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Original Article

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Opening of the inward rectifier potassium channel alleviates maladaptive tissue repair following myocardial infarction

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Abstract

Activation of the inward rectifier potassium current (IK1) channel has been reported to be associated with suppression of ventricular arrhythmias. In this study, we tested the hypothesis that opening of the IK1 channel with zacopride (ZAC) was involved in the modulation of tissue repair after myocardial infarction. Sprague-Dawley rats were subject to coronary artery ligation and ZAC was administered intraperitoneally (15 μg/kg/day) for 28 days. Compared with the ischemia group, treatment with ZAC significantly reduced the ratio of heart/body weight and the cross-sectional area of cardiomyocytes, suggesting less cardiac hypertrophy. ZAC reduced the accumulation of collagen types I and III, accompanied with decrease of collagen area, which were associated with a reduction of collagen deposition in the fibrotic myocardium. Echocardiography showed improved cardiac function, evidenced by the reduced left ventricular end-diastolic dimension and left ventricular end-systolic dimension, and the increased ejection fraction and fractional shortening in ZAC-treated animals (all $P < 0.05$ vs. ischemia group). In coincidence with these changes, ZAC up-regulated the protein level of the IK1 channel and down-regulated the phosphorylation of mammalian target of rapamycin (mTOR) and 70-kDa ribosomal protein S6 (p70S6) kinase. Administration of chloroquine alone, an IK1 channel antagonist, had no effect on all the parameters measured, but significantly blocked the beneficial effects of ZAC on cardiac repair. In conclusion, opening of the IK1 channel with ZAC inhibits maladaptive tissue repair and improves cardiac function, potentially mediated by the inhibition of ischemia-activated mTOR-p70S6 signaling pathway via the IK1 channel. So the development of pharmacological agents specifically targeting the activation of the IK1 channel may protect the heart against myocardial ischemia-induced cardiac dysfunction.

Key words: cardiac repair, inward rectifier potassium channel, zacopride, mTOR-p70S6, myocardial infarction

Introduction

Potassium channels, the most widely distributed type of ion channel in mammalian species, are classified as voltage-gated and ligand-gated channels. Activation of potassium channels regulates the secretion of hormones or neurotransmitters and can control the shape of the action potential waveform [1]. Inwardly rectifying potassium channels are specific subsets of potassium-selective ion channels. During action potentials, the inward rectifier current (IK1) through the potassium channel plays a crucial role in maintaining resting membrane potential and excitability in the heart [2,3]. Down-regulated expression and function of the IK1 channel have been associated with a variety of cardiac diseases including hypertension,

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cardiac hypertrophy, heart failure, and cardiomyopathies by prolonging action potential duration. Accordingly, treatment with β-receptor blockers improves heart function, partially by reversing the down-regulated IK1 channel [4,5]. Therefore, the exploration of novel pharmaceutical agents by targeting the modulation of the IK1 channel may provide a potential opportunity in the treatment of heart diseases.

Zacopride (ZAC), a potent and selective antagonist at the 5-hydroxytryptamine receptor (5-HT3R) and an agonist at the 5-HT4R, has strong anti-emetic effects and also stimulates aldosterone secretion via activating the 5-HT4R on the adrenal glands [6]. Our previous study demonstrated that ZAC is also a selective agonist at the inwardly rectifying channel in cardiomyocytes by activating the Kir2.1 subunit [7,8]. ZAC shortens the duration of the action potential and hyperpolarizes the resting membrane potential by moderately increasing the current density of the IK1. In the *in vivo* models, it has also been found that ZAC suppresses aconitine-triggered or infarction-induced ventricular arrhythmias [7,9].

Maladaptive tissue repair after myocardial infarction is a major determinant in the development of left ventricular dysfunction. It is characterized by alterations in the morphologic and interstitial changes in ventricular architecture, ultimately resulting in heart failure [10]. The cellular events involved include infiltration of macrophages, proliferation of myofibroblasts, and deposition of interstitial collagen [11]. Experimental studies and clinical observations have demonstrated that repression of maladaptive tissue repair is a desirable therapeutic target for improving cardiac function and clinical outcomes [12]. A recent study has reported that addition of ZAC into the St. Thomas cardioplegia significantly improves left ventricular function after ischemia/reperfusion in the isolated rat heart model [13]. Although others and our lab have previously shown that ZAC works as a selective IK1 channel agonist [7,13], it is unknown whether opening of the IK1 channel with ZAC can provide beneficial effects on tissue repair following myocardial infarction. Recent studies have shown that the mammalian target of rapamycin (mTOR) and 70-kDa ribosomal protein S6 kinase (p70S6K) are involved in tissue repair and fibrosis [14,15]. Therefore, the purpose of this study was to test the hypothesis that stimulation of the IK1 channel with ZAC protects the heart against maladaptive cardiac repair after ischemia by down-regulating mTOR and p70S6K expressions. The effects of ZAC on collagen deposition, fibrotic tissue formation, cardiac function and the IK1 channel protein expression were examined. To address the potential mechanisms of ZAC on cardiac repair, the protein expressions of phosphorylated mTOR (p-mTOR) and phosphorylated p70S6K (p-p70S6K) induced by opening IK1 channel were also analyzed.

Materials and Methods

Ethical approval

The animals were purchased from the Experimental Animal Center of Shanxi Medical University. The animal procedures conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (eighth edition, revised, 2011) and were approved by the Bioethics Committee of Shanxi Medical University.

