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Original Article

CGRP inhibits vascular smooth muscle cell proliferation through suppressing ERK1/2 signaling pathway

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Abstract: Vascular smooth muscle cell (VSMC) plays an important role in cardiovascular system disease occurrence and development. Calcitonin gene related peptide (CGRP) is an important regulatory protein that regulates enzymes activity, influences ion channel switch, and participates in the reconstruction of structural protein. CGRP was used to treat injured CGRP and test its impact on phenotype transformation and cell proliferation, aiming to explore the protective effect of CGRP on arterial injury. Rat VSMC was induced by angiotensin II (Ang II) and divided into three groups. The experimental group received CGRP treatment, the control was injured without intervention, and the blank group was conventional cultured VSMC. VSMC phenotype marker α-SMA and osteopontin (OPN), and ERK1/2 phosphorylation were tested by Western blot. Cell cycle, apoptosis, and proliferation were analyzed by flow cytometry. α-SMA expression significantly elevated, while OPN level obviously reduced in VSMC after CGRP intervention ($P < 0.05$). Cell percentage in S phase and proliferation index in experimental group markedly declined compared with control after 6 h, 12 h, and 24 h ($P < 0.05$). Cell percentage in S phase and apoptosis index gradually decreased in experimental group following CGRP intervention time extension ($P < 0.05$). ERK1/2 and p-ERK1/2 proteins in experimental group were obviously lower than that in control ($P < 0.05$) and declined following treatment time elongation ($P < 0.05$). CGRP may regulate VSMCs phenotype and restrain cell proliferation via inhibiting ERK1/2 signaling pathway activation.

Keywords: CGRP, VSMC, ERK1/2

Introduction

Calcitonin gene related peptide (CGRP) belongs to the endogenous vascular dilation protein peptide that can regulate the function of cardiovascular system. It distributes extensively and plays an important role in stabling cardiovascular system. CGRP plays its biological function mainly via regulating important signaling pathways in the body. Among them, it can elevate cAMP concentration and enhance the activity of cAMP dependent protein kinase in the cytoplasm of various cells. It can regulate ion channel switch and structure protein expression by increasing the enzyme activity [1, 2]. It was reported that CGRP plays a promoting role in vascular endothelial cell proliferation and differentiation, inducing a large amount of neovascularization [3]. It was found that CGRP can suppress vascular smooth muscle cell (VSMC) differentiation and proliferation together with fetal bovine serum and angiotensin II (Ang II) [4]. VSMC proliferation participates in the occurrence and development of cardiovascular disease and vascular hyperplastic disease, such as hypertension, atherosclerosis, and vascular restenosis [5]. CGRP can act on vascular active suppression material including Ang II and inflammatory factors such as interleukin 1 to regulate VSMC phenotype transformation and promote VSMC proliferation and differentiation [6-8]. In this study, we used Ang II to damage rat VSMC and provide CGRP intervention. Cell apoptosis and proliferation were analyzed by flow cytometry, while VSMC phenotype marker α-SMA and osteopontin (OPN), and ERK1/2 phosphorylation were tested by Western blot to analyze the protective effect of CGRP on artery injury.

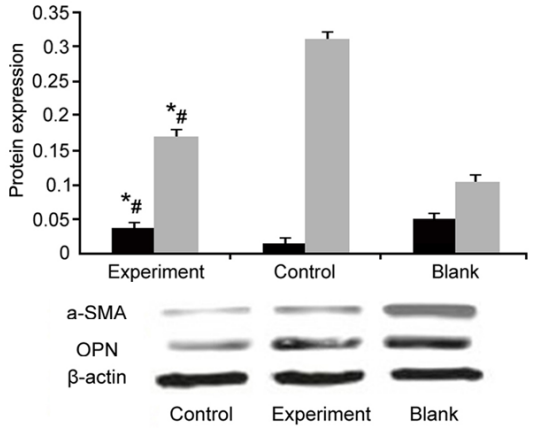

Figure 1. α-SMA and OPN expression changes in VSMC. *P < 0.05 compared with control. #P < 0.05 compared with blank group.

Materials and methods

Experimental cells

VSMC was purchased from Beyotime (China).

Reagents and instruments

RPMI 1640 medium and fetal calf serum was got from Gibco (USA). Ang II and CGRP were from Sigma (USA). Primary antibodies for ERK1/2 and p-ERK1/2, and goat anti rabbit secondary antibody were from Cell Signaling Technology (USA).

