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Angiotensin II induces C-reactive protein expression through ERK1/2 and JNK signaling in human aortic endothelial cells

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ABSTRACT

Background: Atherosclerosis is an inflammatory disease in the vessel. As an inflammatory cytokine, C-reactive protein (CRP) participates in atherogenesis. Although angiotensin II (AngII) is known to evoke inflammatory response in vascular endothelial cells (VECs), there is no direct evidence to demonstrate the proinflammatory effect of AngII on VECs through CRP. The present study focused on effect of AngII on CRP expression and the signal pathway in human aortic endothelial cells (HAECs).

Methods and results: mRNA and protein expression was identified by RT-PCR and Western blot, respectively. Reactive oxygen species (ROS) were observed by a fluorescence microscope. The results showed that AngII significantly increased mRNA and protein expression of CRP in HAECs in time- and concentration-dependent ways. Anti-IL-1β and anti-IL-6 neutralizing antibodies did not affect AngII-induced CRP expression. Losartan reduced AngII-induced CRP expression in mRNA and protein levels in HAECs. Losartan and TIFA decreased AngII-stimulated ROS generation, and antioxidant NAC completely abolished AngII-induced CRP expression in HAECs. The further study indicated that losartan, NAC, PD98059, SP600125 significantly inhibited ERK1/2 and JNK phosphorylation, and PD98059, SP600125, PDTC completely antagonized AngII-induced CRP expression in HAECs.

Conclusions: The present study demonstrates that AngII has ability to induce CRP expression in HAECs through AT_1-ROS-ERK1/2 and JNK-NF-κB signal pathway, which strengthens understanding of the proinflammatory and proathroscerotic actions of AngII.

1. Introduction

Although pathogenesis of atherosclerosis is not completely understood, increasing evidence has demonstrated that atherosclerosis is an inflammatory disease. Thus, the elevated levels of circulating inflammatory markers, like C-reactive protein (CRP), tumor necrosis factor-α, interleukin-6 (IL-6) and cellular adhesion molecules, are associated with the increased risk of cardiovascular diseases [1]. Among the markers, CRP has been identified as a non-specific but sensitive marker of the acute inflammatory response. CRP is not only an important predictor of atherothrombotic events, myocardial infarction and stroke, but also a direct participator in the formation and development of atherosclerosis [2]. It is well known that CRP modulates the activities and expressions of multiple factors implicated in atherogenesis, such as enhancing the expressions of IL-8 and lectin-like oxidized low-density lipoprotein receptor-1, and decreasing endothelial nitric oxide synthase (eNOS) expression and prostacyclin generation in human aortic endothelial cells (HAECs) [3–6]. Recent studies show that CRP is also expressed in vascular smooth muscle cells (VSMCs), vascular endothelial cells (VECs) and atherosclerotic lesions [7–10].

Angiotensin II (AngII), the major bioactive peptide hormone of rennin–angiotensin system, is involved in pathogeneses of various cardiovascular diseases including hypertension, atherosclerosis and heart failure and so on [11]. AngII is able to stimulate release of monocyte chemoattractant protein-1 (MCP-1), plasminogen activator inhibititor-1 (PAI-1), and intercellular adhesion molecule-1 (ICAM-1) in endothelial cells, which contribute to the development of atherosclerosis [12–14]. Our previous work suggested that AngII may induce CRP expression in rat VSMCs [15].

Although AngII is known to evoke the inflammatory responses in VECs, there is no direct evidence to demonstrate the proinflammatory effect of AngII on HAECs through CRP. Therefore, the present study focused on effect of AngII on CRP expression in HAECs and the related signal pathway, especially emphasizing reactive oxygen species (ROS)-mitogen-activated protein kinase (MAPK)-NF-κB signaling.

