

Angiotensin II induces monocyte chemoattractant protein-1 expression by increasing reactive oxygen species-mediated activation of the nuclear factor- κ B signaling pathway in osteoblasts

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Abstract. The present study investigated the effect of angiotensin II (Ang II) on monocyte chemoattractant protein-1 (MCP-1) expression and the underlying mechanism in osteoblasts. MCP-1 expression levels were analyzed by ELISA and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Ang II type 1 receptor (AT1R) expression levels was examined by RT-qPCR, western blotting and immunostaining. In addition, the nuclear factor (NF)- κ B signaling pathway was investigated via western blot analysis. Reactive oxygen species (ROS) were also detected by flow cytometry and fluorescent microscopy. The results of the present study indicated that Ang II upregulated MCP-1 expression in osteoblasts, which was mitigated by agonists of the AT1R, including olmesartan, a ROS scavenger N-acetylcysteine (NAC), ammonium pyrrolidinedithiocarbamate (PDTC) and nuclear factor (NF)- κ B, but not by the Ang II type 2 receptor antagonist, PD123319. Furthermore, Ang II increased the generation of ROS and activated the NF- κ B signaling pathway. These effects of Ang II were blocked by olmesartan, NAC and PDTC, but not by PD123319 in osteoblasts. In conclusion, these data indicated that Ang II enhanced ROS production and activated

NF- κ B signaling via AT1R, thus upregulating MCP-1 expression in osteoblasts.

Introduction

Angiotensin II (Ang II), a primary effector in the renin-angiotensin system (RAS), regulates cell growth and differentiation, blood pressure, fluid and electrolyte homeostasis, and cytokine production (1). Ang II is crucial for renal and cardiovascular function (2), and previous studies have demonstrated that Ang II induces receptor activator of nuclear factor- κ B ligand (RANKL) expression in osteoblasts, leading to osteoclast activation and accelerated osteoporosis (3-5). During bone metabolism, monocyte chemoattractant protein-1 (MCP-1) secreted by osteoblast is important for osteoclast activation (6). However, whether Ang II upregulates MCP-1 expression in osteoblasts remains to be investigated.

Ang II stimulates MCP-1 expression in endothelial cells, vascular smooth muscle cells (VSMCs) and monocytes. Ang II upregulates MCP-1 expression in rat glomerular endothelial cells by activating the NAD(P)H oxidase-dependent nuclear factor (NF)- κ B signaling pathway (7). Furthermore, Ang II enhances MCP-1 expression in proximal tubular cells by activating reactive oxygen species (ROS)-mediated signaling (8). In addition, Ang II through its Ang II receptor type 1 (AT1R) promotes ROS generation in hepatocytes (9). Previous studies have indicated that ROS activates NF- κ B signaling (10). However, whether Ang II also induces ROS production which activates the NF- κ B signaling pathway, resulting in upregulated MCP-1 expression in osteoblasts, remains to be clarified.

The present study used the MC3T3-E1 mouse osteoblastic cell line to investigate the hypothesis that Ang II induces ROS production to activate the NF- κ B signaling pathway, and upregulates MCP-1 expression in osteoblasts.

Materials and methods

Materials. α -Modified minimum essential medium (α -MEM), fetal bovine serum (FBS), streptomycin and penicillin

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Abbreviations: Ang II, angiotensin II; AT1R, angiotensin II type 1 receptor; AT2R, angiotensin II type 2 receptor; MCP-1, monocyte chemoattractant protein-1; RANKL, receptor activator of nuclear factor- κ B ligand; RAS, renin-angiotensin system; ROS, reactive oxygen species

Key words: angiotensin II, osteoblasts, monocyte chemoattractant protein-1, reactive oxygen species, nuclear factor- κ B pathway

Table I. Primers sequences for reverse transcription-quantitative polymerase chain reaction.

Target	Primer sequence (5'-3')	GenBank no.
MCP-1	F: TCACCTGCTGCTACTCATTC R: ATGTCTGGACCCATTCCTTC	NM_011333.3
AT1R	F: CTCTTGCTGCCTCGTCTACC R: TGCAGCAGCGTCTGATGATG	NM_009642.4
β -actin	F: AGGCCAACCGTGAAAAGATG R: TGGCGTGAGGGAGAGCATAG	NM_007393.3

AT1R, Angiotensin II type 1 receptor; MCP-1, Monocyte chemoattractant protein-1; F, forward; R, reverse.

were purchased from Gibco; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Ang II, PD123319, N-acetylcysteine (NAC), 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) and ammonium pyrrolidine thiocarbamate (PDTTC) were obtained from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). A rabbit anti-mouse AT1R (ab18801), anti-mouse I κ B kinase (IKK) β (ab32135) and anti-mouse phosphorylated (p)-IKK β antibodies (ab59195) were purchased from Abcam (Cambridge, MA, USA). In addition, rabbit polyclonal anti-mouse NF- κ Bp65 (ab16502) and p-NF- κ Bp65 (ab86299) antibodies were obtained from Abcam. Rabbit polyclonal anti-mouse I κ B α (sc371), and mouse monoclonal anti-mouse β -actin antibody (sc47778), were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA).

