Angiotensin II Induces Inflammatory Response Partly Via Toll-Like Receptor 4-Dependent Signaling Pathway in Vascular Smooth Muscle Cells

Yuanyuan Ji¹, Juntian Liu¹, Zhidong Wang² and Na Liu¹

¹Department of Pharmacology, School of Medicine, ²Department of General Surgery, The Second Affiliated Hospital, Xi'an Jiaotong University, Xi'an, China

Key Words

Angiotensin II • Toll-like receptor 4 • Inflammation • Atherosclerosis • Vascular smooth muscle cells

Abstract

Angiotensin (Ang II) plays an important role in atherosclerosis through proinflammatory effect. Toll-like receptor 4 (TLR4) may mediate inflammatory response. It is unknown whether TLR4 mediates the proinflammatory effect of Ang II. Thus, we observed the role and signaling pathway of TLR4 in Ang II-induced inflammation in rat vascular smooth muscle cells (VSMCs). Ang II and LPS stimulated TNF- α secretion and inhibited 6-keto-PGF_{1 α} production, upregulated MMP-9 and downregulated PPAR γ and PPAR α in rat VSMCs. Ang II also distinctly upregulated TLR4 expression in the cells. Pretreatment of the cells with anti-TLR4 antibody prior to Ang II stimulation significantly diminished the effects of Ang II. These suggest that Ang II stimulates VSMCs to produce inflammation through regulation of the proinflammatory and the antiinflammatory factors via TLR4-dependent mechanism. The further investigations showed that AT1 receptor antagonist losartan or ERK1/2 inhibitor PD098059 inhibited

Ang II-induced TLR4 expression, TLR4 inhibitor prevented Ang II-induced IP-10 expression, anti-IP-10 antibody partly abolished Ang II-induced PKC increase, and PKC inhibitor chelerythrine suppressed Ang II-induced NF- κ B expression. These demonstrate that TLR4-mediated proinflammatory effect of Ang II in VSMCs involves AT1/ERK1/2/TLR4/IP-10/ PKC/NF- κ B pathway. Our results provide the evidence that Ang II induces inflammatory response involved in pathogenesis of atherosclerosis partly via TLR4-dependent signaling pathway in VSMCs.

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Introduction

Angiotensin II (Ang II) is a main peptide hormone of rennin-angiotensin system, and plays an important role in pathogenesis of cardiovascular diseases including atherosclerosis, myocardial infarction and hypertension [1]. Ang II may cause endothelial dysfunction, vascular smooth muscle cells (VSMCs) proliferation and migration, monocyte chemoattraction and inflammation in vascular tissue. Furthermore, Ang II is able to stimulate vascular cells to produce cytokines (e.g. CRP, IL-6, VCAM-1,

MCP-1) and growth factors (e.g. PDGF, bFGF, VEGF) via receptor-mediated way and subsequent activation of multiple intracellular signaling pathways, which participate in vascular inflammatory responses and cardiovascular disorders [2]. Although atherosclerosis is a multifactorial disease, more and more investigations focus on the inflammatory process in atherosclerosis in the recent years. Now increasing evidences support that atherosclerosis is a chronic inflammatory and immunity-related disease [3-5].

The toll-like receptor (TLR) family has been identified as a major component of pathogen-associated molecular-pattern-recognition molecules [6]. So far, eleven members of the TLR family have been described. Activation of TLRs can initiate the innate and sequentially the adaptive immune system, both responsible for inflammation [7]. Among members of the TLR family, TLR4 is a central signaling receptor mediating inflammatory response. The bacterial lipopolysaccharide (LPS), as an exogenous ligand for TLR4, activates nuclear factor- κ B (NF- κ B) to result in secretion of proinflammatory cytokines, such as TNF- α via TLR4-dependent inflammatory signaling pathway [8, 9]. Recent investigations suggest a possibility that TLRs also play a crucial role in pathogenesis of atherosclerosis. A number of evidences show that there exists a marked expression of TLR4 in human atherosclerotic plaque [10], and ox-LDL and LPS up-regulate TLR4 expression in macrophages and VSMCs [11, 12], suggesting a strong association between TLR4, inflammatory, immune responses and atherosclerosis.

