

Role of reactive oxygen species in angiotensin II: induced receptor activator of nuclear factor- κ B ligand expression in mouse osteoblastic cells

Yanqiu Zhang · Yongtao Zhang · Jianqiang Kou ·
Chunsheng Wang · Kunzheng Wang

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Abstract Angiotensin II (Ang II) has been shown to induce receptor activator of nuclear factor- κ B ligand (RANKL) expression in osteoblasts associated with its effect on reactive oxygen species (ROS) production. The objective of the present study was to investigate the potential pathways by which Ang II induces RANKL expression and the role of ROS in Ang II-induced RANKL expression in mouse osteoblastic MC3T3-E1 cells. Treatment with Ang II induced RANKL expression in a dose- and time-dependent manner in osteoblasts, which was attenuated by pre-treatment with an AT1 receptor antagonist (olmesartan), ROS scavenger (*N*-acetylcysteine, NAC), or the ERK inhibitor (U0126), but not with AT2R antagonist (PD123319). Furthermore, Ang II enhanced AT1R and NAD(P)H oxidase (NOX) p22^{phox} and p67^{phox} expression and activity in osteoblasts. In addition, Ang II promoted ROS production, which was mitigated by pre-treatment with olmesartan or a NOX inhibitor (diphenylethiodonium, DPI), but not with PD123319 or U0126, in osteoblasts. Moreover, Ang II enhanced the ERK1/2

phosphorylation, which was abrogated by pre-treatment with olmesartan, NAC, DPI, or U0126 in osteoblasts. These results suggest that Ang II, through its AT1R, enhanced NOX activity and ROS production, and activated the ERK pathway to up-regulate RANKL expression in osteoblasts *in vitro*.

Keywords Angiotensin II · Osteoblast · RANKL · NAD(P)H oxidase · Reactive oxygen species · ERK1/2

Introduction

The bone remodeling depends on a balance between the bone formation and resorption [1]. Osteoblasts are important for the bone formation while osteoclasts are crucial for the bone resorption. Breakdown of the balance is associated with the bone-related diseases. Indeed, aberrant activation of osteoclasts is associated with pathological bone resorption in rheumatoid arthritis and osteoporosis. Previous studies have shown that interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α) are important factors to regulate the differentiation and survival of osteoblasts and osteoclasts [2, 3]. Receptor activator of nuclear factor- κ B ligand (RANKL), a member of the TNF family, is secreted by osteoblasts. In the presence of macrophage colony stimulating factor (M-CSF), RANKL regulates the differentiation, maturation, and activation of osteoclasts through its receptor, RANK, on the surface of osteoclast precursors [4, 5].

It is well known that RANKL expression can be up-regulated by several osteotropic factor/resorption stimuli, including transforming growth factor- β (TGF- β), 1,25-(OH)₂D₃, parathyroid hormone, basic fibroblast growth factor (BFGF), IL-1 β , and prostaglandin E2 to promote

