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Inhibitory Effects of CGRP on Vascular Smooth Muscle Cell Proliferation: Role of Caveolae/caveolin-1/ERK$_{1/2}$ Signal Pathway*

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Abstract Caveolae and caveolin-1 participate in the transportation of cholesterol and cell signal transduction. Our previous studies showed that the inhibitory effect of calcitonin gene-related peptide (CGRP) on the vascular smooth muscle cells (VSMCs) was related to decreasing the activity of extracellular signal-regulated kinase (ERK)$_{1/2}$ and increasing the expression of caveolin-1. In the present study, we investigated the role of caveolae and caveolin-1 in proliferation of VSMCs and whether there are interaction between the caveolin-1 and ERK$_{1/2}$ in the inhibitory effect of CGRP signal pathway. VSMCs were prepared from thoracic aorta of male Sprague-Dawley rat by the classic explants method, the passage 3 ~ 10 VSMCs were used for the present study. 10% fetal bovine serum (FBS) was employed as a stimulus for the proliferation of VSMCs. β-Cyclodextrin or filipin was used to deplete cholesterol in the caveolae. Proliferation of VSMCs was estimated by methylthiazoletrazolium (MTT) assay and Flow Cytometry. Western blotting and co-immunoprecipitation were used to determine interaction of p-ERK$_{1/2}$ or caveolin-1. Results showed that CGRP significantly inhibited VSMC proliferation and down-regulated phosphorylation of ERK$_{1/2}$. Incubation of VSMCs with β-cyclodextrin or filipin promoted cells proliferation, up-regulated phosphorylation of ERK$_{1/2}$, attenuated the inhibitory action of CGRP on VSMC proliferation and decreased caveolin-1 expression. Pretreatment with CGRP increased the direct binding of cavolin-1 with phosphorylated(p-) ERK$_{1/2}$ but not non-phosphorylated ERK$_{1/2}$ in the presence of 10%FBS. Our results revealed that caveolae and caveolin-1 may contribute to the inhibitory effect of CGRP on the VSMC proliferation, and the mechanism may be related to the deceased nuclei translocation of p-ERK$_{1/2}$ because of the increased binding of caveolin-1 with p-ERK$_{1/2}$.

Key words caveolae, caveolin-1, calcitonin gene-related peptide, extracellular signal-regulated kinase$_{1/2}$, vascular smooth muscle cell, proliferation
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Caveolae, the cholesterol-rich domain located within the cell membrane, participates in multiple cellular processes including cholesterol homeostasis, vascular transport, and signal transduction. Caveolin-1, a main scaffolding protein invaginating caveolae, may function to recruit multiple signaling molecules and to integrate membrane signal transduction[1]. Extracellular signal-regulated kinase (ERK)$_{1/2}$, a member of mitogen-activated protein kinase (MAPK) family, is compartmentalized within caveolae in which the activity of ERK$_{1/2}$ is inhibited by caveolin-1 [2]. It is reported that targeted downregulation of caveolin-1 is sufficient to drive cell transformation and hyperactivate the ERKs signal cascade [3]. Another

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study reported that cyclic stretch transiently induced translocation of caveolins from caveolae to noncaveolar membrane sites in vascular smooth muscle cells (VSMCs) and induced ERKs activation[4]. Disruption of caveolae by methyl-β-cyclodextrin (β-CD) enhanced ERKs activation by decreasing the caveolar caveolin and accumulating the noncaveolar caveolin[4], suggesting caveolin-1 inhibits the activity of ERKs by stabilizing the molecules in an inactive conformation within caveolae.

Calcitonin gene-related peptide (CGRP), a potent vasodilatory neuropeptide, plays a role in modulation of total peripheral resistance[5-6]. Our previous studies revealed that the depressor effects of losartan, perindoprile and rutaecarpine on hypertensive rats were mediated by increase of CGRP synthesis and release *in vivo*[6-7]. Experiments *in vitro* show that the proliferation of VSMCs is inhibited by CGRP and the signal pathways involves the decrease of p-$ERK_{1/2}$ and increase of caveolin-1[8-9]. Based on the previous data, the present study sought to determine whether there is an interaction between caveolae or caveolin-1 and $ERK_{1/2}$ signaling in the inhibitory effect of CGRP on the VSMC proliferation.

1 Materials and methods

The protocol of this study was approved by University Ethics Committee for Animal Experimentation of University of South China. All procedures were performed in accordance with the Council on Animal Care at University of South China and the National Research Council's Guide for Human Care and Use of Laboratory Animals.