Cell culture. All MC3T3-E1 cells (American Type Culture Collection, Manassas, VA, USA) were grown to ~80% confluence and were routinely grown overnight in 10% FBS supplemented with α -MEM containing 100 U/ml penicillin and 100 μ g/ml streptomycin at 37°C in 5% CO₂ and 95% air. In order to determine the effects of Ang II on MCP-1 expression in the MC3T3-E1 cells, the cells were treated with 10⁻⁹~5 M Ang II or without Ang II (control group) for 24 h at 37°C and were treated with 10⁻⁶ M Ang II for varying durations (0, 3, 6, 12, 18 and 24 h) at 37°C. Furthermore, cells were in the presence of 10⁻⁶ M Ang II or absence of Ang II which served as the control group; cells were then separated into groups that were pretreated with 10⁻⁵ M olmesartan (AT1R blocker), 10⁻⁵ M PD123319 (AT2R blocker), 10⁻³ M N-acetylcysteine (NAC, a scavenger of free radicals), or 5x10⁻⁶ M PDTTC (the NF- κ B inhibitor) for 24 h at 37°C. The cells were harvested for subsequent analysis.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from different groups of MC3T3-E1 cells by TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and the cDNA was generated using the Revert Aid™ First Strand cDNA Synthesis kit (Fermentas; Thermo Fisher Scientific, Inc.) according to manufacturer's protocol. The relative levels of MCP-1 and AT1R gene mRNA transcripts to control β -actin in different groups of cells were determined using the cDNA as the template, the SYBR Green 1 PCR master mix (Takara Bio, Inc., Otsu, Japan), under the following conditions: 95°C for 30 sec, 95°C for 5 sec, and 60°C for 30 sec for 40 cycles and 95°C for 15 sec in an ABI 7300 Real-time

PCR system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The primer sequence are presented in Table I. The data were normalized to the control β -actin expression and analyzed by 2^{- $\Delta\Delta$ C_q} method (11). All assays were performed in quintuplicate.

ELISA. The levels of MCP-1 in the supernatants of cultured MC3T3-E1 cells were determined by ELISA using a specific kit (ab100721), according to the manufacturer's protocol (Abcam). The limitation of MCP-1 detection was 10 pg/ml.

Western blot analysis. The different groups of MC3T3-E1 cells were washed with ice-cold PBS, and lysed in radioimmunoprecipitation assay buffer (Thermo Fisher Scientific, Inc.). The cell lysates were centrifuged at 10,000 x g for 20 min at 4°C. The concentration of protein was quantified by the Bicinchoninic Acid protein assay reagent (Pierce; Thermo Fisher Scientific, Inc.). The lysates (20 μ g/lane) were subjected to 5% and 10% SDS-PAGE, which was performed using a constant voltage of 90 V for 120 min. Following this, proteins were transferred onto polyvinyl difluoride membranes. The membranes were blocked with 5% fat-free milk and probed with anti-AT1R (1:600), anti-NF- κ Bp65 (1:1,000), anti-p-NF- κ Bp65 (1:1,000), anti-I κ B α (1:1,000), anti-IKK β (1:1,000), anti-p-IKK β (1:500) and anti- β -actin (1:100) primary antibodies respectively, and incubated overnight at 4°C. Following three washes with PBS, the bound antibodies were detected with horseradish peroxidase-conjugated mouse anti-rabbit immunoglobulin G (IgG; 1:3,000, sc2357) secondary antibody, which was obtained from Santa Cruz Biotechnology, Inc., at room temperature for 2 h. The bands were visualized by an Enhanced ECL Chemiluminescent Substrate kit (JC-PC001, Jingcai Biotechnology Co. Ltd., Xi'an, China). The relative levels of each target protein to the control β -actin were analyzed using Quantity One v4.52 software (Bio-Rad Laboratories, Inc.).

