Angiotensin II Induces the Expression of C-reactive Protein via MAPK-Dependent Signal Pathway in U937 Macrophages

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Key Words

Angiotensin II • C-reactive protein • Macrophages • Atherosclerosis • Inflammation

Abstract

Atherosclerosis is an inflammatory disease in the vessel wall. As an inflammatory molecule, C-reactive protein (CRP) participates in all stages of atherosclerotic process. Although angiotensin II (Ang II) can stimulate the vascular cells to produce CRP, it is unknown whether Ang II induces CRP expression in macrophages. The present study was to observe effect of Ang II on CRP production and the related signal pathway in U937 macrophages so as to provide more evidence for the proinflammatory action of Ang II. The results showed that Ang II significantly increased mRNA and protein expression of CRP in U937 macrophages in time- and concentration-dependent manners. AT₁ receptor blocker losartan blocked Ang II-induced CRP expression in mRNA and protein levels in U937 macrophages. Losartan and complex II inhibitor TIFA decreased Ang II-stimulated reactive oxygen species (ROS) generation, and antioxidant NAC completely abolished Ang II-induced CRP expression in U937 macrophages. The further study indicated

that losartan, NAC, MEK1/2 inhibitor PD98059, p38MAPK inhibitor SB203580 obviously inhibited ERK1/2 and p38MAPK phosphorylation, and PD98059, SB203580 and NF- κ B inhibitor PDTC reduced Ang II-induced mRNA and protein expression of CRP in U937 macrophages. These demonstrate that Ang II is capable of inducing CRP generation in macrophages via AT₁-ROS-ERK1/2/p38MAPK-NF- κ B signal pathway, which contributes to better understanding of the proinflammatory and proatherosclerotic actions of Ang II.

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Introduction

Numerous experimental studies and clinic observations have suggested that systemic inflammation or local inflammation in the vascular wall is connected with atherogenesis. The inflammation features production of a variety of inflammatory cytokines, such as interleukin-1 (IL-1), interleukin-6 (IL-6), tumor necrosis factor- α

(TNF- α) and C-reactive protein (CRP), and activation of the inflammatory cells like monocytes/macrophages [1-4]. Among the inflammatory cytokines, CRP shows the strongest association with increased risk of cardiovascular events. CRP is not only an inflammatory marker, but also an active player in the formation and development of atherosclerosis [5]. CRP stimulates the expressions of adhesion molecules [6] and matrix metalloproteinase-1 [7], activates the complement system [8] and impedes vasorelaxation [9]. In addition, CRP is able to mediate uptake of low density lipoprotein by macrophages in the vascular intima, thereby promoting formation of foam cells [10].

Angiotensin II (Ang II), the most active component of the renin-angiotensin system, plays multiple roles in atherogenesis. In addition to stimulating proliferation of vascular smooth muscle cells (VSMCs) [11] and activating vascular endothelial cells (VECs) [12], Ang II produces a significant proinflammatory action in the vascular wall. It is known that Ang II stimulates releases of IL-6, TNF- α , interferon- γ , monocyte chemoattractant protein-1 (MCP-1), promotes ROS production, and upregulates expressions of CX3CR1, TLR4, matrix metalloproteinases and myeloperoxidase in macrophages [13-16]. Our previous investigations confirmed that Ang II may induce CRP expression in human aortic endothelial cells (HAECs) and VSMCs [17, 18].

Although Ang II is recognized to evoke the inflammatory responses in macrophages, there is no direct evidence to demonstrate the proinflammatory effect of Ang II on macrophages through CRP. Therefore, the present study was to probe the effect of Ang II on CRP expression in U937 macrophages and the molecular mechanisms, particularly focusing reactive oxygen species (ROS)-mitogen-activated protein kinases (MAPK)-NF- κ B signal pathway.

Materials and Methods

Chemicals and antibodies

Ang II, PD98059, SB203580, SP600125, pyrrolidine dithiocarbamate (PDTC), thenoyltrifluoroacetone (TIFA), N-acetylcysteine (NAC) and diphenyleneiodonium (DPI) were obtained from Sigma-Aldrich (St. Louis, MO, USA). PD123319 was purchased from TOCRIS (Bristol, UK). Losartan was from Merck (Merck Corp., Darmstadt, Germany). Human CRP ELISA kit was bought from Alpha Diagnostics (San Antonio, TX, USA). ROS assay kits, antibodies against phospho-ERK1/2, ERK1/2, phospho-p38 and p38MAPK were ordered from the Beyotime Institute of Biotechnology (Jiangsu, China). BCA

protein assay kit was supplied by Pierce (Rockford, IL, USA).