Our previous study demonstrates that Ang II induces secretion of C-reactive protein (CRP) to produce inflammation in VSMCs via activating Ang II type 1 receptor (AT1) and subsequent extracellular signal-regulated kinase 1/2 (ERK1/2) pathway [13]. But, it is unknown whether TLR4-dependent signaling pathway is involved in the proinflammatory effect of Ang II. Therefore, the present study was designed to probe the role and signaling pathway of TLR4 in Ang II-induced inflammation involved in atherosclerotic process.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin were purchased from Gibco BRL (Carlsbad, CA). Ang II, LPS from *Escherichia coli* 0111:B4, PD123319, PD098059 and chelerythrine were provided by Sigma (St. Louis, MO, USA). Losartan was

produced by Merck (Merck Corp., Darmstadt, Germany). Polyclonal anti-rat TLR4 antibody, anti-IP-10 antibody, anti-PKC antibody and anti-NF- κ B antibody were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against PPAR γ , PPAR α , IP-10 and MMP-9 were obtained from ABCAM (Cambridge, UK). Monoclonal anti-TLR4 antibody (MTS510) was from eBioscience (San Diego, CA). Rat TNF- α ELISA kit was purchased from Bender (Bender MedSystems, CA, USA) and rat 6-keto prostaglandin F $_{1\alpha}$ (6-keto-PGF $_{1\alpha}$) ELISA kit was supplied by Cayman (Ann Arbor, MI, USA).

VSMC culture

The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Male Sprague-Dawley rats (weight 120-160 g) were obtained from the Laboratory Animal Institute of School of Medicine of Xi'an Jiaotong University. The experiment was granted by the Ethics Review Board of Xi'an Jiaotong University. VSMCs were isolated from the thoracic aorta of rats according to the previously described method [14]. The cells were grown in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 μ g/ml streptomycin in a humidified atmosphere of 5% CO $_2$ and 95% air at 37 °C until they exhibited a typical "hill and valley" growth pattern. Identity of VSMCs was confirmed by the morphological examination and immunocytochemical staining for α -actin. The cells between passages 3 and 10 were used for all experiments. When the cells were grown to confluence, the medium was changed to serum free medium for an additional 24 h culture before the experiments.

Enzyme-linked immunosorbent assay (ELISA) for TNF- α and 6-keto-PGF $_{1\alpha}$

VSMCs were seeded into 6-well plates at a density of 5×10^6 cells/well, and then incubated with Ang II or LPS at the indicated concentrations for the indicated time. In the another experiment, the cells were pretreated with anti-TLR4 antibody (1 and 5 μ g/ml) for 1 h and subsequently stimulated with 100 nM Ang II for 24 h. TNF- α and 6-keto-PGF $_{1\alpha}$ levels in the supernatant were measured with ELISA kits according to the manufacturer's instructions.

Confocal microscopy

VSMCs were plated on cover slips and grown to confluence, and then treated with Ang II or LPS in the indicated concentrations. After that, the cells were fixed in 4% formaldehyde-PBS for 15 min. The cell membrane was fenestrated with 0.3% Triton-100-PBS, and nonspecific binding sites were blocked with 10% goat serum. The cells were incubated with rabbit anti-rat TLR4 or anti-rat MMP-9 antibody and then, incubated with the secondary antibody conjugated to fluorescein isothiocyanate (FITC) or rhodamine. Finally, the slides were observed under confocal microscopy.

Western blot analysis

VSMCs were washed, lysed, and homogenized in 10 mM Tris-HCl (pH 7.4) containing 0.1% sodium dodecylsulfate and a protease inhibitor cocktail (Roche, Germany). Protein

Gene	Primer sequence	Accession number	Expected size (bp)
TLR4	5'-GGCATCATCTTCATTGTCCTTG -3', 5'-AGCATGTCTCCTCCCACTCG-3	NM_019178	111
PPAR γ	5'-GGAAGCCCTTTGGTGACTTTATGG -3', 5'-GCAGCAGGTTGTCTTGGATGTC-3'	NM_013124	174
PPAR α	5'-CGGGTCATACTCGCAGGAAAG-3', 5'-TGGCAGCAGTGGAAGAATCG-3'	NM_013196	155
MMP-9	5'-CCCTACTGCTGGTCCTTCTGAG-3', 5'-AATTGGCTTCCTCCGTGATTGCG-3'	NM_031055	162
IP-10	5'-CGTTCTCTGCCTCGTGCTG-3', 5'-ATGCGGACAGGATAGACTTGC-3'	NM_139089	157
PKC	5'-TCGCTGCTGGTGCTGAGTC-3', 5'-GTGCTCCTCTCCTCGGTTGTC-3'	NM_017171	161
β -actin	5'-ATCGGCAATGAGCGGTTCC-3', 5'-AGCACTGTGTTGGCATAGAGG-3'	NM_031144	149