Immunostaining. The cells were pre-treated with 10⁻⁵ M olmesartan (an AT1R blocker) for 30 min, and exposed to Ang II (10⁻⁶ M) for 12 h. The cells were fixed with 10% methanol at room temperature, blocked with rabbit serum in PBS for 60 min at room temperature, and subsequently stained with an anti-AT1R antibody (1:500) overnight at 4°C. Following three washes with PBS, the cells were incubated with goat anti-rabbit IgG secondary antibody for 2 h at 37°C (1:200, cw0159), which was obtained from ComWin Biotech Co., Ltd. (Beijing, China). The cells were washed with PBS for three

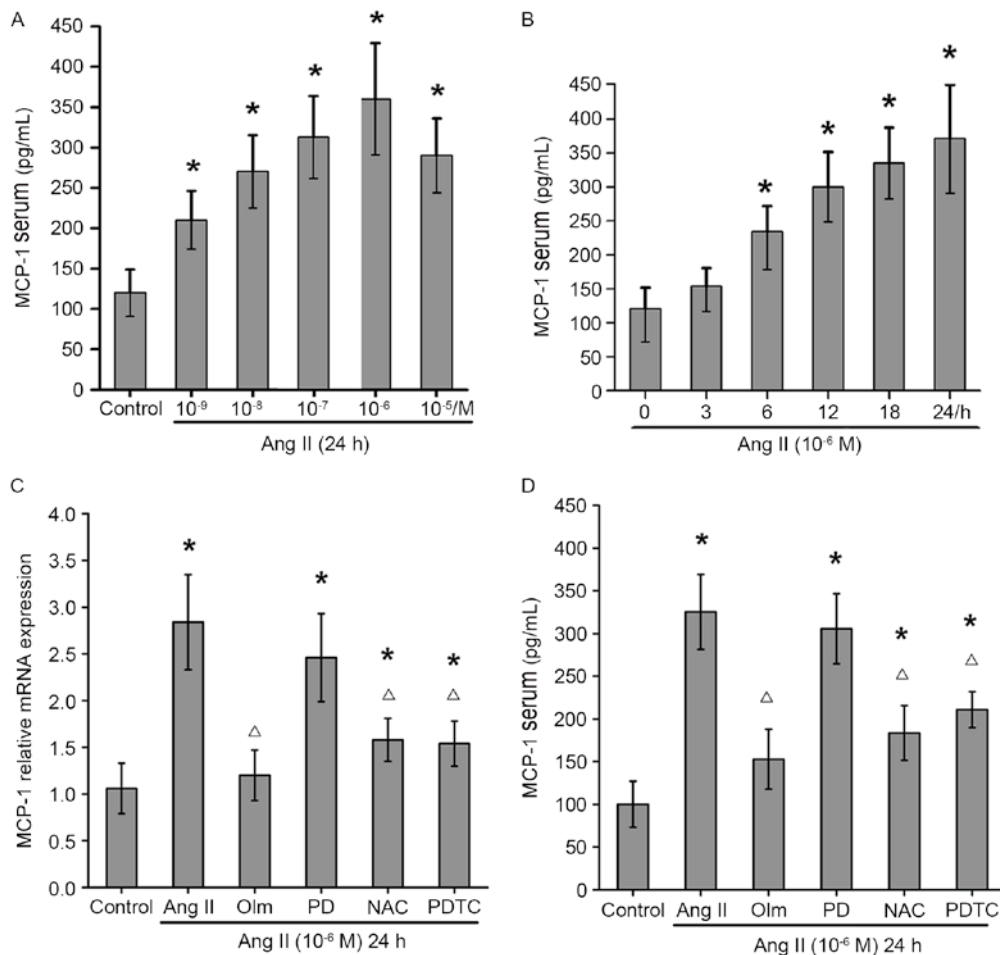


Figure 1. Effects of Ang II on MCP-1 expression in osteoblasts. MCP-1 expression levels following (A) various doses of Ang II, and (B) treatment with 10⁻⁶ M Ang II for 0, 3, 6, 12, 18 and 24 h. (C) mRNA expression and (D) serum levels of MCP-1 following treatment with Ang II and various inhibitors, as assessed by reverse transcription-quantitative polymerase chain reaction and ELISA, respectively. Data are expressed as the mean ± standard deviation of five independent experiments. *P<0.05 vs. control; ^ΔP<0.05 vs. Ang II. Ang II, angiotensin II; MCP-1, monocyte chemoattractant protein-1; Olm, olmesartan; PD, PD123319; NAC, N-acetylcysteine; PDTC, ammonium pyrrolidine thiocarbamate.

times prior to staining with phycoerythrin-streptavidin and DAPI (Sigma-Aldrich; KGaA). Images were obtained under a fluorescent microscope at excitation wavelengths of 550 and 340 nm (BX50; Olympus Corporation, Tokyo, Japan).