Surgical preparation of animals

Male Sprague-Dawley (SD) rats weighing 200–250 g were anesthetized with an intraperitoneal injection of chloral hydrate (0.3 g/kg) and mechanically ventilated with oxygen-enriched room air using a rodent ventilator. Left thoracotomy was performed at the fourth intercostal space, and the pericardium was gently opened to expose the heart. Myocardial infarction was induced by ligation of the left coronary artery (LCA) with a silk suture. At the end of the surgical operation, the incisions were closed in layers. The chest and endotracheal tubes were removed after the spontaneous breathing was recovered. Rats that died within 24 h after the surgery were excluded from the study.

Experimental protocol and group

The SD rats were randomly assigned into six groups and the duration of the experiment was set as 4 weeks for all groups. (i) Sham (n = 10): rats underwent a thoracotomy without ischemia; (ii) sham + ZAC (n = 10): rats received intraperitoneal ZAC injection (a selective IK1 channel agonist; Tocris, Bristol, UK) at a dose of 15 μg/kg/day; (iii) ischemia (n = 13): rats were subject to a permanent LCA ligation without reperfusion; (iv) ZAC treatment (n = 17): ZAC was injected at a same dose in the sham group after LCA ligation; (v) chloroquine (Chlor, an IK1 channel antagonist; Sigma, St Louis, USA) treatment (n = 13): Chlor was injected intraperitoneally at a dose of 7.5 μg/kg/day after LCA ligation; (vi) ZAC + Chlor (n = 14): ZAC and Chlor were injected intraperitoneally at a same dose as in the ZAC and Chlor groups, respectively. The doses of ZAC and Chlor used in this study were primarily based on previous studies showing that ZAC has anti-arrhythmic effect [7] and Chlor blocks the inhibitory action of the IK1 channel activation with ZAC on arrhythmia [16].

Calculation of heart/body weight ratio and left ventricle/body weight ratio

At the end of the experimental period in each group, the rat was euthanized and the heart was removed. The atria and right ventricular free wall were trimmed away, and the left ventricle (LV) was weighed. The heart weight/body weight (HW/BW) ratio and the LV weight/body weight (LVW/BW) ratio were calculated based on individual BW.

Measurement of infarct size and calculation of myocyte cross-sectional area

The myocardial samples from all groups were fixed in 10% phosphate-buffered formalin solution and embedded in paraffin wax for histological analysis. Cryosections (6-μm thick) were made using a Microtome cryostat (Leica, Wetzlar, Germany). The LV tissue of the infarcted heart was carefully dissected into three parts, i.e. the infarct zone, the border zone and the non-infarct zone. The boundary length of the infarcted and non-infracted endocardial and epicardial surface were traced with a planimeter digital image analyzer. Infarct size (%) was calculated as the ratio of average scar circumferences in the endocardium and the epicardium relative to the whole LV average circumferences. Rats only with infarct size >30% of the whole LV were selected for analysis. Myocyte cross-sectional area (CSA, a robust indicator of myocyte diameter) was determined from the non-infarct zone after the tissue sections were stained with hematoxylin-eosin. A total of 100 cardiomyocytes in each group were averaged using a microscope (Olympus, Tokyo, Japan) under a high-powered field (HPF) (×400 magnification).

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Immunohistochemistry and Masson's trichrome staining

Collagen deposition and fibrosis formation in the non-infarcted myocardium were evaluated using immunohistochemistry and Masson's trichrome staining. The expression of collagens I and III of LV samples from the non-infarct zone was evaluated by immunohistochemical staining. In brief, sections were incubated with the respective primary antibodies: a rabbit anti-collagen I polyclonal antibody and a rabbit anti-collagen III polyclonal antibody (1:200; Abcam, Cambridge, UK). The positive levels of collagens I and III staining were analyzed by a computer-assisted morphometry (Aperio Technologies, Vista, USA). Masson's trichrome staining was used to evaluate fibrotic tissue in the myocardium. The staining protocol produces collagen blue, nuclei black, and myocytes red. Fibrosis was quantified by the computer-assisted image analysis software (Aperio Technologies). The value was expressed as a ratio of the positively stained area to the total area.

Hemodynamic assessment

At the end of the experiment, rats were anesthetized and a 2-F micromanometer-tipped catheter was inserted into the LV cavity through the right carotid artery using a microtip pressure transducer (Taimeng Software Co., Chengdu, China) for hemodynamic measurements. LV end-diastolic pressure (LVEDP), LV end-systolic pressure (LVESP), the maximal rate of LV pressure developments ($\pm dp/dt_{max}$) and the heart rate (HR) were determined and averaged from 10 consecutive beats using a recording system (Taimeng Software Co.).

Detection of cardiac function by echocardiography

Echocardiography was used to assess left ventricular function by a two-dimensional (2D) guided M-mode ultrasound system (10S probe; Vivid 7, GE, Milwaukee, USA) via a linear transducer. M-mode tracing of the LV was obtained from the parasternal long-axis view to measure LV end-diastolic diameter (LVEDD), LV end-systolic diameter (LVESD), fractional shortening fraction (FS) and the ejection fraction (EF). All measurements were averaged over three consecutive cardiac cycles by a blinded observer at the end of the study.

