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Apelin-13 Promotes Monocyte Adhesion to Human Umbilical Vein Endothelial Cell Mediated by Phosphatidylinositol 3-Kinase Signaling Pathway*

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Abstract Previously we reported that G protein-coupled receptor APJ endogenous ligand apelin-13 induced adhesion of monocytes to human umbilical vein endothelial cells (HUVECs).Now we investigated whether phosphatidylinositol 3-kinase (PI3K) signaling pathway mediated monocytes (MCs) adhesion to HUVECs induced by apelin-13. Human umbilical vein endothelial cell line ECV304 was cultured in DMEM medium and the monocyte cell line THP-1 was cultured in 1640 medium. Myeloperoxidase(MPO) assay was used to identify effects of monocytes adhesion to HUVECs. Expression of vascular cell adhesion molecule (VCAM) -1, phospho-PI3K and PI3K were detected by Western blotting. Apelin-13 promoted PI3K phosphorylation in concentration-dependent and time-dependent manner, which reached the peak at 1 μmol/L and 30 min respectively. The PI3K inhibitor LY294002 inhibited PI3K phosphorylation and the expression of VCAM-1 in HUVECs induced by apelin-13. And the PI3K inhibitor LY294002 inhibited the MCs adhesion to HUVECs induced by apelin-13. It can be concluded that apelin-13 promoted monocytes adhesion to HUVECs through VCAM-1 mediated by PI3K signaling pathway.

Key words apelin, cell adhesion, PI3K, APJ, VCAM-1, monocyte, endothelial cells
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APJ is a G protein-coupled receptor (GPCRs) discovered by O' Dowd in 1993[1-2]. In 1998, Tatemoto K, *et al*.[3] isolated a peptide from bovine stomach extracts named apelin which had been shown to act as an endogenous ligand for the APJ.

Human APJ gene is coded by 377 amino acids, including 7 transmembrane α helices[2]. APJ receptor shares significant homology with the angiotensin (Ang) Ⅱ type 1 receptor[1], but in fibroblast cells and CHO (Chinese hamster ovary) cells APJ does not bind angiotensin Ⅱ which indicates that angiotensin Ⅱ is not endogenous ligand for APJ. Human apelin gene locates on chromosome Xq25-26.1, the gene encodes preproapelin. Both amino acid sequence and mRNA expression distribution analyses revealed similarities between apelin and angiotensin Ⅱ, suggests they share related physiological roles[4]. The apelin preprotein consists of 77 amino acid residues, while the active sequence apelin in the C-terminal regions[5]. Preproapelin is decomposed to isoforms and apelin-13 and apelin-36 are the main isoforms. Different isoforms are distributed in different tissues and play various

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important physiological roles *in vivo* or *in vitro*[4, 6]. The preproapelin gene of human, bovine, rat and mouse share high homology and conservation[4]. Lung, testicle and womb are mainly distributed with apelin-13 and breast is distributed both with apelin-13 and apelin-36. There are receptor binding sites of apelin in human coronary artery, main artery and saphenous veins. The distribution of apelin and arginine vasopressin (AVP) in central nervous system is very similar, which indicates that the function of apelin and AVP is close. In addition, the distribution of apelin and preproapelin in cardiovascular, brain and kidney is very similar to Ang Ⅱ and angiotensinogen. Meanwhile both apelin and Ang Ⅱ are the substrate of angiotensin converting enzyme 2 (ACE2). ACE2 can break down apelin-36 and apelin-13 to shorter segments such as apelin-31, apelin-28, apelin-19, apelin-17 and apelin-12 and break down Ang Ⅱ to Ang1-7. Based on these results, we can anticipate that apelin, like angiotensin Ⅱ, may have an important role in the regulation of cardiovascular homeostasis.

