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Full Length Research Paper

Protective effect of catalpol on isoproterenol-induced myocardial injury in Wistar rats

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The neuroprotective effects of catalpol had been well demonstrated in many studies. Nevertheless, little work was done to investigate the cardioprotective effects of catalpol. This study was designed to explore whether catalpol protected myocardium against isoproterenol (ISO)-induced myocardial injury through attenuating oxidative stress and suppressing inflammation. Histopathological changes were assessed by hematoxylin and eosin staining. Results show that catalpol could effectively suppress the histopathological changes including myocardial structure damage and leucocyte infiltration. Oxidative stress and antioxidant status were also analysed. Results show that catalpol could significantly attenuate the increase of myocardial malondialdehyde (MDA) and renew the activities of superoxide dismutase (SOD). Furthermore, the results of real time reverse transcription polymerase chain reaction (RT-PCR) and Western-blot analysis showed that catalpol could inhibit the upregulation of the expressions of tumor necrosis factor-*a* (TNF-*a*) and interleukin-1*β* (IL-1*β*) caused by ISO. In conclusion, our data suggest that catalpol had a protective effect against ISO-induced cardiotoxicity in rat, by maintaining endogenous antioxidant enzyme activities and alleviating inflammatory response. This study provides novel insights that catalpol treatment could be an approach for the prevention of ischemic heart disease.

Key words: Catalpol, isoproterenol, inflammation, oxidative stress, myocardial injury.

INTRODUCTION

Ischaemic heart disease (IHD) has emerged as a major world health problem and it is predicted that by the year 2020 this disease will persist as the major and the most common threat to human life (Anonymous 1997). The pathogenic mechanism of myocardial ischaemic damage is still not completely understood, but some evidence from experimental and clinical studies indicates that its pathogenesis is mainly associated with oxidative stress, calcium overload, apoptosis and inflammatory response (Sharma et al., 2011; Shintani-Ishida and Yoshida, 2011; Liao et al., 2011). Drug therapy is still the main method for preventing and treating ischaemic heart disease, although coronary angioplasty has gradually become an important therapy approach.

Catalpol is a kind of iridoid glycoside which is extracted from the roots of *Rehmannia glutinosa,* possessing a wide range of biological and pharmacological activities, including anti-tumor, anti- inflammation and anti-apoptosis properties. Its neuroprotective effect has been well studied (Zhang et al., 2010; Cai et al., 2011), but studies on its cardioprotective effect are little. Our previous study had found that catalpol could exert the cardioprotective effects against DOX-induced toxicity without affecting the antitumor activity of this anthracycline (Xing and Yanbin, 2012). In this work, we evaluated the protective effects of catalpol on ISO-induced myocardial injury.

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Abbreviations: ISO, Isoproterenol; **RT-PCR,** reverse transcription polymerase chain reaction; **TNF-*a*,** tumor necrosis factor-*a*; **IL-1*β*,** interleukin-1*β*; **MDA,** malondialdehyde; **SOD,** superoxide dismutase.

MATERIALS AND METHODS

Adult male Wistar rats weighing 180 to 200 g (total n = 32) were

purchased from the Experimental Animal Center of China Medical University, China and housed in a room with temperature of 21 to 25°C, relative humidity of 50 to 60% and a 12-h light / 12-h dark cycle. Animals had free access to pellet food and tap water. The animal protocols were approved by the Institutional Animal Care and Use Committee of China Medical University.

Model and groups

Myocardial injury was induced according to previous report (Rona et al., 1959; Loh et al., 2007). Model rats were subcutaneously injected with 85 mg/kg ISO once daily for 2 days. Rats were randomly divided into four groups (n = 8 in each group): Group 1: Control group; Group 2: catalpol group; Group 3: ISO group; Group 4: ISO + catalpol group.

Catalpol (10 mg/kg body weight) was administered intra-peritoneally to the rats of Groups 2 and 4 for 10 days. Rats in Groups 1 and 3 received the same volume of distilled water. The rats of Groups 3 and 4 received subcutaneous injection of isoproterenol (85 mg/kg body weight) on the 9th and 10th day. All rats were anaesthetized 24 h after the last ISO injection; the animals were sacrificed by cervical decapitation, then blood samples and hearts were collected for next experiments.