Protein expressions of the IK1, p-mTOR, and p-p70S6K determined by western blot analysis

Tissue samples from the border zone were homogenized and the supernatant protein concentration was determined with the BCA protein assay reagent kit (Cwbiotech, Beijing, China). Sodium dodecyl sulfate-polyacrylamide gel electropheresis was performed using 30 μg of protein, which was then electrotransferred to nitrocellulose membranes. The membranes were first incubated with 5% non-fat milk in Tris-buffered saline (TBS). After being washed three times in 0.1% Tween 20-TBS, the membranes were incubated with following antibodies: rabbit anti-IK1 polyclonal antibody (1:200; Santa Cruz Biotech, Santa Cruz, USA), rabbit anti-p-mTOR polyclonal antibody (1:2000; Sigma), rabbit anti-p-p70S6K monoclonal antibody (1:1000; Cell Signaling, Beverly, USA), or anti-β-actin antibody (1:2000; Cwbiotech) at 4°C overnight. Bound antibody was detected by horseradish peroxidase conjugated anti-rabbit IgG (1:5000; Cwbiotech). Bands were detected with chemiluminescent detection reagent (Applygen Technologies Int., Beijing, China). β-actin was used as a protein-loading standard control. Digital images were processed using a digital gel imaging and analysis system (Jeda Technologies, Shenzhen, China). The final results were calculated as a ratio of intensity from each band relative to that of β-actin.

Chlor plasma concentration determined by high-performance liquid chromatography

The kinetics of Chlor concentration in plasma after the administration was measured using a high-performance liquid chromatography (HPLC). Male SD rats weighing 200–250 g were intraperitoneally administered Chlor (7.5 μg/kg/day) for 28 days. One hour after the administration of Chlor on days 1, 7, 14, 21 and 28, six rats from the Chlor-treated group and one from the control group were lightly anesthetized with ether. Blood samples were withdrawn by cardiac puncture, then transferred into lithium-heparin tubes and centrifuged (208 *g*, 10 min). The separated plasma was stored at −20°C until analysis. Plasma samples (0.5 ml each) were analyzed by HPLC according to previously reported procedures [17].

Statistical analysis

All statistical analyses were performed using SPSS software (Version 11.5; SPSS, Inc., Chicago, USA). Data were expressed as the mean ± SD. Differences in mortality among groups were analyzed by the χ^2-test. Quantitative data were analyzed by one-way ANOVA. Multiple comparisons were performed using the least significant different test. $P < 0.05$ was considered statistically significant.

Results

Changes in ratios of HW/BW and LVW/BW, CSA, and infarct size

The ratios of HW/BW and LVW/BW were significantly higher in the ischemia group compared with those in the sham group (all $P < 0.05$, respectively). Treatment with ZAC reduced the ratios of HW/BW and LVW/BW relative to the ischemia at the end of 4-week study (**Fig. 1A**). Consistent with these changes, the CSA of cardiomyocytes, an indicator of cell hypertrophy, was significantly increased by 63.4% ± 15.1% relative to the sham group (**Fig. 1B**) in the non-infarcted myocardium in the ischemia group, which was also significantly reduced in the ZAC group (20.2% ± 4.8%; $P < 0.05$), suggesting an inhibition of cardiac hypertrophy by ZAC. Treatment with Chlor only, an IK1 channel antagonist, did not show any changes of these parameters relative to the ischemia, but significantly blocked the inhibitory effects of ZAC on HW/BW and LVW/BW ratios (**Fig. 1A**) as well as CSA (**Fig. 1B**) compared with the ZAC group. These results suggest that the IK1 channel antagonist itself has no effect on cardiac hypertrophy, and the inhibition of ZAC on hypertrophy is mediated by activating IK1 channel. Four weeks after coronary artery ligation, infarct size on the cross-sectional slices of the LV was averaged to be 42.5% ± 3.2%, 38.6% ± 4.2%, 42.1% ± 3.8%, and 40.7% ± 4.1% in the ischemia, ZAC, Chlor, and ZAC + Chlor groups, respectively. No statistical difference was found among these groups. In addition, there was no statistical difference in the survival rate among all groups (χ^2-test, $x^2 = 10.503$, $P = 0.062$).

Collagen deposition and fibrotic tissue formation

As shown in **Fig. 2**, collagens I and III were not constitutively expressed in the sham and sham+ZAC groups. Four weeks of infarction caused a significant expression of both collagens I and III

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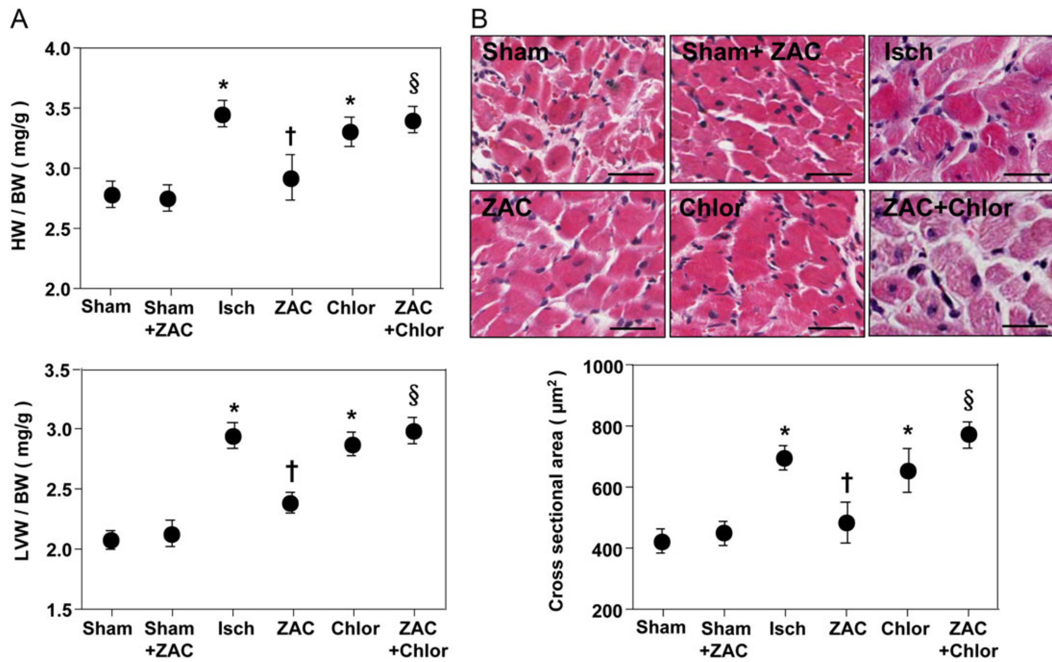

