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ORIGINAL ARTICLE

Hyperinsulinemia impairs functions of circulating endothelial progenitor cells

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Abstract

Aims Circulating endothelial progenitor cells (EPCs) play a key role in maintaining endothelial function. Dysfunction of EPCs is associated with the cardiovascular complication of diabetes. The purpose of this study is to investigate the direct effects of hyperinsulinemia on EPCs and the underlying mechanisms.

Methods EPCs isolated from healthy adults were cultured with various concentrations of insulin (control group, without insulin; physiological insulin group, 10 nM insulin and hyperinsulinemia group, 100 nM insulin) with or without phosphatidylinositol-3-kinase (PI3-K) inhibitor (LY294002, 5 μM), endothelial nitric oxide synthase (eNOS) inhibitor (L-NG-nitro-arginine methyl ester (L-NAME), 100 μM), sodium nitroprusside (SNP, 25 μM), p38 mitogen-activated protein kinase(MAPK) inhibitor (SB203580, 5 μM) or extracellular signal-regulated kinases (ERK) 1/2 inhibitor (PD98059, 10 μM). Proliferation, tube formation, and apoptosis of EPCs were determined. Expressions of eNOS, PI3-K, protein kinase B (Akt), p38 MAPK, and ERK 1/2 were assessed.

Results Hyperinsulinemia caused a significant decrease in proliferation and tube formation abilities than control group. Hyperinsulinemia increased apoptosis rate of EPCs than control group. Furthermore, hyperinsulinemia downregulated phosphorylation of eNOS, PI3-K and Akt, and upregulated phosphorylation of p38 MAPK and ERK. SNP could restore impaired tube formation induced by hyperinsulinemia. P38 MAPK inhibitor but not ERK inhibitor could decrease apoptosis induced by hyperinsulinemia.

Conclusion Hyperinsulinemia impaired EPCs' tube formation ability by downregulation of PI-3K/Akt/eNOS pathway. Hyperinsulinemia induced apoptosis of EPCs via upregulation of p38 MAPK.

Keywords Endothelial progenitor cell · Diabetes mellitus · Insulin · Nitric oxide

Introduction

Circulating endothelial progenitor cells (EPCs), which are precursors of endothelial cells, participate in vasculogenesis and contribute to endothelium repair by replacement of damaged endothelial cells [1]. Accumulating evidences testify that type 1 and type 2 diabetes mellitus impair functions of EPCs, such as proliferation, adhesion, migration, tube formation and secretion of angiogenic cytokines [2–4]. Vascular complications are the leading cause of morbidity and mortality in patients with diabetes [3]. Although the pathogenesis of vascular complications is multifactorial, EPC dysfunction plays a key role in it. Different from type 1 diabetes, type 2 diabetes is a constellation of disorders that includes hyperglycemia, insulin resistance and hyperinsulinemia [5]. Previous studies [5–8] demonstrated that insulin has a "double-phase" effect on atherogenesis. At physiological concentration, insulin has a vascular protective effect. But at hyper-physiological concentration, insulin stimulates proliferation of vascular smooth muscle cells and triggers inflammation. Although hyperinsulinemia has been shown to be a risk factor for the development of atherosclerotic

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cardiovascular disease, there is no study to explore whether hyperinsulinemia impairs functions of EPCs.

We presume that hyperinsulinemia may impair functions of EPC. To testify this hypothesis, 100 nM insulin was added to cell culture medium to simulate hyperinsulinemia in vitro [9]. Then we examined the direct effects of hyperinsulinemia on proliferation, tube formation and apoptosis of EPCs. And, more importantly, we tried to investigate the underlying mechanisms of EPC dysfunction induced by hyperinsulinemia.

Methods

Cell culture and EPC identification

EPCs were isolated from blood donated with written consent by healthy volunteers (average age 46.42 years, range 32–56 years; seven men, eight women). The study protocol was approved by the ethics committee of Qinhuangdao first hospital. Mononuclear cells (MNCs) were isolated from peripheral blood and cultured as previously described [10]. In briefly, 10^6 cells/cm^2 PB-MNCs were cultured on fibronectin-coated six well chamber in MCBD/F12 medium with supplements (10% FBS, VEGF10ng/ml, bFGF 10 ng/ml, IGF 10 ng/ml, EGF 10 ng/ml, heparin 10 U/ml, and antibiotics) (Gibco) at 37 °C in a 5% CO_2 incubator. After changing medium on day 2, medium was replaced every 3 days. Colonies of EPC emerged 5–7 days after start of MNC culture.

Flow cytometric analysis of EPC was performed as previously described [10]. Cells cultured for 10 days were collected and incubated with the following antibodies: CD14-FITC (Coulter), CD34-PE (Coulter), CD45-FITC (Coulter), CD105-PE (Laboratories), CD133-PE (Milltenyi), VEGFR2-PE (R&D Systems), vWF-FITC (SEROTEC), IgG1-FITC isotype controls (Pharmingen), IgG1-PE isotype controls (Pharmingen). Then, cells were analyzed by fluorescence-activated cell sorting (FACS) using a FACS Calibur flow cytometer and Cell Quest software (BD Biosciences).

EPCs were characterized by uptake of a 1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine-labeled low-density lipoprotein (Dil-acLDL; Molecular Probes) and by binding to FITC-labeled ulex europaeus agglutinin-1 (UEA-1, Sigma) as previously described [10].

Passage 1 EPCs were detached by trypsin and cultured in serum-free medium (SFM) for 24 h. Then cells were randomly exposed to one of the following treatments for 24 h: control group (0 nM insulin), physiological insulin group (10 nM insulin), hyperinsulinemia group (100 nM insulin). Some experiments were performed by pre-treating the cells with phosphatidylinositol-3-kinase (PI3-K) inhibitor (LY294002, 5 μM), nitric oxide (NO) synthase inhibitor (L-NG-nitro-arginine methyl ester (L-NAME), 100 μM), NO subtrate (sodium nitroprusside (SNP), 25 μM), p38 mitogen-activated protein kinase (MAPK) inhibitor (SB203580, 5 μM) and extracellular signal-regulated kinase 1/2 (ERK1/2) inhibitor (PD98059, 10 μM) [11, 12]. Inhibitors were added to medium for 1 h before insulin stimulation.

