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Original Article

14-3-3 mediates apelin-13-induced enhancement of adhesion of monocytes to human umbilical vein endothelial cells

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To investigate whether apelin-13 induced THP-1 monocytes (MCs) adhesion to ECV304 human umbilical vein endothelial cells (HUVECs) via 14-3-3 signaling transduction pathway and the potential novel physiological function and signaling transduction pathway of apelin-APJ, HUVECs ECV304 were cultured in DMEM and MCs THP-1 were cultured in RPMI 1640 medium. Monocyte adhesion and the expression of vascular cell adhesion molecule-1 (VCAM-1) and 14-3-3 were measured with monocyte adhesion assay and western blot analysis. Data showed that apelin-13 increased adhesion of MCs to HUVECs in a concentration- and time-dependent manner, which reached their peaks at 1 μM and 12 h, respectively. Similarly, apelin-13 induced the expression of HUVECs adhesion molecule, VCAM-1, in a concentration- and time-dependent manner, reached their peaks at 1 μM and 12 h, respectively. Apelin-13 induced the expression of 14-3-3 in a concentration- and time-dependent manner, which reached their peaks at 1 μM and 5 min, respectively. Furthermore, the potent 14-3-3 inhibitor difopein significantly reduced the expression of 14-3-3 and VCAM-1 in apelin-13 stimulated HUVECs, and difopein significantly inhibited the effect of apelin-13 on induction of MCs adhesion to HUVECs. These data suggested that 14-3-3 mediated the induction of adhesion of MCs to HUVECs by Apelin-13.

Keywords apelin; APJ; cell adhesion; 14-3-3; VCAM-1; monocyte; endothelial cell

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Introduction

APJ [putative receptor protein related to the angiotensin (Ang) receptor AT1], an orphan G protein-coupled receptor (oGPCR), was first identified in a human gene [1]. Orphan GPCRs are GPCRs that do not yet have natural ligand or endogenous ligand [2]. Apelin, a peptide recently isolated from bovine stomach extracts, has been shown to act as an endogenous ligand for the APJ receptor [3]. The pre-proteins consist of 77 amino acid residues, with the apelin active sequence in the C-terminal regions [4]. Because the C-terminal portion of pre-proapelin is rich in basic amino acid residues, endogenous Apelin may be processed in tissues in several forms, including apelin-36 and apelin-13 [5]. When compared with other forms of apelin, apelin-13 shows a greater biological activity, which suggests that apelin-13 might be the main endogenous ligand for the APJ receptor [5]. Apelin is a novel peptide that acts through the APJ receptor, to share similarities with the Ang II-Ang II type 1 (AT1) receptor pathway. It is a peripheral vasodilator, powerfully inotrope and may affect central fluid homeostasis [6,7]. Animal and human studies suggested that it might play a role in the pathogenesis of heart failure by modulating the harmful effects of Ang II [8,9]. The diastolic reactivity of apelin in *ex vivo* vascular rings of SHR (spontanously hypertensive rat) is reduced and the effect is mediated by NO pathway and the ERK1/2 pathway [10]. High levels of both APJ and apelin mRNA are found in cardiac myocytes, vascular smooth muscle, and endothelial cells of large conduit arteries, coronary vessels, and endocardium of the right atrium [11–13]. The structure of apelin family is highly conserved between species, suggesting an important physiological role.

Members of the 14-3-3 protein family are ubiquitously expressed small acidic proteins with a molecular mass of approximately 30 kDa [14]. Seven highly conserved 14-3-3 protein isoforms (β, γ, ε, η, σ, τ, ζ) exist in mammals, which form homo- and heterodimers [15]. Generally, 14-3-3 proteins function as adaptors that bind to their target proteins in a phosphorylation-dependent manner. Consensus motifs for binding are RSxpSxP and RxxxpSxP, with phosphothreonine capable of replacing phosphoserine.