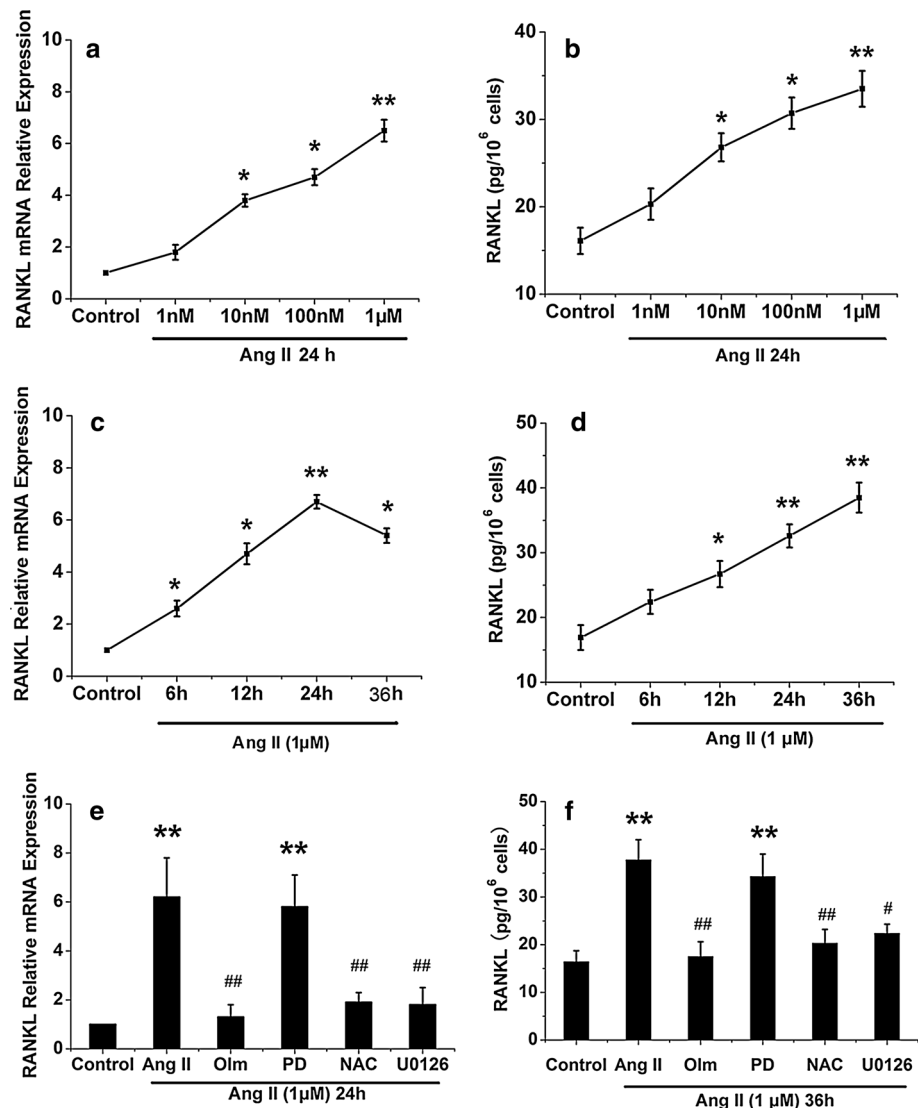
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Y. Zhang · Y. Zhang · C. Wang · K. Wang (✉)
Department of Orthopaedics, The Second Affiliated Hospital of
Xi'an Jiaotong University, Xi'an 710004, Shaanxi, China
e-mail: drkunzhengwang@126.com

Y. Zhang
Department of Physical Education, Xi'an Shiyou University,
Xi'an 710065, Shaanxi, China

Y. Zhang · J. Kou
Department of Orthopedics, The Affiliated Hospital of Qingdao
University, Qingdao 26000, Shandong, China

Fig. 1 Effects of Ang II treatment on RANKL expression in osteoblasts. MC3T3-E1 cells were pre-treated in triplicate with, or without, the AT1R antagonist (olmesartan, Olm, 10 μ M), the AT2R antagonist (PD123319, PD, 10 μ M), the scavenger of free radicals (*N*-acetylcysteine, NAC, 1 mM) or the MEK inhibitor (U0126, 50 μ M) for 30 min and exposed to the indicated concentrations of Ang II for 24 h or Ang II (1 μ M) for the indicated time periods. The relative levels of RANKL mRNA transcripts and protein in the different groups of cells were characterized by quantitative RT-PCR and ELISA (36 h after treatment with Ang II). Data are expressed as the mean \pm SD of per group of cells from three separate experiments. **a–d** The dose and time effects of Ang II on RANKL expression. **e, f** The effect pre-treatment with individual inhibitors on Ang II-induced RANKL expression. * $p < 0.05$ and ** $p < 0.01$ versus the control; # $p < 0.05$ and ## $p < 0.01$ versus the cells treated with Ang II alone



osteoclast differentiation [4, 6]. Angiotensin II (Ang II) is cleaved from angiotensin I (Ang I) by angiotensin-converting enzyme (ACE), and Ang II can regulate the bone metabolism through its angiotensin types 1 and 2 receptors (AT1R and AT2R) [7–13]. Ang II has been shown to induce RANKL expression and to promote the differentiation and activation of osteoclasts [8, 14]. However, the precise signal pathways by which Ang II regulates RANKL expression in osteoblasts have not been clarified.

