Acta Biochim Biophys Sin 2010, 42: 396–402 | © The Author 2010. Published by ABBS Editorial Office in association with Oxford University Press on behalf of the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. DOI: 10.1093/abbs/gmq035.

Original Article

PI3K/Akt signaling transduction pathway is involved in rat vascular smooth muscle cell proliferation induced by apelin-13

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Vascular smooth muscle cells (VSMCs) were prepared from thoracic aortas of male Sprague–Dawley rats by the explant method to observe VSMC proliferation via phosphoinositide 3 kinase (PI3K)/Akt signaling transduction pathway induced by apelin-13. Expression of PI3K, phospho-PI3K, phospho-Akt, ERK1/2, phospho-ERK1/2 and cyclin D1 was detected by western blot analysis. Results showed that apelin-13 promoted the expression of phospho-PI3K and phospho-Akt in dose- and time-dependent manner. PI3K inhibitor LY294002 significantly decreased the expression of phospho-PI3K, phospho-Akt, phospho-ERK1/2, and cyclin D1 induced by apelin-13. The Akt inhibitor 1701-1 significantly diminished the expression of phospho-Akt, phospho-ERK1/2, and cyclin D1 stimulated by apelin-13. MTT assay results showed that PI3K inhibitor LY294002 and Akt inhibitor 1701-1 significantly inhibited the VSMC proliferation induced by apelin-13. Apelin-13 promoted VSMC proliferation through PI3K/Akt signaling transduction pathway.

Keywords apelin-13; APJ; PI3K/Akt; ERK1/2; vascular smooth muscle cell

Received: October 24, 2009 Accepted: March 31, 2010

Introduction

The APJ (angiotensin II receptor-like 1) receptor is a G protein-coupled receptor (GPCR) discovered in 1993. It is an orphan GPCR and its natural ligand has not been identified. The human APJ receptor gene encodes 380 amino acids, including 7 transmembrane helices [1]. APJ receptor shares significant homology with angiotensin II type 1 receptor, but in fibroblast cells, APJ receptor does not bind with angiotensin II, which indicates that angiotensin II is not an endogenous ligand for APJ receptor. Apelin, a peptide isolated from bovine stomach, has been shown to act as an endogenous ligand for the APJ receptor [2].

The apelin pre-proteins consist of 77 amino acid residues, with the apelin active sequence in the C-terminal region [3]. The C-terminal portion of pre-proapelin is rich in basic amino acid and endogenous apelin may be processed into several forms in tissues. These forms include apelin-36 and apelin-13 [4]. Apelin constitutes a novel endogenous peptide system involved in a broad range of physiological functions, such as cardiovascular function, heart development, control of fluid homeostasis, and obesity. APJ receptor and apelin are widely distributed in most tissues in human and animals such as lung, heart, brain, skeletal muscle, kidney, liver, and breast. They are also expressed in human osteoblasts and apelin stimulates proliferation of human osteoblasts. Apelin is an important new stomach peptide with physiological role in the gastrointestinal tract [5]. Apelin can promote cells from G_0/G_1 to S phase and stimulate gastric epithelium proliferation. It is a new mitogenic peptide for endothelial cells [6]. Many researches revealed that apelin and APJ receptor were also expressed in vascular muscle [7–10]. But the effect of apelin on vascular smooth muscle cell (VSMC) is still unclear. Our previous study demonstrated that the expression level of apelin in blood vessels of rats with hypertension was increased (data not shown). We also observed that apelin-13 could promote rat VSMC proliferation and this effect was probably related to cyclin D1 and cyclin E. Apelin stimulated VSMC proliferation mediated by phospho-ERK1/2 expression [11]. Moreover, our previous study showed high expressions of phospho-PI3K, phospho-Akt, phospho-p70S6K, phospho-ERK in hypertrophic myocardium of left ventricular hypertrophy rat model (data not shown), suggesting that apelin phosphorylated ERK1/2 through PI3K/Akt signal pathway.

