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Original Article

The effect of PPAR-γ agonist pioglitazone promotes adhesion of endothelial progenior cells from rat bone marrow

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Abstract: Selective peroxisome proliferator-activated receptor γ (PPAR-γ) agonist affects the functions of endothelial progenitor cells (EPCs). The aim of this study is to explore the effect of selective PPAR-γ agonist pioglitazone (PIO) on endothelial progenitor cell adhesion. Using density gradient centrifugation and differential method of joint cultivate EPCs, EPCs were identified by double fluorescent staining method. EPCs were cultured and identified by the double dyeing method in a medium containing PIO of different concentrations, respectively for 1 μmol/L, 10 μmol/L, 50 μmol/L, 100 μmol/L, 200 μmol/L, EPC adhesion was detected using flow cytometry. On the 7th day, Endothelial progenitor cells are divided into 5 groups, A: a blank control group only added dimathyl sulfoxide dithezone (DMSO) nutrient solution. B: 50 μmol/L PPAR-γagonist PIO; C: 50 μmol/L PPAR-γ agonist PIO and 10 μmol/L PPAR antagonists GW9662; D: 50 μmol/L PPAR-γ agonist PIO and 50 μmol/L phosphatidylinositol-3-kinase/protein kinase B (PI3K/Akt) channel blocker Wortmannin; E: 50 μmol/LPPAR-γ agonist PIO and 20 μmol/L extracellular signal-regulated kinas (ERK) channel blocker PD98059. When PIO at 50 μmol/L concentration, it can affect EPCs adhesion at the greatest degree. The effect disappeared when there are PI3K/Akt channel blocker Wortmannin and PPAR-γ antagonist GW9662, but the effect didn,t disappeare when there is ERK channel blocker PD98059. PIO can increase EPC adhesion. This effect may be mediated by PI3K/Akt signal pathway.

Keywords: Endothelial progenitor cell, pioglitazone, adhesion, PI3K/AKt

Introduction

Endothelial progenitor cells (EPCs) is a kind of immature cells that originate in the bone marrow into the circulation can significantly regulate the cardiovascular function and angiogenesis, EPCs not only participate in human embryonic vascular formation, but also involve in angiogenesis and vascular repair after birth. EPCs are the new dimensions of the vascular biology and can forecast the human vascular disease prognosis independently. In 1997, Asahara et al [1] successfully isolate EPCs from peripheral blood by immune method of magnetic beads, and confirmed that EPCs could induce neovascularization at the level of the ischemic tissue and animal model. Asahara et al [2] define EPCs as unendothelial cells, because they can show the pluripotency, proliferation and differentiate into endothelial cells. EPCs have broad application prospects in vascular regeneration and treatment of ischemic diseases. In recent years, investigators pay close attention to EPCs. PIO is selective peroxisome proliferator-activated receptor γ agonists, can reduce the blood sugar level in patients with type 2 diabetes. In addition to insulin sensitization effect, more and more evidences demonstrate that these drugs can improve vessel function depending on endothelial cells and inflammatory markers of atherosclerosis. The studies show that PIO can increase EPCs number of healthy individuals, but the specific function and molecular mechanism are not clear. Reports on this research are rare. In this study, we observed the effects of PIO on EPC adhesion.

Materials and methods

Experimental animals

Male SD rats of clean grade 30 aged three to four weeks were bought from Henan Province

Laboratory Animal Center. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Henan Provincial People's Hospital.

