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Losartan promotes myocardial apoptosis after acute myocardial infarction in rats through inhibiting Ang II-induced JAK/STAT pathway

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Abstract. – OBJECTIVE: To study the pro-apoptotic effect of Losartan on myocardial cells after acute myocardial infarction (AMI) in rats.

MATERIALS AND METHODS: Before intervention, a total of 48 male Wistar rats were randomly divided into the Sham group (n=12), AMI group (n=12), 5 mg/kg Losartan group (n=12) and 1 mg/kg AG-490 group (n=12). The rats in the Sham group and AMI group received gavage with normal saline, those in the Losartan group received gavage with Losartan for 7 d and those in the AG-490 group were intravenously injected with AG-490 at 30 min before the operation. At 4 d after drug administration, the anterior descending coronary artery was ligated to establish the AMI model in the AMI group and Losartan group, while the same operation was performed, and the anterior descending coronary artery was only threaded in the Sham group. The rats were sacrificed at 24 h after operation. Then, the myocardial apoptosis was detected *via* terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay, and the protein expressions of Janus kinase 2 (JAK2), the signal transducer and activator of transcription 3 (STAT3), the B-cell lymphoma-2 (Bcl-2) and the Bcl-2 associated X protein (Bax) were detected *via* immunohistochemistry and Western blotting. Moreover, the myocardial cells of rats were incubated with angiotensin II (Ang II) at the same concentration at different time points and blocked with Losartan. Finally, the changes in protein expressions of p-JAK2, p-STAT3, Bax, and Bcl-2 were detected *via* Western blotting.

RESULTS: Losartan treatment could increase the number of apoptotic myocardial cells after AMI in rats. In Losartan group, the protein expressions of JAK2, STAT3, and Bcl-2 declined, while the protein expression of Bax was increased, and the Bax/Bcl-2 ratio was also increased, which are consistent with the conditions under the treatment with the JAK-STAT pathway inhibitor AG-490. With the prolonged time of stimulation (5 min, 30 min, 2 h, and 24 h) in myocardial cells using 1.0×10^{-6} mol/L Ang II, the protein expressions of p-JAK2 and p-STAT3 were increased and reached the peak at 24 h. After the application of Losartan, the increased protein expressions of p-JAK2 and p-STAT3 returned to normal levels, and the protein expression of Bax was increased, while that of Bcl-2 was decreased.

CONCLUSIONS: Losartan promotes myocardial apoptosis after AMI in the rats through inhibiting the Ang II-induced JAK/STAT pathway.

Key Words:
Losartan, JAK/STAT, Acute myocardial infarction, Apoptosis.

Introduction

Acute myocardial infarction (AMI) is the most serious type of coronary artery disease (CAD), as well as one of the major causes of death and disability in the world[1]. The renin-angiotensin converting enzyme-aldosterone system (RAAS), being an important system regulating the blood pressure, tissue perfusion, and organ function[2], includes two types of angiotensin converting enzymes (ACE and ACE2) and two types of angiotensin II (AngII) receptors (AT1 and AT2 receptors). The main function of ACE is to catalyze the inactive AngI into biologically active Ang II while inactivating bradykinin.

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Most of the known cardiovascular effects of RAAS are mainly mediated by Ang II, which is involved in vasodilatation and vasoconstriction, water-sodium retention, synthesis and release of aldosterone, cardiovascular hyperplasia and remodeling[3]. At the same time, Ang II leads to AMI by facilitating the vascular endothelium, the production of extracellular matrix, intimal hyperplasia, and formation of pro-inflammatory factors. In AMI, a series of changes occur in the nervous, humoral and endocrine systems, including the activation of RAAS. Moreover, a large number of studies have pointed out that the RAAS disorder has a significant correlation with the occurrence of AMI, and it is clinically found that the serum Ang II is increased in AMI patients[4].

The Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway is an intracellular signal transduction pathway activated by multiplecytokines[5-8], which can be activated by AT1 receptor[9]. The JAK family includes JAK1, JAK2, JAK3, and Tyk2, and they are activated through the interaction among various growth factors and cytokine receptors. JAK-mediated tyrosine phosphorylation of the STAT family can promote these transcription factors to enter into the nucleus and induce the gene transcription. JAK2 plays an important role in the information interaction of the JAK/STAT pathway[10,11]. AG-490 is a selective inhibitor of JAK2[12] that can also inhibit Ang II and the platelet-derived growth factor (PDGF)-induced proliferation of the arterial smooth muscle cells[13]. Moreover, it has been found[14] that JAK1, JAK2, STAT1, and STAT2 are all activated in the heart of rats under acute pressure overload.

