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14-3-3 Is Involved in ERK1/2 Signaling Pathway of Rat Vascular Smooth Muscle Cells Proliferation Induced by Apelin-13*

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Abstract Previously, we found that G protein-coupled receptor APJ endogenous ligand apelin-13 stimulates vascular smooth muscle cells (VSMC) proliferation mediated in part by PKC-PI3K-ERK1/2-cyclinD1 signaling cascades. In this study, Raf-1-14-3-3 signaling in rat VSMCs proliferation stimulated by apelin-13 was further investigated. Cell proliferation was measured with MTT assay. Expression of PI3K, phospho-PI3K, Raf-1, phospho-Raf-1, ERK1/2, phospho-ERK1/2, cyclinD1 and cyclinE were detected by Western blotting. 14-3-3 protein combining with Raf-1 was detected by immunoprecipitation. Here, we demonstrated that apelin-13 increased the expression of 14-3-3, Raf-1 phosphorylation and ERK1/2 phosphorylation in a concentration- dependent and time-dependent manner at 0～4 μmol/L and 0～48 h. 14-3-3 inhibitor Difopein decreased the apelin-13-induced Raf-1 phosphorylation, ERK1/2 phosphorylation, expression of cyclinD1 and cyclinE. Furthermore, apelin-13 promoted the combination of 14-3-3 protein and Raf-1, Difopein significantly inhibited the combination of 14-3-3 and Raf-1 stimulated by apelin-13. Similarly, Difopein significantly inhibited the VSMCs proliferation stimulated by apelin-13. Our results revealed that Raf-1 +14-3-3-ERK1/2 signaling cascades mediated the effect of apelin-13 on rat VSMCs proliferation .

Key words apelin, APJ, 14-3-3, cyclin, ERK1/2, Raf-1, vascular smooth muscle cells
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APJ, a G protein-coupled receptor (GPCR), was first identified in a human gene[1-2]. 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-protein has 77 amino acid residues, with active sequence the apelin in the C-terminal regions[4]. Because the C-terminal portion of preproapelin is rich in basic amino acid residues, endogenous apelin has several forms in tissues including apelin-36 and apelin-13[5]. Compared with other isoforms, apelin-13 shows a greater biological activity, which suggests that apelin-13 might be the main endogenous ligand for the APJ receptor[5]. However, evidences indicate that the cardiovascular

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system is one of the targets of apelin. 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 [6-7]. The structure of apelin family is highly conserved between species, suggesting that it may play important physiological roles.

Evidences suggested that apelin/APJ system may play an important roles in cardiovascular disorder, nerve system, obesy, diabetes, gastrointestinal disorders, inflammation and other tissues[8].

Our previous research showed that apelin-13 promoted ERK1/2 phosphorylation in a concentration-dependent manner. Recently we discovered that apelin/APJ system regulates VSMCs proliferation through PKC-PI3K-ERK1/2-cyclinD1 signaling pathway [9-11]. The diastolic reactivity of apelin in *ex-vivo* vascular rings of spontanously hypertensive rat is reduced and the effect is mediated by nitric oxide(NO) pathway and the ERK1/2 pathway [12]. Our results showed that ERK1/2 is an important signaling protein in apelin/APJ system, and that phosphorylative regulation of ERK1/2 is a key function of biology. But signaling cascade that apelin-13 promotes ERK1/2 phosphorylation remains unclear.

14-3-3 proteins belong to a family consisting of highly conserved proteins, with molecular mass of 27～33 ku, widely expressed acidic polypeptides that spontaneously self-assemble as dimers. 14-3-3 has at least seven mammalian isoforms in all eukaryotic cells (α/β, γ, ε, η, σ, τ (θ) and ζ / δ, α and δ isoforms are the phosphoforms of β and ζ , respectively)[13]. 14-3-3 proteins bind to Raf-1, PKC, KSR, Bcr, ASK and BAD to control cell cycle, cell growth, differentiation, survival, apoptosis, migration and spreading[14-15].