EPC proliferation

Proliferative activity was assayed using a colorimetric MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenol)-2-(4-sulfophenyl)-2H-tetrazolium] assay (Promega, Madison, WI, U.S). EPCs were re-seeded with a 96-well plate (10,000 cells/well) in 100 μl of SFM with different concentrations of insulin. After 24 h, MTS solution was added to each well for 4 h, whereupon light absorbance at 492 nm was detected using an ELISA plate reader (Beckman Coulter).

Tube formation

The tube formation assay was performed to assess the angiogenic potential of EPCs in vitro [13]. After 24-h exposure to insulin, EPCs were treated with 0.25% trypsin–EDTA, and then collected and resuspended in MCBD/DF12 medium with 2% FBS. These cells were seeded (50,000 cells/well) in a 24-well tissue culture plate which had been evenly coated with Matrigel (BD Labware). Seeded cells were incubated at 37 °C in a 5% CO2 incubator for 4 h. Gels were examined using phase-contrast microscopy (leica), and Angiogenesis Analyzer Image J plugin was used to determine the total length of tube-like segment, the total area of tubular structure and number of network junctions in five randomly selected fields.

Apoptosis detection

Harvested EPCs were seeded in a 24-well culture plate and cultured in SFM for 24 h. Then the cells were randomly exposed to different concentration of insulin for 24 h. A DeadEnd™ Colorimetric TUNEL System Detection Kit (Promega, Madison, WI, U.S.) was used for TUNEL detection according to the manufacturer's instructions. The TUNEL-positive cells were counted in five random selected fields under microscope.

Western blot analysis

Total protein of EPCs was extracted with radioimmunoprecipitation assay lysis buffer (Beyotime Biotech, Shanghai, China). Protein concentrations were determined by BCA assay kit (Beyotime Biotech). Equal protein samples (40 μg) were loaded into each well of Pierce Precise Protein gel (Thermo Fisher, Waltham, MA) and were run in 1 × Tris/

HEPES/SDS running buffer at 100 V for 1 h. Proteins were then transferred to polyvinylidene difluoride membranes and blocked with 5% BSA for 2 h at 25 °C. Membranes were then incubated with primary antibodies at 4 °C overnight (1:1000 in 1% BSA/TBS-T) and with secondary antibodies (1:2000 in 1% BSA/TBS-T) at room temperature for 2 h. Membranes were washed two times with TBS-T for 10 min before incubations and once after incubations. The bound complex was detected using the Odyssey Infrared Imaging System (Li-Cor; Lincoln, NE). The images were analyzed using Image Studio Lite version 5.2 (LI-COR), to obtain the integrated intensities. Primary antibodies including anti-phospho-Akt-Ser^{473} (1:1000), anti-Akt (1:1000), anti-phospho-eNOS-Ser^{1177}(1:1000), anti-eNOS (1:1000), anti-ERK1/2 (1:1000); anti-phospho-ERK1/2 (Thr-202/Tyr-204) (1:1000); anti-β-actin(1:5000), anti-PI3-K (1:1000), anti-phospho-PI3-K (1:1000) and horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5000;) were purchased from Cell Signaling Technology (Beverly, MA, USA).

Statistical analysis

SPSS 11.5 statistical analysis software was used to analyze the datasets. All data are presented as means ± SD. Statistical comparisons between several groups were performed using the one-way ANOVA test. A probability value ≤ 0.05 was considered to indicate statistical significance.

Results

Characterization of EPCs

Colonies of EPC emerged 5–7 days after start of MNC culture. The EPCs exhibited "cobblestone" morphology and monolayer growth pattern (see Fig. 1). Laser confocal immunofluorescence displayed that the cells could incorporate DiI-labeled acetylated LDL and bind UEA-1(Fig. 2). FACS analysis revealed that EPCs expressed CD34(14.75 ± 2.88%), CD105(92.31 ± 1.32%), CD106(62.16 ± 11.11%), CD133(9.88 ± 1.48%), VEGFR2(92.63 ± 3.61%), and vWF(52.57 ± 8.62%), but lacked the expression of CD14(0.64 ± 0.45%), CD45(1.79 ± 1.02%), CD54(0.81 ± 0.99%). These results demonstrated that these cells exhibit functional endothelial features. These cells could be defined as EPCs.

Hyperinsulinemia decreased proliferation capacity of EPCs

As shown in Fig. 3, 100 nM insulin significantly decreased EPC' proliferation capacity compared with control group. There were no significant differences of proliferation capacity between control and 10 nM insulin group. Pre-treated with SNP could not restore proliferation capacity impaired by hyperinsulinemia, but pre-treated with SB203580 ameliorated impaired proliferation of EPCs. LY294002 (Inhibitor of PI3-K) or L-NAME had no influence on proliferation of EPCs.

Hyperinsulinemia impaired tube formation ability of EPCs

After 4-h culture, EPCs formed tubular networks (Fig. 4) on matrigel. Using Angiogenesis Analyzer Image J plugin, total length of tubular network, total area of tubular network and number of junctions were measured.