Drugs and chemicals

Catalpol was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing,China), then diluted in physiological saline for treatment. LDH, CK-MB were measured with automatic biochemistry analyzer using commercially available assay kits (Meikang Diagnostics and Olympus Diagnostics, respectively). SOD and MDA assay kits were produced by the Institute of Nanjing Jiancheng Biology Engineering (Nanjing, P. R. China). Protein extraction reagent (RIPA) kit was purchased from Bioteke Corporation (Beijing, P. R. China). ISO was purchased from Sigma Biotechnology (Sigma, USA). All primary and secondary antibodies were purchased from Santa Cruz Biotechnology, USA.

Histopathology

The heart was sliced transversally, and a midventricular slice was fixed in 10% formalin for 24 h, embedded in paraffin, and cut into 5-mm sections for histological assessment. Paraffin sections were stained with hematoxylin and eosin. Then, the stained sections were observed under light microscopy and degree of myocardial injury was graded according to the standard method (Rona et al., 1959).

Assay of creatine kinase (CK)-MB and lactate dehydrogenase (LDH)

To measure the myocardial specific enzymes, including the activities of CK-MB and LDH, blood samples were collected at the end of the experiment. The samples were left at room temperature for 1 h and then centrifuged (2500 g) at 4°C for 15 min. The serum was removed and stored at -80°C for biochemical assay. The activities of CK-MB and LDH were analyzed using commercial kits by employing an automatic biochemistry analyzer (AU-2700, Olympus, Japan).

Assay of malondialdehyde (MDA) and superoxide dismutase (SOD)

The preparation of left ventricle was as follows: One gram of left ventricle was homogenized in 9 volumes of ice-cold saline and centrifuged (4000 g) at 4°C for 15 min. The supernatant was removed and stored at -80°C for analysis. The amount of protein in supernatant was measured according to the previous method (Lowry et al., 1951) using bovine serum albumin as standard. The determination of MDA content in left ventricle was performed according to the previous method (Ohkawa et al., 1979). SOD activity in left ventricle was determined by the nitroblue tetrazolium reduction method (McCord and Fridovich I 1969).

RNA extraction and real time RT-PCR

Total RNA was extracted from myocardial tissue using trizol reagent following the manufacturer's instructions (Invitrogen, Life Technologies, America). RNA concentrations were quantified by spectrophotometry (UV300, Hampshire, England). cDNA was synthesized with Revert Aid H Minus M-muLV reverse transcriptase (Biometra, Goettingen, Germany). Real-time RT-PCR was performed using an ABI 7300 Prism Sequence Detection System and TaqMan primer probes (Applied Biosystems, Foster City, CA). The total reaction volume was 20 µl: 2 µl cDNA, 10 µl SYBR® Premix Ex Taq™, 0.4µl of each primer (10 µM) and 7.2 µl ultrapure water. Cycle parameters were as follows: activation at 95°C for 30 s, 40 cycles of denaturation at 95°C for 5 s and then annealing and extension at 60°C for 31 s. β-Actin was used as an internal control for each sample. The primer sequences for TNF-*a* (215 bp products) were: forward: 5′- CCCAGACCCTCACACTCAGAT -3′; reverse: 5′- TTGTCCCTTGAAGAGAACCTG -3′. The primer sequences for IL-1*β* (241 bp products) were: forward: 5′- CACCTTCTTTTCCTTCATCTTTG -3′; reverse: 5′- GTCGTTGCT-TGTCTCTCCTTGTA -3″. The primer sequences for *β*-actin (150 bp products) were: forward: 5′- GGAGATTACTGCCCTGGCTCCTA -3′; reverse: 5′- GACTCATCGTACTCCTGCTTGCTG -3′.

Western blot assay

The total proteins of myocardium were extracted by 50 mmol/L Tris–HCl (pH 7.4). Samples containing 40 µg of total protein were electrophoresed on 15% polyacrylamide gels (Bio-Rad) for 1 h. The separated proteins were electrophoretically transferred onto a PVDF membrane (Beyotime Biotechnology Inc, China). The transfer time respectively was: TNF-*a*, 20 min; IL-1*β*, 20 min. Then, blocked with 5% defatted milk for 2 h at 37°C. The membrane was incubated with polyclonal rabbit anti-rat TNF-*a*, IL-*β* and*β*-actin antibody (Santa Cruz Biotechnology, USA, diluted at 1:1000), and visualized by the ProtoBlot® II AP system (Promega Corp, USA).

