Bax and Bcl-2 proteins, were determined using western blotting and qRT-PCR. As shown in Figures 8A and B and 9, LC3B, Beclin1, and Bax protein expression levels were elevated in the MI group compared with the sham group ($P < .05$). Diazoxide significantly reduced the levels of the autophagy markers and Bax protein, as shown in the MI + DZ group ($P < .05$). Similar to previous experiments, exposure to LY294002 suppressed the beneficial effects of DZ ($P < .05$). An opposite trend was observed for the apoptosis marker Bcl-2 protein. There were no significant differences in the expression of LC3B, Beclin1, Bax, and Bcl-2 between the MI + 5-HD and MI + 5-HD + LY groups ($P > .05$).

Diazoxide Protects Cardiomyocytes From Ischemic Injury Through the Activation of the AKT/mTOR Signaling Pathway

The AKT/mTOR signaling pathway and its response to mitoK$_{ATP}$ receptor activation in myocardial injury was investigated. As shown in Figure 8C, levels of p-AKT and p-mTOR were significantly elevated in the MI group compared with the sham group ($P < .05$), with even higher levels of these proteins found in the MI + DZ group ($P < .05$). LY294002 co-treatment attenuated these effects ($P < .05$). Consistently, there were no significant differences in the expression levels of these proteins between the MI + 5-HD and MI + 5-HD + LY groups ($P > .05$).

DISCUSSION

Myocardial infarction is the irreversible death of cardiac myocytes caused by a chronic lack of oxygen or fresh blood supply.[18] At present, treatment for AMI is based on myocardial reperfusion, that is, thrombolytic therapy, PCI, or coronary artery bypass grafting. However, timely myocardial reperfusion is a service that is not universally available. This study provides *in vivo* evidence that the degree of myocardial injury and autophagy levels peak at 2 hours post-ischemic injury. In addition, our results indicate that DZ confers protective effects against ischemic myocardial injury by stimulating the AKT/mTOR signaling pathway.

Mitochondria are not only dynamic cardiomyocytes that produce ATP and other metabolites but also signal regulators of cross-talk, and signal integration is involved in