Apoptosis is a mode of programmed cell death that is found at different stages in different cells[15]. Apoptosis is associated with stress-induced myocardial hypertrophy[16], myocardial ischemia-reperfusion injury[17], myocardial infarction[18,19], and chronic heart failure[20,21]. In AMI, the death of myocardial cells results from apoptosis and necrosis.

The ACEI/angiotensin receptor blocker (ARB) can weaken the chronic myocardial remodeling after AMI and improve cardiac function[22]. The clinical application of ACEI/ARB in the treatment of cardiovascular disease protects the cardiovascular function by inhibiting RAAS[23]. Losartan is an ARB commonly used in the treatment of hypertension. Scholars[24,25] have demonstrated that AngII can activate the JAK/STAT pathway, while ARB can inhibit the activity of the JAK/STAT pathway. The present study aims to investigate the effects of Losartan pretreatment on experimental AMI and myocardial apoptosis in rats.

Materials and Methods

Animals and Reagents

A total of 48 male Wistar rats aged 4 weeks old and weighing 100-200 g were provided by the Laboratory Animal Center of Northwest University. They had normal nutritional status and mental conditions. This study was approved by the Animal Ethics Committee of Northwest University Animal Center. B-cell lymphoma-2 (Bcl-2), Bcl-2 associated X protein (Bax), JAK2, STAT3, p-JAK2, and p-STAT3 antibodies were purchased from Sigma (St. Louis, MO, USA), and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining kit QIA33 from Shanghai Shiyi Biotechnology Co., Ltd. (Shanghai, China).

Modeling and Grouping

The rats were weighed before the operation, intraperitoneally injected with pentobarbital sodium (50 mg/kg) for anesthesia, and fixed on a plate in the supine position. The neck and precordial skin were disinfected with 70% alcohol and the hair was shaved off. Then, the neck skin was cut and the muscles were bluntly separated. After tracheal intubation, the small animal ventilator was connected under positive pressure ventilation of 70 times/min, tidal volume of 15 mL, and inspiration/expiration ratio of 1:1. The precordial skin was cut, the muscles were bluntly separated and the chest wall was cut open between the third and fourth intercostal spaces. The anterior descending coronary artery was ligated at 1-2 mm below the junction of the left auricle and pulmonary arterial cone using the 5-0 non-invasive wire. In the Sham group, the anterior descending coronary artery was only threaded but not ligated, and the remaining operations were the same as above. The chest cavity was closed along the third and fourth ribs, the chest muscles were sutured layer by layer, and the chest skin was continuously sutured finally. The chest was squeezed to drain off the gas in the chest cavity for pulmonary reexpansion, the ventilator was withdrawn, and the neck skin was sutured, followed by heat preservation for 8 h.

After the operation, 80,000 U of penicillin sodium was intramuscularly injected for anti-infection for 3 consecutive days after the operation.

Cell Culture

The myocardial cells of the neonatal rats were paved onto a 35 or 60 mm culture dish at a density of $1.0\times10^5/cm^2$ and cultured in the M-199 containing 10% NCS and 0.1 mM bromodeoxyuridine. At 24 h after inoculation, the medium was replaced with the M-199 containing 10% NCS. At 6 h before the experiment, the medium was replaced with the M-199.

TUNEL

The paraffin tissue sections were deparaffinized with xylene, dehydrated with gradient alcohol, and treated with proteinase K working solution at 21-37°C for 15-30 min or permeabilization solution for 8 min. The TUNEL reaction mixture was prepared: 50 μL of TdT + 450 μL of the fluorescein-labeled dUTP solution was added in the treatment group, while only 50 μL of the fluorescein-labeled dUTP solution was added in the negative control group. After slide drying, 50 μL of TUNEL reaction mixture was added onto the specimens in the treatment group, while only 50 μL of the fluorescein-labeled dUTP solution was added onto the specimens in the negative control group. After the slide was covered with the cover glass or sealing membrane, it was reacted in a dark and wet box at 37°C for 60 min. After slide drying, 50 μL of converter-POD was added onto the specimens, and the slide was covered with the cover glass or sealing membrane, followed by the reaction in the dark and wet box at 37°C for 30 min. Then, 50-100 μL of diaminobenzidine (DAB; Solarbio, Beijing, China) substrate was added onto the specimens for the reaction at 15-25°C for 10 min. After counterstaining with hematoxylin or methyl green for a few seconds, the sections were washed wtih tap water, dehydrated with gradient alcohol, transparentized with xylene, and sealed with neutral balsam. After a drop of PBS or glycerol was added, the cells (200-500 cells) were counted and photographed under an optical microscope. Apoptotic index (AI) = the number of positive cells/total number of cells × 100. Then, the average in each group was taken.