Figure 1. The ratios of HW/BW, LVW/BW, and the myocyte CSAs in different experimental groups (A) The HW/BW and LVW/BW were calculated by the tissue weight. (B) The myocyte CSA was measured morphometrically at a HPF. Sham, rats underwent a thoracotomy without ischemia; sham + ZAC, rats received intraperitoneal ZAC injection; ischemia (Isch), rats were subject to a permanent LAD ligation; ZAC treatment, ZAC was injected daily for 4 weeks; Chlor, rats received Chlor treatment daily; ZAC + Chlor, ZAC and Chlor were injected intraperitoneally to the same rat. Values are presented as the mean ± SD (n = 10 in each group). Scale bars: 50 µm. *$P < 0.05$ Isch versus sham; †$P < 0.05$ ZAC versus Isch; §$P < 0.05$ ZAC + Chlor versus ZAC.

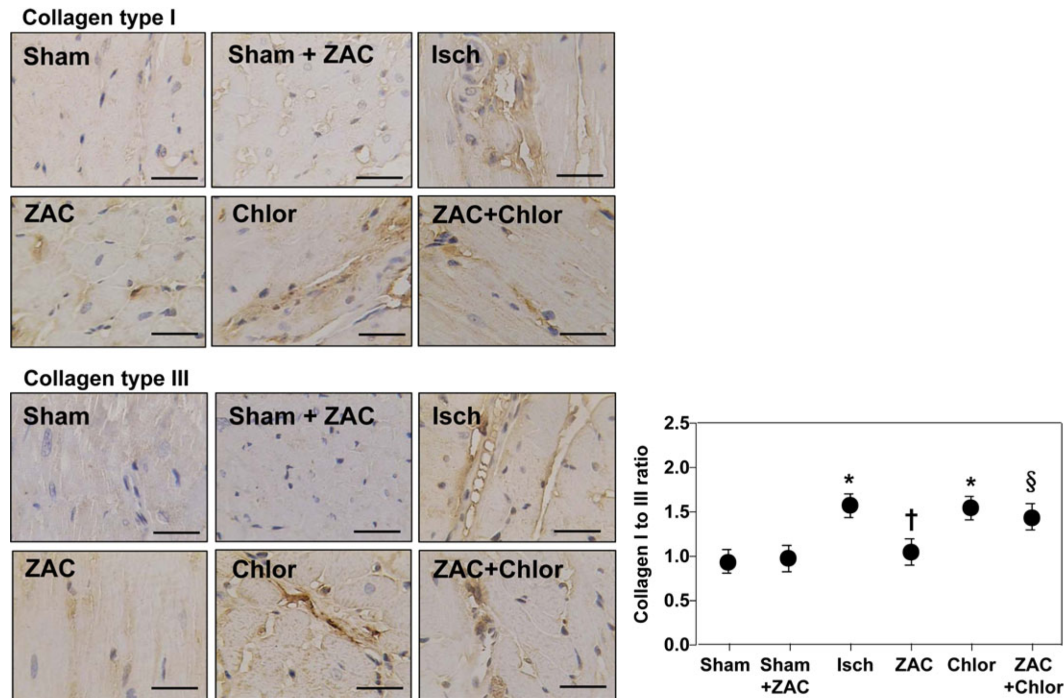

Figure 2. Deposition of collagen types I and III in the non-infarcted myocardium by immunohistochemical staining The graph at the bottom shows the ratios of collagen I over III in different experimental groups. Values are presented as the mean ± SD (n = 10 in each group). Scale bars: 50 µm. Abbreviations are the same as those shown in **Fig. 1**. *$P < 0.05$ Isch versus sham; †$P < 0.05$ ZAC versus Isch; §$P < 0.05$ ZAC + Chlor versus ZAC.

relative to the sham groups in the peri-intracardiac vessels and the intermyocardium. The collagen I/III ratio was significantly increased, suggesting that collagen type I is a main type of collagen deposited in the myocardium. Treatment with ZAC over 4 weeks of infarction significantly reduced accumulation of collagen types I and III relative to the ischemia group. Chlor treatment alone had no effect on the

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production of collagens, but significantly blocked the effect of ZAC (**Fig. 2**). Consistent with enhanced collagen synthesis, the extent of the fibrotic myocardium identified by Masson's trichrome staining was also significantly increased in the ischemia group (**Fig. 3**). Beneficial effect of ZAC on the fibrosis was abolished by Chlor, but Chlor treatment alone showed no effect on ischemia-induced fibrosis (**Fig. 3**).