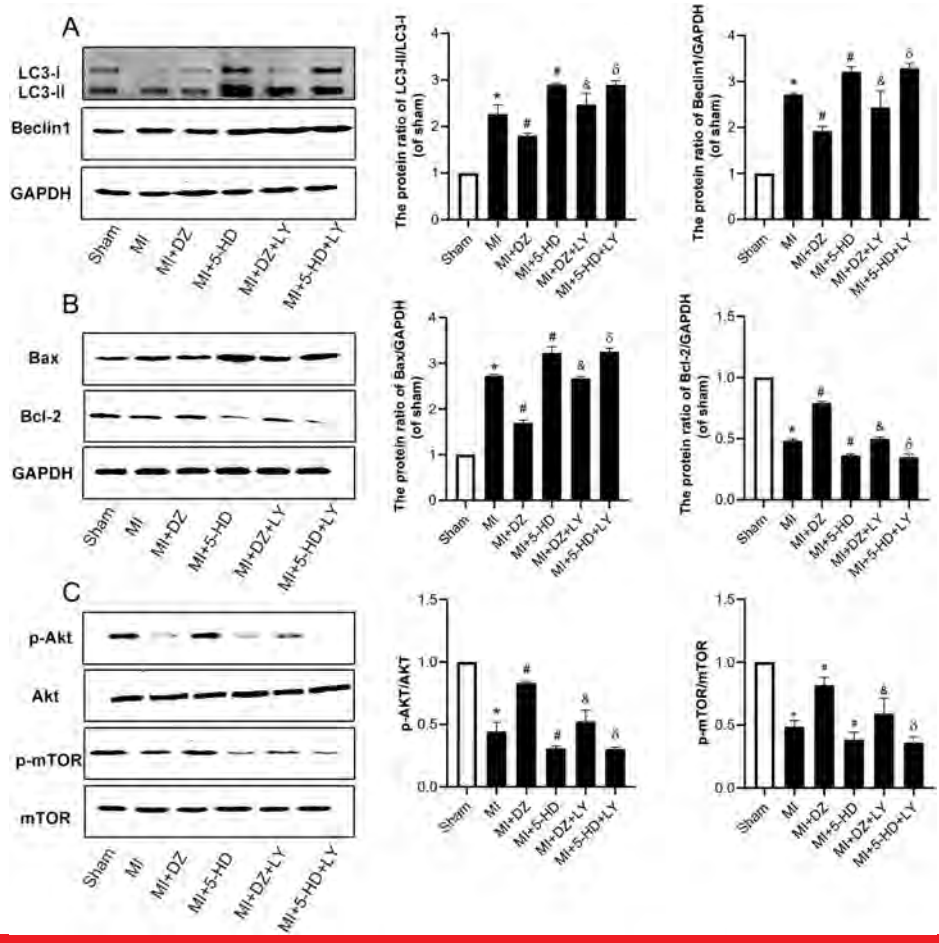

Figure 8. Western blotting analysis of autophagy/apoptosis-associated proteins and the AKT/mTOR pathway in the myocardial tissues of rats at 2 hours after ischemic injury (n = 6); (A) Western blotting bands and analysis of LC3-II and Beclin1; (Bb) Western blotting bands and analysis of Bax and Bc1-2; (C) Western blotting bands and analysis of p-AKT and p-mTOR. $^{*}P < .05$ vs. sham group; $^{\#}P < .05$ vs. MI group; $^{\&}P < .05$ vs. MI + DZ group; $^{\delta}P > .05$ vs. MI + 5-HD group. MI myocardial infarction; 5-HD, 5-hydroxydecanoate.

cardiomyocyte contraction, metabolism, inflammation, and death.[18] Studies have shown that oxidative stress, aberrant mitochondrial fission, defective mitochondrial fusion, and mitotic dysregulation may in combination trigger cardiomyocyte death through pleiotropic effects.[19] mitoK$_{ATP}$ receptors protect cardiomyocytes by inducing mitochondria to produce ROS via signals derived from opioid or bradykinin receptors. The excessive production of mROS in endothelial cells reduces the expression of endothelial NOS, which may cause vasospasm, limiting e blood flow to the reperfused heart.[20] Another putative effect is that the activation of mitoK$_{ATP}$ during ischemia attenuates mitochondrial Ca^{2+} overload and preserves mitochondrial Ca^{2+} homeostasis by regulating matrix volumes.[21] Diazoxide and 5-HD produce activation and inhibition effects, respectively, although these 2 drugs are limited by therapeutic modality specificity.[22] mitoK$_{ATP}$ is thought to deter oxidative stress-induced cardiomyocyte apoptosis. This channel is also involved in pre-apoptotic events, including the depolarization of the mitochondrial membrane, cytochrome c release, and the opening of the mitochondrial permeability transition pore.[23]

Protein kinase B (PKB; also known as AKT) is a serine/threonine kinase that is the cornerstone of cell migration, metabolism, angiogenesis, proliferation, growth, and survival. PKB/AKT exists in 3 mammalian isoforms, which have been characterized in the myocardial tissue as PKB α/AKT1, PKB β/AKT2, and PKB γ/AKT3, among which PKB α and PKB β are the most abundant.[16] Phosphatidylinositol 3-kinase has been shown to activate all 3 PKB subtypes. AKT suppresses pre-mitochondrial apoptosis through its ability of preventing cytochrome c discharge, thereby maintaining the mitochondrial membrane potential.[24-26] Activation of the PI3K/AKT signaling pathway triggers cardiomyocyte apoptosis through downstream targets, including pro-apoptotic Forkhead family of transcription factors, BAD, GSK-3β, and the Bcl-2 family member, caspase-9. IR injury blocks the activation of the PI3K/AKT pathway, protecting cardiomyocytes from IR injury.[27,28]

