

Apelin/APJ signaling promotes hypoxia-induced proliferation of endothelial progenitor cells via phosphoinositide-3 kinase/Akt signaling

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Abstract. Endothelial progenitor cells (EPCs) can adhere to the endothelium at sites of hypoxia/ischemia and participate in the formation of novel vessels through differentiating into endothelial cells (ECs). Apelin is an endogenous ligand for the G protein-coupled receptor APJ, and apelin/APJ signaling has a role in cardiovascular function. The present study aimed to investigate the role of apelin/APJ signaling in the regulation of EPC proliferation under hypoxia. The results showed that hypoxia was able to induce EPC proliferation, accompanied with an upregulation of hypoxia-inducible factor (HIF)-1 α as well as apelin/APJ signaling. Further investigation indicated that siRNA-mediated knockdown of apelin or APJ expression attenuated the hypoxia-induced proliferation of EPCs, suggesting that apelin/APJ signaling has an important role in hypoxia-induced EPC proliferation. Moreover, the phosphoinositide-3 kinase (PI3K)/Akt signaling pathway was found to be involved in the apelin/APJ-mediated EPC proliferation under hypoxia. Based on these findings, the present study suggested that hypoxia-induced upregulation of HIF-1 α promotes the expression of apelin and APJ, which further activate the downstream PI3K/Akt signaling pathway, a key promoter of EPC proliferation. In conclusion, the present study highlighted the role of apelin/APJ in the regulation of EPC proliferation, and apelin/APJ may therefore serve as a potential target for the prevention of hypoxic ischemic injury.

Introduction

Oxidative stress-induced endothelial dysfunction is involved in ischemia/reperfusion injury, which may further cause fatal diseases, including coronary atherosclerosis caused by myocardial infarction and stroke (1,2). It has been well-established that endothelial progenitor cells (EPCs) have a crucial role in angiogenesis of endothelial cells (ECs). They can adhere to the endothelium at sites of hypoxia/ischemia and participate in the formation of novel vessels through differentiating into ECs.

Hypoxia has been shown to have effects on multiple cellular biological processes of EPCs. For instance, hypoxia is able to induce the mobilization of EPCs from the bone marrow into the peripheral blood (3). Furthermore, hypoxia can induce endothelial cells to secrete macrophage migration inhibitory factor (MIF), which may have a crucial role in the recruitment and migration of EPCs to hypoxic tissues (4). Lee *et al* (5) showed that hypoxia inhibited senescence of EPCs from aged individuals. The phosphoinositide-3 kinase (PI3K)/Akt signaling pathway has been found to have an essential role in the regulation of cellular survival, proliferation and migration. Moreover, the activity of the PI3K/Akt signaling pathway is closely associated with hypoxia-induced EPCs. Dai *et al* (6) showed that hypoxia was able to protect against serum withdrawal-induced EPC apoptosis, at least in part via the activation of the PI3K/Akt pathway.

Hypoxia-inducible factor-1 α (HIF-1 α) is a key transcriptional factor, the expression of which can be markedly induced under hypoxia (7). Moreover, HIF-1 α has been shown to promote the proliferation and differentiation of EPCs. Jiang *et al* (8) reported that inhibition of HIF-1 α suppressed the differentiation of EPCs to ECs, as well as the expression of genes which promote neovascularization. However, the regulatory pattern of HIF-1 α under hypoxia in EPCs remains to be fully elucidated.

Apelin is an endogenous ligand for the G protein-coupled receptor APJ. The apelin/APJ pathway has a role in the regulation of diverse biological functions, including cardiovascular function, fluid homeostasis and insulin secretion, via activation

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of various tissue-specific signaling pathways (9-11). Recently, Ye *et al* (12) showed that the downregulation of the serum levels of apelin was associated with acute myocardial infarction-induced mobilization of EPCs, suggesting that apelin may be involved in the regulation of EPCs. Furthermore, apelin deficiency impaired sprouting of EPCs in ischemia-reperfusion injury (13). However, to the best of our knowledge, whether apelin has a role in the regulation of hypoxia-induced EPC proliferation as well as the underlying molecular mechanism have not been investigated, yet.

The present study mainly aimed to investigate the role of apelin/APJ signaling in hypoxia-induced EPC proliferation and the underlying molecular mechanisms were also investigated.