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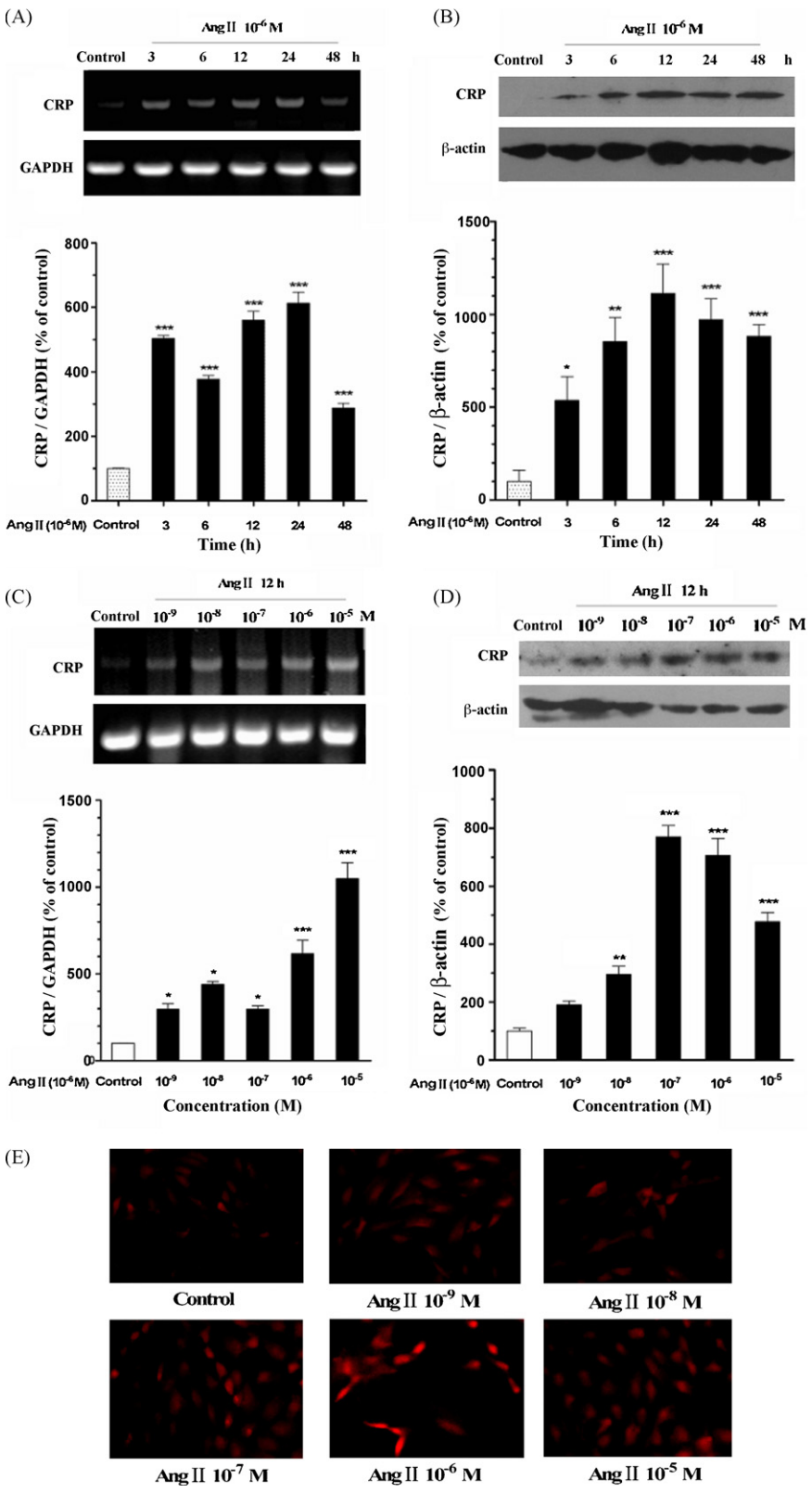

Fig. 1. AngII induces CRP expression in HAECs. (A) Time-dependent increase of CRP mRNA expression, and (B) time-dependent increase of CRP protein expression in HAECs. The cells were treated with 10^{-6} M AngII for the indicated time. Then, mRNA and protein expression of CRP was identified by RT-PCR and Western blot, respectively. (C) Concentration-dependent increase of CRP mRNA expression, and (D and E) concentration-dependent increase of CRP protein expression in HAECs. The cells were treated with the different concentrations of AngII for 12 h. Then, CRP mRNA expression was identified by RT-PCR, and CRP protein expression was assayed by Western blot and fluorescent staining. Results from three independent experiments are expressed as means ± S.E.M. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. control.

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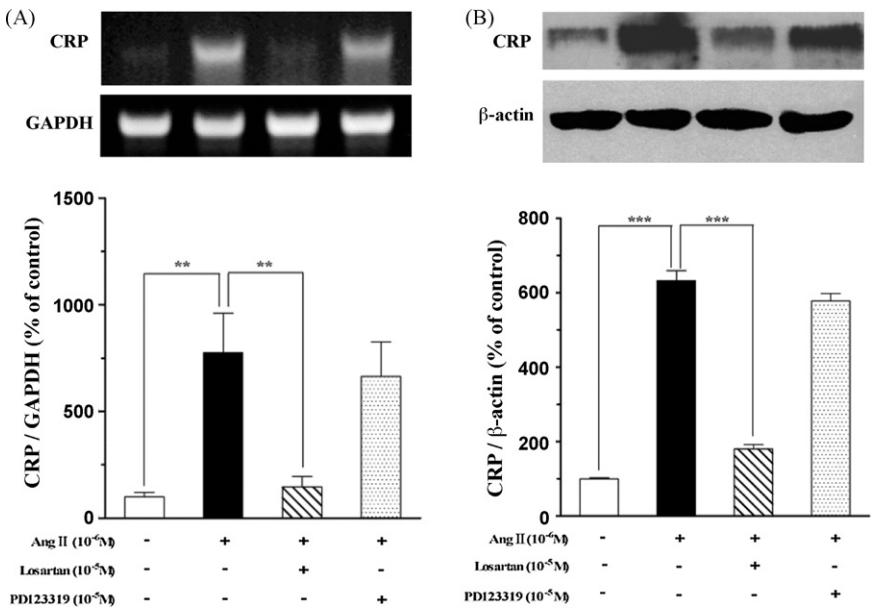