Statistical analysis

All data were analyzed using the program SPSS13.0 for Windows. Data were presented as mean ± SD. For comparison between multiple groups, data were analyzed by ANOVA. Differences were considered to be statistically significant when $P<0.05$.

RESULTS

Catalpol inhibits ISO-induced cardiac injury in rats

Myocardium in the control group rats had normal cardiac muscle fibres without any necrosis (Figure 1A). The morphology of myocardium in the catalpol group had no

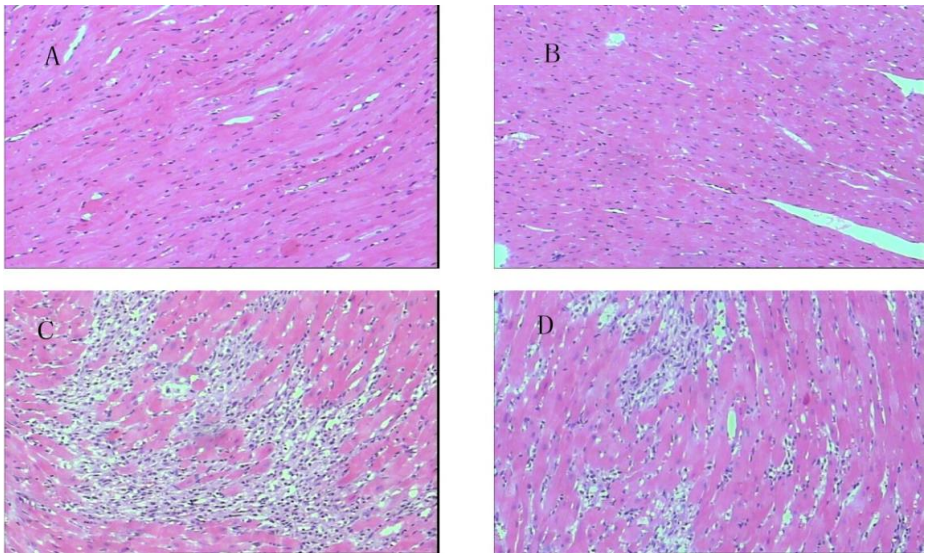

Figure 1. Effects of catalpol on ISO-induced histological changes in the rats myocardium (HE 200×). Rats in the catalpol-treated groups and the ISO + catalpol group were pretreated with intraperitoneal injection of catalpol (10 mg/kg/day) for 10 days. Rats in the control group received physiological saline in an identical fashion with the drug-treated groups instead. Myocardial injury was induced by ISO injection. A :Control group; B: catalpol group; C: ISO group; D: ISO + catalpol group.

changes (Figure 1B). In contrast, the heart tissues in the ISO group were characterized with large scope of necrosis, edema and infiltration of inflammatory cells (Figure 1C). The myocardial lesions in the ISO + catalpol group were similar to those seen in the ISO group. However, the sizes of lesions in the ISO + catalpol group were remarkably smaller when compared with those in the ISO group rats (Figure 1D). In addition, the infiltration of the inflammatory cells was mild. These results indicate that catalpol protected myocardium from ISO-induced injury, which was also confirmed by the pathological grades of all groups rats (Table 1).

Catalpol prevents ISO-induced increases of serum CK-MB and LDH activities

As shown from Table 2, the activities of serum CK-MB and LDH in the ISO group showed significant (p<0.01) increase as compared to the control group. Pretreatment with catalpol significantly prohibited the increase (p<0.01). The results indicate that catalpol prevented ISO-induced myocardial injury.

Effect of catalpol on content of MDA and activities of SOD in myocardial tissue

Table 3 summarizes the levels of myocardial SOD and MDA in all groups. The activities of SOD in the ISO group significantly decreased as compared to the control group (p<0.01). The levels of MDA in the ISO group were significantly increased in comparison with the control group (p<0.01). When compared with the ISO group, the levels of MDA were lower in the ISO + catalpol group (p<0.01). The activities of SOD in the ISO + catalpol group were increased as compared to the ISO group (p<0.01).

Catalpol regulates expressions of inflammatory genes

To investigate the effects of catalpol on expressions of inflammatory genes, real-time RT-PCR analysis was performed. As shown in Figure 2, after ISO injection, the expressions of TNF-*a* and IL-1*β* remarkably increased when compared with the control group (p<0.01). The increases in TNF-*a* and IL-1*β* were reduced significantly by the treatment of catalpol (Figure 2) (p<0.01). However, there was no difference between the control group and the catalpol group. These results suggested that catalpol could inhibit the expressions of inflammatory genes induced by ISO.

