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Effect of NF-κB inhibitor on Toll-like receptor 4 expression in left ventricular myocardium in two-kidney-one-clip hypertensive rats

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Abstract. – OBJECTIVE: To investigate the effects of an inhibitor of NF-κB, PDTC (pyrrolidine dithiocarbamate), on TLR4 (Toll-like receptor 4) expression in the left ventricle of Goldblatt hypertension rats.

MATERIALS AND AND METHODS: Goldblatt rat model of two-kidney, one-clip (2K1C) hypertension was established in 70 healthy male rats. The rats were randomly divided into sham operation group (S group, n=20), non-drug intervention hypertension group (H group, n=25), and PDTC intervention group (P group, n=25). P group was injected with PDTC. The clip was inserted in the left renal artery of H group and P group (2K1C). Eight weeks after the operation, the rats were sacrificed and the samples of the left ventricle were collected. The concentration of AngII in the left ventricle was assessed by radioimmunoassay. RT-PCR was used to examine the mRNA expression of TLR4 in the left ventricle. Immunohistochemistry was adopted to examine the location of TLR4 and NF-κB in the myocardium. Victoria blue-Ponceau staining of Cardiac collagen was used to evaluate the degree of myocardial fibrosis.

RESULTS: Eight weeks after the operation, caudal SBP, meridional end-systolic stress, left ventricular mass index, relative wall thickness, cardiac fibrosis degree, and the concentration of AngII in the left ventricle in P group were significantly lower than those in H group ($p<0.01$). In cardiac myocytes of S group and P group, TLR4 expression was diffused and presumably cytoplasmic. TLR4 mRNA expression in P group was significantly lower than that of H group ($p<0.01$).

CONCLUSIONS: PDTC not only inhibited the activation of NF-κB, but decreased TLR4 expression and AngII content, indicating that the inflammatory signals and oxidative stress mediated by TLR4/NF-κB are involved in the occurrence and development of left ventricular remodeling. Intervention with TLR4/NF-κB and anti-inflammatory and anti-oxidative therapy may be a new target to reverse left ventricular remodeling.

Key Words

Hypertension, Left ventricular remodeling, Toll-Like Receptor 4 (TLR4), NF-κB, Goldblatt rats..

Introduction

Hypertensive left ventricular remodeling is an independent risk factor for cardiovascular and cerebrovascular diseases, as well as the basic mechanism of heart failure. So, looking for common causes of cardiac remodeling in cardiac signaling pathways and exploring the direct protection of target organs has been an important strategy for the prevention and treatment of cardiovascular disease[1]. There is growing evidence that inflammation plays an important role in cardiac remodeling of hypertension[2]. As early as in 1999, researchers noticed that Toll-like receptor 4 (TLR4), a transmembrane signal transduction receptor closely related to inflammation and immunity, had a high level of expression in cardiomyocytes. In normal murine and human myocardium, TLR4 is predominantly diffusely expressed in the cytoplasm of cardiomyocytes, whereas TLR4 expression is up-regulated in locations distant from injured murine myocardial tissue remodeled from the ischemic infarction and in patients with dilated cardiomyopathy, and focal TLR4 is strongly stained at the junction of two or more adjacent cells[3]. Since then, more studies[4-6] have shown that TLR4/NF-κB, a signal transduction pathway closely related to immunity, inflammation and oxidative stress, participates in diseases such as viral myocarditis, sepsis, septic shock, and atherosclerosis. In this work, two-kidney-one-clip Goldblatt rat model of hypertension was used to observe the expression of TLR4 in left ventricular remodeling myocardium. Pyrrolidine dithiocarbamate (PDTC)[3,7,8], the inhibitor of NF-κB, was used to observe changes of TLR4 expression, activation of NF-κB, left ventricular structure, left ventricular myocardial angiotensin II content, and myocardial fibrosis. To provide a new entry point of reversal of hypertensive left ventricular remodeling, the effects of TLR4 signaling pathway on the left ventricular remodeling in hypertensive patients were explored.

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Materials and Methods

Experimental Subjects and Main Reagents

Seventy-five healthy male Sprague-Dawley (SD) rats aged 7-9 weeks and weighing 180 ± 16 grams were provided by Animal Experimental Center, Dalian Medical University (Liaoning, China). The rats were adapted for a week before the experiment, free access to water and food. Pyrrolidine dithiocarbamate (PDTC): NF-κB inhibitor, Sigma-Aldrich (St. Louis, MO, USA). TRIzol: Gibco Co. (Grand Island, NY, USA). RT-PCR Kit: TaKaRa, DRR024A, purchased from Dalian TaKaRa Biological Reagent Co. (Dalian, China). DL2000: DNA marker, a product of TaKaRa, purchased from TaKaRa Biological Reagent Co. (Dalian, China). Angiotensin II (AngII) radioimmunoassay kit: purchased from Beijing Haikerui Radioimmunoassay Center (Beijing, China). Goat anti-mouse TLR4 polyclonal antibody, mouse anti-human NF-κB p65 monoclonal antibody: a product of Santa Cruz Biotechnology (Santa Cruz, CA, USA). SP immunohistochemistry kit, including biotinylated secondary antibody, hydrogen peroxide, blocking serum, horseradish peroxidase-labeled streptavidin: a product of Zymed (San Diego, CA, USA). Victoria Blue, Ponceau S dye: Shanghai Reagent Factory (Shanghai, China). The investigation was approved by the Ethics Committee of The Second Affiliated Hospital of Dalian Medical University.

