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Research paper

Induction of interleukin-8 production by angiotensin II in rat vascular smooth muscle cells ☆

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Abstract

Objective: Interleukin-8(IL-8) represents the prototypical chemokine that is made by a wide variety of cell types. Previously studies have suggested that angiotensin II(Ang II) is involved in atherogenesis through induction of proinflammatory cytokines such as interleukin-6 or monocyte chemoattractant protein-1(MCP-1) in vascular smooth muscle cells(VSMCs), while the role of Ang II on IL-8 expression in VSMCs is poorly studied. **Methods:** In this study, VSMCs were isolated from the thoracic aorta of Sprague-Dawley rats. The expression of smooth muscle α-actin was confirmed by an immunohistochemical method. Semi-quantitative RT-PCR and enzyme-linked immunosorbent assay (ELISA) analyses were conducted to detect IL-8 expression. **Results:** In the present study we found that Ang II significantly increased the expression of IL-8 both at the mRNA and protein levels in rat VSMCs in a dose- and time-dependent manner. **Conclusion:** These findings suggested that Ang II may participate in atherosclerosis through induction of inflammatory mediator in VSMCs.

Key words: Angiotensin II; Interleukin-8; Vascular smooth muscle cells

INTRODUCTION

The renin-angiotensin system has emerged as one of the essential links in the pathophysiology of vascular disease. The most important effect of angiotensin II (Ang II) was considered to be its ability to cause contraction and hyperplasia of vascular smooth muscle cells (VSMCs). Moreover, Ang II has been shown to induce potent inflammatory responses in vascular cells by stimulating the release of cytokines and chemokines [1]. Monocyte chemoattractant protein-1(MCP-1) and interleukin-8(IL-8) are major chemokines belong to C-C and C-X-C chemokine subfamilies respectively[2,3]. Understanding the complex relationship between pro-inflammatory factors and vascular cells may contribute to the development of novel strategies for the treatment of atherosclerosis and other chronic inflammatory diseases. Our previously study has shown that MCP-1 and its receptor are involved in the anti-inflammatory effects of angiotensin receptor antagonist in spontaneously hypertensive rats[4]. In the present study we have observed the effect of Ang II on IL-8 production in VSMCs, which may help to further our understanding of the potent pro-inflammatory effect of Ang II.

MATERIALS AND METHODS

Cell isolation and culture

Rat VSMCs were prepared from the thoracic aorta of 12-week-old male Sprague-Dawley rats(Slaccas, Shanghai Institute for Biological Sciences, China). The study was approved by the Ethics Committee of Shanghai Jiaotong University School of Medicine. Animal handling followed the Declaration of Helsinki and the Guiding Priciples in the Care and Use of Animals. The aorta was excised rapidly and immersed in Dulbecco's Modified Eagle's Medium(DMEM; Gibco/BRL, USA) and cleaned carefully of connective tissue and adherent fat. Isolated aorta was longitudinally cut open,

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and the endothelium was removed by gently rubbing the intimal surface with sharp scissors. Denuded aorta was cut into approximately 3 mm sections and placed intimal side down into 60mm dishes. DMEM containing 20% fetal bovine serum(FBS; HyClone, USA), 100 U/ml penicillin and 100 μg/ml streptomycin(Gibco/BRL) was gently added to cover the tissues without disturbing the orientation of the explants and cultured at 37℃ in a humidified atmosphere of 95% air and 5% CO_2. VSMCs were allowed to grow out from the explants after 7-10 days, after which the tissues were removed. After confluence was reached, cells were harvested using 0.25% trypsin/EDTA(Gibco/BRL), grown in 60 mm dishes, and passaged twice a week by harvesting, using 0.25% trypsin/EDTA and seeding at a 1:2 ratio in 10 mm dishes. All experiments were conducted at cell passage 3 to 5. Prior to stimulation, 95% confluent VSMCs were serum-starved overnight by incubation in DMEM with 0.1% FBS. Cell cultures were incubated at 37℃ and 5%CO_2 in the presence or absence of Ang II(Sigma Chemical Co., USA) for the indicated time.