EPCs treated with 10 nM insulin demonstrated increased network formation, compared with control group. However, networks formed by EPCs treated with 100 nM insulin

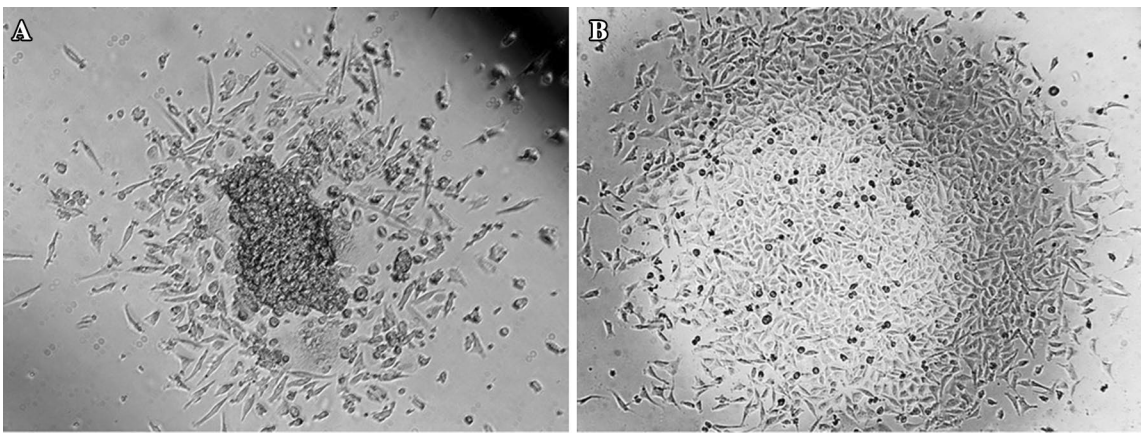


Fig. 1 Colony of cultured EPCs. **a** Six days after culture, colony of EPCs appeared (100× magnification). **b** Fifteen days after culture, cobblestone-like EPCs were grown to confluence (50× magnification)

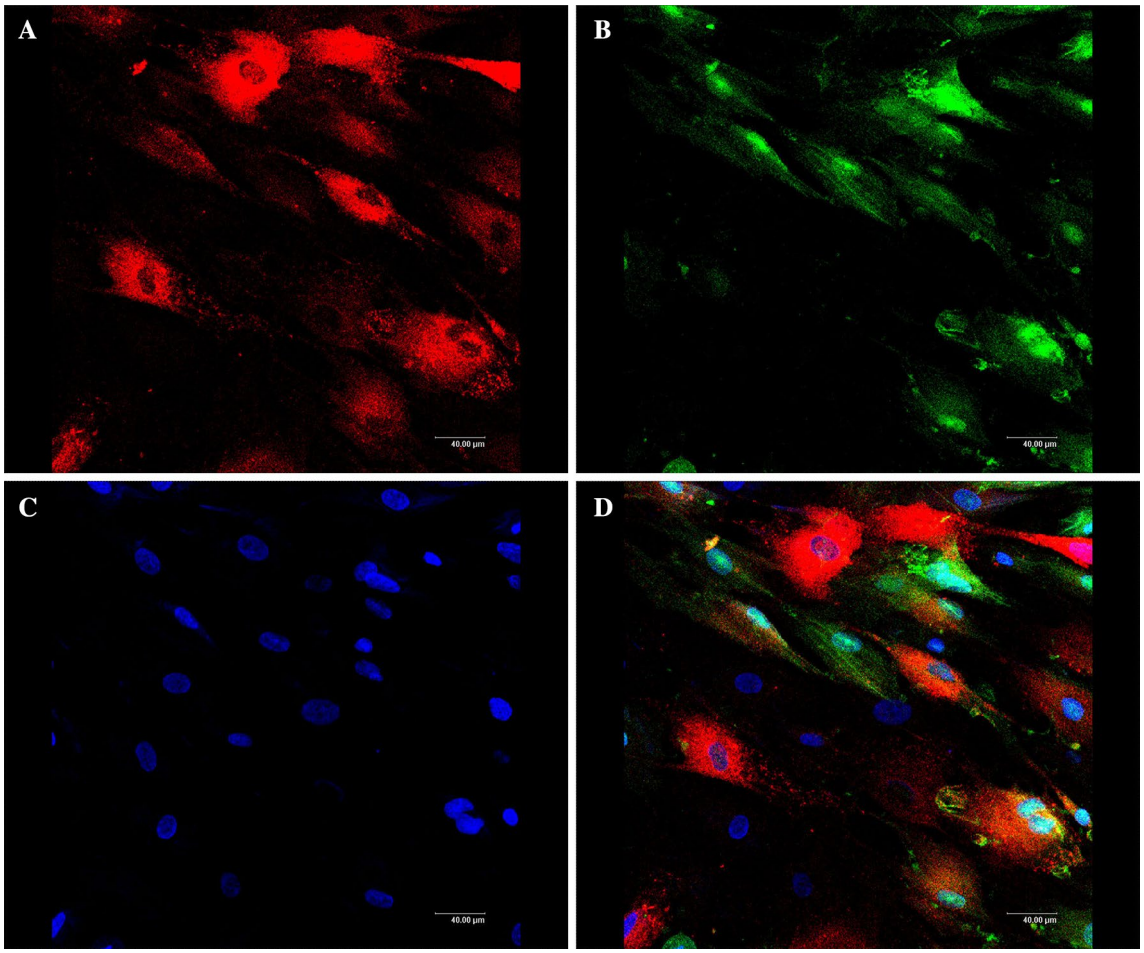


Fig. 2 Identification of cultured EPC by laser confocal microscope. Most cells were shown to simultaneously endocytose DiI-acLDL and bind fluorescein isothiocyanate UEA-1. **a** Red fluorescence showed cells could endocytose Dil-ac- LDL; **b** green fluorescence showed cells could bind UEA-1; **c** blue fluorescence represented cell nucleus; **d** emerged image showed EPCs could simultaneously endocytose Dil-ac-LDL and bind UEA-1 (laser confocal microscope, 400× magnification bar = 40 μm)

displayed lower numbers of junctions, total length and total area compared with control group. This effect could be restored by SNP. LY294002 and L-NAME both decreased tube formation ability of EPCs.

Pre-treating with inhibitor of p38 MAPK or inhibitor of ERK could not restore tube formation ability of EPCs cultured with 100 nM insulin.

Hyperinsulinemia decreased eNOS phosphorylation via downregulation of PI3-K/Akt pathway

As shown in Fig. 5, although 10 nM insulin increased eNOS phosphorylation, 100 nM insulin decreased eNOS phosphorylation of EPCs.