Experimental methods

Conventional cell culture: VSMCs were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum and maintained in 5% CO_2 and 37°C. The cells were digested by 0.25% trypsin and seeded in 24 well plate at 10^5/ml. The cells were incubated in medium containing 1% fetal calf serum for 24 h when the cell fusion reached 80%.

Ang II induction: VSMC single cell suspension was seeded in 24-well plate at 5×10^3/ml. When the cell fusion reached 80%, the cells were incubated in medium containing 0.1% FBS and treated by 100 nmol/L Ang II.

Grouping

Experimental group: VSMC proliferation model was established by Ang II induction. It was treated by 10 nmol/L CGRP for 6 h, 12 h, and 24 h.

Control group: VSMC proliferation model was established by Ang II induction. It was treated by equal amount of normal saline with CGRP in experimental group for 6 h, 12 h, and 24 h.

Blank group: VSMC cultured in serum free medium without Ang II or CGRP intervention.

Western blot

Total protein was extracted from VSMC and separated by SDS-PAGE. After transferred to NC membrane, the protein was blocked by skim milk for 2 h. Next, the membrane was incubated with primary antibody at 4°C overnight, and then incubated in secondary antibody for 2 h. At last, the membrane was developed to analyze the absorbance. β-actin was selected as internal reference.

Flow cytometry

VSMC was centrifuged and collected at 1×10^9/L in centrifuge tube. After washed by PBS and resuspsended in 75% ethanol for 12 h. The VSMC was stained by PI and incubated avoid of light for 30 min. At last, the cells were analyzed on flow cytometry to calculate cell proliferation index (PI) and apoptosis index. $PI = (S + G_2M)/(G_0G_1 + S + G_2M)$.

Data analysis

SPSS 17.0 software was adopted for data analysis. The data was depicted as mean ± standard deviation. Enumeration data was tested by chi-square test, while measurement data was compared by t test. P < 0.05 was considered as statistical significance.

Results

α-SMA and OPN expression changes in VSMC

α-SMA expression reduced, while OPN level increased in VSMC after Ang II induction compared with blank group (P < 0.05), suggesting that VSMC transformed from contractile phenotype to synthetic phenotype. α-SMA expression significantly elevated, while OPN level obviously reduced in VSMC after CGRP intervention (P < 0.05), indicating that CGRP intervention apparently suppress VSMC phenotype transformation (**Figure 1**).

Table 1. Cell cycle, PI, and apoptosis index comparison

Index	Experimental group			Control group			Blank group		
	6 h	12 h	24 h	6 h	12 h	24 h	6 h	12 h	24 h
G_0-G_1 (%)	84.6±1.3	82.2±2.6	77.1±1.3	84.7±2.0	82.1±1.4	77.0±1.5	94.3±0.3	93.1±0.4	94.0±1.5
S (%)	9.5±0.7$^{*,\#}$	8.0±1.4$^{*,\#,\&}$	6.8±2.4$^{*,\#,\&,@}$	10.1±0.7	9.0±2.2	6.5±3.2	2.9±0.2	3.7±0.2	2.5±3.2
G_2-M (%)	5.9±1.9	9.6±1.2	15.2±2.1	5.3±1.8	9.0±1.2	16.6±4.0	2.9±0.6	3.0±0.1	3.6±0.2
Apoptosis rate (%)	1.4±0.4	2.1±0.4	3.0±0.7	1.0±0.2	1.9±0.2	2.8±0.3	0.8±0.2	0.9±0.1	0.8±0.1
PI	0.12±0.01$^{*,\#}$	0.15±0.01$^{*,\#,\&}$	0.18±0.02$^{*,\#,\&,@}$	0.15±0.02	0.18±0.03	0.23±0.03	0.06±0.02	0.07±0.01	0.05±0.03

$^{*}P < 0.05$, compared with control. $^{\#}P < 0.05$, compared with blank. $^{\&}P < 0.05$, compared with 6 h. $^{@}P < 0.05$, compared with 12 h.