Preparation and Grouping of 2K1C, Goldblatt Rat Model of Hypertension

Body weight (BW) and basal caudal arterial pressure were measured and echocardiography was performed in all rats before the operation. The rats were randomly divided into operation group and sham operation group (S group, n = 20). The operation group was given intraperitoneal anesthesia with 20% urethane (1 g/kg). After shearing, disinfecting and opening, the upper left renal artery was separated. The left renal artery was clipped with a 0.3 mm diameter silver pincer to establish partial stenosis. The sham operation group (S Group) was performed with the same procedure, but the silver clip was not placed. The caudal arterial pressure was measured weekly in all rats and echocardiography was performed every other week. After two weeks, fifty rats from operation group with a caudal arterial pressure ≥150 mmHg or more than 30 mmHg above basal blood pressure were selected and randomly divided into hypertension non-drug intervention group (H group, n = 25) and hypertension PDTC intervention group (P group, n = 25). P group received 100 $mg \cdot kg^{-1} \cdot d^{-1}$ subcutaneous injection of PDTC for 6 weeks after two weeks of operation. H group and S group were given an equal volume of saline subcutaneously. At the week 8 after the operation, the tail artery pressure and echocardiography were measured, and then rats were sacrificed and left ventricular myocardium was taken. The cross-section of left middle ventricle directly underwent frozen section. The section was fixed with acetone for 15 minutes and then stored in a refrigerator at 4°C for future use. 4 mm × 4 mm myocardial block was taken at the same position and fixed in 10% neutral formalin to prepare paraffin section. The remaining myocardial tissue was reserved in -80°C refrigerator.

Tail Artery Pressure Measurement

Rats in each group were placed in a warm incubator at 40°C for 15 minutes. The caudal artery cuff (RBP-1B rat tail artery blood measure meter: produced by Sino-Japanese Friendship Institute of Clinical Medicine) was connected in a sober and quiet state to measure the tail artery pressure, continuously recorded 3 times to calculate the average.

Determination of Cardiac Structure

Rats in each group were examined under ultrasound after anesthetized by inhalation of 20% ether (ultrasound diagnostic instrument: SONOLINE prima produced by Siemens, Munich, Germany, probe frequency 7.5 MHz). The left ventricular (LV) long axis transection at mitral valve tip level was taken and the left ventricular end-systolic dimension (LVESD), left ventricular end-diastolic dimension (LVEDD), LV posterior wall thickness at end-systole (LVPWS), LV posterior wall thickness at end-diastole (LVPWD), interventricular septal thickness at end-systole (IVSS), and interventricular septal thickness at end-diastole (IVSD) were measured by M ultrasound under the guidance of two-dimensional image. Five consecutive cardiac cycles were measured and the mean values were obtained. LV mass (LVM), LV mass index (LVMI), relative wall thickness (RWT), and Meridional end-systolic wall stress (MESS) were calculated[9].

Each parameter was calculated as:

$$LVM=1.05\times[(LVEDD+2\times LVPWD)^3-LVEDD^3]$$

$$LVMI=LVM/BW$$

$$RWT=(LVPWD+IVSD)/LVEDD$$

$$MESS=0.334\times SBP\times LVESD/\{LVPWS+[1+(LVPWS/LVESD)]\}$$

Determination of AngII Content in Myocardial Tissue

200 mg of myocardial tissue were obtained and 2 ml 1M acetic acid was added, boiled for 15 minutes, cooled and homogenized, centrifuged 3000 rpm at 4°C for 20 minutes and the supernatant was obtained. The content of angiotensin II (AngII) in myocardial tissue was determined by radioimmunoassay.

Myocardial Collagen Staining and Determination

Victoria blue-Ponceau red staining[10]: myocardial tissue was embedded in paraffin, 4 μm section, routine dewaxing to water. After slightly washed with 70% ethanol, the section was immersed in Victoria Blue dye for 15 minutes, differentiated in 90% ethanol for few seconds, washed twice with distilled water, and stained with Ponceau red dye droplets for 5 minutes; anhydrous ethanol was directly used to differentiate and dehydrate, xylene was used to clear, resinene was used to seal. After staining, collagen fibers were red, myocardial cells were yellow, and nuclei and blood cells were green. The collagen volume fraction (CVF) and perivascular collagen area (PVCA) were measured by the computer image analysis system. Calculation method: CVF = collagen area / total area, where collagen area did not include perivascular collagen. PVCA = collagen area around the lumen of the myocardial small arteries / arterial lumen area.