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But in some cases interaction has been shown to be independent of phosphorylation. Binding of 14-3-3 proteins often sequesters the target protein in a particular subcellular compartment and the release of 14-3-3 proteins then allows the target to relocate. This relocation is often due to the exposure of an intrinsic subcellular targeting sequence masked by the 14-3-3 protein dimer. Binding of 14-3-3 proteins can also induce conformational changes of the target protein or may have a scaffolding function [14,15]. 14-3-3 proteins bind to a variety of signal transduction, checkpoint control, and apoptotic pathway proteins including RAF, PKC, Cdc25C, KSR, Bcr, ASK and BAD [16–19]. 14-3-3 controls cell cycle, cell growth, differentiation, survival, apoptosis, migration and spreading. Recent studies have revealed new mechanisms and new functions of 14-3-3, giving us more insights into this fascinating and complex family of proteins. Cell adhesion molecules are glycoproteins expressed on cell surface and play an important role in inflammatory as well as neoplastic diseases. There are four main groups: the integrin family, the immunoglobulin superfamily, selectins, and cadherins. Vascular adhesion molecule-1 (VCAM-1) is one member of the immunoglobulin superfamily and its expression is enhanced in endothelial and intimal smooth muscle cells of atherosclerosis-prone regions of aorta [20,21]. Together with other adhesion molecules and chemoattractants, VCAM-1 mediates leukocyte adhesion to the endothelial cells and infiltration of the neointima [22–25]. So far, little is known about the cell signaling transduction of the apelin-APJ system. Previous research has suggested that apelin activates the mitogen-activated protein kinase pathway in a Ras independent manner. Our recent research indicated that apelin-13 promoted cell proliferation [11] and ERK1/2 phosphorylation through PKC and 14-3-3 protein in rat VSMCs (unpublished data). In this study, we investigated the effects of apelin-13 induced monocytes (MCs) adhesion to human umbilical vein endothelial cells (HUVECs) via 14-3-3 signaling transduction pathway.

Materials and Methods

Materials

Antibodies against apelin-13 (057-19) and VCAM-1 (sc-8304) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, USA). Antibodies against 14-3-3 were purchased from Abcam Ltd (Cambridge, UK). Difopein was purchased from Tocris Cookson Ltd (Bristol, UK). The ECL reagent was purchased from Amersham Life Science (Arlington Heights, USA). HUVECs (ECV304) and MCs (THP-1) were purchased from Xiangya School of Medicine, Central South University (Hengyang, China).

HUVECs and MCs culture

HUVECs (ECV304) [26] 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), 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) [26] were cultured in RPMI 1640 under the same conditions as in HUVEC culture.

Monocyte adhesion assay

HUVECs were cultured in 96-well plates at 37°C for 24 h. On the day of treatments, cells were washed with warm PBS and cultured in DMEM with or without apelin or difopein. Cells were cultured for 24 h and washed with warmed PBS. THP-1 cells (5×10^6 cells/ml) were then added to HUVECs in DMEM and allowed to adhere at 37°C 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 assay, as described previously [27]. 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 μl PBS containing 0.5% hexadecyltrimethyl ammonium bromide for 30 min at room temperature. Next, 250 μl warmed *O*-dianisidine dihydrochloride (0.2 mg/ml in PBS, pH 6.0) containing 0.4 mM H_2O_2 was added. After 20 min of incubation at 37°C, OD at 450 nm 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 difopein or apelin+difopein at 37°C before the addition of THP-1 cells. Then, the adhesion assay was performed as described.

Western blot analysis

After treatment, HUVECs were washed twice with ice-cold PBS and lysed in HEPES buffer (20 mM, pH 7.4) containing 150 mM NaCl, 100 mM NaF, 10 mM EDTA, 10 mM $Na_4P_2O_7$, 2 mM sodium orthovanadate, 1 mM PMSF, 2 mg/ml aprotinin, 20 mM leupeptin, and 1% Triton X-100 for 10 min on ice. After clarification of the cell lysates by centrifugation at 13,000 *g* for 15 min, the supernatants were collected and their protein concentrations were determined using a bicinchoninic acid (BCA) protein assay kit (Hyclone Pierce, Logan, USA). Aliquots containing 30 μg of protein were electrophoresed in 10% SDS-polyacrylamide gels and transferred to polyvinylidene

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difluoride membranes. The membranes were blocked in Tris-buffered saline (10 mM Tris–HCl, pH 7.6, 0.15 M NaCl) containing 5% non-fat milk and 0.1% Tween-20 for 1 h at room temperature. The proteins were analyzed with the following antibodies: anti-VCAM-1, anti-14-3-3. The membranes were incubated with a primary antibody diluted in blocking solution for 2 h, and then incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin G as the secondary antibody. Peroxidase activity was detected by enhanced chemiluminescence and analyzed by densitometry using a densitometer and an imager.