Cell separation and EPCs cultivation

SD rats were sacrificed and dislocated in ethanol with a volume fraction of 75% for 15 min. We removed the tibial and femoral, as well as the attached muscles in aseptic conditions. The femur was immersed in Phosphate buffer saline (PBS) to remove the tibia. PBS in a 1 mL syringe was used to flush the bone marrow until the marrow cavity became white in the clean bench. Repeated percussions were performed on the bone marrow until it became a single-cell suspension. The cell suspension was carefully added to an equal volume of rat lymphocyte separation medium of liquid to form a clear interface. In the centrifuge, the tube cells were divided into four layers from top to bottom after cetifugation at 2000 r/min for 20 min. The first layer is plasma and PBS, the second layer is mononuclear cells, the third layer is lymphocyte separation medium of liquid and the last layer is red blood cells. Using a pipette, we added 5 volumes of PBS, thoroughly mixed it, centrifuged at 1600 r/min for 14 min, centrifuged at 1400 r/min for 12 min, centrifuged at 1200 r/min for 10 min, and the cells were washed three times. The mononuclear cells were then collected. Mononuclear cells were adjust to (2.5-5) × 10^9/L with a volume fraction in 10% fetal calf serum (FBS) Endothelial Cell Basal Medium-2 (EBM-2). We took 3 mL of the solution and seeded it in a T25 flask coated with 25 μg of human fibronectin. After 48 h, the medium was changed to remove the adherent cells. The suspension cells re-inoculated in the package were placed another T25 flask containing 25 μg of human fibronectin. Under cultivation at 37°C, the volume fraction was 5% CO_2 and the humidity was saturated. Cell morphology was observed using an inverted microscope (olympus, German) daily. The medium was changed after 72 h. After every 2 day, the medium was changed once.

EPCs activity assay

The cell membranes of dead cells are incomplete, cell membrane permeability increased. Dye will penetrate into the cells of death. However, the living cell membrane is integrity, Dye cannot penetrate into cells. Using this principle, we test the activity of cells with trypan blue staining. After culturing for 7 d, the EPCs were washed with PBS for three times, adding to Pancreatic enzyme-Ethylene Diamine Tetraacetic Acid (EDTA) digestive juices, then become single-cell suspension. Cell suspension 0.2 mL, 0.4% trypan blue 0.5 mL, nutrient solution 0.3 mL, 0.4% trypan blue as colouring agent, count living cells and dead cells using blood cell counting plate, living cell undyed, colorless. Dead cells dyed blue, Repeat 3 times and average. Formula Living cell rate (%) = (the total number of cells-number of dead cells)/ total number of cells × 100%.

EPCs growth curve drawing

After culturing for 7 d, made the cells into cell suspension after digested by pancreatin and put in the fresh medium. We counted the cells by the count plate. And then adjusted the cell number to 1 × 10^8/L, we inoculated them in 96-well culture plate. Added cell suspension 100 μl in each hole, we inoculated 42 holes. An alternate 14 holes, Added 100 μl medium without cells in every hole. At 37°C, volume fraction of 5% CO_2, put them into the incubator to cultivate. Every day at the same time blotted the supernatant, then added 80 μl fresh nutrient solution, 20 μl 0.5% 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution. After 4 h blotted the supernatant, added 150 μl DMSO in every hole, shaking slowly on the swing bed for 10 min. Measure light absorption value of each hole at 490 nm using Thermo Labsystems. zero setting use the light absorption value of hole only added medium as blank control. Take 3 holes on average. We measured for 14 days continuously, changing the liquid every 2 days. We drawn the Growth Curve, incubation time as horizontal axis, light absorption value as vertical axis.

EPCs identification

After culturing for 7 d, the cells were washed twice with PBS, diluted in a complete medium with 1 mL of 10 mg/L DiI-acetylated low-density lipoprotein (DiL-ac-LDL) (Sigma, USA), and then incubated in the incubator for 4 h. The culture medium was then removed, and the cells were washed three times with PBS. The 40 g/L paraformaldehyde solution was fixed 20 min

