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Original Article

Adiponectin mediates endothelial progenitor cell proliferation through mTOR-STAT3 signaling pathway

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Abstract: Adiponectin and endothelial progenitor cells (EPCs) were both related to cardiovascular disease. Patients with cardiovascular disease are characterized by hypoadiponectinemia and EPCs number decrease in the circulation. Adiponectin is correlated with EPCs. It was found that adiponectin can promote EPCs proliferation, whereas mTOR/STAT3 signaling pathway is closely associated with cell proliferation, differentiation, and apoptosis. Thus, this study aimed to clarify the role of mTOR/STAT3 signaling pathway in adiponectin promoting EPCs proliferation by culturing EPCs in umbilical cord blood and detecting p-mTOR and p-STAT3 protein level. Ficoll density gradient centrifugation was applied to collect mononuclear cells from human umbilical cord blood. Flow cytometry was performed to identify cell phenotype after adherent culture screening. CCK8 was used to test cell proliferation, and Western blot was used to detect p-mTOR and p-STAT3 protein expression level. To further verify mTOR/STAT3 effect, the cells were divided into control, rapamycin group, adiponectin group, and rapamycin + adiponectin group. The cells proportion with $CD34^+$, $CD133^+$, $CD31^+$, and KDR^+ positive surface markers were 68.12±7.68%, 18.65±4.24%, 5.42±6.43%, and 86.25±7.56%, respectively. Adiponectin may promote EPCs proliferation with time and dose dependent. P-mTOR and p-STAT3 protein level increased following adiponectin concentration. mTOR inhibitor rapamycin can suppress adiponectin induced EPCs proliferation. Rapamycin weakened adiponectin facilitating effect on p-mTOR and p-STAT3 protein expression in EPCs. Adiponectin can regulate EPCs proliferation through mTOR/STAT3 signaling pathway.

Keywords: Adiponectin, mTOR, STAT3, endothelial progenitor cell

Introduction

Endothelial progenitor cells (EPCs) is a kind cells characterized by proliferation, differentiation, and self-renew. They are the precursor of endothelial cells that can differentiate into mature endothelial cells [1]. EPCs were first found by Asahara from bone marrow in 1997 [2]. They are involved in blood vessel formation in the fetal period and neovascularization after birth. EPCs play an important role in cardiovascular disease associated with blood vessel damage occurrence and its prevention. For instance, Schmidt *et al.* discovered that EPCs transplantation could be used for the prevention and treatment of vascular restenosis [3]. EPCs number reduction in the circulation is a risk factor for cardiovascular disease that can increase the incidence [4].

Adiponectin is a newly discovered hormone protein secreted by fat cells with endogenous biological activity [5]. Adiponectin is rich in the circulation that accounts for about 0.01% of human plasma protein. Adiponectin can act through a variety of signaling pathways by binding with its receptor. Researchers suggested that adiponectin has multiple functions such as anti-atherosclerosis, anti-inflammation, anti-diabetes, anti-metabolic syndrome, and regulating glycometabolism [6-10]. Therefore, adiponectin level is closely associated with obesity, insulin resistance, and coronary heart disease [11, 12]. Current, hypoadiponectinemia is an independent risk factor of cardiovascular disease [13]. At the same time, it also can be used as a predictor for cardiovascular disease prognosis [14, 15].

Table 1. Adiponectin effect on EPCs proliferation

Concentration	24 h	48 h	72 h
0 ug/ml	1.00±0.032	1.00±0.033	1.00±0.031
5 ug/ml	1.04±0.068	1.15±0.046*	1.20±0.040*
10 ug/ml	1.13±0.054*	1.34±0.032*	1.37±0.045*
20 ug/ml	1.26±0.057*	1.44±0.043*	1.48±0.042*
50 ug/ml	1.35±0.064*	1.52±0.031*	1.53±0.041*

*P < 0.05, compared with control.