Based on this research and our previous studies, we infer that apelin-13 may stimulate rat VSMC proliferation probably through apelin/APJ-PI3K/Akt-ERK1/2-cyclin D1 signal transduction pathway.

Materials and Methods

Cell culture and reagents

VSMCs from the thoracic aortas of 7–8-week-old male Sprague–Dawley rats were prepared using previous method [12] and cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Gaithersburg, USA) supplemented with 20% fetal bovine serum (FBS) at 37°C with 5% CO_2. The medium was refreshed every 3–5 days and transferred by 2.5 g/l trypsin digestion when the cells were grown to 70–80% confluence. The cells were spindle-shaped or long fusiform shaped under the inverted microscope and displayed a typical 'hill and valley' morphology. Immunohistochemical staining with a monoclonal antibody against β-actin confirmed that there were no co-cultured fibroblasts. Only VSMCs from passages 6–10 were used for experiments.

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was purchased from Amresco (Solon, USA). Bicinchoninic acid (BCA) protein assay kit was purchased from Hyclone (Logan, USA). Polyvinylidene difluoride (PVDF) membranes were purchased from Millipore Biosciences (Billerica, USA). Anti-ERK1/2 antibody [ERK1/2 (K-23)] and anti-phospho-ERK1/2 [pERK 1/2(E-4) antibody] were purchased from Santa Cruz Biotechnology (Santa Cruz, USA). Anti-PI3K antibody (PI3K P85 antibody), anti-phospho-PI3K antibody [phospho-PI3K P85 (Tyr458)/p55 (Tyr199) antibody], anti-Akt antibody (Akt antibody), anti-phospho-Akt antibody (phospho-Akt (Ser473) were purchased from Cell Signaling Technology (Beverly, USA). Anti-cyclin D1 antibody and horseradish peroxidase-conjugated (HRP) goat anti-rabbit IgG were purchased from Boster Biological Technology (Wuhan, China).

Cell proliferation assay

Cells at the exponential phase of growth from passages 6–10 were chosen to measure the extent of cell proliferation by the MTT assay. After being digested by trypsin, the cells were suspended in DMEM containing 10% FBS, and seeded in 96-well plate at a density of 5×10^4 cells/well (200 μl/well), and grouped randomly, 4–6 parallel wells in one group, and cultured at 37°C with 5% CO_2. When grown to 70–80% confluence, cells were synchronized in DMEM containing 0.1% FBS for 24 h and then pretreated with LY294002 or 1701-1 for 1 h, incubated with apelin-13 for 24 h. Following the designated incubation interval, 20 μl of MTT was added to each well of the 96-well plates and incubated for 4 h. After removal of the medium from each well, 150 μl of dimethylsulfoxide (DMSO) was added and incubated for 30 min at 37°C. The accumulated formazan was then dissolved in DMSO and the absorbance at 570 nm was measured.

Western blot analysis

After being digested by trypsin, the cells were made into single cell suspension and placed into different 75-ml culture flask at the density of 1×10^5 cells/ml, and cultured at 37°C with 5% CO_2. When grown to 70–80% confluence, the cells were synchronized in DMEM containing 0.1% FBS for 24 h. After treatment, the VSMCs were washed with ice-cold PBS for three times, lysed with RIPA lysis buffer [50 mM Tris–HCl, pH 7.4, 150 mM sodium chloride, 10 mM NP-40, 5 mM deoxycholic acid, 1 mM sodium dodecyl sulfate (SDS), 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride] for 5–10 min on ice. Crude lysates were sonicated and centrifuged at 15,000 *g* for 30 min at 4°C. The supernatants were collected and their protein concentrations were determined using a BCA protein assay kit. Samples were boiled for 5 min and aliquots containing 40 μg of protein per lane were electrophoresed in 10% SDS-polyacrylamide gel, and then transferred to PVDF membranes. Membranes were blocked in Tris-buffered saline (50 mM Tris–HCl, pH 7.6, and 150 mM sodium chloride) containing 5% non-fat milk and 0.1% Tween-20 for 1 h at room temperature. The proteins were analyzed with the following primary antibodies: anti-PI3K antibody, anti-ERK1/2 antibody, anti-phospho-PI3K antibody, anti-Akt antibody, anti-phospho-Akt antibody, anti-phospho-ERK1/2 antibody, and anti-cyclin D1 antibody. All primary antibodies were diluted with 1% non-fat milk. The membranes were incubated with a primary antibody diluted in blocking solution for 2 h at 37°C, and washed with TBST for four times, 10 min each. Then the membranes were incubated with the secondary antibody, HRP conjugated goat anti-rabbit immunoglobulin G, for 1 h at room temperature, and washed with TBST for three times, 10 min each. Peroxidase activity was detected by enhanced chemiluminescence and analyzed by densitometry using a densitometer and an imager.