So far, little is known about the cell signaling transduction of the apelin/APJ system. Previous researches suggested that apelin activates the mitogen-activated protein kinase pathway in a Ras-independent manner in Chinese hamster ovary (CHO) cells[16]. We presume that PKC-Raf-1-ERK1/2 cascade of Ras-independent may be involved in GPCR APJ function. 14-3-3 may combine to Raf-1 and phosphorylate Raf-1. Recently we reported that 14-3-3 mediated the induction of adhesion of THP-1 monocytes (MCs) to human umbilical vein endothelial cells (HUVECs) by apelin-13 [17]. It suggests that 14-3-3 signaling pathway may be involved in VSMC proliferation induced by apelin-13. Our results indicated that apelin-13 increases Raf-1 phosphorylation and 14-3-3 expression, promotes 14-3-3 and Raf-1 combination to induce ERK1/2 phosphorylation, increases rat VSMCs proliferation.

1 Materials and methods

1.1 Cell culture and chemicals

Cell cultures of vascular smooth muscle cells (vascular smooth muscle cells) from the thoracic aortas of 7～8-week-old male Sprague-Dawley rats were prepared by an explant method and cultured in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS)[9] in a 5% CO_2 humidified-atmosphere incubator at 37℃ until they displayed a typical "hill and valley" morphology. Immunohistochemical staining with a monoclonal antibody against α-actin confirmed that there were no co-cultured fibroblasts. Only vascular smooth muscle cells from passages 5 to 8 were used. The cells were grown to 70%～80% confluence and then rendered quiescent by incubation with DMEM containing 0.1% FBS for 24 h. The following materials were supplied as indicated:[pGlu1]-Apelin-13 (Human, Bovine) (Phoenix Pharmaceuticals, Inc.); rabbit anti-ERK1/2, mouse anti-p-ERK1/2 and rabbit anti-β-actin (Santa Cruz Biotechnology, Inc.); Difopein (Tocris Cookson Ltd.); MTT (Amresco, Inc.); Protein A-Agarose (Merck Biosciences, Inc.); rabbit phospho-Raf (Ser259) and rabbit Raf-1 antibody (Cell Signaling Technology, Inc.); rabbit anti-14-3-3 (Abcam Ltd.); rabbit anti-cyclinD1 and rabbit anti-cyclinE (Boster Biotechnology); Male Sprague-Dawley rats (Department of Zoology of University of South China).

1.2 Western blotting

VSMCs were harvested and lysed in RIPA lysis buffer(50 mmol/L pH 7.4 Tris-HCl, 150 mmol/L NaCl, 10 mmol/L NP-40，5 mmol/L deoxycholic acid，1 mmol/L SDS，1 mmol/L EDTA). Cellular protein was loaded and separated on sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) and transferred to a PVDF membrane (Millipore) by the standard electric transfer protocol. The membrane was blocked and probed with primary antibodies, then incubated with horseradish peroxidase-labeled second antibody. The primary antibody specificity was detected without antibody.The membrane was then exposed to an

enhanced chemiluminescent system (Pierce) and autoradiography was used to visualize immunoreactive bands. Results analyzed by densitometry using a densitometer and an imager, showed in relative to internal reference β-actin.

1.3 MTT assay

The proliferation potential of cultured VSMCs was determined by measuring the reduction of 3-(4, 5- dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) to formazan as described previously[9].

1.4 Immunoprecipitation and immunoblotting

VSMCs were washed twice with PBS and solubilized with 1 ml cell lysis buffer (50 mmol/L pH 7.6 Tris-HCl, 150 mmol/L NaCl, 1% NP-40, 0.1 mol/L NaF, 10 mmol/L Na_3VO_4, 5 mmol/L EDTA, 1 mmol/L PMSF, and 2U Aprotinin). After incubation for 1 h on ice, lysates were centrifuged at 15 000 r/min for 15 min at 4℃. Two microlitres of phospho-Raf (Ser259) antibody were added to the supernatant and incubated on a rotator for 2 h at 4℃. Following the incubation, Protein A-Agarose beads (20 μl of 50% bead slurry) were added to the mixture and incubated on a rotator for 1 h at 4℃. The beads were collected by centrifugation and washed six times with 1 ml of lysis buffer.