Cell cultures

U937 histiocyte (American Type Culture Collection) is also called resident macrophage [19]. U937 macrophages were cultured in a 5% CO₂ atmosphere in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 25 mM HEPES, penicillin (100 U/ml) and streptomycin (100 μ g/ml). The medium was changed every 2 or 3 days. When the cells were seeded into a 6-well plate at 1×10^6 cells/ml, the medium was changed to the serum free medium for an additional 24 h incubation to render the cells quiescent before adding the stimulus. In the inhibitor experiments, U937 macrophages were exposed to Ang II (10^{-6} M) for 12 h after pretreated with the inhibitors for 1 h.

ELISA

U937 macrophages were incubated in 6-well plates, and stimulated with the different concentrations of Ang II for the indicated time. After the treatment, the cells were washed with PBS, lysed and scraped with 200 μ l of ice-cold RIPA lysis buffer. Then, the cell lysates were centrifuged at 12,000 rpm for 20 min at 4 $^{\circ}$ C, and the supernatant containing the protein fraction was extracted. Protein concentration was quantified with the BCA protein assay kit [20]. Finally, equal amount of protein extract was assayed for CRP by ELISA kit specific for human CRP according to the manufacturer's instruction.

RT-PCR

Total RNA was isolated from U937 macrophages after the treatment using Trizol (Invitrogen, NY, USA) according to manufacturer's instruction. One microgram of isolated RNA from each sample was transcribed into complementary (cDNA) using oligo (dT)₁₈ Primer and RevertAidTM M-MuLV Reverse Transcriptase (Fermentas, St. Leon-Rot, Germany). The cDNA was amplified using 1.5 μ l of each of the CRP forward primer (5'-TCG TAT GCC ACC AAG AGA CAA GAC A-3') and reverse primer (5'-AAC ACT TCG CCT TGC ACT TCA TAC T-3'). This resulted in a 440 bp PCR product (35 amplification cycles of 94 $^{\circ}$ C for 5 min, 94 $^{\circ}$ C for 30 s, 55 $^{\circ}$ C for 30 s, 72 $^{\circ}$ C for 45 s, and 72 $^{\circ}$ C for 5 min). The housekeeping gene (GAPDH) was amplified as an internal control for normalization using the forward primer (5'-GGA TTT GGT CGT ATT GGG-3') and the reverse primer (5'-GGAAGA TGG TGA TGG GAT T-3'). This resulted in a 205 bp product (35 amplification cycles of 94 $^{\circ}$ C for 5 min, 94 $^{\circ}$ C for 30 s, 55 $^{\circ}$ C for 30 s, 72 $^{\circ}$ C for 45 s, and 72 $^{\circ}$ C for 5 min). PCR products were separated on 2% agarose gels. Results were expressed as relative to control.

Measurement of ROS

Intracellular ROS production was detected using the 2', 7'-dichlorodihydrofluorescein diacetate (H₂DCF-DA). To evaluate inhibition of ROS generation, U937 macrophages were pretreated with losartan (10^{-5} M), PD123319 (10^{-5} M), DPI (10^{-5} M), or TIFA (10^{-5} M) for 1 h before exposed to Ang II (10^{-6} M) for 12 h. Then, the cells were loaded with H₂DCF-DA (10 μ M) for 30 min at 37 $^{\circ}$ C, and washed twice with PBS. Finally, the cells were imaged with fluorescence microscope (Olympus

Fig. 1. Ang II stimulates CRP generation in U937 macrophages. (A) Concentration-dependent expression of CRP mRNA, (B) time-dependent expression of CRP mRNA, and (C) concentration- and time-dependent increase of CRP protein in U937 macrophages. The cells were incubated with the different concentrations of Ang II or 12 h or with Ang II (10^{-6} M) for the indicated time. Then, CRP mRNA level was identified by RT-PCR, and CRP concentration was measured by ELISA. Data from three independent experiments are expressed as means $\pm$ S.E.M. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. control.

BX51, Japan). Fluorescence images were acquired at the excitation and emission wavelengths of 488 and 525 nm, respectively.