Materials and methods

Cell culture. EPCs were purchased from Nlunbio (Changsha, China). The EPCs were cultured in endothelial growth medium 2 (EGM-2) supplemented with 20% fetal bovine serum (FBS) both purchased from Life Technologies (Carlsbad, CA, USA) at 37°C in a humidified incubator containing 5% CO₂. For performing cell proliferation analysis, LY294002 (Sigma Aldrich, St. Louis, MO, USA) and 740Y-P (Phoenix Pharmaceuticals, Belmont, CA, USA) were used to treat EPCs in different groups.

Hypoxia treatment of EPCs. Prior to hypoxia treatment, EPCs were cultured in serum-free EGM-2 at 37°C under 5% CO₂ for 12 h. In the MTT assay (Sigma-Aldrich), EPCs were cultured in EGM-2 containing 20% FBS at 37°C under 1% O₂, 94% N₂ and 5% CO₂ for 6, 12, 24 and 48 h, respectively.

Cell proliferation assay. The MTT assay was performed to determine cell proliferation. In brief, MTT (10 mg/ml) was added to the medium. After incubation for 4 h, the reaction was terminated by removal of the supernatant and addition of 100 μ l dimethyl sulfoxide (DMSO; Sigma-Aldrich) to dissolve the formazan product. After 30 min, the optical density (OD) of each well was measured at 570 nm using a plate reader (ELx808; Bio-Tek Instruments, Winooski, VT, USA).

Reverse transcription quantitative polymerase chain reaction (RT-qPCR) analysis. TRIzol reagent (Life Technologies) was used to extract total RNA from EPCs, in accordance with the manufacturer's instructions. Total RNA was reverse transcribed into cDNA by using the PrimeScript RT reagent kit, according to the manufacturer's instructions (Takara Biotechnology Co., Ltd., Dalian, China). In brief, 5 μ l total RNA was mixed with 0.15 μ l of 100 mM dNTPs (with dTTP), 1 μ l (50 units) reverse transcriptase, 1.5 μ l of X10 reverse transcription buffer, 0.19 μ l RNase inhibitor (20 U/ μ l), and nuclease-free H₂O was added to obtain a final volume of 15 μ l. Reverse transcription was performed at 16°C for 30 min, followed by an incubation step at 42°C for 30 min and enzyme inactivation at 85°C for 5 min. The mRNA expression levels were determined using SYBR Premix Ex Taq II, in accordance with the manufacturer's instructions (Takara Biotechnology Co., Ltd.). In brief, 0.2 μ l cDNA solution, 10 μ l PCR mix, 2 μ l gene specific primer and 7.8 μ l nuclease-free H₂O were mixed to obtain a final reaction volume of 20 μ l. The

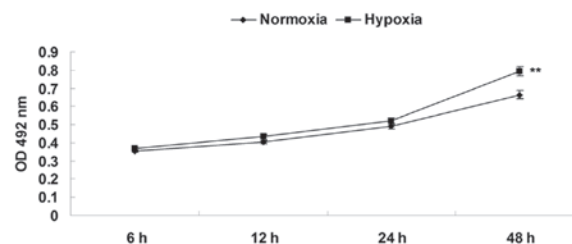


Figure 1. An MTT assay was performed to determine the cell proliferation of endothelial progenitor cells treated with normoxia or hypoxia for 6, 12, 24 and 48 h. Values are expressed as the mean \pm standard deviation. **P<0.01, vs. normoxia. OD, optical density.

reaction conditions for PCR were: Pre-degeneration at 95°C for 1 min followed by 40 cycles of 95°C for 15 sec, 60°C for 15 sec, and a final extension at 72°C for 1 min. The specific primer pairs were as follows: HIF-1 α sense, 5'-GAACGT CGAAAAGAAAAGTCTCG-3' and anti-sense, 5'-CCTTAT CAAGATGCGAACTCACA-3'; apelin sense, 5'-GTCTCC TCCATAGATTGGTCTGC-3' and anti-sense, 5'-GGAATC ATCCAAACTACAGCCAG-3'; APJ sense, 5'-CTCTGGACC GTGTTTCGGAG-3' and anti-sense, 5'-GGTACGTGTAGG TAGCCCACA-3'; GAPDH (internal reference) sense, 5'-GGA GCGAGATCCCTCCAAAAT-3' and anti-sense, 5'-GGCTGT TGTCATACTTCTCATGG-3'. Primers were purchased from Shanghai Shengong Co., Ltd. (Shanghai, China). Independent experiments were repeated three times. The relative expression of mRNA was analyzed using the 2^{- $\Delta\Delta$ Ct} method.