Western Blotting

The tissues extracted were ground with liquid nitrogen, diluted with normal saline, and placed on ice. The supernatant was taken and centrifuged at 4°C for 5 min, and the supernatant was discarded. The precipitate was directly resuspended and lysed in radioimmunoprecipitation assay (RIPA) lysis buffer containing phenylmethylsulfonyl difluoride (PMSF; Beyotime, Shanghai, China), and centrifuged at 16,000 g and 4°C for 15 min. The supernatant was taken for protein quantification. Then the protein was added with loading buffer, denatured *via* heating, subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto a membrane. The membrane was sealed with 5% skim milk for 2 h, incubated with the primary antibody at 4°C overnight, washed with Tris-Buffered Saline and Tween20 (TBST) for 3 times (10 min/time), incubated again with the corresponding secondary antibody at room temperature for 1 h, and washed again with TBST for 3 times (10 min/time). Finally, the protein expression in different specimens was detected by the enhanced chemiluminescence (ECL) method.

Statistical Analysis

The data were expressed as mean ± standard deviation and analyzed *via* paired or unpaired *t*-test. A comparison between multiple groups was done using One-way ANOVA test followed by the post-hoc test (Least Significant Difference). $p<0.05$ suggested the statistically significant difference. Statistical Product and Service Solutions (SPSS) 20.0 software (IBM Corp., Armonk, NY, USA) was used for data analysis, and GraphPad software (Version X; La Jolla, CA, USA) for plotting.

Results

Losartan Increased the Number of Apoptotic Myocardial Cells After AMI and the Protein Expression of Bax in the Infarction Region, and Inhibiting JAK/STAT Pathway Had the Same Effects

In this experiment, a total of 36 rats underwent coronary artery ligation, 29 of whom survived after the operation, and the remaining 12 rats underwent sham operation and were enrolled in the Sham group. After ligation, the myocardial apoptosis was detected *via* TUNEL. It was found that the number of apoptotic myocardial cells was extremely small in the Sham group, while it was increased in the AMI group and had a statistically significant difference compared with that in the Sham group ($p<0.05$). Compared with the AMI

group, the number of apoptotic myocardial cells in the infarction region was further increased in both Losartan group ($p<0.05$) and AG-490 (JAK/STAT pathway inhibitor) group (Figure 1A). Besides, the results of the Western blotting showed that the Bax protein was expressed in all groups, and its expression was higher in the AMI group than that in the Sham group, while it was further increased in both Losartan group and AG-490 group (Figures 1B-1C). Bcl-2 protein was also expressed in all groups, and its expression was significantly higher in the AMI group, Losartan group, and AG-490 group than that in Sham group, while it declined in the Losartan group and in the AG-490 group, compared with that in the AMI group ($p<0.05$) (Figures 1D-1E). The above results indicate that Losartan treatment can promote the expression of Bax (pro-apoptotic gene) and decrease the expression of Bcl-2 (anti-apoptotic gene), and that the inhibition of the JAK/STAT pathway has the same effects.

Losartan Inhibited the Expression of JAK/STAT Pathway in Infarction Region of Rats After AMI

The results of the Western blotting showed that the protein expression of JAK2 after AMI was significantly increased in the AMI group compared with that in the Sham group (Figures 2A-2B), while it was decreased in the Losartan group compared with that in the AMI group ($p<0.05$). Besides, the protein expression of STAT3 was significantly increased in the AMI group, while it significantly declined in the Losartan group compared with that in the AMI group ($p<0.05$) (Figures 2C-2D).

