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Effect of bradykinin on rats with thromboangiitis obliterans through PI3K/Akt signaling pathway

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Abstract. – OBJECTIVE: To explore the effect of bradykinin on rats with thromboangiitis obliterans (TAO) through the phosphatidylinositol 3-hydroxy kinase/protein kinase B (PI3K/Akt) signaling pathway.

MATERIALS AND METHODS: The female Wistar rats were injected with lauric acid *via* the femoral artery to establish the TAO model, and they were randomly divided into control group (healthy rats), model group (TAO rats) and bradykinin group (TAO rats injected with bradykinin B2 receptor-specific inhibitor). The control was set in each group before the operation. The level of serum bradykinin in each group was detected *via* enzyme-linked immunosorbent assay (ELISA), and the reactive oxygen species (ROS) level, Caspase-3 activity and PI3K/Akt protein concentration in vascular tissues were measured *via* ELISA, Western blotting, ROS assay, and Caspase-3 activity assay, respectively. Moreover, the specific therapeutic mechanism of bradykinin was analyzed.

RESULTS: In control group, the intima of the lower extremity venous tissues was smooth, the extima had no evident changes, and there was no inflammatory cell invasion around the arteries and veins. In model group, there was massive inflammatory cell invasion into the lower extremity venous tissues. In bradykinin group, fibrosis and atrophy occurred in venous tissues, the extima was thickened without fibrosis, and there was phagocytosis of neutrophils and mononuclear macrophages around the arteries and veins, as well as massive inflammatory infiltration. The PI3K/Akt protein concentration in lower extremity venous tissues was the highest in control group and the lowest in bradykinin group, and there were statistically significant differences ($p<0.01$). At 24 h after administration of doxorubicin (DOX), the level of ROS in lower extremity venous tissues was higher in bradykinin group than that in model group ($p<0.05$), and it was also higher in model group than that in control group ($p<0.05$). Besides, the activity of Caspase-3 in lower extremity venous tissues was significantly increased in bradykinin group compared with that in model group and control group, while it was slightly higher in model group than that in control group ($p<0.05$).

CONCLUSIONS: The low expression of bradykinin can promote TAO in rats by the mechanism that it inhibits the PI3K/Akt signaling pathway to raise the oxidative stress level, thereby aggravating TAO.

Key Words:
Bradykinin, PI3K/Akt, Thromboangiitis obliterans.

Introduction

Thromboangiitis obliterans (TAO) is a clinically common chronic peripheral vascular disease[1-3], whose main pathophysiological changes are peripheral vascular inflammation and occlusion. It develops stepwise and is manifested as the medium-sized and small-sized artery involvement in four extremities, and extremity ulcer and necrosis may occur in severe cases[4,5]. The pathogenesis of TAO includes immune dysfunction, hormonal regulation disorder, etc., but the traditional conservative treatment has unsatisfactory efficacy, with the amputation rate of patients

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remaining above 80%[6]. Bradykinin belongs to the nonapeptide in the kinin family, whose main component is vasoactive substance, and its main function is to dilate the vessels[7]. Moreover, the content of bradykinin in human plasma is very low under normal conditions, but kininogen can be converted into bradykinin under the action of kallikrein in the case of tissue damage[8]. The main mechanism of action of bradykinin is to directly dilate the vessels and constrict the vessels that produce angiotensin and norepinephrine[9] and promote endogenous vasodilation. Besides, bradykinin can lead to pathophysiological response and physiological protection, and participate in regulating a variety of physiological, pathological and systemic processes, such as vasculitis caused by arterial spasm or thrombosis[10]. The phosphatidylinositol 3-hydroxy kinase/protein kinase B (PI3K/Akt) signal transduction pathway is able to regulate TAO, and the PI3K/Akt signal is a transduction protein involved in intracellular phosphorylation[11]. Munoz-Rodriguez et al[12] studied and showed that the PI3K/Akt signaling pathway regulates the cell physiology and it is one of the major signaling pathways for cell transcription, translation, metabolism, proliferation, migration, and survival. At present, there are few studies on the effect of bradykinin on TAO through regulating the PI3K/Akt signal. In this paper, therefore, the Wistar rat model was established to observe the effects of bradykinin on the level of reactive oxygen species (ROS) and activity of Caspase-3 in lower extremity venous tissues through regulating the PI3K/Akt signaling pathway.

