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Valsartan preconditioning protects against myocardial ischemia–reperfusion injury through TLR4/NF-κB signaling pathway

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Abstract Toll-like receptor 4 (TLR4) activation has been implicated in the pathogenesis of myocardial ischemia/reperfusion (I/R) injury. The activated TLR4 is capable of activating a variety of proinflammatory mediators, such as tumor necrosis factor-a (TNF-a) and interleukin-6 (IL-6). Valsartan as a kind of Angiotensin II type 1 receptor blockers is gradually used for the treatment of ischemic heart disease depending on its anti-inflammation function. Therefore, we hypothesized that valsartan protects against myocardial I/R injury by suppressing TLR4 activation. We constructed the rat model of myocardial I/R injury. The rats were pretreated with valsartan for 2 weeks, and then subjected to 30 min ischemia and 2 h reperfusion. TLR4 and Nuclear factor kappa-B (NF-κB) levels were detected by quantitative real-time PCR and western blot. In order to evaluate myocardial damage, the myocardial infarct size, histopathologic changes, and the release of myocardial enzymes, proinflammation cytokines and Angiotensin II were analyzed by triphenyl tetrazolium chloride (TTC) staining, light microscopy, and enzyme-linked immunosorbent assay (ELISA), respectively. Valsartan preconditioning inhibited TLR4 and NF-κB expressions concomitant with an improvement in myocardial injury, such as smaller infarct size, fewer release of myocardial enzymes, and proinflammation mediators. These findings suggest that valsartan plays a pivotal role in the protective effects on myocardial I/R injury. This protection mechanism is possibly due to its anti-inflammation function via TLR4/NF-κB signaling pathway.

Keywords Valsartan · Reperfusion injury · Inflammation · Toll-like receptors · Nuclear factor kappa-B

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Introduction

Myocardial reperfusion therapy has become the optimal therapeutic strategy for ischemic heart disease, which can preserve myocardial viability and function by reversing myocardial ischemia and limiting the infarct size [1–3]. However, the subsequent ischemia/reperfusion (I/R) injury may reduce the therapeutic benefit [4]. Myocardial I/R is a complex pathophysiological process that involves various factors and pathways [5, 6]. Inflammatory response is considered to be a major cause of I/R-induced tissue injury [7]. There is comprehensive experimental and clinical evidence that anti-inflammatory actions attenuate I/R injury [8, 9].

Toll-like receptor 4 (TLR4) as a member of pattern recognition receptors plays an important role in the induction of inflammatory response by recognition of exogenous pathogen-associated molecular patterns and endogenous ligands [10, 11]. Activation of TLR4 is linked to expression of proinflammatory cytokines and activation of nuclear factor kappa B (NF-κB) signaling pathways in several cell types [10, 12, 13]. More recently, it was reported that both TLR4 knockout and TLR4 mutant mice exhibit less myocardial injury and inflammation after I/R compared to wild-type counterparts [14, 15]. In our previous study, we also

found that TLR4 expression positively correlated with the levels of tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) in models of myocardial I/R rats [16].

Angiotensin II type 1 receptor blockers (ARBs) are extensively used for the treatment of cardiovascular disease as blood pressure-lowering agents [17–19]. Furthermore, more and more clinical and experimental studies have suggested that the cardioprotective effects of ARBs may extend to mechanisms beyond blood pressure lowering, such as anti-inflammation, anti-atherosclerosis, target organ protection, and so on [20–24]. Recently, some studies have demonstrated that ARBs treatment results in the inhibition of NF-κB activity and reduction of proinflammatory cytokines [25]. However, the anti-inflammation mechanism of ARBs and their effects on the proinflammation cytokine levels, TLR4 and NF-κB expressions, are still far from clear. In this study, we investigated the cardioprotective effects of Angiotensin II type 1 receptor blocker, valsartan, in rat myocardial I/R model to explore their anti-inflammation mechanism via TLR4/NF-κB signaling pathway.