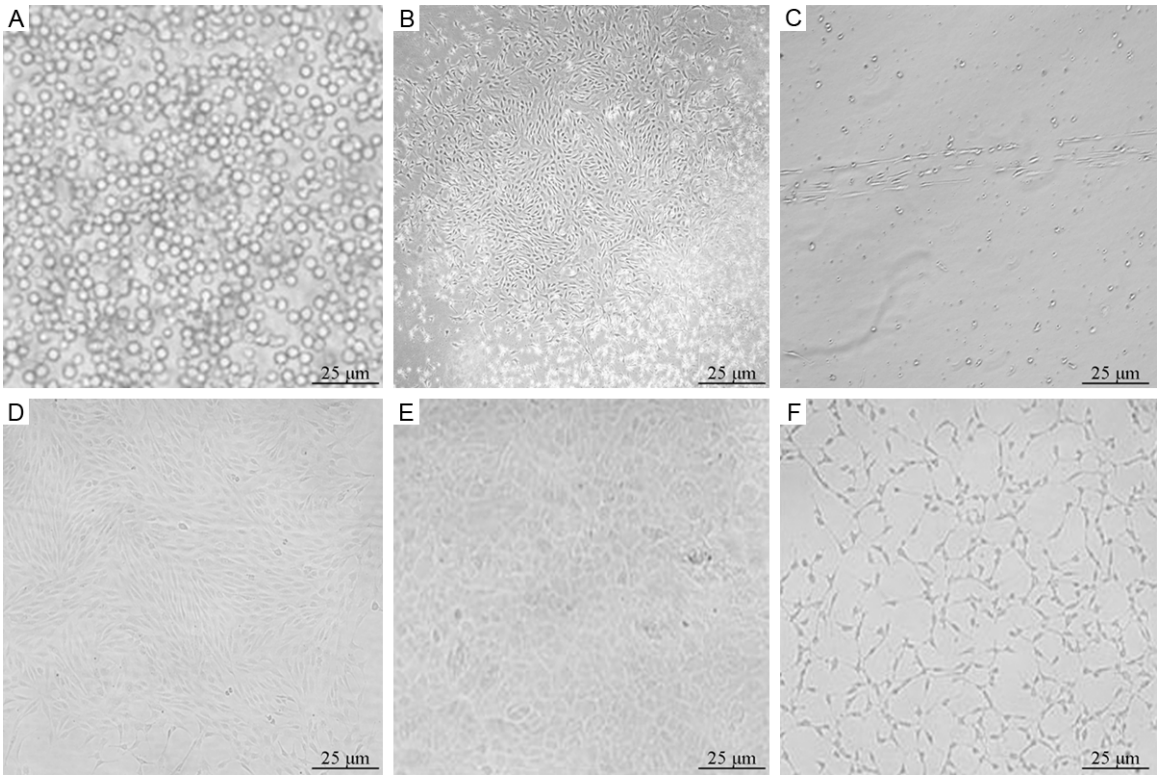

Figure 1. Cells growth at different periods. A. Bone marrow mononuclear cells after primary vaccination were in round and uniform in size (× 200). B. Endothelial progenitor cells after cultured for 7 d, the colony-forming unit could be seen and the cells in the center were round, the cells in the edge were spinal-shaped, and the growth in radial (× 100). C. Endothelial progenitor cells after cultured for 6 d, the cells were growth in linear structure and end-to-end (× 100). D. Endothelial progenitor cells after cultured for 9 d, most of the cells were growth in spindle-shaped (× 200). E. Endothelial progenitor cells after cultured for 14 d, the cells were in cobblestone-like shape (× 100). F. Endothelial progenitor cells after cultured for 16, cells were polygonal and growth in capillary-like (× 100).

