

Original Article

Bicyclol protects cardiomyocytes from apoptosis in ischemia/reperfusion injury via inhibition of TLR4/NF- κ B pathway

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Received June 21, 2016; Accepted August 19, 2016; Epub November 15, 2016; Published November 30, 2016

Abstract: Toll-like receptor 4 (TLR4)-mediated apoptosis plays a critical role in the etiology and pathogenesis of myocardial ischemia/reperfusion (I/R) injury (MIRI). Bicyclol has been shown to possess a variety of pharmacological effects, but its anti-apoptotic property has garnered particular interest. The aim of this study is to elucidate the cardioprotective effect of bicyclol in I/R injury, and to explore the potential mechanisms involving in TLR4-mediated apoptotic cascade. Bicyclol was intragastrically administered in rats for three days before myocardial ischemia was induced at three different dosages: 25 mg/kg, 50 mg/kg and 100 mg/kg. The rat MIRI model was established by 30 min of left anterior descending (LAD) artery occlusion and 4 h of reperfusion. We then evaluated cardiac function using a biotic signal collection and processing system. H&E and Evans blue plus TTC staining were used to observe morphological changes and the infarct size of myocardium, respectively. TUNEL-positive cells were calculated to assess myocardial apoptosis. Quantitative RT-PCR was used to detect TLR4, NF- κ B and TNF- α mRNA expression. Western blotting was performed to measure TLR4, nuclear NF- κ B/p65, TNF- α , Bax, Bcl-2 and caspase-3 proteins levels. We determined that pretreatment with bicyclol improved cardiac function, reduced myocardial infarct size and ameliorated morphological lesions of the myocardium in a dose-dependent manner. In addition, the expression levels of TLR4, NF- κ B and TNF- α were significantly down-regulated following bicyclol pretreatment. This was concomitant with inhibition of cardiomyocyte apoptosis, as evidenced by a decrease in TUNEL-positive cells and the deactivation of the Bax/Bcl-2 dependent apoptotic cascade. Taken together, our findings demonstrate that administration of bicyclol has a cardioprotective effect against I/R injury, possibly through down-regulation of myocardial apoptosis mediated by the TLR4/NF- κ B signaling pathway.

Keywords: Bicyclol, myocardial ischemia/reperfusion, apoptosis, TLR4, NF- κ B

Introduction

Acute myocardial infarction is the most common and most severe form of acute cardiac injury [1]. Early reperfusion treatment yields benefits for the ischemic myocardium but paradoxically results in further damage to viable tissue, which is characterized as myocardial ischemia/reperfusion (I/R) injury (MIRI) [2, 3]. There are complex factors that contribute to its pathogenesis, among which apoptosis has been identified as a major contributing factor in promotion of myocardial dysfunction [4, 5]. As such, many recent investigations have focused on the initiation of the apoptotic process and on identifying interventional methods that may represent potential therapeutic approaches for the reduction of MIRI.

There is much evidence to demonstrate that signaling pathways originated by transmembrane Toll-like receptor 4 (TLR4) and its downstream effector nuclear factor κ B (NF- κ B) regulate the links between extracellular stimuli and alteration in gene expression. The TLR4/NF- κ B axis functions as a critical sensor in inducing the canonical apoptotic cascade [6]. Activated NF- κ B translocates into the nucleus and thus induces the synthesis of tumor necrosis factor-alpha (TNF- α), which has recently emerged as a potent pro-apoptotic cytokine in MIRI [7, 8]. Importantly, myocardial apoptosis can be markedly attenuated through interventional strategies that target TLR4-dependent signaling pathways via a variety of techniques, ranging from antagonist, TLR4 gene deficiency, and small interference RNA technique etc. [9,

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10]. This suggests, and is in agreement with previous studies, that administration of anti-apoptotic drugs which act through a TLR4-mediated mechanism might confer encouraging results for the treatment of MIRI [11, 12].

Bicyclol (4,4'-dimethoxy-5,6,5',6'-bis (methylene-dioxy)-2-hydroxymethyl-2'-methoxy carbonyl biphenyl) is an extensively applied anti-hepatitis drug with clinical efficacy in China [13, 14]. Bicyclol administration has also been shown to possess many pharmacological and pleiotropic organ-protective properties, including protection against renal interstitial fibrosis, neuroprotection against rat ischemic stroke and anti-inflammatory potency in acute lung injury [13-15]. Currently, the novel role of bicyclol in protection against I/R injury has aroused attentions. For instance, Cui and colleagues have confirmed that bicyclol exerts a protective effect on cardiomyocytes through its ability to limit oxidative stress during I/R *in vivo* [16]. Moreover, a current report has further expanded our understanding of bicyclol's biological properties, specifically in terms of its anti-apoptotic effects, through identifying a mechanism that was closely correlated to the inhibition of the mitochondria-associated apoptotic pathway [17]. To the best of our knowledge, there exists neither clear histological or morphological evidence nor appropriate dosage regimen to demonstrate the cardioprotective effect of bicyclol against I/R injury *in vivo*. Neither has the related intracellular processes mediating apoptosis in MIRI been determined. The only prior study to highlight the protective mechanism of bicyclol in ischemic conditions was through the means of down-regulating TLR4-associated signaling pathways [14]. Therefore, the purpose of the present study was to determine the cardioprotective potency of bicyclol during I/R injury, and to identifying the underlying mechanisms associated with the TLR4/NF- κ B-mediated apoptotic cascade.

