Valsartan Prevented Neointimal Hyperplasia and Inhibited SRSF1 Expression and the TLR4–iNOS–ERK–AT1 Receptor Pathway in the Balloon-injured Rat Aorta

Running title: Valsartan and neointimal hyperplasia

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Summary

Valsartan has the potential to attenuate neointimal hyperplasia and to suppress the inflammatory response. This study aimed to evaluate the role of valsartan in neointimal hyperplasia and the toll-like receptor 4 (TLR4)-nitric oxide synthase (NOS) pathway in the balloon-injured rat aorta.Forty-eight Wistar rats were randomly allocated to three groups: sham control (control), balloon-injured group (surgery), and balloon-injured+valsartan-treated group (valsartan). Rats were killed at 14 and 28 days after balloon-injury, and then the aortic tissues were collected for morphometric analysis as well as for measurements of the mRNA or protein expression of angiotensin II, angiotensin II type 1 (AT1) receptor, angiotensin II type 2 (AT2) receptor, TLR4, endothelial nitric oxide synthase (eNOS), inducible NOS (iNOS), serine/arginine-rich splicing factor 1(SRSF1) and extracellular signal regulated kinase (ERK). Valsartan at a dose of 20 mg/kg/day markedly decreased neointimal hyperplasia in the aorta of balloon-injured rats, and significantly reduced the mRNA or protein expression of TLR4, AT1 receptor, SRSF1 and phosphorylated-ERK (p-ERK) as well as the aortic levels of iNOS (all $p < 0.05$). Moreover, valsartan increased the eNOS level and AT2 receptor mRNA and protein expression levels (all $p < 0.05$). Valsartan prevented neointimal hyperplasia and inhibited SRSF1 expression and the TLR4-iNOS-ERK-AT1 receptor pathway in the balloon-injured rat aorta.

Key words: Valsartan; aorta; neointimal hyperplasia; balloon injury; toll-like receptor 4; nitric oxide synthase

Introduction

Percutaneous coronary intervention (PCI) is a generally accepted therapy for treating coronary heart disease. Restenosis remains an important complication with the use of angioplasty and stents. Within 6 months, restenosis occurs in 32-55% of all angioplasties, dropping to 17-41% in the bare metal stent area (Buccheri *et al*. 2016). However, a new complication in-stent restenosis (ISR) arises from neointimal

hyperplasia. Although the use of drug-eluting stents reduce the incidence of ISR to 5%-10% (Paramasivam *et al*. 2020), ISR remains a serious concern as late stent complications and drug eluting stent (DES) and ISR treatment continue to be challenging complications for interventional cardiologists (Alraies *et al*. 2017) .

Neointimal formation after vascular injury is the critical pathological process of ISR. Activated vascular smooth muscle cells (VSMCs) respond to local stimulation and migrate from the media into the lumen of the vessel, participating in the process of restenosis. Serine/arginine-rich splicing factor 1 (SRSF1), an essential splicing factor, promotes VSMC proliferation and injury-induced neointima formation. Vascular injury *in vivo* and proliferative stimuli *in vitro* were indicated to stimulate SRSF1 expression (Xie *et al*. 2017). Moreover, local inflammation after vascular injury also contributes to the development of restenosis (Araújo *et al*. 2015; Joviliano *et al*. 2011; Yoneda *et al*. 2013). Toll-like receptor 4 (TLR4), a member of the pattern recognition receptor family, participates in the inflammatory response after vascular injury (Den Dekker *et al*. 2010; Zou *et al*. 2019), and has been implicated as a potential clinical biomarker in patients who develop in-stent restenosis (Liang *et al*. 2016). Decreased nitric oxide (NO) production due to endothelial denudation leads to VSMC proliferation and migration. Endogenous NO is usually generated by endothelial nitric oxide synthase (eNOS), inducible NOS (iNOS), and neuronal NOS (nNOS) (Forstermann and Sessa 2012). Induction of iNOS production subsequently promotes the inflammatory response and accelerates restenosis (Chyu *et al*. 1999). In contrast, activation of eNOS can decrease neointimal formation after arterial injury (Janssens *et al*. 1998). Therefore, the TLR4-iNOS pathway can provide an emerging target for the prevention of restenosis.