Flow cytometry and fluorescent microscopy. Intracellular ROS was evaluated by flow cytometry and fluorescent microscopy. Briefly, the cells (~80% confluence) were washed with PBS buffer for two times at room temperature and then were pre-treated at 37°C with or without olmesartan (AT1R blocker), PD123319 (AT2R blocker), NAC (scavenger of free radicals) or PDTC (NF-κB inhibitor) for 30 min, and then exposed to Ang II (10⁻⁶ M) for 12 h in 10% FBS medium (Gibco; Thermo Fisher Scientific, Inc.). The cells were washed with fetal calf serum-free medium for two times at room temperature and cultured in a-MEM containing 10 μM DCFH-DA (s0033, Beyotime Institute of Biotechnology, Jiangsu, China) for 30 min at 37°C in 5% CO₂ and 95% air. The cells were then digested with 0.25% pancreatic enzyme (Gibco; Thermo Fisher Scientific, Inc.) and were resuspended in PBS buffer. The contents of intracellular ROS which were detected by dichlorodihydrofluorescein diacetate (s0033; Beyotime Institute of Biotechnology) were analyzed by flow cytometry using

FACSort flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). In addition, the intracellular ROS were analyzed via fluorescent microscopy at an excitation wavelength of 488 nm prior to digestion with 0.25% pancreatic enzyme.

Statistical analysis. All data are presented as the mean ± standard deviation. Multiple comparisons were assessed by analysis of variance followed by post hoc least significant difference test using SPSS version 17.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Ang II induces MCP-1 expression in osteoblasts. To determine the effect of Ang II on MCP-1 expression in osteoblasts, MC3T3-E1 cells were treated with or without various concentrations of Ang II (10⁻⁹-10⁻⁵ M) for 24 h. The results showed that treatment with 10⁻⁹-10⁻⁶ M Ang II increased the levels of MCP-1 in MC3T3-E1 cells in a dose-dependent manner, but treatment with 10⁻⁵ M Ang II slightly reduced the levels of MCP-1, relative to that of 10⁻⁶ M Ang II (Fig. 1A). Furthermore, treatment with 10⁻⁶ M Ang II enhanced MCP-1 expression in