Materials and methods

Animals

Male Sprague–Dawley (SD) rats (SPF class, 220–250 g) were purchased from Tongji Medical School, Huazhong University of Science and Technology (HUST), China. Animals were maintained under standard laboratory conditions at 25 ± 2°C, relative humidity of 50 ± 15%, and normal photoperiod (12 h dark and 12 h light). The procedures for experiments and animal care were approved by the Animal Care and Use Committee of HUST, and conformed to the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health (NIH Publication No. 80-23).

Drugs and preconditioning

Valsartan was kindly supplied by Beijing Novartis Pharma Ltd. (China), and dissolved in distilled water on the day of administration. The rats were randomly divided into four groups each of which contained 13 rats. Groups 1 and 2 were orally treated with vehicle (distilled water) for 2 weeks, which served as a sham control and an ischemia–reperfusion control. Groups 3 and 4 were orally administered with valsartan by gavage at dosages of 5 mg/kg daily (L-Val) and 10 mg/kg daily (H-Val) for 2 weeks before ischemia, respectively.

Myocardial I/R surgical procedure

Surgical ligation of the left anterior descending coronary artery (LAD) was performed similar to the methods described previously. Briefly, the animals were anesthetized with pentobarbital (30 mg/kg IP) [26] and, after tracheotomy, ventilation was provided using a breathing machine at a respiratory rate of 50/min with a tidal volume of 20 ml/kg body weight. Blood pressure was recorded from the left common carotid artery using a pressure transducer, and the heart rate was monitored by an electrocardiogram (ECG) during the procedure. A left parasternal incision was made through the third and fourth ribs, and the pericardium was gently opened to expose the heart. The LAD was ligated using a 6-0 silk suture. Additionally, a medical latex tube (socket, inner diameter, 1.5 mm) was placed between the ligature and the LAD. Myocardial ischemia was induced by compressing the LAD by tightening the ligature around the latex tube. The ECG was monitored for changes in the ST-T segment caused by tightening or loosing the ligature. After 30 min ischemia, the latex tube was removed to reperfuse the myocardium by restoring the coronary circulation. At 2 h post-reperfusion, rats were killed, and parts of the anterior wall of the left ventricular myocardium near the cardiac apex and blood samples were obtained for further analysis. The sham control group underwent the same procedures, with the exception of the induction of myocardial ischemia/reperfusion.

Biochemical studies

Blood serum samples were collected to measure the 3 specific marker enzymes, including the activities of creatine phosphokinase (CK), creatine phosphokinase-isoenzyme (CK-MB), and lactate dehydrogenase (LDH). All these marker enzymes were expressed as U/L using commercial kits (Beijing Kemeidongya Biotechnology Ltd., China).

Determination of infarct size

Five rats were sacrificed for the assessment of infarct area in each group. Two hours after the reperfusion period, rats' hearts were rapidly excised and sliced parallel to the atrioventricular groove into 2-mm-thick sections. The slices were incubated in 1% 2,3,5-triphenyl tetrazolium chloride (TTC) phosphate buffer (pH 7.4) for 20 min at 37°C. Infarct area in each heart determined by a computer-assisted image analysis system (Image-Pro Plus 3.0; Media Cybernetics, Silver Spring, MD) was multiplied by the thickness of the slice to calculate its volume. Infarct size was expressed as a percentage of left ventricular volume (%, infarct size/left ventricular).

Histologic examination

The formalin-fixed, paraffin-embedded sections of myocardial tissues were stained with hematoxylin and eosin and examined under a light microscope (≤400× magnification).

Quantitative real-time PCR analysis

The total RNA from the cardiac muscle samples was extracted and purified using the Trizol reagent kit (Invitrogen, US). Total RNA was reversely transcribed into complementary DNA (cDNA) using the cDNA synthesis kit (TaKaRa, Japan) according to the manufacturer's protocol. The opticon-2 real-time PCR reactor (MJ Research, US) and real-time PCR kit (SYBR® Premix Ex TaqTM, TaKaRa, Japan) were employed based on the manufacturer's instruction. The RT-PCR conditions were 42°C/15 min, 95°C/2 min for reverse transcription; polymerase chain reaction condition included pre-denaturing at 95°C for 10 s, then 40 cycles of 95°C for 5 s, and 60°C for 30 s and 72°C for 1 min. In this experiment, GAPDH and β-actin were used as the housekeeping genes. Levels of TLR4 mRNA and NF-κB mRNA were calculated based on the method of $2^{-\Delta\Delta Ct}$ [27, 28]. The primers were as follows: TLR4, sense primer 5′-AG CCATTGCTGCCAACATCA-3′, antisense primer 5′-GC CAGAGCTACTCAGAAAC-3′. NF-κB, sense primer 5′-G GCAGCACTCCTTATCAA-3′, antisense primer 5′-GGT GTCGTCCCATCGTAG-3′. GAPDH, sense primer 5′-GA CAACTTTGGCTCGTGGA-3′, antisense primer 5′-ATG CAGGGGTTCTGG-3′. β-actin, sense primer 5′-ACG TTGACATCCGTAAAGAC-3′, and antisense primer 5′-G AAGGTGGACAGTGAGGC-3′.