Immunohistochemistry

VSMCs were plated on 1% gelatin-coated coverslips. Fixation involved 4% paraformaldehyde for 15 min at room temperature. Samples were permeabilized with 0.25% Triton-X100(Sigma) for 10 min. Blocking was performed using 1% bovine serum albumin(BSA). An antibody against smooth muscle α-actin(α-SMA, diluted 1:100, Solomon Biotechnology Corp., China) was incubated overnight at 4℃. After an extensive washing, VSMCs were exposed to the Cy3-conjugated secondary antibody(diluted 1:100, Solomon Biotechnology Corp., China) at room temperature for 60 min. DAPI was used to stain nuclei. Samples were mounted with aqueous mounting medium(DAKO, USA). Fluorescence imaging involved use of a Nikon imaging system(Yokohama, Japan).

Semi-quantitative RT-PCR analysis

Total RNA from VSMCs in the presence or absence of Ang II was extracted at indicated times by using TRIzol Reagent(Invitrogen, USA) according to the manufacturer's instructions. cDNA was synthesized from 2 μg of total RNA by M-MLV reverse transcriptase(Invitrogen). The RT product(1 μl) was then amplified by PCR. The specific primers were designed by software primer premier 5.0 and purchased from Shanghai DNA Biotechnology Corporation Ltd (Shanghai China). The primers for IL-8 were 5'-GCACCCAAACCGAAGTCA-3'(sense) and 5'-AAGCCAGCGTTCACCAGA-3'(anti-sense), and the size of the amplified fragment was 169 base pair(bp). The primers for β-actin were 5'-GTAAAGACCT CTATGCCAACA-3'(sense) and 5'-GGACTCA TCGTACTCCTGCT-3'(anti-sense), and the size of the amplified fragment was 227 bp. PCR amplification conditions included an initial denaturation at 95℃ for 5 min, followed by 36 cycles of denaturation at 94℃ for 30 s, annealing at 55-60℃ for 40 s, and 30 s extending at 72℃, and a final extension at 72℃ for 10 min. PCR products were electrophoretically separated on 2% agarose gel and visualized by ethidium bromide staining. The optical densities of the resulting bands were determined by the Quantity One software (Bio-Rad). The densitometric values were normalized by β-actin.

Measurements of IL-8

IL-8 concentrations in the culture supernatants were determined using an enzyme-linked immunosorbent assay(ELISA) kit according to the manufacturer's instructions(Adlitteram Diagnostic Laboratories Inc. USA). The absorbance at 450 nm was measured and their concentrations were determined by interpolation of a standard calibration curve. The lower limit of detection of IL-8 was 1 pg/ml.

Statistical analysis

Data were expressed as means ± SEM. of at least three independent experiments. Statistical analyses involved use of one-way ANOVA or Student's *t*-test. $P < 0.05$ was considered statistically significant.

RESULTS

Characteristic of cultured VSMCs

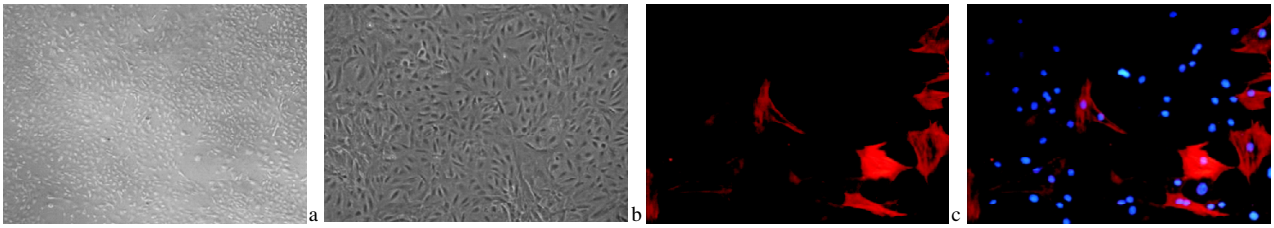
The cells obtained in this study showed the "hills and valleys" growth characteristic of cultured VSMCs (***Fig. 1a***). Cultured VSMCs at passage 3 to 5 were used in the experiment(***Fig. 1b***). The expression of smooth muscle α-actin was confirmed by the immunocytochemical method(***Fig. 1c, d***).