Increasing evidence has indicated that Ang II acts via the AT1R to induce NADPH oxidase (NOX) activation and subsequently leads to over-production of reactive oxygen species (ROS) in cardiomyocytes and renal glomerular endothelial cells [15, 16]. A previous study has shown that ROS can modulate the ERK pathway to induce the expression of RANKL in osteoblasts [17]. However, whether Ang II can also induce NOX activation and ROS production leading to up-regulated RANKL expression in osteoblasts has not been explored.

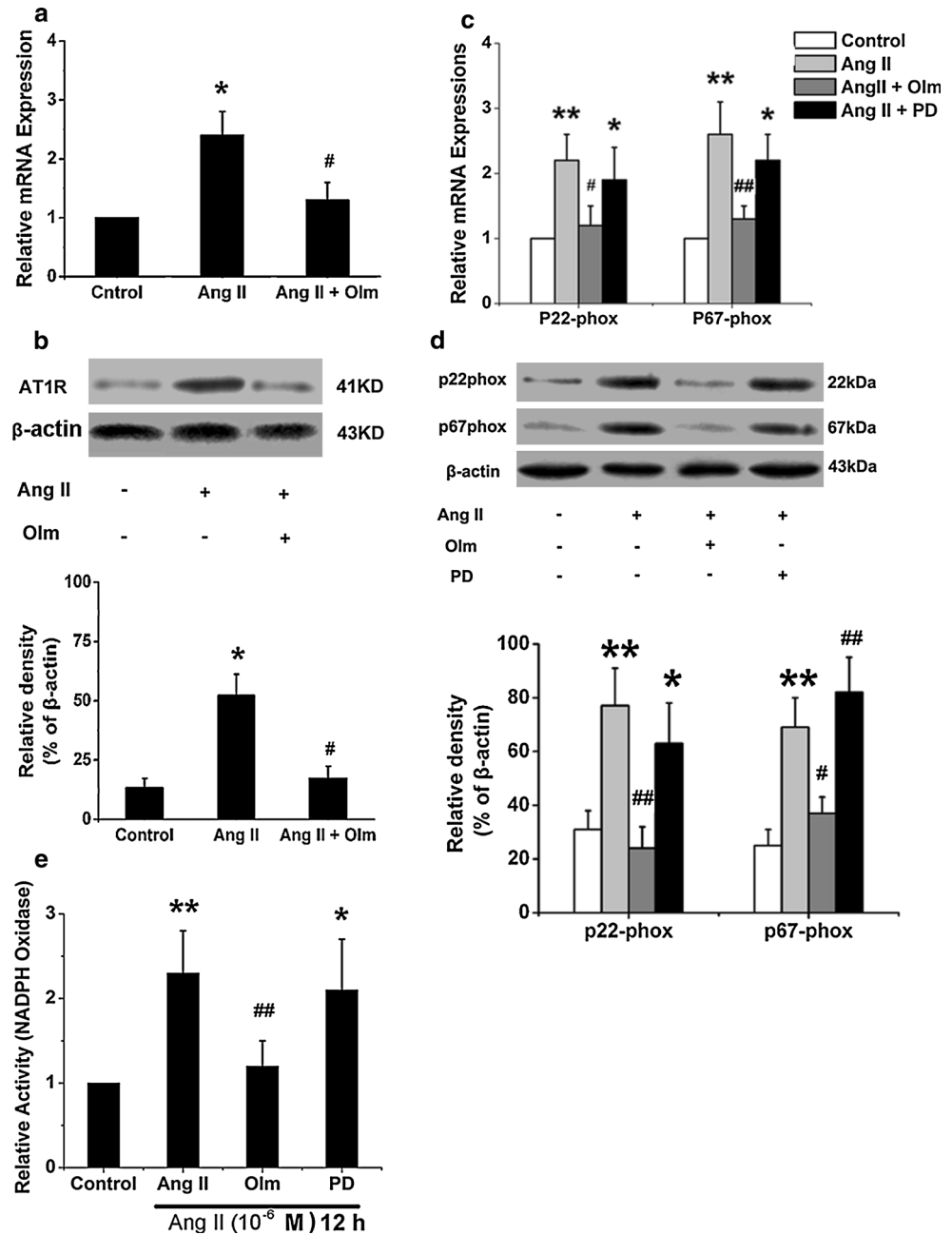
To address this issue, we employed a mouse osteoblastic cell line, MC3T3-E1, to test the hypothesis that Ang II induces NOX activation and ROS production, and up-regulates RANKL expression in osteoblasts.