Western blotting

Cytoplasmic and nuclear protein extracts and membrane fractions were prepared from myocardial tissues as reported previously [29]. Western blotting was performed according to the manufacturer's procedures. Briefly, 50 μg of cytoplasmic or nuclear proteins was separated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membrane. Non-specific binding sites were blocked with 5% non-fat dry milk in Tris-buffer saline (TBS)-0.05% Tween. The membrane was subsequently probed with primary antibody (anti-TLR4 or anti-NF-κB p65, diluted 1:200, Santa Cruz Biotechnology, USA) and incubated in horseradish peroxidase-conjugated secondary antibody (diluted 1:1000, Beijing Zhongshan Biotechnology, China). The protein bands were visualized by an enhanced chemiluminescence system [30], and β-actin was used as an internal control to correct the variations of different samples. The expression level of TLR4/NF-κB p65 was indicated as a ratio of TLR4/NF-κB p65 to β-actin.

Enzyme-linked immunosorbent assay

The titres of TNF-α, IL-6, and Angiotensin II in cardiac muscle samples were measured using enzyme-linked immunosorbent assay (ELISA), and the detailed manipulation process was performed according to the manufacturer's recommendations. The rat TNF-a and IL-6 ELISA kits were purchased from Shanghai Xitang Biotechnology Ltd., China. Cardiac levels of Angiotensin II were determined using commercial kit (Beijing North Biotechnology Ltd., China).

Statistical analysis

Quantitative data were expressed as mean ± SD. For experiments with three or more groups of animals, one-way analysis of variance (ANOVA) was performed, and Student–Newman–Keuls (SNK)-q test was used for selected groups. A P-value of less than 0.05 was considered as statistically significant. Analysis was carried out using Statistical Product and Service Solutions (SPSS) (Version 11.0).

Results

Effects of valsartan on serum marker enzymes

The activities of CK, CK-MB, and LDH in serum were used to monitor the damage of myocardium. Comparing with sham group, CK, CK-MB, and LDH activities in serum of control group markedly increased ($P < 0.05$). After preconditioning with valsartan at dosages of 5 and 10 mg/kg/day, the CK, CK-MB, and LDH levels in L-Val and H-Val groups decreased significantly compared with the control group ($P < 0.05$) (Table 1).

Hemodynamic parameters and infarct size assessment

There was no significant difference in heart rate and mean arterial pressure among the sham, control, L-Val, and H-Val groups (Fig. 1). Infarct size was found to be 37.8 ± 3.5% of the left ventricle in the control group. The infarct size was reduced to 23.7 ± 2.1% and 20.8 ± 2.9% of the left ventricle with treatment of valsartan at dosages of 5 and 10 mg/kg/day, respectively ($P < 0.05$) (Fig. 2). There was no significant difference between the L-Val and H-Val groups ($P = 0.43$, Fig. 2).

Light microscopic findings

The slides of histologic pathology demonstrated that the myocardium of healthy rats kept normal tissue structure and shape. Furthermore, the animals in the valsartan-treated groups had significantly less severe myocardium injury and inflammatory cells infiltration than did those in the control group (Fig. 3).