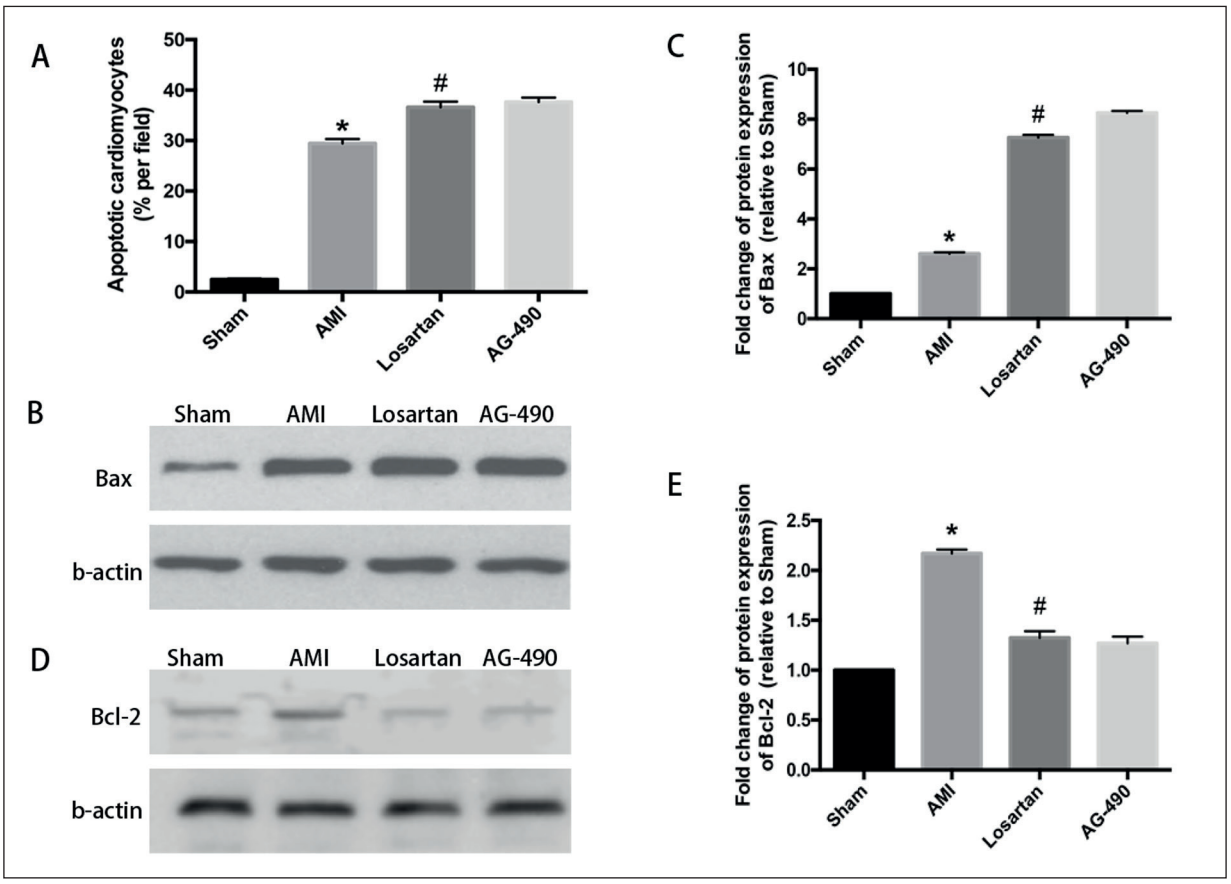

Figure 1. A, TUNEL staining in the infarction region at 24 h after AMI in the Sham group, AMI group, Losartan group, and AG-490 group. The quantitative analysis of apoptosis is TUNEL-positive nuclei/mm^2 in the infarction region, and expressed as mean ± standard deviation. *$p<0.05$ *vs.* Sham group, $^{\#}p<0.05$ *vs.* AMI group. **B, C,** Bax protein content in the infarction region at 24 h after AMI in the Sham group, AMI group, Losartan group, and AG-490 group detected *via* Western blotting and quantitative analysis. *$p<0.05$ *vs.* Sham group, $^{\#}p<0.05$ *vs.* AMI group. **D, E,** Bcl-2 protein content in the infarction region at 24 h after AMI in the Sham group, AMI group, Losartan group, and AG-490 group detected *via* Western blotting and quantitative analysis. *$p<0.05$ *vs.* Sham group, $^{\#}p<0.05$ *vs.* AMI group.