RT-PCR Method to Detect TLR4 mRNA in Left Ventricular Myocardium

100 mg of myocardial tissue were collected and the total RNA was extracted according to the method provided in TRIzol reagent instruction. The upstream primer of TLR4 was: 5'-CGCTTTCAGCTTTGCCTTCATTAC-3', and the downstream primer was 5'-AGCTACTTCCTTGTGCCCTGTGAG-3', and the amplified fragment was 555 bp. The upstream primer of internal reference β-actin was 5'-AACCCTAAGGCCAACCGTGAAAAG-3', the downstream primer was 5'-TCATGAGGTAGTCTGTCAT-3', and the amplified fragment was 241 bp. One-step RT-PCR reaction system: 2.5 μl of 10 × one-step RNA PCR Buffer, 5 μl of $MgCl_2$, 2.5 μl of DNTP, 0.5 μl of RNase inhibitor, 0.5 μl of AMV, 0.5 μl of Taq, 0.5 μl of the upstream primer of TLR4 and 0.5 μl of the downstream primer of TLR4, 0.5 μl of β-actin upstream primer, 0.5 μl of downstream primer of β-actin, 0.5 μl of sample total RNA, 11 μl of RNA free H_2O, and the total system was 25 μl. The reaction conditions were: 50°C 30 minutes, 94°C 2 minutes; 94°C 30 seconds, 60°C 70 seconds, 72°C 90 seconds, 30 cycles, 72°C 10 minutes for extension. RT-PCR product underwent 2% agarose gel electrophoresis. The results of electrophoresis were collected by the computer gel scanning imaging system, and the gray ratio of target band and internal reference band was analyzed as the relative content of gene expression for semi-quantitative analysis.

Immunohistochemistry Detection of the Expression of TLR4 and NF-κB in Myocardium

1) SP method routine immunohistochemical staining.
   Myocardial tissue was paraffin-embedded, sectioned into 4 μm slices, and underwent routine dewaxing to water; 3% H_2O_2 was added dropwise to block endogenous peroxidase; then, the section was placed in 0.01 M pH = 6.0 citrate buffer at 100°C and underwent microwave repair for 10 minutes; the cells were buffered with PBS and blocked with animal serum, incubated at room temperature for 10 minutes. The cells were incubated with a primary antibody against NF-κB P65 (diluted 1:100) for 1 hour in a wet box at 37°C, then added with secondary antibody and incubated in a wet box for 15 minutes; horseradish peroxidase-labeled streptavidin was added dropwise and the cells were incubated at 37°C for 15 minutes; colored with diaminobenzidine (DAB) and terminated with water, then dehydrated, cleared and sealed.
2) Frozen section staining underwent without antigen repair, and the remaining steps were as above. TLR4 primary antibody was diluted 1:100.
3) Observations and analysis of the results: (1) The positive expression of TLR4 protein located in the membrane and cytoplasm, and showed brownish yellow homogeneous. (2) The positive expression of NF-κB protein located in the cytoplasm and nucleus showed brown particles. Under normal conditions, NF-κB was present in the cytoplasm of cells, and moved into the nucleus after activation. In this study, the positive nucleus was counted as the standard. 10 fields in each tissue section were taken randomly under light microscopy (× 400 times). The activation of NF-κB was assessed by counting the percentage of positive cells in cardiomyocytes.

Table I. Rat blood pressure and cardiac structure before operation ($\bar{\chi}$±s).

	S group (n=20)	H group (n=25)	P group (n=25)
SBP (mmHg)	124.17±8.99	122.20±7.64	123.24±6.29
LVMI (mg/g)	2.24±0.52	2.22±0.79	2.23±0.61
LVPWD (mm)	1.62±0.36	1.62±0.38	1.62±0.37
LVEDD (mm)	4.18±1.21	4.16±1.22	4.17±1.21
RWT	0.52±0.04	0.52±0.04	0.52±0.05
MESS (kdyne/cm^2)	30.30±4.15	30.38±2.99	30.44±5.06

Statistical Analysis

SPSS 13.0 statistical software (SPSS Inc., Chicago, IL, USA) was applied for data analysis. Data processing: the experimental results were expressed as mean ± standard deviation ($\bar{x}$± s). One-way ANOVA was used to compare the mean of multiple samples. The comparison between any two means was performed by LSD method. The LSD method was applied in the comparison between two groups. p<0.05 represented that the difference was statistically significant.

Results

Rat Tail Artery Pressure and Cardiac Structure Changes

There were no significant differences in SBP and echocardiography between pre-operation rats (Table I). Eight weeks after the operation, the levels of SBP, LVMI, LVPWD, RWT, and MESS in H group were significantly higher than those in S group (p<0.01), and those in PDTC intervention group were significantly lower than those in H group (p<0.01), but higher than those in S group (p<0.05) (Table II).

AngII Content in Each Group Myocardial Tissue (Table II)

Eight weeks after the operation, the content of AngII in the left ventricular myocardium in H group was significantly higher than that in S group (p<0.01). The AngII content in P group was significantly lower than that in H group (p<0.05), and still higher than that in S group. The differences were statistically significant (p<0.05).