The recent researches showed that apelin might play important roles in regulating cardiovascular homeostasis [7], blood pressure and the pathogenesis of hypertension [8-9] and heart failure [10]. In addition, apelin/APJ system was involved in water intake and fluid retention [11] and pathophysiology of pulmonary hypertension (PH)[12]. Moreover, Zhang *et al*. suggests apelin/APJ signaling pathway played a critical role in the development of the functional vascular network in adipose tissue[13]. Also, based on our previous studies, apelin/APJ could regulate the proliferation of vascular smooth muscle cells through phospho-ERK1/2/cyclinD1 and PI3K/Akt signal pathway[14-15].

Previous studies showed that chronic arterial hypertension could induce the expression of adhesion molecules[16-20], the serum levels of soluble E-selectin, ICAM-1 and VCAM-1 increased in hypertension patients [21]. In hypertrophic myocardial tissue the expression of adhesion molecule ICAM-1 were increased [22-23]. Extracellular signal regulated kinases (ERK) could promote the cell migration and metastasis dependent on cell adhesion molecule L1[24]. Nitric oxide inhibited monocyte adhesion to porcine aortic endothelial cell monolayers [25]. Ang Ⅱ which shares 31% homology with apelin promoted angiogenesis[26-27] and the expression of adhesion molecules VCAM-1 and ICAM-1[28-29] thus stimulated the cell migration. Also, apelin promoted the embryonic and tumor angiogenesis[30-32]. Moreover it was detected adhesion molecules enriched in tumor sites. And adhesion molecules are necessary in cell adhesion. Based on these results we infer that apelin probably has close relation with cell adhesion.

GPCRs signal pathway is involved in cell mgaration and occurrence of cell adhesion[33]. Thereby it is possible that APJ receptor is associated with cell adhesion. According to other researches PI3K proteins were relevant in cell adhesion. Blockade of cell surface α5 integrin expression decreased PI3K activity, inhibited cell attachment[34].

Scavenger receptor 2 regulating the adhesion and migration of monocytes was mediated by PI3K [35]. PI3K inhibitor can decrease cell migration. Migration inhibitory factors could up-regulate the expression of VCAM-1 and ICAM-1 [36]. According to existed researches, PI3K probably mediated monocytes adhesion to endothelial cells.

Furthermore, our laboratory found that apelin-13 increased adhesion of MCs to HUVECs in a concentration- and time-dependent manner and 14-3-3 mediated the induction of adhesion of MCs to HUVECs by apelin-13[37].

Consequently, based on these studies we infer that apelin probably influence the monocytes adhesion to endothelial cells by PI3K signal pathway.

This study is aimed to observe the effects of apelin-13 induced monocytes adhesion to HUVECs *via* PI3K signaling and to confirm the effect of apelin/PI3K signal pathway on monocytes adhesion to endothelial cells. Because of the new physiological function and signaling mechanism, apelin/APJ may become the therapeutic target for hypertension, atherosclerosis and tumors.

1 Methods

1.1 HUVECs and MCs culture

HUVECs (ECV304) (Central South University XiangYa School of Medicine) were cultured in Dulbecco' s modified Eagle' s medium (DMEM) (high glucose L-glutamine, pyridoxine hydrochloride, 110 mg/L sodium pyruvate, 3.7 g sodium bicarbonate) (USA Invitrogen Corporation), supplemented with 10% fetal bovine serum (FBS) under a 5% CO_2 humidified-atmosphere incubator. When the cells were 70% ～80% confluent, they were split after the treatment with 0.25% trypsin and passaged to a fresh culture. The HUVECs were serum starved by changing

the culture media to DMEM containing 0.1% FBS 24 h before experiments.

MCs (THP-1) were cultured in RPMI 1640 under the same conditions as in HUVEC culture.