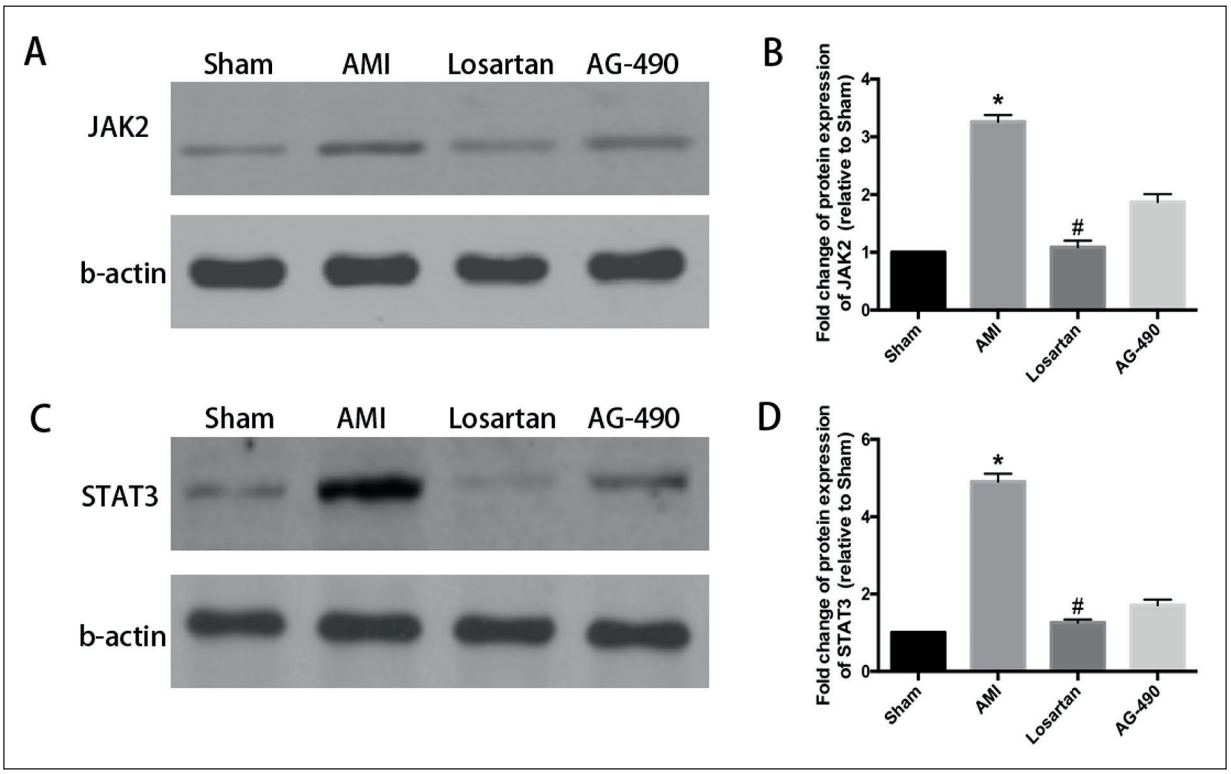

Figure 2. A, B, JAK2 protein content in the infarction region at 24 h after AMI in the Sham group, AMI group, Losartan group, and AG-490 group detected *via* Western blotting and quantitative analysis. **p*<0.05 *vs.* Sham group, #*p*<0.05 *vs.* AMI group. **C, D,** STAT3 protein content in the infarction region at 24 h after AMI in the Sham group, AMI group, Losartan group, and AG-490 group detected *via* Western blotting and quantitative analysis. **p*<0.05 *vs.* Sham group, #*p*<0.05 *vs.* AMI group.

Effects of Ang II on the Expression of JAK/STAT Signaling Pathway After Incubation for Different Time

With the prolonged time of stimulation (5 min, 30 min, 2 h, and 24 h) in myocardial cells using 1.0×10^{-6} mol/L Ang II, the protein expressions of p-JAK2 and p-STAT3 were increased and reached the peak at 24 h, indicating that the expressions of p-JAK2 and p-STAT3 are enhanced in a time-dependent manner under Ang II stimulation (Figure 3A).

Effects of Losartan on Ang II-Stimulated JAK/STAT Signaling Pathway and Expressions of Bax and Bcl-2

The myocardial cells of rats were incubated with Losartan (1 μm/L) first for 24 h, and then co-incubated with Ang II (1.0×10^{-6} mol/L) for 24 h. The results of the Western blotting manifested that the expressions of p-JAK2 and p-STAT3 declined, compared with those under Ang II stimulation alone in myocardial cells ($p<0.05$). However, they had no statistically significant differences compared with the Sham group ($p>0.05$), suggesting that Losartan can completely inhibit the increase in p-JAK2 and p-STAT3 after Ang II stimulation in myocardial cells. After the myocardial cells were incubated with Losartan alone, the expressions of p-JAK2 and p-STAT3 had no evident changes compared with those in the Sham group ($p>0.05$), suggesting that Losartan has no influences on the expressions of p-JAK2 and p-STAT3 in myocardial cells under basal conditions (Figures 3B-3E).