This study found that the post-AMI degree of myocardial injury in rats was greatest 2 hours after ischemic injury, as evidenced by the size of infarct, pathological damage scores, and serum levels of CK-MB and cTnI expression. Infarct size is

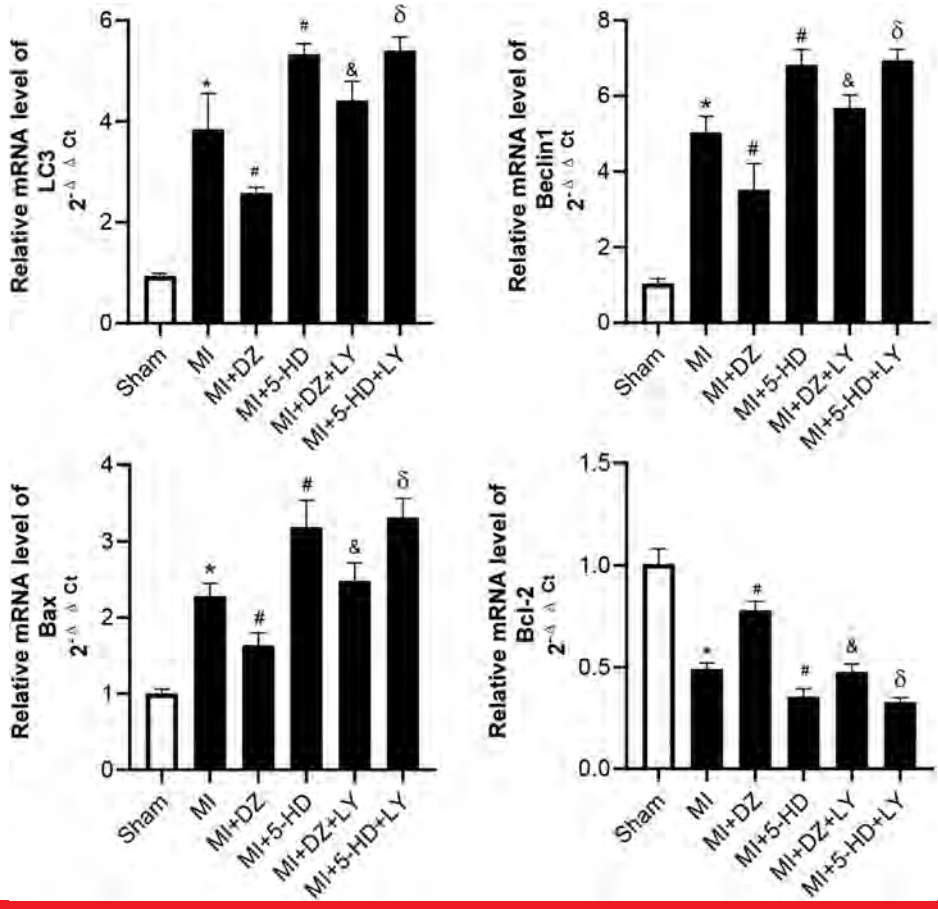

Figure 9. The mRNA expression of autophagy/apoptosis-related proteins in the myocardial tissues of rats at 2 hours after ischemic injury (n = 6). *$P < .05$ vs. sham group; #$P < .05$ vs. MI group; &$P < .05$ vs. MI + DZ group; δ$P > .05$ vs. MI + 5-HD group. MI, myocardial infarction; 5-HD, 5-hydroxydecanoate.