Transfection. Transfection was performed using Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. All plasmids and siRNA were purchased from Nlunbio. Sequences or other information were not provided due to commercial confidentiality. Lipofectamine 2000, siRNA and plasmid were diluted with serum-free medium, respectively. The diluted Lipofectamine 2000 was added into the diluted siRNA or plasmid and incubated for 20 min at room temperature, and then added into the EPC suspension. Subsequently, EPCs were incubated at 37°C, 5% CO₂ for 6 h. After that, the medium was replaced by Dulbecco's modified Eagle's medium with 10% FBS, and cultured for 24 h prior to the following assays.

Western blot analysis. Cells were solubilized in cold radio-immunoprecipitation assay lysis buffer (Sigma-Aldrich). Proteins were separated by 12% SDS-PAGE (Sigma-Aldrich) and transferred onto a polyvinylidene difluoride (PVDF) membrane (Life Technologies), which was then incubated with Tris-buffered saline with Tween 20 (TBST; Sigma-Aldrich) containing 5% skimmed milk at 4°C for overnight. After that, the PVDF membrane was incubated with specific primary antibodies, rabbit polyclonal anti-Apelin (cat. no. ab59469; 1:100) and rabbit polyclonal anti-APJ (cat. no. ab84296; 1:100) and incubated at room temperature for 3 h. After washing with TBST three times, the PVDF membrane was incubated with goat anti-rabbit IgG (cat. no. ab175734; 1:5,000, secondary antibodies at room temperature for 1 h. All antibodies were purchased from Abcam (Cambridge, MA, USA). After washing with TBST three times, an enhanced chemiluminescence kit

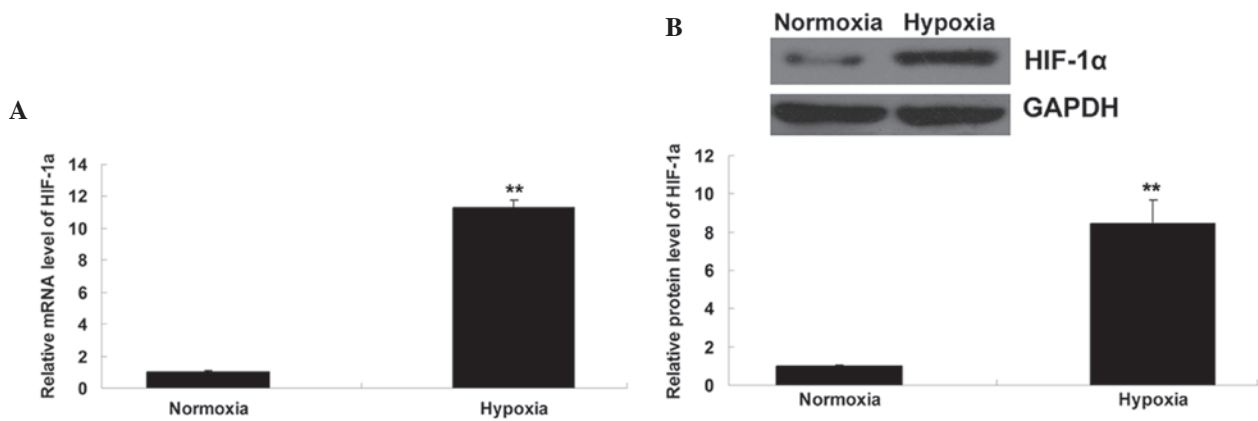


Figure 2. (A) Polymerase chain reaction analysis was performed to examine the mRNA expression of HIF-1 α in EPCs cultured under normoxia or hypoxia. GAPDH was used as an internal reference. ** $P < 0.01$ vs. normoxia. (B) Western blot analysis was performed to examine the protein expression of HIF-1 α in EPCs cultured under normoxia or hypoxia. GAPDH was used as an internal reference. ** $P < 0.01$ vs. normoxia. Values are expressed as the mean \pm standard deviation. HIF, hypoxia-inducible factor; EPC, endothelial progenitor cell.