The protein expression of Bax in myocardial cells stimulated by Ang II and co-incubated with Losartan was remarkably higher than that in the cells stimulated by Ang II alone ($p<0.05$) (Figures 4A-4B). The protein expression of Bcl-2 was increased in myocardial cells stimulated by Ang II alone and myocardial cells co-incubated with Losartan, showing a statistically significant difference compared with the Sham group ($p<0.05$), while it declined in myocardial cells co-incubated with Losartan compared with that in the myocardial cells stimulated by Ang II alone ($p<0.05$) (Figures 4C-4D).

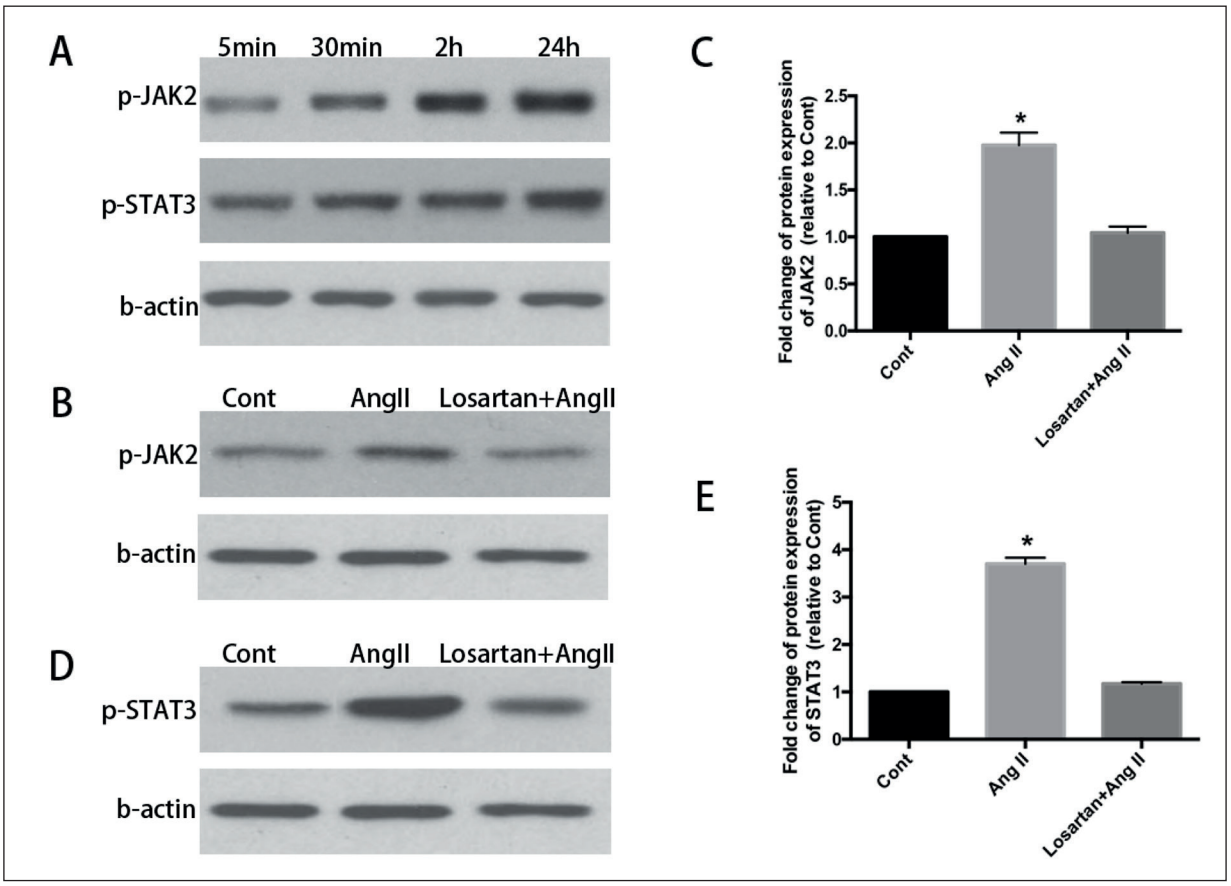

Figure 3. A, P-JAK2 and p-STAT protein content in myocardial cells stimulated by Ang II at 5 min, 30 min, 2 h, and 24 h detected *via* Western blotting. **B, C,** P-JAK2 protein content in myocardial cells stimulated by Ang II for 24 h in the Cont group, Ang II group, Losartan group, and Losartan + Ang II group detected *via* Western blotting. *$p<0.05$ *vs.* Cont group. **D, E,** P-STAT3 protein content in myocardial cells stimulated by Ang II for 24 h in the Cont group, Ang II group, Losartan group, and Losartan + Ang II group detected *via* Western blotting. *$p<0.05$ *vs.* Cont group.

