Research Paper

Losartan Regulates Transforming Growth Factor-Beta and Phosphoinositide 3-Kinase/Protein Kinase B Pathway Inhibits Vascular Remodeling in Aortic Dissection

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To investigate the effect of losartan on vascular remodeling and transforming growth factor-beta and phosphoinositide 3-kinase/protein kinase B pathway. 30 pregnant rats on the 14th d were selected, 20 of which were given hydroxyethyl ethylene diamine 150 mg/kg/d by gavage to induce aortic dissection. They were randomly divided into losartan group, which was given losartan 20 mg/kg/d by gavage, model group, gavaged with the same amount of normal saline, and 10 normal newborn rats were given the same amount of normal saline by gavage as normal group. Hematoxylin and eosin staining was used to measure the thickness and diameter ratio of the aortic media of mice in each group, Masson staining was used to observe the content of collagen fibers in the aorta of mice in each group, elastic fibers in the aorta of mice in each group were stained, and transforming growth factor-beta and phosphoinositide 3-kinase/protein kinase B pathway protein expression. The diameter of aortic lumen in losartan group was reduced than that in model group, and the ratio of the thickness of the middle membrane and the diameter of the aorta in losartan group was raised than that in model group ($p<0.05$). The contents of elastic fibers and collagen fibers in the aorta of losartan group were raised than those in the model group ($p<0.05$). The transforming growth factor-beta 1 protein content in the losartan group rats was reduced than that in the model group ($p<0.01$). The content of protein kinase B protein in the aortic vessels of losartan group was reduced than that of the model group, and phosphorylated-protein kinase B protein was raised than that of the model group ($p<0.01$). Losartan has protective effect on aortic wall of rats with aortic dissection, and the mechanism may be through inhibiting transforming growth factor-beta 1 pathway, which activates downstream phosphoinositide 3-kinase/protein kinase B pathway related proteins, and then inhibits the expansion and progression of aortic dissection.

Key words: Losartan, transforming growth factor-beta, phosphoinositide 3-kinase/protein kinase B, aortic dissection

There is high morbidity and mortality associated with Aortic Dissection (AD), also referred to as AD aneurysm. The incidence is as high as 50 % in patients with acute type dissection. The clinical consequence of AD progression is aortic rupture, which seriously endangers the patient's life[1]. Hypertension, physical trauma, smoking, previous cardiac surgery, male sex and genetic diseases are risk factors for AD[2]. The development of AD is also associated with vascular remodeling, according to pathological studies. Abnormal vascular remodeling is the core cause of neointimal formation, mediator degeneration and cell dysfunction, which leads to atherosclerosis and vascular dilatation and other diseases[3]. Vascular Smooth Muscle Cells (VSMCs) are highly specialized cells that maintain vascular tone and ensure vascular contractility under

Accepted 27 September 2023
Revised 09 December 2022
Received 12 February 2022
Indian J Pharm Sci 2023;85(5):1472-1477

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physiological conditions. AD is associated with abnormal proliferation, apoptosis and phenotype of VSMCs[4]. Losartan belongs to the Angiotensin II Receptor Blocker (ARB) class of antihypertensive drugs, which regulates vasoconstriction and aldehyde sterol secretion by blocking ABR[5]. Recent studies have found that losartan can delay the occurrence and development of AD and aneurysm[6], but its mechanism remains unclear. Existing studies have shown that there is crossover between Renin-Angiotensin-Aldosterone (RAAS) signaling pathway and TGF-β pathway, but little is known about whether ARB drug losartan can block Angiotensin-II (Ang-II) signaling pathway, inhibit the activation of Transforming Growth Factor-Beta (TGF-β) pathway, and regulate vascular remodeling. This study aims to explore the effect of losartan on vascular remodeling and its effect on TGF-β and Phosphoinositide 3-Kinase/Protein Kinase B (PI3K/AKT) pathway in AD rats by constructing AD model.

MATERIALS AND METHODS

Experimental reagents and instruments:

Shanghai Jianglai Biotechnology Co. provided Ang-II; Phosphate Buffer Solution (PBS) buffer, antigen repair solution and antibody diluent were purchased from Shanghai Mengya Biotechnology Co., Ltd; Trizol extraction reagent was purchased from Beijing Mairibo Biotechnology Co., Ltd; the reverse transcription kit was purchased from Takara, Japan; Mitogen-Activated Protein Kinase (MAPK)-14 antibody was purchased from Shanghai Komin Biotechnology Co., Ltd.