Statistic analysis

Data were expressed as the mean ± SD and analyzed using the SPSS 13.0 for Windows software package. Student's *t*-test was performed for comparison of means between two samples and values of $P < 0.05$ were considered as statistically significant.

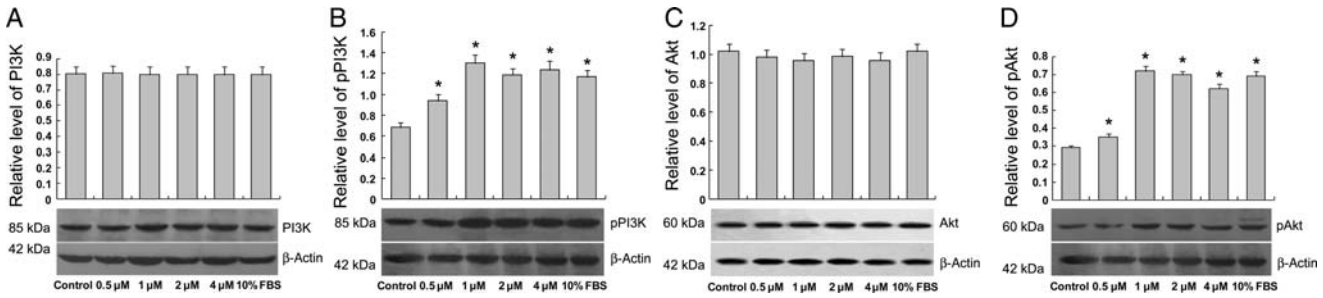

Figure 1 Dose effect of apelin-13 on expression of PI3K, phospho-PI3K, phospho-Akt in VSMCs VSMCs were incubated with apelin-13 at the indicated concentrations and 10% FBS for 4 h. Membranes were probed with anti-PI3K antibody (A), anti-phospho-PI3K P85 (Tyr458)/p55 (Tyr199) antibody (B), anti-Akt antibody (C), and anti-phospho-Akt (Ser473) antibody (D).The data represent the mean ± SD ($n = 3$). *$P < 0.01$ vs. control.

Results

Dose effect of apelin-13 promote expression of PI3K, phospho-PI3K P85 (Tyr458)/P55 (Tyr199), phospho-Akt (Ser473) in VSMCs

VSMCs were incubated with apelin-13 for 4 h at concentrations of 0, 0.5, 1, 2, and 4 μM [11], and 10% FBS. Western blot analysis results showed that apelin-13 promoted expression of phospho-PI3K and phospho-Akt in concentration-dependent manner [**Fig. 1(B,D)**]; the expression level was in peak at 1 μM, and reduced at 2 and 4 μM, but still higher than that in the control group. There were no significant changes in the expression of PI3K and Akt [**Fig. 1(A,C)**].

Time effect of apelin-13 on expression of phospho-PI3K P85 (Tyr458)/P55 (Tyr199), phospho-Akt (Ser473) in VSMCs

To examine the time effect of apelin on expression of phospho-PI3K and phospho-Akt, cells were incubated with 1 μM of apelin-13 for 0, 5, 15, 30, 45, and 60 min. Western blot analysis results showed that apelin-13 promoted expression of phospho-PI3K and phospho-Akt in time-dependent manner (**Fig. 2**); the expression level was in peak at 30 min, while gradually declined, but still higher than that in the control group.