1.1 Drugs and reagents

CGRP, β-cyclodextrin and filipin were the products of Sigma Company (USA). Bicinchoninic acid (BCA) Protein Assay Kit was purchased from HyClone-PIERCE Biotechnology Company (USA). Antibodies of $ERK_{1/2}$ (K-23), p-$ERK_{1/2}$ (E-4), caveolin-1 were obtained from Santa Cruz Biotechnology, Inc. (USA). Acrylamide, N, N-Methylene Bisacrylamide, Tris-Base, Ammonium Persulfate, MTT (methylthiazoletrazolium) and sodium dodecyl sulfate (SDS) were the products of Amresco, Inc. (USA). The secondary antibodies (HRP Goat Anti-Mouse / Rabbit Ig G) were purchased from Boster Company (Wuhan, China). Prestained Protein Marker was purchased from the branch of New England Biolabs Inc. (Beijing, China). Fetal bovine serum (FBS) was purchased from Sijiqing Company (Hangzhou, China).

1.2 Rat vascular smooth muscle cell culture

The rat VSMCs were prepared using a previously described method with modification [8]. Briefly, the thoracic aorta was rapidly excised from healthy male Sprague-Dawley rats (aged 7 ~ 8 weeks), and the medial layer cut into small pieces (0.5 ~ 1 mm^3 cubes). Explants were placed in a culture vial. DMEM (high glucose, L-glutamine, pyridoxine hydrochloride, 3.7 g sodium bicarbonate)supplemented with 20% FBS was used as the initial incubation medium. Then, the explants were incubated at 37℃ with 5% CO_2 in an incubator, to allow vascular smooth muscle cells to migrate out of the explants. When the cells grew fully to 70% ~ 80% of the bottom area of the culture vial, the subculture was carried out in DMEM with 10% FBS following treatment of 0.25% trypsin. Passage 3 ~ 10 vascular smooth muscle cells were used for experiments.

1.3 Determination of cell proliferation

The proliferation of VSMCs was described by cell viability and proliferation index, which were measured by MTT and flow cytometry, respectively, as previously described [8-9]. Briefly, vascular smooth muscle cells (5×10^3/ml, 100 μl/well) were plated in 96-well plantes for 24 h and incubated with DMEM containing 0.1% FBS for 48 h. After vascular smooth muscle cells were incubated with 10% FBS in the absence or presence of CGRP for 24 h, the cells were treated with MTT (0.5 g/L) for 4 h at 37℃. The culture medium was removed from 96-well plates, and DMSO was added to dissolve the formazan in the cells. The metabolized MTT was measured in an enzyme-linked immunosorbent assay reader at 570 nm light-wave. Vascular smooth muscle cell viability or proliferation was expressed as absorbance(A) relative to the control. Meanwhile, changes in the vascular smooth muscle cell cycle were assayed by flow cytometry (Beckman Coulter, USA) as we previously described and the proliferation index (PI) was calculated with the formula: PI=(S+G2/M)/(G+S+G2/M)[9-10].

1.4 Western blotting

Proteins of VSMCs were extracted as previously described[8-10]. Briefly, cells were washed 3 times with PBS and 50 ~ 75 μl cell lysate (50 mmol/L pH 7.4 Tris•HCl, 150 mmol/L NaCl, 1% Triton X-100, 1% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS, 0.1 g/L phenylmethyl sulfonyl fluoride, 1 g/L leupetin)

was added. Cell lysates were prepared by freezing, thawing, scraping, and homogenization on ice. After centrifugation (10 000 *g*) for 30 min (4℃), the supernatant was stored at −80℃ for Western blot analysis.

The protein samples were mixed with sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE) buffer (50 mmol/L Tris-HCl pH 6.8, 2% SDS, 100 mmol/L dithiothreitol, and 0.1% bromophenol blue and 10% glycerol), followed by brief sonicate and boiling for 5 min. Then, 50 μg of the protein samples in each well were subjected to SDS-PAGE (10% separating gel and 5% stacking gel). After electrophoresis, proteins were then transferred to nylon polyvinylidene fluoride (PDVF) membrane (AMRESCO). The membrane was blocked for 2 h at a room temperature with blocking buffer TBST (50 mmol/L pH 7.6 Tris•HCl, 150 mmol/L NaCl, 0.1% Tween 20)containing 5% milk, incubated with phosphorylated (p-) ERK$_{1/2}$, caveolin-1, β-actin antibody (1∶1 000) for 2 h at 4℃, washed 4 times using the TBST buffer, and incubated for 1 h with secondary antibody (1∶1 000). After washing with TBST buffer, the immunoreactive bands in PDVF membrane were visualized by enhanced chemiluminescence. Expression levels of the target protein were determined by densitometry normalized against β-actin.