a major determinant of long-term prognosis.[19] cTnI is a component of the cardiac troponin regulatory complex, which is involved in cardiac muscle contraction and possesses high sensitivity and specificity to AMI.[29] Leakage of the CK-MB isozyme and cTnI from cardiomyocytes leads to elevated serum concentrations, which is reflective of the extent of myocardial injury.[30] In general, biochemical markers in humans were found to be elevated 3-4 days after myocardial ischemia, and substantial cardiomyocyte death occurs during acute ischemic attacks.[31] In our study, we found that the mortality rate was highest in the 2-hour group, indicating that a large number of cardiomyocytes died within 2 hours of the onset of ischemia. The rats that were compensated for the formation of collateral circulation survived within these 2 hours, and the myocardial damage was gradually reversed in the surviving rats, while rats that were not adequately compensated did not survive. Additionally, we noticed that DZ attenuated infarct size and maintained myocardial tissue integrity. 5-Hydroxydecanoate appeared to have an opposite effect to that of DZ, and LY294002 eliminated the beneficial effects of DZ. We also examined mitochondrial structures using a TEM. Mitochondria is the principal energy machine available for generating ATP in cells and is composed of 5 respiratory chain complexes, which are involved in cell metabolism and signal regulation. An intact mitochondria is critical in halting the progression of myocardial ischemic injury. Upon comparison with the sham group, the ultrastructure of the mitochondria in the MI group was found to be distorted, as evidenced by swelling, disintegration, shrinkage, and disappearance of the cristae. However, DZ treatment improved these aberrant morphological changes in the AMI rats. Taken together, DZ protects cardiomyocytes from AMI injury through its action on the PI3K/AKT signaling pathway.

Autophagy is a type II programmed cell death, which is the cornerstone of ischemic injury.[32] Controlled augmentation of the autophagic processes may promote the removal and recycling of damaged proteins and organelles, which can compensate for mitochondrial damage and maintain cell homeostasis.[33] However, the overactivation of the process of autophagy results in self-destruction and cell death and is marked by elevated levels of microtubule-associated protein 1 light chain 3 (LC3) and Beclin1. Many studies have used LC3-II/LC3-I ratios to analyze autophagy.[34] Inhibition of excessive levels of autophagy has been shown to significantly attenuate myocardial injury and apoptosis while improving cardiac function following MI/R injury both *in vivo* and *in vitro*.[35,36] This investigation found that the highest levels of LC3 and Beclin1 expression were at 2 hours after AMI. Diazoxide could decrease the expression levels of LC3 and Beclin1. Meanwhile, we found that the number of

autophagosomes in the DZ group was lower than that of the MI group. The expression of LC3 and Beclin1 and the number of autophagosomes in the DZ + LY294002 group were higher than that of the DZ group. These results prove that DZ prevents excessive autophagy through its action on the PI3K/AKT/mTOR pathway.

Ischemic injury is mainly induced by cell apoptosis. The BCL-2 gene family regulates apoptosis through the mitochondrial pathway. There are 3 known subgroups of the BCL-2 protein family: antiapoptotic proteins (such as BCL-2 and Bcl-xL), pro-apoptotic proteins (such as Bax and Bak), and BH3-only proteins (such as Bid and Bad). Bax and Bcl-2 are the most well-known proteins. Bax promotes apoptosis by stimulating cytochrome c release, activating caspase-9, and forming a dimer with Bcl-2 to inhibit Bcl-2 activity, and the antiapoptotic effect of Bcl-2 prevents the discharge and revitalization of mitochondrial cytochrome c.[37] Apoptosis is inhibited by AKT at the mitochondrial level due to its involvement in preventing cytochrome c release, thereby maintaining mitochondrial membrane potential.[21] Activation of PI3K and its downstream regulator, AKT, exert a protective effect against post-ischemic injury, based on their ability of inhibiting cell apoptosis.[38] These findings are reflected in our study. We found a decrease in Bax expression, an increase in Bcl-2 expression, and suppression of levels of apoptosis in DZ-treated rats. Opposite findings were observed in the 5-HD-treated rats. LY294002 reversed the antiapoptotic effect of DZ on ischemic cardiomyocytes. The mitoK$_{ATP}$ activator, DZ, prevents cardiomyocyte apoptosis in a PI3K/AKT pathway-dependent manner.

