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Effect of calcitonin gene-related peptide on angiotensin II-induced proliferation of rat vascular smooth muscle cells

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Abstract

The present study was designed to examine the effect of calcitonin gene-related peptide (CGRP) on angiotensin II-induced proliferation of cultured rat vascular smooth muscle cells. Vascular smooth muscle cells were grown from explants of Sprague–Dawley rat aorta. Vascular smooth muscle cells (between passages 5 and 10) were incubated with 0.1% neonatal calf serum for 48 h, and then treated with angiotensin II (100 nM) in the absence or presence of CGRP for 24 h. The viability, DNA synthesis and cell cycle of vascular smooth muscle cells were measured. Western blotting was used to determine the activity of intracellular extracellular regulated kinase (ERK1/2). Angiotensin II significantly decreased the viability and proliferation of vascular smooth muscle cells, decreased the proliferation index, and increased the activity of ERK1/2; the effects of angiotensin II were inhibited by CGRP (1–100 nM) in a concentration-dependent manner. In conclusion, CGRP significantly inhibits angiotensin II-induced proliferation of vascular smooth muscle cells, an effect related to a decrease in the activation of mitogen-activated protein kinase pathway.

Keywords: CGRP (calcitonin gene-related peptide); Angiotensin II; Smooth muscle cell, Vascular; Proliferation

1. Introduction

Angiotensin II, an octapeptide hormone, is the active component of the renin–angiotensin system and plays an important role in the mediation of vasomotor tone, cell growth and apoptosis, cell migration and extracelluar matrix deposition, events which are responsible for vascular remodeling and cardiac mass in hypertension (Nishimura et al., 1992). The proliferation of vascular smooth muscle cells induced by angiotensin II involves multiple signal pathways, including ERK1/2, a member of the mitogen-activated protein kinase (MAPK) family (Touyz and Schiffrin, 2000; Duff et al., 1992; Liao et al., 1996).

Calcitonin gene-related peptide (CGRP), a 37-amino acid peptide, is widely distributed in vascular tissues in both the central and the peripheral nervous system (Gibson et al., 1984; Lunberg et al., 1985; Zaidi et al., 1985; Kwasaki et al., 1988). CGRP has complex cardiovascular actions. There is evidence that administration of exogenous CGRP can inhibit excessive thickening of injured vascular intima and prevent restenosis (Dilinur et al., 1997). Our recent work has shown that losartan or perindopril produces significant hypotension and regression of vascular remodeling concomitantly with elevation of the level of CGRP in hypertensive rats (Qin et al., 2003; Xu et al., 2003). Others have found that CGRP inhibits the proliferation of vascular smooth muscle cells induced by fetal bovine serum (Li et al., 1997).

Since angiotensin II is an important contributor to the proliferation of vascular smooth muscle cells and CGRP has an inhibitory effect on the proliferation of vascular smooth muscle cells, in the present study we tested the effect of CGRP on the proliferation of vascular smooth muscle cells induced by angiotensin II. Since the facilitatory effect of angiotensin II on the proliferation of vascular smooth muscle cells involves the MAPK pathway, we also explored whether the inhibitory effect of CGRP on angiotensin-II-induced proliferation of vascular smooth muscle cells is related to inhibition of ERK1/2 activity.

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2. Materials and methods

2.1. Chemicals and drugs

MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-terazolium bromide), angiotensin II and CGRP were purchased from Sigma (USA). Phospho-specific ERK1/2 (p-ERK1/2) antibody was purchased from Cell Signaling Technology (USA). The second antibody (goat anti-mouse) was purchased from DAKO (Denmark). Dulbecco's modified Eagle's medium (DMEM) was purchased from GIBCO BRL (USA). [^{3}H]Thymidine (^{3}H-TdR) was purchased from Atomic Energy Research Institute of China. Dimethylsulfoxide (DMSO) was a product of AMERSCO (USA). Neonatal calf serum was purchased from the Sijiqing Company in Hangzhou of China.