Table 1 Effects of valsartan on CK, CK-MB, and LDH levels in the serum of experimental group animals

Groups	CK (U/L)	CK-MB (U/L)	LDH (U/L)
Sham	2164 ± 205	903 ± 35	862 ± 58
Control	4018 ± 343$^{\triangle}$	2514 ± 298$^{\triangle}$	2345 ± 219$^{\triangle}$
Low-dose valsartan	3019 ± 236$^{\triangle,\star}$	1499 ± 190$^{\triangle,\star}$	1467 ± 137$^{\triangle,\star}$
High-dose valsartan	2854 ± 311$^{\triangle,\star}$	1363 ± 88$^{\triangle,\star}$	1287 ± 117$^{\triangle,\star}$

Values are presented as mean ± SD, $n = 8$. $^{\triangle}$ $P < 0.05$, vs. sham group; * $P < 0.05$, vs. control group

CK creatine phosphokinase, *CK-MB* creatine phosphokinase-isoenzyme, *LDH* lactate dehydrogenase

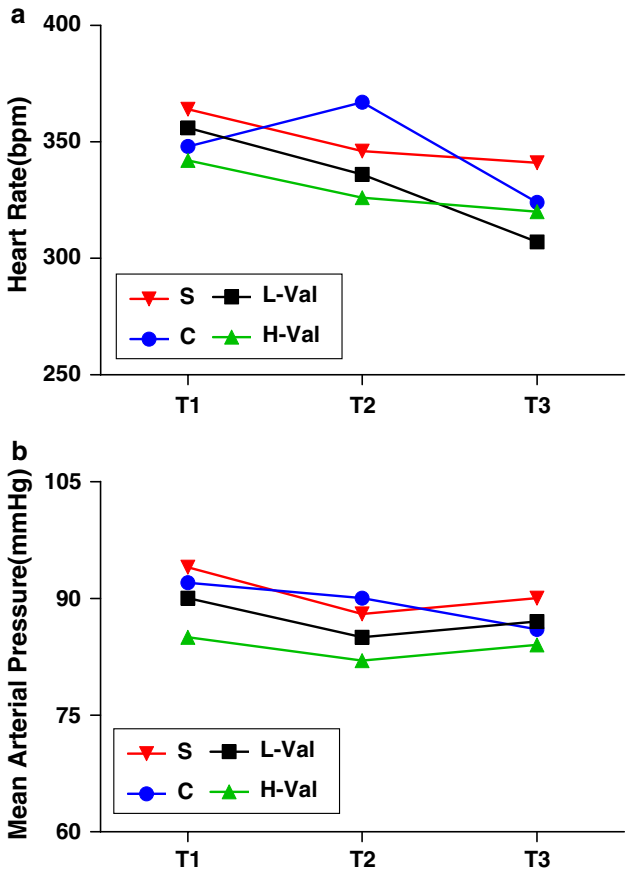

Fig. 1 Hemodynamic parameters before and during myocardial I/R. **a** Heart rate change. **b** Mean arterial pressure change. T1 = before myocardial I/R, T2 = 30 min after myocardial ischemia, and T3 = at the end of myocardial I/R. Mean ± SD, $n = 13$

The role of valsartan in regulating expressions of TLR4 and NF-κB mRNA

The expression of TLR4 mRNA was significantly increased after myocardial I/R compared with sham group ($3.97 \times 10^{-2} \pm 0.63 \times 10^{-2}$ vs. $9.83 \times 10^{-3} \pm 1.28 \times 10^{-3}$, $P < 0.05$). Treatment with valsartan 5 and 10 mg/kg/day could prevent the elevation of TLR4 mRNA level ($2.67 \times 10^{-2} \pm 0.31 \times 10^{-2}$ and $2.29 \times 10^{-2} \pm 0.40 \times 10^{-2}$) after ischemia–reperfusion injury (Fig. 4). However, there was no difference between the groups L-Val and H-Val ($P = 0.26$). The expression of NF-κB mRNA paralleled that of TLR4 in the heart tissues (Fig. 4).