Materials and Methods

Animal Sources and Grouping

A total of 60 normal female Wistar rats of 18 weeks old and weighing about 300 g were purchased from the Animal Laboratory of Nanjing Medical University, and they were randomly divided into control group (healthy rats, n=20), model group (TAO rats, n=20) and bradykinin group (TAO rats injected with bradykinin B2 receptor-specific inhibitor, n=20). This study was approved by the Animal Ethics Committee of Jinzhou Medical University Animal Center.

Establishment of TAO Model

According to the method of Shin-Ichiro Ashida, the female rats were weighed, anesthetized and fixed on a sterile operating table in a supine position. A medial incision was made from the midpoint of groin to the medial knee, and the arteries in the legs were found. The proximal end of artery was clamped using the hemostatic clips, while the distal end was injected with 0.2 mL of lauric acid solution. After 1 min, the clips were loosened, and the skin was sutured when there was no bleeding. In control group, 0.2 mL of 0.9% sodium chloride solution was injected into the femoral artery. After operation, all rats were intraperitoneally injected with 200,000 U of penicillin for infection prevention.

Instruments and Reagents

37°C constant temperature water bath box (Jiangsu Jintan Medical Instrument Factory, Jintan, China), normal saline (Tangshan Jixiang Pharmaceutical, Tangshan, China), microplate reader (Shenzhen Highcreation Technology Co., Ltd., Shenzhen, China), quantitative real-time fluorescence polymerase chain reaction (qRT-PCR) instrument (ABI, Foster City, CA, USA), PCR kit (ABI, Foster City, CA, USA), ABI 2700 PCR instrument (ABI, Foster City, CA, USA), PI3K and Akt primary antibodies (CST, Danvers, MA, USA), 96-well plate and sealing membrane (ABI, Foster City, CA, USA), RNA extraction kit (Qiagen, Hilden, Germany), and bradykinin B2 receptor-specific inhibitor (Sigma-Aldrich, St. Louis, MO, USA).

Blood Specimen Collection and Processing

In model group and bradykinin group, 2 mL of venous blood was drawn from the right hind limb, and centrifuged at 3, 000 rpm and 4°C for 15 min. Then, the supernatant was stored at -20°C. In control group, the blood was sampled at 28 days after the operation. The serum was collected from the caudal vein at 48 h before operation.

Culture of Lower Extremity Venous Tissue Cells

After 100 μL of specimens were taken from each group, they were added into the enzyme-linked immunosorbent assay (ELISA; Novus, Littleton, CO, USA) plate wells and incubated at 37°C for 120 min. The solution in the well was discarded and the well was thoroughly washed with washing liquid for 10 min. Then, 100 μL of primary antibody solution was added into each well, and the ELISA plate was washed at 37°C for 60 min. After that, 100 μL of enzyme-labeled antibody

solution was added into each well, and the ELISA plate was washed again at 37°C for 30 min, then added with 100 μL of substrate solution and let stand in a dark place at 37°C for 15 min. Finally, 100 μL of stop buffer was added to terminate the reaction.

Hematoxylin-Eosin (HE) Staining

A certain number of lower extremity venous tissues were taken, some of which were fixed with 4% paraformaldehyde overnight, dehydrated and embedded in paraffin, followed by induction for 24 h. Then the collagen deposition in vascular tissues in each group was observed via Masson staining, and the vascular tissue structure was detected *via* HE staining (Boster, Wuhan, China). Finally, the sections were observed under a microscope to compare the changes in tissues in each group.

Detection of PI3K/Akt Protein Concentration in Lower Extremity Venous Tissues Via Western Blotting

The lower extremity venous vascular cells in the logarithmic phase were taken from each group, inoculated into a 6-well plate (5×110 cells/mL), incubated for 24 h and digested with 0.25% trypsin. Then the cells were collected, from which the protein was extracted and subjected to ice bath for 30 min and centrifugation at 12,000×g and 4°C for 5 min. The supernatant was taken to detect the protein concentration in each group using the bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). After that, an appropriate number of proteins in each group were taken, separated *via* 10% dodecyl sulfate, sodium salt-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto a polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA) and sealed with 5% skim milk powder at room temperature for 1 h. Then, they were incubated with the primary antibody at 4°C overnight, washed with Tris-Buffered Saline and Tween (TBST), and incubated again with the corresponding fluorescence-labeled secondary antibody (IRDye700/IRDye800) in a dark place at room temperature for 1 h. Finally, the protein band was scanned with Odyssey infrared system and analyzed using Quantity One software.