2.2. Vascular smooth muscle cell culture

Healthy male Sprague–Dawley rats (180–200 g) were obtained from the Laboratory Animal Center, Xiang-Ya School of Medicine, Central South University. Animals received humane care in compliance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH publication 86-23, revised 1986).

Rat vascular smooth muscle cells were prepared using a modification of a previously described method (Li et al., 1997). Rats were anesthetized with sodium pentobarbital (60 mg/kg, i.p.), and the thoracic aorta was rapidly excised and the medial layer was cut into small pieces (0.5–1 mm^3 cubes). The explants were placed in the culture vials. DMEM supplemented with 20% neonatal calf serum was used as the initial incubation medium. The explants were incubated at 37 °C with 5% CO_2 in an incubator, to allow the vascular smooth muscle cells to migrate out of the explants. The subculture was carried out in DMEM with 10% neonatal calf serum after treatment with 0.25% trypsin. Passage 5–10 vascular smooth muscle cells were used for experiments.

2.3. Determination of cell viability

A modified MTT method was used for assaying vascular smooth muscle cell viability. Vascular smooth muscle cells (3×10^4/ml) were plated in 96-well plates for 24 h and incubated with DMEM containing 0.1% neonatal calf serum for 48 h. After vascular smooth muscle cells were incubated with angiotensin II (100 nM) in the absence or presence of CGRP for 24 h, the cells were treated with MTT (0.5 mg/ml) for 4 h at 37 °C. 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. Vascular smooth muscle cell viability is expressed relative to that (OD value) of control.

2.4. DNA synthesis assays

The proliferation of vascular smooth muscle cells was determined by [^{3}H]Thymidine incorporation, an index of DNA synthesis. Vascular smooth muscle cells were plated at an initial density of 3×10^4/well in 24-well trays in DMEM with 10% neonatal calf serum and incubated for 24 h, followed by an additional 24 h with 0.1% neonatal calf serum. The cells were exposed to DMEM with 0.1% neonatal calf serum in the absence or presence of angiotensin II or CGRP for 24 h. [^{3}H]Thymidine (1 μCi/ml) was added during the last 6 h and the cells were harvested onto glass fiber filters. Radioactivity was determined by liquid scintillation counting and presented as counts per minute. All cultures were performed in triplicate. Data are expressed as the percentage inhibition in the angiotensin II group (0.1% neonatal calf serum + 100 nM angiotensin II).

2.5. Cell cycle assays

Changes in the vascular smooth muscle cell cycle were assayed by flow cytometry (Beckman Coulter, USA). Quiescent vascular smooth muscle cells were treated with angiotensin II (100 nM) in the absence or presence of CGRP (1–100 nM) for 24 h. Cells were harvested into tubes and fixed with 70% ethanol, then washed with phosphate-buffered saline (PBS), and assayed by flow cytometer. Proliferation index (PI) was calculated with the formula: $PI=(S+G_2/M)/(G+S+G_2/M)$.

2.6. Determination of ERK1/2 activity

Quiescent vascular smooth muscle cells (2×10^6) grown on 60-mm plates were incubated in 0.1% neonatal calf serum DMEM. After cells were incubated with angiotensin II for 5 min, the reaction was terminated with cold PBS, and then 200 μl cell lysate (50 mM Tris, 150 mM NaCl, 0.1% SDS, 1% Nonidet P-40, 0.1 mg/ml phenylmethyl sulfonyl fluoride, 1 μg/ml leupetin) was added. Cell lysates were prepared by freezing, thawing on ice, scraping, and homogenization. After centrifugation ($10{,}000 \times g$) for 30 min (4 °C), the supernatant was stored at − 80 °C for Western blot analysis.