The potential roles of PI3-K/Akt-related mechanisms were also examined. PI3-K inhibitor (LY294002) and eNOS inhibitor (L-NAME) depressed eNOS phosphorylation. However, SNP could restore eNOS phosphorylation of EPCs.

Hyperinsulinemia (100 nM insulin) depressed PI3-K and Akt phosphorylation (Fig. 6). But 10 nM insulin could increase PI3-K and Akt phosphorylation. These results indicated that hyperinsulinemia decreased eNOS phosphorylation via downregulation of PI3-K/Akt pathway.

Hyperinisulinemia induced apoptosis of EPCs via upregulation of p38 MAPK

There were no differences of apoptosis ratios of EPCs between control group and 10 nM insulin group. But 100 nM insulin group had a significant higher rate of apoptosis compared with control group (Fig. 7). The results showed that apoptosis of EPCs induced by 100 nM insulin could be abrogated by pretreatment with p38 MAPK inhibitor, but not by PD98059, an inhibitor of ERK1/2. Results of western blot

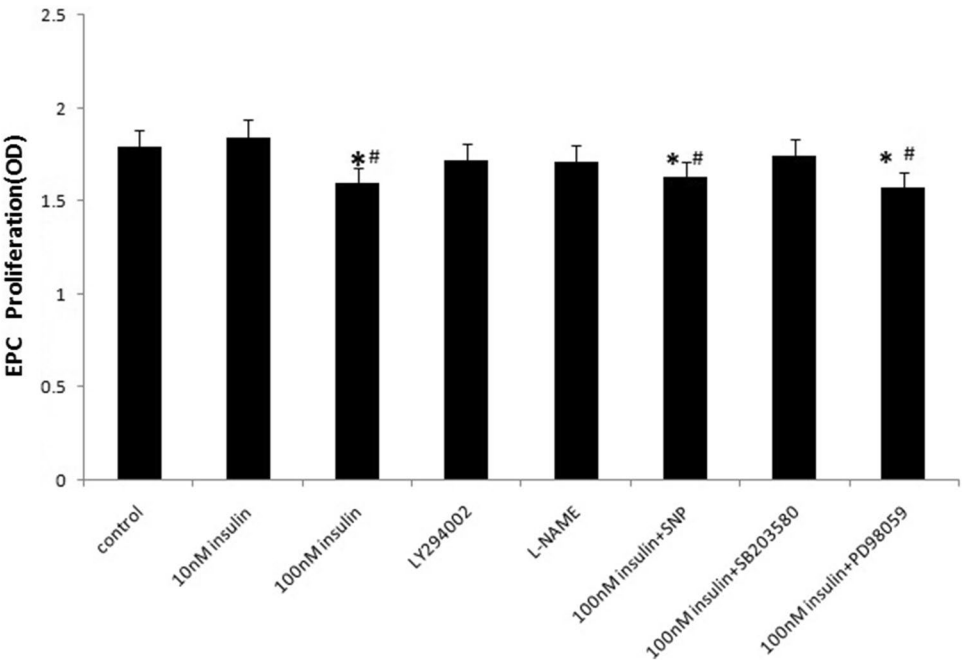


Fig. 3 EPC proliferation. Hyperinsulinemia decreased proliferation of EPCs; p38 MAPK inhibitor (SB203580) could restore the proliferation ability of EPCs impaired by hyperinsulinemia. Cells were incubated with 0 nM insulin, 10 nM insulin, 100 nM insulin, PI3-K inhibitor (LY294002, 5 μM, without insulin), eNOS inhibitor (L-NAME, 100 μM, without insulin), NO subtrate (SNP, 25 μM, plus 100 nM insulin), p38 MAPK inhibitor (SB203580, 5 μM, plus 100 nM insulin) or ERK 1/2 inhibitor (PD98059, 10 μM, plus 100 nM insulin). $N=6$, $^{*}p<0.05$ compared with control; $^{\#}p<0.05$ compared with 100 nM insulin + SNP

displayed that 100 nM insulin increased phosphorylation of p38 MAPK and ERK1/2 (Fig. 8). These results suggested that hyperinsulinemia mediated apoptosis of EPCs via the activation of p38 MAPK.

Discussion

The present study demonstrated that hyperinsulinemia impaired tube formation ability of EPCs via depressing PI3-K/Akt/eNOS pathway. Hyperinsulinemia also induced apoptosis of EPCs via activating p38 MAPK pathway.

Insulin plays an important role in maintaining vascular homeostasis. It not only stimulates NO production of endothelial cells [14], but also mediates endothelin-1 production, which is a strong vasoconstrictor [15]. The dual action of insulin is mediated by PI3-K and MAPK pathways. PI3-K/Akt pathway predominates under physiological conditions and is responsible for activation of eNOS [16]. MAPK pathway is activated under insulin resistance and mediates inflammation, vasoconstriction, and vascular smooth muscle cell proliferation [17]. Previous study [16] demonstrated that insulin resistance and accompanied hyperinsulinemia impaired endothelial function. EPCs contribute to endothelial regeneration and new vessel formation [18]. As a result, EPCs are very critical in maintaining endothelial function. However, it is not clear how hyperinsulinemia disturbed balance of PI3-K/Akt pathway and MAPK pathway, and then impaired function of EPCs.