1.2 Adhesion assay

HUVECs were cultured in 96-well plates at 37℃ for 24 h. On the day of treatments, cells were washed with warm PBS and cultured in DMEM with or without apelin or LY294002. Cells were cultured for 24 h and washed with warmed PBS. THP-1 cells (5 ～106 cells/ml) were then added to HUVECs in DMEM and allowed to adhere at 37℃ under static conditions. The wells were then washed three times with warmed PBS to remove non-adherent cells. Adhesion of MCs was quantified using a modified myeloperoxidase(MPO) assay, as described previously [37]. Briefly, HUVECs plus adherent THP-1 cells were washed twice with PBS without Ca^{2+} and Mg^{2+}(pH 6.0), and subsequently HUVECs plus adhering cells were permeabilized in 50 ml PBS containing 0.5% hexadecyltrimethyl ammonium bromide for 30 min at room temperature. Next, 250 ml warmed O-dianisidine dihydrochloride (0.2 g/L in PBS, pH 6.0) containing 0.4 mmol/L H_2O_2 was added. After 20 min of incubation at 37℃, A_{450} was read. Serial dilutions of neutrophils were used as a standard to calculate the number of adherent THP-1 cells. For the inhibition assay, HUVECs were incubated with LY294002 or apelin + LY294002 at 37℃ before the addition of THP-1 cells. Then, the adhesion assay was performed as described[38].

1.3 Western blotting

To examine the proteins expression in HUVECs induced by apelin-13 (Phoenix Biotech), we used Western-blotting techniques. HUVECs were grown to confluence and synchronized with DMEM containing 0.1% FBS for 12 h, then stimulated with different treating factors as we grouped to assigned time. After stimulation, the supernatant fluids were removed and the monolayer was washed 3 times with phosphate-buffered saline. Cells were placed on ice and lysed using RPIA lysis buffer (50 mmol/L pH7.4 Tris-HCl, 150 mmol/L NaCl, 10 mmol/L NP-40, 5 mmol/L deoxycholic acid, 1 mmol/L SDS, 1 mmol/L EDTA) containing 1 mmol/L phenylmethylsulfonyl fluoride. The lysate was then collected with sterile cell scrapers and sonicated. The resulting cell lysate was centrifuged at 12 000 r/min for 30 min and the supernatant fluid was removed. The protein concentrations were estimated using a bicinchoninic acid protein colorimetric assay. The standardized supernatants were combined with sample buffer, sterile glycerol and stored at –80℃. The cell lysates were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to methanol-activated polyvinylidene difluoride membranes. The membranes were blocked with 5% nonfat dry milk dissolved in TBST (50 mmol/L pH 7.6 Tris-HCl, 150 mmol/L NaCl, 0.1% Tween 20) for 1 h. Immunoblotting was performed using a primary antibody (VCAM-1 (USA Santa Cruz Biotechnology, Inc) and phospho-PI3K (USA Cell Signaling Technology, Inc.) were 1 ∶200 dilution, PI3K (USA Cell Signaling Technology, Inc.) was 1 ∶1 000 dilution. After an overnight incubation at 4℃ or 2 h at 37℃, the membranes were washed three times with TBST and incubated with their respective secondary antibodies coupled to horseradish peroxidase for 1 h at room temperature. The membranes were then washed 6 times with TBST. The immunoreactive bands were visualized using enhanced chemiluminescence and Western blotting detection agents according to the manufacturer's directions[37].

1.4 Data analysis

Data were analyzed by one-way ANOVA followed by the Student-Newman-Keuls test for multiple comparisons or by the unpaired Student's t-test for pairwise comparisons. Data were expressed as the $\bar{x} \pm s$. Statistical significance was defined as $P < 0.05$.

2 Results

2.1 The effects of apelin-13 on phospho-PI3K and PI3K expression in endothelial cells

According to our previous studies, PI3K was involved in vascular smooth muscle cells proliferation induced by apelin-13. To investigate the effect of PI3K on cell adhesion, the following experiments were conducted. HUVECs were stimulated with apelin-13 at the concentration of 0, 0.5, 1, 2, 4 μmol/L and 1 μmol/L Ang Ⅱ [39] for 30 min. The expression of PI3K and phospho-PI3K were detected by Western blotting. The results showed that apelin-13 concentration-dependently induced PI3K phosphorylation and the peak occurred at 1 μmol/L. But apelin-13 had no effect on the expression of PI3K (Figure 1a).