Fig. 2. AngII induces CRP expression in HAECs via AT_1 receptor. HAECs were pretreated with AT_1 receptor blocker losartan or AT_2 receptor blocker PD123319 for 1 h prior to stimulation with AngII for 12 h. Then, mRNA (A) and protein (B) expression of CRP in HAECs was analyzed by RT-PCR and Western blot. Results from three independent experiments are expressed as means ± S.E.M. $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. control.

2. Materials and methods

2.1. Chemicals and antibodies

AngII, PD98059, SP600125, SB203580, *N*-acetylcysteine (NAC), pyrrolidine dithiocarbamate (PDTC), thenoyltrifluoroacetone (TIFA) and diphenyleneiodonium (DPI) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Losartan was from Merk (Merk Corp., Darmstadt, Germany). PD123319 was obtained from TOCRIS (Bristol, UK). Polyclonal anti-human C-reactive protein antibody was provided by ABCAM (Cambridge, UK). Anti-human IL1β neutralizing antibody and anti-human IL-6 neutralizing antibody were ordered from R&D Systems (MN, USA). Antibodies against phospho-ERK1/2, ERK1/2, phospho-JNK and JNK were supplied by Beyotime (Jiangsu, China).

2.2. Cell culture

HAECs were purchased from Cascade Biologics (Oregon, USA), and cultured in Medium 200 (Cascade Biology) supplemented with low serum growth supplement (LSGS, Cascade Biology) containing 2% fetal bovine serum (FBS), 1 μg/ml hydrocortisone, 10 ng/ml human epidermal growth factor, 3 ng/ml basic fibroblast growth factor, 10 μg/ml heparin, 100 U/ml penicillin and 100 μg/ml streptomycin. When grown to 70–80% confluence, HAECs were kept in the serum-free medium for 12 h prior to the experiment, and then stimulated by AngII as indicated. In the inhibitor experiments, HAECs were exposed to AngII (10^{-6} M) for 12 h after pretreated with the inhibitors for 1 h.

2.3. RT-PCR

The total RNA was extracted from HAECs using Trizol (Invitrogen, NY, USA) as described previously [16]. cDNA were synthesized from 1 μg total RNA in 20 μl reaction using oligo $(dT)_{18}$ Primer and RevertAid^{TM} M-MuLV Reverse Transcriptase (Fermentas, St. Leon-Rot, Germany). mRNA expressions of CRP, AngII type 1 receptor (AT_1) and GAPDH were examined by RT-PCR. Sequence-specific PCR primers used were: CRP, forward 5′-TCGTATGCCACCAAGAGACAAGACA-3′, reverse 5′-AACACTTCGCCTTGCACTTCATACT-3′ [10], giving 440 bp PCR product; AT_1, forward 5′-ACTAGGCATCATACGTGACTGTAG-3′, reverse 5′-TGTTGAAAGGTTTGAGTGGG-3′, giving 196 bp PCR product; GAPDH, forward 5′-AACATCATCCCTGCCTCTACTGG-3′, reverse 5′-CTCCGACGCCTGCTTCACC-3′, giving 189 bp PCR product. PCR products were separated by electrophoresis on 2% agarose gel. Expression of mRNA was quantified as relative to internal-control GAPDH.

2.4. Fluorescent staining of CRP

HAECs plated on 6-well culture plates with glass cover slips were starved for 12 h in the serum-free medium prior to the treatment with the indicated concentrations of AngII for 12 h. After that, the cells were fixed with 4% paraformaldehyde for 20 min, washed three times with phosphate-buffered saline (PBS), permeabilized with 0.2% Triton X 100 for 10 min at room temperature, and blocked with 10% goat serum for 30 min. Then, the cells were incubated with rabbit anti-human CRP antibody (1:50 dilution) overnight at 4 °C, and further treated with fluorescence-labeled secondary antibody (1:50 dilution) for 2 h at room temperature. Finally, the slides were observed under a fluorescent microscope (Olympus BX51, Japan).