Angiotensin II, a major mediator of the rennin-angiotensin system, acts through angiotensin II type 1 (AT1) and angiotensin II type 2 (AT2) receptors. Valsartan is regarded as a widely used AT1 receptor

blocker (Huang *et al*. 2014). Our previous studies have shown that valsartan can reduce neointimal hyperplasia of the rat aorta via activating the angiotensin-converting enzyme 2 (ACE2)-angiotensin-(1-7)-Mas axis, inhibiting the AT1 receptor, and upregulating heme oxygenase-1 (Li *et al*. 2012, Li *et al*. 2014, 2016). Valsartan was proved to exert anti-inflammatory effects in clinical (Kintscher *et al*. 2010) and experimental (Iwashita *et al*. 2012, Hadi *et al*. 2015) studies. Previous study suggested that valsartan-eluting stents can inhibit neointimal hyperplasia post-stent implantation by reducing VSMC proliferation and collagen deposition (Guihua *et al*. 2010). Clinically, valsartan has yielded a beneficial effect by preventing in-stent restenosis after stenting (Peters 2008). However, the effects of valsartan on neointimal hyperplasia, the upstream signaling pathways of TLR4 and other proinflammatory cytokines in the balloon-injured rat aorta need to be further explored. Therefore, this study sought to evaluate the role of valsartan in neointimal hyperplasia, the TLR4–NOS pathway and SRSF1 expression in the balloon-injured rat aorta.

Materials and Methods

Drug and reagents

Valsartan was provided by Novartis Pharma Ltd. (Beijing, China). The angiotensin II radioimmunoassay kit was obtained from the Institute of Radioimmunoassay of the People's Liberation Army General Hospital, China. The NOS reagent kit was obtained from the Nanjing Jiancheng Institute of Biological Engineering, China. Polyclonal anti-TLR4, anti-β-actin, anti-AT1, anti-AT2, and antiphospho- ERK were provided by Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA. Polyclonal anti-SRSF1 was provided by Thermo Fisher Inc., Waltham, MA, USA.

Animals and experimental protocol

All the experimental protocols were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of Qingdao University (Number of Ethic Committee: QYFY WILL 25705). A total of 48 adult male Wistar rats weighing 300-350 g were provided by the Qingdao Animal Center, and housed at a temperature at 22 °C and 35 ± 5% humidity under a 12-h dark-light cycle. All rats were fed on a laboratory diet of rat chow and freely accessed to water. These rats were randomly allocated to three groups: sham control group (control group), balloon-injured group (surgery group), and balloon-injured+valsartan-treated group (valsartan group) (n=16 in each group). Neointimal hyperplasia of aortas was established using endothelial denudation with a 2F balloon dilation catheter. Briefly, rats were anesthetized by 0.6% sodium pentobarbital (5ml/kg) intraperitoneally. The balloon catheter entered the thoracic aorta and abdominal aorta through the left common carotid artery. The balloon was inflated and passed with rotation three times in different directions. The success of aortic denudation was determined by injection of 2 mL/kg 0.5% Evan's blue at 60 min after surgery (Li *et al*. 2008). The sham control rats underwent the same surgery except for balloon injury. The valsartan-treated rats received 20 mg/kg/day valsartan (dissoluted in normal saline) via gastric gavage. Treatments started 24 h before balloon injury and continued until day 14 or day 28 post-surgery. The same volumes of 0.9% saline solution were administered to the sham control and surgery rats.

Histological analysis

Rats were anesthetized by 0.6% sodium pentobarbital and sacrificed by cervical dislocation at 14 days and 28 days post-surgery, respectively. Thoracic aortic tissues in length of 4-5 cm were collected immediately. Other harvested aortic tissues were then immediately frozen in liquid nitrogen until a further analysis. Collected aortic tissues were cut into 5-μm sections for hematoxylin-eosin (HE) staining and immunofluorescence. Images of neointimal hyperplasia were visualized under a light microscope at 200×

magnification. Morphometric determinations of the intimal and medial thicknesses were quantified using an image analyzer (JEDA801 series, Science-Technology Development Co., China). The expression of SRSF1 protein was detected in immunofluorescence under a fluorescence microscope (DS-Ri2, Nikon Co., Japan) according to the previous report (Zaqout *et al*. 2020).

Measurement of angiotensin II and NOS in aortic tissues

Collected thoracic aortic tissues (35 mg in each group) were homogenized and centrifuged at 3000 rpm for 20 min, and then the supernatant was collected. The aortic angiotensin II levels were determined by a radioimmunoassay kit, according to the manufacturer's protocol. The iNOS and eNOS levels in aortic tissues were detected using chemical methods in accordance with the manufacturer's instructions.