Detection of ROS Level

A certain number of round cover glasses were taken, soaked in 75% alcohol for 30 min, burned with an alcohol lamp and placed in a 24-well plate. Then, the lower extremity venous tissues were taken from model group and bradykinin group, and prepared into suspension. The suspension in the two groups was inoculated into the cover glasses (100 μL/well) and incubated at 38°C under 6% CO_2 and saturated humidity for 4 h. Subsequently, 100 μL of medium was supplemented in each well for incubation overnight. After drug administration and incubation for 24 h, the cells in both groups were taken and washed twice with PBS. The specific fluorescence probe 2,7-dichlorofluorescein diacetate (DCFH-DA) was diluted to 1 μM and used to stain the cover glasses for 30 min. The fluorescent substance cannot be detected in DCFH-DA, but it can pass through the cell membrane and form DCFH. Then, DCFH binds to ROS in cells to form fluorescent DCF, after which the fluorescence density of cells can be detected. Therefore, the amount of fluorescence can directly reflect the level of ROS in cells.

Caspase-3 Activity Assay

The lower extremity venous tissues were lysed on ice and centrifuged at 4°C. The supernatant was collected, diluted with 50 μL of buffer, added into the 96-well plate and incubated with 5 μL of LEHD-pNA substrate at 37°C for 1 h. 3 repeated wells were set for each specimen. Finally, the optical density (OD) value was measured at 405 nm using a microplate reader.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 16.0 software (SPSS, Chicago, IL, USA) were used for statistical processing of results. All data were expressed as mean ± standard deviation. *t*-test was performed for the difference in indexes among control group, model group, and bradykinin group. $p<0.05$ suggested statistically significant differences. Analysis of variance was used for the intragroup comparison.

Results

Morphology of Lower Extremity Venous Tissues in Each Group

In control group, there were not many changes in the hind limbs, and the muscle cells of the hind limbs were clear and rosy. Pathological sections showed that the arteries and veins were unobstructed, the intima was smooth, the extima had no evident changes, and there was no

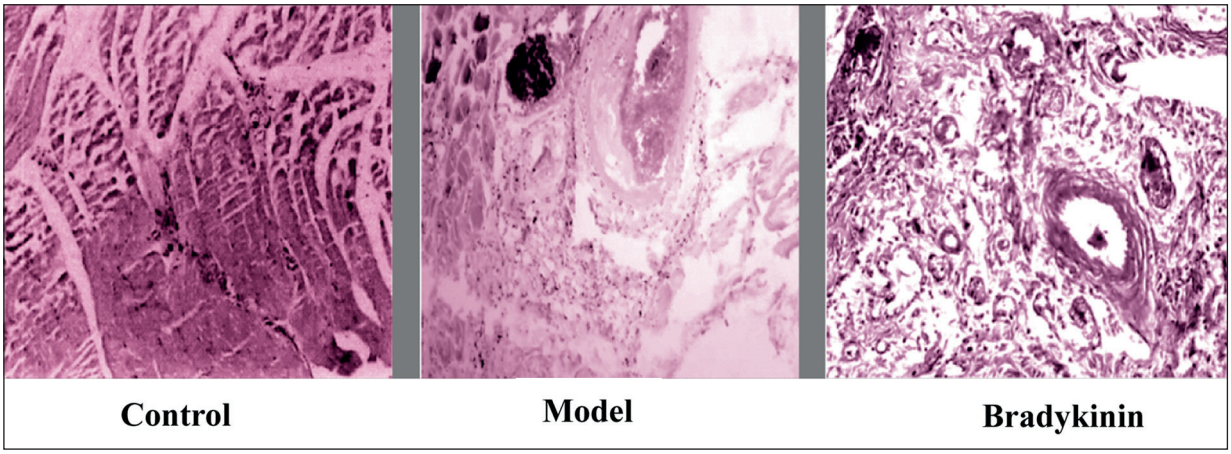

Figure 1. Morphology of lower extremity venous tissues in each group (magnification: 400×).

inflammatory cell invasion around the arteries and veins. After injection of lauric acid solution into the femoral artery in model group, the hind paw began to turn pale and purple, the skin temperature decreased and the artery pulse was weakened and even disappeared. However, no necrotic muscles were observed and there were ischemic changes. Besides, the pathological sections showed the erythrocyte sedimentation in the arteries and veins. In bradykinin group, most of the hind limbs had fallen off, and some rats had the signs of systemic infection. Besides, the lumen occlusion, thrombosis, and medial calcification could be seen, fibrosis and atrophy occurred, the extima was thickened without fibrosis. Moreover, there was phagocytosis of neutrophils and mononuclear macrophages around the arteries and veins, as well as massive inflammatory infiltration (Figure 1).