2.5. Western blot analysis

The treated cells were washed with PBS, lysed by 200 μl ice-cold RIPA lysis buffer supplemented with the protease inhibitor cocktail (Roche, Mannheim, Germany). Protein concentration was measured by BCA protein assay kit (Pierce, Rockford, USA). Equal amount of protein extract (50 μg) was loaded, separated by 12% SDS-PAGE, and blotted onto nitrocellulose membrane. Then, the membranes were incubated with rabbit polyclonal anti-CRP (1:50 dilution), anti-JNK (1:1250 dilution), anti-phospho-JNK (1:1250 dilution), anti-phospho-ERK (1:1000 dilution), anti-ERK (1:1000 dilution), anti-beta actin (1:1000 dilution) antibodies overnight at 4 °C. After washed, the membranes were incubated with anti-rabbit or anti-mouse IgG antibody conjugated with horseradish peroxidase for 2 h at room temperature. The immunostaining was visualized by the enhanced chemiluminescence (Pierce, Rockford, USA).

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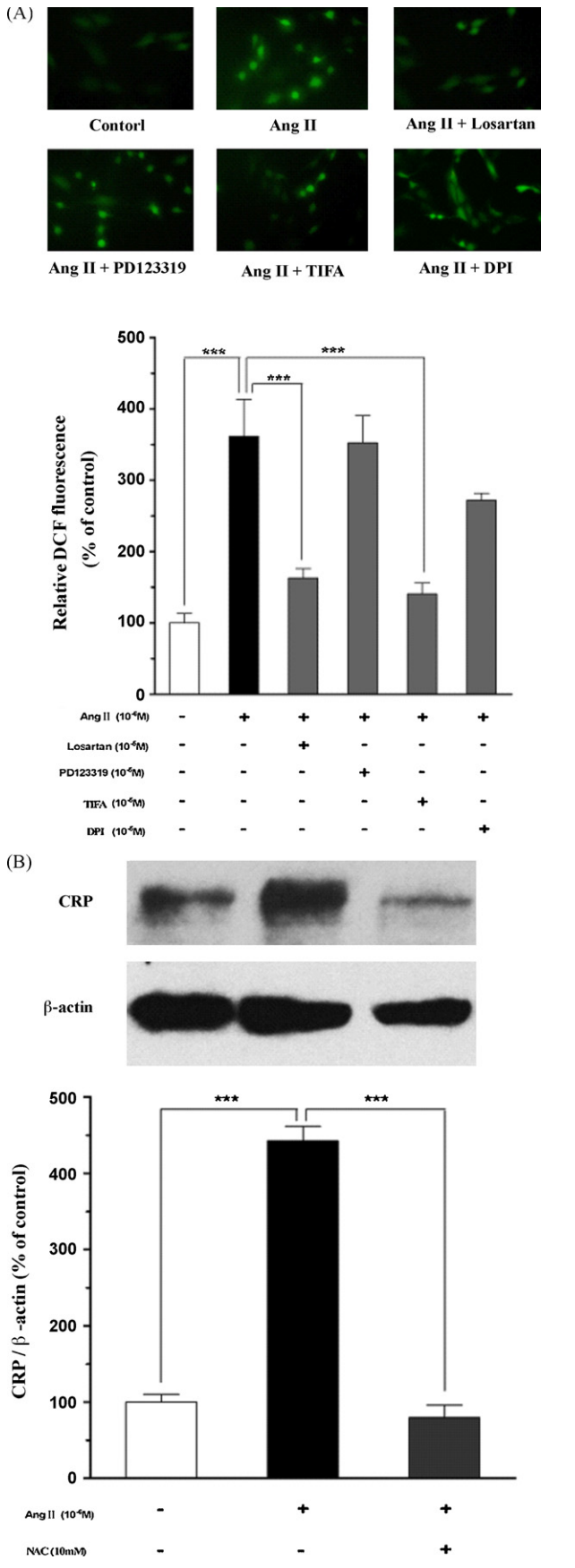

Fig. 3. Involvement of ROS in AngII-induced CRP expression in HAECs. (A) The cells were pretreated with losartan, PD123319, TIFA (complex II inhibitor) or DPI [NAD(P)H oxidase inhibitor] for 1 h prior to stimulation with AngII for 12 h, and then the cells were incubated for 1 h with H_2DCF-DA (10 μM). Finally, the fluorescent intensity was measured by a fluorescence microscope. (B) The cells were incubated with AngII for 12 h after pretreated with antioxidant NAC for 1 h. Then, CRP protein expression was identified by Western blot. Results from three independent experiments are expressed as means ± S.E.M. ***$P < 0.001$ vs. control.