Both of hypoadiponectinemia and EPCs number reduction are related to cardiovascular disease occurrence, while adiponectin level showed close relationship to EPCs number [16, 17]. Adiponectin can reduce cardiovascular disease occurrence by decreasing EPCs apoptosis and making blood vessel repairmen [18]. In addition, it was reported that adiponectin can promote EPCs migration [19] and proliferation [20], but with few mechanism investigation. mTOR/STAT3 signaling pathway is an important pathway cell growth and metabolism that regulates cell proliferation, differentiation, and apoptosis [21, 22]. This study aimed to clarify the role of mTOR-STAT3 signaling pathway in adiponectin promoting EPCs proliferation by cultivation of EPCs in umbilical cord blood and detecting p-mTOR and p-STAT3 protein expression.

Materials and methods

Main instruments and reagents

Umbilical cord blood was collected from healthy puerperal in Yidu Central Hospital Affiliated to Weifang Medical University between June 2014 and April 2015. Lymphocyte separating medium was bought from R&D System (USA). EGM-2MV EPCs medium and fetal bovine serum were bought from Gibco (USA). CCK8 kit was got from Tongren (Japan). FITC labelled $CD34^+$ and $CD31^+$ monoclonal antibodies were from Becton Dickinson. PE labelled $CD133^+$ monoclonal antibody was from MiltenyiBiotec. PE labelled KDR^+ monoclonal antibody was from R&D System. Apoptosis detection kit was bought in Beyotime. Total protein extraction kit was purchased from Shanghai BestBio Biology. Coomassie brilliant blue protein assay kit was bought from Shanghai Majorbio. SDS-polyacrylamide, PBST solution, vertical electrophoresis apparatus, and GIS-2020D gel image analysis system were purchased from Sigma. P-mTOR, p-STAT3 and GAPDH antibodies were from Abcam (USA).

EPCs separation and cultivation

50 ml umbilical cord blood was diluted by equal volume of PBS. After adding to lymphocyte separating medium at 1:1, the mixture was centrifuged at 2000 rpm for 10 min. Then the albuginea layer was transferred to another tube and washed by PBS twice. The cells were seeded in EGM-2MV medium containing 10% FBS at density of 1×10^7 cells and maintained in an incubator at 37°C and 5% CO_2. The medium was changed after 4 days' cultivation. After washing off the suspended cells, the adherent cells were used for the subsequent experiments.

EPCs identification

After cultivated for 7 days, the cells were digested by 0.25% trypsin and stained with FITC-$CD34^+$, PE-$CD133^+$, FITC-$CD31^+$, and PE-KDR^+ antibodies at density of 5×10^5 cells at 4°C in dark for 30 min. After washed by PBS, the cells were identified by flow cytometry.

Cell proliferation assay

The cells were seeded in 96-well plate at density of 1×10^4 cells/well. After 4 hours' adaptive cultivation, the cells were treated with different concentration of adiponectin (0, 5, 10, 20, 50 μg/ml) for different times (24 h, 48 h, 72 h). Each group has five replicate wells. After intervention, the cells were washed by PBS for 3 times and added with 100 μL CCK8 mixture (CCK8 reagent: medium = 1:10) at 37°C for 2 h. Then the plate was read at 450 nm for cell survival rate calculation. Rapamycin was added at 2 h before adiponectin treatment in signal transduction inhibiting assay.

Western blot

Total protein was extracted from cells using cell lysis and quantified by Coomassie brilliant blue protein assay kit. Then the protein was separated by SDS-PAGE electrophoresis and transferred to membrane. The membrane was blocked by PBST containing 5% skim milk powder for 2 h at room temperature. After washed

Table 2. p-mTOR and p-STAT3 protein expression comparison under different concentration of adiponectin treatment

Protein	0 μg/ml	5 μg/ml	10 μg/ml	20 μg/ml	50 μg/ml	*P* value
p-mTOR	0.51±0.08	0.56±0.09*	0.73±0.07*	0.98±0.10*	1.12±0.10*	< 0.001
P-STAT3	0.42±0.07	0.50±0.08*	0.64±0.08*	0.91±0.09*	1.23±0.08*	< 0.001

*P < 0.05, compared with control.