In this current study, culture of EPCs from healthy individuals in hyperinsulinemia medium led to decrease of eNOS phosphorylation, which was associated with the impaired tube formation ability. This effect could be blocked by SNP (NO subtrate), suggesting that suppression of EPC angiogenesis by hyperinsulinemia is mediated by the decrease of NO production. As we know, NO is a key regulator in modulating endothelial function [19]. It not only promotes vasorelaxation but also regulates angiogenesis in response to tissue ischemia. Our results indicated that supplementing SNP increased eNOS phosphorylation and restored impaired tubular network induced by hyperinsulinemia. In line with previous studies [20, 21], we found that physiological concentration of insulin (10 nM) could activate eNOS via phosphorylation at Ser1176 by activation of PI3-K/Akt. We also found that hyperinsulinemia depressed eNOS phosphorylation via depressing PI3-K/Akt. Inhibitor of PI3-K (LY294009) and inhibitor of eNOS (L-NAME) decreased tube length, tube area, number of junctions of EPCs. These findings indicated that PI3-K/Akt/eNOS pathway plays a vital role in regulating angiogenesis of EPCs.

p38 MAPK and ERK1/2 belong to MAPK family. Previous studies testified that p38 MAPK and ERK1/2 are activated in muscle of humans with type 2 diabetes [22, 23]. The p38 MAPK modulates glucose metabolism in skeletal muscle and adipose tissue [24]. The activation of ERK1/2 decreased the responsiveness of insulin gene promoter by elevated glucose levels and induce insulin resistance [25]. Insulin has been identified as an important molecule for regulating p38 MAPK and ERK1/2, but the molecular mechanism has not been fully characterized [26]. In the current study, we detected that hyperinsulinemia activated phosphorylation of p38 MAPK and ERK1/2. However, p38 MAPK

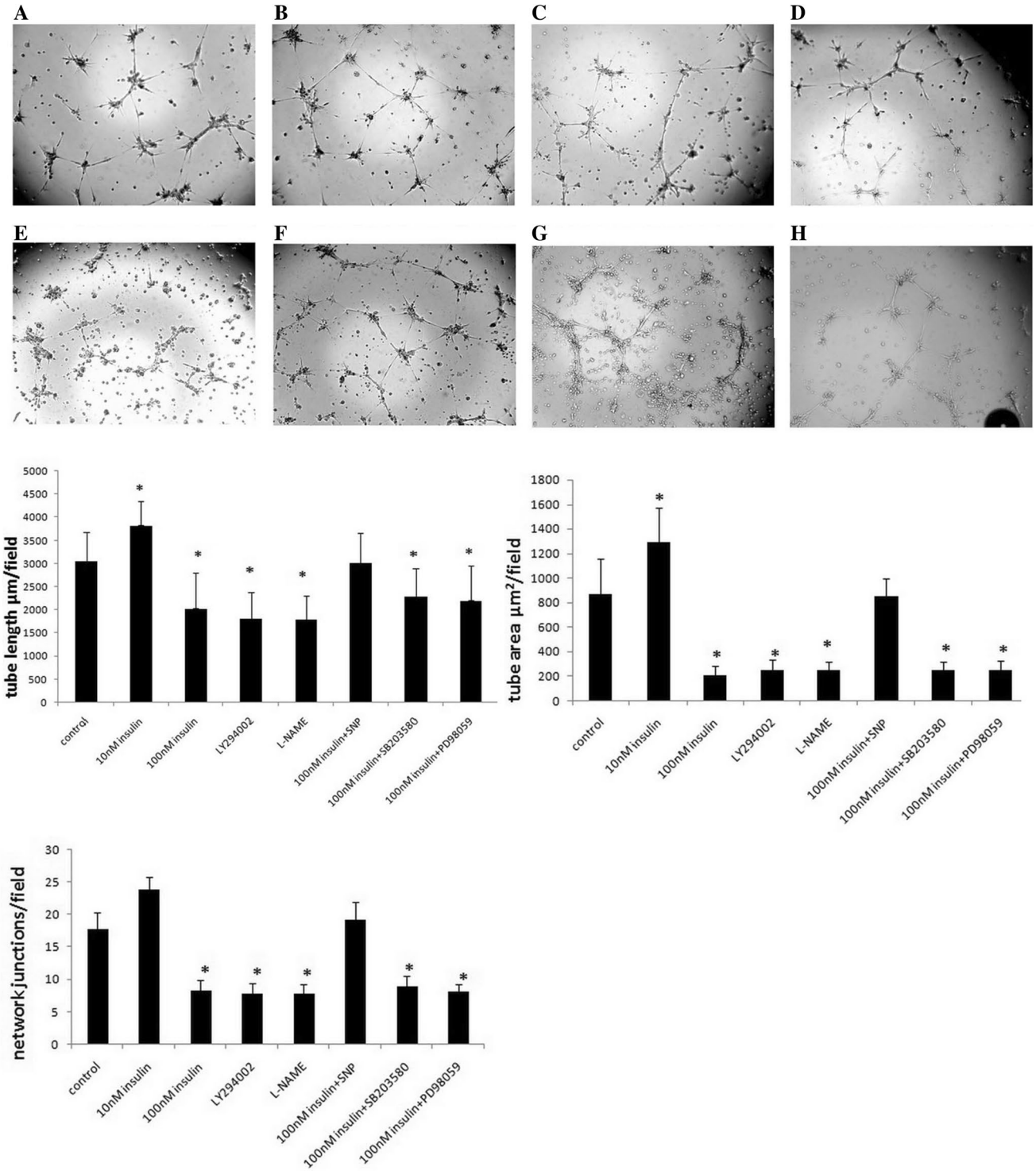


Fig. 4 Tube formation ability of EPCs. Cells were treated with insulin or inhibitors as following. **a** control (without insulin); **b** 10 nM insulin; **c** 100 nM insulin; **d** LY294002; **e** L-NAME; **f** 100 nM insulin + SNP; **g** 100 nM insulin+SB203580; **h** 100 nM insulin + PD98059. Results showed 10 nM insulin could increase tube length, tube area and network junctions of EPCs; 100 nM insulin impaired these abilities of EPCs. SNP could ameliorate tube formation impairment induced by hyperinsulinemia. P38 MAPK inhibitor and ERK1/2 inhibitor could not restore impaired tube formation. Cells incubated with PI3-K inhibitor LY294002 or eNOS inhibitor L-NAME also showed impaired tube formation ability. $N = 4$, $^{*}p < 0.05$ compared with control