1.5 Immunoprecipitation

The quiescent VSMCs were obtained by the same method above. Experimental VMSCs were pretreated with CGRP for 30 min, then 10% FBS added for 10 min. Cell lysates was immunoprecipitated using the pierce classic mammalian immunoprecipitive kit. Briefly, after lysing on the ice, the cell lysates were centrifugated (10 000 *g*) for 30 min at 4℃, supernatant proteins were quantitated with BCA, and equal amount specific (2 μl of p-ERK$_{1/2}$ or ERK$_{1/2}$) antibody added and incubated at 4℃ overnight, followed by adding Protein G agarose beads to form the immune complex. The immunoprecipitated protein was recovered using an elution buffer and centrifugation (4℃). Eluted protein that mixed with the supplied sample buffer (250 mmol/L pH 8.8 Tris-HCl, 500 mmol/L DTT, 10% SDS, 0.5% bromophenol blue, 50% glycerol)were used for SDS-PAGE for testing the caveolin-1. The Protein G resin was regenerated and stored in the spin cups for additional immunoprecipitations.

1.6 Statistical analysis

Data are expressed as $\bar{x}\pm s$. All data were analyzed by one-way ANOVA using the SPSS 13.0 software, followed by an unpaired student Newman- Keulas test. A value of $P < 0.05$ was considered statistically significant.

2 Results

2.1 Inhibitory effect of CGRP on VSMC proliferation

The proliferation of VSMCs was described by cell viability and changes of cell cycle. Pretreatment of the VSMCs with CGRP (0.1, 1, 10, 100 nmol/L) for 30 min attenuated cell proliferation induced by 10% FBS in a dose-dependent manner (Figure 1a). A concentration of 10 nmol/L CGRP significantly inhibited the proliferation of VSMC in a time-dependent manner for the period of 12 h to 48 h ($P < 0.05$) (Figure 1b). Similarity, as shown in Table 1 that 10% FBS significantly increased the percentage of cells in "S"

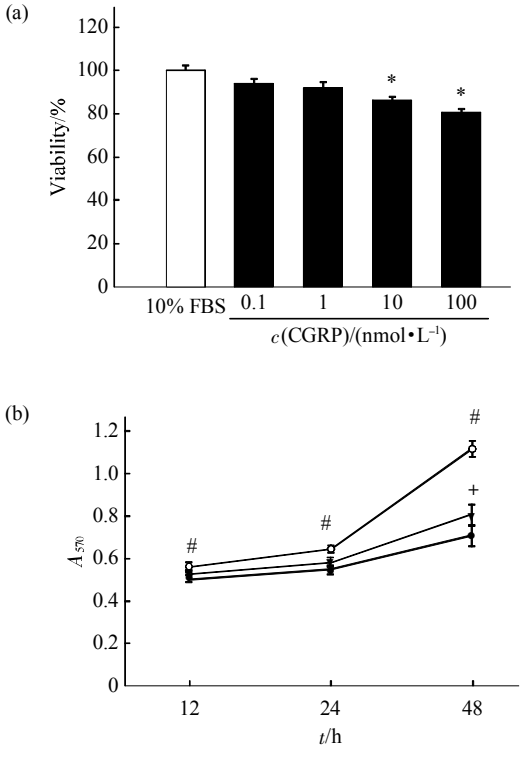

Fig. 1 Effect of CGRP on rat VSMC proliferation

The inhibitory effect of CGRP on the VSMC proliferation was described as cell viability determined by MTT. The inhibitory ratio was calculated by comparing to the 10% FBS and exhibited a dose-effect in 24 h (a) and a time-effect relationship (b). The data was represented as $\bar{x} \pm s$ ($n = 6$). *$P < 0.05$ *vs* 10%FBS, #$P < 0.01$ *vs* Control(0.1%FBS), +$P < 0.05$ *vs* 10%FBS. ●—●: 0.1% FBS; ○—○: 10% FBS;▼—▼: 10% FBS+CGRP.

Table 1 The percent of cell cycle distribution and value of *PI* ($\bar{x} \pm s$, $n = 3$)

Groups		G0/G1(%)	S(%)	G2/M(%)	*PI*
0 h	Control	93.2 ± 0.160	4.00 ± 0.362	2.00 ± 0.150	0.0601 ± 0.0203
12 h	10%FBS	84.2 ± 0.701	7.70 ± 0.353	8.20 ± 0.301	0.159 ± 0.0321*
	+CGRP	89.4 ± 0.302	6.50 ± 0.264	4.10 ± 0.398	0.106 ± 0.0202#
24 h	10%FBS	76.8 ± 0.432	7.30 ± 1.27	5.90 ± 0.253	0.232 ± 0.0813*
	+CGRP	81.9 ± 0.291	4.90 ± 0.263	13.2 ± 1.21	0.181 ± 0.0721#
48 h	10%FBS	68.4 ± 2.33	20.5 ± 0.452	11.1 ± 0.853	0.316 ± 0.0523*
	+CGRP	74.1 ± 0.563	9.90 ± 0.248	16.0 ± 0.435	0.259 ± 0.0192#

G_0/G_1: Ratio of G_0 / G_1 that representative the quiescent VSMCs; S: The phase of DNA synthesis; G_2: The later phase of DNA synthesis; M: The phase of mitotic cells. $PI = (S+G_2M) / (G_0G_1+S+G_2M)$. $^*P < 0.05$ *vs* control (0.1%FBS), $^{\#}P < 0.05$ *vs* 10% FBS.

cycle and "G2" cycle, compared with that in the control group (0.1% FBS). The proliferation index in the 10% FBS group was higher than that of the control group. Pretreatment of CGRP (10 nmol/L) also significantly decreased the proliferation index of VSMCs in the same time-dependent manner as described as MTT assay.