*Reverse transcription polymerase chain reaction (RT-PCR)*TLR4, AT1 receptor, and AT2 receptor mRNA expression levels were detected by the reverse transcription polymerase chain reaction (RT-PCR). Trizol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA from the frozen aortic samples, according to the manufacturer's protocol. The integrity of extracted RNA was confirmed spectrophotometrically at 260 nm and 280 nm wavelengths. GAPDH was chosen as an internal control. The primer sequences were as follows: TLR4 (304 bp), 5'-TGGCATCATCTTCATTGTCC-3' (Forward) and 5'-GTCCACAGCAGAAACCCAGA-3' (Reverse); AT1 receptor (444 bp), 5'-CACCTATGTAAGATCGCTTC-3' (Forward) and 5'-GCACAATCGCCATAATTATCC-3' (Reverse); AT2 receptor (536 bp), 5'-TGAGTCCGCATTTAACTGC-3' (Forward) and 5'-ACCACTGAGCATATTTCTCGGG-3' (Reverse); GAPDH (161 bp), 5'-AGATGGTGAAGGTCGGTGTG-3' (Forward) and 5'-CTTGCCGTGGGTAGAGTCAT-3' (Reverse). The reaction was conducted on an ABI PRISM 7000 Sequence System (Applied Biosystems, Foster City, CA, USA) under the following conditions: 95 °C for 3 min; 98 °C for 10 s, 57 °C for 10 s, and 72 °C for

30 s (total of 40 cycles); then a final extension for 10 min. The amplified PCR product was checked and analyzed by a gel imaging analyzer. Target mRNA expression was normalized to GAPDH mRNA expression for quantification.

Western blot analysis Aortic TLR4, AT1 receptor, AT2 receptor, and p-ERK protein expression levels were detected by Western blot analysis. Briefly, 20 μg of protein was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PGE), and transferred to a polyvinylidene fluoride membrane. The membranes were blocked with nonfat milk, and then incubated with anti-TLR4 (1:100 dilution), anti-AT1 (1:50 dilution), anti-AT2 (1:50 dilution), anti-phospho-ERK (1:100 dilution), or anti-β-actin (1:500 dilution), and subsequently incubated with the secondary antibody at 1:1250 dilution. Protein bands were visualized with an enhanced luminescence reagent and visualized using a Flurochem 9900-50 digital gel imaging system (Alpha Innotech Co., San Leandro, CA, USA). The optical density of each band was normalized to that of β-actin from the same sample.

Statistical analysis

Statistical analysis was performed using Statistical Product and Service Solutions (SPSS) version 16.0 (SPSS, Inc., Chicago, IL, USA). Values were expressed as the mean ± standard deviation (SD). For the values of repeated measurements, two-way analysis of variance was performed. A statistical significance was set as $p < 0.05$.

Results

Effects of valsartan on neointimal hyperplasia in the balloon-injured rat aorta

On 14th day post-surgery, compared to the control (Figure 1A), HE staining showed that most injured surfaces were covered by endothelial cells, and there was a large amount of VSMC proliferation.

In addition, the intima was apparently tortuous, and neointimal hyperplasia was predominant (Figure 1B). On 28th day post-surgery, compared to the control (Figure 1D), endothelial cells almost covered all injured surfaces, and there was obvious VSMC proliferation as well as sustained neointimal hyperplasia. An increased extracellular matrix was the main histological change (Figure 1E).Compared with surgery group, the VSMC proliferation and VSMC migration in valsartan-treated group were obviously attenuated on 14th day (Figure 1C) and 28th day (Figure 1F) post-surgery. The image analysis results showed that valsartan significantly attenuated the intimal (Figure 1G) and medial (Figure 1H) thicknesses, compared to the untreated balloon-injured rats (all $p < 0.01$).

Effects of valsartan on aortic levels of angiotensin II, eNOS, and iNOS

Angiotensin II, eNOS, and iNOS expression levels in the aorta were shown in Figure 2. On days 14 and days 28 post-surgery, the aortic angiotensin II (Figure 2A) and iNOS (Figure 2C) levels of the surgery rats were markedly increased; whereas, the aortic eNOS expression levels (Figure 2B) in the surgery rats were significantly reduced, compared to those of the sham controls (all $p < 0.01$). In contrast, at both 14 and 28 days post-surgery, the administration of valsartan markedly reduced the aortic levels of angiotensin II and iNOS, and increased the aortic levels of eNOS, compared to the untreated balloon-injured rats (all $p < 0.05$). However, still increased angiotensin II and iNOS expression levels, and decreased eNOS expression levels were seen in rat aorta of valsartan group compared with controls (all $p < 0.05$).