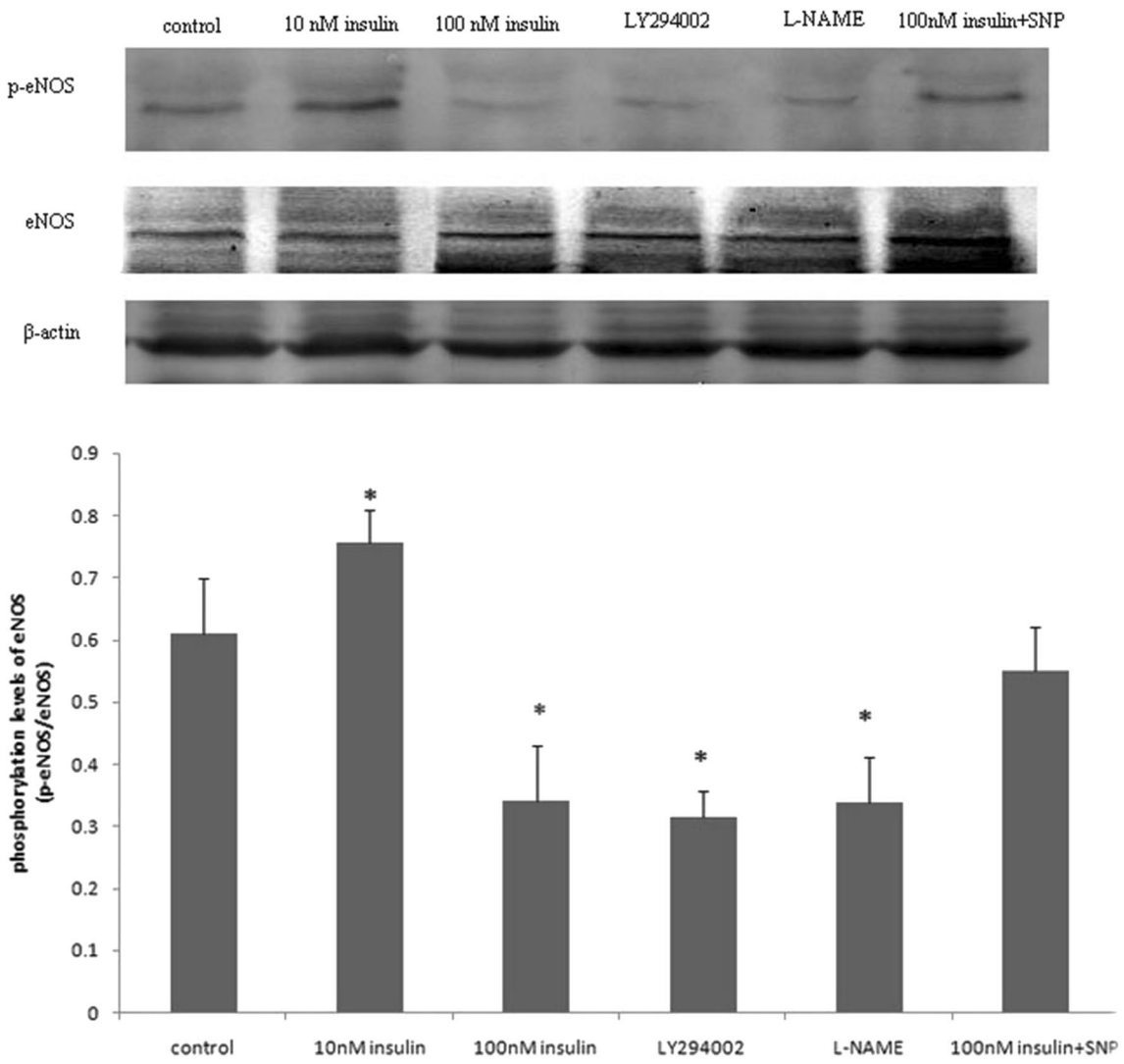


Fig. 5 eNOS phosphorylation of EPCs. 100 nM insulin decreased eNOS phosphorylation level of EPCs. But 10 nM insulin could increase eNOS phosphorylation level of EPCs. LY294002 and L-NAME depressed eNOS phosphorylation. However, SNP could restore eNOS phosphorylation of EPCs. $N=4$, $*p<0.05$ compared with control

and ERK 1/2 activation did not directly influence tube formation of EPCs. Our findings demonstrated that hyperinsulinemia depressed angiogenesis of EPCs via downregulation of PI3-K/Akt/eNOS pathway but not by upregulation of p38 MAPK /ERK1/2 pathway.

In the current study, we found that proliferation of EPCs was depressed by hyperinsulinemia. But mechanism of EPC proliferation is very complicated. In fact, several signaling pathways may involve in cell proliferation. Madeddu et al's study [27] demonstrated that PI3-K-knockout mice had poor proliferation ability of EPCs. Zhang et al's study [28] showed that p38 MAPK modulated EPC proliferation. Our results showed that PI3-K inhibitor and eNOS inhibitor had no direct influence on proliferation of EPCs; p38 MAPK inhibitor (SB 203,580) but not ERK 1/2 inhibitor could ameliorate impaired proliferation induced by hyperinsulinemia. These results indicated that hyperinsulinemia impaired proliferation of EPCs by activating p38 MAPK pathway but not by depressing PI3-K/AKT/eNOS pathway.

In this study, we found that hyperinsulinemia increased apoptosis ratio of EPCs. Apoptosis of EPCs could be largely mitigated by p38 MAPK inhibitor but not by ERK inhibitor. Previous studies [16, 29] has testified that activation of p38 MAPK was related to apoptosis caused by cellular stresses such as ultraviolet light exposure, hyperosmolarity, PH change, cellular injury, and reactive oxygen species (ROS). Our results indicated for the first time that hyperinsulinemia induced apoptosis of EPCs through activating p38 MAPK pathway.