Effect of LY294002 on the expression of phospho-PI3K P85 (Tyr458)/P55 (Tyr199), phospho-Akt (Ser473), ERK1/2, phospho-ERK1/2 (E-4), and cyclin D1 in VSMCs induced by apelin-13

Results showed that apelin-13 can promote the expression of phospho-PI3K, phospho-Akt, phospho-ERK1/2 and cyclin D1 in rat VSMCs. Here, the influence of PI3K inhibitor LY294002 was examined. After being pretreated with 25 μM of LY294002 for 1 h, VSMCs were stimulated with 1 μM apelin-13 for 30 min. Western blot analysis results revealed downregulated expression of phospho-PI3K, phospho-Akt, phospho-ERK1/2, and cyclin D1 (**Fig. 3**). But there was no significant change in ERK1/2, suggesting that LY294002 could inhibit the expression of phospho-PI3K, phospho-Akt, phospho-ERK1/2, and cyclin D1 induced by apelin-13 in VSMCs. There is no effect of DMSO on the expression of phospho-PI3K, phospho-Akt, phospho-ERK1/2, and cyclin D1.

Effect of 1701-1 on the expression of phospho-Akt (Ser473), ERK1/2, phospho-ERK1/2 (E-4), and cyclin D1 induced in VSMCs by apelin-13

Here, the influence of Akt inhibitor 1701-1 was examined. After being pretreated with 10 μM of 1701-1 for 1 h, VSMCs were stimulated with 1 μM apelin-13 for 30 min. Western blot analysis results revealed downregulated expression of phospho-Akt, phospho-ERK1/2, and cyclin D1. There was no significant change in ERK1/2, indicating that 1701-1 could inhibit the expression of phospho-Akt, phospho-ERK1/2, and cyclin D1 induced by apelin-13 in VSMCs (**Fig. 4**). DMSO has no effect on the expression of phospho-PI3K, phospho-Akt, phospho-ERK1/2, and cyclin D1.

Effect of LY294002 on VSMCs proliferation induced by apelin-13

MTT analysis was used to demonstrate the effects of LY294002 on VSMC proliferation induced by apelin-13. There are seven groups in this experiment: control group (0.1% FBS), apelin-13 group, LY294002 + apelin-13 group, LY294002 group, DMSO + apelin-13 group, solvent DMSO group, and 10% FBS positive control group. The concentrations used for apelin-13 and LY294002 were 2 and 25 μM, respectively. VSMCs were incubated with LY294002 for 1 h, and then were treated with apelin-13 for 24 h. VSMC proliferation of LY294002 + apelin-13 group was reduced significantly compared with that of apelin-13-treated group (**Fig. 5**). DMSO had no effect on cell proliferation.

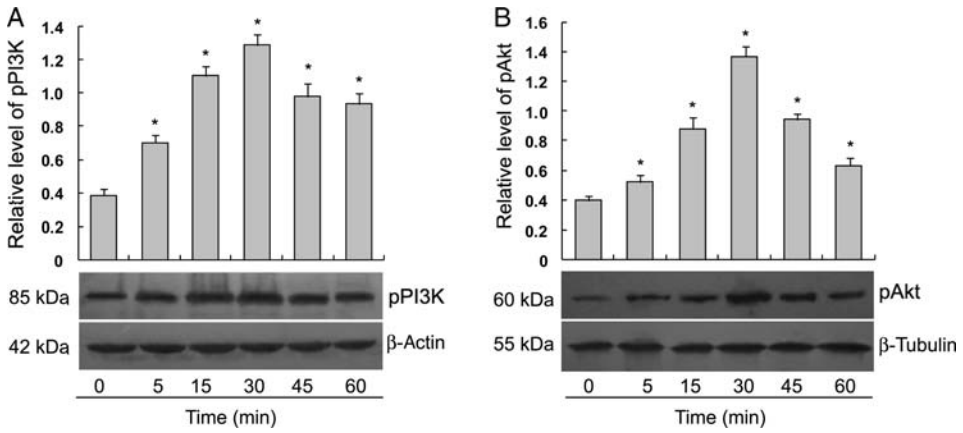

Figure 2 Time effect of apelin-13 on expression of phospho-PI3K, phospho-Akt in VSMCs VSMCs were incubated with 1 μM apelin-13 for indicated time. Membranes were probed with anti-phospho-PI3K P85 (Tyr458)/p55 (Tyr199) antibody (A) and anti-phospho-Akt (Ser473) antibody (B). The data represent the mean ± SD ($n = 3$). *$P < 0.01$ vs. apelin-13.