Discussion

In this research, the changes in the expression of JAK/STAT signaling pathway and its mechanism in myocardial infarction (MI) in rats were mainly explored. AMI is accompanied by necrosis and apoptosis of myocardial cells, and myocardial apoptosis in the infarction region is closely related to early ventricular remodeling[26]. The mechanisms of myocardial apoptosis in AMI are complex, one of which is that myocardial cells can release Ang II under ischemia and stress in AMI, and activate the JAK/STAT pathway in combination with other cytokines[27]. The results of this experiment showed that Ang II could induce an increase in the expression of JAK/STAT pathway, which was increased more significantly with time. Under Losartan treatment, the expression of Ang II declined, and the expression of JAK/STAT pathway was also decreased.

The apoptosis process is accompanied by an increase in the expression of Bax, which promotes the formation of the Bax-Bcl-2 complex, and then, leads to the activation of caspase-3 to further promote apoptosis. Qin et al[28] have found that the Bax protein expression in the myocardium is increased due to heart injury in AMI, thus inducing apoptosis and ventricular remodeling. In this experiment, JAK2 induced the Bcl-2 expression and inhibited the cell death after JAK2 was inhibited by AG-490. Moreover, reports have shown that JAK2 is involved in inhibiting apoptosis, and the anti-apoptotic signal is transmitted by JAK2.

The STAT family is closely related to apoptosis. STAT1 and STAT3 are the most observed,