Statistical analyses

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 mean ± SEM. Statistical significance was defined as $P < 0.05$.

Results

The effects of apelin-13 on cell adhesion

Different concentrations of apelin (0, 0.5, 1, 2, and 4 μM) and Ang II (1 μM) were used and treated with HUVECs and MCs for 24 h. The rates of MCs adhesion to HUVECs are 46.8 ± 1.6%, 60.8 ± 2.2%, 74.2 ± 1.6%, 80.5 ± 3.5%, 80.9 ± 2.3% and 91.2 ± 1.6%, respectively [**Fig. 1(A)**]. The results show that apelin-13 concentration dependently promotes MCs adhesion to HUVECs, which reached its peak at 1 μM. When treated with HUVECs and MCs for 0, 3, 6, 12, 24 and 48 h by apelin-13 (1 μM), the rates of MCs adhesion to HUVECs are 38.7 ± 2.3%, 50.6 ± 2.4%, 59.1 ± 2.4%, 67.9 ± 3.1%, 69.6 ± 2.3% and 70.0 ± 2.5% [**Fig. 1(B)**]. These data suggested that apelin-13 time dependently promotes MCs adhesion to HUVECs, which reached its peak at 12 h.

The effects of apelin-13 on VCAM-1 expression

Different concentrations of apelin-13 (0, 0.5, 1, 2, and 4 μM) and Ang II (1 μM) were used and treated with HUVECs and MCs for 24 h. Western blotting was conducted to detect the expression of VCAM-1. Results showed that apelin-13 concentration dependently promoted the expression of VCAM-1, which reached its peak at 1 μM [**Fig. 2(A)**]. It was also found that apelin-13 time dependently promoted MCs adhesion to HUVECs, which reached its peak at 12 h [**Fig. 2(B)**].

The effects of apelin-13 on 14-3-3 expression

Different concentrations of apelin (0, 0.5, 1, 2, and 4 μM) and Ang II (1 μM) were used and treated with HUVECs and MCs for 30 min. Western blotting was adopted to detect the expression of 14-3-3. Results showed that apelin-13 concentration dependently promoted the expression of 14-3-3, which reached its peak at 1 μM [**Fig. 3(A)**]. Results of western blot assay also showed that apelin-13 time dependently promoted the expression of 14-3-3, which reached its peak at 5 min [**Fig. 3(B)**].

Difopein inhibits apelin-13-induced 14-3-3 and VCAM-1 expression

HUVECs were preincubated by 1 μM difopein (dimeric 14-3-3 peptide inhibitor) for 1 h, and then treated with apelin-13 (1 μM) for 5 min. Results of western blot assay suggested that difopein significantly inhibited the expression of 14-3-3 protein induced by apelin-13 in HUVECs [**Fig. 4(A)**]. For VCAM-1, 1 μM difopein was used and then HUVECs were treated with apelin-13 (1 μM) for 12 h. Results of western blot assay indicated that difopein significantly inhibited the expression of VCAM-1 protein in HUVECs [**Fig. 4(B)**].

Difopein inhibits apelin-13-induced cell adhesion

HUVECs were preincubated by 1 μM difopein for 1 h, and then were added in MCs and apelin-13 (1 μM) was used to treat with HUVECs and MCs for 12 h. The results of

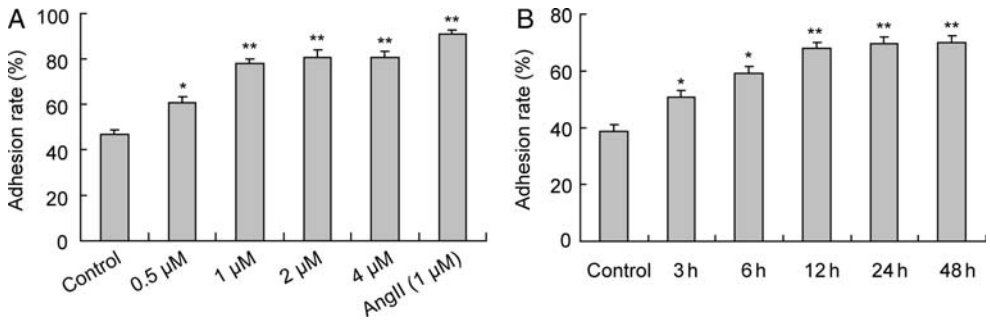

Figure 1 Dose effect of apelin-13 on MCs adhesion to HUVECs (A) Concentration effect of apelin-13 (treating for 24 h) on MCs adhesion rate. (B) Time effect of apelin-13 (1 μM) on MCs adhesion rate. Data are expressed as the mean ± SEM, $n = 6$. *$P < 0.05$ and **$P < 0.01$ vs. control, respectively.

