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Original Paper

Angiotensin II Suppresses Rev-erbα Expression in THP-1 Macrophages via the Ang II Type 1 Receptor/Liver X Receptor α Pathway

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Key Words

Angiotensin II • Rev-erbα • Liver X receptor • Macrophages • Matrix metalloproteinase-9

Abstract

Background/Aims: Angiotensin II (Ang II) regulates the expression of some core clock genes; excess Ang II leads to atherosclerosis advancement. Macrophage Rev-erbα mediates clockwork and inflammation, and plays a role in atherosclerotic lesion progression. However, the role of Ang II in regulating Rev-erbα expression in macrophages remains unclarified. ***Methods:*** We induced THP-1 macrophages by phorbol 12-myristate 13-acetate and investigated the effect of Ang II on Rev-erbα expression via real-time polymerase chain reaction, western blotting and small interfering RNA (siRNA) techniques. The cytotoxicity of the Rev-erbα agonist SR9009 was analyzed using a (3-[4,5-dimethylthiazol-2-yl])-2,5- diphenyltetrazolium bromide assay. ***Results:*** Ang II suppressed Rev-erbα mRNA and protein expression in THP-1 macrophages in a dose and time dependent manner. This effect was mediated via Ang II type 1 receptor (AT1R), and not Ang II type 2 receptor or peroxisome proliferator-activated receptor γ (PPARγ). Consistent with Rev-erbα expression regulated by Ang II, the liver X receptor α (LXRα) protein expression was downregulated in a time-dependent manner after Ang II treatment. The activation or silence of LXRα significantly increased or decreased Rev-erbα expression regulated by Ang II, respectively. This suggests that LXRα is involved in the effect of Ang II on Rev-erbα expression. MMP-9 mRNA expressions were significantly suppressed by SR9009 in THP-1 and RAW264.7 macrophages; moreover, SR9009-treatment significantly reduced Ang II–induced MMP-9 protein expressions in two types of macrophages. ***Conclusion:*** Ang II downregulates Rev-erbα expression in THP-1 macrophages via the AT1R/LXRα pathway.

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Introduction

Acute myocardial infarction (AMI) mainly results from the rupture of vulnerable plaques in coronary vessels. Factors affecting plaque rupture include shear stress, macrophage infiltration, inflammation, and circadian rhythm variation during stress hormone release and infection [1, 2]. Clinical studies have shown that the onset of AMI exhibits an obvious circadian variation [2-4]. It is believed that circadian variation in the incidence of plaque rupture mainly accounts for the characteristic circadian rhythm of AMI. Circadian stress hormone alterations comprise an important factor regulating diurnal variations of plaque rupture [1]. The mechanisms underlying this regulation are unclear; the circadian clock and clock-controlled genes in macrophages may be involved. In fact, previous studies have shown that the secretion of many proinflammatory cytokines associated with plaque rupture is gated by the circadian clock [5, 6].

Matrix metalloproteinases (MMPs) produced by activated cells (e.g., macrophages, smooth muscle cells, and endothelial cells) in inflamed atherosclerotic plaques are thought to weaken plaque caps and consequently promote plaque disruption through direct degradation of the extracellular matrix (ECM) [7]. Clinical and genetic studies have revealed that MMP-2 and MMP-9 are involved in the onset of acute coronary syndrome (ACS) [8-10]. In addition, excessive levels of angiotensin II (Ang II), the major stress hormone in the rennin-angiotensin system (RAS), have been confirmed to accelerate the progression of atherosclerotic plaques toward instability via MMPs induction [11, 12]. These actions are mainly mediated through Ang II type 1 receptor (AT1R) [11, 13, 14] and its downstream signaling molecules, including nuclear factor-kappa B (NF-κB), liver X receptor α (LXRα), Toll-like receptors (TLRs) and peroxisome proliferator–activated receptor γ (PPARγ) [15-17]. Interestingly, Ang II also regulates the mRNA expression of some core clock and clock related genes, such as Period, Dbp, Bmal1, and Rev-erbα, in cultured vascular smooth muscle cells and hearts of rats [18, 19]. However, the association between its effect on clock controlled genes and atherosclerotic plaque development is not well known.