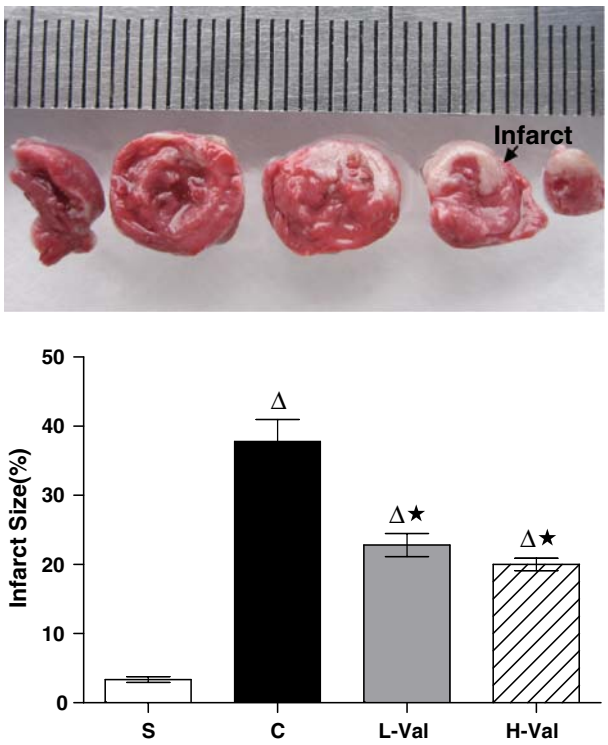

Fig. 2 Myocardial infarct size in the four groups of rats. **a** Representative example of a heart slices from a rat stained with TTC. Infarct areas are not stained by TTC (*white*). **b** The infarct size at the end of reperfusion. *S* sham group, *C* control group, *L-Val* Low-dose valsartan group, and *H-Val* High-dose valsartan group. Mean ± SD, $n = 5$. $^{\triangle}$ $P < 0.05$, vs. S group; * $P < 0.05$, vs. C group

Effects of valsartan on TLR4 and NF-κB p65 proteins expression

Thirty minutes of ischemia and 2 h of reperfusion in control group significantly increased heart TLR4 and NF-κB p65 protein concentrations (1.36 ± 0.25 and 0.98 ± 0.17) compared with sham group (0.58 ± 0.10 and 0.32 ± 0.07, respectively) (P of both <0.05). Furthermore, in valsartan-treated animals, TLR4 (L-Val = 1.02 ± 0.11 and H-Val = 0.96 ± 0.14) and NF-κB p65 (L-Val = 0.73 ± 0.08 and H-Val = 0.60 ± 0.03) activities in tissues from the ischemic area was significantly lower compared with the control group (P both <0.05). However, no significant difference was found between valsartan groups (Fig. 5).