Catalpol regulates expressions of inflammatory cytokine proteins

The Western-blot assay was performed to determine the expressions of TNF-*a* and IL-1*β* proteins in myocardial tissues. As shown in Figure 3, there was no difference in

Table 1. The pathological grades of ISO-induced myocardial injury in rats.

Group	Number	Grade 0	Grade I	Grade II	Grade III	Grade IV
Control	8	8	0	0	0	0
Catalpol	8	8	0	0	0	0
ISO	8	0	0	1	5	2
ISO+catalpol	8	0	3	4	1	0

Rats in ISO + catalpol group were pretreated with intraperitoneal injection of catalpol for 10 days. Rats in the control and catalpol groups received vehicle in an identical fashion with the drug-treated groups instead. Myocardial injury was induced by ISO. The degree of injury was graded according to the standard submitted by Rona et al. (1959). Data were analyzed with the Ridit test method. The pathological grades between the ISO group and ISO + catalpol group were $P < 0.01$.

Table 2. The activities of CK-MB and LDH in serum of all groups.

Group	Number	LDH (U/L)	CK-MB (U/L)
Control	8	260.41±20.01	55.23±6.28
Catalpol	8	251.00±18.48	56.03±6.854
ISO	8	669.47±28.94$^{\#}$	156.74±13.91$^{\#}$
ISO + catalpol	8	456.45±32.4$^{\#}$*	91.29±8.05$^{\#}$*

Catalpol inhibited the increases of lactate dehydrogenase (LDH) and creatine kinase (CK-MB) activities in serum. Serum levels of LDH and CK-MB were measured with automatic biochemistry analyzer (AU-2700, Olympus, Japan). Results were presented as mean±SD. $^{\#}P<0.01$ vs. control group; *$P<0.01$ vs. ISO group.

Table 3. Effects of catalpol on the biochemical parameters in ISO-injured myocardium.

Group	Number	MDA (nmol/mg)	SOD (u/mg)
Control	8	0.89±0.08	8.33±0.71
Catalpol	8	0.91±0.09	8.65±0.59
ISO	8	1.67±0.15$^{\#}$	3.77±0.60$^{\#}$
ISO + catalpol	8	1.08±0.13$^{\#}$*	5.58±0.79$^{\#}$*

Rats in the catalpol-treated group and ISO + catalpol group were pretreated with intraperitoneal injection of catalpol (10 mg/kg/day) for 10 days. Rats in the control group received physiological saline in an identical fashion with the catalpol-treated groups instead. Myocardial injury was induced by ISO. The superoxide dismutase (SOD) activities and malonaldehyde (MDA) levels in myocardial tissue were spectrophotometrically determined. Data were expressed as the mean ± SD. $^{\#}P<0.01$ vs. control group; *$P<0.01$ vs. ISO group.

expressions of TNF-*a* and IL-1*β* proteins between the control and the catalpol group. However, the expressions of TNF-*a* and IL-1*β* proteins significantly increased in the ISO group when compared with the control group ($p<0.01$). In pretreatment with catalpol, the changes were significantly reversed ($p<0.01$). The data demonstrated that catalpol could prevent the increases of TNF-*a* and IL-1*β* proteins induced by ISO.

DISCUSSION

ISO, a synthetic catecholamine and *β*-adrenergic agonist, has been found to induce myocardial injury in rats (Rona et al., 1959; Ponnian et al., 2011) and the pathological process mimics the injury that occurs in human hearts. Therefore, the model of isoproterenol-induced myocardial injury offers a standardized noninvasive technique for studying the effects of various cardioprotective therapeutic attempts. Although, the exact mechanism of ISO-induced myocardial injury has not been fully understood, inflammatory response and oxidative stress have been recognized as possible mechanisms (Zhang et al., 2005; David et al., 2000). In our study, the results confirm previous findings that the lesions induced by ISO were characterized by myocyte necrosis, myofibrillar degeneration and leukocyte infiltration in the light microscope. However, pretreatment with catalpol attenuated these changes.