2.2 Effect of β-CD on the VSMC proliferation

The proliferation of VSMCs was determined by MTT assay. Pretreatment of VSMCs with β-CD (0.1, 1, 5 mmol/L) for 40 min significantly enhanced proliferation of VSMC induced by 10% FBS in a dose-dependent manner ($P < 0.05$) (Figure 2a). When VSMCs were exposed to 5 mmol/L of β- CD for 40 min firstly and then treated with CGRP for 30 min, results showed that the inhibitory effect of CGRP on the proliferation of VSMCs was decreased compared with the non-β-CD treated group($P < 0.05$)(Figure 2b). These results show that disruption of caveolae lowered the inhibitory effect of CGRP on the VSMC proliferation.

2.3 Effect of β-CD on phosphorylation of $ERK_{1/2}$

The effect of caveolae or caveolin-1 on expression or activity of $ERK_{1/2}$ was confirmed by Western blotting. Pretreatment with β-CD(5 mmol/L) or Filipin (8 μmol/L) for 40 min increased the expression of p-$ERK_{1/2}$ induced by 10% FBS for 10 min ($P < 0.05$) (Figure 3). Pretreatment with CGRP (10 nmol/L) for 30 min decreased the phosphorylation of $ERK_{1/2}$ induced by 10% FBS, which effect was abolished by pretreatment with β-CD($P < 0.05$)(Figure 4). However, the expression of $ERK_{1/2}$ in all of groups was not significantly changed.

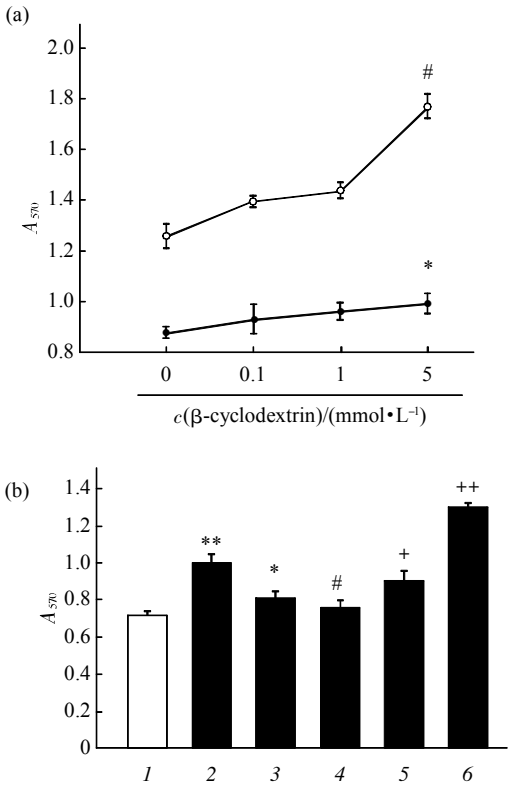

Fig. 2 Effect of β-cyclodextrin on VSMC proliferation

The effect of β-cyclodextrin on the proliferation of VSMCs was determined by MTT. Disruption of caveolae with β-cyclodextrin amplified the proliferation of 10% FBS and displayed a dose-dependent manner in 24 h (a). ●—●: 0.1% FBS; ○—○: 10% FBS. β-CD: β-cyclodextrin, $^*P < 0.05$ *vs* β-CD-untreated cells induced by 0.1% FBS; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs* β-CD-untreated cells induced by 10%FBS. Pretreatment of CGRP(10 nmol/L) for 30 min decreased the inhibitory effect of CGRP on the proliferation of VSMC induced by β-CD and 10% FBS (b). The data was represented as $\bar{x} \pm s$ (n =6), $^*P < 0.05$, $^{**}P < 0.01$ *vs* 0.1%FBS, $^{\#}P < 0.05$ *vs* 10%FBS, $^{+}P < 0.05$ *vs* CGRP, $^{++}P < 0.01$ *vs* CGRP+10%FBS. *1*: 0.1% FBS; *2*: 10% FBS; *3*: CGRP; *4*: CGRP+10% FBS; *5*: β-CD+CGRP; *6*: β-CD+CGRP+10%FBS.

