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Original Article

Effects of valsartan on VEGF expression and PI3K/Akt/mTOR signaling pathway in rats with diabetic retinopathy

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Abstract: Objective: This paper aimed to investigate the effects of valsartan on the expression of vascular endothelial growth factor (VEGF) and the PI3K/Akt/mTOR signaling pathway in rat a model of diabetic retinopathy (RD). Methods: Sixty male Wistar rats were divided into a control group, a valsartan group and a model group (n=20 each) based on random number tables. Diabetic rat models were induced by streptozotocin (STZ). Rats in the valsartan group were treated with valsartan, whereas those in the other two groups were treated with the same amount of normal saline. They were continuously observed for 20 weeks to record changes in their body weights and blood glucose. qPCR was performed to detect the mRNA expression of VEGF, PI3K, AKT and mTOR in rat retinal tissues. Western-blot (WB) was conducted to detect the protein expression of VEGF, p-PI3K, p-AKT, p-mTOR, PI3K, AKT and mTOR in the tissues. Results: Rat body weights increased faster in the control group, but had no obvious changes in the model group. After 4 weeks, the body weights increased significantly in the valsartan group compared with the model group. The blood glucose increased significantly in the model group, but decreased after 4 weeks in the valsartan group. According to qPCR, the mRNA expression of VEGF, PI3K, AKT and mTOR in the model group was significantly higher than that in the control group (all $P<0.001$), with no statistically significant difference in the mRNA expression between the valsartan and control groups. According to WB, the protein expression of VEGF, PI3K, AKT, mTOR, p-PI3K/PI3K, p-AKT/AKT and p-mTOR/mTOR in the model group was significantly higher than that in the control group ($P<0.001$), and the protein expression in the valsartan group was significantly lower than that in the model group ($P<0.001$). Conclusion: In the valsartan group, the rats had significantly reduced blood glucose, significantly increased body weights, effectively relieved adverse symptoms, and significantly decreased post-treatment VEGF expression, which may be due to the effects of PI3K/Akt/mTOR signaling pathway that is regulated by VEGF.

Keywords: Valsartan, diabetic retinopathy, diabetic model, VEGF, PI3K/Akt/mTOR

Introduction

As a common and specific microvascular complication of diabetes mellitus, diabetic retinopathy (DR) affects almost all patients with type I diabetes mellitus and most patients with type II diabetes mellitus, having an influence on the vision of patients to varying degrees [1-3]. Vascular endothelial growth factor (VEGF) is a multifunctional growth factor, which specifically binds to vascular endothelial cells and is involved in the progression of DR [4-6]. Its expression in the eyes of patients with diabetes mellitus is directly proportional to the neovascularization rate and the severity of damage to the blood-retinal barrier [7]. According to a large number of animal experiments with models of DR, at 4 weeks after the successful establishment of a DR model in rats, varying degrees of retinopathy appears; downregulating VEGF can prevent the occurrence of microvascular complications [8, 9]. Valsartan, a selective AT1R receptor blocker that has a low metabolic rate *in vivo*, is less influenced by other drugs, and can lower blood pressure and dilate blood vessels by regulating plasma an-

giotensin II [10, 11]. The activation of retinal AT1R involves abnormal changes in DR microvasculature, so AR-1R antagonists can reduce DR-induced changes in retinal hemodynamics, retinal angiogenesis, microvascular permeability and other undesirable characteristics [12]. Zhang Yunli and other researchers used Wistar rats and induced diabetes models that were then treated with different doses of valsartan. The results showed that this drug could effectively reduce VEGF expression, relieve DR to a certain extent, and protect the retina [13]. The VEGF/PI3K/Akt/mTOR signaling cascade is not only a process that promotes angiogenesis, but is also an important signaling pathway that promotes endothelial cell proliferation and survival and inhibits cell apoptosis. In addition to inducing VEGF overexpression, hypoxic and hyperglycemic states in diabetes can also induce a variety of downstream signal molecules, such as PI3K/Akt/mTOR and other signaling pathways, through KDR, FIT-1 and other receptors. The activation of this pathway can accelerate the migration and proliferation of endothelial cells, form new microvascular lumens, and generate stable blood vessels [14]. In this study, valsartan was used to treat diabetic induced rats, and the protein expression of VEGF, p-PI3K/PI3K, p-AKT/AKT and p-mTOR/TOR was detected, to explore the molecular mechanism of this drug on treating DR.