The samples were mixed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) buffer (50 mM Tris–HCl pH 6.8, 2% SDS, 100 mM dithiothreitol, and 0.1% bromophenol blue and 10% glycerol), followed by brief sonication and boiling for 3 min. Samples of 20 μg protein in each well were subjected to SDS-PAGE (10% separating gel and 5% stacking gel). After electrophoresis was finished, proteins were then transferred to a nylon polyvinylidene fluoride (PDVF) membrane (AMRESCO) as previously described (Liao et al., 1996). The membrane was blocked for 2 h at a room temperature with blocking buffer containing 4% milk, then incubated with phospho-specific ERK1/2 antibody for 2 h at 4 °C, followed by

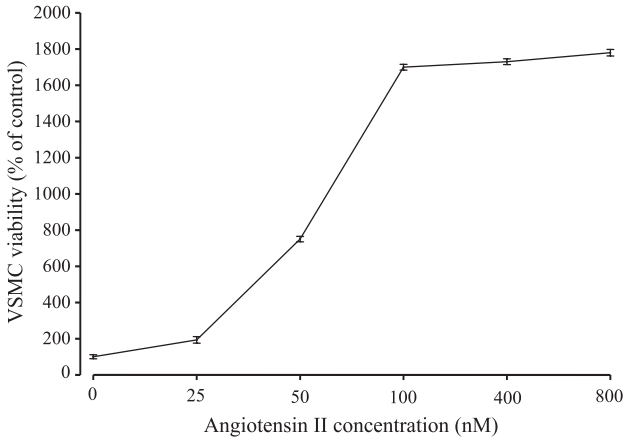

Fig. 1. Effect of angiotensin II on vascular smooth muscle cell viability. VSMC: vascular smooth muscle cell. Values are expressed as means ± S.E.M. ($n=8$).

incubation for 1 h with secondary antibody. Immunoreactive bands were visualized by enhanced chemiluminescence.

2.7. Statistical analysis

All result are expressed as means ± S.E.M. The data were analyzed using one-way analysis of variance and Newman–Keuls–Student's *t*-test. $P<0.05$ was considered significant.

3. Results

3.1. Effects of CGRP on the viability of vascular smooth muscle cells

Angiotensin II increased the viability of vascular smooth muscle cells in a dose-dependent manner (25–100 nM) (Fig. 1). The EC_{50} of angiotensin II was 53.7 ± 4.1 nM. Pretreatment with CGRP (1–100 nM) significantly attenuated the increased viability of vascular smooth muscle cells induced by angiotensin II (100 nM) in a dose-dependent manner (Table 1).

3.2. Effect of CGRP on the synthesis of DNA

Angiotensin II (100 nM) induced DNA synthesis in vascular smooth muscle cells, and CGRP (1–100 nM) dose-dependently inhibited the incorporation of [^{3}H]Thymidine into DNA in vascular smooth muscle cells stimulated by angiotensin II (100 nM) (Table 1).

Table 1
Effect of CGRP on viability and [^{3}H]Thymidine incorporation in DNA of vascular smooth muscle cells in the presence of angiotensin II (100 nM)

	Viability (%)	[^{3}H]Thymidine incorporation (%)
Angiotensin II	100.0 ± 1.3	100.0 ± 4.0
CGRP (1 nM)	88.0 ± 1.0[a]	87.0 ± 5.0[a]
CGRP (10 nM)	81.0 ± 2.4[b]	77.2 ± 5.5[b]
CGRP (100 nM)	70.1 ± 1.2[b]	61.3 ± 4.0[b]

Viability ($n=8$), [^{3}H]Thymidine incorporation ($n=3$). Values are expressed as means ± S.E.M. [a]$P<0.05$, [b]$P<0.01$ vs. Angiotensin II groups.