Effects of valsartan on aortic TLR4, AT1 receptor, and AT2 receptor mRNA expression

RT-PCR analysis showed that, compared to the sham controls, the TLR4 (Figure 3A) and AT1 receptor (Figure 3B) mRNA expression levels were significantly increased in the surgery rats on days 14 and 28 post-surgery (all $p < 0.01$). In contrast, the mRNA expression of AT2 receptor (Figure 3C) was

markedly reduced in the surgery rats ($p < 0.01$). Importantly, treatment with valsartan for both 14 and 28 days markedly reduced the TLR4 and AT1 receptor mRNA expression, and increased the AT2 receptor mRNA expression, compared to the surgery rats (all $p < 0.05$). However, compared to the controls, TLR4 and AT1 receptor expression was still increased, and AT2 was decreased in valsartan group (all $p < 0.05$).

Effects of valsartan on aortic TLR4, AT1 receptor, AT2 receptor, and p-ERK protein expression

Effect of valsartan on aortic TLR4, AT1 receptor, AT2 receptor, and p-ERK protein expression was evaluated at 14 and 28 days after surgery using Western blot assay (Figure 4A). It was shown that aortic TLR4 (Figure 4B), AT1 receptor (Figure 4C), and p-ERK (Figure 4E) protein expressions were markedly upregulated in the surgery rats; whereas, AT2 receptor (Figure 4D) protein expression was markedly decreased, compared to the sham controls (all $p < 0.01$). Treatment with valsartan for both 14 and 28 days markedly reduced the protein expression of TLR4, AT1 receptor, and p-ERK as well as increased the AT2 receptor protein expression, compared to the surgery group (all $p < 0.05$). In addition, compared to the controls, the expression of TLR4, p-ERK and AT1 receptor was still increased, and AT2 receptor was decreased in valsartan group (all $p < 0.05$).

Effects of valsartan on aortic SRSF1 protein expression

SRSF1 protein expression was measured by immunofluorescence assay in control, surgery and valsartan groups at 14 and 28 days after surgery. It was suggested that, aortic SRSF1 expression was increased obviously in the surgery rats (Figure 5B,E) than in the sham controls (Figure 5A,D). When valsartan given for both 14 (Figure 5C) and 28 (Figure 5F) days, SRSF1 expression all decreased significantly in comparison with the surgery group, but it was still higher than the controls.

Discussion

The results of the current study indicated that: 1) valsartan at a dose of 20 mg/kg/day treated balloon-injured rats for both 14 and 28 days significantly inhibited the neointimal hyperplasia and reduced the aortic SRSF1 expression; 2) valsartan decreased the aortic angiotensin II and iNOS levels while increased the aortic eNOS level; and 3) valsartan downregulated the TLR4 and AT1 receptor while upregulated the AT2 receptor mRNA and protein expression. Valsartan also decreased the aortic p-ERK protein expression. These findings suggest that the therapeutic potential of valsartan in attenuating neointimal hyperplasia and inhibiting the TLR4-iNOS-ERK-AT1 receptor pathway and SRSF1 expression in balloon-injured rat aorta.

The proliferation of VSMCs is pivotal to intimal hyperplasia and finally leads to restenosis after angioplasty and atherogenesis (Yuan *et al*. 2017). SRSF1 is the prototypical member of the highly conserved SR protein family that functions in key aspects of mRNA metabolism. SRSF1 is highly expressed in VSMCs and actively involved in the alternative splicing events that shape the transcriptome of proliferative VSMCs (Llorian *et al*. 2016). We found that SRSF1 is upregulated in VSMCs during neointima formation, indicating that SRSF1 promotes VSMC proliferation and neointimal thickening. Furthermore, a previous study has manifested that SRSF1 promotes vascular smooth muscle cell proliferation through a D133p53/KLF5 pathway (Xie *et al*. 2017).