2.6. Measurement of ROS

Intracellular ROS in HAECs was measured using 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) fluorescent labeling method. HAECs in 6-well culture plates were exposed to AngII (10^{-6} M) for 12 h after pretreatment with losartan (10^{-5} M), PD123319 (10^{-5} M), TIFA (10^{-5} M), or DPI (10^{-5} M) for 1 h. Then, the cells were incubated with H_2DCF-DA (10 μM) for 1 h. The fluorescent intensity was measured by a fluorescence microscope (Olympus BX51, Japan) at the excitation wavelengths of 488 nm and emission wavelengths of 525 nm [17].

2.7. Neutralizing experiment

HAECs were pretreated with anti-IL-1β or/and anti-IL-6 neutralizing antibody (0.1 μg/ml) for 1 h before the stimulation with AngII (10^{-6} M) for 24 h [10]. Then, CRP protein expression was assayed by Western blot.

2.8. Statistical analysis

The results were expressed as means ± S.E.M. Differences between groups were determined by one-way ANOVA. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. AngII induces CRP expression in HAECs

Fig. 1A and B showed that AngII at 10^{-6} M significantly increased mRNA and protein expression of CRP in HAECs in time-dependent way. CRP expression was obviously upregulated in 3 h, and reached the maximum in 24 h or 12 h. The results in Fig. 1C and D showed that AngII from 10^{-9} to 10^{-5} M concentration-dependently upregulated mRNA and protein expression of CRP in HAECs. The similar result was achieved with the fluorescent staining of CRP (Fig. 1E).

3.2. AngII induces CRP expression in HAECs via AT_1 receptor

To investigate whether AngII receptors were involved in AngII-induced expression of CRP in HAECs, the cells were stimulated with 10^{-6} M AngII for 12 h after pretreatment with losartan (AT_1 receptor blocker) or PD123319 (AT_2 receptor blocker) for 1 h. As shown in Fig. 2A and B, losartan substantially reduced AngII-induced mRNA and protein expression of CRP in HAECs, while PD123319 had no significant effect on CRP expression.

3.3. Involvement of ROS in AngII-induced CRP expression in HAECs

To determine whether intracellular ROS participates in AngII-induced production of CRP, intracellular ROS generation was observed. As shown in Fig. 3A, AngII significantly increased ROS generation in HAECs. But, pretreatment of the cells with losartan and TIFA (complex II inhibitor) obviously reduced AngII-stimulated ROS generation. Although DPI [NAD(P)H oxidase inhibitor] showed an inhibitory trend on AngII-induced ROS production, there was no significantly statistical difference. Further investigation revealed

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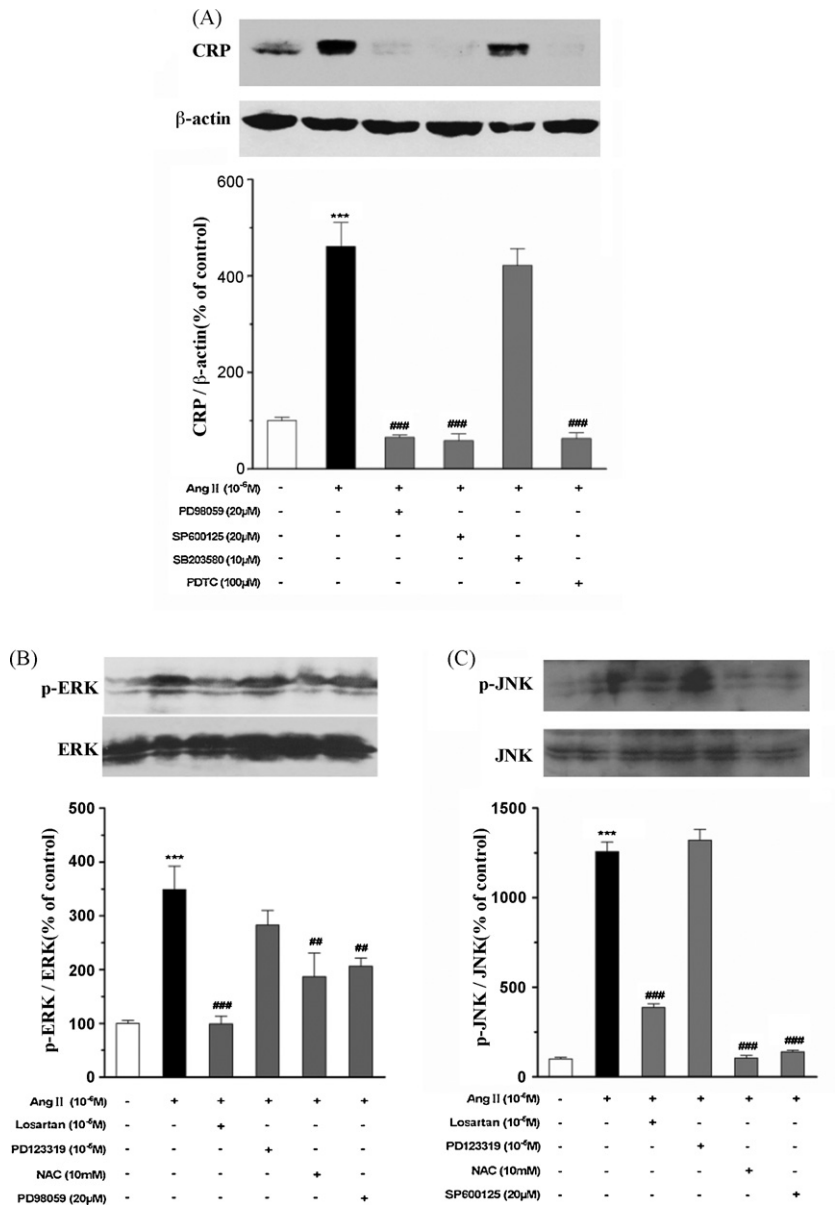