1.5 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 Dose-dependent effects of apelin-13 on expression of 14-3-3, Raf-1 phosphorylation and ERK1/2 phosphorylation in rat VSMCs

VSMCs were incubated for 24 h with apelin-13 at 0, 0.5, 1, 2, 4 μmol/L, 10% FBS or 1 μmol/L angiotensin Ⅱ (Ang Ⅱ). Western blotting results showed that apelin-13 concentration-dependently increased the expression of 14-3-3, Raf-1 phosphorylation and ERK1/2 phosphorylation, but Raf-1 and ERK1/2 expression had no significant changes. The positive control groups of 10% FBS and 1 μmol/L Ang Ⅱ significantly increased the expression of 14-3-3, Raf-1 phosphorylation and ERK1/2 phosphorylation(Figure 1).

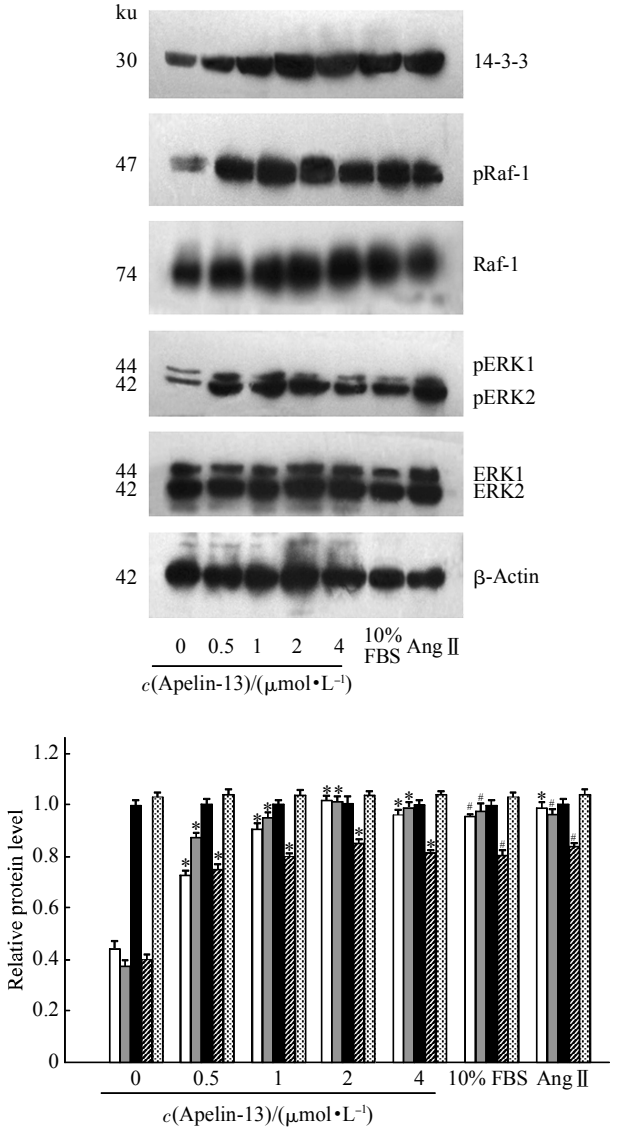

Fig. 1 Concentration effects of apelin-13 on the expression of 14-3-3, phospho-Raf-1, Raf-1, phospho-ERK1/2 and ERK1/2 in VSMCs

VSMCs were serum-starved for 24 h, and then stimulated with apelin-13 for 24 h. Following cell lysis, the lysates were separated by SDS-PAGE and immuno-blotted. The Western blotting was probed with rabbit anti-14-3-3, Raf-1 and phospho-Raf-1 antibodies, mouse anti- phospho-ERK1/2 and rabbit anti-ERK1/2 antibodies. The data represent the $\bar{x} \pm s$ (n=3). $^*P < 0.01$, $^\#P < 0.05$, *vs* apelin-13 (0 μmol/L). □: 14-3-3; ▨: pRaf-1; ■: Raf-1; ▨: pERK1/2; ▨: ERK1/2.