Recently, macrophages were found to exhibit rhythmic expression of several clock genes, including Rev-erbα, a member of the nuclear receptor (NR) superfamily [5]. Rev-erbα, which is highly expressed in adipose tissue, muscle cells, and macrophages, regulates circadian rhythm, lipid, and glucose metabolism. It also plays important roles in inflammation and diseases, such as diabetes and atherosclerosis, through repressing transcription of target genes. Several studies have found that Rev-erbα is an important molecular component of the biological clock and a critical regulator of proper circadian timing [20, 21]. Moreover, Rev-erbα may be a transcriptional link between circadian rhythmicity and cardiometabolic disease [22]. Recent studies have outlined the importance of Rev-erbα in atherosclerotic lesion progression. For example, in low-density lipoprotein (LDL) receptor–deficient mice, decreased Rev-erbα expression in hematopoietic cells led to increased atherosclerotic lesion size in the aortic root [23]. Consistent with this study, administration of synthetic Rev-erbα agonists was found to inhibit aortic atherosclerosis development in these mice [24]. Furthermore, *in vitro* research has shown that Rev-erbα suppresses MMP-9 gene expression in macrophages by inhibiting the transcription of enhancer-derived RNAs (eRNAs) [25], indicating a potential mechanistic connection between Rev-erbα activity and plaque disruption.

Ang II influences Rev-erbα expression in hearts of rats and has been shown to be involved in the regulation of MMPs expression in macrophages; however, the precise mechanisms by which Ang II controls Rev-erbα expression in macrophages and its relationship with MMPs regulation remain unknown.

In this study, we aimed to determine the effect of Ang II on Rev-erbα expression and investigate whether Rev-erbα could modulate Ang II-induced MMP-9 and MMP-2 expression in macrophages. We found that Ang II significantly downregulated Rev-erbα expression via the AT1R/LXRα signaling pathway. Moreover, our findings indicated that Ang II-induced upregulation of MMP-9 expression was suppressed by stimulation of Rev-erbα.

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Materials and Methods

Materials

Ang II, phorbol 12-myristate 13-acetate (PMA), the PPAR-γ agonist pioglitazone, the PPAR-γ antagonist GW9662, the LXRα agonist T0901317, the NF-κB antagonist pyrrolidine dithiocarbamate (PDTC) and the Ang II type 2 receptor (AT2R) antagonist PD123319 were purchased from Sigma-Aldrich (St. Louis, MO, USA). SR9009 was purchased from MedChemExpress (Monmouth Junction, NJ, USA), and the AT1R antagonist valsartan was from Novartis (China). The cell culture medium RPMI 1640 and DMEM were from Corning (NY, USA), and fetal bovine serum (FBS) were from ScienCell (Carlsbad, CA, USA). TRIzol reagent for RNA isolation was from Invitrogen (Shanghai, china), and the one-step reverse transcription-polymerase chain reaction (RT-PCR) Kit and the SYBR Green 1 Real-Time RT-PCR kit were from Roche (Basel, Basel-Stadt, Switzerland). The rabbit monoclonal antibodies against human Rev-erbα, human LXRα and human MMP-9 were purchased from Abcam Inc (Cambridge, MA, USA), and the mouse monoclonal antibody against β-actin was from ABclonal Inc (Cambridge, MA, USA). Anti-mouse/anti-rabbit conjugated antibodies were purchased from Zhongshan Golden Bridge Bio Co (Beijing, China). The Nuclear and Cytoplasmic Protein Extraction Kit, BCA Protein Assay Kit, and Enhanced Chemiluminescence (ECL) Kit were from Beyotime Institute of Biotechnology (Shanghai, China).

Cell culture

The human monocytic leukemia cell line THP-1 (Stem Cell Bank, Chinese Academy of Sciences) was incubated in RPMI 1640 medium with 10% FBS in a humidified 95% air/5% CO_2 atmosphere at 37 °C. Then, cells were seeded onto a six-well plate at 1×10^6 cells/mL. In accordance with the literature [26], 160 nM/mL PMA was added to the culture to induce macrophages differentiation. After 48 h, the plates were washed thrice with 1 mL of phosphate-buffered saline (PBS). Adherent THP-1 macrophages were evaluated by CD68 immunofluorescence labeling to identify differentiation of THP-1 monocytes to macrophages. The RAW264.7 macrophage cell line (Shanghai Institute for Biological Science, the Chinese Academy of Sciences, Shanghai, China) was cultured in DMEM supplemented with 10% FBS and the culture medium was replaced every 2-3 days. For subsequent experiments, the cells were cultured in serum-free medium for an additional 24 h and then maintained in complete culture medium for the next stimulus.