Table 1. Primers used for real-time PCR analysis

concentration was determined with the BCA protein assay kit (Pierce, Rockford, IL). Twenty microgram of protein sample was resolved on 12% SDS-PAGE gels and transferred onto a polyvinylidene difluoride (PVDF) membrane in semi-dry system (Bio-Rad, Hercules, CA). The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20, and incubated with specific antibodies against TLR4 (1:200), MMP-9 (1:400), PPAR γ (1:400), PPAR α (1:100), IP-10 (1:5000), PKC (1:400), NF- κ B (1:400) and β -actin (1:400). In the experiments of cell-associated proteins, β -actin was used as loading control. Reagents (Pierce) for strengthening chemiluminescence were applied to the blots and the light signals were detected by X-ray film. Optical density of the bands was scanned and quantified with Gel Doc 2000 (Bio-Rad). Data were normalized against those of the corresponding β -actin. Results were expressed as relative to control.

Quantitative real-time and traditional polymerase chain reaction

Total RNA was isolated with TRIzol reagent kit (Invitrogen, CA, USA). cDNA was synthesized from 1 μ g of total RNA by using Revert AidTM First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany) and following the manufacturer's instructions. Real-time polymerase chain reaction (PCR) was performed with the SuperScript III Platinum SYBR-Green One-Step qRT-PCR kit (Invitrogen) on a Mx3000P QPCR System (Stratagene, LaJolla, CA, USA) following the manufacturer's instructions. The samples were run in triplicate. Primers for rat TLR4, MMP-9, PPAR γ , PPAR α , IP-10, PKC and β -actin were designed with Beacon designer v 4.0 (Premier Biosoft, USA) (see Table 1 for the sequences). β -actin was used as an internal control. Traditional PCR was performed according to the manufacturer's instructions. RT-PCR products were analyzed by electrophoresis through 2% agarose gel containing ethidium bromide. A melting point dissociation curve generated by the instrument was used to confirm that only a single product was present. Quantization of relative gene expression was calculated by the comparative Ct method ($2^{-\Delta\Delta C_t}$) as described by the manufacturer. Results were expressed as relative to control.

Fig. 1. Ang II- and LPS-induced TNF- α production in rat VSMCs. VSMCs were incubated with Ang II or LPS for 12, 24, 36 or 48 h. TNF- α concentration in the supernatant was measured by the enzyme-linked immunosorbent assay (ELISA). (A) TNF- α production in VSMCs elicited by Ang II. (B) TNF- α production in VSMCs elicited by LPS. Data are presented as the mean $\pm$ SEM (n=3).

Statistical analysis

Data are expressed as mean $\pm$ SEM. Statistical evaluation was performed by Student *t* test and one- or two-way ANOVA followed by Dunnett test. A probability value of < 0.05 was considered significant.

Fig. 2. Ang II- and LPS-induced 6-keto-PGF_{1α} production in rat VSMCs. VSMCs were incubated with Ang II or LPS for 12, 24, 36 or 48 h. 6-Keto-PGF_{1α} concentration in the supernatant was measured by ELISA. (A) 6-keto-PGF_{1α} production in VSMCs elicited by Ang II. (B) 6-keto-PGF_{1α} production in VSMCs elicited by LPS. Data are presented as the mean±SEM (n=3).

Results

Ang II and LPS increase TNF-α and decrease 6-keto-PGF_{1α} productions in VSMCs

Because of TNF-α and 6-keto-PGF_{1α} productions in VSMCs and the demonstrated roles that they play in vascular inflammation and atherosclerosis, it is necessary to investigate whether Ang II exerts an effect on TNF-α and 6-keto-PGF_{1α} in the cultured rat VSMCs. Incubation of the cells with Ang II (10⁻⁹ to 10⁻⁶ M) or LPS (50 to 200 ng/ml) for 12, 24, 36 or 48 h increased TNF-α production, but decreased 6-keto-PGF_{1α} level in time- and concentration-dependent manners (Fig.1 and 2).