2.2 Time-dependent effects of apelin-13 on expression of 14-3-3,Raf-1 phosphorylation and ERK1/2 phosphorylation in rat VSMCs

VSMCs were incubated with apelin-13(2 μmol/L) for 0, 0.5, 1, 2, 4, 6, 12, 24 and 48 h. Western blotting results showed that apelin-13 increased expression

of 14-3-3, Raf-1 phosphorylation, and ERK1/2 phosphorylation, peaking at 4 h, and then gradually declining. But Raf-1 and ERK1/2 had no significant changes (Figure 2).

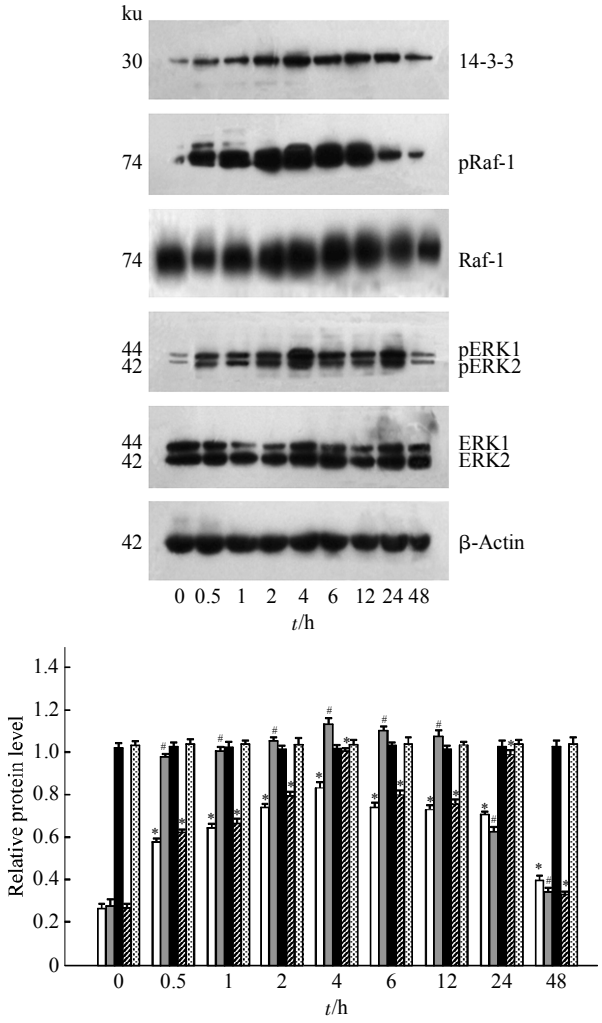

Fig. 2 Time effects of apelin-13 on the expression of 14-3-3 protein, phospho- Raf-1, Raf-1, phospho-ERK1/2 and ERK1/2 in VSMCs

Following cell lysis, the lysates were separated by SDS-PAGE and immunoblotted. The Western blotting was probed with rabbit anti-14-3-3, Raf-1 and phospho-Raf-1 antibodies, mouse anti-phospho-ERK1/2 and rabbit anti-ERK1/2 antibodies. The data represent the $\bar{x} \pm s$ (n=3). $^{*}P<0.01$, $^{\#}P<0.05$ *vs*. apelin-13(2 μmol/L, 0 h). □ : 14-3-3; ▩ : pRaf-1; ■ : Raf-1; ▨ : pERK1/2; ▦ : ERK1/2.

2.3 Effects of 14-3-3 inhibitor Difopein on Raf-1 phosphorylation, ERK1/2 phosphorylation, expression of cyclinD1 and cyclinE induced by apelin-13 in rat VSMCs

VSMCs were pre-incubated for 1 h with 1 μmol/L Difopein, the competitive inhibitor of 14-3-3-ligand interactions[18], was followed by treatment with 2 μmol/L apelin-13 for 4 h. Western blotting results showed that Difopein inhibited 14-3-3 protein expression induced by apelin-13 and decreased Raf-1 phosphorylation, ERK1/2 phosphorylation, expression of cyclinD1 and cyclinE induced by apelin-13. However, Raf-1 and ERK1/2 expression had no significant change(Figure 3). DMSO had no effect on protein expression.