HUVECs were stimulated with 1 μmol/L apelin-13 for 0, 5, 15, 30, 45 and 60 min respectively. The expression of PI3K and phospho-PI3K were detected by Western blotting. The results showed that apelin-13

time-dependently induced PI3K phosphorylation in HUVECs and the peak occurred at 30 min. But apelin-13 had no effect on the expression of PI3K (Figure 1b).

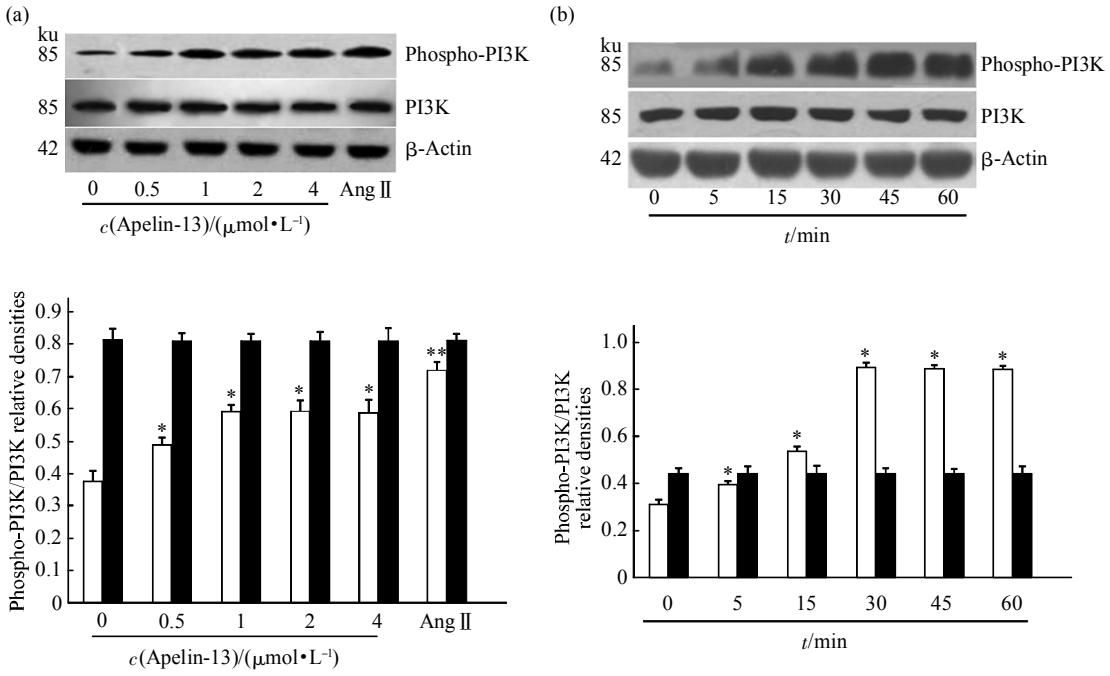

Fig. 1　Dose(a) and time(b) effect of apelin-13 on expression of phospho-PI3K and PI3K in endothelial cells

(a) Treated for 30 min. The data represent the $\bar{x} \pm s$ (n=3), $^*P < 0.05$,$^{**}P < 0.01$ *vs*. apelin-13 0 μmol/L. (b) Apelin-13, 1 μmol/L. The data represent the $\bar{x} \pm s$ (n=3), $^*P < 0.05$ *vs*. apelin-13 0 min. □: p-PI3K; ■: PI3K.

2.2　PI3K inhibitor LY294002 inhibits the phosphorylation of PI3K and expression of VCAM-1 induced by apelin-13 in endothelial cells

After 1 h pretreated with PI3K inhibitor LY294002 at the concentration of 25 μmol/L, HUVECs were stimulated with 1 μmol/L apelin-13 for 12 h and phospho-PI3K, VCAM-1 in HUVECs were detected by Western blotting. The results showed that LY294002 significantly inhibited the expression of phospho-PI3K and VCAM-1 induced by apelin-13. And LY294002 and solvent control group DMSO had no effect on the expression of PI3K (Figure 2).