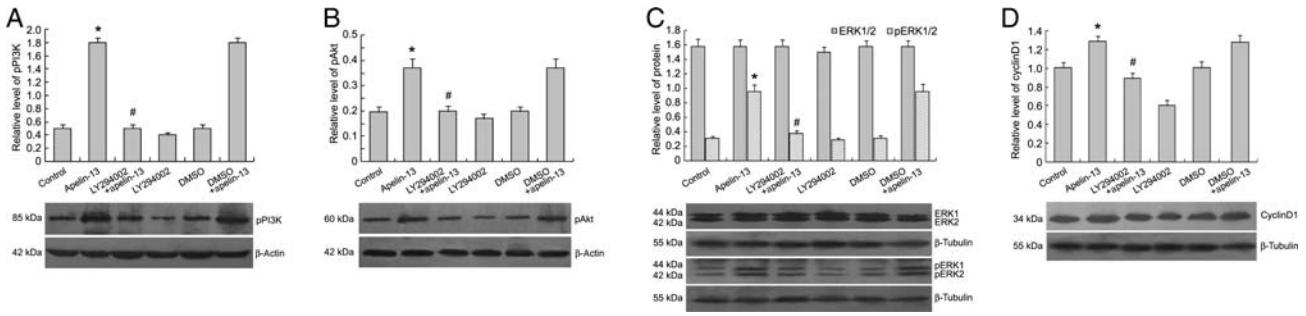

Figure 3 Effect of PI3K inhibitor LY294002 on the expression of phospho-PI3K, phospho-Akt, ERK1/2, phospho-ERK1/2, and cyclin D1 induced by apelin-13 VSMCs were treated with LY294002 (25 μM) or DMSO for 1 h and then stimulated with or without apelin-13 (1 μM) for 30 min. Membranes were probed with anti-phospho-PI3K P85 (Tyr458)/p55 (Tyr199) antibody (A), anti-phospho-Akt (Ser473) antibody (B), anti-ERK1/2 antibody and anti-phospho-ERK1/2 (E-4) antibody (C), and anti-cyclin D1 antibody (D). The data represent the mean ± SD ($n = 3$). *$P < 0.01$ vs. control; $^{\#}P < 0.01$ vs. apelin-13.