Cell treatment

To analyze Rev-erbα expression due to the effect of Ang II on THP-1 macrophages, cells were stimulated with Ang II (10^{-9}, 10^{-8}, 10^{-7}, and 10^{-6} M) for different periods (6, 12, and 24 h). To identify the receptor subtypes involved in angiotensin II receptor blocker (ARB)-mediated regulation of Rev-erbα expression, macrophages were preincubated with the AT1 receptor blocker valsartan (10 μM, non-PPARγ–activating ARB), the AT2 receptor blocker PD 123319 (10 μM), the PPARγ agonist pioglitazone (10 μM), and the PPARγ antagonist GW9662 (10 μM) for 2 h before Ang II treatment. Next, we investigated the effects of the NF-κB inhibitor PDTC (10 μM) and LXRα agonist T0901317 (1 μM) on Rev-erbα expression induced by Ang II in THP-1 macrophages by preincubation for 30 min and 24 h, respectively, before Ang II addition. In addition, cells were transfected with LXRα siRNA for 72 h before Ang II addition and pretreated with T0901317 (1 μM) for 24 h, separately. To confirm the role of LXRα in Rev-erbα expression downregulated by Ang II, cells were treated with Ang II (10^{-6} M) for different periods (6, 12, and 24 h). Then, THP-1 Macrophages and RAW264.7 macrophages were stimulated with Ang II to induce MMPs expression and cotreated with SR9009 (10 μM) for 12 h. To determine the role of Rev-erbα in MMP-9 protein expression induced by Ang II, THP-1 macrophages were preincubated with the AT1 receptor blocker valsartan (10 μM) for 2 h and the LXR agonist T0901317 (1 μM) for 24 h, before Ang II-treatment, and subsequently cotreated with SR9009 (10 μM) and Ang II for 12 h, and transfected with LXRα siRNA for 72 h before Ang II addition, and pretreated with T0901317 (1 μM) for 24 h, separately. Unless stated otherwise, 10^{-6} M Ang II was used for 12 h.

(3-[4, 5-Dimethylthiazol-2-yl])-2, 5-diphenyltetrazolium bromide (MTT) assay

The cytotoxicity of SR9009 was analyzed by MTT. THP-1 macrophages were seeded into 96-well plates at 1×10^5 cells per well with 100 μL of RPMI-1640 medium. THP-1 macrophages were then stimulated with SR9009 (1, 5, 10, 50 μM) for 12 h; the cells incubated in 0.1% dimethyl sulfoxide (DMSO) were defined as the control group. MTT (5 mg/mL) reagent was added into each well. After incubation for 4 h, the medium

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was siphoned off and replaced with DMSO. The absorbance in each well was measured at 540nm using a microplate reader.

Real-time PCR

Total RNA was extracted from macrophages with TRIzol reagent according to the manufacturer's protocol. RNA quality was examined using an Experion Automated Electrophoresis System (BioRad, Hercules, CA, USA). First-strand cDNA was generated using reverse transcriptase (RT) and oligo dT primers (Transcriptor First Strand cDNA Synthesis Kit, Roche) and was then subjected to real-time RT-PCR amplification (PrimeScript RT reagent Kit Perfect Real Time, Roche). The following primers were used: hRev-erbα: 5′-GAC ATG ACG ACC CTG GAC TC-3′ (forward), 5′-GCT GCC ATT GGA GTT GTC AC-3′ (reverse); hMMP-9: 5′- AGT CCA CCC TTG TGC TCT TC-3′ (forward), 5′-ACT CTC CAC GCA TCT CTG C-3′ (reverse); and hMMP-2: 5′-ACC TGG ATG CCG TCG TGG AC-3′ (forward), 5′-TGT GGC AGC ACC AGG GCA GC-3′ (reverse); mMMP-9: 5′- CTG GAC AGC CAG ACA CTA AAG-3′ (forward), 5′-CTC GCG GCA AGT CTT CAG AG-3′ (reverse). Human and mouse β-actin (housekeeping gene) were amplified as an internal control for normalization. hRev-erbα, hMMP-9, hMMP-2, mMMP-9, and β-actin expression was assessed via SYBR Green 1 fluorescence real-time RT-PCR. The CFX96 Real-Time PCR detection system (Bio-Rad) was used to directly monitor the PCR.

RNA interference experiment

Three small interfering RNA (siRNA) fragments against human LXRα and the nonsilencing siRNAs were synthesized by Ribobio (Guangzhou, China). THP-1 macrophages (1×10^6 cells/well) were transfected with each siRNA using ribo- FECTTMCP Reagent (Ribobio). After 72 h, transfection efficiency was measured by western blotting. The second siRNA fragment (5′- TCA TCA AGG GAG CGC ACT A -3′, LXRα-siRNA) targeting LXRα suppressed LXRα expression by 70%. Next, cells were treated with 10^{-6} M Ang II for 12 h, then harvested.