PI3K/Akt Protein Concentration in Lower Extremity Venous Tissues in Each Group Detected Via Western Blotting

Western blotting was performed for the PI3K/Akt protein in lower extremity venous tissues in the three groups. According to the analysis of gray level, the PI3K/Akt protein concentration in lower extremity venous tissues was the highest in control group and the lowest in bradykinin group, and there were statistically significant differences (p<0.01) (Figures 2, 3 and 4).

ROS Level in Lower Extremity Venous Tissues

To detect whether oxidative stress is associated with the cell damage caused by TAO, doxorubicin

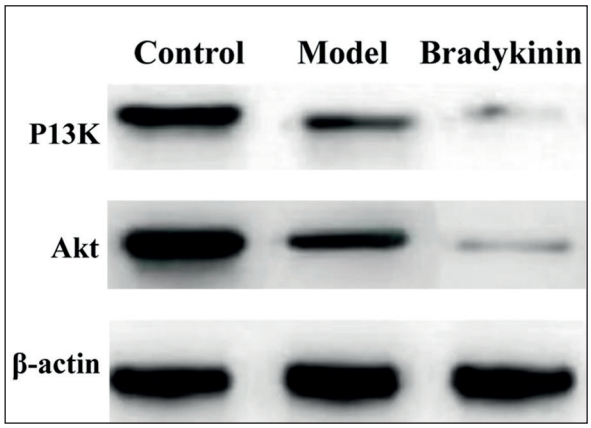

Figure 2. PI3K/Akt protein concentration in lower extremity venous tissues in each group.

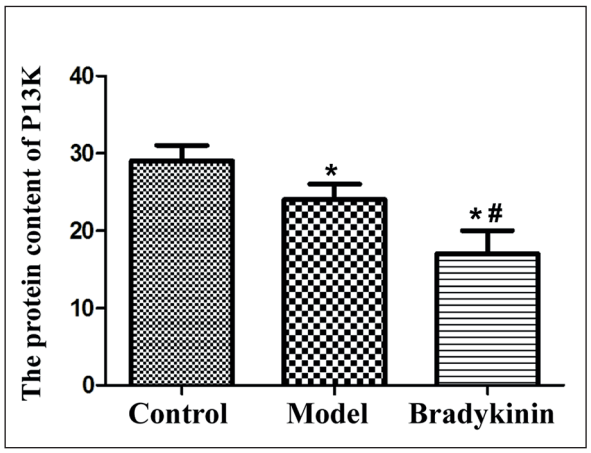

Figure 3. Bar chart of PI3K protein in lower extremity venous tissues in each group. *p<0.05 *vs.* control group, #p<0.05 *vs.* model group.

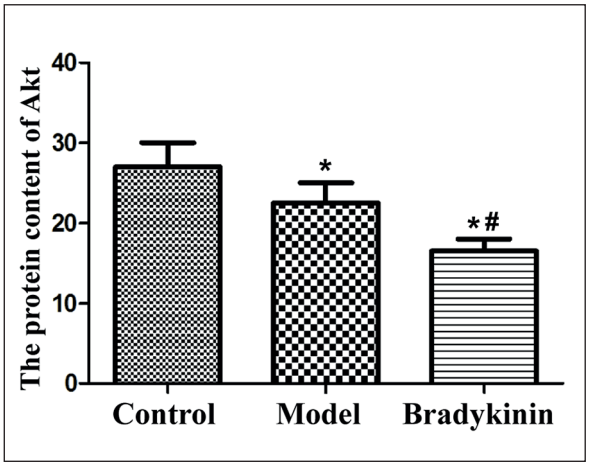

Figure 4. Bar chart of Akt protein in lower extremity venous tissues in each group. *p<0.05 *vs.* control group, #p<0.05 *vs.* model group.