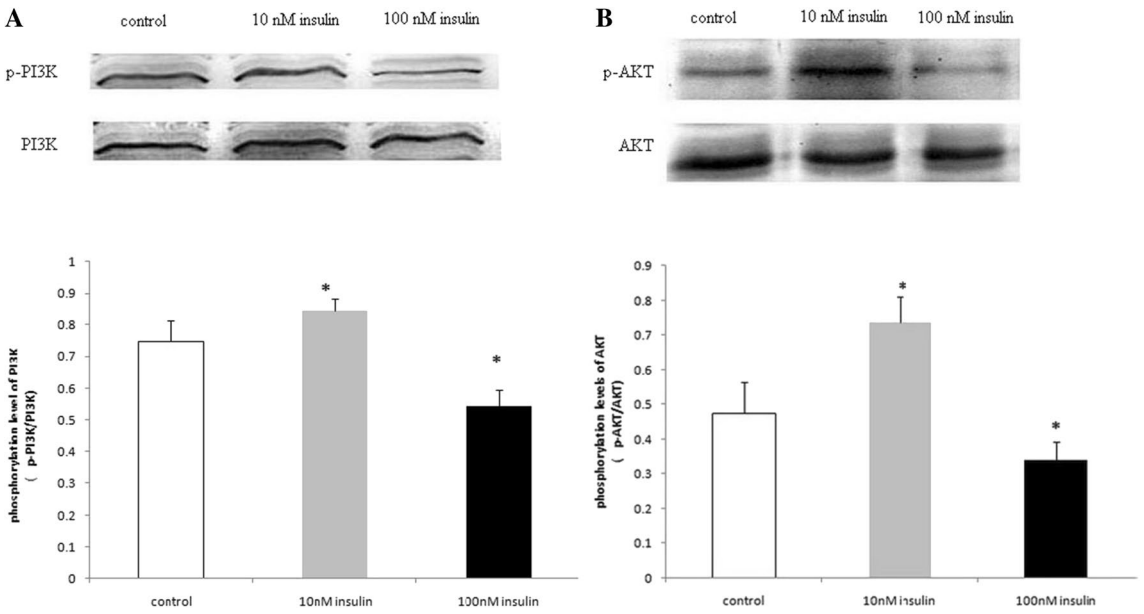


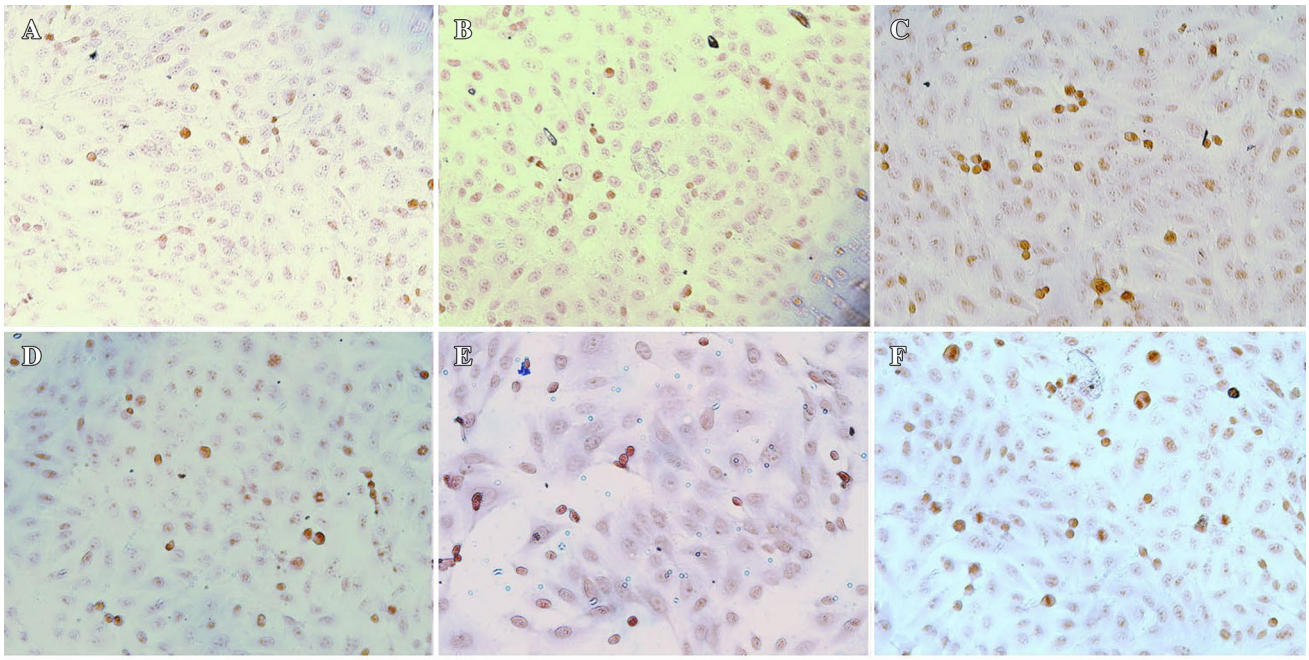
Fig. 6 PI3-K/AKT pathway. 10 nM insulin activated PI3-K/AKT phosphorylation but100 nM insulin depressed PI3-K/AKT phosphorylation. **a** PI3-K phosphorylation levels were decreased in 100 nM insulin group. **b** AKT phosphorylation levels were decreased in 100 nM insulin group. $N=4$, $^{*}p<0.05$ compared with control

Limitations

First, this is a cell culture-based study. We only simulated hyperinsulinemia without hyperglycemia. It will be much more complicated in human body. Cross reaction of hyperinsulinemia and hyperglycemia is unknown. Second, EPC dysfunction induced by hyperinsulinemia is related with course of disease. Long-term exposure to hyperinsulinemia may have different results. Third, hyperinsulinemia is related to inflammation and oxidative stress [30]. Previous study [31] testified that production of oxidants, such as H_2O_2 could activate p38 MAPK pathway and lead to impairment of endothelial cells. But we did not explore inflammation and oxidative stress induced by hyperinsulinemia.