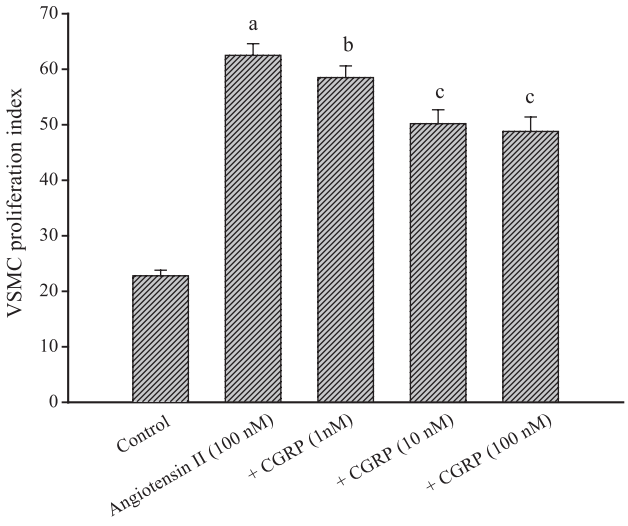

Fig. 2. Effect of CGRP on the proliferation index of vascular smooth muscle cells. VSMC: vascular smooth muscle cell. PI (proliferation index)=(S + G_2/M)/(G + S + G_2/M). Values are expressed as means ± S.E.M. ($n=5$). [a]$P<0.05$ vs. Control, [b]$P<0.05$, [c]$P<0.01$ vs. Angiotensin II groups.

3.3. Effect of CGRP on cell cycle of vascular smooth muscle cells

Angiotensin II (100 nM) significantly increased the percentage of cells in "S" cycle and "G_2" cycle, compared

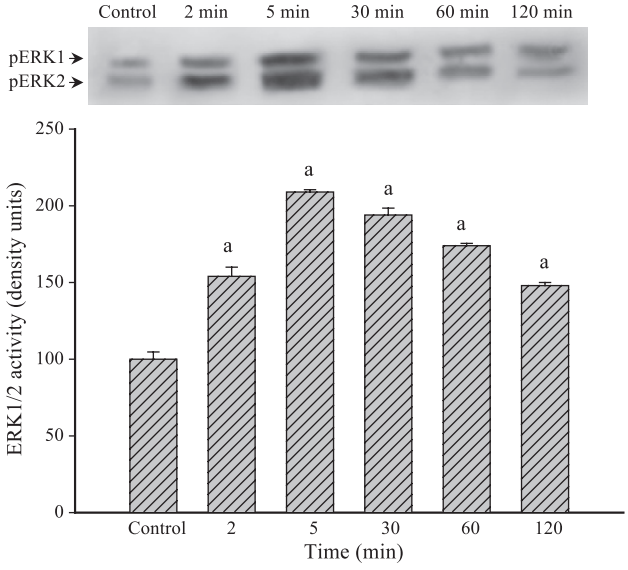

Fig. 3. Effect of angiotensin II on the activity of intracellular ERK1/2 in vascular smooth muscle cells. The activity of p-ERK1/2 was determined by Western blotting. Optical densities were quantitated and the results are shown in the bar graph under the Western blot of p-ERK1/2 products. Values are means ± S.E.M. ($n=3$). [a]$P<0.01$ vs. Control (0.1% neonatal calf serum).