Materials and methods

Experimental animals

Male Sprague-Dawley (S-D) rats (SPF grade, weighing 220-250 g) were supplied by the animal experiment centre of Wuhan University (Wuhan, China). All rats were maintained under controlled conditions with a normal photoperiod of 12/12 h light/dark cycle at 24°C, a rela-

tive humidity of 60% and free access to food and water. The experimental procedures and animal care were performed in compliance with the Guidelines for Animal Experimentation at the Institutional Animal Care and Use Committee of Wuhan University, which were also conducted under the Guide for the Care and Use of Laboratory Animals by the National Institute of Health.

Drugs and animal pretreatments

Bicyclol (C₁₉H₁₈O₉; purity >98%) was kindly provided by Beijing Union Pharmaceutical Manufactory (China), and dissolved in polyethylene glycol 400 (PGE400) [18]. All rats were randomized into a sham operated group (SO group), a myocardial I/R injury group (I/R group) and a bicyclol-pretreated group for the dose-effect relationship study. In the SO and I/R groups, rats were orally administered with vehicle (PEG400) by a gavage for 3 days and served as a sham control and an I/R control. In bicyclol-treated groups, animals received a gavage of bicyclol at three different dosages of 25 mg/kg (L-Bic group), 50 mg/kg (M-Bic group) and 100 mg/kg (H-Bic group) for three consecutive days prior to I/R injury. Each group contained 12 rats. The doses and duration regimes of bicyclol pretreatment were determined by previous data by Cui *et al* [16] and data from the pretests.

Establishments of myocardial I/R injury models

Animals in the I/R group and the bicyclol-pretreated groups were subjected to experimental coronary artery LAD ischemia for 30 min and reperfusion for 4 h (to represent I/R) as previously described [5, 16]. In detail, rats were anesthetized with sodium pentobarbital at a dose of 30 mg/kg (i.p.), and were ventilated via a breathing machine at 70 breaths/min and a tidal volume of 20 ml/kg. An electrocardiogram (ECG) in combination with a computer-based EP system was used to detect alternation of ST-T segment and heart rate (LEAD-2000B; Jinjiang Ltd., China). Body temperature of rats during and after surgical procedures was retained at 36-37°C via a homeothermic blanket. A thoracotomy between the third and fourth ribs on the left side was performed. After rat heart exposure, a 6-0 silk suture was used to ligate the LAD. In addition, a socket of

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medical latex tube (inner diameter = 1.5 mm) was gently placed between the LAD and the ligature. In this way, the reversible ischemia was induced by tightening the silk for 30 min, and then loosening of the knot restored blood supply for 4 h. The elevation of the ECG ST-T segment, the pale appearance in the apex of the heart and abnormal wall motion were all considered phenotypes of myocardial ischemia. Animals in the SO group were treated with the same procedures except that the silk was not tied. At 4 h post-reperfusion, all rats underwent hemodynamic measurements and then four rats in each group were maintained to assess the myocardial infarct size. All other rats were subjected to the hemodynamic evaluation and then immediately sacrificed. Left ventricular tissue near the heart apex and blood samples were collected for corresponding determinations.

Evaluations of hemodynamics

Following 4 h after I/R, a pressure transducer was cannulated into the rat left ventricular cavity via the right common carotid artery for a hemodynamic evaluation [19]. Heart rate (HR), left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP) and maximal rise/fall rate of left ventricular pressure ($\pm dp/dt_{\max}$) were measured to determine the cardiac function by a data acquisition system (BL420F-Powerlab, Taimeng Technology Co., Ltd., China).

Measurements of myocardial infarct size

Myocardial infarct size was measured by Evans blue plus 2,3,5-triphenyl tetrazolium chloride (TTC) double staining 4 h after the reperfusion procedure [5, 19]. Once the LAD was occluded again, 1.5 ml of 2% Evans blue dye (Sigma-Aldrich, St Louis, MO, USA) was injected into the jugular vein to stain viable non-ischemic tissue a blue colour. After being removed and washed with saline, the entire heart was frozen at -80°C for 5 min. Then, the heart was cut into five 2 mm thick transverse slices, which were then incubated in 1.5% TTC at 37°C (Sigma, St. Louis, MO, USA). Twenty minutes later, the ischemic but still viable area (the area at risk, AAR) was dyed red with TTC, while non-stained necrotic myocardium appeared pale white. Image-Pro Plus 5.0 software (Media Cybernetic, USA) was used to measure the

infarct size and AAR. The ratio of infarct size to AAR was calculated and represented as a percentage.