Conclusion

Hyperinsulinemia decreased proliferation, tube formation abilities of EPCs and increased apoptosis of EPCs. Hyperinsulinemia also depressed PI3-K/Akt/eNOS pathway and activated p38 MAPK/ERK pathway. Supplementing SNP could restore impaired network induced by hyperinsulinemia. Inhibitor of PI3-K decreased tube length, tube area, number of junctions of EPCs. However, inhibitor of p38 MAPK could not restore impaired network induced by hyperinsulinemia. These results indicated that hyperinsulinemia depressed angiogenesis of EPCs via downregulation of PI3-K/Akt/eNOS pathway but not by upregulation of p38 MAPK pathway.



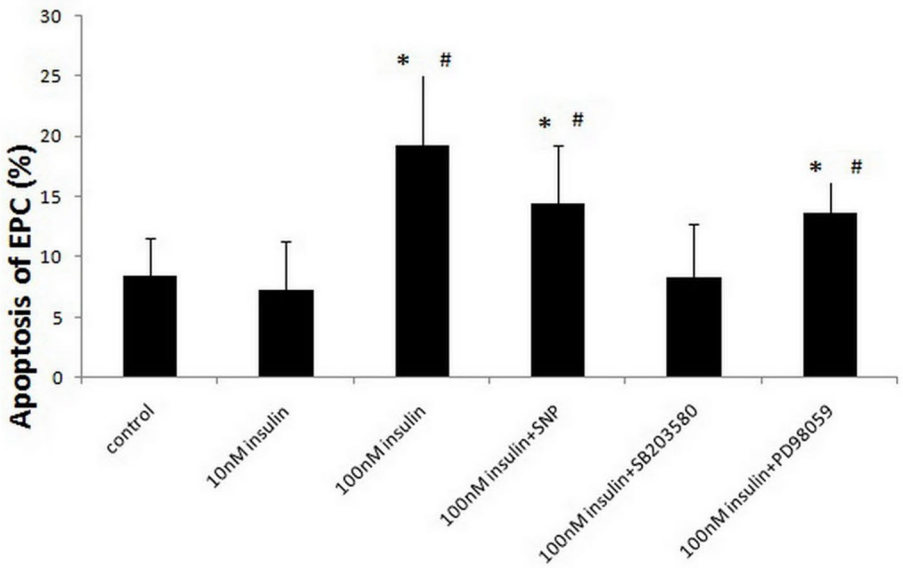


Fig. 7 Apoptosis of EPCs induced by hyperinsulinemia. Cells were treated with **a** control (without insulin); **b** 10 nM insulin; **c** 100 nM insulin; **d** 100 nM insulin plus SNP (25 mM); **e** 100 nM insulin plus SB203580 (5 μM); **f** PD98059 (10 μM). Apoptosis was evaluated by Terminal dUTP nick end labeling (TUNEL) staining. Brown particles indicate $TUNEL^+$ nuclei (×200 final magnification). 100 nM insulin increased apoptosis rate of EPCs, SB203580 could decrease apoptosis of EPCs induced by hyperinsulinemia. $N=4$, $^*p<0.05$ compared with control. $^{\#}p<0.05$ compared with 100 nM insulin + SB203580

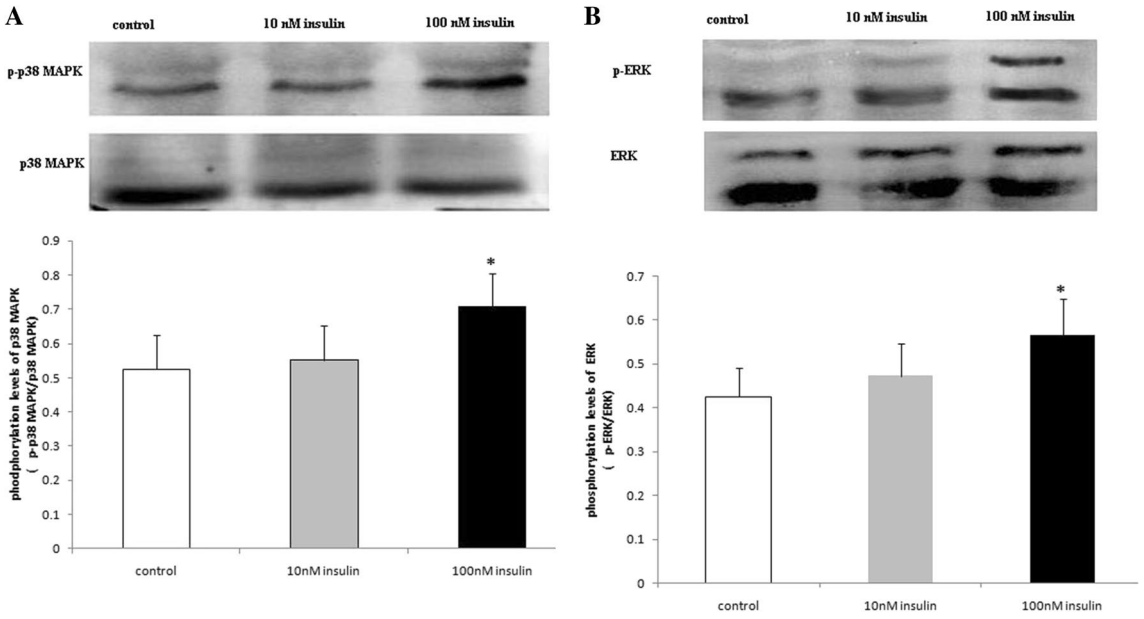


Fig. 8 p38 MAPK and ERK1/2 pathway. Hyperinsulinemia activated p38 MAPK and ERK1/2 phosphorylation. **a** p38 MAPK phosphorylation levels were increased in 100 nM insulin group. **b** ERK 1/2 phosphorylation levels were increased in 100 nM insulin group. $N=4$, * $p<0.05$ compared with control

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Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

Ethical approval This study was appoved by ethical committee of Qinhuangdao First hospital (2016D005).

Informed consent All participants provided informed consent prior to their participation.

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