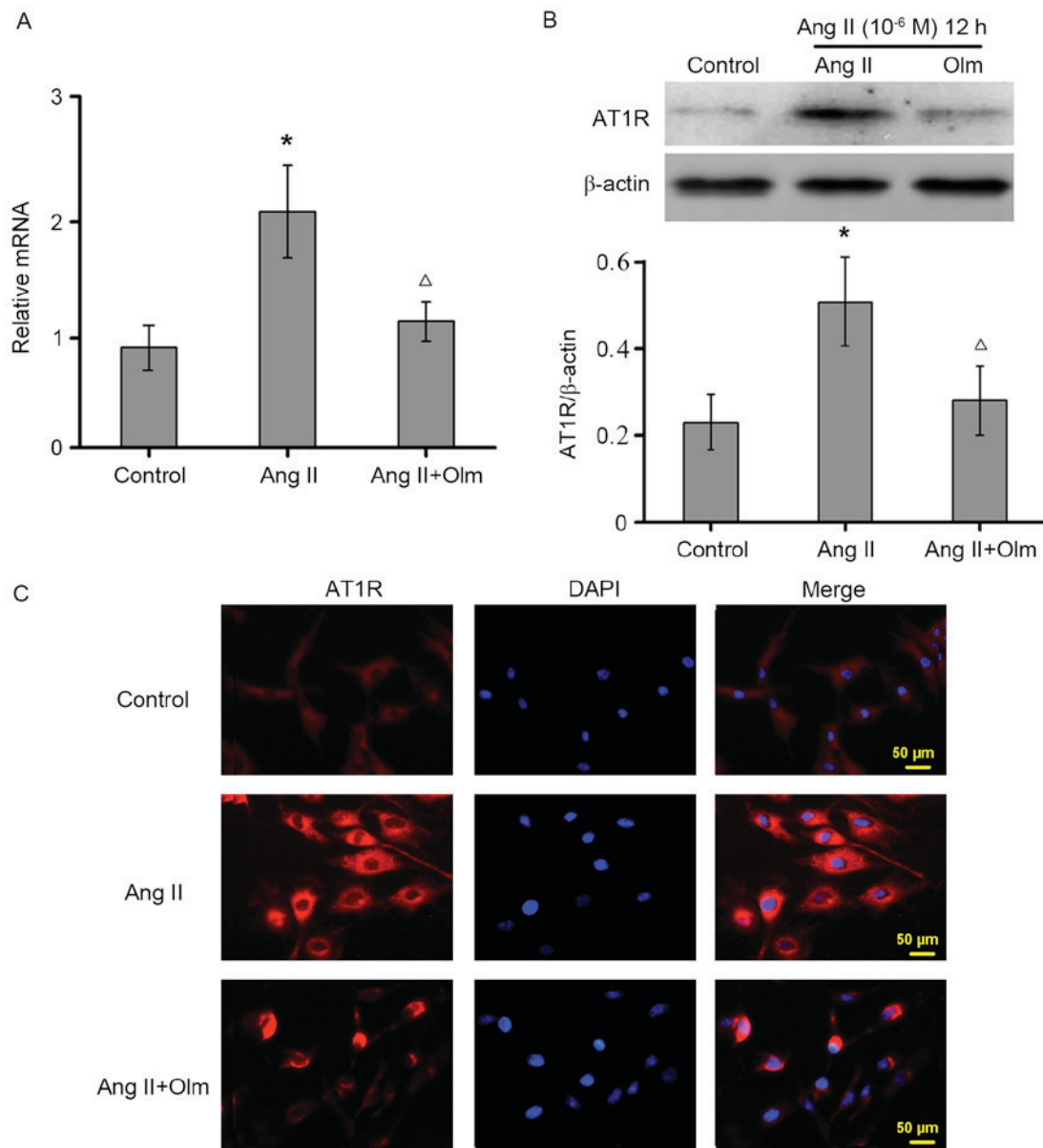


Figure 2. Effects of Ang II on AT1R expression in osteoblasts. Osteoblasts were pre-treated with olmesartan (10^{-5} M) for 30 min and exposed to Ang II (10^{-6} M) for 12 h. (A) mRNA and (B) protein expression levels of AT1R, as determined by reverse-transcription-quantitative polymerase chain reaction and western blot analysis, respectively. Data are presented as the mean \pm standard deviation. * $P < 0.05$ vs. control; $^{\Delta}P < 0.05$ vs. Ang II. (C) Immunofluorescence images of AT1R. Ang II, angiotensin II; AT1R, angiotensin II type 1 receptor; Olm, olmesartan.

MC3T3-E1 cells in a time-dependent manner (Fig. 1B). The enhanced effect of Ang II on MCP-1 mRNA (Fig. 1C) and serum (Fig. 1D) expression levels was completely abrogated by pre-treatment with olmesartan (an AT1R blocker), NAC (a scavenger of free radicals) or PDTC (a NF- κ B inhibitor), but not by PD123319 (an AT2R antagonist). Therefore, AT1R-associated ROS production and associated NF- κ B signaling pathways may be important for Ang II-induced MCP-1 expression in osteoblasts.

Ang II enhances AT1R expression in osteoblasts. To determine whether Ang II induces AT1R expression in osteoblasts, the levels of AT1R expression were measured by RT-qPCR and western blot assays. Treatment with Ang II upregulated AT1R mRNA (Fig. 2A) and protein (Fig. 2B) expression levels in osteoblasts, which was completely blocked by the

selective AT1R antagonist of olmesartan. A similar pattern of AT1R expression was detected by immunofluorescent assay (Fig. 2C). Thus, Ang II upregulates AT1R expression in osteoblasts *in vitro*.

Ang II induces ROS production via AT1R in osteoblasts. To examine the effects of Ang II on ROS production in osteoblasts, MC3T3-E1 cells were pre-treated with or without olmesartan, PD123319, NAC or PDTC, and then exposed to Ang II (10^{-6} M) for 12 h. The contents of intracellular ROS were determined by flow cytometry (Fig. 3A and B) and immunofluorescent assays (Fig. 3C). The results indicated that treatment with Ang II significantly increased the levels of intracellular ROS in osteoblasts, which was abrogated by pre-treatment with olmesartan or NAC, but not PD123319 or PDTC (Fig. 3). These data suggested that Ang II increased