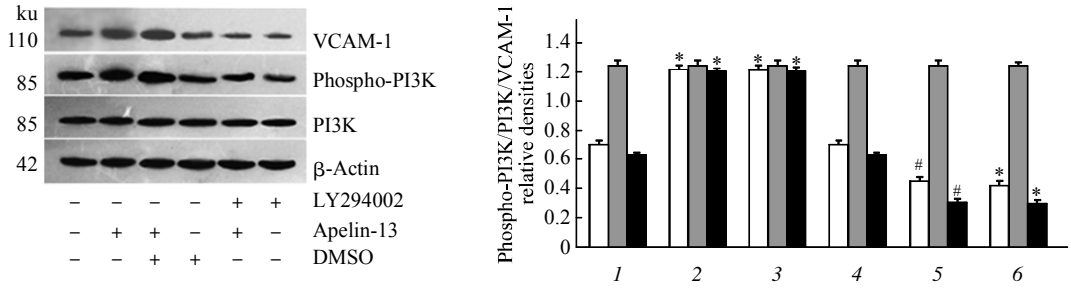

Fig. 2　PI3K inhibitor LY294002 inhibits the apelin-13-induced phosphorylation of PI3K and expression of VCAM-1 in endothelial cells

The data represent the $\bar{x} \pm s$ (n=3), $^*P < 0.05$ *vs*. control, $^{\#}P < 0.05$ *vs*. apelin-13. □: p-PI3K; ▩: PI3K; ■: VCAM-1. *1*: Control; *2*: Apelin-13; *3*: Apelin-13+DMSO; *4*: DMSO; *5*: Apelin-13+LY294002; *6*: LY294002.

2.3 PI3K inhibitor LY294002 inhibits apelin-13-induced monocytes adhesion to endothelial cells

To confirm the effect of PI3K inhibitor on MCs adhesion to HUVECs induced by apelin-13, HUVECs were pretreated with PI3K inhibitor LY294002 [40-41] at concentration of 25 μmol/L for 1 h. And then THP-1 cells suspension was added to HUVECs and stimulated with 1 μmol/L apelin-13 for 12 h. *A* values were taken at 450 nm. And the results showed that LY294002 significantly inhibited the MCs adhesion to HUVECs induced by apelin-13. Solvent control group DMSO had no effect on MCs adhesion to HUVECs (Figure 3).

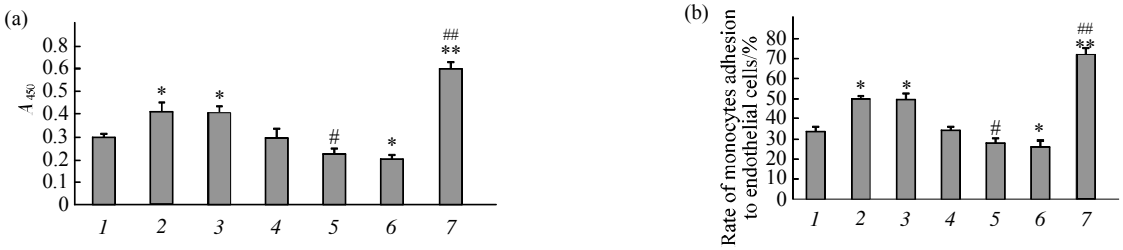

Fig. 3 Effect of PI3K inhibitor LY294002 on MCs adhesion to HUVECs induced by apelin-13

HUVECs and MCs were pretreated with PI3K inhibitor LY294002 for 1 h. And then THP-1 cells suspension was added to HUVECs, with or without stimulation with apelin-13 for 12 h. *A* values (a) were taken at 450 nm and the adhesion rates were illustrated (b). The data represent the $\bar{x} \pm s$ (n=6). $^{*}P < 0.05$,$^{**}P < 0.01$ *vs*. control, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs*. apelin-13. *1*: Control; *2*: Apelin-13; *3*: Apelin-13+DMSO; *4*: DMSO; *5*: Apelin-13+LY294002; *6*: LY294002; *7*: Ang Ⅱ.