Ang II regulates TNF-α and 6-keto-PGF_{1α} productions in VSMCs through TLR4

To explore whether effects of Ang II on TNF-α and 6-keto-PGF_{1α} are mediated through TLR4, we further observed change of the effects of Ang II at the same concentrations after pretreatment of VSMCs with anti-TLR4 antibody. The results indicated that Ang II increased TNF-α to 98.07±4.07 pg/ml from 37.08±2.12 pg/ml in control group, and reduced 6-keto-PGF_{1α} to 1628±89.87 pg/ml from 3713±68.28 pg/ml in control group. After blocking TLR4 with anti-TLR4 antibody (1 and 5 μg/ml) followed by Ang II stimulation, TNF-α concentration was significantly decreased to 77.05±5.02 pg/ml and 60.93±7.62 pg/ml, and 6-keto-PGF_{1α} level was increased to 2113±135.1 pg/ml and 3021±191.3 pg/ml, respectively (Fig. 3), suggesting that TLR4 mediates the effects of Ang II on TNF-α and 6-keto-PGF_{1α} in VSMCs.

Fig. 3. Ang II regulates TNF-α and 6-keto-PGF_{1α} productions through TLR4 in rat VSMCs. VSMCs were pretreated with anti-TLR4 antibody for 1 h and stimulated with Ang II for 24 h, and concentrations of TNF-α and 6-keto-PGF_{1α} in the supernatant were measured by ELISA. (A) TNF-α production. (B) 6-keto-PGF_{1α} production. Data are presented as the mean±SEM (n=3). ***P<0.001 vs control; #P<0.05, ##P<0.01, ###P<0.001 vs Ang II.

Fig. 4. Immunofluorescent analysis of TLR4 and MMP-9 expressions induced by Ang II and LPS in rat VSMCs. VSMCs were incubated with Ang II or LPS for 24 h and then, TLR4 and MMP-9 expressions were identified with immunocytofluorescence and observed under confocal microscope. (A) TLR4 expression. (B) MMP-9 expression. The shown is representative of three separate experiments. The scale bar represents 100 μ m.

Ang II and LPS induce MMP-9 expression in VSMCs

To examine whether Ang II induces MMP-9 expression in rat VSMCs, the cells were incubated with Ang II (10^{-9} to 10^{-6} M) or LPS (50 to 200 ng/ml) for 24 h and then, MMP-9 expression was identified with immunocytofluorescence and observed by confocal microscope. The result showed that Ang II and LPS elicited MMP-9 expression in VSMCs in a concentration-dependent manner (Fig. 4B).

Under suggestion of the above-mentioned results with the immunofluorescent method, we observed effects of Ang II and LPS on protein and mRNA expression of MMP-9 in VSMCs. As shown in Fig. 5, Ang II and LPS upregulated protein and mRNA expression of MMP-9 in a concentration-dependent manner.

Ang II induces MMP-9 expression in VSMCs through TLR4

The above-mentioned results show that Ang II may induce MMP-9 expression. To assess role of TLR4 in the process, we further evaluated the effects of anti-TLR4

antibody pretreatment on MMP-9 expression in Ang II-stimulated VSMCs. As shown in Fig. 6, 1 and 5 μ g/ml anti-TLR4 antibody remarkably attenuated the Ang II-induced protein and mRNA expression of MMP-9. Consequently, these results demonstrate that Ang II induces MMP-9 expression through TLR4 signaling pathway.

Ang II and LPS reduce PPAR γ and PPAR α expressions in VSMCs

As PPAR γ and PPAR α are negative regulators of inflammatory response, we also observed effects of Ang II and LPS on protein and mRNA expressions of PPAR γ and PPAR α in VSMCs. As shown in Fig. 7, Ang II and LPS downregulated protein and mRNA expressions of PPAR γ and PPAR α in a concentration-dependent manner.