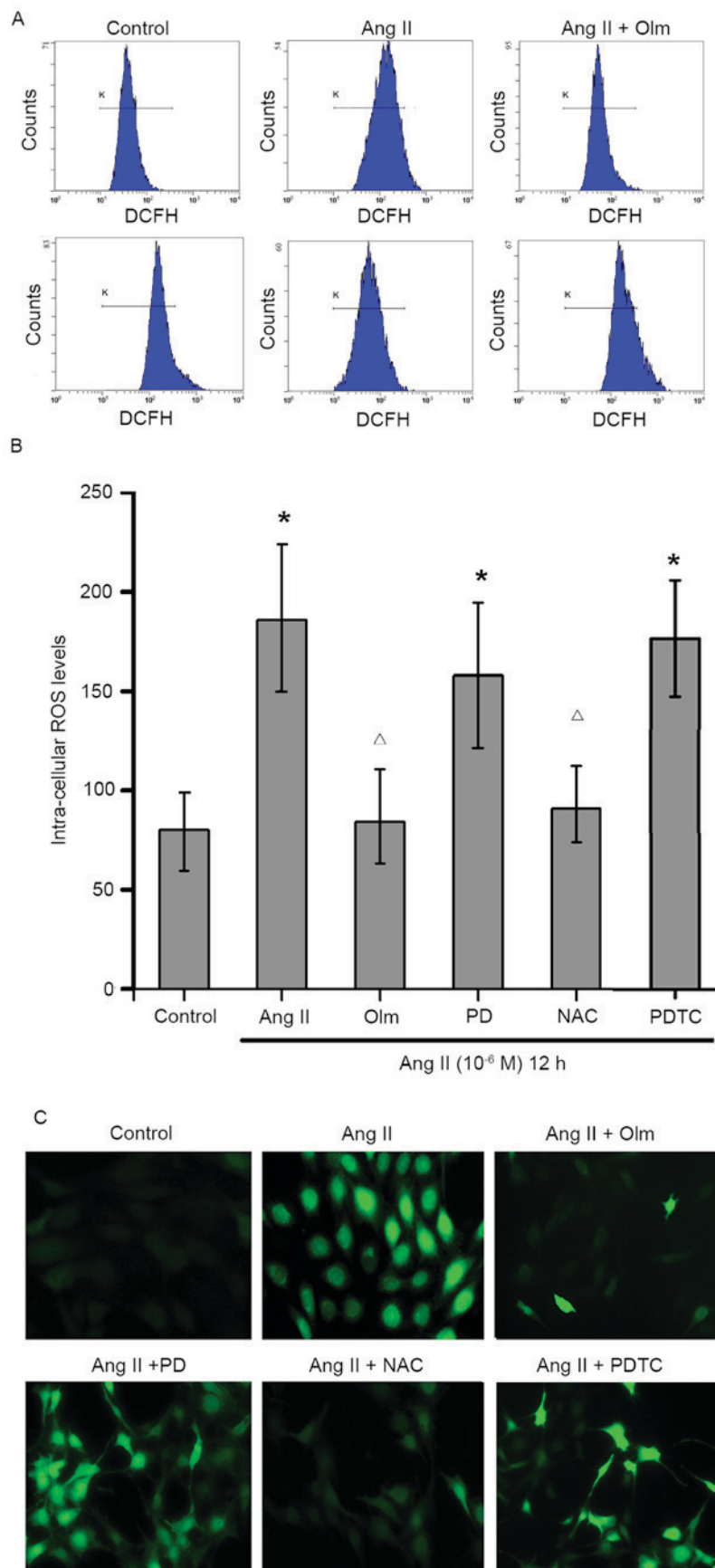


Figure 3. AngII induces ROS production in osteoblasts. Osteoblasts were pre-treated with Olm (10 μ M), PD (10 μ M), NAC (1 mM) or PDTC (5 μ M) for 30 min and exposed to Ang II (10⁻⁶ M) for 12 h. The levels of intracellular ROS were determined by flow cytometry and fluorescent microscopy. (A) Flow cytometry analysis of intracellular ROS. (B) Quantitative analysis of the levels of intracellular ROS. Data are presented as the mean \pm standard deviation. *P<0.05 vs. control; Δ P<0.05 vs. Ang II. (C) Fluorescent microscopy analysis. Olm, olmesartan; PD, PD12319; NAC, N-acetylcysteine; PDTC, ammonium pyrrolidine thiocarbamate; ROS, reactive oxygen species; Ang II, angiotensin II.