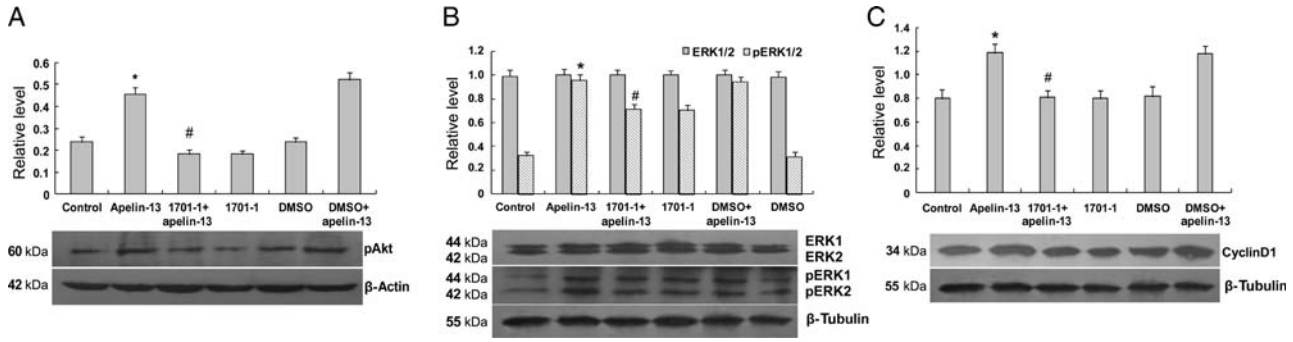

Figure 4 Effect of Akt inhibitor 1701-1 on the expression of phospho-Akt, ERK1/2, phospho-ERK1/2, and cyclin D1 induced by apelin-13 VSMCs were treated with 1701-1(10 μM) or DMSO for 1 h and then stimulated with or without apelin-13(1 μM) for 30 min. Membranes were probed with anti-phospho-Akt (Ser473) antibody (A), anti-ERK1/2 antibody and anti-phospho-ERK1/2 (E-4) antibody (B), and anti-cyclin D1 antibody (C). The data represent the mean ± SD ($n = 3$). *$P < 0.01$ vs. control; $^{\#}P < 0.01$ vs. apelin-13.

Effect of 1701-1 on VSMCs proliferation induced by apelin-13

MTT analysis was used to illustrate the effects of 1701-1 on VSMCs proliferation induced by apelin-13. There are seven groups in this experiment: control group (0.1% FBS), apelin-13 group, 1701-1 + apelin-13 group, 1701-1 group, DMSO + apelin-13 group, solvent DMSO group, and 10% FBS positive control group. The concentrations used for apelin-13 and 1701-1 were 2 and 10 μM, respectively. VSMCs were incubated with 1701-1 for 1 h, and then were treated with apelin-13 for 24 h. VSMC proliferation in 1701-1 + apelin-13 group was reduced

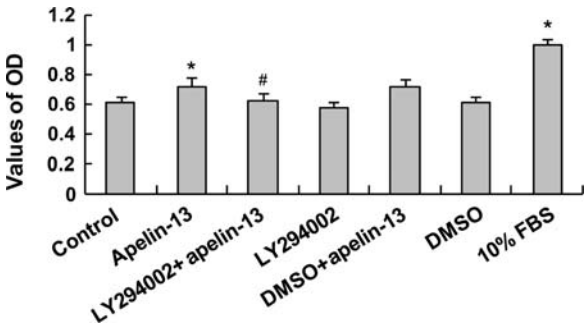

Figure 5 Effect of PI3K inhibitor LY294002 on VSMC proliferation induced by apelin-13 VSMCs were incubated with or without LY294002 (25 μM) for 1 h, and then incubated with apelin-13 (2 μM) for 24 h. LY294002 + apelin-13 group was reduced significantly compared with apelin-13-treated group. The data represent the mean ± SD ($n = 3$). $^{*}P < 0.01$ vs. control; $^{\#}P < 0.01$ vs. apelin-13.

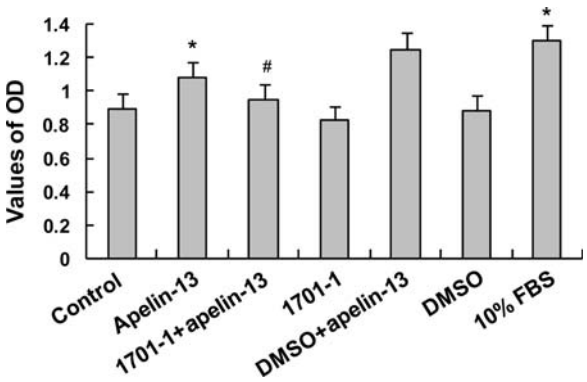

Figure 6 Effect of Akt inhibitor 1701-1 on VSMC proliferation induced by apelin-13 VSMCs were incubated with or without 1701-1 (10 μM) for 1 h, and then incubated with apelin-13 (2 μM) for 24 h. 1701-1 + apelin-13 group was reduced significantly compared with apelin-13-treated group. The data represent the mean ± SD ($n = 3$). $^{*}P < 0.01$ vs. control; $^{\#}P < 0.01$ vs. apelin-13.

significantly compared with that of apelin-13-treated group (**Fig. 6**). DMSO had no effect on cell proliferation.