Fig. 4. AngII induces CRP expression in HAECs through AT_1-ROS-MAPK-NF-κB signal pathway. (A) AngII-induced CRP protein expression in HAECs through ERK1/2- and JNK-NF-κB signal pathway. The cells were pretreated with PD98059 (MEK1/2 inhibitor), SP600125 (JNK inhibitor), SB203580 (p38 MAPK inhibitor) or PDTC (NF-κB inhibitor) for 1 h before exposure to AngII for 12 h. Then, CRP protein expression was assayed by Western blot. (B and C) Involvement of AT_1 and ROS in AngII-induced activation of ERK1/2 and JNK in HAECs. The cells were preincubated with losartan, PD123319, NAC, PD98059 or SP600125 for 1 h before stimulation with AngII for 1 h. Then, expressions of ERK1/2 and JNK, phosphorylated ERK1/2 and JNK were detected by Western blot. Results from three independent experiments are expressed as means ± S.E.M. $^{***}P < 0.001$ vs. control. $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ vs. AngII alone.

that antioxidant NAC completely abolished AngII-induced CRP protein expression in HAECs (Fig. 3B).

3.4. AngII induces CRP expression in HAECs through AT_1-ROS-MAPK-NF-κB signal pathway

Following stimulation of HAECs with AngII for 12 h, CRP protein expression was significantly increased. However, pretreatment of the cells with PD98059 (MEK1/2 inhibitor), SP600125 (JNK inhibitor), or PDTC (NF-κB inhibitor) for 1 h completely antagonized AngII-induced CRP expression. But, p38 MAPK inhibitor SB203580 did not show the similar effect (Fig. 4A).

The above-mentioned results indicate that AT_1 receptor, ROS, ERK and JNK molecules participate in AngII-induced CRP expression in HAECs. To elucidate whether AngII receptors and ROS mediated AngII-induced activation of ERK1/2 and JNK in HAECs, phosphorylated ERK1/2 and JNK were determined. The results displayed that a marked increase of the phosphorylated ERK1/2 and JNK was detected following stimulation of the cells with AngII. However, pretreatment of the cells with losartan, NAC, PD98059 or SP600125 for 1 h prior to exposure to AngII for 1 h significantly inhibited AngII-induced phosphorylation of ERK1/2 and JNK (Fig. 4B and C).

3.5. Effect of IL-1β and IL-6 on AngII-induced CRP expression in HAECs

Since IL-1β and IL-6 are known to stimulate CRP production in human endothelial cells [8,10], AngII-induced CRP expression could be related to these cytokines. Therefore, we observed AngII-induced CRP expression in the absence or presence of IL-1β and IL-6. As seen from Fig. 5A, IL-1β and IL-6 alone induced CRP protein expression in HAECs. In the combined stimulation with IL-1β