Materials and methods

Experimental animals

Two-month old male Wistar rats were purchased from the Vital River Laboratory Animal Technology Co., Ltd. For ensuring the consistency of the study, they weighed 220-250 g and had free access to water and food during the period of feeding and observation.

Major reagents

Streptozotocin (STZ; Sigma, USA), valsartan (Beijing Novartis Pharma Co., Ltd., China), rabbit anti-rat VEGF, p-PI3K/PI3K, p-AKT/AKT, p-mTOR/TOR, β-actin primary antibody, HRP-labeled goat anti-rat and rabbit (Abcam, UK), Western blot-related reagents (RIPA, BCA, etc.) (Thermo, USA), Invitrogen TRIzol reagents (Thermo, USA), reverse transcription kits (Takara, Japan), qPCR kits (TsingKe Biological Technology, China).

Major experimental instruments

The Western blot (WB) system and a gel imaging analyzer (Bio-Rad, USA), a protein analyzer (BioDrop, UK), a low-temperature high-speed centrifuge (Eppendorf, Germany), a blood glucose meter (Roche, Germany), a real-time fluorescence quantitative PCR instrument (Bio-Rad, USA), an ultraviolet spectrophotometer (Thermo, USA).

Animal modeling, grouping and medication

The experimental rats were randomly divided into the control, model and valsartan groups (n=20 each). All rats were adaptively fed in the animal feeding room for one week. After fasting for 12 hours, they were intraperitoneally injected with STZ, which was dissolved in a newly prepared sodium citrate buffer solution (0.01 mol/L and pH=4.6), at 60 mg/kg once to induce diabetes models. Those in the control group were injected with the same amount of sodium citrate buffer solution. At 72 hours after treatment, blood was extracted from the caudal vein of the rats, and the blood glucose concentration was detected with a blood glucose meter. If the concentration was ≥16.7 mmol/L, the modeling was considered successful. After the successful modeling, the rats in the valsartan group were intragastrically administered with 40 mg/kg of valsartan every day, while the rest rats were treated with the same amount of normal saline.

Determination of fasting blood glucose and observation of signs

After the successful modeling, changes in the rats' fasting blood glucose and body weights were observed at the 1st, 4th, 8th, 12th, 16th and 20th weeks, with corresponding indicators recorded.

Western blot

At 20 weeks after the successful modeling, the surviving rats were anesthetized with excessive pentobarbital sodium, killed and dissected, so as to remove and carefully grind the retina. After that, the product was mixed with a proper amount of precooled RIPA lysis buffer. After a 5-minute ice bath, the mixture was centrifuged in a low temperature centrifuge at 12,000 r/min for 15 minutes, and then the supernatant

Table 1. qPCR primer sequence

Gene	Forward prime (5'-3')	Reverse prime (5'-3')
VEGF	CAAACCTCACCAAAGCCAGC	GCGCTTTCGTTTTTGACCCT
PI3K	ATACTTGATGTGGCTGACG	CAATAGGTTCTCGGCTTT
AKT	TTTATTGGCTACAAGGAACG	AGTCTGAATGGCGGTGGT
mTOR	AGATACGCCGTCATTCCT	GCTCAAACACCTCCACCT
β-actin	TCAGGTCATCACTATCGGCAAT	AAAGAAAGGGTGTAAAACGCA

Note: VEGF: vascular endothelial growth factor.

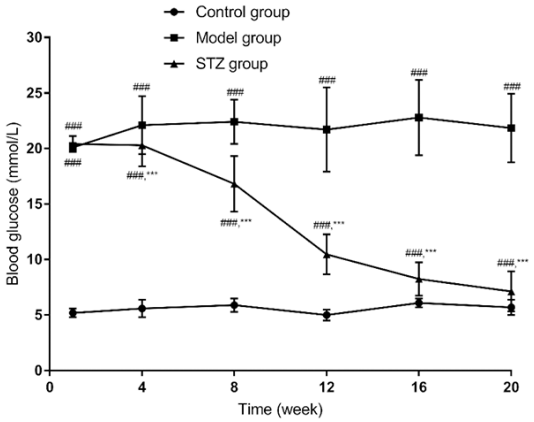

Figure 1. Blood glucose of rats at different time points. Compared with the control group, ###P<0.001; compared with the model group, ***P<0.001.