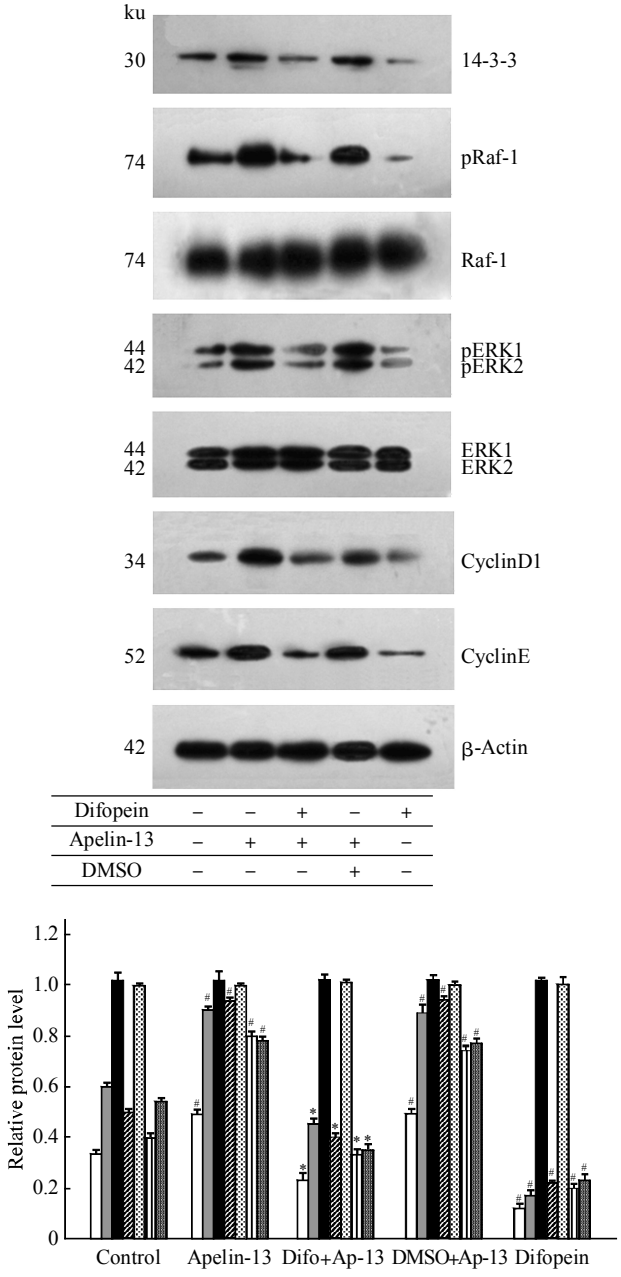

Fig. 3 Effects of Difopein, a competitive inhibitor of 14-3-3-ligand interactions, on 14-3-3 protein, phospho-Raf-1, Raf-1, phospho-ERK1/2, ERK1/2, cyclinD1 and cyclinE activities induced by apelin-13 in VSMCs

After serum-starvation for 24 h, VSMCs were incubated with or without Difopein for 1 h, and then incubated with apelin-13 (2 μmol/L) for 4 h. DMSO +apelin-13 treatment shows no significant differences from apelin-13 treatment. The data in the bottom panel represent the $\bar{x} \pm s$ (n=3). $^{*}P < 0.01$ *vs*. apelin-13, $^{\#}P < 0.05$ *vs*. control. □ : 14-3-3; ▩ : pRaf-1; ■ : Raf-1; ▨ : pERK1/2; ▦ : ERK1/2; ▥ : CyclinD1; ▧ : CyclinE.

2.4 Combination between 14-3-3 and Raf-1 in rat VSMCs induced by apelin-13

There were five groups in this experiment: control group, apelin-13(2 μmol/L) group, Difopein(1 μmol/L)+ apelin-13(2 μmol/L) group, DMSO+apelin-13(2 μmol/L) group and Ang Ⅱ (1 μmol/L) positive control group. The immunoprecipitation results suggested that apelin-13 promoted the combination of 14-3-3 and Raf-1. Furthermore, 14-3-3 inhibitor Difopein significantly inhibited the apelin-13-induced combination of 14-3-3 and Raf-1 (Figure 4).