(DOX) was injected into rats in the three groups. Then, the changes in ROS content were detected and observed using the specific fluorescence probe DCFH-DA at 24 h after drug administration. As shown in Figures 5 and 6, at 24 h after administration of DOX, the level of ROS in lower extremity venous tissues was higher in bradykinin group than that in model group (p<0.05), and it was also higher in model group than that in control group (p<0.05).

Caspase-3 Activity in Lower Extremity Venous Tissues

The activity of Caspase-3 in lower extremity venous tissues was significantly increased in bradykinin group compared with that in model group and control group, while it was slightly higher in model group than that in control group (p<0.05) (Figure 7).

Discussion

TAO is an occlusive disease showing the non-atherosclerotic and inflammatory occlusion in the medium-sized and small-sized arteries and veins in four extremities. Mainly manifested as the distal venous and arterial necrosis of the hands and feet, accompanied by ischemic neuritis[13], as well as the occlusive and immuno-inflammatory changes in the vascular wall. TAO is also characterized by the long course and recurrent onset[14]. Therefore, the therapeutic effect in the early stage of TAO is more evident, and the treatment should focus on the prevention and treatment of lower extremity thrombosis, control of ischemic pain, treatment of ischemic ulcer and recovery of blood vessels in affected extremities[15]. The typical clinical symptoms of TAO are swelling and pain in the distal medium-sized and small-sized arteries and veins in the extremities, and serious conditions after activity. Phlebitis can cause irreversible damage to the patients and lead to failure of multiple organs, such as the brain and lung[16]. TAO widely occurs, but mainly in Asia and roughly distributed in the Middle East, Southeast Asia, and the Far East[17]. The majority of TAO patients are young people. Once diagnosed, TAO should be treated in time to improve the ischemic symptoms of limbs, save the limbs and prevent amputation caused by necrosis[18].

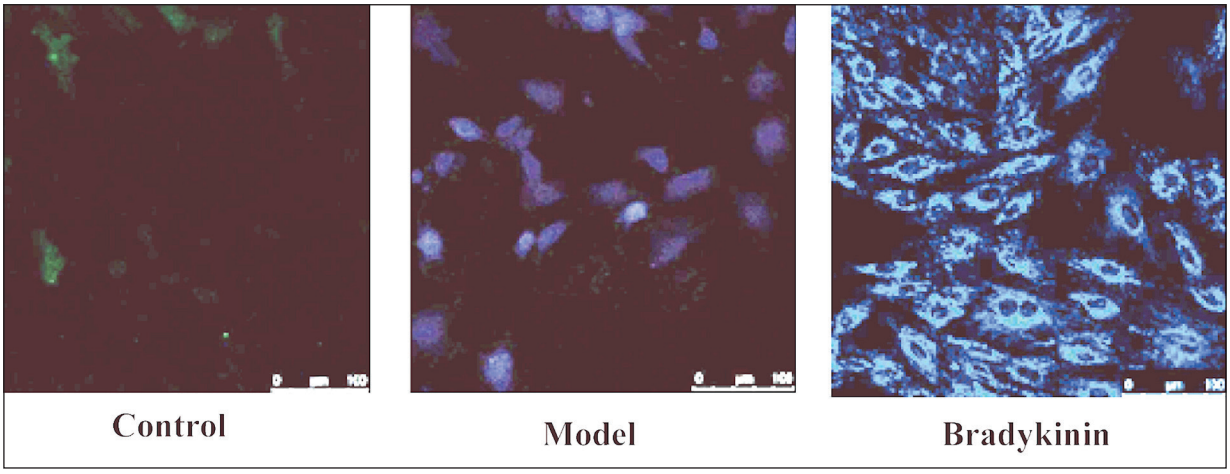

Figure 5. ROS level in lower extremity venous tissues in each group.

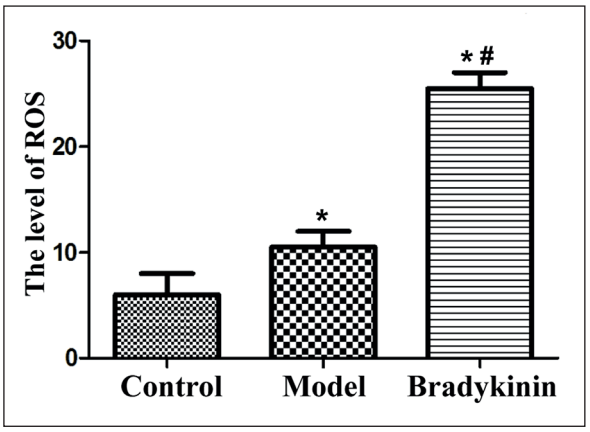

Figure 6. Bar chart of ROS level in lower extremity venous tissues in each group. *p<0.05 *vs.* control group, #p<0.05 *vs.* model group.