Inflammation causes the initiation and development of restenosis. Increasing evidence indicates that inflammatory factor TLR4 activates the gene expression of proinflammatory cytokines (Goulopoulou *et al*. 2016). As an important transmembrane receptor in the human immune response, TLR4 is widely expressed in cardiomyocytes, endothelial cells, VSMCs, leukocytes, macrophages, etc. Importantly, a higher mRNA and protein expression of TLR4 and its downstream signaling proteins was found in restenosis patients (Liang *et al*. 2016). TLR4 recognizes and responds against lipopolysaccharides (LPS),

and activation of TLR4 by LPS induces the release of inflammatory cytokines and the stimulation of the nuclear factor kappa B (NF-κB) signaling pathway, ultimately initiating or promoting atherosclerotic lesions (Song *et al*. 2017). Everolimus-eluting stents reduce PCI induced increase in the TLR4 expression on the surface of monocytes (Shokri *et al*. 2015). Notably, TLR4 also plays an important role in nonbacterial inflammatory responses (Vallejo 2011), and may serve as a link between inflammation and atherosclerotic lesions. TLR4 activates p38 mitogen-activated protein kinase (MAPK) and ERK1/2 signaling through myeloid differentiation factor 88 (MyD88) and TIR-domain-containing adapter-inducing interferon-β (TRIF) in VSMCs to regulate VSMC functions (Lee *et al*. 2016). Therefore, inhibition of TLR4 and its upstream signaling molecules provides therapeutic potential for treating restenosis. In the current study, on days 14 and 28 post-surgery, histological analysis demonstrated rat aortic neointimal hyperplasia in balloon-injured rats which was associated with the increased mRNA and protein expression of TLR4. Treatment with valsartan markedly decreased the TLR4 mRNA and protein expression, concomitant with a reduction in neointimal hyperplasia. Our findings reveal that targeting TLR4 may have therapeutic potential for attenuating neointimal hyperplasia. Endothelial denudation is a prominent characteristic of vascular injury associated with angioplasty. Vascular injury leads to a decreased eNOS production (Hu *et al*. 2019). In an attempt to offset the absence of eNOS, there is a compensatory upregulation of iNOS within VSMCs (Kibbe, 1999). Inducible NOS contributes to the pathophysiology of inflammatory diseases. In addition, increased TLR4 expression can promote the production of iNOS by increasing the expression of proinflammatory cytokines (Heo *et al*. 2008). The current study showed the increased aortic levels of iNOS and decreased aortic levels of eNOS in the untreated balloon-injured rats. Accordingly, abnormal aortic levels of iNOS and eNOS are reversed by the administration of valsartan. Angiotensin II promotes VSMC proliferation and migration, which

contribute to the progression of neointimal hyperplasia. Angiotensin II acts via AT1 receptor and AT2 receptor. Apart from its vascular effects, angiotensin II also triggers an inflammatory response in human VSMCs (Granger *et al*. 2004, Ji et al. 2009). TLR4 upregulation by angiotensin II contributes to inflammation and endothelial dysfunction (de Batista *et al*. 2014; Hernanz *et al*. 2015). Angiotensin Ⅱ also increased SRSF1 expression at the mRNA and protein levels in a time-dependent manner (Xie *et al*. 2017). ERK1/2 has been demonstrated to be involved in the pathological process of restenosis after coronary vascular injury (Sarov-Blat *et al*. 2010). Activation of ERK can promote VSMC proliferation or migration as well as accelerate neointimal hyperplasia. It is confirmed that Klotho inhibits proliferation and migration of angiotensin II-induced VSMCs by modulating NF-κB p65, protein kinase B (Akt), and ERK signaling activities (Yu *et al*. 2018). Valsartan is a highly selective AT1 receptor blocker (Huang *et al*. 2014). In this study, we found that it can downregulate the expression of SRSF1 in balloon-injured aorta, and this is not reported before. Consistent with our study, valsartan has been shown to inhibit high-glucose-induced TLR4 expression (Kintscher *et al*. 2010) and myocardial ischemia-reperfusion injury associated with TLR4 expression (Yang *et al*. 2009). Also, our study indicated that valsartan markedly reduces the aortic angiotensin II level, SRSF1 expression, AT1 receptor expression, and ERK protein phosphorylation as well as increases AT2 receptor expression.

In conclusions, this study indicated that the administration of valsartan for both 14 and 28 days after surgery reduced neointimal hyperplasia, and inhibited SRSF1 expression and the TLR4-iNOS-ERK-AT1 receptor pathway in the balloon-injured rat aorta.