Determinations of morphological changes

At the end of reperfusion, the left ventricular tissue near the heart apex was collected and fixed in 4% paraformaldehyde for 24 h. Then, the samples underwent the standard protocol for paraffin embedded sections. Approximately 5 μm thick paraffin slices were then subjected to hematoxylin & eosin (H&E) staining to measure morphological changes using an optical microscope (200 \times magnification) as described by Yang *et al* [20].

Assessments of TUNEL-positive cardiomyocytes

The terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was utilized to evaluate the number of apoptotic cells among cardiomyocytes [5]. At 4 h post-reperfusion, the collected heart samples were fixed with 4% paraformaldehyde, dehydrated, and then paraffin-embedded. TUNEL staining was performed with an assay kit (Roche, Basle, Switzerland) according to the manufacturer's instructions. Five microscopic fields were randomly chosen to count the TUNEL-positive cells, and photographs were obtained under 400 \times magnification. Scale bar represented 20 μm . Apoptotic index (AI) was calculated as follows: AI = TUNEL-positive cells/total cells $\times 100\%$.

Western blotting analysis

After I/R injury, the left ventricular tissue near the heart apex in all groups was obtained. Then, total and nuclear protein extractions were performed by the methods of Yang *et al*, and according to the manufacturer's instruction [20]. Fifty μg of proteins per lane were separated by 12% SDS-polyacrylamide gel electrophoresis, and transferred to a PVDF membrane. Membranes loaded with proteins of interest were blocked with Tris buffer saline (TBS)-0.1% Tween-20 containing 5% w/v nonfat dry milk. Then, blots were probed with primary antibodies (Proteintech Group, Chicago, IL, USA) against TLR4 (1:600), NF- κB /p65 (1:1500), TNF- α (1:600), Bax (1:1000), caspase-3 (1:800) and Bcl-2 (1:1500), followed by incubation with the

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Table 1. Effects of bicyclol on hemodynamic alternation after MIRI

Group	LVSP (mmHg)	LVEDP (mmHg)	+dp/dt _{max} (mmHg/s)	-dp/dt _{max} (mmHg/s)
SO	127.32 ± 3.23	3.03 ± 0.07	7499.21 ± 222.96	5346.75 ± 83.79
I/R	97.09 ± 1.01***	5.40 ± 0.25***	5188.41 ± 118.78***	3149.79 ± 85.86***
L-Bic	99.55 ± 1.11	5.32 ± 0.13	5171.82 ± 106.12	3216.41 ± 61.99
M-Bic	108.02 ± 0.93**	4.69 ± 0.17**	6341.16 ± 121.52#	4032.85 ± 36.40#
H-Bic	114.65 ± 1.72#,&	4.14 ± 0.09#,&	6847.75 ± 127.05#,&	4280.48 ± 33.25#,&

Data were presented as means ± SEM, n = 6 per group. ***P<0.001 vs. SO group; **P<0.01 and #P<0.001 vs. I/R group, respectively; &P<0.05 vs. M-Bic group. LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure; ± dp/dt_{max}, maximal rise/fall rate of left ventricular pressure.

horseradish peroxidase-conjugated secondary antibody. Each band was developed with an enhanced chemiluminescence system. GAPDH and Lamin B were employed as internal controls for cytoplasmic and nuclear NF-κB/p65 proteins, respectively.

Quantitative real-time PCR

Total RNA from heart samples were extracted and purified using TRIzol reagent (Takara, Osaka, Japan). Four µg of RNA was reverse-transcribed into cDNA using a commercial synthesis kit (Invitrogen) in accordance with manufacturer's instruction. Real-time PCR was performed with the SYBR green/fluorescein qPCR Master Mix kit (Bio-rad). The β-actin gene was used as an internal control. Data were calculated according to the comparative quantification method ($2^{-\Delta\Delta Ct}$). Sequence-specific primers utilized to amplify genes of interest are listed as follows: TLR4, forward 5'-TATCGGTGGTCA-GTGTGCTT-3' reverse 5'-CTCGTTTCTACCCAG-TCCT-3' NF-κB, forward 5'-AAGCACAGATACCAC-TAAGACGCA-3' reverse 5'-TTCAGCCTCATAGAA-GCCATCC-3' TNF-α, forward 5'-TAGAGGTGTCG-TCCCATCGT-3' reverse 5'-GGGCTTGTCCTCGA-GTTTT-3' β-actin, forward 5'-CACGATGGAGGG-GCCGGACTCATC-3' reverse 5'-TAAAGACCTCTA-TGCCAACACAGT-3'.

Statistical analysis

Quantitative data were analyzed using SPSS software (Version 13.0), and expressed as the means ± SEM. Statistical comparison between groups were determined by ANOVA and followed with a SNK-q test. A p value less than 0.05 was considered statistically significant.

Results

Treatment with bicyclol ameliorates cardiac function following I/R injury

Hemodynamic assessment was used to directly determine the effects of bicyclol on cardiac function after MIRI.