In this investigation, the rat model was established. After injection of lauric acid solution into the femoral artery in model group, the hind paw began to turn pale and purple, and the local skin temperature decreased. However, no necrotic muscles were observed and there were ischemic changes. Besides, the pathological sections showed the erythrocyte sedimentation in the arteries and veins. In bradykinin group, most of the hind limbs had fallen off, and some rats had the signs of systemic infection. In control group, there were not many changes in the hind limbs, and the muscle cells of the hind limbs were clear and rosy. According to the pathological sections, the arteries and veins were unobstructed, the intima was smooth, the extima had no evident changes, and there was no inflammatory cell invasion around the arteries and veins. The

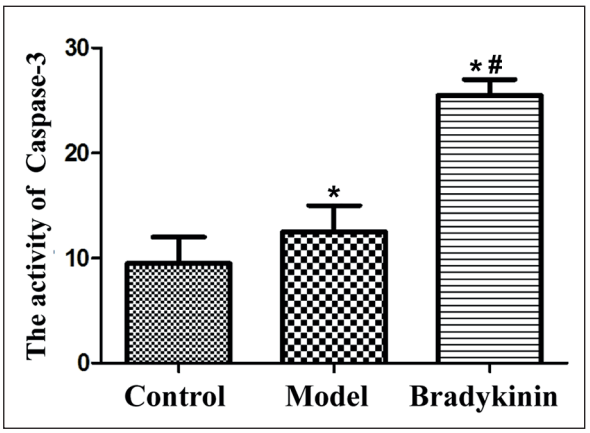

Figure 7. Comparison of Caspase-3 activity in lower extremity venous tissues. *p<0.05 *vs.* control group, #p<0.05 *vs.* model group.

results of Western blotting showed that the PI3K/Akt protein concentration in lower extremity venous tissues was the highest in control group and the lowest in bradykinin group, and there were statistically significant differences (p<0.01). The experimental results revealed that the bradykinin B2 receptor-specific inhibitor could lower the PI3K/Akt protein concentration in lower extremity venous tissues, suggesting that bradykinin can promote the PI3K/Akt protein expression[19,20]. PI3K/Akt plays a regulatory role in TAO, and during the pathogenetic process, InR and Chio are phosphorylated, and PI3K/Akt and p60 enter the cell membrane and are activated[21]. At the same time, the phosphatidylinositol is produced and the phosphatase activity of bradykinin inhibits the kinase activity of PI3K/Akt[22], during which the downstream inflammatory factors are also inhibited, simultaneously. The bradykinin B2 receptor-specific inhibitor is an inhibitor of the PI3K/Akt signaling pathway and inhibiting this signaling pathway further aggravates TAO[23]. In addition, bradykinin is an important regulator of PI3K/Akt signaling pathway, and the increased level of bradykinin can promote the PI3K/Akt signaling pathway and inhibit the tissue apoptosis of TAO, exerting a protective effect in TAO[24].

At 24 h after administration of DOX, the level of ROS in lower extremity venous tissues was higher in bradykinin group than that in model group (p<0.05), and it was also higher in model group than that in control group (p<0.05). Besides, the activity of Caspase-3 in lower extremity venous tissues was significantly increased in bradykinin group compared with that in model group and control group (p<0.05). The above findings indicate that the increased bradykinin can raise the oxygen content in vascular tissues of rats and inhibit the activity of Caspase-3 in cells[25]. Anderson et al[26] studied and found that after bradykinin intervention, PI3K/Akt regulates and reduces the levels of downstream inflammatory factors TNF-α, IL-6, and IL-1β, thereby playing an important role in cardiovascular protection[27].

Conclusions

In summary, the low expression of bradykinin can promote TAO in rats, whose mechanism is to inhibit the PI3K/Akt signaling pathway to raise the oxidative stress level, thereby aggravating TAO.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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