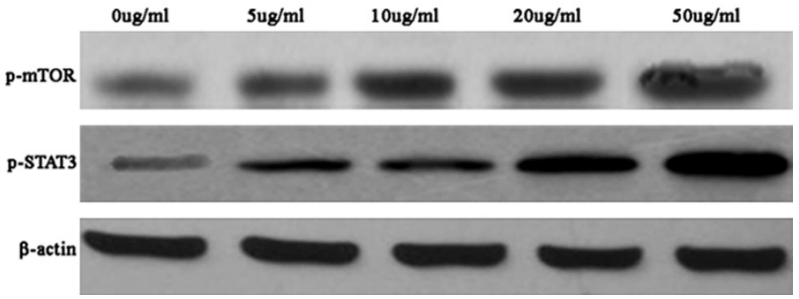

Figure 1. Adiponectin impact on p-mTOR and p-STAT3 protein expression in EPCs.

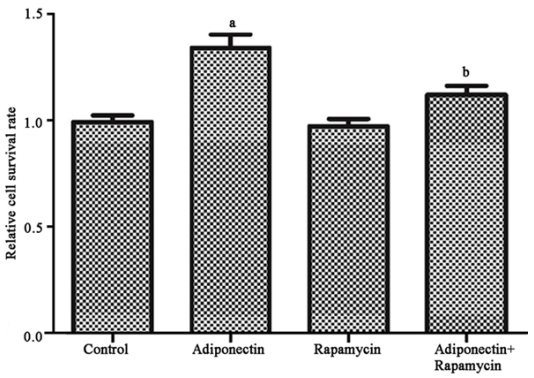

Figure 2. mTOR participated in adiponectin promoting EPCs proliferation. a. P < 0.05, compared with control; b. P < 0.05, compared with adiponectin group.

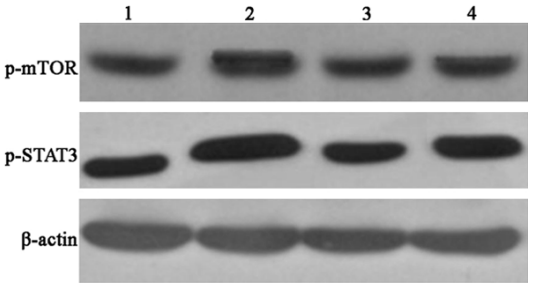

Figure 3. p-mTOR and p-STAT3 protein expression comparison. 1, control; 2, adiponectin group; 3, rapamycin group; 4, rapamycin + adiponectin group.

by PBST for 3 times, the membrane was incubated with primary antibody at 4°C overnight. After that, the membrane was incubated with secondary antibody in PBST containing 2.5% skim milk powder for 60 min. The bind was detected by chemiluminiscence method and analyzed by GIS-2020D gel image analysis system.

Statistical analysis

All statistical analyses were performed using SPSS13.0 software. Results were presented as means and standard deviation. Differences between multiple groups were analyzed by one-way ANOVA or LSD test, inspection level α = 0.05.

Results

EPCs phenotype identification

Flow cytometry was applied to identify the adherent cells after 7 days' cultivation. The cells proportion with $CD34^+$, $CD133^+$, $CD31^+$, and KDR^+ positive surface markers were 68.12±7.68%, 18.65±4.24%, 5.42±6.43%, and 86.25±7.56%, respectively.

Adiponectin impact on EPCs proliferation

Different concentration and time of adiponectin was used to treat EPCs, and CCK8 assay was performed to confirm its role on EPCs proliferation (**Table 1**). The results showed that adiponectin promotes EPCs proliferation with time and dose dependent. Of which 50 μg/ml adiponectin acts for 48 h and 72 h presented most significant effect on cell proliferation.