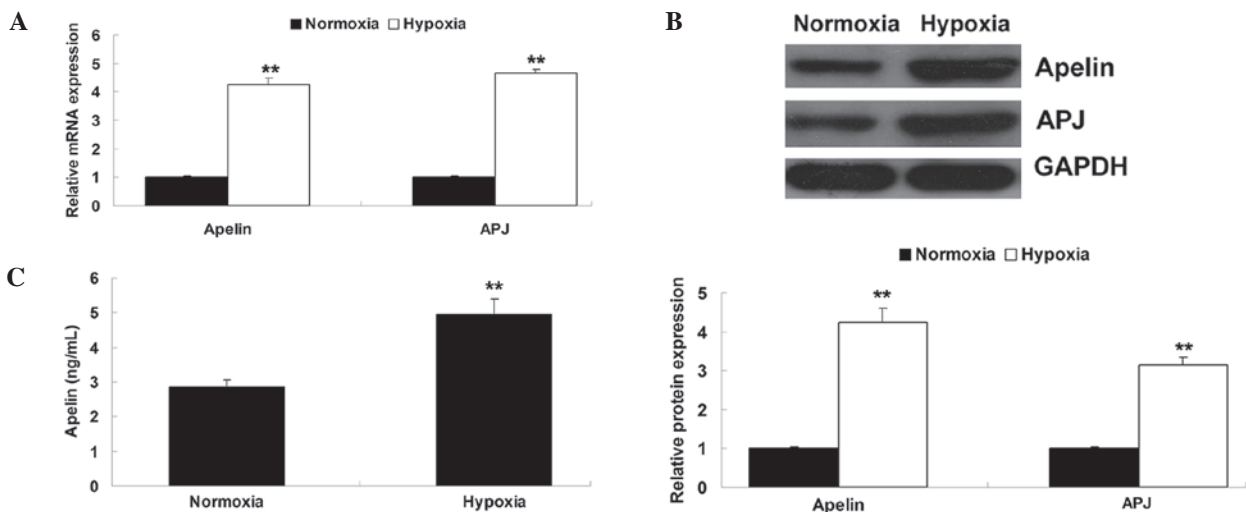


Figure 3. (A) Polymerase chain reaction was performed to examine the mRNA expression of apelin and APJ in EPCs cultured under normoxia or hypoxia. GAPDH was used as an internal reference. ** $P < 0.01$ vs. normoxia. (B) Western blot analysis was performed to examine the protein expression of apelin and APJ in EPCs cultured under normoxia or hypoxia. GAPDH was used as an internal reference. ** $P < 0.01$ vs. normoxia. (C) ELISA was performed to examine the secretion levels of apelin in EPCs cultured under normoxia or hypoxia. ** $P < 0.01$ vs. normoxia. Values are expressed as the mean \pm standard deviation. EPC, endothelial progenitor cell.

(Pierce Chemical, Rockford, IL, USA) was used to perform chemiluminescent detection. The relative expression of protein was determined by Image-Pro plus software 6.0 (Media Cybernetics, Inc., Rockville, MD, USA) represented as the density ratio versus GAPDH. The film was purchased from Kodak (Tokyo, Japan).

Statistical analysis. Values are expressed as the mean \pm standard deviation of three independent experiments. Statistical analysis of differences between values was performed using one-way analysis of variance with SPSS 17 software (SPSS, Inc., Chicago, IL, USA). $P < 0.05$ was considered to indicate a statistically significant difference between values.

Results

Hypoxia promotes EPC proliferation. First, the proliferation of EPCs was determined under hypoxia and normoxia by

using an MTT assay. As shown in Fig. 1, after culturing under hypoxia for 6, 12, 24 and 48 h, the relative proliferation of EPCs was notably upregulated compared with that of EPCs cultured in normoxia. These findings indicated that hypoxia promoted EPC proliferation.

Hypoxia induces upregulation of HIF-1 α and apelin/APJ signaling. The expression levels of HIF-1 α were determined in each group, and it was found that hypoxia induced upregulation of HIF-1 α mRNA and protein expression in EPCs (Fig. 2A and B). As apelin and APJ are known to be regulated by HIF-1 α , their expression levels in EPCs cultured in hypoxia or normoxia were then determined. As shown in Fig. 3A and B, the mRNA and protein levels of apelin and APJ were significantly upregulated in EPCs cultured in hypoxia. In addition, ELISA was performed to examine the secretion of apelin. As demonstrated in Fig. 3C, the secretion levels of apelin in hypoxia-treated EPCs were also upregulated