Ang II modulates PPAR γ and PPAR α expressions in VSMCs through TLR4

To assess role of TLR4 in Ang II-downregulated PPAR γ and PPAR α expressions in VSMCs, we further evaluated the effects of anti-TLR4 antibody pretreatment

Fig. 5. Ang II- and LPS-induced protein and mRNA expression of MMP-9 in rat VSMCs. VSMCs were treated with Ang II or LPS for 24 h and then, protein (A) and mRNA (B) expression of MMP-9 was analyzed by western blot, RT-PCR and quantitative real-time PCR, respectively. Data are presented as the mean±SEM (n=3). ** P <0.01, *** P <0.001 vs control.

Fig. 6. Ang II-induced MMP-9 expression through TLR4 in rat VSMCs. VSMCs were pretreated with anti-TLR4 antibody for 1 h and subsequently stimulated with Ang II for 24 h. Protein (A) and mRNA (B) expression of MMP-9 was analyzed by western blot, RT-PCR and quantitative real-time PCR, respectively. Data are presented as the mean±SEM (n=3). *** P <0.001 vs control; # P <0.05, ## P <0.01, ### P <0.001 vs Ang II.

on PPAR γ and PPAR α expressions in Ang II-stimulated VSMCs. As shown in Fig. 8, protein and mRNA expressions of PPAR γ and PPAR α in response to Ang II were upregulated in the cells pretreated with anti-TLR4 antibody, suggesting that Ang II downregulates PPAR γ and PPAR α expressions through TLR4 signaling pathway.

Ang II and LPS induce TLR4 expression in VSMCs

To examine whether Ang II directly induces TLR4 expression in rat VSMCs, the cells were incubated with Ang II (10^{-9} to 10^{-6} M) or LPS (50 to 200 ng/ml) for 24 h and then, TLR4 expression was identified with immunocytofluorescence and observed by confocal microscope. The result showed that Ang II and LPS increased TLR4 expression in VSMCs in a concentration-

dependent manner (Fig.4A).

On the basis of the above-mentioned results with the immunofluorescent method, we further observed effects of Ang II and LPS on protein and mRNA expression of TLR4 in VSMCs. As shown in Fig. 9, Ang II and LPS upregulated protein and mRNA expression of TLR4 in a concentration-dependent manner. The results confirm that Ang II and LPS are able to activate TLR4.

Ang II activates TLR4 through AT1 receptor and subsequent ERK1/2 in VSMCs

To certify which AT receptor subtype and whether ERK1/2 is involved in Ang II- induced TLR4 activation in VSMCs, the cells were stimulated with Ang II (100 nM) for 24 h after pretreatment for 30 min with AT1 receptor antagonist losartan (1 μ M), AT2 receptor

Fig. 7. Ang II- and LPS-downregulated protein and mRNA expressions of PPAR γ and PPAR α in rat VSMCs. VSMCs were treated with Ang II or LPS for 24 h and then, protein and mRNA expressions of PPAR γ and PPAR α were analyzed by western blot, RT-PCR and quantitative real-time PCR, respectively. (A and B) Protein expressions. (C and D) mRNA levels. Data are presented as the mean $\pm$ SEM (n=3). * P <0.05, ** P <0.01, *** P <0.001 vs control.

antagonist PD123319 (10 μ M) and ERK1/2 inhibitor PD098059 (1 and 10 μ M) alone or in different combinations. As described in Fig. 10A and 11A, Ang II-induced protein and mRNA expression of TLR4 were significantly inhibited by pretreatment of the cells with losartan or PD098059 but not PD123319, and the inhibitory effect was more obvious when the cells were pretreated with the combination of losartan, PD123319 and PD098059. These confirm that Ang II activated TLR4 in VSMCs through AT1 receptor and subsequent ERK1/2.

Involvement of TLR4 in Ang II-induced IP-10 expression in VSMCs

To probe role of TLR4 in Ang II-induced IP-10 expression, VSMCs were preincubated with anti-TLR4 antibody for 1 h prior to Ang II stimulation for 24 h. As shown in Fig. 10B and 11B, anti-TLR4 antibody partially but significantly blunted Ang II-induced protein and mRNA expression of IP-10, illustrating that TLR4 mediates expression of downstream molecule IP-10 in the

proinflammatory signaling pathway of Ang II in VSMCs.