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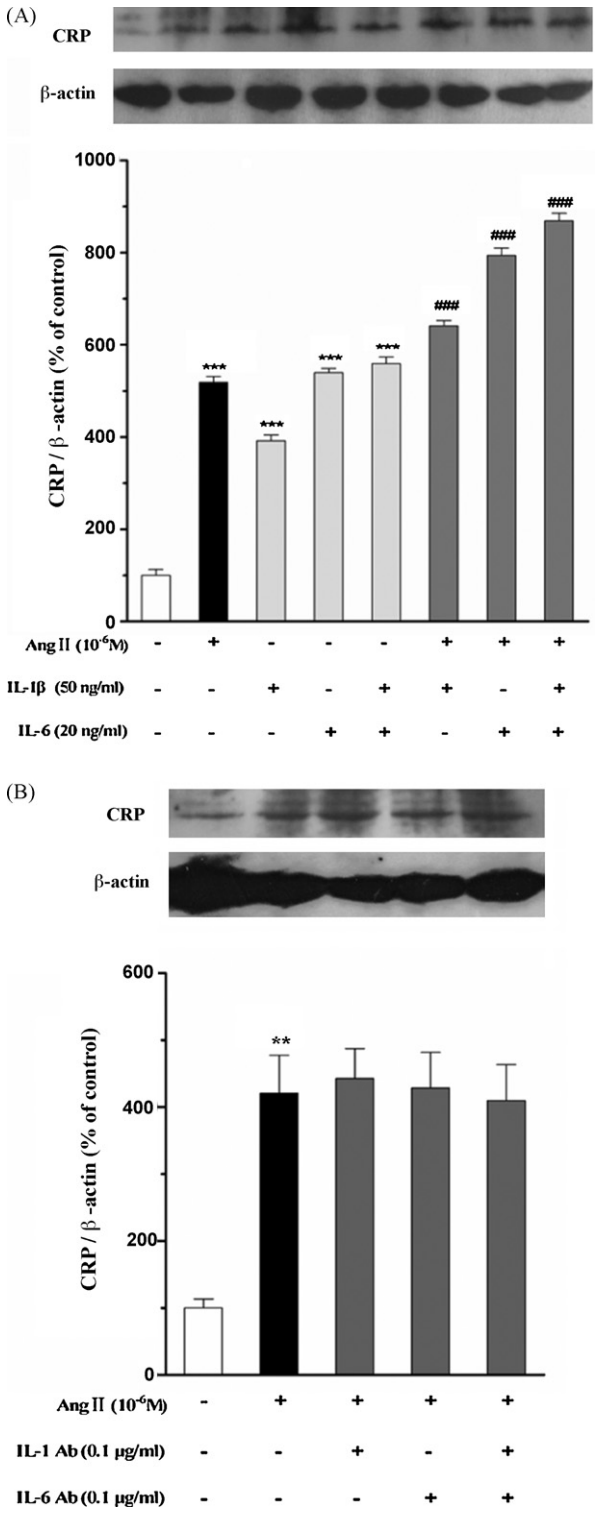

Fig. 5. AngII-induced CRP protein expression is independent of IL-1β and IL-6 in HAECs. (A) Effect of IL-1β and IL-6 on AngII-induced CRP expression in HAECs. The cells were treated for 24 h with IL-1β or/and IL-6 in the absence or presence of AngII. (B) Effect of anti-IL-1β and anti-IL-6 neutralizing antibodies on AngII-induced CRP expression in HAECs. The cells were pretreated for 1 h with anti-IL-1 or/and anti-IL-6 neutralizing antibody before the stimulation with AngII for 24 h. Then, CRP protein expression was assayed by Western blot. Results from three independent experiments are expressed as means ± S.E.M. $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. control. $^{###}P < 0.001$ vs. AngII alone.

or/and IL-6 and AngII, CRP expression in HAEC was significantly increased in comparison to AngII alone.

Further experiment illustrated that anti-IL-1β or anti-IL-6 neutralizing antibody alone or in combination was unable to influence AngII-induced CRP protein expression in HAECs (Fig. 5B).

4. Discussion

At present, most scientists support the concept that vascular inflammation is a key of atherosclerotic lesion, formation, progression and eventual rupture. As a representative marker of the inflammatory response, CRP directly participates in all stages of atherosclerotic process. Although hepatocytes and non-hepatic tissues including neurons, renal epithelium and respiratory tract are believed to be contributors to circulating CRP in plasma [18–20], the growing evidence has identified that the vascular cells including VSMCs and VECs can also secrete CRP. Moreover, the locally produced CRP in the vessel wall may play a direct and essential role in the whole inflammatory process of atherosclerosis and the development of cardiovascular complications [21].

A great number of evidence from animal and clinical experiments supports the prominent role of hypertension in pathogenesis of atherosclerosis. As a potent proinflammatory agent in the vessel wall, AngII has been widely accepted as a pivotal mediator in hypertension-induced arteriosclerosis [22]. Several in vivo studies demonstrate that transiently or chronically subcutaneous infusion of AngII activates expressions of cell adhesion molecules and chemokines, and amplifies atherosclerosis in apolipoprotein E-deficient mice, which distinctly suggest a strong association between AngII, inflammation and atherosclerosis [22,23].