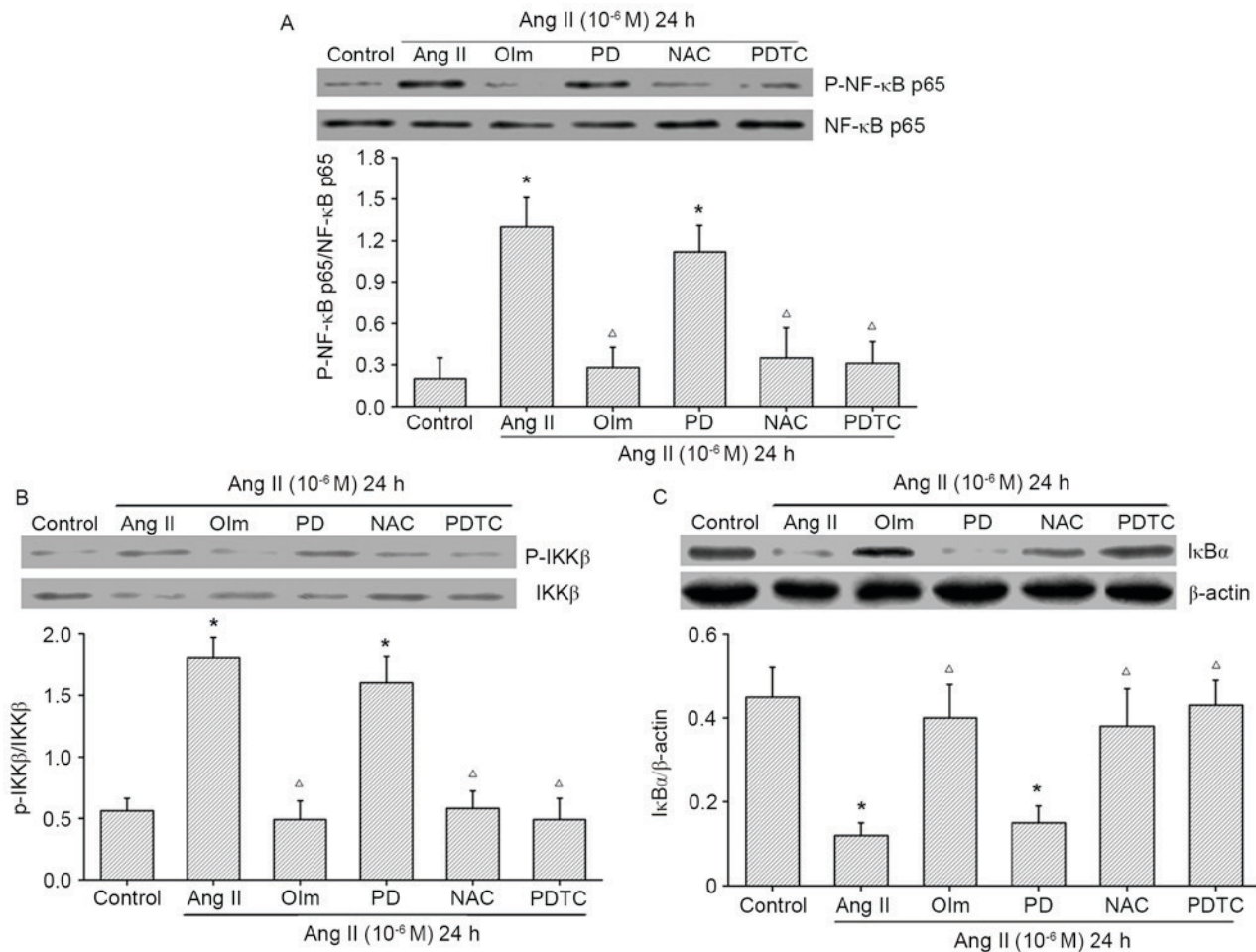


Figure 4. Effect of Ang II on NF-κB activation in osteoblasts. Osteoblasts were pre-treated with Olm (10 μM), PD (10 μM), NAC (1 mM) or PDTC (50 μM) for 30 min, and exposed to Ang II (10⁻⁶ M) for 24 h. Representative western blot images and quantification of (A) NF-κBp65 phosphorylation, (B) IKKβ phosphorylation and (C) IκBα expression, as assessed by western blotting. Data are presented as the mean ± standard deviation. *P<0.05 vs. control; ^ΔP<0.05 vs. Ang II. Olm, olmesartan; PD, PD12319; NAC, N-acetylcysteine; PDTC, ammonium pyrrolidine thiocarbamate; Ang II, angiotensin II; p, phosphorylated; IKK, IκB kinase; NF, nuclear factor.

ROS production by activating AT1R, but is independent of NF-κB signaling in osteoblasts.