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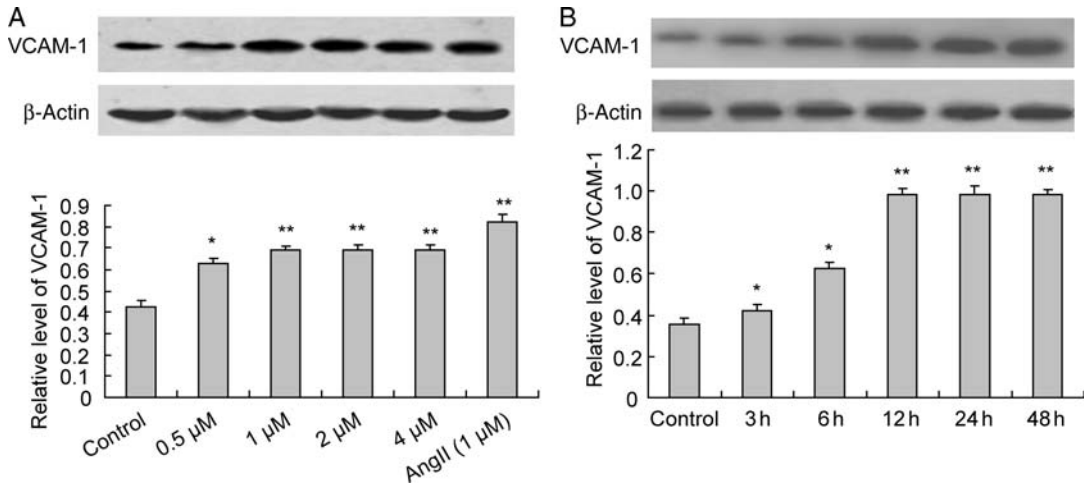

Figure 2 Effect of apelin-13 on expression of adhesion molecule VCAM-1 in HUVECs (A) Concentration effect of apelin-13 (treating for 24 h) on VCAM-1 expression. (B) Time effect of apelin-13 (1 μM) on VCAM-1 expression. Data are expressed as the mean ± SEM, $n = 3$. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. control, respectively.

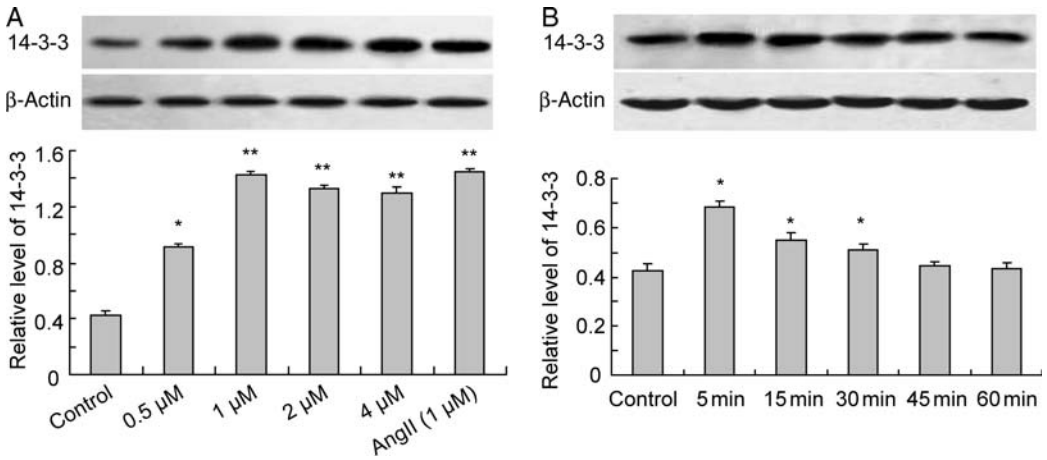

Figure 3 Effect of apelin-13 on expression of 14-3-3 protein in HUVECs (A) Concentration effect of apelin-13 (treating for 30 min) on 14-3-3 protein expression. (B) Time effect of apelin-13 (1 μM) on 14-3-3 protein expression. Data are expressed as the mean ± SEM, $n = 3$. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. control, respectively.