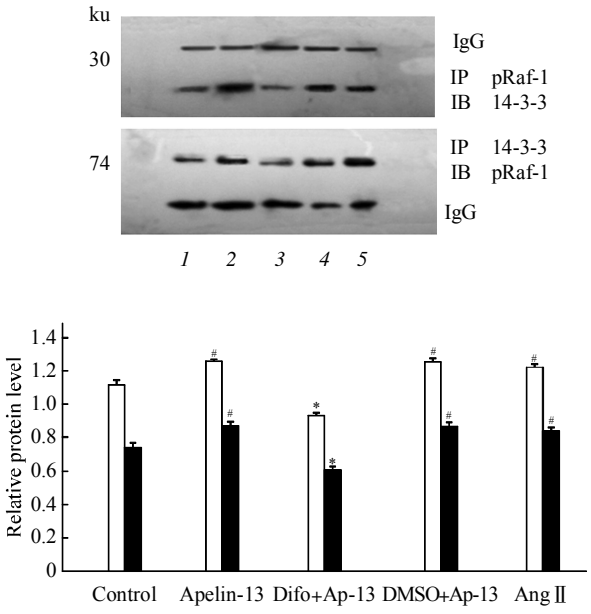

Fig. 4 Effects of Difopein on the combination of 14-3-3 protein and Raf-1 in VSMCs

After serum-starvation for 24 h, VSMCs were incubated with or without Difopein for 1 h, and then incubated with apelin-13 (2 μmol/L) for 4 h. DMSO +apelin-13 treatment shows no significant differences from apelin-13 treatment. The data in the bottom panel represents the $\bar{x} \pm s$ (n=3). *P < 0.01 *vs*. apelin-13, #P < 0.05 *vs*. control. □: 14-3-3; ■: pRaf-1. *1*: Control; *2*: Apelin-13; *3*: Apelin-13; *4*: Difopein+Apelin-13; *5*: DMSO+Ang Ⅱ.

2.5 Effects of 14-3-3 inhibitor Difopein on rat VSMCs proliferation induced by apelin-13

MTT analysis was used to illustrate the effects of Difopein on VSMCs proliferation stimulated by apelin-13. Six groups were used for this experiment: control group, apelin-13 group, Difopein +apelin-13 group, solvent DMSO group, DMSO+apelin-13 group and Ang Ⅱ positive control group. The concentrations used for apelin-13, Difopein and Ang Ⅱ were 2 μmol/L, 1 μmol/L and 1 μmol/L respectively. VSMCs were preincubated with Difopein for 1 h, or with apelin-13 for 24 h (Figure 5). As showed by MTT ananylsis, Difopein significantly reduced VSMCs proliferation induced by apelin-13. Again, resolution DMSO had no effect on cell proliferation.

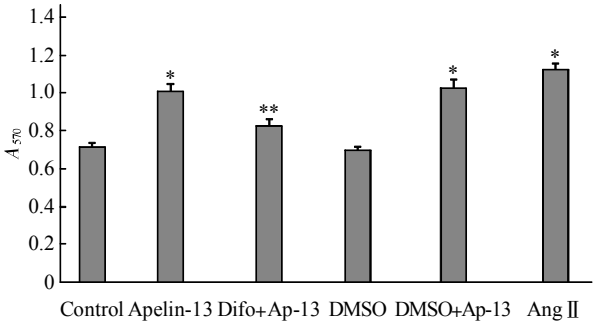

Fig. 5 Effects of Difopein on VSMCs proliferation induced by apelin-13

Cells at passages three were plated in 96-well plates. After synchronization for 24 h, the cells were incubated with or without Difopein or DMSO for 1 h, and then incubated with apelin-13(2 μmol/L) for 24 h. The data represents the $\bar{x} \pm s$ (n=6). *P < 0.01 *vs*. control, **P < 0.01 *vs*. apelin-13.

3 Discussion

APJ shares significant homology with the angiotensin receptor AT1, suggests that apelin/APJ system may participate in VSMCs proliferation similar to Ang Ⅱ /AT1 system in PKC-Raf-1 cascade of Ras-independent pathway[16]. Previously we found that PKC-Raf-1 cascade involved in rat VSMC proliferation induced by apelin-13 [10]. Apelin was reported to stimulate MC3T3-E1 cell proliferation *via* the JNK and PI3K/Akt signaling pathways[19]. In addition, apelin can enhance human osteoblast proliferation, and the APJ/PI3K/Akt pathway is involved in the proliferation. These findings suggest that apelin may be a mitogenic agent for human and animal tissue cells [20]. It is an available evidence that APJ deficiency is preventative against oxidative stress-linked atherosclerosis[21–22].