Discussion

Apelin, produced and released by vascular endothelial cells, is a cardiovascular activity regulating substance with very important pathological and physiological functions by binding and activating the APJ receptor. The distribution of apelin in tissues indicates that apelin signal pathway is involved in a broad range of physiological activities. The APJ receptor was first identified in the endothelial cells of embryo blood vessel-forming phase [13,14]. The APJ receptor is highly expressed in endothelial cells, and endothelial cells respond to apelin by phosphorylating the key effectors related to cell proliferation and cell migration. Moreover, apelin is a mitogenic factor to endothelial cells [6], to present the characteristics of promoting angiogenesis [15]. Apelin might decrease pro-inflammatory cytokine and chemokine activation [16].

It is shown that apelin is a potent angiogenic factor required for vascular development of the frog embryo [17,18], taking an important part in normal and abnormal vascular development. Apelin and APJ receptor are required for cardiovascular development in *Xenopus laevis* [19]. In addition, recent research indicated that apelin is characterized as a novel adipose-expressed factor, which is upregulated in rodent and human obesity and influences cardiovascular function, as well as insulin secretion [20]. The apelin/APJ system contributes to portosystemic collateralization and splanchnic neovascularization in portal hypertensive rats [21]. Apelin and APJ receptor are expressed in human osteoblasts and apelin enhances human osteoblast proliferation, which APJ/PI3K/Akt pathway is involved in the proliferation response [5]. Apelin and APJ receptor are expressed in the gastrointestinal (GI) tract, and intestinal inflammation can increase intestinal hypoxia-inducible factor and apelin expression that induce epithelial proliferation [22]. Moreover, apelin is a neuropeptide that co-localizes with vasopressin (AVP) in magnocellular neurons and is involved in body fluid homeostasis [23]. Apelin may play a role in inflammation and cardiovascular tone [24].

It has been verified that APJ receptor and apelin are expressed in VSMC. Apelin combining with the APJ receptor at endoderm can activate nitric oxide synthase to release NO and induce vasodilation to decrease the average blood pressure in rat [25–28].

The similarities between apelin and angiotensin II in both gene sequence and mRNA distribution suggested that they played similar physiological roles [29]. In this study, we found that apelin-13 induced rat VSMC proliferation in concentration- and time-dependent manners with a peak at 1 μM and 4 h of incubation, respectively. This suggests that apelin-13 can promote rat VSMC proliferation and the phospho-ERK1/2-cyclin D1 signal pathway is involved in this process. This function of apelin-13 is similar to angiotensin II.

APJ receptor in granulosa cells may be involved in the appearance of the cell apoptosis, and luteinizing hormone stimulates the expression of apelin and APJ receptor in theca cells [30]. We previously examined the effect of apelin-13 on cell cycle distribution by using flow cytometry. The results showed that cells in the G_0/G_1 phase of apelin-13-treated group decreased to 81.5% compared with the serum-free group and cells in the S phase increased from 3.4 to 12.5%. These data suggested that apelin-13 promoted cells from G_0/G_1 phase to S phase [11].

ERK1/2, a key molecule to cell proliferation, stimulates the expression of cyclin protein and promotes cell cycle progress. Among all known cyclin proteins, cyclin D1 is