Myocardial Collagen Staining Results of Each Group (Table III)

Eight weeks after the operation, a small amount of red collagen deposition occurred in the outer membrane of the coronary artery in the myocardium of rats in S group and did not extend to the periphery (Figure 1). A large number of red collagen fiber depositions were abnormally seen in the intima adventitia of coronary artery of H group, and the depositions extended to the gap of surrounding myocardium (Figure 2). A large number of collagen fiber depositions can be observed in the intima adventitia of the myocardial coronary artery of P group (Figure 3). Statistical analysis showed that CVF and PVCA in H group

Table II. Rat blood pressure, cardiac structure and Ang II content in myocardium tissue 8 weeks after operation ($\bar{\chi}$±s).

	S group (n=20)	H group (n=25)	P group (n=25)
SBP (mmHg)	120.17±8.03	172.80±10.61**	139.48±8.96$^{▲▲*}$
LVMI (mg/g)	2.09±0.19	2.98±0.25**	2.35±0.24$^{▲▲*}$
LVPWD (mm)	1.70±0.21	2.32±0.28**	1.98±0.23$^{▲▲*}$
LVEDD (mm)	4.85±1.33	4.35±1.25**	4.61±1.30$^{▲▲*}$
RWT	0.50±0.05	0.70±0.09**	0.59±0.07$^{▲▲*}$
MESS (kdyne/cm^2)	30.06±9.02	41.74±12.56**	34.87±10.14$^{▲▲*}$
AngII (ng/g)	5.11±1.54	8.10±2.64**	5.96±2.50$^{▲*}$

Note: Compared with S group $^{*}p$<0.05, $^{**}p$<0.01; compared with H group $^{▲}p$<0.05, $^{▲▲}p$<0.01.

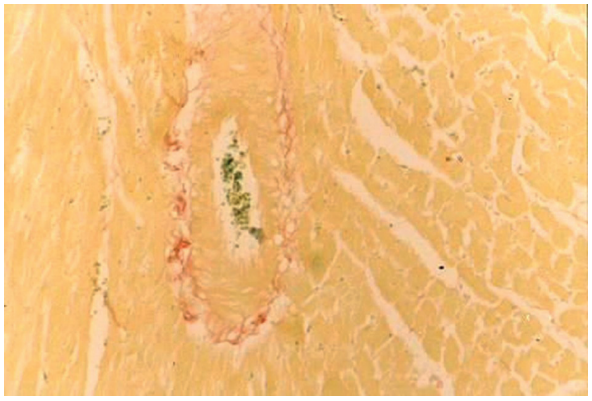

Figure 1. Myocardial collagen staining in S group (× 200).

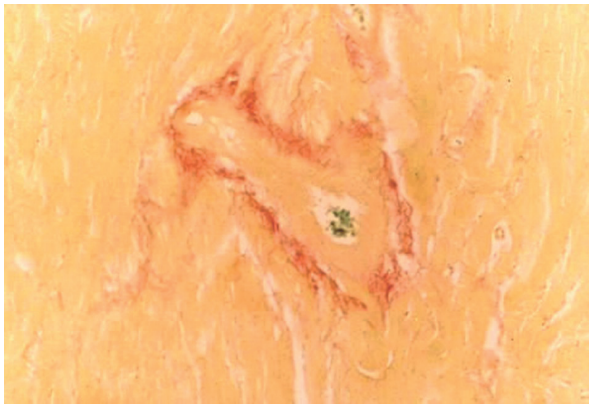

Figure 2. Myocardial collagen staining in H group (× 200).

were significantly higher than those in S group (p<0.01). CVF and PVCA in P group were significantly lower than those in H group (p<0.01), and still higher than those in S group. The differences were statistically significant (p< 0.05).

TLR4 RT-PCR Results

TLR4 mRNA was expressed in all myocardial tissues of rats, and the gray level ratio of TLR4 band to β-actin band in each sample was taken as the relative content of TLR4 mRNA expression in each sample. Statistical analysis showed that TLR4 mRNA level in H group increased 1.72 times (absorbance ratio 0.523 ± 0.124 *vs.* 0.915 ± 0.243, p<0.01) in comparison to that in S group. TLR4 mRNA level in P group decreased significantly compared with that in H group (absorbance ratio 0.915 ± 0.243 *vs.* 0.712±0.147, p<0.01) but still higher than that in S group (0.712 ± 0.147 *vs.* 0.523 ± 0.124, p<0.05) (Figure 4).

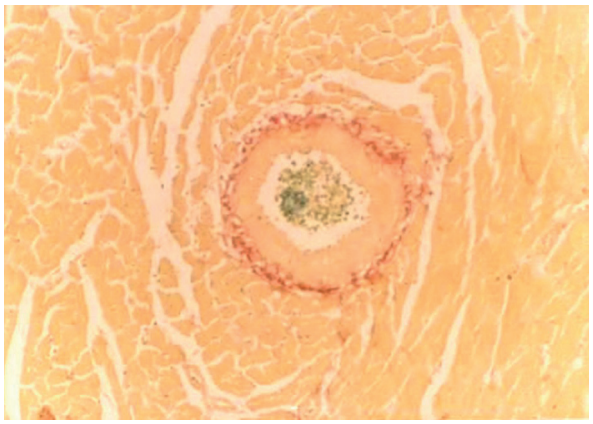

Figure 3. Myocardial collagen staining in P group (× 200).