Table 2. ERK1/2 and p-ERK1/2 protein expression in VSMC

Group	ERK1	ERK2	pERK1	pERK2
Experiment				
6 h	0.187±0.013$^{*,\#}$	0.326±0.018$^{*,\#}$	0.155±0.007$^{*,\#}$	0.194±0.008$^{*,\#}$
12 h	0.172±0.011$^{*,\#,\&}$	0.278±0.015$^{*,\#,\&}$	0.103±0.004$^{*,\#,\&}$	0.166±0.003$^{*,\#,\&}$
24 h	0.135±0.009$^{*,\#,\&,@}$	0.213±0.013$^{*,\#,\&,@}$	0.078±0.002$^{*,\#,\&,@}$	0.092±0.002$^{*,\#,\&,@}$
Control				
6 h	0.198±0.016^{1}	0.438±0.021^{1}	0.186±0.012^{1}	0.212±0.015^{1}
12 h	0.187±0.017^{1,2}	0.315±0.018^{1,2}	0.147±0.008^{1,2}	0.186±0.009^{1,2}
24 h	0.165±0.014^{1,2,3}	0.274±0.015^{1,2,3}	0.102±0.004^{1,2,3}	0.134±0.005^{1,2,3}
Blank	0.018±0.007	0.011±0.002	0.003±0.002	0.002±0.001

$^{*}P < 0.05$, compared with control. $^{\#}P < 0.05$, compared with blank. $^{\&}P < 0.05$, compared with 6 h. $^{@}P < 0.05$, compared with 12 h. $^{1}P < 0.05$, compared with Blank. $^{\#}P < 0.05$, compared with blank. $^{2}P < 0.05$, compared with 6 h. $^{3}P < 0.05$, compared with 12 h.

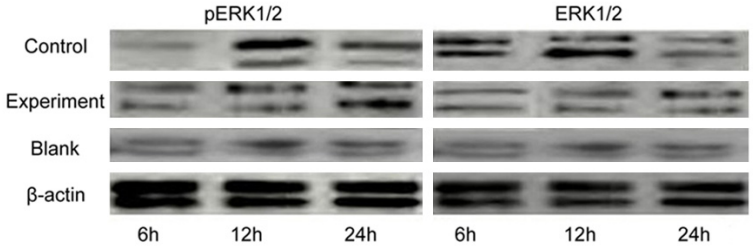

Figure 2. ERK1/2 and p-ERK1/2 protein expression in VSMC.

Cell cycle, PI, and apoptosis index comparison

It was showed that cell percentage in S phase and PI in experimental group markedly declined compared with control after 6 h, 12 h, and 24 h ($P < 0.05$). Cell percentage in S phase and PI gradually decreased in experimental group following CGRP intervention time extension ($P < 0.05$) (**Table 1**).

ERK1/2 and p-ERK1/2 protein expression in VSMC

ERK1/2 and p-ERK1/2 proteins in experimental group were obviously lower than that in control ($P < 0.05$). Their expressions gradually declined following treatment time elongation ($P < 0.05$) (**Table 2; Figure 2**).

Discussion

VSMC maintains its structure and function stability mainly by regulating the balance between proliferation and apoptosis, which is mediated through a series of signaling pathways. It involves in the formation of antithrombus material, timely response to cell damage, and regulating materials exchange inside and outside of blood vessels [9, 10]. As a key nerve related peptide, CGRP is an important microvascular diastolic agent with powerful functions that participates in the occurrence and development of nervous system and cardiovascular system disease. CGRP can play a vasodilation role via restraining VSMC differentiation and proliferation, contract myocardial cells, and promote vascular endothelial cell proliferation [11-13]. Angiotensin converting enzyme inhibitor is involved in reverse vascular reconstruction. It is not only related to its inhibition on renin angiotensin system, but also can enhance the function of sensory nerve to increase CGRP synthesis and release [14]. This study analyzed the impact of CGRP on VSMC

proliferation and phenotype transformation, and related mechanism by establishing Ang II induced VSMC proliferation model and applying CGRP intervention.

VSMC abnormal differentiation, proliferation, apoptosis, and even the structure changes of extracellular matrix can lead to vascular remodeling, which is the basis of the development of cardiovascular disease [15]. For patients with hypertension disease for many years, Ang II excessive secretion may promote VSMC hypertrophy and hyperplasia, aggravating vascular remodeling [16]. In this study, VSMC phenotype marker protein was detected. VSMC contractile phenotype α-SMA protein declined, whereas synthetic phenotype OPN obviously increased after Ang II induction. CGRP intervention enhanced α-SMA level and declined OPN expression, suggesting that CGRP obviously restrain VSMC phenotype transformation induced by Ang II.