was carefully removed to determine the protein content. WB was carried out according to the experimental steps [15]. An equal amount of the protein was electrophoresed with SDS-PAGE and transferred to the membrane, which was decolorized at room temperature (TBST buffer solution) and then placed in 5% skim milk (prepared by 0.5% TBST) for blocking at room temperature for 1 hour. After the primary and secondary antibodies were diluted, the color-developing solution was added to the membrane, which was exposed after 1-2 minutes. Appropriate time was selected for exposure, with the dilution concentrations of the antibodies being 1:200 and 1:5,000, respectively. The protein expression of VEGF, p-PI3K, PI3K, p-AKT, AKT, p-mTOR and TOR was detected with β-actin as the internal reference protein. ImageJ was used to statistically quantify the gray values of protein bands [16].

Real-time fluorescence quantitative PCR (qRT-PCR)

The rat retinal tissues were dissected under aseptic conditions, and total RNA was extracted from the tissues by the Trizol method, with the operating steps conducted according to the kit instructions. The RNA was measured for its purity and quantified by the ultraviolet spectrophotometer. A260 nm/A280 nm between 1.8 and 2.2 indicating that RNA quality was qualified and could be used in reverse transcription experiments. The RNA (1,000 ng) was used for the reverse transcription to synthesize cRNA, with the specific operating steps conducted based on the instructions of the Takara reverse transcription kit. The cDNA was prepared in 10 μL of the reaction system and amplified with the real-time fluorescence quantitative PCR instrument: predenaturation at 95°C for 30 s, amplification and extension at 95°C for 15 s, and annealing at 60°C for 30 s. The above three steps were cycled for 40 times. Primers were designed according to sequences of rat PI3K, AKT, mTOR and β-actin that were provided by Genebank. With β-actin as the internal reference, the relative gene expression was calculated by $2^{-\Delta\Delta CT}$ method. Primers were synthesized by Tsing-Ke Biological Technology. Primer sequences are shown in the **Table 1**.

Statistical analysis

SPSS 22.0 was used to analyze the data. Measurement data were expressed as mean ± standard deviation ($\bar{x}$ ± sd), and one-way analysis of variance was used for comparison between groups, while Bonferroni test was used for pairwise comparison between multiple groups. When P<0.05, the difference was statistically significant.

Results

Changes in blood glucose

Fasting blood glucose was measured at different time points after successful modeling. Its concentration at the 20th week was (5.7±0.7) mmol/L in the control group, and was relatively stable. The concentration at the 20th week was (21.9±3.1) mmol/L in the model group, and was relatively high. In the valsartan group, the concentration began to decrease at the 4th week and was (7.1±1.8) mmol/L at the 20th week. See **Figure 1** and **Table 2**.

Table 2. Blood glucose of rats at different time points (mmol/L)

Group	1 w	4 w	8 w	12 w	16 w	20 w
Control group	5.2±0.4	5.6±0.8	5.9±0.6	5.0±0.5	6.1±0.4	5.7±0.7
Model group	20.1±0.4$^{\#\#\#}$	22.1±2.6$^{\#\#\#}$	22.4±2.0$^{\#\#\#}$	21.7±3.8$^{\#\#\#}$	22.8±3.4$^{\#\#\#}$	21.9±3.1$^{\#\#\#}$
Valsartan group	20.4±0.7$^{\#\#\#}$	20.3±1.9$^{\#\#\#,***}$	16.8±2.5$^{\#\#\#,***}$	10.5±1.8$^{\#\#\#,***}$	8.3±1.5$^{\#\#\#,***}$	7.1±1.8$^{\#\#\#,***}$

Note: Compared with the control group, $^{\#\#\#}$P<0.001; compared with the model group, ***P<0.001.

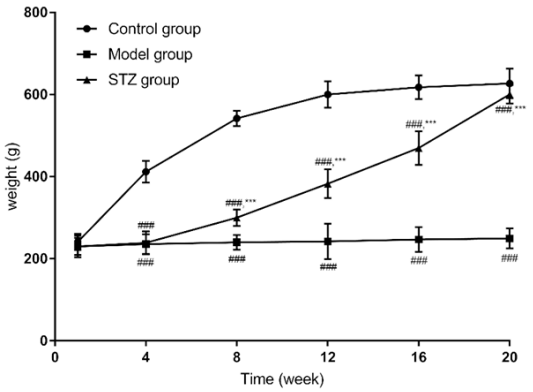

Figure 2. Changes in body weights of rats at different time points. Compared with the control group, $^{\#\#\#}$P<0.001; compared with the model group, ***P<0.001. STZ: streptozotocin.