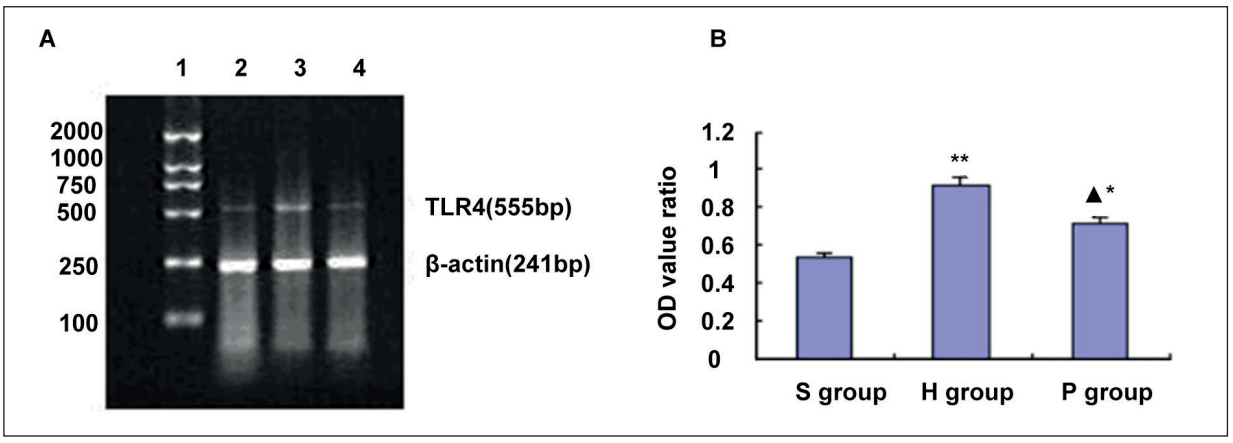

Figure 4. TLR4 mRNA expression. **A**, Agarose gel electrophoresis of TLR4 and β-actin PCR products. 1: DNA marker, 100bp, 250bp, 500bp, 750bp, 1000bp, 2000bp from bottom to top; 2: S group, 3: H group, 4: P group. The target gene TLR4 was amplified 555 bp in experiment, and fragment of ß-actin amplified was 241bp. **B**, Ratio of absorbance values of TLR4 to β-actin PCR amplification bands. Note: Compared with S group *p<0.05, **p<0.01; compared with H group ▲p<0.05, ▲▲ p<0.01.

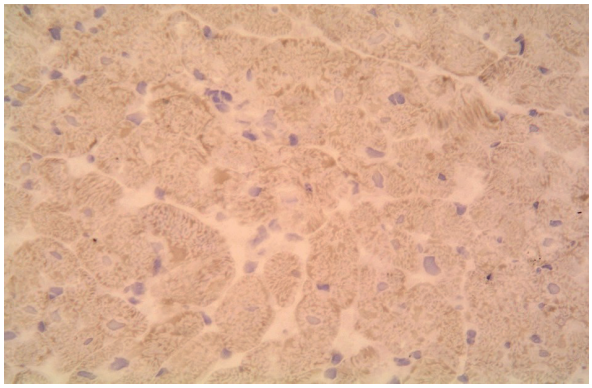

Figure 5. Immunohistochemical staining of TLR4 in myocardial tissue of S group (× 400).

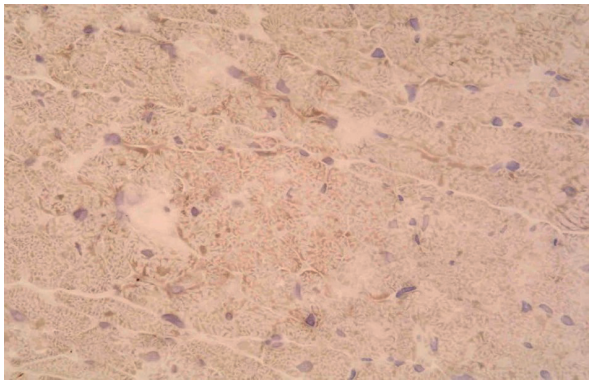

Figure 7. Immunohistochemical staining of TLR4 in myocardial tissue of P group (× 400).

Immunohistochemistry Results

1) TLR4 staining site: H group was mainly in the myocardial cell membrane, especially cluster-like expression occurred close to two or more adjacent myocardial cells, but there was also a small amount of expression occurred in the cytoplasm in half of the samples. In S and P group, TLR4 was mainly located in the cytoplasm with diffuse distribution, brownish yellow homogeneous, and minimal expression in the membrane (Figures 5-7).
2) NF-κB staining site: NF-κB was normally presented in the cytoplasm, and activated into the nucleus. Immunohistochemical staining showed nuclear staining. It was found in this experiment that more nuclei can be seen brown in group H; S group cytoplasm is stained light, almost no nuclear staining; in P group, a small amount of brown nuclei can still be seen (Figures 8-10). The statistical results of nuclear staining were as follows: the percentage of cardiomyocytes with NF-κB positive nucleus in H group (15.0%) was significantly higher than that in S group (1.4%) ($p<0.01$); the percentage in P group (6.1%) was significantly lower than that in H group (15.0%) ($p<0.01$), but still significantly higher than that in S group (1.4%) ($p<0.05$) (Table III).