Detection of LDH released into the blood stream from the damaged tissue was another effective diagnostic and prognostic criterion for myocardial injury. In the present

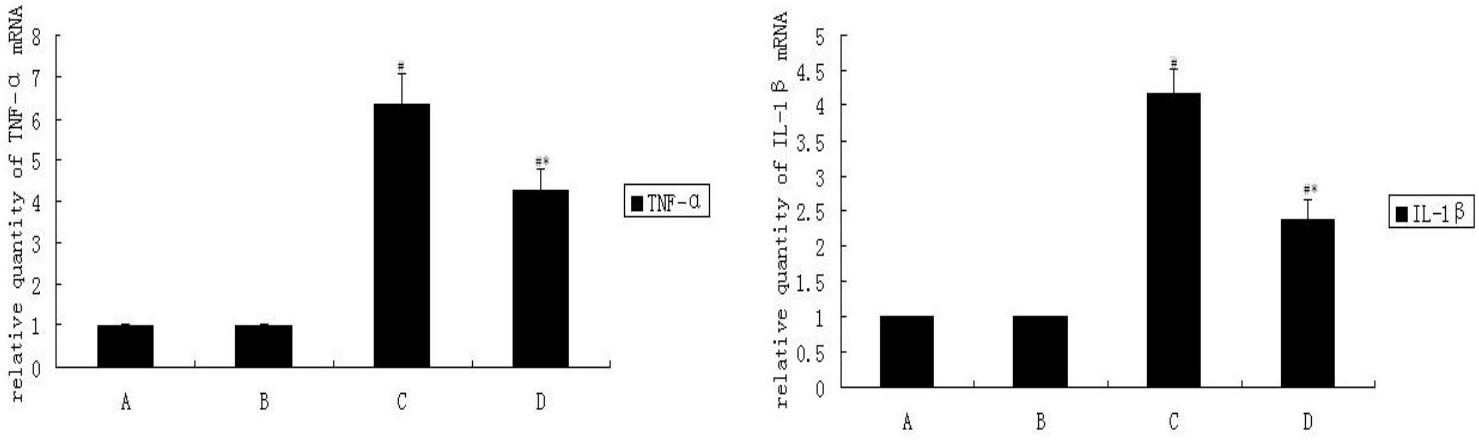

Figure 2. TNF-*a* and IL-1*β* mRNA expressions in rats myocardium. The expressions of tumuor necrosis factor (TNF)-*a* and interleukein (IL)-1*β* mRNA were detected by real time reverse transcription-polymerase chain reaction (RT-PCR). A: Control group; B: catalpol group; C: ISO group; D: ISO + catalpol group. $^{\#}P$<0.01 vs. control group, $^{*}P$<0.01 vs.ISO group.