Changes of hemodynamic parameters at 4 weeks after infarction

Hemodynamic parameters measured among different groups were shown in **Fig. 4**. There was no group difference in cardiac function between the sham and the sham+ZAC groups during the course of the observation. However, 4 weeks of infarction caused a significant reduction in LVESP and $\pm dP/dT_{max}$, and an elevation in LVEDP in the ischemia group relative to the sham group. Following daily treatment with ZAC, hemodynamic parameters were significantly improved compared with those in the ischemia group. Administration of Chlor alone had no effect on these parameters, but significantly blocked the beneficial effects of ZAC on improvements of cardiac systolic and diastolic function (**Fig. 4**).

Improvement of cardiac function 4 weeks after infarction

As shown in **Fig. 5**, LV dimension including LVEDD and LVESD remained unchanged in the sham and sham + ZAC groups throughout the study period. Compared with these parameters in the sham group, 4 weeks of myocardial infarction caused a significant increase of LVEDD and LVESD, indicating a progressive LV decompensatory dilatation. This enlargement of LV chamber was consistent with hemodynamic deterioration and substantial reduction in cardiac function. Consistent with these changes, FS and EF were decreased by 31% and 35%, respectively, relative to the sham group, suggesting cardiac contractile dysfunction. However, LV enlargement and systolic dysfunction were significantly improved by the treatment of ZAC compared with the ischemia (**Fig. 5**; all $P < 0.05$). Administration of Chlor did not show any effect when it was given alone, but significantly blocked the beneficial effects of ZAC on the LV function (**Fig. 5**). There was no difference in HRs among groups during the course of the experiment (data not shown).

Activating the IK1 channel and mTOR-p70S6 signaling pathway

As shown in **Fig. 6**, the expression of IK1 protein was significantly reduced and the protein level of mTOR was phosphorylated relative to the sham group at the end of 4-week infarction. In coincidence with these changes, the p70S6K, a hallmark of activation by mTOR, was also phosphorylated in the ischemia group. Treatment with ZAC prevented the IK1 channel protein from down-regulation and also inhibited the expression of p-mTOR and p-p70S6K. Chlor treatment alone had no effect on the expressions of IK1 protein, mTOR and p70S6K, but significantly blocked the IK1 expression up-regulated by ZAC, consistent with an inhibition of ZAC on down-regulation of mTOR and p70S6K, suggesting that there is a link among IK1, mTOR, and p70S6K in the regulation of myocyte remodeling by ZAC. Down-regulation of p70S6K expression with ZAC was consistent with its inhibition in myocyte hypertrophy, suggesting that p70S6K acts as a key trigger of protein synthesis responsible for cell hypertrophy.

Dynamic plasma Chlor level

To ensure that Chlor can efficiently block the effect of ZAC on the IK1 channel, Chlor plasma concentration during Chlor injection

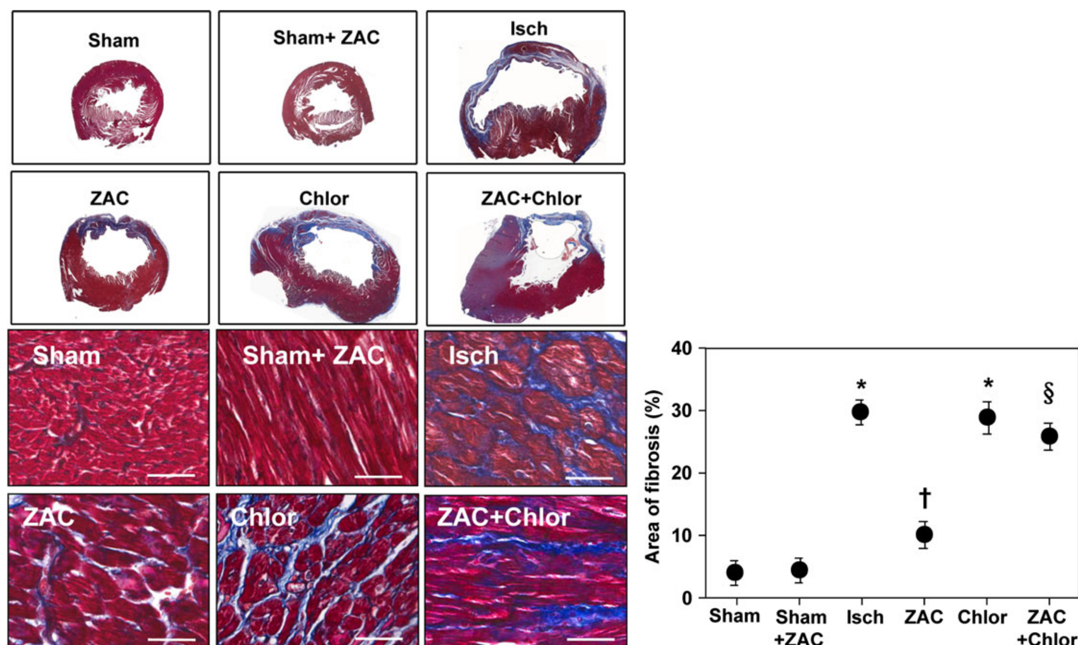

Figure 3. Representative images of gross morphology (top panel) of left ventricle (LV) and collagen deposition in non-infarction zone (bottom panel) of LV tissues in rats 4 weeks post-MI by Masson's staining Cardiomyocytes and collagen fibers were stained as red and blue, respectively. Fibrotic area was calculated as percentage of total area in each HPF. Scale bars: 50 μm. Values are presented as the mean ± SD ($n = 10$ in each group). Abbreviations are the same as those shown in **Fig. 1**. *$P < 0.05$ Isch versus sham; †$P < 0.05$ ZAC versus Isch; §$P < 0.05$ ZAC + Chlor versus ZAC.