14-3-3 proteins as an adaptor or "chaperone molecule" are mainly cytoplasmic molecules [23–24]. Autieri *et al*. [25] indicated that 14-3-3γ expression increases in response to vessel damage and proliferative signals and may implicate a role for the γ isoform of 14-3-3 in VSMC activation and metabolism. In light of these reports of 14-3-3 protein involvement in cell proliferation, 14-3-3 protein may be considered a hallmark of vascular restenosis pathological and

physiological functions. Recently we found that 14-3-3 mediated the induction of adhesion of monocytes to human umbilical vein endothelial cells by apelin-13[17]. Here we found that 14-3-3 may play an important role in VSMC proliferation stimulated by apelin-13. But which subtype of 14-3-3 involved in VSMCs proliferation induced by apelin-13 will be studied in future.

The p44/42 MAPK (ERK1/2) pathway is an important signal transduction pathway in G1-S phase progression and cell proliferation[26–29]. In addition, the crystal structure for two 14-3-3 isoforms has been solved and revealed a putative PKC binding site and a phosphorylation site within a consensus motif for CDKs (cyclin-dependent kinases)[30–31]. More evidences included our data show that ERK1/2 signal pathway is a key regulator in apelin/APJ system physiology functions[9–11, 16, 32].

Moreover, aplein is a mitogenic factor to endothelial cells [33], to present the characteristics of promoting angiogenesis [34]. The apelin/APJ system contributes to portosystemic collateralization and splanchnic neovascularization in portal hyper- tensive rats [35]. It is shown that apelin is a potent angiogenic factor required for cardiovascular development of the frog embryo[36–37] and *Xenopus laevis*[38], as an important part in normal and abnormal vascular development.

Apelin modulates vascular tone *in vivo*, causing a reduction in blood pressure when infused into rats[39–40] and vasodilation of resistance vessels when infused into the human forearm [41], both responses mediated primarily by nitric oxide. *In vitro*, apelin causes vasodilation of human splanchnic artery, largely *via* a nitric oxide-dependent mechanism [42]. Apelin also causes vasoconstriction of human saphenous vein [43] and mammary artery [44] *in vitro* by a direct action on vascular smooth muscle. These data support a role for the apelin system in modulation of vascular tone, where apelin released from endothelial cells would act on apelin receptors on the endothelium to cause vasodilation or on underlying smooth muscle cells to cause vasoconstriction.

In human, apelin peptide is up-regulated in atherosclerotic coronary artery[22], and both apelin and its receptor are up-regulated in aortic valve stenosis, a process that displays some hallmarks of atherosclerosis[45]. Apelin also prevents aortic aneurism formation in mice[46]. Spontaneously hypertensive rats have decreased cardiovascular apelin receptor and apelin mRNA and protein compared with control rats [47]. There is also evidence for a role in pulmonary hypertension [48] and portal hypertension [35]. It is noteworthy that apelin is able to cause vasodilation by a prostanoid-dependent mechanism in blood vessels from patients with atherosclerotic heart disease [44]. This suggests that apelin may have beneficial vasodilatory effects even in patient groups that display a degree of endothelial dysfunction.