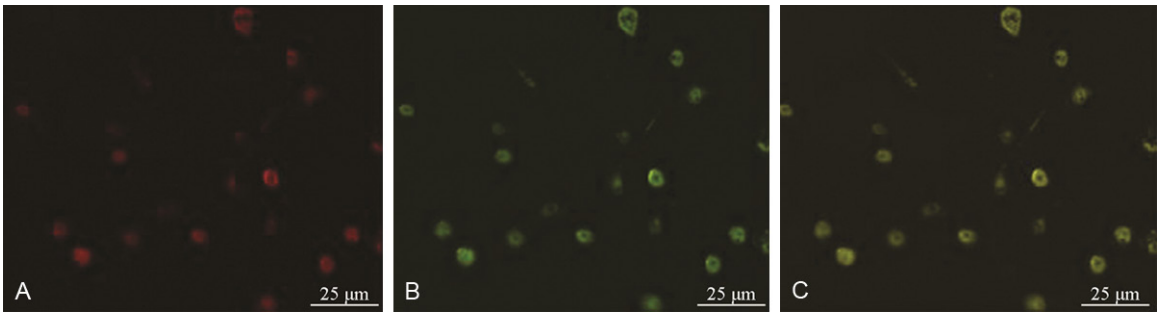

Figure 2. Endothelial progenitor cells after being cultured for 7 days, detected via double fluorescence staining (× 100). A. EPCs that swallowed the DiI labeled acetylated low-density lipoprotein show red (*arrows*). B. EPCs that swallowed the FITC-labeled Ulex europaeus agglutinin 1 show green (arrows). C. Overlay of figure a and b show yellow (*arrows*).

after washing three times with PBS. After the addition of diluted PBS, 1 mL of 10 mg/L Fluorescein-Ulex Europeaus Lectin-1 (FITC-UEA-1) (Sigma, USA) was incubated at room temperature for 1 h. In the absence of DiL-ac-LDL and FITC-UEA-1, the cells were used as the negative control group. After washing with PBS three times, observations were made and pictures were obtained using a laser scanning confocal microscope (olympus, German).

Group of Experiments, observe the effection of different concentration of PIO on EPCs adhesion.

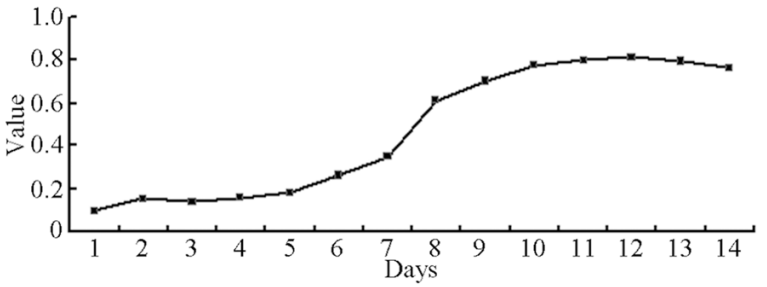

Figure 3. EPCs growth curve determined by MTT method. (n = 9). The growth process included three growth periods: (1) incubation period: at 1-4 days, cells repair the damage caused by separation and passage, and adapt to the new environment. (2) Logarithmic growth: at 5-10 days, Growth accelerated, a logarithmic growth. (3) the stagnation: at 11-14 days, growth plateau, cells no longer proliferate, grown.

Table 1. Different concentration impact on endothelial progenitor cells 24 hours detecting adhesion ($\bar{x}$ ± s, n = 6)

Concentration (μmol/L)	Adhesion EPCS
0	21.5 ± 3.3
1	21.6 ± 3.5
10	31.8 ± 4.7
50	43.1 ± 5.1
100	44.0 ± 5.7
200	46.6 ± 6.1

Table 2. Different concentrations of pioglitazone culturing 24 hours and then compared with different groups

Comparing with different group	*P* value
Blank *vs* 1 μmol/L	0.065
Blank *vs* 10 μmol/L	0.006
Blank *vs* 50 μmol/L	0.000
Blank *vs* 100 μmol/L	0.000
Blank *vs* 200 μmol/L	0.000
10 μmol/L *vs* 50 μmol/L	0.000
50 μmol/L *vs* 100 μmol/L	0.536
50 μmol/L *vs* 200 μmol/L	0.612
100 μmol/L *vs* 200 μmol/L	0.672