Adiponectin impact on p-mTOR and p-STAT3 protein expression in EPCs

Based on cell proliferation assay results, different concentration of adiponection (0, 5, 10, 20, 50 μg/ml) was used to treat the cells for 48 h. As shown in **Table 2** and **Figure 1**, compared with control group, p-mTOR and p-STAT3 pro-

Table 3. p-mTOR and p-STAT3 protein expression intensity comparison

Protein	Control	Adiponectin	Rapamycin	Adiponectin + rapamycin
p-mTOR	0.52±0.06	0.86±0.07[a]	0.54±0.07	0.58±0.06[b]
P-STAT3	0.48±0.07	1.13±0.06[a]	0.49±0.08	0.60±0.09[b]

a, $P < 0.05$, compared with control; b, $P < 0.05$, compared with adiponectin group.

tein level increased obviously in adiponectin group with concentration dependent.

mTOR inhibitor impact on adiponectin promoting EPCs proliferation

To observe mTOR inhibitor regulating effect on adiponectin promoting EPCs proliferation, the cells were divided into control group, 50 μg/ml adiponectin 24 h group, 1 ng/ml rapamycin group, and rapamycin + adiponectin group. As shown in **Figure 2**, rapamycin showed no significant impact on EPCs proliferation, while adiponectin promoted EPCs proliferation. mTOR inhibitor rapamycin treatment suppressed adiponectin promoting effect on EPCs proliferation, suggesting that mTOR participated in adiponectin promoting EPCs proliferation.

mTOR inhibitor impact on adiponectin induced p-mTOR/p-STAT3 protein expression

Western blot was applied to test different treatment effect on p-mTOR and p-STAT3 protein expression (**Figure 3** and **Table 3**). Rapamycin did not have obvious impact on p-mTOR and p-STAT3 protein expression in EPCs ($P > 0.05$). p-mTOR and p-STAT3 protein expression increased markedly after adiponectin treatment for 24 h ($t = 6.39$, $P = 0.001$; $t = 12.21$, $P < 0.001$), whereas their levels declined obviously after rapamycin treatment ($t = 5.26$, $P = 0.003$; $t = 8.49$, $P < 0.001$). It revealed that mTOR participated in adiponectin promoting EPCs proliferation.

Discussion

mTOR) is a kind of highly conservative serine/threonine protein kinase belonging to phosphatidyl inositol kinase related protein kinase family. mTOR activation can induce downstream signaling molecules activity and expression elevation, leading to cell growth, differentiation, proliferation and inhibiting apoptosis [23]. STAT3 is easy to be activated by phosphorylated mTOR, resulting in mTOR/STAT3 signaling pathway formation. mTOR/STAT3 signaling pathway is closely related to cell growth metabolism [24]. For example, ghrelin can suppress Th17 cells differentiation through the mTOR/STAT3 signaling pathway [25], glycyrrhizic acid can inhibit leukemia cell proliferation by blocking AKT/mTOR/STAT3 signaling pathway [26]. Studies found that adiponectin can promote EPCs proliferation [20], but the specific mechanism is still unclear. Therefore, this research intended to explore the role of mTOR/STAT3 signaling pathway in adiponectin promoting EPCs proliferation. In addition, for rapamycin can completely block mTOR activation easily, we adopted rapamycin to verify the role of mTOR/STAT3 signaling pathway in adiponectin promoting EPCs proliferation.

Our results showed that adiponectin can significantly promote EPCs proliferation with obvious time and dose dependent. These results were consistent with R. Shibata's report [20], and this study further confirmed the effect of adiponectin on promoting EPCs proliferation.

In order to further understand the mechanism of adiponectin on promoting EPCs proliferation, we explored mTOR/STAT3 signaling pathway. By using different concentration of adiponectin on EPCs for 48 h, we found that p-mTOR and p-STAT3 protein expression level increased significantly compared with control, prompting that mTOR/STAT3 signaling pathway may be involved in adiponectin promoting EPCs proliferation. We further adopted mTOR inhibitor rapamycin to block mTOR/STAT3 signaling pathway to observe its effect and found that EPCs proliferation was obviously suppressed in adiponectin + rapamycin group compared with adiponectin group. Meanwhile, p-mTOR and p-STAT3 protein expression also significantly reduced, further confirming that mTOR/STAT3 signaling pathway participated in adiponectin promoting EPCs proliferation.

This investigation found that adiponectin can regulate EPCs proliferation through mTOR/STAT3 signaling pathway, providing basis for the related mechanism research. Following the deepening of the research, a growing number of signaling pathways were found. Since the

mechanism of signaling pathway is extremely complex, the upstream and downstream molecules of mTOR/STAT3 signaling pathway needs further discussion.

Disclosure of conflict of interest

None.

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