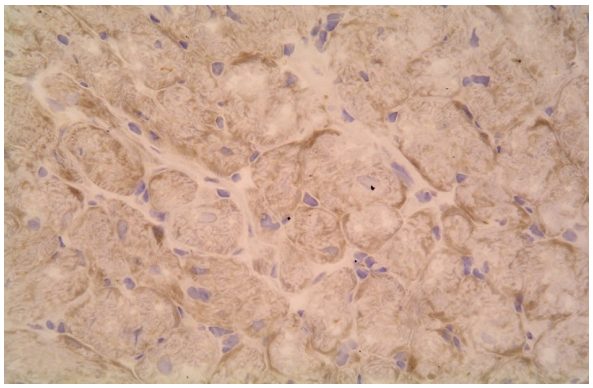

Figure 6. Immunohistochemical staining of TLR4 in myocardial tissue of H group (× 400).

Discussion

In 1997, Medzhitov et al[11] first isolated the homolog of Drosophila Toll in the human body, which was firstly called human Toll protein, and later named human toll-like receptor, namely, TLR4 (Toll-like receptor 4). It has been confirmed that TLR4 is a portal protein that conducts LPS (Lipopolysaccharide) signal to cells[12]. Toll proteins are widely expressed in insects, plants, and animals. Frantz et al[3] demonstrated that TLR4 is expressed in non-specific immune cells, including cardiomyocytes and microvascular endothelial cells, and that TLR4 mRNA expression is elevated after LPS stimulation.

At present, the research on the cardiovascular diseases of TLRs receptor family mainly focuses on the pathogenesis of diseases such as viral myocarditis, septicemia, septic shock, and atherosclerosis, which are closely related to inflammation. It is tended to be considered that there is a fully functional congenital immune system in the adult mammalian myocardium[4-6,13]. Cardiomyocytes can express media and effectors of innate immunity in response to the stimulation of classical pathogen-associated molecular patterns (PAMPs) (such as LPS, viral particles), including proinflammatory cytokines IL-1β, TNF, inducible nitric oxide synthase Enzymes (iNOS) and chemokines; the heart expresses at least two pat-

Table III. Comparison of CVF, PVCA and NF-κB nuclear positive rate 8 weeks after surgery.

	S group (n=20)	H group (n=25)	P group (n=25)
CVF (%)	2.47±0.25	4.37±0.31**	3.21±0.27▲▲*
PVCA (%)	13.56±2.78	30.76±3.90**	18.47±3.14▲▲*
NF-κB nucleus positive rate (%)	1.4±0.56	15.0±4.05**	5.8±1.58▲▲*

Note: Compared with S group *p<0.05, **p<0.01; compared with H group ▲p<0.05, ▲▲p<0.01.

tern recognition receptors (PRRs) corresponding to PAMPs: CD14, TLR2, TLR4. Thus, it has been suggested by some scholars[14,15] that the heart itself has a non-specific immune system which can be non-specifically activated by DAMPs caused by various reasons, and the dysregulation of non-specific immune responses may be involved in the pathophysiological processes of many cardiovascular diseases.

In an animal model of myocardial hypertrophy induced by aortic ligation, researchers[16,17)] found that the TLR4 gene-deficient mice had a significantly lower cardiac index (cardiac weight/body weight) and cardiomyocytes than wild-type mice. Blocking TLR4/NF-κB signaling pathway can significantly reduce cardiac hypertrophy and cardiomyocyte apoptosis induced by pressure load; it can also reduce the sensitivity of myocardial cells to the inflammatory response and improve cardiac function. We found that TLR4 mRNA expression was significantly increased in left ventricular remodeling myocardium of Goldblatt rats, which was 1.72 times that of the sham group (p<0.01).

Immunohistochemistry showed that TLR4 was homogenized and dispersed in cardiomyocytes in the sham-operation group and mainly located in the cytoplasm. In the myocardial cells of Goldblatt rats, TLR4 showed clustered expression, mainly adjacent to two or more cardiomyocytes. As a transmembrane signal transduction protein, the expression of TLR4 directly affects the mediator effect of immune inflammatory of itself.) TLR4 signal activation is the body's protective response to injury stimuli, but it continually enhanced TLR4 expression. Its signaling pathways were involved in the occurrence and development of hypertensive left ventricle remodeling, and they increased the susceptibility of the myocardium to damage and stimulation by activating the primary immune mechanisms and inflammatory responses in the damaged myocardium.