IP-10 mediates Ang II-induced PKC expression in VSMCs

To further explore role of IP-10 in Ang II-induced PKC expression, VSMCs were pretreated with a specific inhibitor anti-IP-10 antibody and subsequently stimulated with Ang II. As shown in Fig. 10C and 11C, Ang II-induced protein and mRNA expression of PKC was substantially prevented by anti-IP-10 antibody, manifesting that IP-10 is required for Ang II-induced PKC expression in VSMCs.

Involvement of PKC in Ang II-induced NF- κ B expression in VSMCs

Among several transcription factors in the cells, NF- κ B correlates closely with inflammatory response. Ang II-induced inflammatory cytokines is believed to be associated with NF- κ B activation, which may be the downstream event of ERK activation in VSMCs [15]. In this study, we investigated role of PKC in Ang II-

Fig. 8. Ang II-downregulated expressions of PPAR γ and PPAR α through TLR4 in rat VSMCs. VSMCs were pretreated with anti-TLR4 antibody for 1 h and subsequently stimulated with Ang II for 24 h. Protein and mRNA expressions of PPAR γ and PPAR α were analyzed by western blot, RT-PCR and quantitative real-time PCR, respectively. (A) Protein expressions. (B) mRNA levels. Data are presented as the mean $\pm$ SEM (n=3). * P <0.05 vs control; # P <0.05, ## P <0.01 vs Ang II.

Fig. 9. Ang II- and LPS-induced protein and mRNA expression of TLR4 in rat VSMCs. VSMCs were treated with Ang II or LPS for 24 h and then, protein(A) and mRNA(B) expression of TLR4 was analyzed by western blot, RT-PCR and quantitative real-time PCR, respectively. Data are presented as the mean $\pm$ SEM (n=3). * P <0.05, ** P <0.01, *** P <0.001 vs control.

induced NF- κ B expression. VSMCs were preincubated with a specific PKC inhibitor chelerythrine prior to Ang II (100 nM) stimulation. The results showed that chelerythrine significantly suppressed the total NF- κ B protein expression, thus implying Ang II-induced NF- κ B expression possibly through activating PKC pathway (Fig. 10D).

Discussion

Ang II and LPS, clearly predictive of clinical atherosclerosis, are the sources of inflammatory response. Ang II is able to induce inflammatory response by activating NF- κ B and stimulating expression of chemokines [16]. TNF- α not only is an inflammatory mediator, but also enhances release of the other inflammatory mediators and expression of adhesion

molecules. Although roles of PGI $_2$ in inflammation are controversial, it produces anti-atherosclerotic effect via vasodilation, inhibiting platelet aggregation and VSMCs proliferation [17]. In this experiment, TNF- α and PGI $_2$ were used as two critical factors relevant to vascular inflammation and atherosclerosis. The results revealed that Ang II and LPS at the concentrations used stimulated TNF- α secretion and inhibited 6-keto-PGF $_{1\alpha}$ production of (a stable degraded product of PGI $_2$) in rat VSMCs, suggesting that Ang II and LPS stimulate VSMCs to produce inflammatory response via TNF- α . Besides, the results also suggest that Ang II and LPS make contribution to atherosclerosis through decreasing PGI $_2$ in addition to their proinflammatory effect.

Recently, several lines of researches have showed that MMP-9 plays an important role in atherogenesis by promoting degradation of the extracellular matrix [18, 19]. Within the vulnerable atheromatous plaques, particularly

Fig. 10. Mechanisms of Ang II-induced inflammatory response via TLR4-dependent signaling pathway in rat VSMCs by western blot analysis. (A) Ang II-induced TLR4 protein expression through AT1 receptor and ERK1/2. (B) Involvement of TLR4 in Ang II-induced IP-10 protein expression. (C) IP-10 mediates Ang II-induced PKC protein expression. (D) PKC is involved in Ang II-induced NF- κ B expression. CH indicates PKC inhibitor chelerythrine. Data are presented as the mean $\pm$ SEM (n=3). ** P <0.01, *** P <0.001 vs control; # P <0.05, ## P <0.01, ### P <0.001 vs Ang II.

in the inflamed shoulder and fibrous caps, there exists strong MMP-9 expression [20]. Inflammatory mediators such as Ang II have been shown to increase MMP-9 expression in VSMCs [21]. Consistent with this, we also demonstrated that Ang II and LPS upregulated MMP-9 expression in rat VSMCs.