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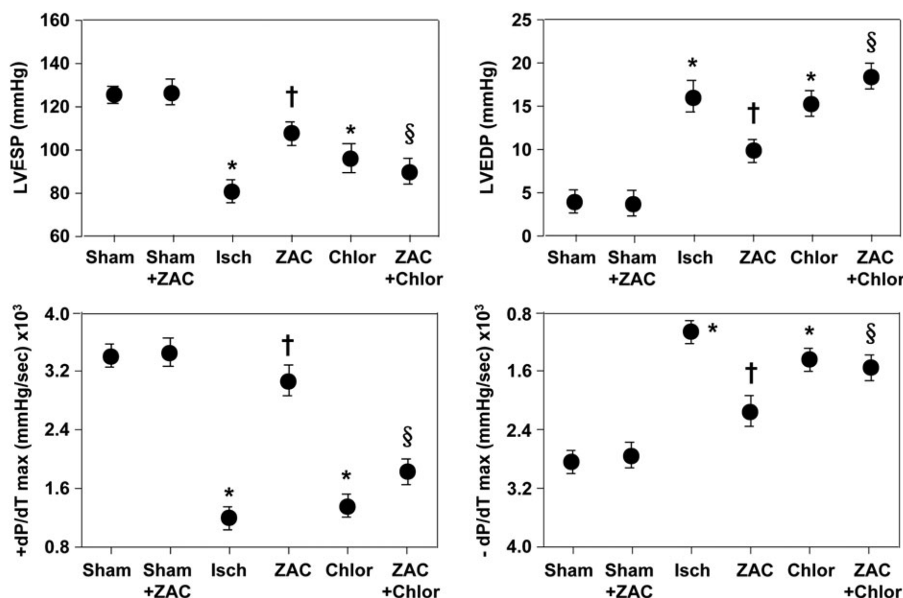

Figure 4. Hemodynamic parameters from different experimental groups LVESP, left ventricular end-systolic pressure; LVEDP, left ventricular end-diastolic pressure; and $\pm dP/dT_{max}$, the maximal rate of LV pressure developments. Values are presented as the mean ± SD ($n = 6$ in each group). Abbreviations are the same as those shown in **Fig. 1**. *$P < 0.05$ Isch versus sham; †$P < 0.05$ ZAC versus Isch; §$P < 0.05$ ZAC + Chlor versus ZAC.

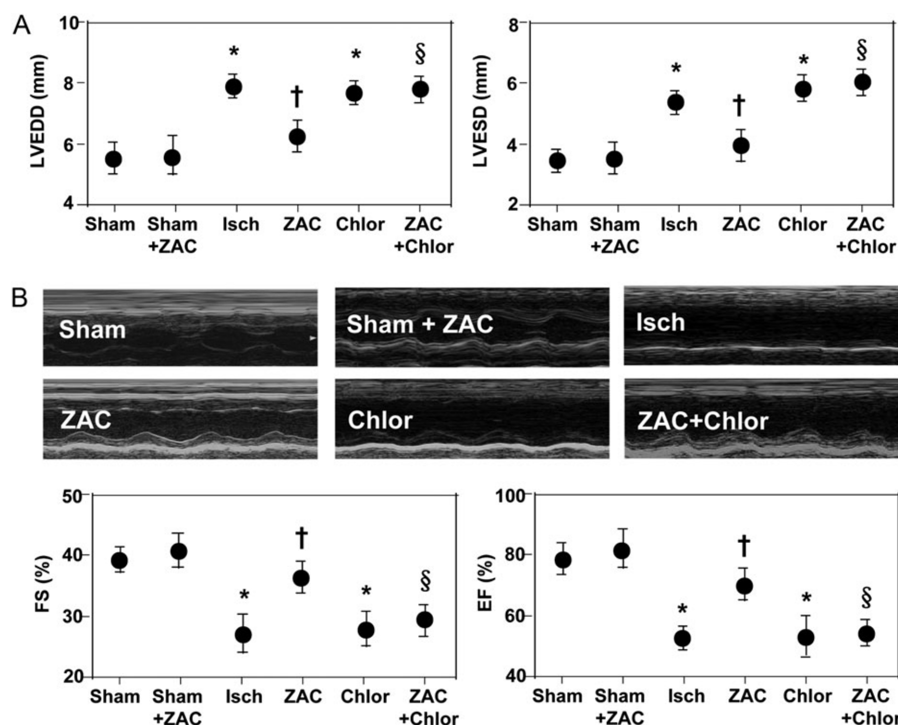

Figure 5. Left ventricular volume and function detected by echocardiography (A) The quantitative parameters of the volume and function. (B) Typical M-mode echocardiographic images. The volume was measured from diastolic and systolic diameter dimension and cardiac systolic function was detected from the M-mode echocardiographic images by measuring fraction shortening and EF. Values are presented as the mean ± SD ($n = 6$ in each group). LVEDD, end-diastolic diameter dimension; LVESD, left ventricular end-systolic diameter dimension; FS, fractional shortening; EF, ejection fraction. Other abbreviations are the same as those shown in **Fig. 1**. *$P < 0.05$ Isch versus sham; †$P < 0.05$ ZAC versus Isch; §$P < 0.05$ ZAC + Chlor versus ZAC.

was measured using HPLC. Plasma concentrations of Chlor were 0.17, 0.26, 0.32, 0.50, and 0.57 μM on days 1, 7, 14, 21, and 28, respectively, suggesting that the plasma concentration of Chlor rose steadily during the experiment.