Sterile and clean table was purchased from Shanghai Lianqiao Biotechnology Co., Ltd; Inverted microscope was purchased from Guangzhou Mingmei Photoelectric Technology Co., Ltd; Polymerase Chain Reaction (PCR) instrument was purchased from Blu-ray Biotechnology Co., Ltd; micro pipette was purchased from Shanghai Huzhen Industrial Co., Ltd; vertical electrophoresis apparatus Shenzhen Kangchuyuan Co., Ltd; the gel imaging analysis system was purchased from Beijing Mingtai Jiaxin Technology Co., Ltd; automatic paraffin embedding machine was purchased from Shanghai Yuyan Scientific Instrument Co., Ltd.

Establishment and grouping of experimental animal models:

Eighteen Sprague-Dawley (SD) rats, aged 8 w and pregnant for 14 d, weighing (220-240) g, were purchased from Beijing Hufukang Biotechnology Co., Ltd. and housed in 22°-24°, 50 %-60 % humidity, the light was alternating with darkness, and the rats were fed freely with water and food for 1 w. This animal experiment was approved by the Ethics Committee of our hospital. 12 pregnant SD rats were selected and given hydroxyethyl ethylenediamine 150 mg/kg/d by gavage to induce AD newborn rats. Sixteen young rats were randomly selected and given Ang receptor antagonist losartan 20 mg/kg/d by gavage as Losartan Group (LG), additionally, 10 young rats were gavaged with normal saline at the same rate as the Model Group (MG). The Normal Group (NG) consisted of 6 neonatal rats that were gavaged with the same amount of normal saline.

Detection methods:

Western blot: The protein expression of TGF-β and PI3K/AKT pathway in aorta of rats was detected using Western blot. Aortic tissue samples were collected from rats in each group, and 300 µl Radioimmunoprecipitation Assay (RIPA) lysate was added, and protein quantification was performed by Bicinchoninic Acid (BCA) kit. The extracted protein solution was added to 10 µl loading buffer, denaturated at 100° for 5 min the gel was prepared according to the 12 % Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) discontinuous gel in the reagent preparation, and 1× electrophoresis buffer was added to the electrophoresis tank for electrophoresis. The SDS-PAGE gel after electrophoresis was removed and transferred to the Polyvinylidene Difluoride (PVDF) membrane. The gel was blocked by shaking for 1 h at room temperature, and then 1:500 diluted MAPK14 polyclonal antibody was added at 4° overnight. Washed membranes with Tris-Buffered Saline with 0.1 % Tween® 20 Detergent (TBST) for 3 times, 10 min at a time, and the corresponding secondary antibody was added and incubated on a shaker for 1 h at room temperature, washed membranes with TBST for 3 times (10 min at a time), and the bands were analyzed by gel imaging analysis system.

HE staining: HE staining was used to measure the ratio of aortic media thickness and diameter of rats in each group. The rat's aortic tissue was sectioned and fixed with 10 % formalin, and the tissue was kept at 4° overnight. We rinsed the aortic tissue twice with xylene for 10 min each, dehydrated the tissue with ethanol gradients, and then transparentize it twice in ethanol gradients for 24 h. Transparent tissue soaked in soft wax; put the wax-soaked mice into the embedding device injected with wax liquid, so that the tissue and wax liquid together solidify; after embedded in paraffin, sections were sectioned, and then Hematoxylin and Eosin (H&E) staining was performed. The staining effect of sections was observed using a microscope, and then the sections were sealed with neutral resin. Image-Pro Plus 6.0 software was used to measure the aortic diameter and media thickness of rats in each group, and the ratio was calculated.

Masson staining: Masson staining was used to observe the collagen fiber content in aorta of rats in each group. Paraffin sections were dried, immersed in Wiegert's hemoxylin solution, using 1 % hydrochloric acid alcohol solution, stained with acid reared-Ritchon-orange yellow G solution, and rinsed with 0.2 % Acetic Acid (HAc), differentiated with phosphomolybdic acid solution, rinsed with 0.2 % HAc, rinsed with aniline blue, rinsed with 0.2 % HAc, rinsed with 95 % ethanol, dehydrated, transparent, resin glue sealed. Collagen fibers appear blue under a microscope.

Aortic elastic fiber staining of rats in each group: Paraffin sections were dried, dewaxed with xylene and ethanol, soaked with Wiegert elastic fiber staining solution, differentiated with hydrochloric acid ethanol, soaked with Van Gieson staining solution, differentiated with 95 % ethanol, dehydrated with 100 % genetic, transparent and resin glue sealed, and black or dark blue elastic fiber tail was observed under the microscope.