As shown in **Table**

1, worse levels of LVEDP, LVSP and ± dp/dt_{max} of the left ventricle were observed in post-I/R groups relative to the SO group (P<0.001). However, bicyclol pretreatment in the M-Bic and H-Bic groups remarkably ameliorated LVEDP, LVSP (P<0.01 vs. I/R group) and ± dp/dt_{max} (P<0.001 vs. I/R group) levels. In particular, visible improvements of these parameters were observed in the H-Bic group compared with those in the M-Bic group (P<0.05). Values were alike between the I/R and L-Bic groups (P>0.05). Moreover, there was no significant difference in heart rate (HR) among the five groups (data not shown).

Bicyclol pretreatment attenuates histopathological lesion and infarct size of the myocardium

To further define the morphologic evidence of the protective effects of bicyclol in MIRI, we measured the pathological lesion and infarct size of the myocardium. Heart samples were harvested after the I/R procedure, and following H&E staining they were examined under a light microscope (**Figure 1**). In the SO group, myocardial cells and the interstitium did not show any obvious histopathological alterations. Heart tissue isolated from the I/R group displayed widespread striated cardiac muscle disorder, local swelling of the myocardium, myocardial necrosis, interstitial hemorrhage, intercellular space widening and monocyte infiltration. Heart tissue from the L-Bic group were pathologically similar to that in the I/R group. However, rats pretreated with medium- and high-bicyclol doses showed slight edema of the myocardium, mild disorder changes and only a partially ruptured cardiac muscle fiber, as well as a decrease in the amount of monocyte infiltration.

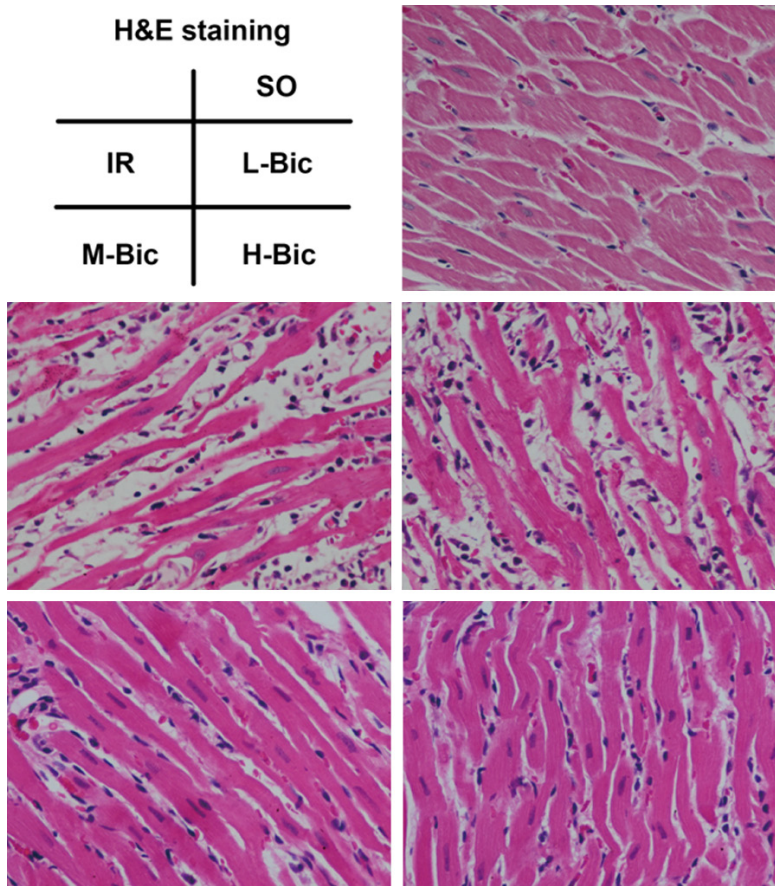


Figure 1. Bicyclol attenuates myocardial histopathological lesions caused by I/R injury. Representative photographs of H&E-stained myocardial tissues in each group (original magnification $\times 200$).

For the assessment of myocardial infarct size (**Figure 2**), the necrotic area was determined to be $44.31 \pm 1.96\%$ of the AAR in the I/R group. Bicyclol treatment at medium and high dosages strongly attenuated this elevated infarct size to $32.37 \pm 1.19\%$ of the AAR and $25.25 \pm 1.19\%$ of the AAR (M-Bic and H-Bic vs. IR, p both <0.001), respectively. However, the low-dosage bicyclol-treated group showed no significant difference in necrotic area when compared with the I/R group ($P>0.05$). This would suggest that bicyclol's inhibitory effect on myocardial infarct size is dose-dependent (H-Bic vs. M-Bic, $P<0.01$).