Protein isolation and western blot analysis

Treated THP-1 macrophages were lysed in radioimmunoprecipitation assay (RIPA) buffer and phenylmethanesulfonyl fluoride (PMSF) in ice for 30 min. The protein deposit was obtained after centrifugation at 12, 000 *g* for 30 min. Nucleic protein was extracted using the Nuclear and Cytoplasmic Protein Extraction Kit. The protein concentration was determined with the BCA protein assay kit, using bovine serum albumin (BSA) as a standard. Protein samples (40 μg) were used for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) on 10% polyacrylamide gels and were transferred to a polyvinylidene difluoride (PVDF) membrane for 0.5 h at 15 V. Membranes were blocked in 5% non-fat milk solution in Tris-buffered saline (TBS) buffer with 0.5% Tween 20 for 1 h at 22°C. Then, the membranes were incubated overnight at 4°C with the specific primary antibodies anti-Rev-erbα (1:2, 000), anti-LXRα (1:3, 000), and anti-MMP-9 (1:4, 000). They were then washed three times in Tris-buffered saline with 0.1% Tween 20 (10 min for each wash) before incubation with the fluorescent-labeled secondary anti-rabbit antibody (1:8, 000) for 1 h at 22°C. β-actin antibody was used as a loading control. Immunoreactive bands were detected using the ECL Kit according to the manufacturer's instructions.

Statistical analysis

The SPSS13.0 software was used to analyze data from all experiments. Groups of data were compared with analysis of variance (ANOVA), followed by Student– Newman–Keuls multiple comparison tests. Data have been provided as mean ± standard deviation (SD) values. $P < 0.05$ was considered significant.

Results

Assessment of macrophages differentiated from THP-1 cells

THP-1 macrophages were identified by immunofluorescence. After PMA treatment for 48 h, THP-1 macrophages have high expression of CD68 on their surface (data not shown), which demonstrated that THP-1 macrophages were successfully established.

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Rev-erbα expression in THP-1 macrophages was suppressed by Ang-II stimulation

Real time RT-PCR and western blotting showed that Rev-erbα expression was significantly suppressed ($P < 0.05$) at the mRNA and protein levels (Fig. 1A), respectively, in THP-1 macrophages after incubation with different concentrations of Ang II. This suppression was maximum at the highest concentration of Ang II. Rev-erbα mRNA and protein expression levels at 6 h were significantly lower than those in the control group and reached a bottom at 12 h (Fig. 1B).

Involvement of Ang II receptor subtypes in Ang II–induced downregulation of Rev-erbα

The antagonists valsartan and PD123319 were used to determine the roles of AT1R and AT2R in Ang II-induced downregulation of Rev-erbα expression. Preincubation with the AT1 receptor antagonist valsartan could significantly attenuate the effect of Ang II on Rev-erbα expression in macrophages ($P < 0.01$). However, blockade of AT2R did not influence the impact of Ang II on Rev-erbα expression (Fig. 2).

Previous studies have demonstrated that PPARγ agonism results from AT1 receptor blockade in the vasculature and that Rev-erbα can be upregulated by PPARγ in adipocytes [15, 27]. Therefore, to determine whether the effect of Ang II on macrophage Rev-erbα expression is mediated by AT1R blockade or PPARγ activation, THP-1 macrophages were treated with the full PPARγ agonist pioglitazone and the PPARγ antagonist GW9662. Ang II–