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Conflicts of interest

The authors declare that they have no competing interests, and all authors should confirm its accuracy.

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Figure legends

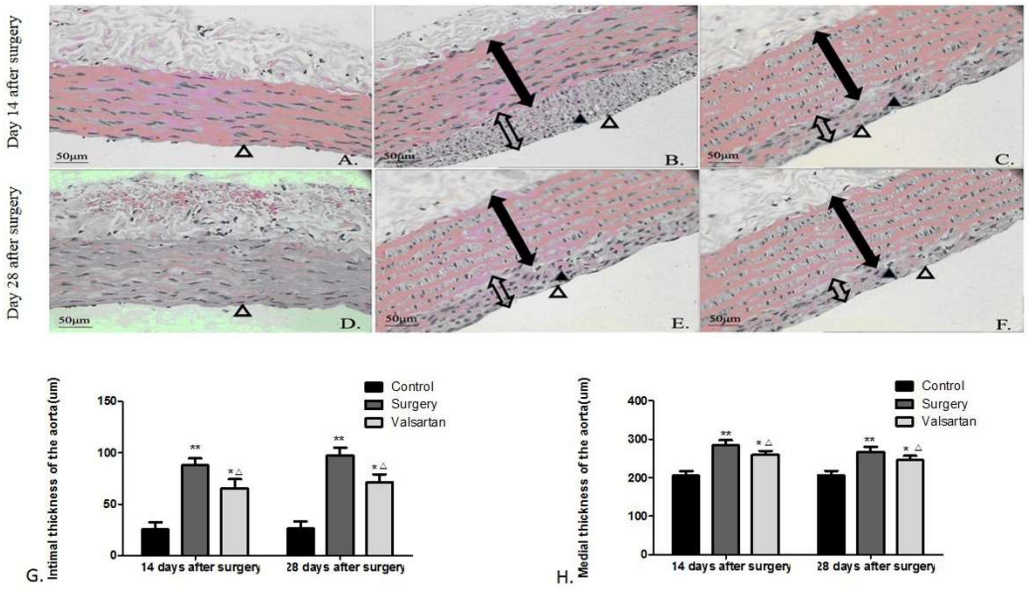

Figure 1 Representative hematoxylin-eosin staining images of neointimal hyperplasia in different groups (magnification, ×200). The sham control rats exhibited a normal intima and media of the aorta at 14 (A) and 28 (D) days after surgery; the balloon-injured rats showed hyperplasic intima with endothelial cells and proliferative VSMCs (B and E); the valsartan-treated rats showed less intimal and medial hyperplasia (C and F). Image analysis of the intimal (G) and medial (H) thicknesses. Data are shown as the mean ± SD (n = 6). $^{*}p < 0.05$, $^{**}p < 0.01$ *vs* the control group, and $^{\triangle}p < 0.05$ *vs* the surgery group. The solid line with double arrow represented media and the empty line with double arrow represented intima. The solid triangle represented proliferative VSMCs and empty triangle represented endothelial cells.