Bicyclol alleviates the amount of TUNEL-positive cardiomyocytes

To determine if bicyclol has an effect on myocardial apoptosis caused by I/R injury, TUNEL staining was performed to visualize the apoptotic cell population. The AI, which is repre-

nted as the ratio of TUNEL-positive cells to total cardiomyocytes, was remarkably increased in all I/R-treated groups compared to the SO group ($P<0.001$) (**Figure 3A** and **3B**). Bicyclol pretreatment at medium and high doses mitigated the increase in the number of apoptotic cells in comparison with that in the I/R group. The M-Bic and H-Bic groups showing a separate 30.37% and 40.02% reduction in AI relative to the IR group (p both <0.001), while the L-Bic group showed no significant difference ($P>0.05$) (**Figure 3B**). This suggests that myocardial apoptosis induced by I/R can be strikingly ameliorated by bicyclol in a dose-dependent manner (H-Bic vs. M-Bic, $P<0.05$).

Bicyclol mitigates the Bax/Bcl-2-dependent apoptotic cascade

To further validate that bicyclol reduces cardiomyocyte apoptosis, and to determine the potential molecular mechanisms underlying this reduction, Western blot analysis was performed to measure the protein levels of cytoplasmic Bax, Bcl-2 and caspase-3 (**Figure 4A** and **4B**). Our results demonstrated that rats subjected to I/R showed strong elevation in the levels of Bax and cleaved caspase-3 proteins (0.88 ± 0.05 and 0.68 ± 0.03 , respectively) relative to the SO group (0.33 ± 0.02 and 0.17 ± 0.01 , respectively, p both <0.001). Upon pretreatment with bicyclol at medium- and high-doses, reductions in Bax (M-Bic = 0.67 ± 0.03 and H-Bic = 0.57 ± 0.03) and cleaved caspase-3 (M-Bic = 0.43 ± 0.03 and H-Bic = 0.37 ± 0.03) protein levels were observed in comparison with that from the I/R group (M-Bic vs. IR, $P<0.01$; H-Bic vs. IR, $P<0.001$). However, the level of Bcl-2 was contrary to those observed for Bax and cleaved caspase-3. Furthermore, there were no differences in any of the protein levels measured between the L-Bic and I/R groups ($P>0.05$).

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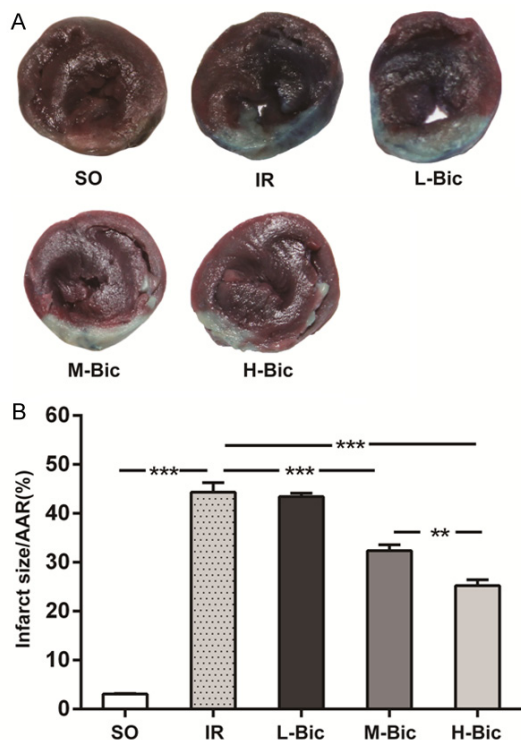


Figure 2. Effects of bicyclol on reducing myocardial infarct size in I/R injury. A: Representative pictures showing myocardial tissue cross-sectioned after Evans Blue combined with TTC staining. B: Graphs depicting the comparisons of infarction size/AAR between groups. Values were means \pm SEM, $n = 4/\text{group}$, ** $P < 0.01$, *** $P < 0.001$.

Bicyclol down-regulates the TLR4-mediated signaling pathway

To determine which signaling pathways are involved in the mechanism, in which bicyclol prevents apoptosis, we detected the protein and mRNA expression levels of TLR4, NF- κ B and TNF- α . As demonstrated in **Figure 5A**, the mRNA expression levels of TLR4, NF- κ B and TNF- α were profoundly higher in the I/R group relative to the SO group (4.15 ± 0.44 vs. 1.00 ± 0.05 , 4.43 ± 0.31 vs. 1.00 ± 0.05 and 4.89 ± 0.27 vs. 1.00 ± 0.04 , respectively, p all < 0.001). Administration of bicyclol repressed TLR4 (M-Bic = 2.25 ± 0.18 and H-Bic = 1.60 ± 0.33), NF- κ B (M-Bic = 2.91 ± 0.20 and H-Bic = 2.10 ± 0.32) and TNF- α (M-Bic = 2.74 ± 0.16 and H-Bic = 1.62 ± 0.31) mRNA expression (M-Bic vs. IR, $P < 0.01$; H-Bic vs. IR, $P < 0.001$). No significant difference in mRNA expression was observed between the I/R and L-Bic groups ($P > 0.05$).