This study compared cell cycle, PI, and apoptosis rate in VSMC. Cell percentage in S phase and PI in experimental group markedly declined compared with control after 6 h, 12 h, and 24 h. Cell percentage in S phase and PI gradually decreased in experimental group following CGRP intervention time extension. It showed that CGRP suppressed Ang II induced VSMC cell proliferation, while exhibited no significant impact on VSMC apoptosis.

CGRP receptor is a kind of G protein coupled receptor. Previous study pointed out that CGRP receptor existed in a variety of cells and plays its important biological function mainly through activating MAPK signaling pathway [17]. This study tested ERK1/2 and p-ERK1/2 proteins in VSMC. ERK1/2 and p-ERK1/2 proteins in experimental group were obviously lower than that in control. Their expressions gradually declined following treatment time elongation. ERK, induced by a variety of cytokines and growth factors, mainly mediates cell proliferation and cell cycle. It plays a promoting role in cell differentiation and proliferation, of which the key factor is ERK1/2 [18]. It was revealed that ERK1/2 sustained activation may increase cell death, which mainly relies on the degree of ERK1/2 activation. It suggested that ERK1/2 continuous activation may facilitate cell survive or induce cell death through a series of signaling pathways and related downstream molecules [19, 20]. Our results showed that Ang II enhanced ERK1/2 phosphorylation in VSMC, while CGRP treatment inhibited Ang II induced protein changes and ERK1/2 phosphorylation. It indicated that CGRP suppression on VSMC proliferation and phenotype transformation may be related to inhibiting ERK1/2 signaling pathway. It was pointed out that ERK1/2 signaling pathway may affect VSMC cell growth, proliferation, and migration [21]. Basic experiments suggested Bowman's capsule artery injury can induce significant ERK1/2 activation and sustain for 2 weeks. Inhibiting ERK1/2 activation degree in this process can influence neovascularization [22]. Moreover, sulfur dioxide treatment can negatively regulate VSMC proliferation via inhibiting ERK/MAPK signaling pathway to regulate cAMP/PKA signal [23].

Conclusion

Ang II can induce VSMC proliferation. CGRP intervention can significantly inhibit VSMC phenotype transformation, obstruct protein synthesis in proliferative VSMC, and inhibit the mitosis process by inhibiting ERK1/2 phosphorylation. Further in-depth investigation is needed to clarify the mechanism of CGRP and its interaction with Ang II.

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Disclosure of conflict of interest

None.

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References

[1] Alexander MR, Owens GK. Epigenetic control of smooth muscle cell differentiation and pheno-