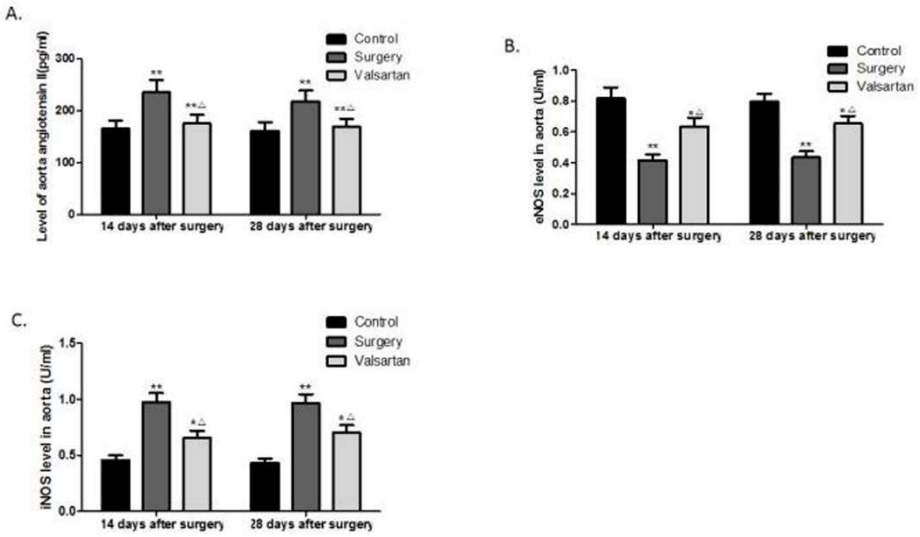

Figure 2 Effects of valsartan on aortic angiotensin II (A), eNOS (B), and iNOS (C) levels. Data are shown as the mean ± SD (n = 6). $^{*}p < 0.05$, $^{**}p < 0.01$ *vs* the control group, and $^{\triangle}p < 0.05$*vs* the surgery group.

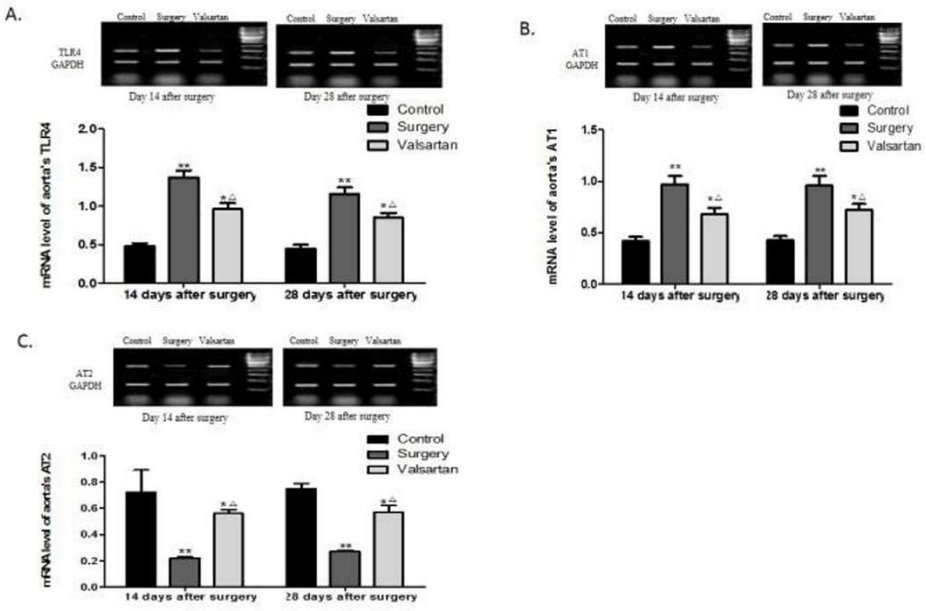

Figure 3 Effects of valsartan on the aortic TLR4, AT1 receptor, and AT2 receptor mRNA expression. RT-PCR analyses of the aortic TLR4 (A), AT1 receptor (B), and AT2 receptor (C) mRNA expression following valsartan treatment in rats. GAPDH was used as an internal control. Data are shown as the

mean ± SD (n = 6). $^{*}p < 0.05$, $^{**}p < 0.01$ *vs* the control group, and $^{\triangle}p < 0.05$ *vs* the surgery group.