Fig. 1. Rev-erbα mRNA and protein expression is downregulated by angiotensin (Ang) II in THP-1 macrophages. (A) Rev-erbα mRNA and protein expression was downregulated by Ang II in a concentration-dependent manner. THP-1 macrophages were treated in 12 h with different concentrations of Ang II (10^{-9}, 10^{-8}, 10^{-7}, and 10^{-6} M). (B) Rev-erbα mRNA and protein expression was downregulated in 6, 12, and 24 h after Ang II (10^{-6} M) stimulation. The findings at different time points (6, 12, and 24 h) were examined. Rev-erbα expression was analyzed by western blotting (a) and real-time reverse transcription-polymerase chain reaction (b). Data from three independent experiments are shown relative to the controls and have been presented as mean ± SD values. *P<0.05 vs. control, **P<0.01 vs. control.
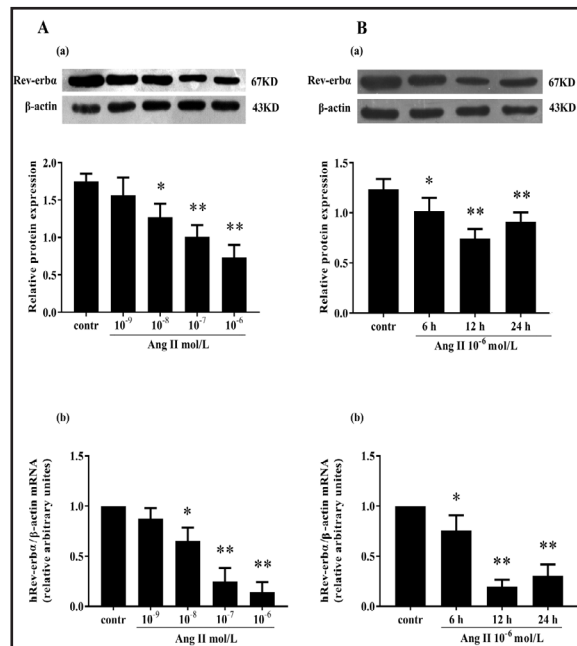
Fig. 2. Ang II receptor subtypes are involved in Ang II–induced downregulation of Rev-erbα. THP-1 macrophages were preincubated with the AT1 receptor blocker valsartan (10 μM, non-PPARγ-activating ARB), AT2 receptor blocker PD123319 (10 μM), the PPARγ agonist pioglitazone (10 μM), and the PPARγ antagonist GW9662 (10 μM) for 2 h before Ang II (10^{-6} M)-treatment. RPMI 1640 medium served as the negative control. Representative results from western blotting for Rev-erbα are shown. Data from three independent experiments are shown relative to the controls and have been presented as mean ± SD values. *P<0.01 vs. control, #P<0.01 vs. Ang II.

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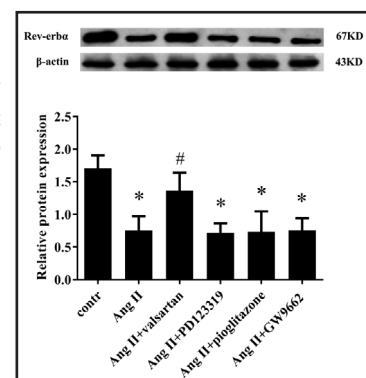
Fig. 3. Downregulation of Rev-erbα expression by Ang II is dependent upon liver X receptor α (LXRα) suppression. (A) LXRα siRNA significantly downregulated protein expression of LXRα in THP-1 macrophages. THP-1macrophages were transfected with LXRα siRNA for 72 h. (B) LXRα was involved in Rev-erbα protein expression following Ang II treatment. THP-1 macrophages were treated by either the synthetic LXR ligand T090137 (1 µM) for 24 h or the antioxidant NF-κB inhibitor PDTC (10 µM) for 30 min , or transfected with LXRα siRNA in 72 h before Ang II stimulation in 12 h, respectively. (C) LXRα protein expression in THP-1 macrophages after incubation with different time of Ang II (10⁻⁶M). THP-1 macrophages were

treated with Ang II (10^{-6} M). The findings at different time points (6, 12, and 24 h) were examined. Representative results from western blotting for Rev-erbα are shown. Data from three separately performed experiments are shown relative to the controls and have been presented as mean ± SD values. *P<0.01 vs. control, ※P<0.01 vs. control, #P<0.05 vs. Ang II,§P<0.01vs. Ang II.
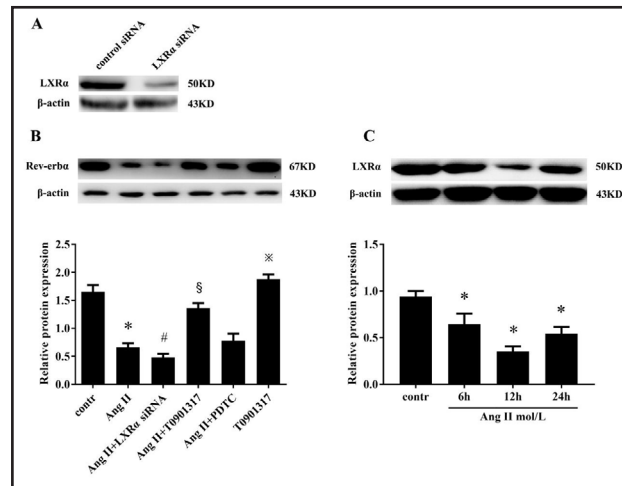
induced downregulation of Rev-erbα was unaffected by either the PPARγ agonist or PPARγ antagonist in THP-1 macrophages (Fig. 2).