3 Discussion

Atherosclerosis is a chronic inflammatory process that is characterized by the formation of plaques consisting of foam cells, immune cells, vascular endothelial cells (ECs), smooth muscle cells (SMCs), platelets, extracellular matrix, a lipid-rich core with extensive necrosis and fibrosis of surrounding tissues [42-43]. Numerous documents showed the role of vascular adhesion molecules in the development and progression of atherosclerosis. Increased expression of adhesion molecules by the activated endothelium is a critical feature of atherosclerosis. The expression of VCAM-1 is induced by arterial endothelial cells in response to accumulation of cholesterol within the intima of aortas [44]. VCAM-1-and ICAM-1-dependent recruitment of immune cells through intimal neovasculature may participate in atherosclerosis[45].

Phosphatidylinositol 3-kinase (PI3K) is a member of lipid kinase family and it was considered as an effector of protein tyrosine kinase (PTK) [46]. PI3K are identified as 3 classes (class Ⅰ, Ⅱ, Ⅲ) according to substrate specificity and activated pathway[47]. *In vivo*, class Ⅰ PI3K can phosphorylate PIP2 to PIP3 and class Ⅲ promote phosphatidylinositol to PtdIns P3 but the function of class Ⅱ PI3K is controversial, it probably acts on both PIP3 and PtdIns P3 [48]. Sato *et al.* [49] indicated 3-phosphoinositide-dependent protein kinase-1 (PDK1) and played a central role in activating the protein kinase A, G, and C subfamily. In particular, PDK1 played an important role in regulating the Akt survival pathway by phosphorylating Akt on Thr-308. This suggested that its activity is regulated by binding to other proteins, such as protein kinase C-related kinase-2 (PRK2), p90 ribosomal protein S6 kinase-2 (RSK2), and heat-shock protein 90 (Hsp90).

Apelin, shown to be an adipokine secreted from the adipocytes, has been suggested to be involved in the regulation of food intake and energy homeostasis and prevent obesity [50]. It was reported that apelin stimulated central neurons that may play roles in the regulation of gastric acid, and hypothalamic neurons that may play roles in the maintenance of body fluid homeostasis as well as other physiological functions[51]. Also apelin was involved in the regulation of blood vessel diameter during angiogenesis [52] and the maturation of bovine corpus luteum [53]. Moreover apelin was a mitogenic peptide for HUVECs and activation of endothelial cell proliferation linked to p70S6K though PI3K pathway[54].

Endothelin-1 mediated vascular inflammation and neointima formation following vascular injury and promoted vasoconstriction and cell proliferation [55]. Also, apelin/APJ signaling participated in retinal vascularization in a cooperative manner with VEGF or FGF2, and contributed to normal ocular development[56]. It is reported that APJ deficiency is preventative against oxidative stress-linked atherosclerosis [57]. In

addition, the elevation in apelin expression during hypoxia and inflammation in the gastrointestinal tract functions in part to stimulate epithelial cell proliferation[58].

Our previous study showed that apelin-13 promoted the expression of VCAM-1 in time and dose dependent manner and the peak reached at 12 h and 1 μmol/L respectively, increased monocytes adhesion to HUVECs [37]. Now we found that PI3K inhibitor LY294002 significantly inhibited the effect of apelin-13 on promoting the monocytes adhesion to HUVECs. This result suggested that apelin-13 promoted monocytes adhesion to HUVECs mediated by phospho-PI3K. Data showed that apelin-13 promoted the expression of phospho-PI3K in time and dose dependent manner and LY294002 significantly inhibited the expression of phospho-PI3K and VCAM-1 in HUVECs induced by apelin-13. These results implied that apelin-13 regulated PI3K phosphorylation to influence the monocyte adhesion to HUVECs. Previously we reported that 14-3-3 signal protein mediated the induction of adhesion of MCs to HUVECs by apelin-13 [37]. In this paper we found apelin-13 induced monocytes adhesion to human umbilical vein endothelial cells by PI3K signal pathway. We presume that 14-3-3 probably combine with PI3K to modulate the activation of PI3K in this process as a chaperon protein.The whole procession is illustrated in Figure 4.