The PI3K/AKT signaling pathway is a classic proliferation and antiapoptotic signaling pathway.[39] Activation of this pathway is integral for ensuring cell survival. mTOR is the principal downstream modulator of the PI3K/AKT pathway.[40] This study found that DZ treatment upregulated AKT and mTOR phosphorylation, while attenuating AMI-induced myocardial injury, in a rat model of AMI. In contrast, LY294002 treatment downregulated AKT and mTOR phosphorylation and reversed the positive effects exerted by DZ. Terashima et al[21] found that pretreatment with DZ increased PI3K-receptor-dependent phosphorylation of GSK-3β in an *in vitro* IR model and prevented myocardial necrosis by activating mitoK$_{ATP}$ channels. However, our study differed significantly in its *in vivo* design and more closely mimicked real-life clinical scenarios.

We investigated the pathological and autophagic features of the rats post-AMI across various time windows to simulate clinical scenarios of delayed reperfusion and found that autophagic activity was the highest and myocardial tissue damage was the most serious at 2 hours after AMI, resulting in the highest mortality rate as well. The compensatory effect of collateral circulation exerts a significant benefit on myocardial injury 2 hours after the onset of MI. Therefore, in the absence of emergency PCI within 6 hours following an AMI, effort should be made to rescue damaged cardiomyocytes within 2 hours before the onset of any lasting damage.

In addition, we found that myocardial injury and the expression levels of p-AKT and p-mTOR increased or decreased when DZ or 5-HD alone was used, respectively. The addition of LY294002 had no effect on the 5-HD group, and the results show that the addition of this agent to the DZ group could activate the AKT/mTOR pathway, which is consistent with the findings of Wang et al[41] However, this finding is contrary to previously published results, which found that PI3K/AKT was an upstream activator of mitoK$_{ATP}$.[42] In response to this controversial result, we hypothesized that mitoK$_{ATP}$ may not only be regulated by AKT/mTOR, but its opening and closing may also be mediated by other pathways during an AMI. Interestingly, this speculation is consistent with the findings of Terashima et al[21], who postulated that mitoK$_{ATP}$ channel-mediated myocardial protection post-ischemic injury is only partially achieved through the PI3K/AKT pathway. Hu et al[2] suggested that the PI3K/AKT pathway may proceed both upstream and downstream of mitoK$_{ATP}$. We hypothesized that the AKT/mTOR pathway and mitoK$_{ATP}$ channels may not be linearly linked and may in fact be more interconnected that initially speculated.

Study Limitations

First, this study was limited to an investigation on the AKT/mTOR pathway and mitoK$_{ATP}$ channels for the treatment of AMI. mitoK$_{ATP}$ may exert its effects on acute myocardial ischemia through other signaling pathways. Moreover, the involvement of additional interactions between the AKT/mTOR pathway and other pathways activated by the mitoK$_{ATP}$ channel cannot be disregarded. Second, both DZ and 5-HD were used to activate and inhibit the mitoK$_{ATP}$ channel, respectively. These reagents remain to be limited in their levels of specificity. Third, the potential species differences in AKT/mTOR signaling should be considered.

CONCLUSION

This investigation demonstrated that the degree of myocardial tissue injury and autophagic activity are the highest at 2 hours post-ischemic injury in rat models of AMI. Therefore, in the absence of standard medical treatment following an acute myocardial infarction, drugs should be administered as soon as possible to rescue damaged cardiomyocytes. It is worthy to note that the findings of our experiments are based on *in vivo* animal models and not directly in a clinical setting of ST-segment elevation myocardial infarction. We evaluated the cardioprotective effects of mitochondrial ATP-sensitive potassium channels at a molecular mechanistic level using autophagy agonists and inhibitors. The mitoK$_{ATP}$ activator, DZ, directly or indirectly decreases levels of autophagy and apoptosis by activating the AKT/mTOR pathway, thereby ameliorating myocardial ischemia injury.

Ethics Committee Approval: The Ethics Committee for the Experimental Use of Animals at Guangxi Medical University (Guangxi, China) on May 3, 2021 (no. 202004022) reviewed and approved all experimental protocols used in this investigation.

Peer-Review: Externally peer-reviewed.