Discussion

Maladaptive tissue repair with subsequent cardiac dysfunction after myocardial infarction is a hallmark of the syndrome of heart failure in patients and ultimately progresses to an abnormal cellular state

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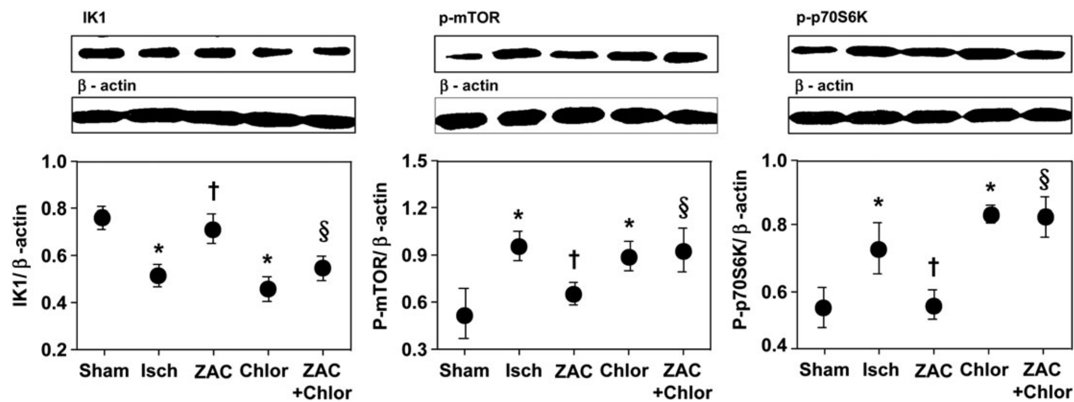

Figure 6. Protein expressions of the IK1 channel, p-mTOR, and p-p70S6K Proteins were detected by western blot analysis. All bands in different experimental groups were normalized by β-actin as a standard of protein-loading control. The results were calculated as the ratios of intensity of the bands to that of β-actin as illustrated at bottom panels. Values are presented as the mean ± SD (n = 6 in each group). Abbreviations are the same as those shown in **Fig. 1**. $^{*}P < 0.05$ Isch versus sham; $^{\dagger}P < 0.05$ ZAC versus Isch; $^{\S}P < 0.05$ ZAC + Chlor versus ZAC.

precipitating energetic, electrical, and mechanical dysfunction. It occurs regardless of the degree of infarction and involves myocardial fibers in both infarcted and non-infarcted regions [11]. Much has been learned of the ligand-gated adenosine triphosphate (K_{ATP}) channel (i.e. mitochondrial K_{ATP} channel) in cardiacprotection after myocardial infarction. Many studies have demonstrated that the selective mitochondrial K_{ATP} channel agonists reduce infarct size, enhance cardiac function, and suppress ventricular arrhythmias. The mechanisms of protection proposed include the inhibition of reactive oxygen species production and Ca^{2+} accumulation, as well as improvement in mitochondrial energy production and enhancement the expression of potassium channel protein [18–20]. Our previous study reported that opening of the IK1 channel with ZAC effectively suppresses ventricular arrhythmias [7]. In the present study, results showed that treatment with ZAC over 4 weeks of myocardial infarction significantly reduced collagen deposition and fibrosis and improved cardiac function. The protection may largely be associated with enhanced expression of IK1 channel protein, and reduced expressions of p-mTOR and p-p70S6K, which was blocked by the IK1 channel antagonist Chlor. These results suggest that the selective opening of the IK1 channel is a potential pharmacological target in the treatment of ischemic heart diseases.

Reduction of cardiomyocyte swelling during myocardial ischemia may be a potential mechanism of cardioprotection. Previous studies have reported that down-regulation of the IK1 channel expression is associated with prolongation of ventricular action potentials, which is common finding in animal models of cardiac hypertrophy and patients with idiopathic dilated cardiomyopathy [21–23]. We have previously shown that the IK1 channel stabilizes the resting membrane potential of ventricular myocytes during phase 4 and contributes to the terminal portion of phase 3 repolarization [7]. In this regard, opening of the K_{ATP} channels has been associated with action potential shortening in modulation of cell swelling-mediated atrial cell volume [24]. The results presented here demonstrated that ZAC may normalize ventricular repolarization and maintain electrical functioning through the transcriptional up-regulation of repolarizing the IK1 channel. However, it is necessary to check how prolongation of action potential duration (i.e. the relationship between the IK1 channel and Ca^{2+}-dependent modulation of membrane potential [25]) leads to pressure overload-induced cardiac hypertrophy using an animal model of transverse aorta constriction.