Effect of Ang II on IL-8 expression in VSMCs

Ang II significantly up-regulated the mRNA level of IL-8 in VSMCs in a dose-dependent manner, with maximal activity at 10^{-7} M Ang II(***Fig. 2a***). Moreover, the mRNA level of IL-8 was increased in a time-dependent manner in the presence of Ang II at 10^{-7} M (***Fig. 2b***). At the same time, addition of Ang II for 24h resulted in a dose-dependent increase in IL-8 levels in the conditioned medium(***Fig. 3a***). Furthermore, the basal IL-8 level was increased in a time dependent manner in the presence of Ang II at 10^{-7} M(***Fig. 3b***).

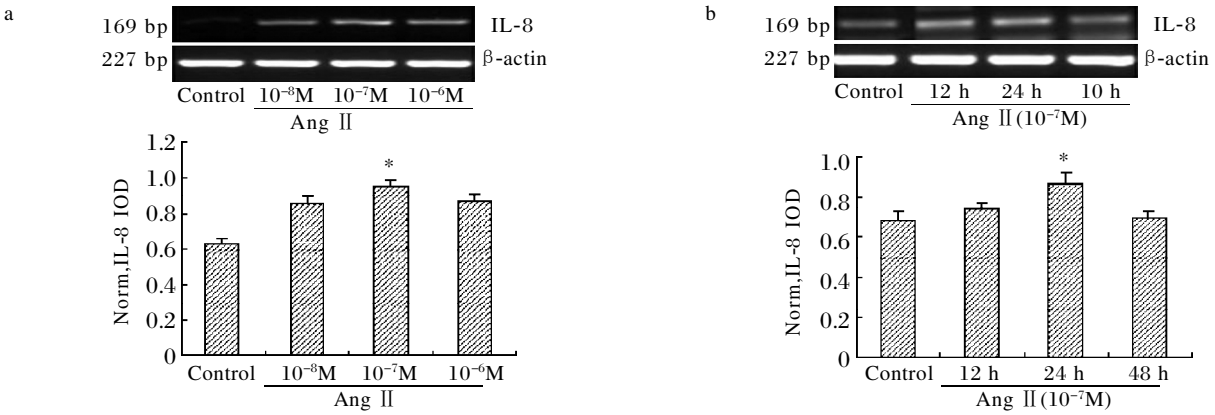
DISCUSSION

Hypertension is one of the risk factors for atherosclerosis. Since the inflammation status of a patient makes a substantial contribution to hypertension, it may be that inflammatory cytokines form the link between hypertension and atherosclerosis[5]. It is well



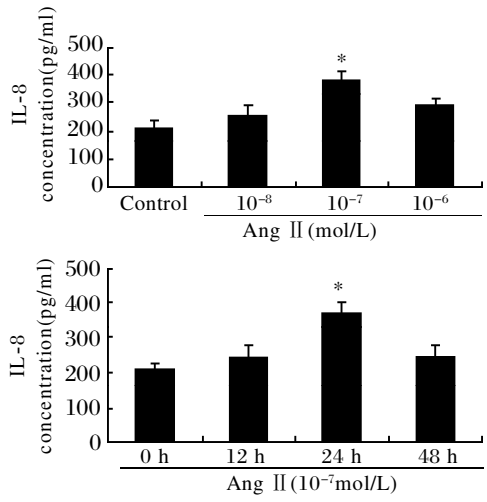
The cells obtained from rat thoracic aorta showed the "hills and valleys" growth characteristic of cultured VSMCs (a).Cultured VSMCs at passage 4 (b).VSMCs stained with smooth muscle α-actin(red fluorescence, (c).Double images of smooth muscle α-actin and DAPI(blue fluorescence, d).