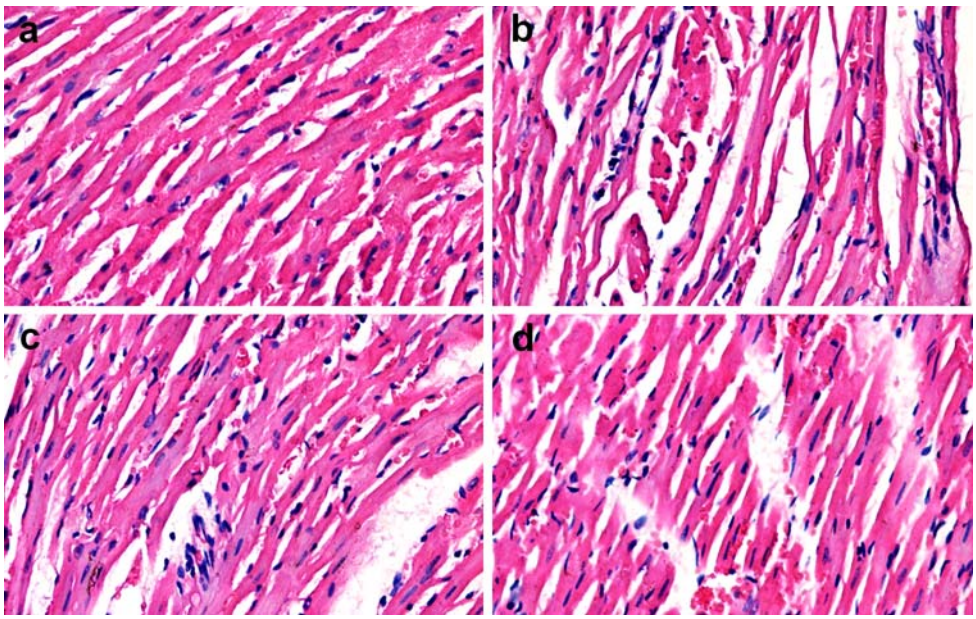

Fig. 3 Histopathological detection of heart tissue changes in rats (magnification, ×400). **a** Sham group, the myocardial fibers are arranged in an orderly manner. **b** Control group, myocardial fibers are partially ruptured and lysed. **c** Low-dose valsartan group (L-Val), light edema is observed in the interstitial tissues. **d** High-dose valsartan group (H-Val), the histopathological changes are similar to L-Val

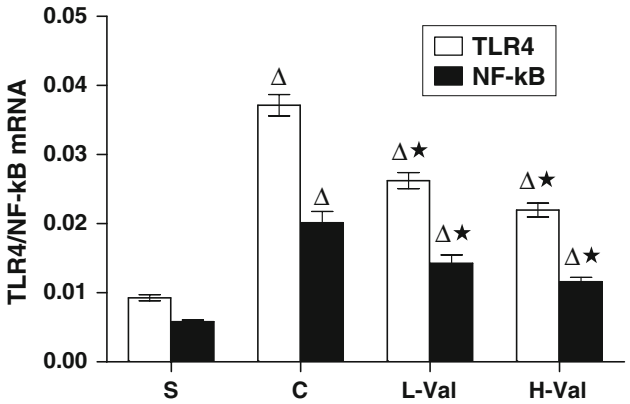

Fig. 4 Expressions of TLR4 and NF-κB mRNA in the different experimental groups. *S* sham group, *C* control group; *L-Val* Low-dose valsartan group, and *H-Val* High-dose valsartan group. Mean ± SD, $n = 8$. $^{\triangle}$ $P < 0.05$, vs. S group; * $P < 0.05$, vs. C group

Valsartan inhibited production of TNF-α and IL-6

The effects of valsartan on inflammatory cytokine expression in heart tissues of rats were analyzed by ELISA (TNF-α and IL-6). Comparing with sham group, Myocardial I/R induced a dramatic increase in the concentrations of TNF-α (41.13 ± 3.15 vs. 20.01 ± 1.87 pg/mg, $P < 0.05$) and IL-6 (57.66 ± 3.42 vs. 30.18 ± 2.09 pg/mg, $P < 0.05$) in the control group. Preconditioning with valsartan inhibited TNF-α (L-Val = 31.37 ± 1.46 pg/mg and H-Val = 28.36 ± 2.42 pg/mg) and IL-6 (L-Val = 42.60 ± 4.16 pg/mg and H-Val = 36.18 ± 1.75 pg/mg) production compared with control group (P both <0.05) (Fig. 6). In addition, the valsartan inhibitory effects of TNF-α and IL-6 were dose dependent (H-Val vs. L-Val, P both <0.05).

Angiotensin II levels in tissue

Angiotensin II concentration in control group was more than twice the concentration in the heart of the sham group rats ($S = 46.76 \pm 3.76$ vs. $C = 98.53 \pm 7.47$ pg/mg, $P < 0.05$). Comparing with the control group, only a tendency to increase the angiotensin II levels was observed in the valsartan groups (L-Val = 103.21 ± 10.73 pg/mg, H-Val = 111.86 ± 8.89 pg/mg, respectively).

Discussion

Clinically, reperfusion is the definitive treatment for acute coronary syndromes, especially for acute myocardial infarction. However, I/R injury which is characterized by a significant inflammatory response may lead to cardiac function recovery time delay. In this study, we showed that the pretreatment of valsartan before myocardial ischemia profoundly attenuated reperfusion injury by its action on inflammatory factors and related TLR4/NF-κB pathway in a rat myocardial I/R model. Myocardial enzyme release, infarct size, and morphological changes were significantly improved after reperfusion in valsartan-treated groups.