In addition, the protein levels of TLR4, nuclear NF- κ B/p65 and TNF- α exhibited a similar pat-

tern of expressions. As shown in **Figure 5B**, compared to the SO group, heart tissue samples isolated from the I/R group exhibited significant up-regulation of TLR4, nuclear NF- κ B/p65 and TNF- α proteins (p all < 0.001). Furthermore, in medium- and high-dosages of the bicyclol-treated animals, the expression levels of TLR4 (M-Bic and H-Bic vs. IR, p both < 0.01), nuclear NF- κ B/p65 and TNF- α (M-Bic and H-Bic vs. IR, p both < 0.001) proteins were significantly decreased compared with the I/R group. However, no significant differences were observed in protein expression levels between the L-Bic and the I/R groups ($P > 0.05$).

Discussion

Previous studies have demonstrated that myocyte death from apoptosis is a critical mediator of MIRI [4, 5]. In the present study, we provide, for the first time, *in vivo* evidence that administration of bicyclol can markedly inhibit myocardial apoptosis and thus attenuate I/R injury in a dose-dependent manner via mediating the expression of apoptotic molecules (Bax, Bcl-2 and caspase-3). The mechanism of protection is most likely linked to the repression of the TLR4 and NF- κ B signaling pathway. Our findings suggest that bicyclol possesses a novel therapeutic potential for MIRI through the inhibition of myocardial apoptosis.

Bicyclol is a synthetic analog of the Chinese herb *Fructus schisandrin*, which has been widely used in the treatment of hepatitis [18, 21]. Recently, a number of studies have also confirmed the pleiotropic organ-protective properties of bicyclol, as treatment that may provide protection against kidney and brain injury resulting from I/R through the clearance of free radicals [22, 23]. However, little is known about its potential cardio-protective effect in MIRI. In the present study, we provide clear hemodynamic and histological evidence that administration of medium (50 mg/kg) and high (100 mg/kg) doses of bicyclol during I/R markedly attenuates myocardial damage *in vivo*, as measured by the amelioration of cardiac function and histological lesion as well as decreases in myocardium infarcted size. However, treatment with low-dose of bicyclol (25 mg/kg) shows no advantages in terms of myocardial injury, suggesting that bicyclol possesses a protective effect during MIRI in a dose-dependent manner. To exploit the underlying mecha-

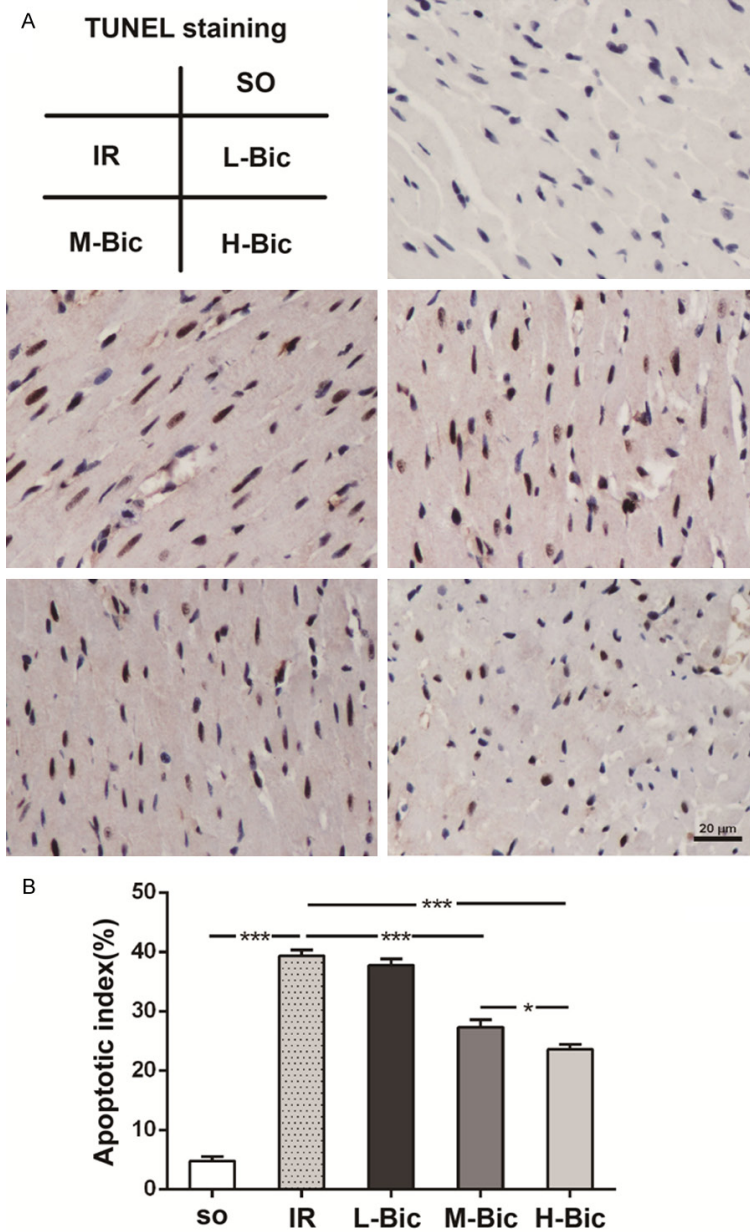


Figure 3. Bicyclol alleviates the amount of TUNEL-positive cardiomyocytes observed in I/R injury. Representative photomicrographs of TUNEL-stained cardiomyocytes (A) and graphs showing the mean apoptotic index in five groups (B). Scale bar represents 20 μ m. Data were expressed as the means \pm SEM, n = 6/group, * P <0.05, *** P <0.001.

nism of this cardioprotection, we focused on bicyclol's anti-apoptotic potency.