Fig. 1 Characteristics of cultured VSMCs *in vitro*



Ang II significantly up-regulated the mRNA levels of IL-8 in a dose-dependent manner, with maximal activity at 10^{-7} M Ang II (a). The mRNA levels of IL-8 were increased in a time-dependent manner in the presence of Ang II at 10^{-7} M (b). Results expressed as integrated optical density(IOD) after normalization to β-actin (right panel; a, b) $^{*}P < 0.05$ vs. control.

Fig. 2 Dose- and time-dependent effect of Ang II on IL-8 expression at the mRNA levels in VSMCs



IL-8 concentrations in the culture supernatants were determined using ELISA analysis. Ang II significantly increased the IL-8 levels in a dose-dependent manner, with maximal activity at 10^{-7} M Ang II (upper panel, a) $^{*}P < 0.05$ vs. control(Ang II at 0 M). The concentrations of IL-8 were increased in a time-dependent manner in the presence of Ang II at 10^{-7} M (lower panel, b) $^{*}P < 0.05$ vs. control (Ang II at 10^{-7} M for 0 h).

Fig. 3 Dose- and time-dependent effect of Ang II on IL-8 expression at the protein level in VSMCs

known that the renin-angiotensin-aldosterone system is involved in the pathophysiology of hypertension. Historically, the most important effect of Ang II was considered to be the neurohormonal regulation of arterial blood pressure[1]. Recent studies have suggested that Ang II is also involved in atherogenesis through induction of proinflammatory cytokines such as interleukin-6 or MCP-1 in VSMCs[6,7], while the role of Ang II in IL-8 expression in VSMCs is poorly studied.

Previously studies showed that IL-8 is produced by many cells, including neutrophils, monocytes, macrophages, mast cells, vascular endothelial cells, stromal cells and epithelial cells[8-10]. In the present study we found that Ang II significantly increased the expression of IL-8 both at the mRNA and at the protein levels in rat VSMCs in a dose- and time-dependent manner. The effect of Ang II induced-IL-8 expression in the present study is in agreement with the other report which demonstrate that human VSMCs treated with Ang II also increase IL-8 production[11]. Recently Kim *et al* found that the expression levels of Ang II-induced IL-8/CXCL8 were significantly higher in VSMCs from spontaneously hypertensive rats than in VSMCs from Wistar-Kyoto rats[12]. These findings suggest that Ang II has an atherogenic role associated with IL-8 synthesis which may account for the link between hypertension and atherosclerosis.

IL-8 is important in the regulation of an acute

inflammatory response. A unique component of IL-8 is its relative longevity at sites of acute inflammation[3]. There are multiple examples of increased levels of IL-8 in human diseases, including heart disease[13-16]. Romuk *et al* reported that the levels of IL-8 were higher in unstable coronary heart disease patients in comparison to the stable coronary heart disease patients[13]. Furthermore, several studies confirmed that IL-8 is expressed in the intima and by macrophage-derived foam cells within atherosclerotic lesions[17,18]. With the increasing evidence that IL-8 can participate in the pathological process of atherosclerosis, it may be possible to develop a strategy to inhibit the production of IL-8 in the treatment of atherosclerosis. Ito *et al* reported that fluvastatin decreased the basal and Ang II-induced IL-8 production in human VSMCs[11]. Our previous observation showed that AT1 receptor antagonist treatment significantly decreased the gene expression of MCP-1 in the thoracic aorta, and decreased the serum protein levels of MCP-1 in SHR[4]. Whether an AT1 receptor antagonist is effective in decreasing the IL-8 production in VSMCs should be further investigated.

In conclusion, we demonstrated that Ang II increased IL-8 production in rat VSMCs in vitro. These findings suggest that Ang II may participate in atherosclerosis through induction of IL-8 in VSMCs.

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