Western blot

After the treatments, U937 macrophages were washed with ice-cold PBS. The whole cell lysates were prepared in lysis buffer supplemented with the protease inhibitor cocktail (Roche, Mannheim, Germany) and phosphatase inhibitors. Protein concentration was measured by BCA protein assay kit. Equal amount of protein extract (50 μ g) was loaded, separated by 10% SDS-PAGE, and blotted onto nitrocellulose membrane. The membranes were incubated with anti-ERK (1:1000 dilution), anti-phospho-ERK (1:1000 dilution), anti-phospho-p38 (1:1000 dilution), and anti-p38 (1:1000 dilution) antibodies overnight at 4 $^{\circ}$ C. After washed, the membranes were detected with a horseradish peroxidase-conjugated anti-rabbit second antibody (1:7000 dilution) or anti-mouse second antibody (1:8000 dilution) followed by enhanced chemiluminescence (Pierce, Rockford, USA).

Statistical analysis

All values were shown as mean $\pm$ S.E.M. Statistical significance between groups was assessed by one-way ANOVA,

followed by Tukey's multiple comparison tests. A value of $P < 0.05$ was considered statistically significant.

Results

Ang II induces CRP expression in U937 macrophages

Fig. 1A showed that Ang II at 10^{-7} M to 10^{-5} M evidently upregulated CRP mRNA expression in U937 macrophages in a concentration-dependent manner compared to control. The maximal response of 4.9 folds over control was reached at a concentration of 10^{-6} M. The results in Fig. 1B showed that Ang II also increased mRNA expression of CRP in U937 macrophages in time-dependent fashion. CRP mRNA expression was obviously upregulated in 6 h, and reached the maximum 48 h after Ang II stimulation.

The results from ELISA indicated that the unstimulated U937 macrophages exhibited a low basal CRP release of 2.92-3.25 ng/ml in the protein extracts.

Fig. 2. Ang II induces CRP expression in U937 macrophages via AT_1 receptor. The cells were pretreated with AT_1 receptor antagonist losartan (10^{-5} M) or AT_2 receptor antagonist PD123319 (10^{-5} M) for 1 h prior to stimulation with Ang II (10^{-6} M) for 12 h for mRNA expression or for the indicated time for protein generation. Then, mRNA expression (A) and protein level (B) of CRP were analyzed by RT-PCR and ELISA, respectively. Data from three independent experiments are expressed as means $\pm$ S.E.M. *** P <0.001 vs. Ang II alone.

Fig. 3. Involvement of ROS in Ang II-induced CRP expression in U937 macrophages. (A) Effect of the different inhibitors on Ang II-stimulated generation of ROS. The cells were stimulated with Ang II (10^{-6} M) for 12 h after pretreatment with losartan (10^{-5} M), PD123319 (10^{-5} M), DPI [NAD(P)H oxidase inhibitor, 10^{-5} M] or TIFA (complex α inhibitor, 10^{-5} M) for 1 h, and then, incubated for 30 min with H_2DCF -DA (10μ M). Finally, the cells were observed under fluorescence microscope. (B) Effect of NAC and TIFA on Ang II-induced mRNA expression of CRP. The cells were stimulated with Ang II (10^{-6} M) for 12 h after pretreatment with NAC (antioxidant, 10 mM) or TIFA (10^{-5} M) for 1 h. Then, CRP mRNA expression was assayed by RT-PCR. Results from three independent experiments are expressed as means $\pm$ S.E.M. ** P <0.01 and *** P <0.001 vs. Ang II alone.

Ang II at 10^{-8} M up to 10^{-5} M caused an apparent time- and concentration-dependent increase of CRP production in U937 macrophages as compared to control. CRP level reached a maximum after stimulating the cells with Ang II at 10^{-5} M for 48 h, which was 3.97 times over baseline (Fig. 1C).

Ang II induces CRP expression in U937 macrophages via AT_1 receptor

To test the role of Ang II receptors in Ang II-induced CRP expression in U937 macrophages, the cells were pretreated with AT_1 receptor blocker losartan or AT_2 receptor blocker PD123319 for 1 h before Ang II

Fig. 4. MAPK and NF- κ B mediate Ang II-induced expression of CRP in U937 macrophages. The cells were pretreated with PD98059 (MEK1/2 inhibitor, 20 μ M), SP600125 (JNK inhibitor, 20 μ M), SB203580 (p38MAPK inhibitor, 10 μ M) or PDTC (NF- κ B inhibitor, 100 μ M) for 1 h prior to stimulation with Ang II (10^{-6} M) for 12 h. Then, mRNA expression (A) and protein level (B) of CRP were detected by RT-PCR and ELISA, respectively. Data from three independent experiments are expressed as the mean $\pm$ S.E.M. **P<0.01 and ***P<0.001 vs. Ang II alone.