Emerging evidences suggest that PPARs has the ability to counterregulate inflammatory response plays essential roles in both immunity and metabolic control [22]. PPARs activation may limit inflammation and atherosclerosis by altering expression of the master transcription factor NF- κ B to result in inhibition of inflammatory target genes [23-25]. As described in our previous study, Ang II induces production of a few of inflammatory mediators (e.g. CRP, IL-6) in rat VSMCs to accelerate atherosclerosis [13]. In this study, we also observed effect of Ang II on PPARs in rat VSMCs involved in antiinflammatory mechanisms in addition to the proinflammatory mediators. The results showed that Ang II and LPS downregulated PPAR γ and PPAR α expressions in VSMCs. Therefore, the results implicate that Ang II produces inflammatory response via both stimulating production of the proinflammatory mediators and diminishing the antiinflammatory activity of PPAR γ and PPAR α .

Proinflammatory effect of Ang II has firmly been established through our previous and present experiments as well as other studies and but, the proinflammatory mechanisms and signaling pathway of Ang II, particularly in cardiovascular system, remain to be elucidated. Accumulative investigations have indicated that TLR4 is a central signaling receptor mediating inflammatory response. Moreover, TLR4 are intensely expressed in human atherosclerotic vessels [10], and deletion of TLR4 gene is shown to reduce atherosclerosis in apolipoprotein E knockout mice [26]. Human epidemiological data also demonstrate that an Asp299Gly TLR4 polymorphism, which attenuates receptor signaling, is associated with a lower risk of atherosclerosis and acute coronary events [27]. Therefore, we presumed that TLR4 could mediate the proinflammatory effect of Ang II, and examined effect of Ang II on TLR4 expression in VSMCs with immunocytofluorescent, western blot, RT-PCR and quantitative real-time PCR analyses. The result found that Ang II distinctly upregulated TLR4 expression in rat VSMCs *in vitro*, which was also demonstrated in rats receiving the subchronic infusion of Ang II (data not shown). These provide a strong evidence for the direct involvement of TLR4 in Ang II-induced inflammation.

It has been well known that LPS is one of important

Fig. 11. Mechanisms of Ang II-induced inflammatory response via TLR4-dependent signaling pathway in rat VSMCs by RT-PCR and quantitative real-time PCR analyses. (A) Ang II-induced TLR4 mRNA expression through AT1 receptor and ERK1/2. (B) Involvement of TLR4 in Ang II-induced IP-10 mRNA expression. (C) IP-10 mediates Ang II-induced PKC mRNA expression. Data are presented as the mean $\pm$ SEM (n=3). ** P <0.01, *** P <0.001 vs control; # P <0.05, ## P <0.01, ### P <0.001 vs Ang II.

inflammatory mediators. LPS activates NF- κ B to cause secretion of inflammatory cytokines, such as TNF- α , via TLR4-dependent inflammatory signaling pathway [8, 9]. In this study, LPS was used as a positive stimulant of TLR4 expression. We found that LPS also upregulated TLR4 expression in VSMCs, which is consistent with the previous studies.

In the view of the finding that Ang II is able to stimulate TLR4 expression in VSMCs in vitro and in vivo, it is essential to study whether regulation of inflammatory mediators by Ang II is related to TLR4. The further investigation showed that the pretreatment of VSMCs with TLR4 inhibitor (anti-TLR4 antibody) prior to Ang II stimulation significantly diminished the effects of Ang II on generations of TNF- α and PGI₂, expressions of MMP-9, PPAR γ and PPAR α . These imply that proinflammatory effect of Ang II in VSMCs is TLR4-dependent. In addition, TLR4 also contributes to the pathophysiology of plaque instability by mediating Ang II-induced MMP-9 expression in VSMCs in a certain degree. Consequently, TLR4 is closely involved in Ang II-induced inflammatory response and cardiovascular disorders.