Statistical methods:

Statistical Package for the Social Sciences (SPSS) 20.0 software package was used to analyze the data in this study, and all measurement data were expressed as ($\bar{x}$±s). Independent sample t-test was used to analyze the mean between two groups, and Analysis of Variance (ANOVA) was used to compare the mean between multiple groups. $p<0.05$ was considered as statistically significant. Compared with the normal group, *$p<0.05$ and compared with the model group, $^{\#}p<0.05$.

RESULTS AND DISCUSSION

The active vascular lumen diameter of the MG was raised than that of the NG, while the ratio of media thickness to aortic media thickness was reduced than that of the NG ($p<0.05$), the diameter of active vascular lumen in LG was reduced than that in MG, while the ratio of media thickness to aortic media thickness was raised than that in MG ($p<0.05$) as shown in Table 1.

The contents of aortic elastic fibers and collagen fibers in the MG were reduced than those in the NG, and the contents of aortic elastic fibers and collagen fibers in the LG were raised than those in the MG ($p<0.05$) as shown in Table 2.

The protein content of TGF-β1 in aortic vessels of rats in the MG was raised than that in the NG, and the protein content of TGF-β1 in aortic vessels of rats in the LG was reduced than that in the MG ($p<0.01$) as shown in Table 3.

The protein content of AKT in aorta of MG was raised than that of NG, and p-Akt protein was reduced than that of NG. The levels of AKT protein in aortic vessels of rats in LG were reduced than those in MG, and p-Akt protein was raised than that in MG ($p<0.01$) as shown in Table 4.

TABLE 1: EFFECT OF LOSARTAN ON AORTIC MEDIA THICKNESS AND DIAMETER RATIO IN RATS ($\bar{x}$±s)

Group	n	Thickness of media (μm)	Active lumen diameter (mm)	Ratio of medium thickness to diameter (μm/mm)
Normal	6	135.54±12.87	1.89±0.21	71.71±5.87
Model	6	74.65±8.53*	2.57±0.26*	29.04±3.36*
Losartan	6	117.68±9.73*#	1.96±0.22*#	60.04±4.71*#

Note: *$p<0.05$ and $^{\#}p<0.05$

TABLE 2: EFFECTS OF LOSARTAN ON ELASTIC FIBERS AND COLLAGEN FIBERS IN RAT AORTA (x̄±s)

Group	n	Content of elastic fiber	Content of collagen fiber
Normal	6	2.24±0.25	2.54±0.21
Model	6	1.24±0.11*	1.16±0.13*
Losartan	6	1.79±0.20*#	2.18±0.19*#

Note: *p<0.05 and #p<0.05

TABLE 3: COMPARISON OF TGF-β1 PROTEIN CONTENT IN AORTIC VESSELS OF RATS (x̄±s)

Group	n	TGF-β1 protein
Normal	6	0.36±0.04
Model	6	1.35±0.12*
Losartan	6	0.89±0.10*#
F		26.54
p		<0.001

Note: *p<0.01 and #p<0.01

TABLE 4: EXPRESSION OF SMAD2/SMAD4 PROTEIN IN AORTIC VESSELS OF RATS (x̄±s)

Group	n	Akt protein	p-Akt protein
Normal	6	0.35±0. 06	0.89±0.09
Model	6	0.86±0.07*	0.24±0.07*
Losartan	6	0.53±0.04*#	0.75±0.08*#
F		12.45	19.65
p		<0.001	<0.001

Note: *p<0.01 and #p<0.01

Blood flows into the aortic wall through the rupture in the inner lining of the aorta, causing the middle layer to separate from the advent, forming two cavities. A false lumen and aorta are filled with blood, resulting in anatomical hematomas[7]. The tube wall is enlarged, resulting in cardiac tamponade or massive thoracic bleeding. Surgical repair and interventional therapy are required for the treatment of AD. Drug therapy has not been successful in clinical trials. There is still no clear understanding of the pathological mechanism leading to AD[8]. Therefore, elucidation of the molecular mechanisms underlying the AD progression is essential for the development of effective prevention and treatment approaches. Aortic media degeneration is an important pathological basis of AD, including smooth muscle cell apoptosis, elastic fiber breakage, collagen fiber degradation, inflammatory cell infiltration, etc.[9]. In this study, the elastic fibers and collagen fibers contents in the aorta of the MG were reduced than those of the NG, which was like previous research. Aortic media degeneration is associated with oxidative stress, as evidence is accumulating. The delicate balance between Reactive Oxygen Species (ROS) production and elimination is disrupted by excessive oxidative stress. Increased ROS concentrations damage biofilms, leading to lipid peroxidation, C protein denaturation, calcium overload inside cells, and reduced enzyme activity[10].