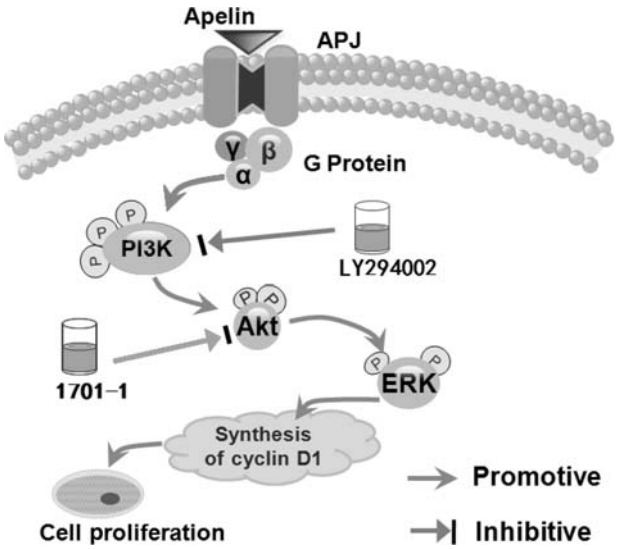

Figure 7 Molecular mechanism of VSMC proliferation induced by apelin-13 Apelin combining with APJ receptor causes PI3K phosphorylation, which activates Akt and its downstream signal molecule ERK1/2, thus promotes the synthesis of cyclin D1 and finally induces cell proliferation. This can be verified by the results of treating with PI3K inhibitor LY294002 and Akt inhibitor 1701-1. LY294002 and 1701-1 can obviously inhibit the proliferation induced by apelin-13.

shown to be the most important in regulating G_1 to S check-point and cyclin E is the second important one [31].

Our previous studies indicated that apelin could promote the expression of phospho-ERK1/2 in VSMCs [32]. Besides, cyclin D1 increased in serum-free culturing VSMCs stimulated by apelin-13. Meanwhile, apelin-13 could promote the expression of cyclin E. This proves that apelin-13 advances cell cycle progress by stimulating the expression of cyclin D1 and cyclin E. But how does apelin induce phosphorylation of ERK1/2 and proliferation in VSMCs? Based on our previous research, we investigated whether PI3K and Akt will involve in phosphorylating ERK1/2 induced by apelin.

Our study shows that apelin-13 can induce the expression of phospho-PI3K and phospho-Akt. Phospho-PI3K, phospho-Akt, phospho-p70S6K, and phospho-ERK1/2 expression is increased in the hypertrophic myocardium of the left ventricular hypertrophy rat model (data not shown). These results suggested that apelin-13 induced rat VSMC proliferation by PI3K and Akt phosphorylating ERK1/2. To verify this hypothesis we observed the effect of PI3K inhibitor LY294002 and Akt inhibitor 1701-1 on cell proliferation induced by apelin-13. Results show that LY294002 and 1701-1 obviously inhibited VSMC proliferation. Furthermore, we found that PI3K and Akt inhibitors decreased the expression of phospho-PI3K, phospho-Akt, phospho-ERK1/2, and cyclin D1. These results further supported that PI3K/Akt signal transduction pathway mediates VSMC proliferation induced by apelin-13. The process is illustrated in **Fig. 7**. Also, we found that the density unit of pERK1/2 caused by Akt inhibitor 1701-1 is higher than that of DMSO-treated or control group [**Fig. 4(B)**]. This suggests that other molecules and signal pathways are probably involved in the induction of pERK1/2.

The APJ receptor is a G protein-coupled receptor [13]. According to Masri *et al*. [6], apelin-mediated phosphorylation of p70S6K in CHO cells is blocked by G_i inhibitor pertussis toxin (PTX). And apelin causing extracellular acidification in CHO-A10 cells was transduced via G_i [4]. Then whether and how is G protein involved in regulating VSMC proliferation and what is the function of G_i in VSMC proliferation induced by apelin-13? More research is needed to explore it.

In summary, some preliminary understanding of the biological functions and novel mechanistic insights of the role of apelin-APJ signaling during VSMC proliferation have been presented. The effect of apelin-13 promoting rat VSMC proliferation has a close relationship with PI3K/Akt-ERK1/2 signal pathway. Because VSMC proliferation plays a critical role in the pathogenesis of atherosclerosis, it is reasonable to consider the apelin-APJ-PI3K/Akt-ERK1/2 pathway as a potential therapeutic target. However, whether there is any other signal pathway mediating the activity of apelin-inducing VSMC proliferation remains to be further explored.

Funding

This work was supported by the grants from the National Natural Science Foundation of China (no. 3090577), Hengyang Municipal Bureau of Science and Technology (2009KJ14), and the Scientific Research Foundation for the Returned Overseas Chinese Scholars, State Education Ministry.

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