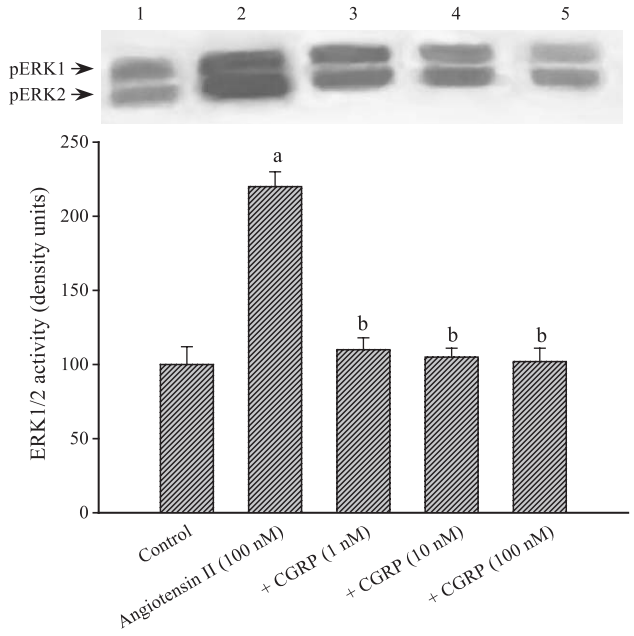

Fig. 4. Effect of CGRP on the activity of ERK1/2 of vascular smooth muscle cells in the presence of angiotensin II. The activity of ERK1/2 was determined by Western blotting. Optical densities were quantitated and the results are shown in the bar graph under the Western blot of p-ERK1/2 products. Ang II: angiotensin II. 1: Control (0.1% neonatal calf serum), 2: Ang II 100 nM, 3: 100 nM Ang II + 1 nM CGRP, 4: 100 nM Ang II + 10 nM CGRP, 5: 100 nM Ang II + 100 nM CGRP. Values are means ± S.E.M. ($n = 4$). [a]$P < 0.01$ vs. Control, [b]$P < 0.01$ vs. Angiotensin II group.

with that in the control group (0.1% neonatal calf serum). The proliferation index in the angiotensin II group was higher than that of the control group. However, CGRP (1–100 nM) significantly decreased the proliferation index of vascular smooth muscle cells in the presence of angiotensin II (100 nM) (Fig. 2).

3.4. Effect of CGRP on the activity of ERK1/2

ERK1/2 activity was significantly activated by angiotensin II. The peak activity of ERK1/2 was reached after 5 min and was sustained for 120 min (Fig. 3). Pretreatment with CGRP suppressed the increased activity of ERK1/2 induced by angiotensin II (100 nM) in a dose-dependent manner (Fig. 4).

4. Discussion

The proliferation of vascular smooth muscle cells is an important pathophysiological event in many cardiovascular diseases, such as hypertension and arteriosclerosis, and after percutaneous transluminal coronary angioplasty (Ross, 1993). Hyperplasia or hypertrophy of vascular smooth muscle cells is mainly responsible for vascular remodeling, which contributes to maintain high pressure in chronic hypertension. Angiotensin II, besides regulating vascular tone, participates in the mediation of cell growth and extracellular matrix deposition. As has been reported previously (Duff et al., 1992; Liao et al., 1996), in the present study angiotensin II induced the proliferation of vascular smooth muscle cells, suggesting that angiotensin II is an important contributor to vascular smooth muscle cell proliferation.

CGRP, the predominant neurotransmitter in cardiovascular sensory nerves, is a potent vasodilator. It has been demonstrated that many blood vessels are innervated by both sympathetic and capsaicin-sensitive sensory nerves, and it has been suggested that capsaicin-sensory nerves may play a role in the modulation of total peripheral resistance (Uddmann et al., 1986; Wisskirchen et al., 1999). In recent studies, we have found that endogenous CGRP is involved in the depressor effect and regression of vascular remodeling of losartan or perindopril in renohypertensive rats. Others have shown that CGRP prevents restenosis of balloon-denuded endothelium (Dilinur et al., 1997). In the present study, CGRP reduced the viability and DNA synthesis of vascular smooth muscle cells, and decreased the increased proliferation index of vascular smooth muscle cells induced by angiotensin II. The present results, along with previous findings that CGRP inhibited the proliferation of vascular smooth muscle cells induced by fetal bovine serum (Li et al., 1997), support the hypothesis that CGRP has an inhibitory effect on the proliferation of vascular smooth muscle cells.

The mechanism underlying the inhibitory effect of CGRP on vascular smooth muscle cell proliferation is unclear. It has been reported that CGRP inhibits the proliferation of vascular smooth muscle cells concomitantly with an elevation of the second-messenger, cAMP (Li et al., 1997). It is well know that ERK1/2, the important protein kinases in cell proliferation, are activated by many growth factors via different upstream signal proteins. It has been reported that angiotensin II activates ERK1/2 via PKC-ζ by cross-talk with the tyrosine kinase receptor pathway (Liao et al., 1997). The present results showed that CGRP reduced the increased activity of ERK1/2 induced by angiotensin II. These findings suggest that the inhibitory effect of CGRP on the proliferation of vascular smooth muscle cells involves the MAPK signaling pathway.

In summary, the present results suggest that CGRP inhibits the proliferation of vascular smooth muscle cells induced by angiotensin II, and that the intracellular signaling pathway underlying the antiproliferative effect of CGRP may be related to the inhibition of ERK1/2 activity.

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