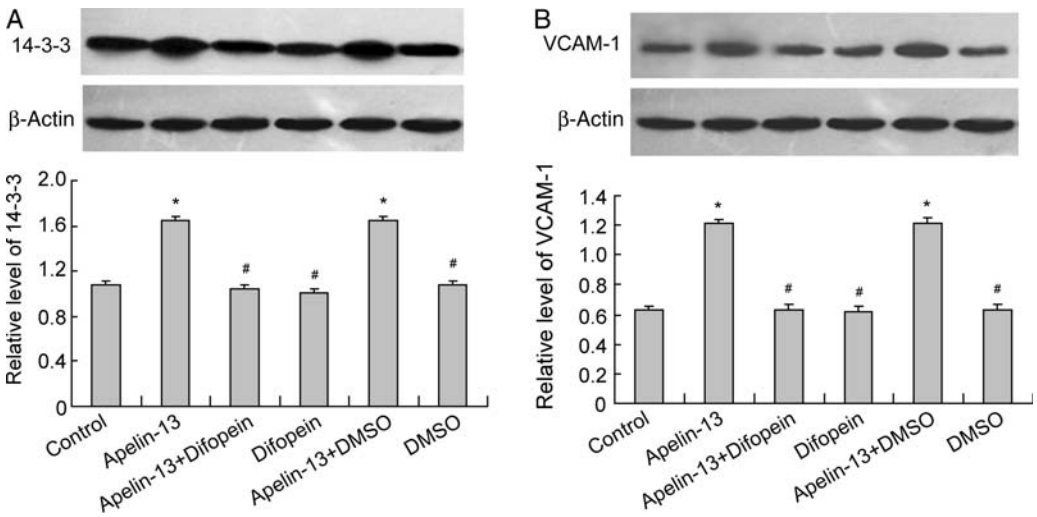

Figure 4 Effect of 14-3-3 inhibitor difopein on the expression of 14-3-3 protein (A) and adhesion molecule VCAM-1 (B), respectively, in HUVECs induced by apelin-13 Data are expressed as the mean ± SEM, $n = 3$. $^{*}P < 0.05$ vs. control and $^{\#}P < 0.05$ vs. apelin-13 group.

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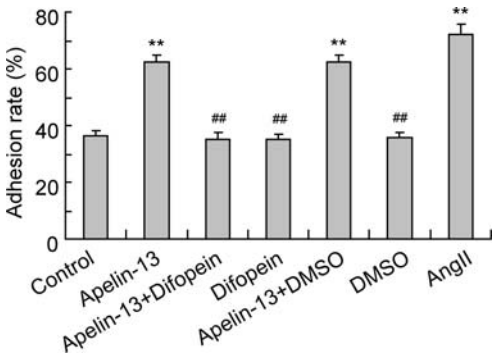

Figure 5 Effect of 14-3-3 inhibitor difopein on MCs adhesion to HUVECs induced by apelin-13 Data are expressed as the mean ± SEM, $n = 6$. The concentration of apelin-13 was 1 μM and treating time is 12 h. $^{**}P < 0.01$ vs. control and $^{\#\#}P < 0.01$ vs. apelin-13 group.

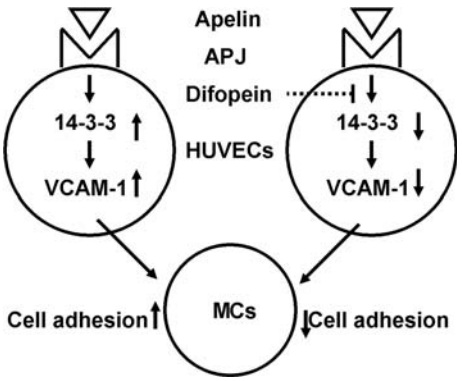

Figure 6 Schematic representation of a model explaining how 14-3-3 mediates apelin-13-induced enhancement of MCs adhesion to HUVECs and 14-3-3 inhibitor difopein significantly inhibits the effect of MCs adhesion to HUVECs induced by apelin-13.

monocyte adhesion assay demonstrate that difopein significantly inhibits the effect of apelin-13 induced MCs adhesion to HUVECs (**Fig. 5**).