Author Contributions: W.W. and Q.F.C. conceived the hypothesis, Q.Z. and L.D.Z. performed a literature search, chose related

studies, participated into data analysis, and wrote the manuscript. Others participated in experimental operation and data collection. W.W. and Q.F.C. contributed to checking the acquired data and revising the manuscript. All authors read and approved the final draft.

Acknowledgments: None.

Declaration of Interests: The authors declare that they have no competing interest.

Funding: This study was supported by the National Nature Science Foundation of China (grant no. 81860346 and 81560318); Training Project of "139" Program for High-level Medical Talents in Guangxi (no. G201903034); Nanning Qingxiu District Science and Technology Project (no. 2020046); and Guangxi Medical and Health Suitable Technology Development Project (no. S2017021). The funders were not involved in study design, in data collection and analysis, or in the writing of this manuscript.

REFERENCES

1. Tong S, Zhang L, Joseph J, Jiang X. Celastrol pretreatment attenuates rat myocardial ischemia/reperfusion injury by inhibiting high mobility group box 1 protein expression via the PI3K/Akt pathway. *Biochem Biophys Res Commun*. 2018;497(3):843-849. [CrossRef]
2. Hu X, Wu B, Wang X, et al. Minocycline attenuates ischemia-induced ventricular arrhythmias in rats. *Eur J Pharmacol*. 2011;654(3):274-279. [CrossRef]
3. Thomas CJ, Lim NR, Kedikaetswe A, et al. Evidence that the MEK/ERK but not the PI3K/Akt pathway is required for protection from myocardial ischemia-reperfusion injury by 3′,4′-dihydroxyflavonol. *Eur J Pharmacol*. 2015;758:53-59. [CrossRef]
4. Li Z, Li J, Zhu L, et al. Celastrol nanomicelles attenuate cytokine secretion in macrophages and inhibit macrophage-induced corneal neovascularization in rats. *Int J Nanomedicine*. 2016;11:6135-6148. [CrossRef]
5. Noma A. ATP-regulated K+ channels in cardiac muscle. *Nature*. 1983;305(5930):147-148. [CrossRef]
6. Garlid KD, Paucek P, Yarov-Yarovoy V, et al. Cardioprotective effect of diazoxide and its interaction with mitochondrial ATP-sensitive K+ channels. Possible mechanism of cardioprotection. *Circ Res*. 1997;81(6):1072-1082. [CrossRef]
7. Pain T, Yang XM, Critz SD, et al. Opening of mitochondrial K(ATP) channels triggers the preconditioned state by generating free radicals. *Circ Res*. 2000;87(6):460-466. [CrossRef]
8. Gourine AV, Molosh AI, Poputnikov D, Bulhak A, Sjöquist PO, Pernow J. Endothelin-1 exerts a preconditioning-like cardioprotective effect against ischaemia/reperfusion injury via the ET(A) receptor and the mitochondrial K(ATP) channel in the rat in vivo. *Br J Pharmacol*. 2005;144(3):331-337. [CrossRef]
9. Imani A, Faghihi M, Sadr SS, Keshavarz M, Niaraki SS. Noradrenaline reduces ischemia-induced arrhythmia in anesthetized rats: involvement of alpha1-adrenoceptors and mitochondrial K ATP channels. *J Cardiovasc Electrophysiol*. 2008;19(3):309-315. [CrossRef]
10. Jafari M, Ghadami E, Dadkhah T, Akhavan-Niaki H. PI3k/AKT signaling pathway: erythropoiesis and beyond. *J Cell Physiol*. 2019;234(3):2373-2385. [CrossRef]
11. Sulaiman D, Li J, Devarajan A, et al. Paraoxonase 2 protects against acute myocardial ischemia-reperfusion injury by modulating mitochondrial function and oxidative stress via the PI3K/Akt/GSK-3β RISK pathway. *J Mol Cell Cardiol*. 2019;129:154-164. [CrossRef]