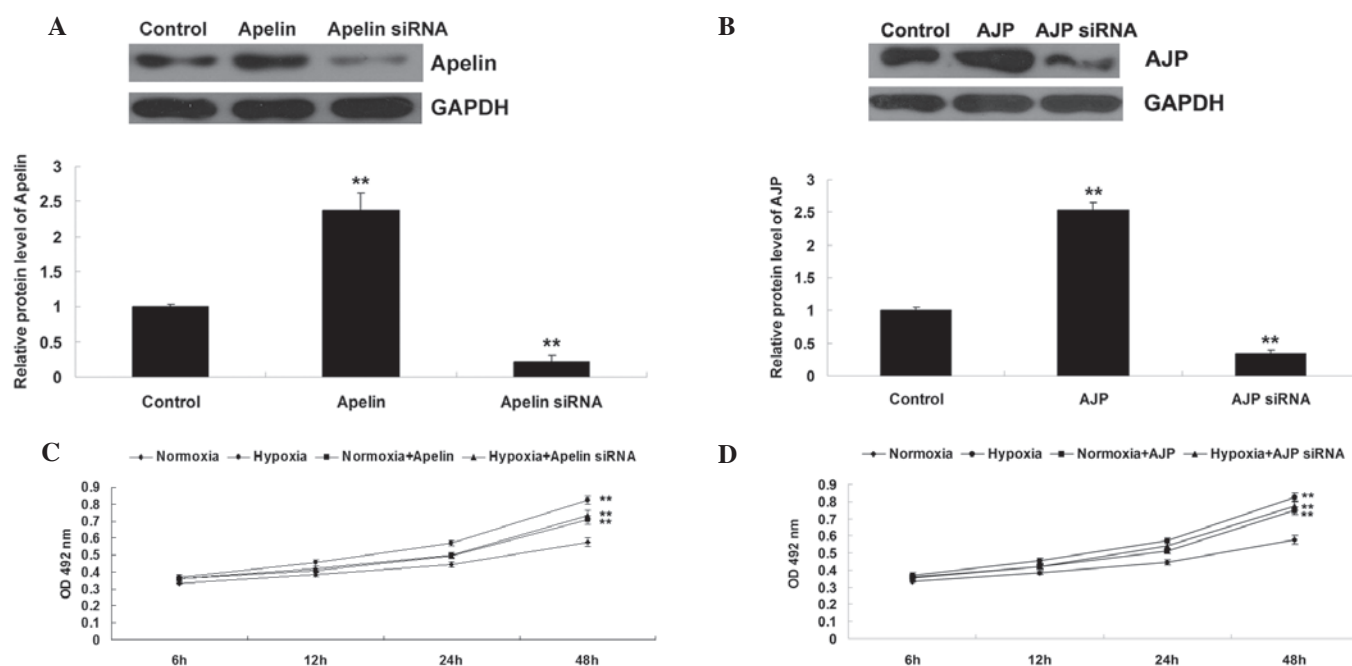


Figure 4. (A) Western blot analysis was performed to examine the protein expression of apelin in EPCs transfected with apelin plasmid or apelin siRNA, respectively. (B) Western blot analysis was performed to examine the protein expression of APJ in EPCs transfected with APJ plasmid or APJ siRNA, respectively. GAPDH was used as an internal reference. Control cells were not transfected. ** $P < 0.01$ vs. control. (C) An MTT assay was performed to determine the cell proliferation of EPCs in each group at 6, 12, 24 and 48 h. Groups: Normoxia, EPCs cultured under normoxia; Hypoxia, EPCs cultured under hypoxia; Normoxia + Apelin, EPCs transfected with apelin plasmid and cultured under normoxia; Hypoxia + Apelin siRNA, EPCs transfected with apelin siRNA and cultured under hypoxia. ** $P < 0.01$, vs. normoxia. (D) An MTT assay was performed to determine the cell proliferation of EPCs in each group at 6, 12, 24 and 48 h. Groups: Normoxia, EPCs cultured under normoxia; Hypoxia, EPCs cultured under hypoxia; Normoxia + APJ, EPCs transfected with APJ plasmid and cultured under normoxia; Hypoxia + APJ siRNA, EPCs transfected with APJ siRNA and cultured under hypoxia. ** $P < 0.01$, vs. normoxia. Values are expressed as the mean \pm standard deviation. EPC, endothelial progenitor cell; siRNA, small interfering RNA; OD, optical density.

compared to those in EPCs cultured under normoxia. These findings suggested that HIF-1 α as well as its downstream apelin/APJ signaling were upregulated in hypoxia-treated EPCs.