*Downregulation of Rev-erbα expression after Ang II treatment was dependent upon LXRα suppression*

NF-κB and LXRα, which are downstream of AT1R in the inflammation response in macrophages, are activated and suppressed, respectively, by Ang II [28]. Therefore, we need investigate whether NF-κB activation and/or LXRα suppression are involved in the downregulation of Rev-erbα expression by Ang II. Our results indicated that Rev-erbα expression in T0901317 treated THP-1 macrophages was significantly higher than that in the control group ($P < 0.01$). Preincubation with T0901317 could significantly upregulate the expression of Rev-erbα suppressed by Ang II ($P < 0.01$ vs. Ang II). However, modulation of Rev-erbα by Ang II was not affected by the antioxidant NF-κB inhibitor PDTC (Fig. 3B).

Then, the effect of LXRα silencing on Rev-erbα protein expression was examin- ed. SiRNAs targeting LXRα and the nonsilencing (control) siRNA were established, LXRα protein expression in LXRα siRNA group was significantly lower compared with that in control siRNA group, (Fig. 3A). As expected, LXRα silencing increased the inhibitory effect of Ang II on Rev-erbα expression ($P < 0.05$ vs. Ang II). To confirm the role of LXRα in Rev-erbα expression downregulated by Ang II, we assessed LXRα protein expression in THP-1 macrophages after incubation with different time of Ang II (10⁻⁶M). Consistent with Rev-erbα expression downregulated by Ang II, LXRα protein expression treated by Ang II was downregulated at 6 h and reached a trough at 12 h, respectively (Fig. 3C). These results demonstrated that regulation of Rev-erbα expression by Ang II was dependent upon LXRα expression in THP-1 macrophages.
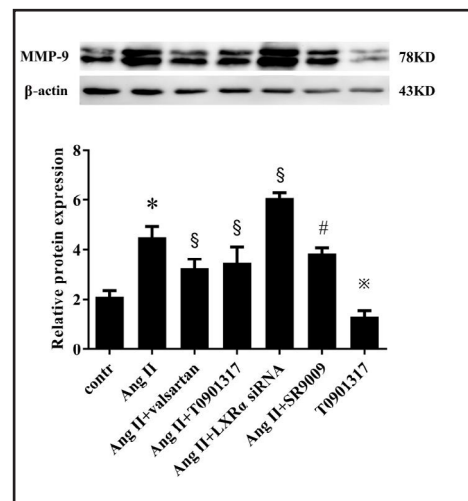
Effect of the Rev-erbα agonist on MMPs mRNA expression in macrophages

To determine whether SR9009 was toxic to THP-1 macrophages, cell viability was monitored by the MTT assay. THP-1 macrophages were stimulated with SR 9009 (1, 5, 10, and 50 µM) for 12 h. The viability of THP-1 macrophages was not significantly affected by SR9009 at 1–10 µM, indicating the absence of significant cytotoxicity at these concentrations in THP-1 macrophages (Fig. 4A).

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Fig. 4. Effect of the Rev-erbα agonist on matrix metalloproteinases (MMPs) mRNA expression in macrophages. (A) Effect of different concentrations of SR9009 on the viability of THP-1 macrophages. THP-1 macrophages were stimulated in 12 h with different concentrations of SR9009 (1, 5, 10, and 50 μM). Representative results from the MTT assay for THP-1 macrophage viability are shown. Data from three separately performed experiments are shown relative to the controls. (B) Effect of the Rev-erbα agonist on hMMP-2 mRNA expression in THP-1 macrophages. (C) Effect of the Rev-erbα agonist on hMMP-9 mRNA expression in THP-1 macrophages. (D) Effect of the Rev-erbα agonist on mMMP-9 mRNA expression in RAW264.7 macro-