In the present study, a rat model of permanent coronary artery occlusion without reperfusion was selected. There was no statistic difference in infarct size among all groups after 4 weeks of ligation, excluding a possibility that the size of infarction may affect an evaluation of IK1 activation on cardiac repair and function among different groups. It has been reported that the mTOR is a key intermediary in multiple mitogenic signaling pathways, and plays a critical role in modulating proliferation and angiogenesis in normal and pathological conditions. These modulations can be blocked by rapamycin, a highly specific inhibitor of mTOR. The upstream molecules responsible for regulation of mTOR are phosphatidylinositol 3-kinase and protein kinase B (PKB), which determine the subsequent phosphorylation of downstream substrate, i.e. the 70-kDa ribosomal protein p70S6K [14,26]. The p70S6K is known to regulate cell growth by initiating protein translation and synthesis [20]. Therefore, the phosphorylation of p70S6K may represent an important target to reflect pharmacodynamic effects of ZAC on cardiac hypertrophy and tissue repair. A previous study has shown the beneficial effect of K_{ATP} channel agonists on cardiac repair through inhibiting the phosphorylation of p70S6K [20]. Because both K_{ATP} and IK1 channels belong to the family of inwardly rectifying potassium channels and have significant structural similarity [1], we proposed that these two channels may share the same molecular mechanisms underlying protection against maladaptive tissue repair after infarction. In the present study, results showed that along with the up-regulation of the IK1 channel protein, the levels of p-mTOR and p70S6K were significantly reduced by ZAC. We postulated that the IK1 channel initiates an upstream inhibiting signal on p70S6K through mTOR, because blockade of the IK1 channel with Chlor not only inhibited ZAC up-regulated IK1 protein expression, but also enhanced the phosphorylation of mTOR and p70S6K. It has previously been identified that Chlor works as a potent blocker of inward rectifier potassium channels [16,27]. In the present study, Chlor administered at a dosage of 7.5 μg/kg/day was found to yield an accumulative plasma concentration within 4 weeks and effectively block the IK1 channel throughout the experiment. These results suggest a possibility that opening of the IK1 channel is associated with the down-regulation of phosphorylation of p70S6K, which is mainly driven by p-mTOR, and that the inhibitory pathway initiated by activated IK1 channel is transmitted to p70S6 kinase via mTOR. Despite the difference in other possible signaling targets,

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there has been relatively general agreement that activation of protein kinases by G protein-coupled receptor induces many of the features of hypertrophy. Myocardial hypertrophy has been associated with both ventricular and supraventricular arrhythmias. We have previously reported that ZAC-inhibited ventricular arrhythmias is blocked by protein kinase A (PKA) inhibitor, but is not altered by protein kinase C (PKC) and protein kinase G (PKG) blockers [8]. However, it is necessary to further address whether ZAC promoted-cardiac repair is associated with modulation of protein kinases.

Synthesis and deposition of collagens are known to induce the fibrosis predisposing the heart to undergo maladaptive tissue repair and heart failure [28]. Activated cardiac fibroblasts synthesize and release collagens among many other extracellular matrix-related proteins. Collagen types I and III represent most of the total newly synthesized collagens after myocardial infarction [29]. In the present study, results showed that the types I and III collagens are scarcely expressed in the non-ischemic myocardium in sham and sham + ZAC groups, but their expressions are significantly increased after 4 weeks of coronary occlusion, which is a time frame that the necrotic myocytes are entirely replaced by fibrous tissue [30]. Coincident with reduction in the fibrotic tissue identified by Masson's trichrome staining, the current study demonstrated that daily ZAC treatment significantly down-regulates the expression of the collagen types I and III.

Profound phenotypic changes occur as monocytes transit from the blood vessels to the interstitial myocardium after myocardial infarction. Monocytes undergo a series of changes to become macrophages after entering infarcted tissue through the vascular endothelium. Macrophages are known to stimulate proliferation of fibroblasts to myofibrobalsts that are involved in the production of collagens [31]. A recent study using a patch-clamp experiment demonstrated that gene expression of outward rectifier K^+ currents is reduced in isolated fibroblasts from human atrial tissue with chronic atrial fibrillation, implicating an enhanced differentiation of fibroblasts into myofibroblasts. The mechanisms underlying the outward rectifier K^+ current-modulated cell proliferation may be associated with changes in resting membrane potential that affects the progression of cell cycle, regulation of cell volume and activation of downstream Ca^{2+}-dependent signaling pathways [32]. However, at present, we do not know whether stimulation of the IK1 channel with ZAC reduces collagen production through inhibiting macrophage migration and fibroblast differentiation. Further experiments are needed to characterize the populations of fibroblast and myofibroblast in myocardial tissue sections by targeting mTOR-p70S6K signaling pathways, which might provide an insight into the impact of the IK1 channel on tissue fibrosis [33]. Excessive collagen deposition and tissue fibrosis have been associated with development of cardiac dysfunction [34]. As demonstrated in the present study, cardiac dysfunction after 4 weeks of infarction was associated with increased myocyte size and myocardial fibrosis. In coincidence with an inhibition of collage deposition in the non-infarcted region, stimulation of the IK1 channel with administration of ZAC over 4 weeks suppressed cardiac hypertrophy, evidenced by decreased ratios of HW/BW and LVW/BW and cardiomyocyte CSA. Furthermore, ZAC significantly increased LVESP and $\pm dP/dT_{max}$, and reduced LVEDP. Detection of global cardiac function by echocardiography also revealed that LVEDD and LVESD are decreased, and FS and EF are increased, indicating less dilation of the heart and better recovery of cardiac function.

In summary, opening of the IK1 channel with ZAC attenuates maladaptive tissue repair and improves global cardiac function after myocardial infarction. The underlying mechanisms may primarily involve activation of the IK1 channel and suppression of mTOR-p70S6K signaling pathways. However, the specificity of ZAC in stimulating a direct signaling link between mTOR-p70S6K and cardiac repair through the IK1 channel needs to be further addressed using either the rapamycin to specifically inhibit mTOR activation or the mTOR-knockout model. Given the fact that the IK1 channel has been reported with suppression of ventricular arrhythmias [7,8] and augmentation of cardiac function as shown in the present study, pharmacological stimulation of the IK1 channel may open another 'window of opportunity' in the treatment of patient with heart failure.

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