Discussion

Apelin, a newly identified Ang II homologue, has been shown to act as an endogenous ligand of the orphan receptor APJ. In addition to the limited homology between apelin and Ang II, there is sequence homology between the APJ and AT1 receptor (54% in the transmembrane regions), and the anatomical distribution of both receptors and peptides overlaps in the cardiovascular system. However, the functions of apelin are independent of AT1 receptor and APJ does not show specific binding of Ang II [28]. More intriguingly, Apelin and Ang II have similar degradation pathways, and both are substrates of angiotensin-converting enzyme 2 (ACE2), which is a critical regulator of the renin–angiotensin system (RAS) and counteracts the function of ACE and Ang II [29–31]. All this implies that apelin and Ang II have the same or similar biological functions. As a predominant effector of RAS, Ang II exerts various deleterious effects via AT1 receptor by promoting vascular contraction, impairing insulin signaling pathways, stimulating productions of growth factor, reactive oxygen species (ROS) and adhesions molecules, causing cardiovascular hypertrophy, endothelial dysfunction and insulin resistance [32–34]. In vascular smooth muscle cells, apelin induced nicotinamide-adenine dinucleotide phosphate oxidase subunit expression. Apelin also induced vascular smooth muscle cell proliferation, which was inhibited by superoxide dismutase or diphenyleneiodonium. The apelin-APJ system is a mediator of oxidative stress in vascular tissue, and a critical factor in atherogenesis under high-cholesterol dietary conditions. APJ deficiency is preventative against oxidative stress-linked atherosclerosis [35]. In animal models of atherosclerosis, vascular cell adhesion molecule-1 (VCAM-1) mediates adhesion, rolling, and tethering of mononuclear leukocytes and facilitates their transmigration to the developing atherosclerotic plaque. VCAM-1 expression is not constitutive, but is present at atherosclerosis-prone sites, even before macroscopic disease is apparent, with persistent expression in more advanced lesions. VCAM-1 is therefore a potentially useful marker for atherosclerosis from early stages. In our study, we found that apelin-13 increased the adhesion of MCs to HUVECs in concentration dependence and time dependence; similarly, apelin-13 induced the expression of HUVECs adhesion molecule VCAM-1 in concentration dependent and time dependent. These results suggested that apelin-APJ system may relate to atherosclerosis, inflammation, and tumor induced by cell adhesion.

Phosphoinositide 3-kinases (PI3Ks) are lipid kinases that phosphorylate inositol phopholipids at the 3′-OH of inositol ring, acting through the downstream kinase AKT, regulate a number of cellular processes such as cell growth and survival [36]. There are some researches about the relation between PI3K and cell adhesion [37–39]. In our study, 14-3-3 mediated the effect of apelin-13-induced MCs adhesion to HUVECs and the 14-3-3 inhibitor difopein significantly reduced the expression of 14-3-3 and VCAM-1 in apelin-13 stimulated HUVECs. But, what is the relationship between 14-3-3 and PI3K? The interaction of PI3K with 14-3-3 can regulate a variety of cellular functions, including spreading, migration, proliferation, and apoptosis [40,41]. So they may also regulate cell adhesion through binding, but the specific molecular mechanism is unclear.

Many organisms contain multiple 14-3-3 isoforms, however, so far, it remains unclear whether isoforms have specialized biological functions. Are these based on temporal and tissue-specific expression regulation and/or isoform-specific ligand binding? In addition, ambiguous and conflicting data have been reported on the specific

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subcellular distribution of isoforms [42]. In our study, we found that 14-3-3 mediated the effect of apelin-13-induced MCs adhesion to HUVECs, but it is still not clear, which isoform mediates the effect and how to mediate the effect. What are the 14-3-3 isoforms involved in the process of cell adhesion? The resolving of these issues will help us further understand the molecule mechanisms of endothelial cell adhesion monocyte and may provide a number of intervention measures for clinical immune inflammatory diseases. In our study, we found 14-3-3 mediated the effect of apelin-13 induced MCs adhesion to HUVECs, which is the new physiological function and signaling pathway of apelin-APJ (**Fig. 6**). Further investigation will enrich the knowledge about the physiological significance of the apelin-APJ signaling in the cardiovascular system.

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