Recently, Bao *et al* have elucidated that treatment with bicyclol provides a protective effect on HepG2 cells by its action to limit mitochondria-associated apoptotic cascade [17]. However, there is little evidence demonstrating the efficacy of bicyclol treatment during I/R or

the possible molecular mechanism of its protection through regulation of myocardial apoptosis. I/R injuries remain the major detriment to ischemic cardiomyocytes following reperfusion treatment [3, 4]. Possible reasons behind this form of damage might be the activation of inflammatory pathways, oxidative stress and/or programmed cell deaths as well as, including apoptosis and autophagy [5, 24, 25]. Apoptosis is a type I programmed cell death, performed as the final arbiters and thus plays an indispensable role in inducing MIRI [5, 25, 26]. The Bax/Bcl-2-dependent cascade, which triggers mitochondria-mediated apoptosis, is regarded as the most pivotal signal in inducing intrinsic apoptotic cascade [25-27]. Balance between pro-apoptotic Bax and anti-apoptotic Bcl-2 proteins, along with the subsequent activation of caspase-3, which acts as the direct initiator in the induction of apoptosis, synergistically results in cell apoptosis in MIRI [9]. As set forth, the therapeutic interventions focusing on Bax/Bcl-2-associated signaling have attracted attention because of the protective effect against I/R injury [27, 28]. Here, we demonstrate that bicyclol pretreatment with 50 mg/kg and 100 mg/kg significantly decreased the number of TUNEL-positive cardiomyocytes. We also show that there was a reversal in the Bax/Bcl-2 balance following bicyclol treatment, as evidenced by an increased level of Bcl-2 and a decreased level of Bax, which was accompanied by the down-regulation of caspase-3. Taken together, we propose that bicyclol possesses a cardioactive effect against I/R injury by preventing apoptosis through the inhibition of the Bax/Bcl-2-dependent apoptotic cascade.

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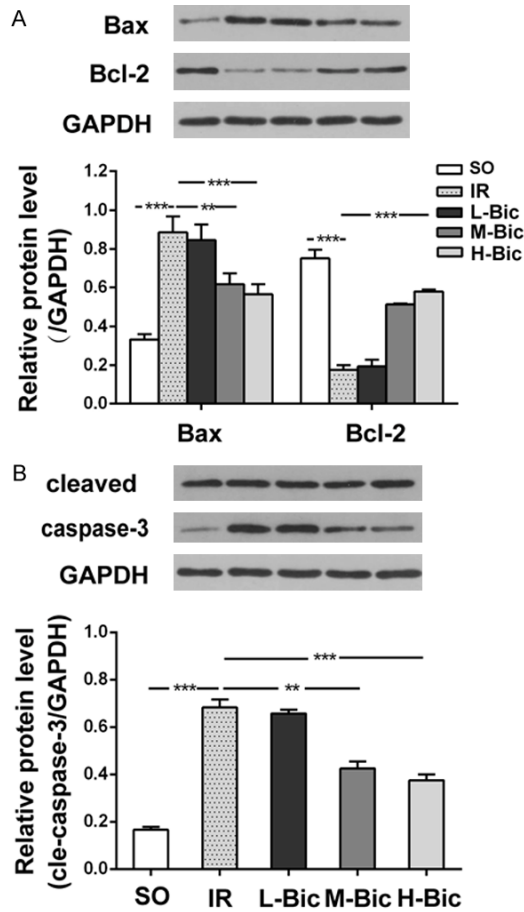


Figure 4. Bicyclol mitigates the Bax/Bcl-2-dependent apoptotic cascade in MIRI. Representative Western blots of Bax, Bcl-2 (A) and cleaved caspase-3 (B) proteins. The corresponding densitometric analyses were plotted as bar graphs. Data were normalized to the GAPDH signal. Values were presented as the means \pm SEM. $n = 6$ /group, $**P < 0.01$, $***P < 0.001$.