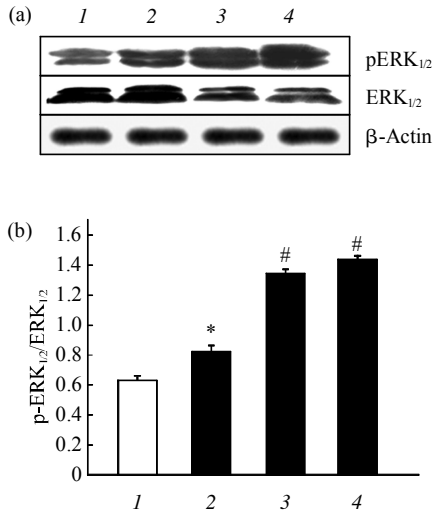

Fig. 3 Effect of β-cyclodextrin on the phosphorylation of ERK$_{1/2}$ induced by FBS

Disruption of caveolae by β-cyclodextrin or filipin exerted the VSMC proliferation and expression of p-ERK$_{1/2}$ induced by 10% FBS. β-CD: β-cyclodextrin. (a) The activity of ERK$_{1/2}$ was determined by Western blotting. (b) Optical relative densities were quantitated and the results are shown in the bar graph under the ratio of p-ERK$_{1/2}$ to ERK$_{1/2}$. The data was represented as $\bar{x} \pm s$ (n = 3). *P < 0.05 *vs* Control (0.1% FBS), #P < 0.05 *vs* 10% FBS. *1*: Control (0.1% FBS); *2*: 10% FBS; *3*: β-CD+10% FBS; *4*: Filipin+10%FBS.

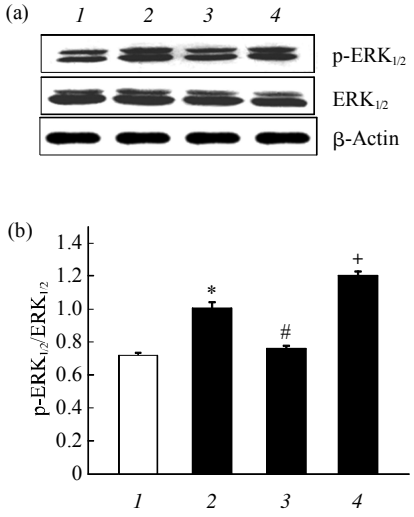

Fig. 4 Effect of CGRP on the phosphorylation of ERK$_{1/2}$ induced by FBS on the caveolae-deprived condition

Disruption of caveolae abolished the inhibitory effect of CGRP on the phosphorylation of ERK$_{1/2}$. β-CD: β-cyclodextrin. (a) The activity of ERK$_{1/2}$ was determined by Western blotting. (b) Optical relative densities were quantitated and the results are shown in the bar graph under the ratio of p-ERK$_{1/2}$ to ERK$_{1/2}$. The data was represented as $\bar{x} \pm s$ (n = 3), *P < 0.05 *vs* Control (0.1% FBS), #P < 0.05 *vs* 10% FBS, +P < 0.05 *vs* CGRP+10% FBS; *1*: Control (0.1% FBS); *2*: 10% FBS; *3*: CGRP+10% FBS; *4*: β-CD + CGRP + 10%FBS.

2.4 Effect of CGRP on expression of caveolin-1 in the presence of β-CD

The time-effect relationship of 10% FBS on the VSMC is shown in Figure 5. Quiescent VSMCs were incubated with 10% FBS for 0, 1, 4, 8, 16, 24 h, respectively. Results showed that expression of caveolin-1 was significantly decreased at 4 h to 8 h, then restored to normal level at 16 h and 24 h. Pretreatment of VSMC with β- CD or filipin for 40 min enhanced the down regulation of caveolin-1 expression induced by 10% FBS for 4 hours(P < 0.05) (Figure 6). Treatment of cell with CGRP or 10% FBS decreased the expression of caveolin-1 compared with control, respectively. However, incubation of cells with CGRP and 10% FBS together significantly increased the expression of caveolin-1. But, incubating cells with β- CD and CGRP (10 nmol/L) for 40 min and 30 min one after another decreased caveolin-1