Endothelial activation and dysfunction play the important roles in atherogenesis through multiple ways, including proinflammatory action. It is reported that VECs participate in the vascular inflammatory responses by expressing MCP-1, PAI-1, tissue factor and ICAM-1 under AngII stimulation [12–14]. CRP decreases eNOS and prostacyclin, and increases IL-8, ICAM-1 and vascular cell adhesion molecule-1 through Fcgamma receptors CD32 and CD64 in HAECs [24].

In the present study, we found that AngII at concentration used stimulated HAECs to produce CRP in mRNA and protein levels, which is consistent with our previous finding in VSMCs [15]. This result enriches the proinflammatory effects of AngII on VECs, and further support the inflammatory role of VECs in atherosclerosis.

The known evidence shows that there exists AT_1 receptor in VECs [25], and AngII promotes CRP production by AT_1 receptor in VSMCs [15]. Therefore, AngII possibly regulates the inflammatory response of VECs via AngII receptors. In the experiment, the specific AT_1 receptor blocker losartan almost completely abolished AngII-induced mRNA and protein expression of CRP in HAECs, while AT_2 receptor blocker PD123319 did not change the effect of AngII on CRP expression, suggesting that AngII induces CRP expression in HAECs via AT_1 receptor.

It is well known that ROS mediate production of the inflammatory cytokines through the multiple signal pathways including MAPK signaling, and AngII may stimulate ROS production in the vascular cells [26]. In this study, we confirm that AngII can stimulate ROS generation in HAECs via AT_1/mitochondria-derived pathway, but not NAD(P)H oxidase system, since AT_1 receptor antagonist losartan and mitochondrial respiratory complex II inhibitor TIFA remarkably antagonized AngII-stimulated generation of ROS, whereas AT_2 receptor antagonist PD123319 and NAD(P)H oxidase inhibitor DPI did not show the significant inhibitory effect. These data are consistent with the others' results [26,27]. Furthermore, we also found that antioxidant NAC completely inhibited AngII-induced CRP protein expression. These

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indicate that AngII-induced CRP expression in HAECs involves the intracellular ROS, and AT_1 receptor mediates AngII-stimulated ROS production.

MAPK activation is associated with the vascular inflammation in atherosclerosis. AngII-elicited ROS signaling has been implied to mediate MAPK activation [28]. In the present study, we observed that AngII activated ERK1/2 and JNK phosphorylation. As AT_1 receptor blocker losartan and antioxidant NAC significantly inhibited AngII-induced ERK1/2 and JNK phosphorylation, it is suggested that AngII-activated ERK1/2 and JNK phosphorylation is mediated by AT_1 receptor and ROS. This is also consistent with the report of Hsu et al. that ROS-mediated phosphorylation of ERK1/2 is involved in AngII-stimulated production of ET-1 in VECs [26]. Moreover, activation of ERK1/2 and JNK is necessary for AngII-induced CRP expression in HAECs, since MEK1/2 inhibitor PD98059 and JNK inhibitor SP600125 evidently antagonized AngII-induced CRP protein expression. But, the effect does not involve p38MAPK. The results confirm that AngII induces CRP expression through ERK1/2- and JNK-dependent signal pathway in HAECs.

Several lines of evidence indicate that ROS and MAPK are implicated in activation of NF-κB [29]. MAPK is considered to be an upstream event of NF-κB activation, and AngII is able to promote NF-κB activation [30] in VECs. In this study, NF-κB is involved in AngII-induced protein expression of CRP, since PDTC, a selective NF-κB inhibitor, also completely inhibited CRP protein expression in HAECs in response to AngII.

Although AngII has been verified to stimulate CRP generation in HAECs, we do not exclude the possibility that other inflammatory cytokines, such as IL-1β and IL-6, mediate the process. Our another experiment revealed that IL-1β and IL-6 induced CRP protein expression in HAECs, which further supports the previous findings presented by Singh et al. and Venugopal et al. [8,10]. Furthermore, IL-1β or/and IL-6 plus AngII produced an additional effect on CRP expression in HAECs. Despite all this, AngII-induced CRP expression in HAECs is independent of IL-1β and IL-6, because pretreatment of the cells with anti-IL-1 or anti-IL-6 neutralizing antibody did not affect AngII-induced CRP expression.

In conclusion, the present study demonstrates that AngII has ability to induce CRP expression in HAECs through AT_1-ROS-ERK1/2 and JNK-NF-κB signal pathway, which strengthens understanding of the proinflammatory and proathroscerotic actions of AngII.

Acknowledgement

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