Myocardial I/R injury is an antigen-independent pathological process. Various intra- and extra-cellular molecules, such as high mobility group box 1 protein (HMGB1), TNF- α , MAPKs and NF- κ B, participate in its development and are expressed during its occurrence [9, 29, 30]. Upon binding with extracellular ligations, TLR4, as an important signal transduction sensor in MIRI, leads to the activation of downstream cascade of events. In particular, interaction with Myd88 precedes the nuclear translocation of NF- κ B, which enables up-regulation of inflammation-related genes (IL-6 and TNF- α) [31, 32]. The activation of the TLR4/NF- κ B/TNF- α (IL-6) signaling pathway has been widely accepted as the canonical pro-inflammatory action in inducing I/R injury [31, 32]. Recent

focus has turned to the innovative function of this signaling axis in mediating the interior apoptotic cascade through maintaining Bax/Bcl-2 balance and activating caspase-3 [6, 7, 33]. Moreover, pharmacological inhibition of TLR4 and/or NF- κ B as well as TNF- α , also significantly limits myocardial apoptosis and results in the attenuation of I/R injuries [7, 34, 35]. Prior study has highlighted the protective mechanism of bicyclol in ischemic condition through means of down-regulating the TLR4-associated signaling pathways [14]. Consistent with previous studies, we provide the novel findings that elevated expression of TLR4, nuclear NF- κ B subunit p65 and cytokine TNF- α were presented during I/R injury and that bicyclol pretreatment robustly diminishes this up-regulation of the TLR4/NF- κ B/TNF- α pathway. This is in parallel with a lower number of TUNEL-positive cardiomyocytes and the limitation of the Bax/Bcl-2-dependent apoptotic cascade following bicyclol pretreatment. As such, our findings identify a new role for the TLR4/NF- κ B signaling pathway beyond pro-inflammatory action in MIRI. In addition, this could be regarded as a potential drug target as to the pathological mediatory effect involved in the pro-apoptotic process.

In summary, our findings offer proof of concept that the administration of bicyclol plays an anti-apoptotic role and has a cardioprotective effect in I/R injury; furthermore, the possible mechanism of action might be due to the down-regulation of the Bax/Bcl-2-dependent apoptotic cascade mediated by the TLR4/NF- κ B signaling pathway. We provide a fundamental and pharmacological method of bicyclol pretreatment in the amelioration of MIRI, although further studies are still needed to clarify the causative association between bicyclol and the anti-apoptotic action involved in the TLR4/NF- κ B axis. Possible approaches may include signaling pathway specific inhibitors or small interfering RNAs to further test this idea.

Acknowledgements

This work was supported by the National Natural Science Foundation of China [81200156, 81200088, 81470387 and 81570331] and the Fundamental Research Funds for the Central Universities [20120141120079]. We thank

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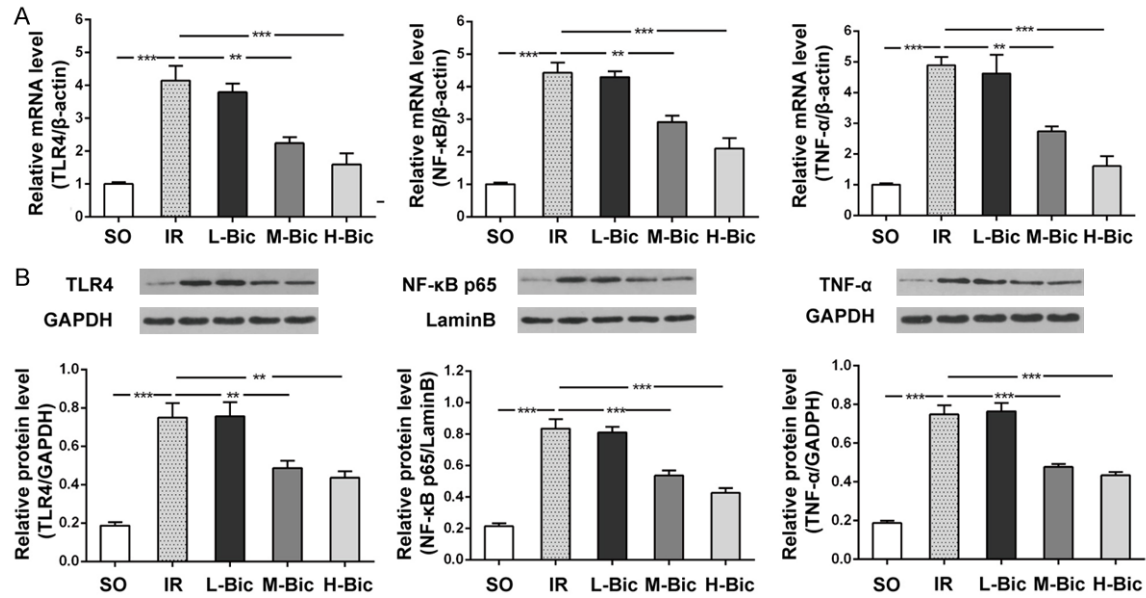


Figure 5. Bicyclol down-regulates the TLR4-mediated signaling pathway in MIRI. A: Quantitative RT-PCR showing mRNA expression of TLR4, NF-κB and TNF-α. B: Western blots of TLR4, nuclear NF-κB/p65, TNF-α and the respective bar graphs to illustrate densitometric quantitative measurements. The results were expressed as the means ± SEM. n = 6/group, **P<0.01, ***P<0.001.

Yan-Hong Tang, Xi Wang, Teng Wang, Ping Hu from Renmin Hospital of Wuhan University for excellent technical assistance.

Disclosure of conflict of interest

None.

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