PIO powder accurately weighed and dissolved in DMSO, stirred with a magnetic stirrer, and kept at room temperature until completely dissolved. The obtained solution was then used with the medium that was diluted to the desired concentration. After EPCs culturing for 7 d, they were cultured in DMEM without phenol red and serum pass the night, washing three times with PBS, then added 0.25% trypsin, 2-5 min at 37°C. When observed Cells becoming round, refraction sexual enhancement or falling off, add serum to suspend the digestion, the cells were centrifuged for 5 min at 1000 r, washed three times with PBS and count. EPCs were cultured in a medium containing PIO of different concentrations: 0, 1 μmol/L, 10 μmol/L, 50 μmol/L, 100 μmol/L, 200 μmol/L. Also a blank control group was prepared. Cultured again after 7 d, the cells were culture with DMEM which without phenol red and serum overnight. Collected the adherent cells and inoculated 24 hole plate coated with human fibronectin. PIO was added to different final concentration: 0, 1, 10, 50, 100 and 200 μmol/L and the cells were cultured for 24 h at 37°C. The adherent cells were digested with 0.25% trypsin and put into 24 hole plate coated with human fibronectin. After cultured for 30 min at 37°C, the adherent cells were counted. After culturing for 30 min, fixed with 2% paraformaldehyde, Giemsa stain, Randomly selected 3 field of microscope (400 times), counting the adherent cells.

Molecular mechanism

We dissolved GW9662, Wortmannin and PD98059 in DMSO according to the specifications. The above reagents were diluted to the desired concentration before use. After culturing for 7 d, the EPCs were divided into five groups randomly: group A: added the DMSO culture media only; group B: added PPAR-γ ligand PIO with a final concentration of 50 μmol/L; group C: added PPAR-γ ligand PIO with a final concentration of 50 μmol/L and 10 μmol/L of PPAR-γ antagonist GW9662; group D: added PPAR-γ ligand PIO and PI3K/Akt channel blocker Wortmannin with the same final concentration of 50 μmol/L; the last group (group E): added both 50 μmol/L of PPAR-γ ligand PIO and 20 μmol/L of ERK channel blocker PD98059. Experimental method as above, each set of experiments were repeated three times.

Statistical analysis

We used the SPSS17 statistical software, measurement data $\bar{x}$ ± s, and major indicators for

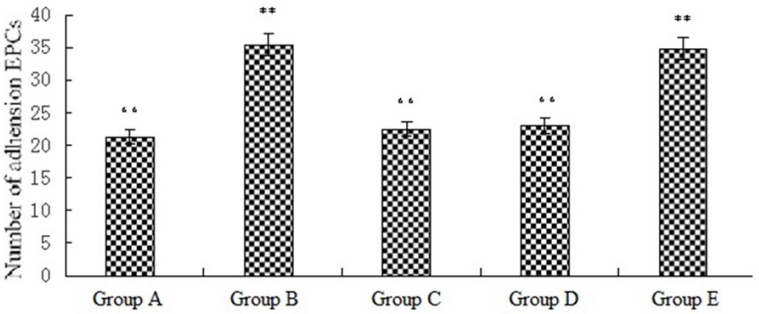

Figure 4. EPCs ability of MS rat bone marrow after joining different channel blockers. Effects of different channel blockers (GW9662, Wortmannin, PD98059) on pioglitazone-interfered adhesion of rat bone marrow endothelial progenitor cells. EPCs cultures were exposed to 50 μmol/ pioglitazone, 10 μmol/L GW9662, 50 μmol/L Wortmannin and 20 μmol/L PD98059. Observe the number of adhesien EPCs. ($\bar{x}$ ± s, n = 6). VS group A, *P < 0.05; **P < 0.01; VS group B, ΔP < 0.05; ΔΔP < 0.01.