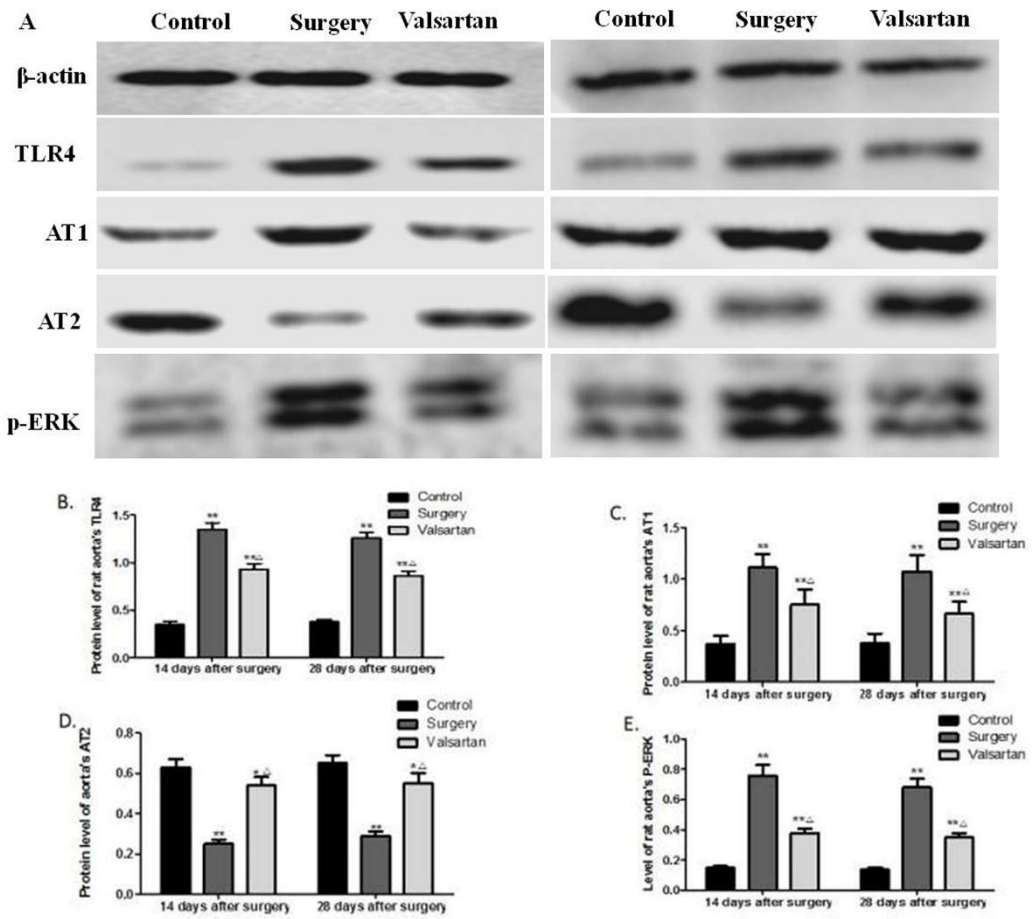

Figure 4 Effects of valsartan on aortic TLR4, AT1 receptor, AT2 receptor, and p-ERK protein expression. At 14 and 28 days after surgery, protein expression of TLR4, AT1 and AT2 receptors, and p-ERK was evaluated by Western blot assay (A). Quantifications of TLR4 (B), AT1 receptor (C), AT2 receptor (D), and p-ERK (E) protein expression relative to that of β-actin. Data are shown as the mean ± SD (n = 6). $^{*}p < 0.05$, $^{**}p < 0.01$ *vs* the control group, and $^{\triangle}p < 0.05$*vs* the surgery group.

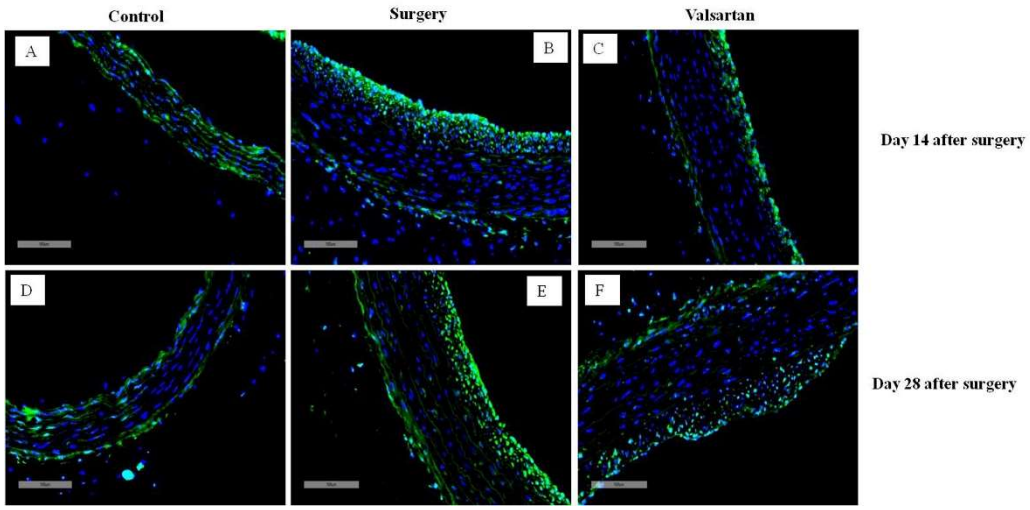

Figure 5 Effects of valsartan on protein expression of SRSF1. Protein expression of SRSF1 was determined using immunofluorescence in 14 days (A, B, C) and 28 days (D, E, F).