Apelin/APJ signaling has a role in hypoxia-induced EPC proliferation. The detailed role of apelin/APJ signaling in hypoxia-induced EPCs proliferation was investigated. Four groups were established: The normoxia group as a control, a hypoxia group, a normoxia + apelin group and a hypoxia + apelin siRNA group. After transfection of the EPCs with apelin plasmid or siRNA, respectively, the expression levels of apelin were determined using western blotting, and the results showed satisfactory transfection efficiency (Fig. 4A). After that, the role of APJ in the regulation of hypoxia-induced EPC proliferation was investigated, for which four groups were established: A normoxia group as the control, a hypoxia group, a normoxia + APJ group and a hypoxia + APJ siRNA group. After transfection of EPCs with APJ plasmid or siRNA, respectively, the expression levels of APJ were determined using western blotting, and the results showed satisfactory transfection efficiency (Fig. 4B). Subsequently, EPC proliferation was determined in each group by using the MTT assay. As shown in Fig. 4C, the proliferation of EPCs in the normoxia + apelin group was higher than that in the normoxia group, and the proliferation of EPCs in the hypoxia + apelin siRNA group was lower than that in the hypoxia group. Furthermore, as shown in Fig. 4D, the proliferation of EPCs in the normoxia + APJ group was

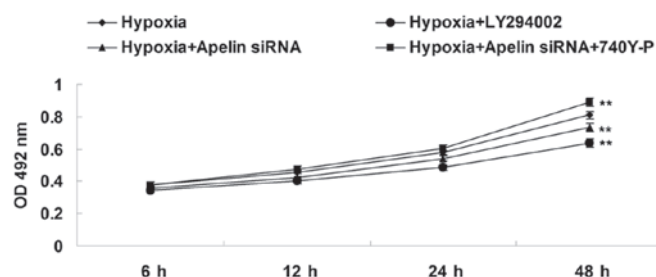


Figure 5. An MTT assay was performed to determine the cell proliferation of EPCs in each group at 6, 12, 24 and 48 h. Groups: Hypoxia, EPCs cultured under hypoxia; Hypoxia + Apelin siRNA, EPCs transfected with apelin siRNA and cultured under hypoxia; Hypoxia + LY294002, EPCs treated with LY294002 and cultured under hypoxia; Hypoxia + Apelin siRNA + 740Y-P, EPCs transfected with apelin siRNA, treated with 740Y-P and cultured under hypoxia. ** $P < 0.01$, vs. hypoxia. Values are expressed as the mean \pm standard deviation. OD, optical density; siRNA, small interfering RNA; EPC, endothelial progenitor cell.

higher than that in the normoxia group, and the proliferation of EPCs in the hypoxia + APJ siRNA group was lower than that in the hypoxia group. These findings suggested that apelin/APJ signaling has a role in hypoxia-induced EPC proliferation.

PI3K/Akt pathway is involved in apelin/APJ-mediated hypoxia-induced EPC proliferation. The present study further determined the underlying molecular mechanism by which apelin/APJ mediated EPC proliferation under hypoxia. To determine whether the PI3K/Akt pathway was

involved in the apelin/APJ-mediated EPC proliferation, PI3K inhibitor LY294002 and PI3K agonist 740Y-P were used. Four groups were set as follows: The hypoxia group, the hypoxia + LY294002 group, the hypoxia + apelin siRNA group and the hypoxia + apelin siRNA + 740Y-P group. As shown in Fig. 5, the proliferation of EPCs in the hypoxia + LY294002 group was lower than that in the hypoxia group, suggesting that inhibition of PI3K/Akt signaling suppressed hypoxia-induced EPC proliferation. In addition, the proliferation of EPCs in the hypoxia + apelin siRNA + 740Y-P group was higher than that in the hypoxia + apelin siRNA group, suggesting that upregulation of PI3K/Akt signaling reversed the inhibitory effect of apelin siRNA on hypoxia-induced EPCs proliferation. These results suggested that the PI3K/Akt pathway acts as a downstream effector of apelin/APJ signaling in EPCs, and is involved in the apelin/APJ-mediated EPC proliferation under hypoxia.

Discussion

The present study showed that hypoxia induced upregulation of HIF-1 α and apelin/APJ signaling in EPCs. Increased apelin/APJ further activated PI3K/Akt signaling, which was involved in the upregulation of EPC proliferation. The findings of the present study highlighted the importance of apelin/APJ signaling in the regulation of EPC proliferation under hypoxia.