Our study indicated that apelin-13 promoted the expression of 14-3-3, Raf-1 phosphorylation and ERK1/2 phosphorylation in concentration-dependent and time-dependent manner with a peak at 2 μmol/L and 4 h. Difopein inhibited Raf-1 and ERK1/2 phosphorylation, expression of cyclinD1 and cyclinE induced by apelin-13. 14-3-3 inhibitor Difopein significantly inhibited the VSMCs proliferation stimulated by apelin-13. Apelin-13 promoted the combination of 14-3-3 and Raf-1, and Difopein significantly inhibited 14-3-3 and Raf-1 combination induced by apelin-13. Thus, Raf-1 +14-3-3- ERK1/2 cascades involved in apelin-13-induced VSMC proliferation. However, the specific isoform of PKC and 14-3-3 that plays a role in the apelin/APJ/PKC/Raf-1/14-3-3/ERK1/2/Cyclins signal transduction pathway remains to be determined. On stimulation of cells, the 14-3-3 dimer is bound to the Ser259 phosphorylation sites of Raf-1 [49]. Zhang *et al.* [18] showed that an unphosphorylated synthetic peptide, R18 effectively inhibited the interaction of ligands with 14-3-3 in the binding groove of 14-3-3. Two R18 peptide motifs [50] linked by an 11-mer peptide (GAAGLDSADGA, called difopein) also bind very tightly to the 14-3-3 binding groove [51]. These investigation suggest that apelin-13 increase Raf-1 phosphorylation, promote the combination of 14-3-3 and Raf-1, stimulate ERK1/2 phosphorylation in rat VSMCs (Figure 6). It may be involved in cardiovascular diseases from insulin maintenance and pulmonary hypertension that apelin induced VSMCs proliferation[48, 52]. Apelin/APJ system may be a potential therapeutic target for vascular hyperplastic disease.

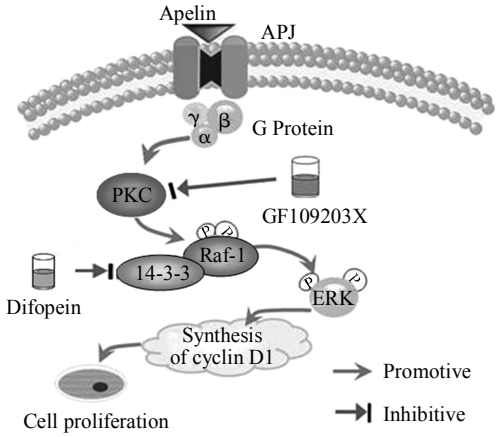

Fig. 6　Raf-1+14-3-3/ERK1/2 signal cascade mediated rat VSMCs proliferation induced by apelin-13

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14-3-3 参与 apelin-13 促进大鼠血管平滑肌细胞增殖 ERK1/2 信号途径研究 *

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摘要　本室以前已经报道了 G 蛋白偶联受体 APJ 的内源性配体多肽，apelin-13，通过激活 ERK1/2 促进大鼠血管平滑肌细胞增殖．本文研究 14-3-3 信号蛋白是否参与 apelin-13 促进大鼠血管平滑肌细胞增殖 ERK1/2 信号途径，探讨 apelin/APJ 系统的细胞信号转导机制．组织贴块法培养大鼠胸主动脉 VSMCs；Western blotting 方法检测 14-3-3、pRaf-1、Raf-1、pERK1/2、ERK1/2、cyclinD1、cyclinE 的表达； MTT 方法观察 14-3-3 抑制剂 Difopein 对 VSMCs 的增殖作用；免疫共沉淀方法检测 14-3-3 和 Raf-1 蛋白复合物的形成．Western blotting 方法结果显示，apelin-13 (0、0.5、1、2、4 μmol/L)浓度依赖性刺激大鼠 VSMCs 14-3-3 表达、Raf-1 和 ERK1/2 磷酸化，以 2 μmol/L最为明显；2 μmol/L apelin-13 时间依赖性刺激大鼠 VSMCs 14-3-3 表达、Raf-1 和 ERK1/2 磷酸化，在 4 h 增加最为显著； 14-3-3 蛋白抑制剂 Difopein 明显抑制 apelin-13 诱导的 Raf-1 磷酸化、ERK1/2 磷酸化、cyclinD1 及 cyclinE 表达； 免疫共沉淀方法发现 apelin-13 诱导 14-3-3 与 Raf-1 结合增加，而 Difopein 明显抑制两者结合；MTT 法显示 Difopein 明显抑制 apelin-13 诱导的血管平滑肌细胞增殖．上述结果表明，Apelin-13 通过 14-3-3/Raf-1 复合物 -ERK1/2 信号转导通路促进大鼠血管平滑肌细胞增殖．

关键词　apelin，APJ，14-3-3，血管平滑肌细胞，ERK1/2，Raf-1
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