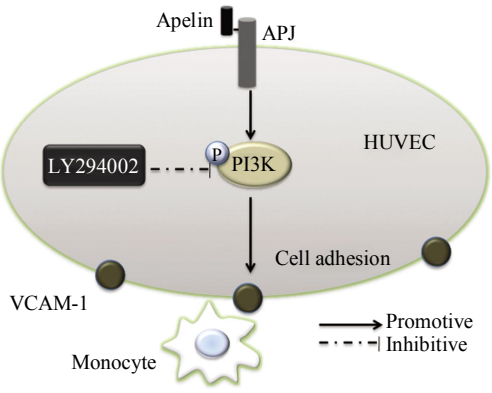

Fig. 4 Molecular mechanism of monocytes adhesion to HUVECs induced by apelin-13/PI3K signaling pathway

Apelin combining with APJ receptor induces PI3K phosphorylation and expression of VCAM-1, thus promotes the monocytes adhesion to HUVECs. These effects can be verified by treating with PI3K inhibitor LY294002. LY294002 can obviously inhibit the adhesion activity and the expression of VCAM-1 induced by apelin-13. PI3K signaling pathway is probably involved in the procession that monocytes adhesion to vascular endothelial cells.

In vivo, normal cells rarely express adhesion molecule such as VCAM-1. But it was found VCAM-1 and ICAM-1 expressed at atherosclerotic plaque and tumor. It should be noted that this study has examined only the cells adhesion cultured *in vitro*. The basic level of adhesion of momocytes and HUVECs is high in our experiment. It is probably due to the endothelial cells cultured *in vitro* suffered slight impairment and the lacking of fluid shear stress of blood enhanced the adhesion activity. It will need further study to confirm the effect of apelin-13 on monocytes adhesion to isolated vascular endothelium and *in vivo* response. Some studies showed that apelin inhibited macrophage infiltration in blood vessels. However, our data indicate that apelin seems to be proatherogenic.We speculated that the the function of APJ subtypes play different roles in different tissue and target sites. And the apelin content could probably cause different reaction. Also, different animal species probably respond differently to apelin. More researches are needed to discover the facts.

In conclusion, our study demonstrated that apelin could promote monocytes adhesion to HUVECs. Furthermore we showed the promoting effect of apelin acted by PI3K signaling. Apelin-13 promoted monocytes adhesion to HUVECs through VCAM-1 mediated by PI3K signal cascades. While the effect of blocking APJ receptor and the detailed signaling pathway involved in this process will be explored in our further study.

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PI3K 信号通路介导 apelin-13 促进单核细胞-血管内皮细胞黏附的研究 *

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摘要 本室以前已经报道 G 蛋白偶联受体 APJ 的内源性配体多肽 apelin-13 促进单核细胞 - 血管内皮细胞黏附，本文研究 PI3K 信号途径是否参与 apelin-13 促进单核细胞 - 血管内皮细胞黏附，探讨 apelin/APJ 系统的细胞信号转导机制. MPO 方法检测细胞黏附；Western blot 方法检测 PI3K、VCAM-1 的表达. Western blot 方法结果显示，apelin-13 (0、0.5、1、2、4 μmol/L) 浓度依赖性刺激血管内皮细胞 PI3K 磷酸化，以 1 μmol/L最为明显；1 μmol/L apelin-13 时间依赖性促进血管内皮细胞 PI3K 磷酸化，在 30 min 增加最为显著； PI3K 抑制剂 LY294002 明显抑制 apelin-13 诱导的 VCAM-1 表达和单核细胞 - 血管内皮细胞黏附. 上述结果表明，PI3K 信号途径介导 apelin-13 促进单核细胞 - 血管内皮细胞黏附.

关键词 apelin，APJ，PI3K，血管内皮细胞，单核细胞
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