Changes in body weights

The rat's body weights at different time points were measured after successful modeling. The body weights increased rapidly and were (627.1±36.2) g at the 20th week in the control group. The body weights increased slowly and were (249.6±24.7) g at the 20th week in the model group. In the valsartan group, the body weights began to increase at the 4th week and were (600.4±22.9) g at the 20th week. See **Figure 2**.

Changes in mRNA expression of VEGF and PI3K/AKT/mTOR

The mRNA expression of VEGF, PI3K, AKT and mTOR in the model group was significantly higher than that in the control group. The mRNA expression in the valsartan group was significantly lower than that in the model group. The difference between groups was statistically significant. See **Table 3**.

Protein expression of PI3K/AKT/mTOR signaling pathway

The protein expression of VEGF, p-PI3K/PI3K, p-AKT/AKT, and p-mTOR/mTOR in the model group was significantly higher than that in the control group. The protein expression of p-PI3K/PI3K, p-AKT/AKT and p-mTOR/mTOR in the valsartan group was significantly lower than that in the model group. The protein expression of PI3K, AKT and mTOR in the model group was significantly higher than that in the control group, and the protein expression in the valsartan group was significantly lower than that in the model group. The difference between groups was statistically significant. See **Figures 3-5**.

Discussion

According to recent studies, the development and progression of DR are related to the abnormal expression of certain growth factors and proteins [17]. Valsartan can relieve retinal microvascular changes, disorders of blood glucose and lipids and other adverse symptoms in DR modled rats, and can inhibit the rats' VEGF expression [18, 19], but the specific mechanism is still unclear. This study focused on the PI3K/AKT/mTOR signaling pathway to explore the molecular mechanism of this drug in treating DR.

In this study, a rat model of DR was induced by a single intraperitoneal injection of STZ. If the blood glucose concentration was ≥16.7 mmol/L, the modeling was considered successful. As reported by clinical studies, the effective control of blood glucose can alleviate the adverse symptoms of DR [20, 21]. Valsartan has a great effect on controlling blood glucose in patients with DR, with a low plasma clearance rate and a satisfactory effect [22, 23]. In this study, blood glucose in the DR rats decreased at the 4th week after valsartan interventions, suggesting that this drug can effectively reduce the rats' blood glucose.

Some studies have conducted electrophysiology in the retna, and found that valsartan can act on AT1R and eventually relieve DR-induced retinal disorders, with superior efficacy to that

Table 3. The mRNA expression of VEGF, PI3K, AKT and mTOR in each group

Group	n	VEGF	PI3K	AKT	mTOR
Control group	20	1.05±0.12	1.08±0.06	1.02±0.14	0.98±0.10
Model group	20	1.52±0.13###	1.29±0.15###	1.62±0.09###	1.58±0.18###
Valsartan group	20	1.14±0.17***	1.10±0.10***	1.10±0.13***	1.03±0.12***

Note: Compared with the control group, ###P<0.001; compared with the model group, ***P<0.001. VEGF: vascular endothelial growth factor.

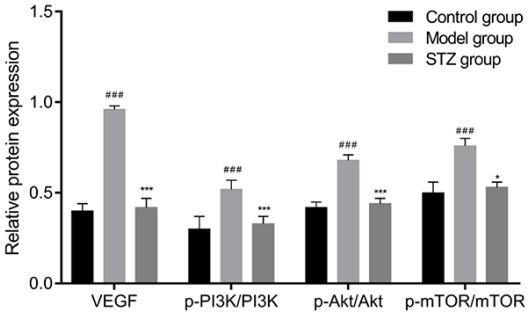

Figure 3. Relative protein expression of VEGF, p-PI3K/PI3K, p-AKT/AKT and p-mTOR/mTOR. Compared with the control group, ###P<0.001; compared with the model group, ***P<0.001, *P<0.05. VEGF: vascular endothelial growth factor; STZ: streptozotocin.