phages. macrophages were treated with the Rev-erbα agonist SR9009 (10 μM), used either alone or along with Ang II (10^{-6} M). MMP-2 and MMP-9 mRNA expression was analyzed by real-time RT-PCR from three and four independent experiments, respectively. Data are shown relative to the controls and have been presented as mean ± SD values. *$P<0.05$ vs. control, **$P<0.01$ vs. control, #$P<0.05$ vs. Ang II. hMMP - human MMP, mMMP - mouse MMP.
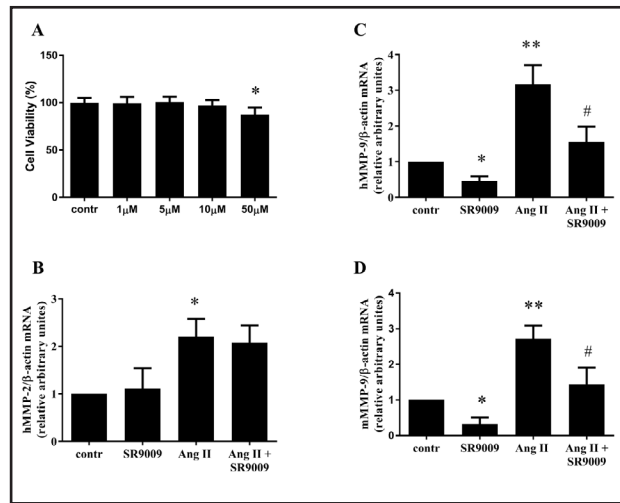
Fig. 5. Rev-erbα expression in relation to MMP-9 protein expression induced by Ang II in THP-1 macrophages. MMP-9 protein expression was analyzed by western blotting. Seven groups (control, Ang II, Ang II + valsartan, Ang II + T0901317, Ang II + LXRα siRNA, Ang II + SR9009, and T0901317) were examined after Ang II treatment for 12 h. Data from three independent experiments are expressed relative to the controls and have been presented as mean ± SD values. *$P<0.05$ vs. control, ※$P<0.01$ vs. control, #$P<0.05$ vs. Ang II, §$P<0.01$ vs. Ang II.

The effect of the Rev-erbα agonist on MMPs expression was examined by real-time RT-PCR. THP-1 macrophages were treated with the Rev-erbα agonist SR9009, used either alone or along with Ang II. Human MMP-9 (hMMP-9) mRNA expression in SR9009-treated THP-1 macrophages significantly decreased after Ang II stimulation ($P < 0.05$; Fig. 4C). Expression of hMMP-9 mRNA also decreased when cells were treated with SR9009 alone, without Ang II stimulation ($P < 0.05$). The effect of SR9009 was further confirmed on mouse MMP-9 (mMMP-9) mRNA expression in RAW264.7 macrophages (Fig. 4D). However, MMP-2 mRNA expression in THP-1 macrophages was unaffected by SR9009 treatment (Fig. 4B).

Rev-erbα expression in relation to MMP-9 protein expression induced by Ang II
THP-1 macrophages in conditioned media were subjected to different treatments and accordingly divided into the control, Ang II, Ang II + valsartan, Ang II + T0901317, Ang II + LXRα siRNA, Ang II + SR9009 and T0901317 groups before western blotting (Fig. 5). MMP-9 protein expression in the Ang II–treated group was significantly higher than that in the

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control group ($P < 0.05$). This regulation could be depressed by treatment with valsartan, T0901317, and SR9009, respectively. However, LXRα siRNA treatment significantly increased MMP-9 protein expression in THP-1 macrophages treated with Ang II ($P < 0.01$ vs. Ang II). As expected, MMP-9 protein expression in the T0901317–treated group was significantly lower than that in the control group ($P < 0.01$). Thus, MMP-9 protein expression was related to Rev-erbα expression.

Discussion

To our knowledge, the current study is the first to show that Ang II stimulation can decrease Rev-erbα expression in THP-1 macrophages and that this effect is mainly mediated via the AT1R /LXRα signaling pathway. Moreover, the agonist of Rev-erbα markedly reduced MMP-9 expression under Ang II stimulation in macrophages.

The circadian rhythm is a biological process that is controlled by clock signals such as light, food, and hormones. As a clock-controlled gene, Rev-erbα is also believed to be entrained and modulated by humoral factors including hormones in serum [29]. Ang II, a stress hormone, is an attractive candidate for clock cues because it is released into the serum in daily cycles and can induce clock gene expression in smooth muscle cells and cardiomyocytes [18, 19]. Herichová et al. found that 28-day infusion of Ang II modified Rev-erbα mRNA expression in hearts of Wistar rats [19]. We also found that Rev-erbα expression was regulated by Ang II in THP-1 macrophages. These findings expand our understanding of the role of RAS in clock and clock-controlled genes expression.

The effect of Ang II on clock gene expression has been confirmed to be predominantly mediated via AT1R [18]. Therefore, we investigated the functional role of AT1R in the regulation of Rev-erbα gene expression. Our results showed that Ang II regulated Rev-erbα expression through AT1R in THP-1 macrophages. However, previous research has demonstrated that Ang II downregulates PPAR-γ expression and that AT1R blockade leads to PPAR-γ stimulation in the vasculature [15]. Therefore, the effect of PPAR-γ should also be considered while studying the effect of Ang II on Rev-erbα expression. Here, we found that the effect of PPAR-γ in macrophages was not involved in the regulation of Rev-erbα expression by Ang II. However, PPAR-γ has been reported to induce Rev-erbα expression during murine adipocyte differentiation [27]. These contradictory results indicate that the regulation of Rev-erbα expression is pathway-specific in different cell lines.