Activation of TLR4 triggers several intracellular signaling pathways, the most important of which is the activation of the transcription factor NF-κB[13,14]. NF-κB is one of the key factors that regulate gene transcription. It is widely expressed in immunocompetent cells, endothelial cells, smooth muscle cells, cardiomyocytes, and fibroblasts. NF-κB has two subunits, P50 and P65 dimer, which exist in the cytoplasm in an inactive form. Inhibitors kappa B (IκBs) control the activation of NF-κB. They bind to NF-κB dimers and conceal the NF-κB nuclear localization sequence.

Various stimulators activate NF-κB by phosphorylating and activating Inhibitor-kappa B kinase, resulting in the IκB phosphorylation and dissociation from NF-κB. Thus, NF-κB is translo-

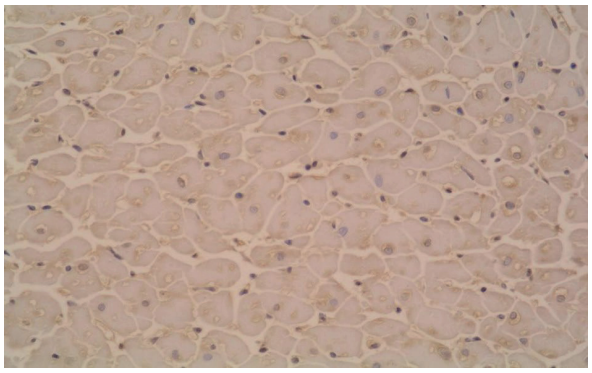

Figure 8. Immunohistochemical staining of NF-Kb in myocardial tissue of S group (× 400).

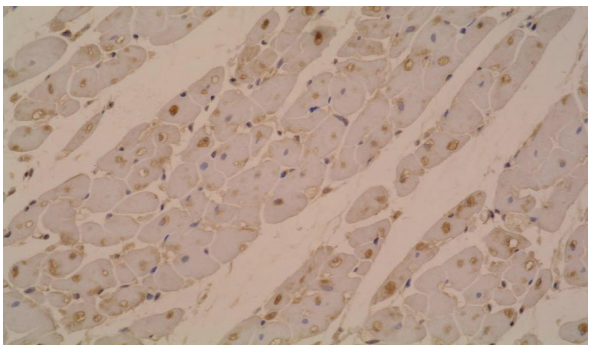

Figure 9. Immunohistochemical staining of NF-Kb in myocardial tissue of H group (× 400).

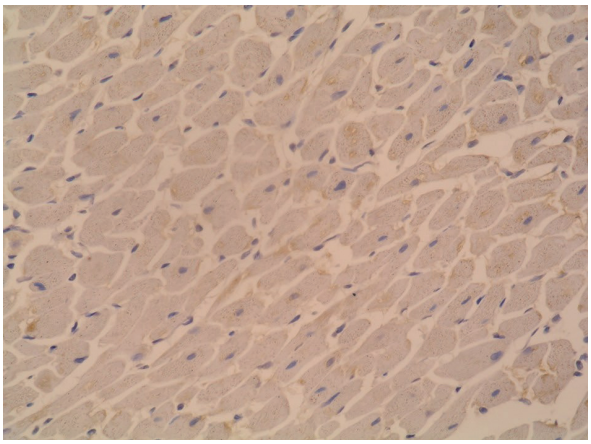

Figure 10. Immunohistochemical staining of NF-Kb in myocardial tissue of P group (× 400).

cated from the cytoplasm to the nucleus, binds to target genes and activates a series of target genes involved in cardiovascular pathophysiology, including transcription of cytokines, angiotensinogen, chemokines, leukocyte adhesion molecules, genes that regulate cell proliferation, and cell survival. So, it plays a key regulatory role in signal transduction of stress load, mechanical stretch, phenylephrine, endothelin, AngII, and other factors that promote cardiac hypertrophy[16]. The results of this experiment showed that NF-κB activation was significantly increased in left ventricular remodeling myocardium of Goldblatt rats, the rate of the positive nucleus was significantly higher than that of sham operation group ($p<0.01$), and the expression of TLR4 in the membrane was increased significantly. Although the correlation of this spatial location cannot essentially indicate that TLR4 activates NF-κB, it suggests that enhanced TLR4 expression is associated with the increase in NF-κB activation in cardiomyocytes and that TLR4 is involved in hypertrophic rat cardiac hypertrophy inflammatory activation.

Madej et al[18] showed that in the serum of patients with essential hypertension without inflammation, dyslipidemia, intercellular adhesion molecule and monocyte chemoattractant, protein levels were significantly higher than those in the normal control group. Haller et al[19] also detected perivascular interstitial infiltration of mononuclear macrophages in myocardial tissue of spontaneously hypertensive rats (SHRs), suggesting that inflammation is involved in the pathophysiology of hypertension. Scholars[20-22] reported that renin and angiotensin-double-transgenic rats (dTGR) showed severe hypertension with significant infiltration of mononuclear macrophages around the heart blood vessels, adhesion molecule activation, and fibrinoid necrosis, accompanied by a significant increase in myocardial NF-κB activity.