Fig. 5. Ang II induces CRP expression in U937 macrophages through AT₁-ROS-MAPK signal pathway. The cells were stimulated with Ang II (10^{-6} M) for 1 h after pretreated with losartan (10^{-5} M), PD123319 (10^{-5} M), NAC (10 mM), PD098059 (20 μ M) or SB203580 (10 μ M) for 1 h. Then, expressions of ERK1/2 and p38MAPK, phosphorylated ERK1/2 and p38MAPK were measured by Western blot. Data from three independent experiments are expressed as the mean $\pm$ S.E.M. **P<0.01 and ***P<0.001 vs. Ang II alone.

stimulation. The results showed that AT₁ receptor but not AT₂ receptor mediated Ang II-induced CRP expression in U937 macrophages, since losartan almost abolished Ang II-induced mRNA and protein expression of CRP, while PD123319 did not produce a significant effect on CRP expression (Fig. 2A and 2B).

Involvement of ROS in Ang II-induced CRP expression in U937 macrophages

To determine whether intracellular ROS participate in Ang II-induced production of CRP, intracellular ROS generation was observed. Exposure of U937 macrophages to Ang II resulted in a noticeable raise of DCF fluorescence as compared to control, whereas losartan and TIFA (complex II inhibitor) obviously attenuated the effect.

Although DPI [NAD(P)H oxidase inhibitor] tended to depress Ang II-stimulated ROS production, there was no statistical significance (Fig. 3A). Further investigation revealed that antioxidant NAC and TIFA significantly inhibited Ang II-induced mRNA expression of CRP in U937 macrophages (Fig. 3B).

MAPK and NF- κ B mediate Ang II-induced expression of CRP in U937 macrophages

To investigate if the MAPK and NF- κ B pathway is involved in Ang II-induced expression of CRP in U937 macrophages, the cells were stimulated with 10^{-6} M Ang II for 12 h after pretreatment with PD98059 (MEK1/2 inhibitor), SB203580 (p38MAPK inhibitor), SP600125 (JNK inhibitor), or PDTC (NF- κ B inhibitor) for 1 h.

As shown in Fig. 4A and 4B, PD98059, SB203580 and PDTC substantially reduced Ang II-induced mRNA and protein expression of CRP in U937 macrophages, while SP600125 had no significant effect on CRP expression.

Ang II induces CRP expression in U937 macrophages through AT₁-ROS-MAPK signal pathway

The above-mentioned results indicate that AT₁ receptor, ROS, p38MAPK and ERK1/2 participate in Ang II-induced expression of CRP in U937 macrophages. To elucidate whether Ang II receptors and ROS mediated Ang II-induced activation of p38MAPK and ERK1/2 in U937 macrophages, the phosphorylated p38 and ERK1/2 were determined. Western blot analysis revealed that a marked increase of the phosphorylated p38 and ERK1/2 was detected following stimulation of the cells with Ang II. However, pretreatment of the cells with losartan, NAC, PD98059 or SB203580 for 1 h prior to exposure of the cells to Ang II for 1 h markedly inhibited Ang II-induced phosphorylation of p38 and ERK1/2 (Fig. 5A and 5B).

Discussion

The growing evidences demonstrate that atherosclerosis is an inflammatory disease, and inflammatory cytokines play important roles in atherogenesis. As a representative marker of the inflammatory response, CRP participates in all stages of atherosclerotic process, and provides a pivotal link between inflammation and atherogenesis [21]. Although hepatocytes, kidney and neurons are believed to be contributors of circulating CRP in plasma [22-24], the existed evidences have suggested that VSMCs, VECs and monocytes/macrophages also secrete CRP [2, 18, 20]. 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.

Ang II, one of the main vasoactive peptides, contributes not only to pathogenesis of hypertension but also to inflammatory process of many diseases, such as atherosclerosis, coronary heart disease, stroke, brain ischaemia, diabetes mellitus. Ang II exerts its proinflammatory action through AT₁ receptor. Therefore, AT₁ receptor blockers have been successfully used in treatment of a pathologic inflammatory process of the diseases. For example, Pavlatou et al report that candesartan is able to relieve the systemic inflammatory responses in patient

with diabetes mellitus type 2 by decreasing serum levels of IL-6, MCP-1 and CRP [25]. In addition, hypertensives and prehypertensives show a higher serum level of CRP compared with normotensives, which suggests an association between elevated serum level of CRP and complications of hypertension like atherosclerosis [26].