Table 3. Comparing of adding in different channel blockers, and then detecting endothelial progenitor cells migration after 24 hours culture ($\bar{x}$ ± s, n = 7)

	A group	B group	C group	D group	E group
50 μmol/l pioglitazone	-	+	+	+	+
10 μmol/l GW9662	-	-	+	-	-
50 μmol/L Wortmannin	-	-	-	+	-
20 μmol/L PD98059	-	-	-	-	+

Comparing with A group, VS A group, *P < 0.05; **P < 0.01; Comparing with B group, VS B group, $^{\Delta}$P < 0.05; $^{\Delta\Delta}$P < 0.01.

normal analysis. The two groups were compared using Analysis of Variance (ANOVA). Two groups of comparisons were made using T-test of homogeneity of variance and heterogeneity of variance for corrective t-tests. The groups were compared using one-way ANOVA. *P* < 0.05 was considered statistically significant.

Results

EPCs morphology

The freshly isolated EPCs were round with varying sizes. The average for each rat was 1 × 10^8 cells. Three days from the outset, two adherent cells in the adherent culture were round tapered, and with pseudopodia extending into the spindle, spindle-shaped, polygonal, and spindle-like. Some cells that were cultured for 2 d exhibited adherent deformation. Cell cell proliferation was not obvious in the first 4 days, from days 5 to 10, rapid proliferation, visible colony, and linear structure formation were observed. At day 10, confluence was up to 80%. Round cells surrounded by spindle-shaped cells were found in the center of the colony center. After 10 days, cell proliferation slowed down. From days 14 to 16, majority of the cells were polygonal, showed microvascular-like growth, and formed into a typical cobblestone. Cell fusion reached 80%, and digestion and passage ratio 1:2 (**Figure 1**).

Fluorescence staining

EPCs engulfed DiL-ac-LDL and FITC-UEA-1. The laser scanning confocal microscope revealed the appearance of yellow fluorescence. The yellow fluorescent cells are differentiated progenitor cells (**Figure 2**).

EPCs vitality

We do trypan blue staining as above method, about 96% of EPCs survived after 7 days.

EPCs growth curve

Results of growth curve measured by MTT demonstrated that cells are colony growth. The growth process can be roughly divided into three periods: 1) incubation period: Cell growth incubation period is usually 1-4 days, cells repair the damage caused by separation and passage, and adapt to the new environment. 2) Logarithmic growth: at 5-10 days, Growth accelerated, a logarithmic growth. 3) the stagnation: at 11-14 days, Growth plateau, cells no longer proliferate, grown stagnate. Cell growth curve showed that EPCs growth and proliferation of in vitro cultured rat bone marrow are well, biological performance is stable (**Figure 3**).

Different concentrations of PIO's influence on the adhesion of EPCs

Compared with the blank group, PIO with a concentration of 1 μmol/L had no significant effect on promoting EPCs adhesion (P > 0.05). After 24 h, the PIO with concerntrations of 10, 50,

100, and 200 μmol/L promoted the adhesion of EPCs cultured for seven days. The promoted effect of 50, 100, and 200 μmol/L PIO on EPC adhesion was not statistically significant. Optimum adhesion promotion was attained at a concentration of 50 μmol/L (**Tables 1** and **2**).

Molecular mechanism

Compared with group A, group C and group D had no effect on EPCs adhedion. Compared with group A, group B and group E had effect on EPCs adhesion. Comparing group B with group E, there was no statistical differences in the EPCs adhesion. Comparing group B with group A, comparing group C with group D, the difference were both significant. These findings indicate that the promotion of PIO on EPC adhesion might be mediated through the PI3K/Akt signalling pathway (**Figure 4**; **Table 3**).

Discussion

PIO is TZDS insulin sensitization agent, the selective PPAR-γ activator, is common drugs in treatment of type 2 diabetes. PPAR-γ is mainly expressed in fat tissue, but also have low level expression in the heart, skeletal muscle, kidney and liver tissues [3]. PPAR-γ plays an important role in glucolipid metabolism and energy adjusting [4], but recent studies have found that TZDs drugs have the anti-inflammatory, immune resistance, protecting vascular endothelium, resistance to atherosclerosis, reduce blood press and other insulin sensitization effect [5, 6]. EPCs in the development of cardiovascular system disease plays an important role, discussing PPAR-γ agonist effects on the biological functions of EPCs, is beneficial to further expound the role of PPAR-γ agonist mechanisms. These provide new theoretical basisfor further research of EPCs in the prevention and treatment of atherosclerosis and ischemic diseases.