The critical role of EPCs in angiogenesis under hypoxia/ischemia has been widely revealed. It has been well established that hypoxia/ischemia triggers EPCs to migrate from the bone marrow into the peripheral blood (14). After migration to the site of hypoxic/ischemic tissues, EPCs can differentiate into ECs and participate in the formation of novel vessels. HIF-1 α is a key determinant of oxygen-dependent gene regulation in angiogenesis, which has been shown to be involved in EPC proliferation and differentiation (8,15). For instance, several studies found that inhibition of HIF-1 α was able to inhibit the expression of vascular endothelial growth factor (VEGF), VEGF receptor 2, endothelial nitric oxide synthase as well as NO production, and suppress the differentiation of EPCs to ECs (8,15). On the contrary, overexpression of HIF-1 α was reported to promote the differentiation of EPCs to ECs (16). The present study showed that under hypoxia, the expression of HIF-1 α was significantly upregulated in EPCs, consistent with the results of other studies (4,5). Furthermore, the present study found that the expression of apelin was upregulated under hypoxia in EPCs. In fact, the association between HIF-1 α and apelin has been elucidated by previous studies. Ronkainen *et al* (17) showed that the upregulation of apelin under hypoxia was abolished by the HIF-inhibitory PAS protein in cardiomyocytes, suggesting that the expression of apelin was regulated by hypoxia in cardiac myocytes via the HIF pathway. Furthermore, Glassford *et al* (18) showed that HIF-1 α regulated hypoxia-induced expression of apelin in adipocytes. However, to the best of our knowledge, the association between HIF-1 α and apelin in EPCs has never been reported. As the results of the present study showed that hypoxia induced the upregulation of HIF-1 α as well as apelin, it was hypothesized that the upregulation of HIF-1 α induced by hypoxia may further promote the expression of apelin in EPCs.

Apelin/APJ has been found to be involved in a wide range of physiological and pathological functions in the cardiovascular system (19). For instance, apelin has a crucial role in the regulation of cell proliferation in vascular smooth muscle cells (VSMCs) (19). Moreover, inhibition of apelin expression markedly inhibited EC proliferation in pathological retinal angiogenesis (20). Furthermore, apelin/APJ was found to increase angiogenesis and improve cardiac functional recovery post-myocardial infarction (21). These findings suggested that Apelin has a key role in the regulation of cell proliferation in the cardiovascular system. Recently, apelin was reported to be involved in the mobilization of EPCs after acute myocardial infarction (12). However, whether apelin mediates EPCs proliferation has never been investigated, to the best of our knowledge. The present study showed that siRNA-mediated inhibition of apelin/APJ signaling markedly attenuated EPC proliferation induced by hypoxia, indicating that apelin/APJ signaling acts as a downstream regulator in hypoxia-induced EPC proliferation.

Furthermore, it was found that inhibition of PI3K/Akt signaling suppressed hypoxia-induced EPC proliferation, while activation of PI3K/Akt signaling reversed the inhibitory effect of apelin/APJ signaling knockdown on hypoxia-induced EPC proliferation. These results confirmed that PI3K/Akt signaling was the downstream effector of apelin/APJ signaling in the regulation of EPC proliferation induced by hypoxia. Kleinz and Baxter (22) showed that the protective effect of apelin on myocardial reperfusion injury was independent of PI3K/Akt signaling. However, Tao *et al* (23) reported that apelin protected the heart against ischemia-reperfusion injury through activation of PI3K/Akt signaling, via inhibition of endoplasmic reticulum stress-dependent apoptosis activation. Moreover, another study also showed that apelin protected against ischemic cardiomyocyte apoptosis via activation of the PI3K/Akt pathway (24). Furthermore, Liu *et al* (25) showed that apelin was able to promote VSMC proliferation through activation of PI3K/Akt signaling. In fact, most studies demonstrated that PI3K/Akt signaling acted as a downstream effector of apelin, consistent with the findings of the present study.

In conclusion, the present study reported that hypoxia enhanced the expression of HIF-1 α , which led to the upregulation of apelin/APJ signaling, and further activated the downstream PI3K/Akt signaling, an important promoter of EPC proliferation. Therefore, apelin/APJ may serve as a potential target for the prevention of hypoxic/ischemic injury in the cardiovascular system.

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