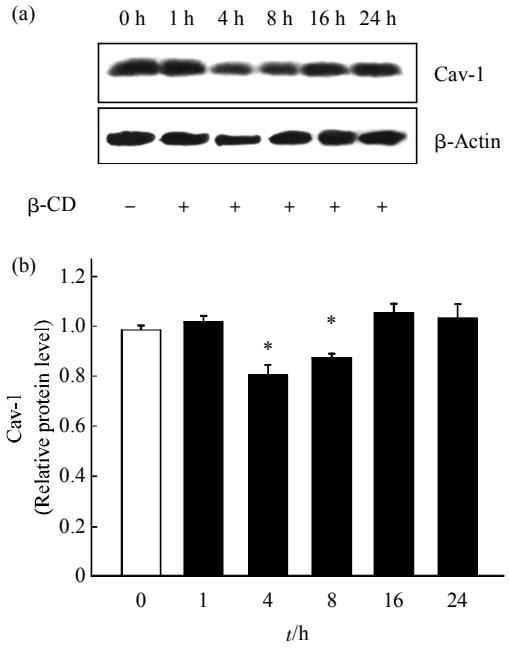

Fig. 5 Effect of β-cyclodextrin on expression of caveolin-1 in VSMC

Quiescent VSMCs were incubated with 10% FBS for 0, 1, 4, 8, 16, 24 hours, respectively. The expression of caveolin-1 decreased when incubated for 4 to 8 hours, then restored to normal level when incubated for 16 and 24 hours. Cav-1: caveolin-1. β-CD: β-cyclodextrin. (a) The expression of caveolin-1 was determined by Western blotting. (b) The optical density of caveolin-1 was quantitated and the results are shown in the bar graph under the ratio of caveolin-1 to β-actin. The data was represented as $\bar{x} \pm s$ (n = 3), *P < 0.05 *vs* 0, 1, 16, 24 h.

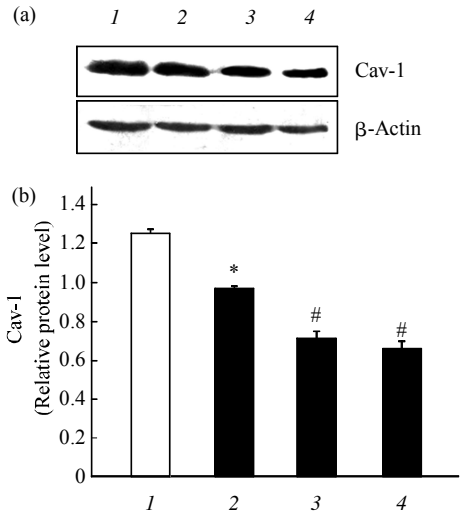

Fig. 6 Effect of β-cyclodextrin or filipin on expression of caveolin-1 induced by 10%FBS

The expression of caveolin-1 in the VSMCs was decreased when cells caveolae was interrupted using β-CD or filipin. β-CD: β-cyclodextrin. (a) The expression of caveolin-1 protein was determined by Western blotting. (b) Optical density of caveolin-1 was quantitated and the results are shown in the bar graph under the ratio of caveolin-1 to β-actin. The data was represented as $\bar{x} \pm s$ (n = 3). *P < 0.05 *vs* Control (0.1%FBS), #P < 0.05 *vs* 10%FBS. *1*: Control(0.1%FBS); *2*: 10%FBS; *3*: β-CD+10% FBS; *4*: Filipin+10%FBS. Cav-1: caveolin-1.

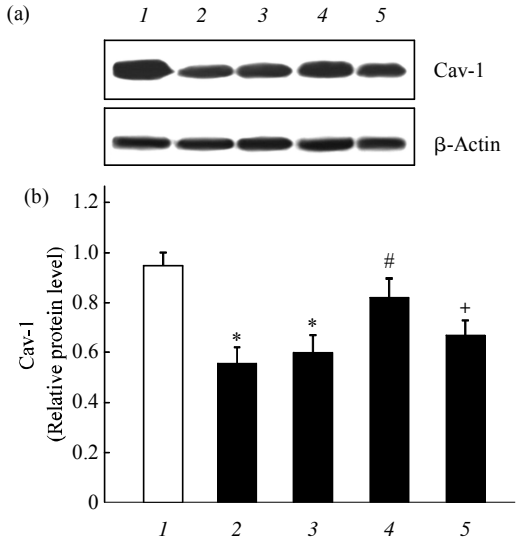

Fig. 7 Effect of CGRP on expression of caveolin-1 in the β-cyclodextrin-treated VSMCs

Treatment of VSMCs with CGRP (10 nmol/L) decreased the expression of caveolin-1 in the disruption condition of caveolae. β-CD: β-cyclodextrin. (a) The expression of caveolin-1 protein was determined by Western blotting. (b) Optical density of caveolin-1 was quantitated and the results are shown in the bar graph under the ratio of caveolin-1 to β-actin. Cav-1: caveolin-1. The data was represented as $\bar{x} \pm s$ (n = 3), *P < 0.05 *vs* Control (0.1% FBS), #P < 0.05 *vs* 10%FBS, +P < 0.05 *vs* CGRP +10% FBS. *1*: Control (0.1% FBS); *2*: CGRP; *3*: 10% FBS; *4*: CGRP+10%FBS; *5*: β-CD + CGRP + 10%FBS.

expression again(P < 0.05)(Figure 7).