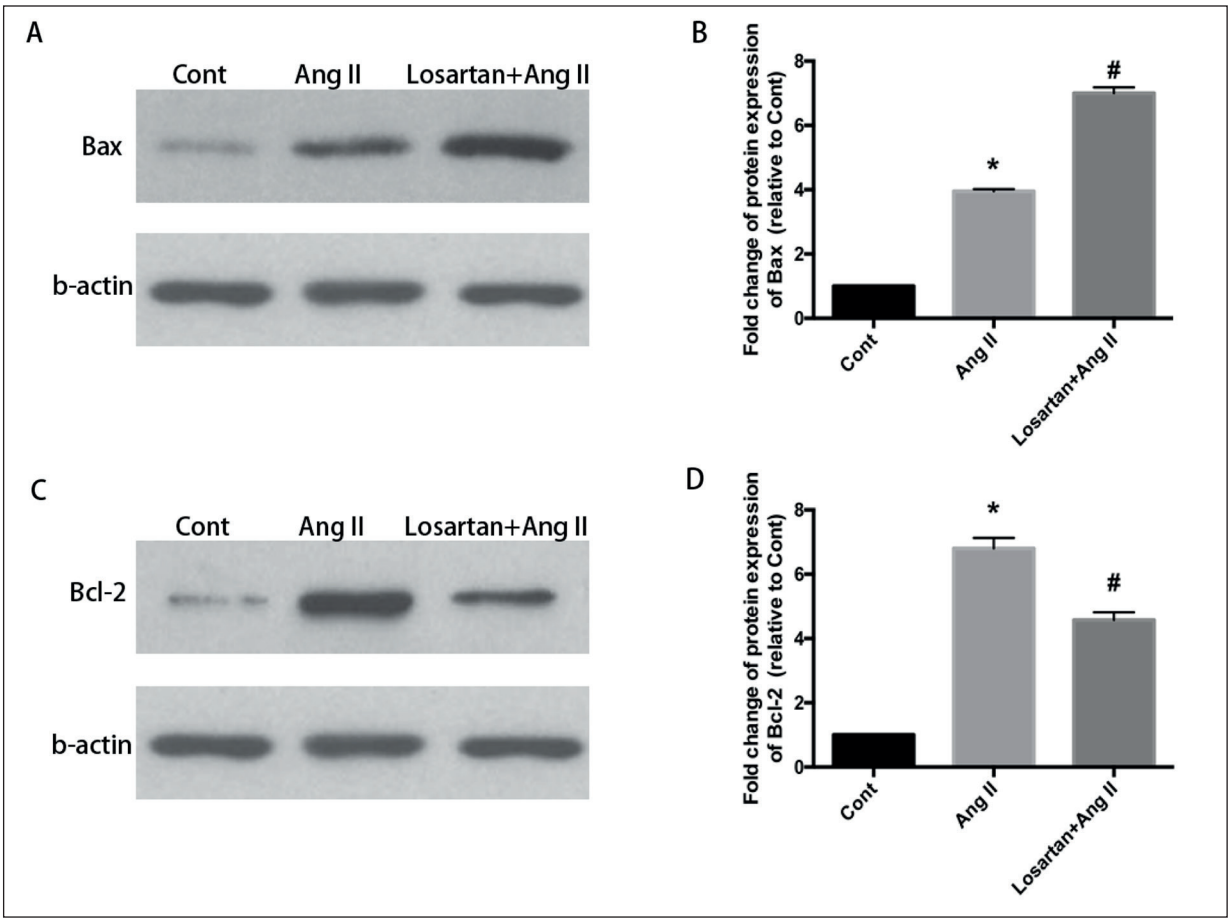

Figure 4. A, B, Bax protein content in myocardial cells stimulated by Ang II for 24 h in Cont group, Ang II group, Losartan group and Losartan + Ang II group detected *via* Western blotting. **p*<0.05 *vs.* Cont group, #*p*<0.05 *vs.* Ang II group. **C, D,** Bcl-2 protein content in myocardial cells stimulated by Ang II for 24 h in Cont group, Ang II group, Losartan group and Losartan + Ang II group detected *via* Western blotting. **p*<0.05 *vs.* Cont group, #*p*<0.05 *vs.* Ang II group.

the former of which promotes apoptosis, and the latter of which inhibits apoptosis. The degree and time of STAT1 and STAT3 activation determine whether apoptosis occurs. Some researchers have demonstrated that p-STAT1 and p-STAT3 have increased the expressions in mice with acute cardiac hypertrophy, during which p-STAT1 is transiently increased to promote myocardial apoptosis by reducing the expression of anti-apoptotic proteins Bcl-2 and Bcl-x. Moreover, p-STAT3 is persistently increased to exert an anti-apoptotic effect on myocardial cells[29]. According to the studies on the myocardial ischemia-reperfusion injury, both STAT1 and STAT3 are activated by related cytokines within several minutes to hours, but STAT1 is activated later than STAT3. Moreover, they are activated at different time points: STAT3 in ischemic stage and STAT1 in reperfusion stage, and the ratio between them may determine the balance between survival and death of myocardial cells induced by myocardial ischemia-reperfusion, ultimately determining the severity of the myocardial ischemia-reperfusion injury. STAT1 promotes myocardial apoptosis by up-regulating the expressions of caspase-1 and apoptotic membrane surface molecules (Fas and FasL)[30]. The mechanism of action of STAT3 is to inhibit apoptosis by up-regulating Bcl-2 and down-regulating Bax and caspase-3[31]. In this investigation, it was found that the JAK/STAT expression in the infarction region of AMI rats was increased, and the Bax protein expression was also increased, raising the number of apoptotic myocardial cells. The Losartan treatment inhibited the JAK/STAT expression in the infarction region, decreased the expression of Bcl-2, and further increased the expression of Bax, so the Bax/Bcl-2 ratio was further increased, aggravat-

ing myocardial apoptosis. The consistent results were obtained under AG-490 treatment, and the trends in cell experiments and animal researches were the same.

In MI, myocardial apoptosis and resulting ventricular remodeling are of important significance in the direction of the outcome of cardiac function after MI. On the one hand, myocardial apoptosis is an acute response of myocardial cells to MI, which plays an important role in the degree of heart damage and retention of cardiac function in MI. On the other hand, it is also involved in early and late ventricular remodeling, which has a non-negligible influence on cardiac dilatation and impaired cardiac function after MI. As a member of the ACEI/ARB family and a common antihypertensive drug, Losartan can effectively reduce the expression of Ang II. In this experiment, it was found that Losartan reduced Ang II and inhibited the Ang II-induced expression of JAK/STAT pathway, thereby suppressing the anti-apoptotic effect of JAK/STAT pathway, and further increasing the level of myocardial apoptosis in MI. However, the detailed mechanism of Losartan in inhibiting the JAK/STAT pathway requires further studies, and the role of ACEI/ARB in MI patients needs to be carefully considered to avoid risk.

Conclusions

We found that Losartan promotes myocardial apoptosis after AMI in rats by inhibiting the Ang II-induced JAK/STAT pathway.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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