AD rats can be induced using Ang-II perfusion. As a result of the production of ROS in vascular cells and inflammatory cells, Ang-II can cause oxidative stress injury to the vascular wall, promoting the formation of AD[11]. Again through the effects on blood pressure and blood vessel membrane involved in the course, it also can promote the sympathetic nerve endings release norepinephrine, enhance the central sympathetic discharge activity, causing increased blood pressure, lead to aortic intima hyperplasia, accelerate the degradation of extracellular matrix and apoptosis, and lead to endometrial lesions, the tear film and the occurrence of AD[12]. In addition, Ang-II can stimulate endothelial cells to secrete endothelin, activate mononuclear factor. VSMCs are the main cellular components of aortic vascular media. Some scholars have found that Ang-II can

participate in the vascular loss process, repair and remodeling by upregulating the expression of osteopontin in VSMCs[13]. Ang-II receptor blockers can significantly reduce phosphorylated extracellular signal-regulated kinases level in aortic media, thereby delaying the progression of AD[14]. Losartan, as an Ang-II receptor blocker, has a significant blocking effect on the expansion of false lumen and pathological changes of media in AD[15]. In this study, the active lumen diameter of rats in the LG was reduced than that in the MG, while the ratio of tunica media thickness to aortic tunica media thickness was raised than that in the MG, indicating that losartan had a certain repair effect on AD.

It has been found that Ang II can effectively activate TGF-β signaling, which plays a biological role mainly through the classical pathway SMAD protein related pathway and the non-classical pathway PI3K/Akt pathway. Existing studies have clarified the mechanism of Mothers against Decapentaplegic Homolog 3 (SMAD3), Extracellular Signal Regulated Kinase (ERK)/MAPK signaling pathway and Matrix Metalloproteinase (MMP)-2 and MMP-9 involved in AD formation by regulating extracellular matrix degradation, and TGF-β phosphorylates SMAD2/3 protein through activated TGFβR2 homodimer. It promotes the formation of SMAD2/3-SMAD4 complex, activates target genes, participates in cell growth and apoptosis regulation, and participates in the occurrence and development of AD[16]. However, the role of non-classical pathways such as PI3K/Akt signaling pathway in AD remains unknown. PI3K/Akt signaling pathway is thought concerning the mediation of cell proliferation and apoptosis in mammalian cells. Hypoxia induces the proliferation and differentiation of Platelet-Activating Factor (PAF) through PI3K/Akt/p70S6K signaling pathway, and induces hypoxic pulmonary vascular remodeling in rats[17]. In mice, the Phosphatase and Tensin Homolog (PTEN) inhibitor BPV inhibits myocardial infarction by activating PI3K/Akt/VEGF signaling pathways, reducing cardiomyocyte apoptosis and promoting angiogenesis[18]. Small interfering Ribonucleic Acid (siRNA) targeting Serine-Arginine Protein Kinase 1 (SRPK1) enhances the proliferation and vascular remodeling of VSMCs and inhibits VSMCs apoptosis in aneurysm rats by inhibiting PI3K/Akt pathway activation, thus enhancing the vascular remodeling ability of aneurysms[19]. Curcio *et al.*[20] found that total inhibition of Akt in aged animals resulted in decreased VSMCs proliferation and increased vascular apoptosis rate. Meanwhile, Liu *et al.*[21] confirmed the effects of PI3K inhibitor LY294002 and Akt inhibitor 1701-1 on Apelin-13-induced cell proliferation, indicating that apelin-13 induces rat VSMCs proliferation through PI3K/Akt signaling pathway. Aside from PI3K pathway genes, Akt also mediates B-cell lymphoma 2 (Bcl-2) associated proteins, Glycogen Synthase Kinase-3 beta (GSK3β) or Murine Double Minute 2 (MDM2)[22]. Akt also phosphorylates BAD (a member of the pro-apoptotic Bcl-2 family), thereby reducing the pro-apoptotic function of BAD[23]. In this study, it was found that the protein content of AKT in aortic vessels of the MG was raised than that of the NG, and the protein of p-Akt was reduced than that of the NG. The protein content of AKT in aorta of LG was reduced than that of MG, and the protein of p-Akt was raised than that of MG. This suggests that losartan can significantly inhibit TGF-β1 signaling pathway, and then activate PI3K/Akt pathway, which can delay the progression of AD.

In conclusion, losartan has a protective effect on the aortic vascular wall of AD rats, which may be through inhibiting TGF-β1 pathway activation, activating downstream PI3K/Akt pathway related proteins, and then inhibiting the expansion and progression of AD.

Conflict of interests:

The authors declared no conflict of interests.

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