2.5 CGRP enhanced the interaction between caveolin-1 and p-ERK$_{1/2}$

The interaction of caveolin-1 and p-ERK$_{1/2}$ or ERK$_{1/2}$ was determined by the immunoprecipitation and Western blot assays. ERK$_{1/2}$ or p-ERK$_{1/2}$ was precipitated with its antibody, then Western blotted with anti-caveolin-1. The data showed that compared with the 10% FBS group, pretreatment of CGRP significantly increased the binding mass of caveolin-1 and p-ERK$_{1/2}$ but not the binding mass of caveolin-1

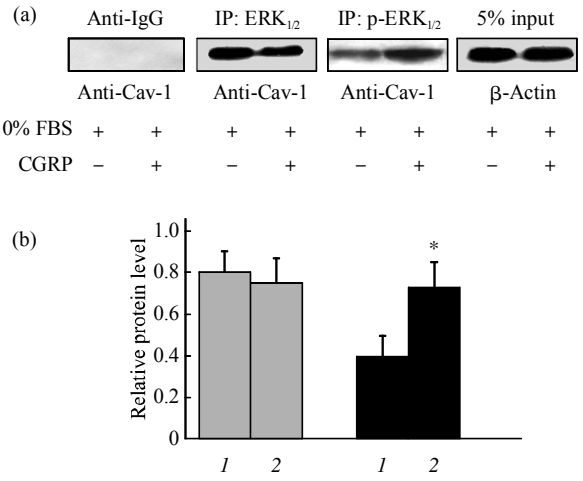

Fig. 8 Caveolin-1 interaction with p-ERK$_{1/2}$ in VSMCs

Quiescent VSMCs were incubated with 10%FBS in absence or presence of CGRP (10 nmol/L) for 10 min, 500 μg of the cell total proteins were extracted for immunoprecipitation. The extracted proteins of p-ERK$_{1/2}$ and ERK$_{1/2}$ were immunoprecipitated with their antibodies (take mouse polyclonal IgG as a control) then Western blot tested the caveolin-1, the Western blot bands were showed (a), the optical densities were quantitated and the results were shown in the bar graph under the ratio of imunocomplex p-ERK$_{1/2}$ or ERK$_{1/2}$ and caveolin-1 to β-actin (b). Cav-1: caveolin-1. The data was represented as $\bar{x} \pm s$ (n = 3). *P < 0.05 *vs* 10% FBS. ▭: ERK$_{1/2}$-Cav-1; ■: p-ERK$_{1/2}$-Cav-1. *1*: 10% FBS; *2*: +CGRP.

and ERK$_{1/2}$(P < 0.05, Figure 8).

3 Discussion

Our present data showed that treatment of VSMCs with β-cyclodextrin or filipin not only augmented the proliferation of VSMCs induced by 10% FBS but also lowered the inhibitory effect of CGRP on the cell proliferation. These facts suggest that caveolae may play an indispensable role in

assembly as sites for receptors and in turn affect the membrane signal transduction or cellular signal pathway. It has been demonstrated that CGRP inhibits proliferation or migration of VSMCs induced by growth factors and vascular activated peptides such as platelet-derived growth factor, endothelin-1 and angiotensin Ⅱ [8-9, 11-12]. In this experiment, CGRP also inhibited proliferation of VSMC induced by 10% FBS displayed a dose-dependent and time-dependent manner as before reported [10] (Figure 1 and Table 1). CGRP receptor is a kind of G-protein coupled receptor and activated by CGRP to initiate intracellular signaling. Like angiotensin Ⅱ type Ⅰ receptor [13] and transforming growth factor-beta type Ⅰ receptor ALK1 [14], CGRP receptor may assemble within caveolae when it is activated, in return, caveolae have a role in the fidelity and dynamics of receptor activation, which phenomena was reported by others in the similar studies[15-17].

It is well-known that MAPK family mainly mediate the extra-cellular signal to cell plasma and nuclear and that essentially regulate all stimulated cellular processes, such as proliferation, differentiation, stress response, apoptosis and more. ERK$_{1/2}$, a main member of MAPKs, is stimulated by various extracellular stimuli. The signals of these stimuli are then transferred by the cascade's components to a large number of targets at distinct subcellular compartments, which in turn induce and regulate a large number of cellular processes [18-19]. Morphological studies show that ERK$_{1/2}$ is concentrated in the plasma membrane caveolae *in vivo* using immunoelectron microscopy. In addition, constitutive activation of the ERK$_{1/2}$ cascade is sufficient to reversibly down-regulate caveolin-1 mRNA and protein expression [20]. Moreover, it is reported that caveolin-1 expression can inhibit signal transduction from the ERK$_{1/2}$ cascade both *in vitro* and *in vivo*, and this inhibitory activity was mapped to caveolin-1 residues 32 ～95 [21]. Our previous study showed that the effect of CGRP on proliferation of VSMC induced by angiotensin Ⅱ was accompanied by the increases in caveolin-1 expression, suggesting that negative regulation exists between ERK$_{1/2}$ activation and caveolin-1 protein expression in the signal transduction of CGRP receptor[9]. In the present study, disruption of caveolae by β-cyclodextrin increased expression of p-ERK$_{1/2}$ (Figure 3, Figure 4) and magnified the effect of downregulation of caveolin-1 induced by CGRP (Figure 7). The interaction of caveolin-1 and MAPK signal cascades was also demonstrated in another investigation showing that high glucose-induced caveolin-1 and integrinβ1 activation can stimulate mouse embryonic stem cell proliferation through the modification of focal adhesion signaling pathways and p38MAPK [22]. Moreover, direct binding of caveolin-1 and ERK$_{1/2}$ was enhanced by CGRP further supporting the concept that caveolin-1 inhibits the signal transduction from the ERK$_{1/2}$ cascade by acting as a natural endogenous inhibitor of both MEK and ERK. Recently, others reported that IFITM1, a cell surface molecule, mediates the inhibitory effect of interferon-γ on the Chang liver and BEL-7404 cells *via* enhancing the inhibiting effect of caveolin-1 on ERK activation [23]. Taken together with these data, we predicted that caveolae and caveolin-1 play a pivotal role in the inhibitory effect of CGRP on proliferation of VSMC, and the mechanism may be related to the CGRP increase the directly binding of caveolin-1 and p-ERK$_{1/2}$ within the caveolae resulting in inhibition of the nucleus transformation of p-ERK$_{1/2}$ in the cytoplasm, which were summarized and pictured in the Figure 9. A better understanding of the signal pathway of VSMC proliferation may provide a clue for the development of novel therapeutic drugs to prevent vascular restenosis or remodeling.