The most important finding in this study was that valsartan administration decreased TLR4 mRNA and protein expression, which were upregulated in the ischemia–reperfusion rats heart. The effect of TLR4 in myocardial I/R injury is supported by evidence of cardiovascular dysfunction in bacterial sepsis through TLR4 signaling [31, 32]. Under sterile conditions, however, noninfectious endogenous ligands released from injured cells or tissue fragments seem to initiate the inflammatory reaction by inducing TLR4

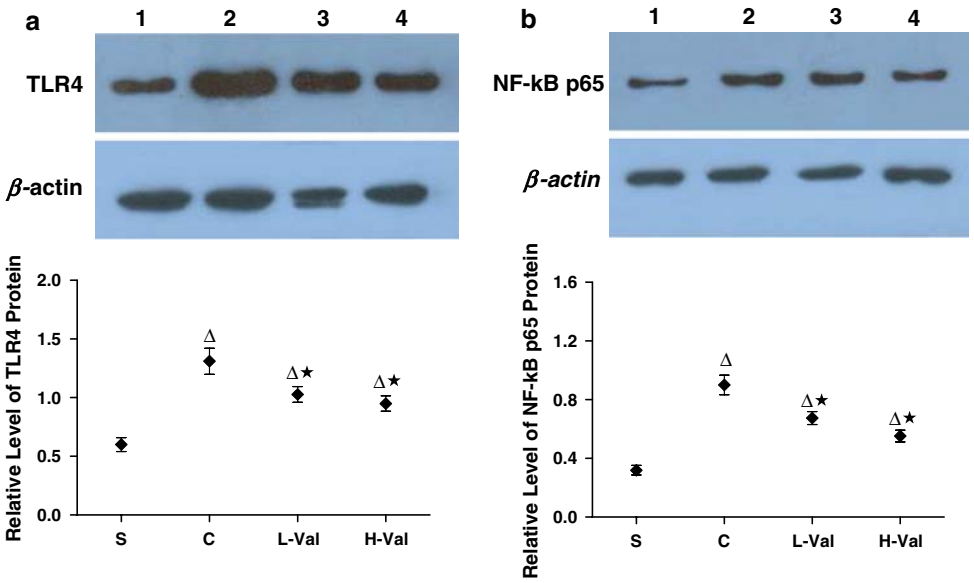

Fig. 5 Effects of in vivo valsartan treatment on TLR4 and NF-κB p65 protein expressions after ischemia–reperfusion. **a** TLR4 protein expression. **b** NF-κB p65 protein expression. Original representative western blots are reported in the *upper panels* and relative levels of TLR4/NF-κB p65 in the *lower panels*. (1) heart in non-ischemic conditions; (2) heart subjected to ischemia–reperfusion; (3) heart subjected to ischemia–reperfusion from rats treated with 5 mg/kg/day valsartan; (4) heart subjected to ischemia–reperfusion from rats treated with 10 mg/kg/day valsartan. *S* sham group, *C* control group, *L-Val* Low-dose valsartan group, and *H-Val* High-dose valsartan group. Mean ± SD, $n = 8$. $^{\triangle}$ $P < 0.05$, vs. S group; * $P < 0.05$, vs. C group

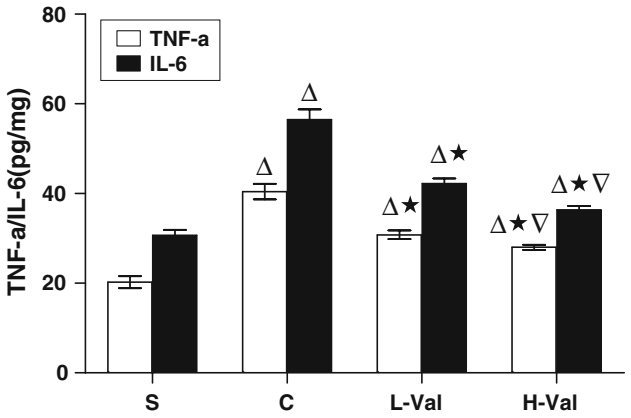

Fig. 6 Effects of valsartan on TNF-a and IL-6 production in heart tissues. *S* sham group, *C* control group, *L-Val* Low-dose valsartan group, and *H-Val* High-dose valsartan group. $^{\triangle}$ $P < 0.05$, vs. S group; * $P < 0.05$, vs. C group; $^{\triangledown}$ $P < 0.05$, vs. L-Val group. Values are presented as mean ± SD, $n = 8$