The regulation of NRs on Rev-erbα in different types of cells and tissues has been studied. Previous research has indicated that Rev-erbα expression is downregulated by glucocorticoids via their receptor mechanism in both rats and human primary hepatocytes [30]. Basic research also has shown that PPARα and PPARγ induce Rev-erbα expression in liver and adipose tissues, respectively [27, 31]. Moreover, Fontaine et al. found that LXRs regulate Rev-erbα in human macrophages [32]. Consistent with these previous studies, our current results indicated that LXRα regulates Rev-erbα protein expression following Ang II treatment in human macrophages. The regulatory mechanism of LXRα for Rev-erbα has been confirmed by recent research demonstrating that LXRα binds as a heterodimer with the retinoid X receptor α (RXRα) to the human Rev-erbα promoter and induces the transcriptional activity of Rev-erbα [32]. In addition, recent research has demonstrated that LXRα, one of the NRs controlling cholesterol homeostasis and inflammation, is inhibited by Ang II in macrophages [28]. Collectively, these evidences indicate that the regulation of Ang II on Rev-erbα expression is LXRα-dependent, at least in part, in THP-1 macrophages.

The circadian variation of atherosclerotic lesion rupture might be attributed, at least in part, to circadian oscillation of genes relevant to the ECM [33] and circadian fluctuation of MMPs [34]. Moreover, mutation of circadian genes has been shown to lead to vascular stiffness via MMPs dysregulation [35]. Thus, molecular circadian clock dysfunction is proposed to be involved in the progression of atherosclerotic plaque. In the current study, our results showed that Rev-erbα is involved in regulating MMP-9 expression in human and mouse

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macrophages. Thus, we speculate that Rev-erbα may serve to integrate ECM degradation functions of MMP-9 and circadian signals in macrophages. Moreover, the inhibitory effect of Rev-erbα on MMP-9 expression induced by Ang II suggests that the increase in Rev-erbα expression could relieve degradation of the ECM in atherosclerotic plaques, and thus mediates, at least in part, the Ang II effect on atherosclerotic progression. In a recent study, AT1R antagonism was found to repress intimal neovascularization in atherosclerotic plaques in apolipoprotein E–deficient mice, partly by reducing inflammatory effects mediated by TLRs and MMPs activation [14]. In addition, human TLR-4 promoter activity is inhibited by Rev-erbα, which binds with the LXRα response element site in the TLR-4 promoter [31]. Together, these data provide some indication regarding how NR Rev-erbα influences the development of RAS-induced atherosclerotic plaques, suggesting that Rev-erbα might be a potential therapeutic target for vulnerable plaque.

Studies on rats and mice have indicated that Rev-erbα expression reaches a peak during the daytime and trough in the nighttime in peripheral tissues and organs, such as the liver, heart, immune system components and adipose tissues [5, 19, 36]. The morning peak incidence of cardiovascular events has a significant implication that circadian variation of Rev-erbα might correlate with the attack of ACS. Although a direct functional role of Rev-erbα in ACS has not yet been shown, our observation raises the possibility that Rev-erbα in plaques might influence pathogenesis of ACS by its effect on MMPs. Thus, further research on circadian variation of Rev-erbα expression in atherosclerotic plaques is required to improve understanding of the relation between Rev-erbα and ACS.

Rev-erbα and retinoic acid receptor-related orphan receptors-α (RORα), which recognize and compete for the same response elements in the genome, regulate the expression of genes involved in immune function, muscle metabolism, and lipid and glucose homeostasis [37]. Recent research has revealed that this dual regulation is related to gene expression of proinflammatory cytokines in macrophages [38]. Therefore, further research is required to determine the competitive relationship of the two receptors in MMP-9 regulation in macrophages. However, Zhang et al. have recently suggested that the tissue-specific non-clock genes targeted by Rev-erbα are regulated independent of competition from RORα [39]. In addition, further investingations are required to elucidate the involvement of other clock-related genes controlled by Ang II on MMPs expression.

We have to point out that this study has examined the effect of Ang II on Rev-erbα expression only in THP-1 macrophages and further research need be performed in peritoneal macrophages or bone marrow-derived mouse macrophages.

In summary, we have shown that Ang II can downregulate Rev-erbα expression in THP-1 macrophages via AT1R and that the LXRα pathway plays an important part in this effect. In addition, Rev-erbα is involved in Ang II–regulated MMP-9 expression in macrophages, which may provide a new therapeutic target for the treatment of atherosclerotic plaques.

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Disclosure Statement

No conflict of interests exists.

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