typic switching in vascular development and disease. Annu Rev Physiol 2012; 74: 13-40.
[2] Xie C, Ritchie RP, Huang H, Zhang J, Chen YE. Smooth muscle cell differentiation in vitro: models and underlying molecular mechanisms. Arterioscler Thromb Vasc Biol 2011; 31: 1485-1494.
[3] Curlik J, Kolesar M, Durza O, Hiller E. Dandelion (taraxacum officinale) and agrimony (agrimonia eupatoria) as indicators of geogenic contamination of flysch soils in eastern slovakia. Arch Environ Contam Toxicol 2016; 70: 475-486.
[4] Avila G, Aguilar CI, Ramos-Mondragon R. Sustained CGRP1 receptor stimulation modulates development of EC coupling by cAMP/PKA signalling pathway in mouse skeletal myotubes. J Physiol 2007; 584: 47-57.
[5] Gomez D, Owens GK. Smooth muscle cell phenotypic switching in atherosclerosis. Cardiovasc Res 2012; 95: 156-164.
[6] Chattergoon NN, D'Souza FM, Deng W, Chen H, Hyman AL, Kadowitz PJ, Jeter JR Jr. Antiproliferative effects of calcitonin gene-related peptide in aortic and pulmonary artery smooth muscle cells. Am J Physiol Lung Cell Mol Physiol 2005; 288: L202-211.
[7] Almeida RD, Manadas BJ, Melo CV, Gomes JR, Mendes CS, Graos MM, Carvalho RF, Carvalho AP, Duarte CB. Neuroprotection by BDNF against glutamate-induced apoptotic cell death is mediated by ERK and PI3-kinase pathways. Cell Death Differ 2005; 12: 1329-1343.
[8] Xu Hong-Tao YQ, Wag Cai-Ying, et al. Influence of calcitonin gene-related peptide on the proliferation and phenotype transformation of vascular smooth muscle cells. Acta Anatomica Sinica 2007; 38: 452-456.
[9] Steiner D, Lampert F, Stark GB, Finkenzeller G. Effects of endothelial cells on proliferation and survival of human mesenchymal stem cells and primary osteoblasts. J Orthop Res 2012; 30: 1682-1689.
[10] Kaigler D, Silva EA, Mooney DJ. Guided bone regeneration using injectable vascular endothelial growth factor delivery gel. J Periodontol 2013; 84: 230-238.
[11] Xie Jun NX, Kou Ling, et al. Changes of plasma vasoactive peptides in chronic heart failure patients and its significance. Prog Mod Biomed 2011; 11: 4138-4140.
[12] Lu Zai-ying ZN-s. Medicine seventh edition ed. Beijing: People Health Press; 2008.
[13] Merrill GF, Kurth EJ, Hardie DG, Winder WW. AICA riboside increases AMP-activated protein kinase, fatty acid oxidation, and glucose uptake in rat muscle. Am J Physiol 1997; 273: E1107-1112.
[14] Yu XJ, Li CY, Xu YH, Chen LM, Zhou CL. Calcitonin gene-related peptide-effort of human HaCat keratinocytes by activation of MAP kinases. J Cell Biol Int 2009; 33: 1144-8.
[15] Poittevin M, Lozeron P, Hilal R, Levy BI, Merkulova-Rainon T, Kubis N. Smooth muscle cell phenotypic switching in stroke. Transl Stroke Res 2014; 5: 377-384.
[16] Shi B, Long X, Zhao R, Liu Z, Wang D, Xu G. The Transplantation of mesenchymal stem cells to their the human receptor activity-modifying protein 1 gene little cardiac function and inhibits neointimal effort in the carotid angioplasty and myocardial infarction rabbit model. J Exp Biol Med (Maywood) 2014; 239: 356-365.
[17] Han N, Zhang DY, Wang TB, Zhang PX, Jiang BG. Calcitonin gene-related peptide induces proliferation and monocyte chemoattractant protein-1 expression via extracellular signal-regulated kinase activation in rat osteoblasts. Chin Med J (Engl) 2010; 123: 1748-1753.
[18] Kawanami Y, Morimoto Y, Kim H, Nakamura T, Machida K, Kido T, Asonuma E, Yatera K, Yoshii C, Kido M. Calcitonin gene-related peptide stimulates proliferation of alveolar epithelial cells. Respir Res 2009; 10: 8.
[19] Tahtamouni LH, Alqurna NM, Al-Hudhud MY, Al-Hajj HA. Dandelion (taraxacum officinale) decreases male rat fertility in vivo. J Ethnopharmacol 2011; 135: 102-109.
[20] Cady RJ, Glenn JR, Smith KM, Durham PL. Calcitonin gene-related peptide promotes cellular changes in trigeminal neurons and glia implicated in peripheral and central sensitization. Mol Pain 2011; 7: 94.
[21] Yang L, Sakurai T, Kamiyoshi A, Ichikawa-Shindo Y, Kawate H, Yoshizawa T, Koyama T, Iesato Y, Uetake R, Yamauchi A, Tanaka M, Toriyama Y, Igarashi K, Shindo T. Endogenous CGRP protects against neointimal hyperplasia following wire-induced vascular injury. J Mol Cell Cardiol 2013; 59: 55-66.
[22] Han Y, Deng J, Guo L, Yan C, Liang M, Kang J, Liu H, Graham AM, Li S. CREG promotes a said smooth muscle cell phenotype and reduces neointimal formation in a balloon-injured rat carotid artery. J Cardiovasc Res 2008; 78: 597-604.
[23] Liu D, Huang Y, Bu D, Liu AD, Holmberg L, Jia Y, Tang C, Du J, Jin H. Sulfur dioxide inhibits vascular smooth muscle cell proliferation via suppressing the Erk/MAP kinase pathway mediated by cAMP/PKA signaling. Cell Death Dis 2014; 5: e1251.