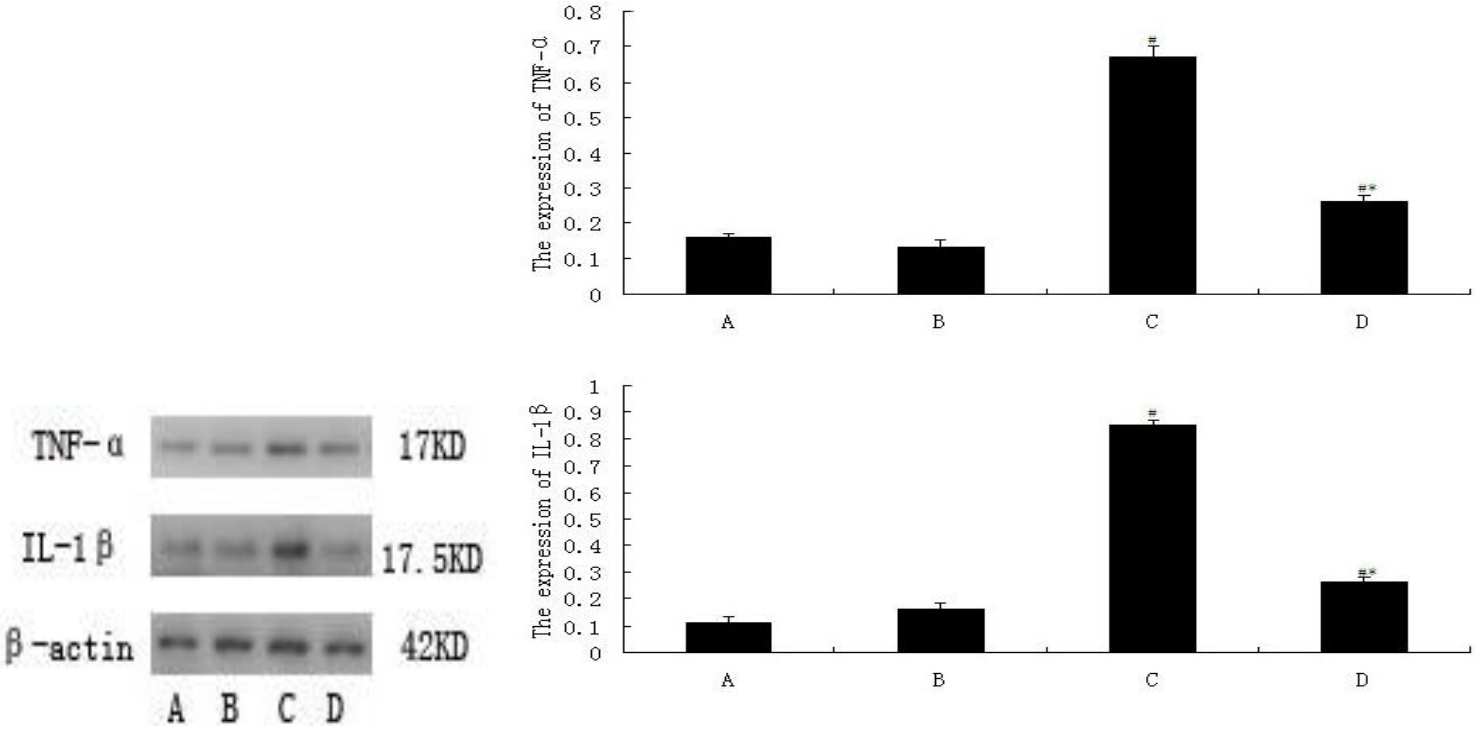

Figure 3. The expressions of TNF-*a* and IL-1*β* proteins in the rats. The TNF-*a* and IL-1*β* proteins were detected by Western blot. A: Control group; B: catalpol group; C: ISO group; D: ISO + catalpol group. $^{\#}P$<0.01 vs. control group; $^{*}P$<0.01 vs. ISO group.

study, we observed the increased activities of serum CK-MB and LDH in the ISO group. The results were consistent with previous reports (Ahmed et al., 2004). Pretreatment with catalpol prevented the increase of serum CK-MB and LDH. These results suggest that catalpol has protective effects on ISO-induced myocardial injury.

Oxidative stress has been implicated in the pathogenesis of many cardiovascular diseases. In the present study, ISO caused obvious oxidative stress in rats heart as evidenced by reduction of myocardial SOD activities, and increase of myocardial MDA. These changes have also been described previously (Ithayarasi and Devi, 1997), and strongly suggests overwhelming superoxide

radical generation and lipid peroxides formation following ISO treatment (Rathore et al., 2000). From comparison between the ISO group and the ISO + catalpol group, we found that catalpol prevented isoproterenol-induced oxidative stress. The observed protective effects might have been mediated through antioxidant effect of catalpol.

Increasing evidences support that inflammation plays an important role in cardiovascular disease, including myocardial injury or silent myocardial ischemia and acute coronary syndromes (Pasqui et al., 2006; Suzuki et al., 2007; Parveen et al., 2011). Many studies have described ISO stimulation induced myocardial proinflammatory cytokine TNF-*a* and IL-1*β* expressions (Feng and Li, 2010; Kumar et al., 2009). On the other hand, other studies have found that *β*-adrenergic blockade treatment could exert salutary effects on myocardial injury which is accompanied by selective reductions in myocardial expression of TNF-*a* and IL-1*β*. Therefore, inhibition of TNF-*a* and IL-1*β* has been considered an important approach to protect myocardial damage (Prabhu et al., 2000). To further study the protective effect of catalpol on myocardial injury, the expressions of TNF-*a* and IL-1*β* mRNA and protein in myocardial tissues were examined. The results show that the expressions of TNF-*a* and IL-1*β* increased significantly in ISO group, while pretreatment with catalpol can significantly inhibit the expressions of TNF-*a* and IL-1*β*, suggesting that anti-inflammation was one of the potential mechanisms by which catalpol protected myocardium from damage in rats.

In summary, we presented the first evidence that catalpol has a significant effect on the protection of the heart against isoproterenol induced myocardial injury, which is associated with its anti-oxidant and anti-inflammatory effects. This finding suggests that catalpol may be a promising agent for the treatment of cardio-vascular disease.

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