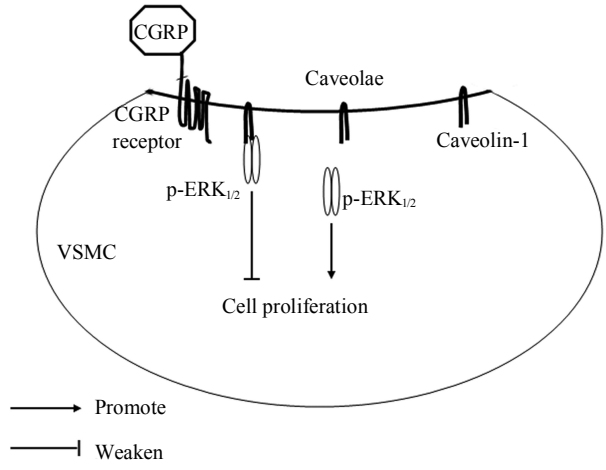

Fig. 9 The effect of CGRP on the interaction of caveolin-1 and p-ERK$_{1/2}$ pathway in VSMC proliferation

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Caveolae/caveolin-1/ERK$_{1/2}$ 信号通路在降钙素基因相关肽抑制血管平滑肌细胞增殖中的作用 *

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摘要　业已证明，Caveolae 及其蛋白 caveolin-1 参与了细胞膜的胆固醇转运和细胞膜的信号转导．我们前期工作发现降钙素基因相关肽(CGRP)抑制血管平滑肌细胞(VSMC)增殖的信号通路与抑制 ERK$_{1/2}$ 活性和上调 caveolin-1 表达有关．本文研究 Caveolae 及 caveolin-1 在 CGRP 抑制 VSMC 增殖中的作用，进一步研究 caveolin-1 表达增加是否有直接抑制 ERK$_{1/2}$ 信号激酶活性的作用．采用大鼠主动脉贴块法培养 VSMC，取 3～10 代 VSMC 用于实验，10%小牛血清(FBS)用于刺激 VSMC 增殖，用 β- 环糊精(cyclodextrin)或菲律宾菌素(filipin)剥夺胆固醇破坏 Caveolae 结构；MTT 法和流式细胞仪用于检测细胞增殖；蛋白质印迹和免疫共沉淀法分别用于检测目的蛋白的表达或蛋白质间相互作用．结果显示，CGRP 呈时间和浓度依赖性显著抑制 10%FBS 诱导的 VSMC 增殖．细胞 Caveolae 结构的破坏能降低 CGRP 抑制 VSMC 增殖作用，同时也增加了 ERK$_{1/2}$ 的磷酸化；β- 环糊精孵育细胞能降低 caveolin-1 的表达．免疫共沉淀发现 10%FBS 和 / 或 CGRP 共同孵育细胞对非磷酸化 ERK$_{1/2}$ 与 caveolin-1 的结合无差别，但 10%FBS 能降低磷酸化 ERK$_{1/2}$ 与 caveolin-1 的结合，CGRP 预孵育细胞能增加这两者的相互作用．结果揭示，Caveolae 及 caveolin-1 可以正调控 CGRP 抑制 VSMC 增殖作用，其机制可能与 CGRP 增加 caveolin-1 与 p-ERK$_{1/2}$ 在 Caveolae 的结合，并抑制 p-ERK$_{1/2}$ 核转位作用有关．

关键词　Caveolae，caveolin-1，降钙素基因相关肽，细胞外信号调节酶，血管平滑肌细胞，增殖
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