12. Vander Heiden MG, Plas DR, Rathmell JC, Fox CJ, Harris MH, Thompson CB. Growth factors can influence cell growth and survival through effects on glucose metabolism. *Mol Cell Biol*. 2001;21(17):5899-5912. [CrossRef]
13. Henshall DC, Araki T, Schindler CK, et al. Activation of Bcl-2-associated death protein and counter-response of Akt within cell populations during seizure-induced neuronal death. *J Neurosci*. 2002;22(19):8458-8465. [CrossRef]
14. Zi C, Zhang C, Yang Y, Ma J. Penehyclidine hydrochloride protects against anoxia/reoxygenation injury in cardiomyocytes through ATP-sensitive potassium channels, and the Akt/GSK-3β and Akt/mTOR signaling pathways. *Cell Biol Int*. 2020;44(6):1353-1362. [CrossRef]
15. Xin BR, Li P, Liu XL, Zhang XF. Visfatin relieves myocardial ischemia-reperfusion injury through activation of PI3K/Akt/HSP70 signaling axis. *Eur Rev Med Pharmacol Sci*. 2020;24(20):10779-10789. [CrossRef]
16. Linares-Palomino J, Husainy MA, Lai VK, Dickenson JM, Galiñanes M. Selective blockade of protein kinase B protects the rat and human myocardium against ischaemic injury. *J Physiol*. 2010;588(12):2173-2191. [CrossRef]
17. Guan BF, Dai XF, Huang QB, et al. Icariside II ameliorates myocardial ischemia and reperfusion injury by attenuating inflammation and apoptosis through the regulation of the PI3K/AKT signaling pathway. *Mol Med Rep*. 2020;22(4):3151-3160. [CrossRef]
18. Zhou H, Ren J, Toan S, Mui D. Role of mitochondrial quality surveillance in myocardial infarction: from bench to bedside. *Ageing Res Rev*. 2021;66:101250. [CrossRef]
19. Zhu H, Tan Y, Du W, et al. Phosphoglycerate mutase 5 exacerbates cardiac ischemia-reperfusion injury through disrupting mitochondrial quality control. *Redox Biol*. 2021;38:101777. [CrossRef]
20. Wang J, Toan S, Zhou H. New insights into the role of mitochondria in cardiac microvascular ischemia/reperfusion injury. *Angiogenesis*. 2020;23(3):299-314. [CrossRef]
21. Terashima Y, Sato T, Yano T, et al. Roles of phospho-GSK-3β in myocardial protection afforded by activation of the mitochondrial K ATP channel. *J Mol Cell Cardiol*. 2010;49(5):762-770. [CrossRef]
22. Lim KH, Javadov SA, Das M, Clarke SJ, Suleiman MS, Halestrap AP. The effects of ischaemic preconditioning, diazoxide and 5-hydroxydecanoate on rat heart mitochondrial volume and respiration. *J Physiol*. 2002;545(3):961-974. [CrossRef]
23. Bodiga S, Zhang R, Jacobs DE, et al. Protective actions of epoxyeicosatrienoic acid: dual targeting of cardiovascular PI3K and KATP channels. *J Mol Cell Cardiol*. 2009;46(6):978-988. [CrossRef]
24. Uchiyama T, Engelman RM, Maulik N, Das DK. Role of Akt signaling in mitochondrial survival pathway triggered by hypoxic preconditioning. *Circulation*. 2004;109(24):3042-3049. [CrossRef]
25. Marinovic J, Ljubkovic M, Stadnicka A, Bosnjak ZJ, Bienengraeber M. Role of sarcolemmal ATP-sensitive potassium channel in oxidative stress-induced apoptosis: mitochondrial connection. *Am J Physiol Heart Circ Physiol*. 2008;294(3):H1317-H1325. [CrossRef]
26. Yin H, Chao L, Chao J. Adrenomedullin protects against myocardial apoptosis after ischemia/reperfusion through activation of Akt-GSK signaling. *Hypertension*. 2004;43(1):109-116. [CrossRef]
27. Zhu M, Feng J, Lucchinetti E, et al. Ischemic postconditioning protects remodeled myocardium via the PI3K-PKB/Akt reperfusion injury salvage kinase pathway. *Cardiovasc Res*. 2006;72(1):152-162. [CrossRef]