From the results mentioned above, we conclude that Ang II induces production of the proinflammatory mediators and downregulates expression of the negative inflammatory regulators to cause inflammatory response via TLR4. However, the concrete TLR4-mediated

proinflammatory signaling pathway of Ang II is still unclear. As indicated in previous study, Ang II binding to AT1 receptors in VSMCs is linked to ERK1/2- dependent pathways [13, 28]. Other reports have recently demonstrated that the stimulation of TLRs leads to the activation of several mitogen-activated protein kinase (MAPK) pathways, suggesting that TLRs have or share a common signaling pathway with MAPK [29]. It is also recognized that the MAPK family members p38, JNK and ERK are the activated downstream of TLRs to induce the production of cytokines and inflammatory mediators [30]. However, interplay between the ERK1/2 signaling pathway and TLR4 remains further determined. The results in the present study showed that Ang II-induced TLR4 expression in VSMCs was regulated by ERK signaling pathway through the AT1 receptor, because AT1 receptor antagonist losartan or PD098059, which specifically inhibits the upstream activator of ERK1/2, significantly suppressed Ang II-induced TLR4 expression both in protein and in mRNA levels, but AT2 receptor antagonist PD123319 did not have the effect. On the basis of the present results, it is most likely that Ang II activates TLR4 by a AT1-ERK1/2-dependent pathway in VSMCs. Although reason for the discrepancies between the published and the present results is still unclear, the result suggests that there possibly exists a crosstalk between the ERK1/2 signaling pathway and TLR4 activation,

which is helpful to understand the key link between the inflammatory pathway and the immune system.

TLR4 agonist specifically promotes production of the chemokine interferon- γ (IFN- γ) inducible protein 10 (IP-10), which is also associated to immune responses. IP-10 is an IFN- γ inducible gene and a CXC chemokine produced by different types of cells in response to IFN- γ and microbial products [31]. But, it is unknown whether TLR4 is able to activate IFN- γ specific signaling pathways, such as the interferon-responsive factors and IP-10 in Ang II- induced inflammation. In the present study, pretreatment of VSMCs with the TLR4 inhibitor effectively prevented Ang II-induced protein and mRNA expression of IP-10. Accordingly, the finding further supports the involvement of TLR4 in IP-10 signaling pathway that, in turn, appears to be necessary for the TLR4-mediated proinflammatory signaling transduction of Ang II (Ang II /AT1/ERK1/2/TLR4/IP-10).

Protein kinase C (PKC) is a central component in signaling pathways that regulate numerous cellular processes in both adaptive, innate immunity and inflammation [32, 33]. In addition, PKC is implicated as an important positive regulator in cell proliferation, especially during the process of angiogenesis [34], and as a critical component of the TLR4 signaling pathway [35]. The full complement of downstream signal events that are initiated by Ang II is still not fully understood. It is tempting to speculate that IP-10 could function at a point common to both TLR4 and PKC cascades. The present experiment confirms the involvement of IP-10 in Ang II-TLR4-mediated PKC activation, because anti-IP-10 antibody almost completely abolished the Ang II-induced mRNA and protein increase of PKC. Hence, IP-10 lies upstream from PKC in the signal cascades (Ang II/AT1/ERK1/2/TLR4/IP-10/PKC).

NF- κ B is a multi-tasking transcription factor implicated in an array of normal biological phenomena as well as different states of pathology such as cell growth and cell death, atherosclerosis, and innate immunity including inflammation [36]. The PKC-mediated nuclear translocation of NF- κ B has been demonstrated [37]. Furthermore, in the MyD88-independent TLR4 signaling pathway, NF- κ B is also subsequently activated [38].

Fig. 12. A scheme for signaling pathway relevant to Ang II-induced inflammatory response in VSMCs.

Upon the activation, NF- κ B translocates from cytoplasm to nucleus, and functions as a transcription factor to induce expression of cytokines including IL-1, IL-6, TNF- α and IFN- γ [39]. In this study, Ang II-induced NF- κ B expression was conspicuously suppressed by specific PKC inhibitor chelerythrine, providing the proof that PKC is a critical component for Ang II-dependent NF- κ B expression in VSMCs.

In summary, the present study for the first time demonstrates that Ang II induces inflammatory response involved in pathogenesis of atherosclerosis partly via TLR4-dependent signaling pathway in VSMCs. Ang II activates TLR4 through AT1 receptor and subsequent ERK1/2 in VSMCs. Then, the TLR4 mediates expression of downstream molecule IP-10, which induces PKC expression in turn. Finally, NF- κ B is activated and translocates from cytoplasm to nucleus to initiate expression of cytokines involved in inflammatory response by PKC (Fig. 12). These findings may be important in terms of understanding the potential role of the TLR4-dependent signaling pathway in modulating inflammatory events in atherosclerosis.

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