In this study, we dispose healthy rats bone marrow EPCs in vitro, find that after impacting with concentrations of 10 μmol/L, 50 μmol/L, 100 μmol/L, 200 μmol/L PIO 24 h, has the promoting effect on cultured EPCs for 7 days.

Phosphatidylinositol 3-kinase/protein kinase B (PI3/Akt) signaling pathway is involved in cell cycle regulation and commencement of cell survival regulation apoptosis. In normal circumstances, phosphatidylinositol 3-kinase activation arising from the lipid product of 3, 4-diphosphate phosphatidylinositol of [PI (3, 4) P2] and 3, 4, 5-triphosphate phosphatidylinositol muscle alcohol [PI (3-5) P3], which acts as a second messenger, binds to and activates a variety of target proteins within the cell, induces the formation of a signaling cascade complex, and ultimately regulates cell proliferation, differentiation, survival, and adhesion [7]. PI3K is activated in two ways. One method is through the activation and phosphorylation of tyrosine residues of growth factor receptors or the connection of protein interaction, which causes dimer conformational change.

The other method is through the direct binding of Ras and p 110 to the cause of P13K activation [8] PI3K activation generates the second messenger of PIP3 at the plasma membrane. PIP3 combines with the intracellular phosphoinositide-dependent protein kinase (phosphoinositide dependent protein kinase-1, PDK-1) and signaling protein Akt, thereby activating Akt. The activation of Akt occurs through the phosphorylation or inhibition of its target proteins Bad, caspase9, NF-κB, GSK-3, p21, Cip1, and p27 Kip7. Cell proliferation is then adjusted, followed by differentiation, apoptosis, adhesion and migration.

The decrease in EPC adhesion rate stops in the presence of PPAR-γ antagonist GW9662 and PI3K/Akt blocker Wortmannin. Adhesion changes in the presence of the ERK channel blocker PD98059. This finding shows that PPAR-γ activators may impact the adhesion of EPCs through the activation of the PI3K/Akt signaling pathway. This effect is independent of the insulin-sensitizing effect. EPCs are mainly distributed in the bone marrow, bone marrow EPCs are closely linked with the surrounding stromal cells, release activity by physical or chemical stimulation. Previous research has shown that a variety of factors such as promote angiogenesis growth factors, vascular endothelial growth factor (VEGF), angiopoietins-1, stroma cell-derived factor-1 (SDF-1), Erythropoietin (EPO), Glucagon peptide-1 (GLP-1) etc [9-12]; Angiotensin enzyme inhibitors (ACEI) [13-16], Angiotensin II receptor antagonist (ARB) [17-24], statins etc [25-29]; estrogen etc [30-33]. can further mobilize bone marrow EPCs, strengthen the EPC proliferation, migration, adhesion and homing to surrounding tissues. Regulatory mechanism of EPCs is com-

plex in vivo and in vitro, PI3K/Akt activation is essential in mediation of EPCs function and number. Research shows that the above factors affect the biological activities of EPCs through PI3K/Akt pathway.

More evidence has confirmed EPCs important function in the cardiovascular system. PIO can promote the adhesion of rat bone marrow EPCs, it may regulate the adhesion through PI3K-Akt signaling pathways. And the adhesion ability is very important for EPCs homing, angiogenesis and other functions. But the detailed mechanism is not clear, we believe that as the research on molecular mechanism of PI3K-Akt pathway going further, PI3K-Akt pathways-target drug is expected to be applied to improve blood vessel function, prevent and treat the cardiovascular disease.

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Disclosure of conflict of interest

None.

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