28. Shu L, Zhang W, Huang C, Huang G, Su G. Troxerutin protects Against myocardial ischemia/reperfusion injury via Pi3k/Akt pathway in rats. *Cell Physiol Biochem*. 2017;44(5):1939-1948. [CrossRef]
29. Li HX, Han SY, Ma X, et al. The saponin of red ginseng protects the cardiac myocytes against ischemic injury in vitro and in vivo. *Phytomedicine*. 2012;19(6):477-483. [CrossRef]
30. Qiao Z, Ma J, Liu H. Evaluation of the antioxidant potential of Salvia miltiorrhiza ethanol extract in a rat model of ischemia-reperfusion injury. *Molecules*. 2011;16(12):10002-10012. [CrossRef]
31. Tan Y, Mui D, Toan S, Zhu P, Li R, Zhou H. SERCA overexpression improves mitochondrial quality control and attenuates cardiac microvascular ischemia-reperfusion injury. *Mol Ther Nucleic Acids*. 2020;22:696-707. [CrossRef]
32. Ke J, Yao B, Li T, Cui S, Ding H. A2 adenosine receptor-mediated cardioprotection against reperfusion injury in rat hearts is associated with autophagy downregulation. *J Cardiovasc Pharmacol*. 2015;66(1):25-34. [CrossRef]
33. Xiao J, Zhu X, Ji G, et al. Ulinastatin protects cardiomyocytes against ischemiareperfusion injury by regulating autophagy through mTOR activation. *Mol Med Rep*. 2014;10(4):1949-1953. [CrossRef]
34. Xuan F, Jian J, Lin X, et al. 17-Methoxyl-7-Hydroxy-Benzene-Furan chalcone ameliorates myocardial ischemia/reperfusion injury in rat by inhibiting apoptosis and autophagy via the PI3K-Akt signal pathway. *Cardiovasc Toxicol*. 2017;17(1):79-87. [CrossRef]
35. Yao T, Ying X, Zhao Y, et al. Vitamin D receptor activation protects against myocardial reperfusion injury through inhibition of apoptosis and modulation of autophagy. *Antioxid Redox Signal*. 2015;22(8):633-650. [CrossRef]
36. Zheng Y, Gu S, Li X, et al. Berbamine postconditioning protects the heart from ischemia/reperfusion injury through modulation of autophagy. *Cell Death Dis*. 2017;8(2):e2577. [CrossRef]
37. Zhang Y, Liu D, Hu H, Zhang P, Xie R, Cui W. HIF-1α/BNIP3 signaling pathway-induced-autophagy plays protective role during myocardial ischemia-reperfusion injury. *Biomed Pharmacother*. 2019;120:109464. [CrossRef]
38. Zhang KR, Liu HT, Zhang HF, et al. Long-term aerobic exercise protects the heart against ischemia/reperfusion injury via PI3 kinase-dependent and Akt-mediated mechanism. *Apoptosis*. 2007;12(9):1579-1588. [CrossRef]
39. Bai J, Wang Q, Qi J, et al. Promoting effect of baicalin on nitric oxide production in CMECs via activating the PI3K-AKT-eNOS pathway attenuates myocardial ischemia-reperfusion injury. *Phytomedicine*. 2019;63:153035. [CrossRef]
40. Zhang G, Wang Q, Wang W, et al. Tempol protects Against acute renal injury by regulating PI3K/Akt/mTOR and GSK3β signaling cascades and afferent arteriolar activity. *Kidney Blood Press Res*. 2018;43(3):904-913. [CrossRef]
41. Wang Y, Ahmad N, Kudo M, Ashraf M. Contribution of Akt and endothelial nitric oxide synthase to diazoxide-induced late preconditioning. *Am J Physiol Heart Circ Physiol*. 2004;287(3):H1125-H1131. [CrossRef]
42. Hausenloy DJ, Yellon DM. Survival kinases in ischemic preconditioning and postconditioning. *Cardiovasc Res*. 2006;70(2):240-253. [CrossRef]