As the main inflammatory cells, monocytes and monocyte-derived macrophages have been recognized as the cellular hallmark of atherosclerosis [27]. Monocytes are recruited to the vascular wall during the process of atherogenesis where they differentiate into macrophages and acquire the capacity to synthesize an array of heparin-binding cytokines, chemokine, and growth factors, which contribute to the development of atherosclerosis [28].

In the present study, we found that Ang II induced mRNA and protein expression of CRP in U937 macrophages in time- and concentration-dependent manners, which is consistent with our previous results in VSMCs and HAECs [17, 18]. This result further enriches the proinflammatory action of Ang II.

It is known that there exists Ang II receptors in macrophages [29], and Ang II up-regulates CRP expression in VSMCs and HAECs through AT₁ receptor [17, 18]. Therefore, Ang II receptors possibly mediate the proinflammatory effect of Ang II in macrophages. In the experiment, pretreatment of U937 macrophages with the specific AT₁ receptor blocker losartan almost completely abolished Ang II-induced increase of CRP in mRNA and protein levels. But, the specific antagonist of AT₂ receptor, PD123319, did not alter the effect of Ang II on CRP. These demonstrate that Ang II induces CRP expression in macrophages via AT₁ receptor.

It is reported that ROS mediate production of the inflammatory cytokines through the multiple signal pathways including MAPK signaling, and Ang II stimulates peroxide production in monocytes/macrophages predominantly through activation of AT₁ receptor [15]. The results from the present experiment indicated that Ang II-induced ROS generation in U937 macrophages involved an AT₁/mitochondria-derived pathway, but not NAD(P)H oxidase system, since AT₁ receptor antagonist losartan and mitochondrial respiratory complex α inhibitor TIFA remarkably antagonized the stimulatory effect of Ang II on ROS production, whereas AT₂ receptor antagonist PD123319 and NAD(P)H oxidase inhibitor DPI did not show the significant inhibitory effect. These data are also consistent with the study of Han et al in HAECs [18]. In addition, we also found that antioxidant NAC completely inhibited Ang II-induced mRNA expression of CRP. These imply that Ang II stimulates CRP generation

in macrophages via AT₁ receptor mediated ROS signaling.

A great number of investigations document that the MAPK family members (p38MAPK, JNK and ERK) are important in regulating the production of inflammatory cytokines and mediators [30]. In the present study, we found that MEK1/2 inhibitor PD98059 and p38MAPK inhibitor SB203580 substantially reduced Ang II-induced mRNA and protein expression of CRP in U937 macrophages, while JNK inhibitor SP600125 had no significant effect on CRP expression, implicating that Ang II-induced CRP expression depends on ERK1/2 and p38MAPK signaling in U937 macrophages. This finding is not entirely the same as previous results in HAECs, which indicate that Ang II induces CRP expression through ERK1/2- and JNK-dependent signal pathway [18]. This different result suggests that CRP expression is cell-specific and pathway-specific [14]. In addition, we also found that AT₁ receptor blocker losartan and antioxidant NAC efficiently blocked Ang II-induced phosphorylation of ERK1/2 and p38MAPK in U937 macrophages, certifying that AT₁ receptor and ROS mediate Ang II-activated phosphorylation of ERK1/2 and p38MAPK. This is consistent with the results of Tanifuji et al, who report that ROS-mediated phosphorylation of ERK1/2 is involved in Ang II-stimu-

lated production of MCP-1 [31]. Taken together, Ang II induces CRP expression in macrophages via AT₁-ROS-ERK1/2/p38MAPK signal pathway.

At present, the NF- κ B pathway is considered to be an important “bridge” between Ang II and its proinflammatory actions in atherosclerosis, and MAPK is an upstream event of NF- κ B activation [32]. Moreover, Ang II can enhance NF- κ B activity in macrophages [14]. Our further study displayed that NF- κ B participated in Ang II-induced CRP expression in U937 macrophages, since the selective NF- κ B inhibitor PDTC remarkably antagonized Ang II-stimulated CRP production.

In conclusion, our data demonstrate the ability of Ang II to initiate an inflammatory response by stimulating CRP production in macrophages, which is mediated through AT₁-ROS-ERK1/2/p38MAPK-NF- κ B signal pathway. The finding may contribute to better understanding of the proinflammatory and proatherosclerotic actions of Ang II.

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