Ang II induces NF-κB activation in osteoblasts. As NF-κB activation is important for Ang II to enhance MCP-1 expression, the levels of the NF-κB activation in the different groups of cells were examined by western blot assays. Treatment with Ang II significantly increased NF-κB p65 and IKKβ phosphorylation (Fig. 4A and B, respectively), but decreased the levels of IκBα protein expression (Fig. 4C) in MC3T3-E1 cells. However, the enhanced effect of Ang II on the NF-κB activation was completely abrogated by pre-treatment with olmesartan, NAC, or PDTC, but not with PD12319 in osteoblasts (Fig. 4). Therefore, Ang II may promote ROS production via its AT1R, and subsequently activate the NF-κB signaling pathway, leading to enhanced MCP-1 expression in osteoblasts.

Discussion

The present study examined the effect of Ang II on MCP-1 expression and potential mechanisms underlying the action of Ang II in osteoblasts. These data indicated that treatment with Ang II significantly upregulated MCP-1 synthesis in

osteoblasts, which was abrogated by pre-treatment with the AT1R inhibitor olmesartan, scavenger of free radicals (NAC), and the NF-κB inhibitor PDTC, but not the AT2R antagonist PD12319. These data demonstrated that Ang II enhances MCP-1 generation in MC3T3-E1 cells via its AT1R, dependent on ROS/NF-κB signaling.

Ang II is a potent stimulator of osteoclastic bone resorption (12,13). Although Ang II does not target osteoclasts (4), it may stimulate RANK and interleukin-6 expression in osteoblasts, which promotes osteoclast maturation, leading to osteoporosis (3,4,14). The present study demonstrated that Ang II enhances MCP-1 expression in osteoblasts, which previous studies have revealed should promote osteoclast maturation and activation (15,16). Therefore, these findings may provide novel insight into regulation of Ang II on bone metabolism and homeostasis.

Ang II can bind to AT1R and AT2R, which are expressed by osteoblasts (17,18). The present study demonstrated that the enhanced effect of Ang II on MCP-1 expression was completely mitigated by pre-treatment with the AT1R antagonist olmesartan, but not with the AT2R antagonist PD12319, indicating that Ang II enhances MCP-1 expression in osteoblasts via AT1R, but not AT2R. These data were

consistent with a previous study (17), supporting the notion that Ang II induces MCP-1 expression, dependent on the AT1R (7). Furthermore, treatment with Ang II was revealed to upregulate AT1R expression in osteoblasts, consistent with previous findings (5,19). Upregulated AT1R may serve as a positive feedback mechanism to enhance the effect of Ang II on MCP-1 expression and other bioactivities in osteoblasts.

Ang II may induce ROS production in vascular smooth muscle cells (20). To understand the molecular mechanisms underlying the action of Ang II, the present study investigated the effect of Ang II on ROS production in osteoblasts. Treatment with Ang II stimulated ROS production in osteoblasts, which was abrogated by pre-treatment with an AT1R antagonist and the ROS scavenger NCA. These data suggested that Ang II stimulated ROS production in osteoblasts via AT1R, which was similar to the results of our previous experiment (5). As high levels of ROS can activate NF- κ B signaling (21) and pre-treatment with PDTC mitigates Ang II-induced MCP-1 expression in osteoblasts, the present study examined the effect of Ang II on NF- κ B activation in osteoblasts. It was demonstrated that treatment with Ang II enhanced NF- κ B activation, evidenced by increased ratios of NF- κ Bp65 and IKK β phosphorylation and decreased levels of I κ B α expression in osteoblasts. The enhanced effect of Ang II on the NF- κ B activation was completely blocked by pre-treatment with an AT1R antagonist, an NF- κ B inhibitor and the ROS scavenger NCA in osteoblasts. These novel data indicated that Ang II stimulated ROS production via its AT1R, which activated NF- κ B signaling, leading to upregulated MCP-1 expression in osteoblasts. MCP-1 is important for regulating osteoclast maturation activation, and the increased levels of MCP-1 expression by osteoblasts may promote osteoclast activation and osteoporosis. Therefore, the Ang II/AT1R/MCP-1 axis may be a novel target for intervention of osteoporosis.

In conclusion, these data indicated that Ang II stimulated ROS production via AT1R, and activated the NF- κ B signaling pathway, leading to upregulated MCP-1 expression in osteoblasts. These findings may provide novel insights into understanding the action of Ang II in regulating bone metabolism and homeostasis, and may facilitate the development of novel therapies for osteoporosis.

Acknowledgements

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