signaling [11, 33, 34]. Downstream of TLR4 signaling could occur through c-Jun N-terminal kinases (JNKs), p38 kinases, and NF-κB pathway. NF-κB is a key transcription factor in TLR4-mediated MyD88-dependent signaling pathway and plays a pivotal role in stimulating inflammatory cytokines, chemokines, and adhesion molecules secretion in myocardial I/R [35, 36]. In our study, we found the expression of NF-κB and proinflammatory cytokines (TNF-α, IL-6) was paralleled by TLR4 in the rat's heart, suggested that TLR4 plays an important role in recognizing endogenous ligands and triggering the inflammatory response in the process of myocardial I/R.

Valsartan, one of the most widely used renin–angiotensin system (RAS) inhibitors, along with its well-established actions on blood pressure, also exerts an anti-inflammatory effect [23]. As reported in hypertension patients, valsartan therapy could reduce the plasma level of serum inflammatory markers, such as IL-1 and high-sensitivity C reactive protein [37]. Interestingly, the results of our study revealed that valsartan administration could improve myocardial histopathological damage, reduce myocardial infarct size, and relieve inflammatory cytokines release by downregulating expression of TLR4/NF-κB mRNA and protein. Cytosolic enzymes leak into the intracellular space, where there is a cell membrane damage [38]. Therefore, enzyme analysis has proved considerably valuable in the diagnosis of myocardial infarction. Increased levels of CK, CK-MB, and LDH are well-known diagnostic markers of myocardial injury. In this study, marked elevations in the levels of these enzymes in the serum of the control group indicated that the occurrence of severe myocardial cell membrane damage compared with the sham group. Treatment with valsartan resulted in a significant reduction in the levels of these enzymes close to normal level compared with the control group, suggesting that valsartan can protect myocardial cells. Of course, the clinical setting where valsartan pretreatment might apply should be mentioned. Our results may be appropriate for those who develop an acute coronary syndrome while receiving ARBs for hypertension, heart

failure, or diabetes as they might benefit from the cardioprotective effect of ARBs during reperfusion therapy.

This study demonstrated that myocardial I/R associated with a rise in cardiac Angiotensin II level. Moreover, such cardiac regulations were not prevented by Angiotensin II type 1 receptor blocker using valsartan. Our results were in agreement with previous data [39]. Several articles, however, reported opposite results. Silvestre et al. [40] reported that losartan prevented the myocardial infarction induced rise in tissue Angiotensin II level in rats. Therefore, the specific regulatory mechanism of the locally generated Angiotensin II is far from clear. We presumed that the elevated Angiotensin II in valsartan groups was mainly due to the effect of negative feedback after Angiotensin II type 1 receptor being blocked.

It is unclear why TNF-α and IL-6 concentration reduction exhibited a dose-dependent manner in valsartan-treated groups. In addition, we could find that TLR4/NF-κB mRNA and protein changes had no significant statistical difference between low-dose valsartan group and high-dose valsartan group. One possible explanation is that valsartan as Angiotensin II type 1 receptor blocker has another regulatory mechanism preventing inflammatory cytokines production. Recently, Dai and colleagues [41] reported that losartan could exert anti-inflammatory effects in spontaneously hypertensive rats by inhibition of PI3K/Akt. Furthermore, Angiotensin II receptor type I blockers are known to have direct anti-inflammatory actions. The direct effect is likely through their free radical scavenging properties, which are derived from their phenolic moiety [42]. Therefore, we presumed that protection by valsartan against myocardial I/R injury may be mediated partly by prevention of inflammatory mediators through the TLR4/NF-κB pathway.

In summary, our data contributed to a better understanding of the role of ARB in inhibiting the inflammatory response in the ischemia–reperfusion-induced myocardial injury. The increased production of TNF-α and IL-6 was associated with the elevated expression of TLR4/NF-κB in rat myocardial I/R model, and valsartan pretreatment could suppress their overexpression in heart tissues. Considering the important role in inflammation, a therapeutic approach involving the TLR4-mediated NF-κB signaling pathway might constitute a new strategy for myocardial ischemia–reperfusion injury.

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