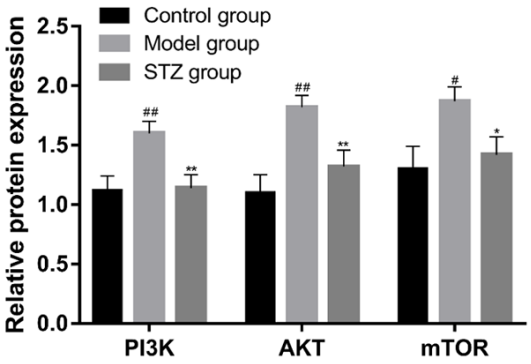

Figure 4. Relative protein expression of PI3K, AKT and mTOR. Compared with the control group, ##P<0.01, #P<0.05; compared with the model group, **P<0.01, *P<0.05. STZ: streptozotocin.

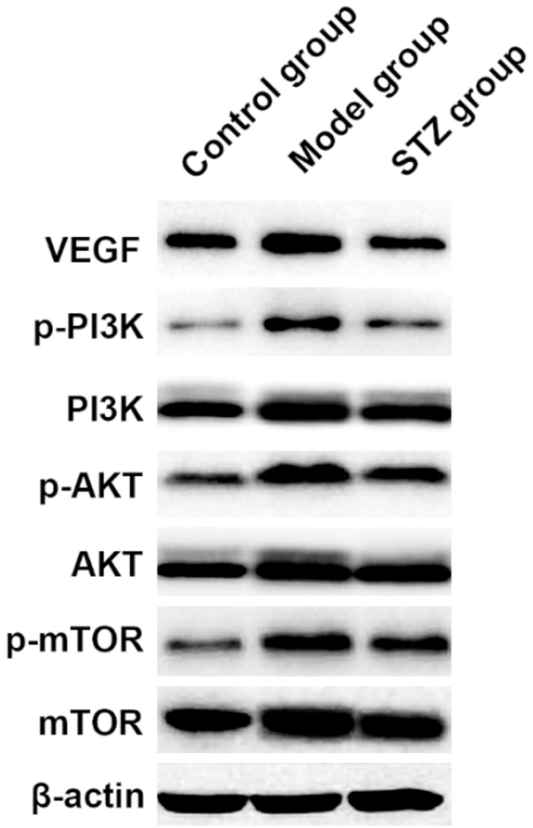

Figure 5. The protein expression of VEGF, p-PI3K, PI3K, p-AKT, AKT, p-mTOR and mTOR. VEGF: vascular endothelial growth factor.

of atenolol [24]. Zhang Yunli and other researchers selected Wistar rats to establish diabetes models, and treated the diabetic rats with different doses of valsartan [13]. The results showed that after valsartan interventions, VEGF expression in the rat's retina significantly decreased, and the effect was the most obvious with large doses. This suggests that this drug could reduce VEGF expression and help relieve DR. In our study, VEGF expression in the retina of diabetic modeled rats (treated with valsartan) was significantly lower than that of untreated diabetic rats, which indicates that valsartan can alleviate the adverse symptoms of DR by reducing VEGF expression.

The PI3K/AKT/mTOR pathway is widely involved in cell growth, survival, proliferation, angiogenesis and other important physiological processes [25]. In this study, qPCR was performed to detect the mRNA expression of VEGF, PI3K, AKT and mTOR in the rat retina. The results showed that the mRNA expression in the

model group was significantly higher than that in the control group, with no statistically significant difference in the mRNA expression between the valsartan and control groups. According to the WB, the protein expression of p-PI3K/PI3K, p-AKT/AKT, p-mTOR/mTOR, PI3K, AKT and mTOR in the model group was significantly higher than that in the control group, while the protein expression in the valsartan group was similar to that in the control group. This reveals that valsartan may downregulate VEGF expression and then regulate DR through the PI3K/AKT/mTOR signaling pathway.

The deficiencies and prospects of this study are that this study has mainly explored the effects of valsartan on signaling pathways of retinopathy, but the PI3K signaling pathway was not intervened with directly to determine the dependence of this drug on this pathway. Therefore, related experiments will be carried out in the future.

In summary, valsartan has been found to effectively reduce blood glucose, and intervene in DR through downregulating the VEGF-mediated PI3K/AKT/mTOR signaling pathway, which provides an experimental basis for intervention in DR.

Disclosure of conflict of interest

None.

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