After 3 weeks of treatment with immunosuppressant CsA or endothelin receptor antagonist Bosentan, the blood pressure was significantly reduced, myocardial remodeling was partially reversed, and cardiac monocyte/macrophage infiltration and vascular cell proliferation were significantly reduced. Further work found that both CsA and Bosentan decreased the DNA-binding activity of NF-κB and inhibited the expression of IL-6 and iNOS, while the vasodilator hydralazine could not decrease the activity of NF-κB in the dTGR myocardium, and it decreased blood pressure. Therefore, it has less effect on the myocardial remodeling and perivascular macrophage infiltration, adhesion molecules, and integrin expression.

In this research, Goldblatt rat model was based on the increase of Ang II, activation of circulatory and local RAS systems. AngII itself is not only a proinflammatory mediator, but also promotes the occurrence of oxidative stress as an intrinsic oxidant. TLR4 expression and NF-kB activation in the left ventricular remodeling myocardium were detected to be increased. NF-κB is known to be a key factor mediating tissue damage by many proinflammatory cytokines, chemokines, iNOS, and reactive oxygen species, indicating that NF-κB-mediated inflammatory signals and oxidative stress are involved in the occurrence and development of left ventricular remodeling. The myocardial damage caused by a variety of factors may play a role in the occurrence and development of left ventricular remodeling in hypertension. However, TLR4 may be activated as a portal protein of the innate immune system and may be involved in the process of myocardial remodeling through the production of proinflammatory cytokines through the NF-κB-dependent pathway.

We found that, with the pressure load and local AngII content decreased, the expression of TLR4 also decreased significantly after the application of PDTC, suggesting that pressure overload and AngII may be involved in the regulation of TLR4 expression. Sustained stress load and AngII may not directly activate TLR4, but it will inevitably lead to other factors that up-regulate TLR4 expression. Furthermore, the development of left ventricular remodeling in hypertension is a complex process *in vivo*, with mechanical stretch, oxidative stress, and activation of neurohumoral factors, all of which may cause tissue damage to a certain degree and

may activate TLR4. Therefore, they trigger the cell signaling cascade and, ultimately, activate the transcription factor NF-κB-dependent signaling pathway to activate cells, starting a series of immune inflammatory response.

Left ventricular remodeling manifested as an increase of myocardial weight, not only hypertrophy of myocardial cells, but also hyperplasia of interstitial fibrosis; from the cellular and molecular level, it mainly includes three aspects: the extracellular stimulus signals, intracellular signal transduction, and activation of gene transcription in the nucleus; it is the result of hypertrophic signals that induce changes in gene expression in the nucleus. Among them, the intracellular signal transduction pathway is a coupling between extracellular stimulus and gene activation in the nucleus, so blocking the key signaling pathway becomes an important therapeutic target to prevent myocardial hypertrophy. It has been confirmed[8] that PDTC is an inhibitor of NF-κB activation both *in vitro* and *in vivo*, and its mechanism may be related to metal chelation, thiol modification, oxygen free radical scavenging, and antioxidative activity.

In this study, NF-κB activation was significantly decreased after subcutaneous injection of PDTC 100 $mg \cdot kg^{-1} \cdot d^{-1}$ for 6 weeks. Although the indexes of blood pressure, MESS, LVMI, RWT and CVF, PVCA and myocardial AngII in the experimental group were higher than those in the sham operation group ($p<0.05$), they were significantly reduced in comparison with the hypertension of non-drug intervention group (H group, $p<0.01$). The results were similar to Mervaala et al[21], indicating that antioxidant PDTC can partially reduce blood pressure and partially reverse left ventricular remodeling. It is suggested that intervention of NF-κB and anti-inflammatory and anti-oxidative therapy may become new targets for the treatment of myocardial remodeling in hypertension. The *in vitro* results of Frantz et al[3] suggest that NF-κB not only mediates downstream signaling transduction of TLR4, but a certain level of NF-κB activity is also necessary for maintaining the expression of TLR4. It was also found in this report that PDTC can inhibit the activation of NF-κB while reducing the expression of TLR4, and NF-κB is a downstream molecule of TLR4, mutual coordination and mutual promotion process may exist between TLR4 and NF-κB. It is still not clear whether NF- κB-induced cytokines can regulate the expression of TLR4 in turn and also cause the activation of NF-κB, thus forming a cascade of the amplification process, or the TLR4 gene itself has NF-κB binding sites. The specific activation and termination mechanisms of the signaling pathway still need to be further explored.

Conclusions

We showed that TLR4 expression, NF-κB activation, and AngII content increased in left ventricular remodeling myocardium of Goldblatt rats. Antioxidant PDTC decreased TLR4 expression and AngII content, and reversed left ventricular remodeling while inhibiting NF-κB activation, suggesting that TLR4/NF-κB-mediated inflammatory signals and oxidative stress are involved in the occurrence and development of left ventricular remodeling. Intervention with TLR4/NF-κB and anti-inflammatory and anti-oxidative therapy may be new targets of reversing left ventricular remodeling in hypertension.

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Conflict of Interest

The authors have no conflicts of interest to declare.

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