Materials and methods

Materials

α -Modified minimum essential medium (α -MEM), fetal bovine serum (FBS), penicillin and streptomycin were purchased from Gibco (Gibco BRL, Grand Island, New York, USA). Ang II, olmesartan, PD123319, *N*-acetylcysteine (NAC), diphenylethiodonium (DPI), U0126 and 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) were purchased from Sigma (St. Louis, MO, USA). Rabbit polyclonal anti-mouse p22-phox, and polyclonal anti-mouse AT1R were purchased from Abcam (Cambridge,

Fig. 2 Effects of Ang II treatment on AT1R and NOX expression activity in osteoblasts. MC3T3-E1 cells were pre-treated in triplicate with, or without, the AT1R antagonist (olmesartan, Olm, 10 μ M), or the AT2R antagonist (PD123319, PD, 10 μ M), and exposed to Ang II (1 μ M) for 24 h. The relative levels of AT1R, NOX p22^{phox} and p67^{phox} mRNA transcripts and protein expressions in the different groups of cells were characterized by quantitative RT-PCR and Western blot assays. The NOX activity was determined by a chemiluminescence assay. Data are expressed as the mean \pm SD of per group of cells from three separate experiments. **a** The relative levels of AT1R mRNA transcripts. **b** The relative levels of AT1R protein. **c** The relative levels of NOX p22^{phox} and p67^{phox} mRNA transcripts. **d** The relative levels of NOX p22^{phox} and p67^{phox} proteins. **e** The NOX activity. * $p < 0.05$ versus the control; # $p < 0.05$ versus the cells treated with Ang II alone



MA, USA). Rabbit polyclonal anti-mouse p67^{phox}, anti-mouse ERK1/2, and rabbit polyclonal anti-mouse phospho-ERK1/2 antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). Goat polyclonal anti-mouse β -actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell cultures

MC3T3-E1 cells (5×10^4 cells/cm²) were routinely grown overnight in 10 % FCS α -MEM containing 100 units/ml of penicillin and 100 μ g/ml of streptomycin at 37 $^{\circ}$ C in

5 %CO₂ and 95 % air. The cells were starved in FCS-free medium for 12 h and treated with, or without, Ang II (1 nM–1 μ M), AT1R blocker (10 μ M, olmesartan), AT2R blocker (10 μ M, PD123319), NOX inhibitor, (5 μ M, DPI), scavenger of free radicals (1 mM, NAC), or MEK inhibitor (50 μ M, U0126) for varying periods. The cells were harvested and extracted for the next analysis.

Real-time quantitative PCR analysis

Total RNA was extracted from cultured MC3T3-E1 cells by TRizol reagent (Invitrogen, Carlsbad, USA) and

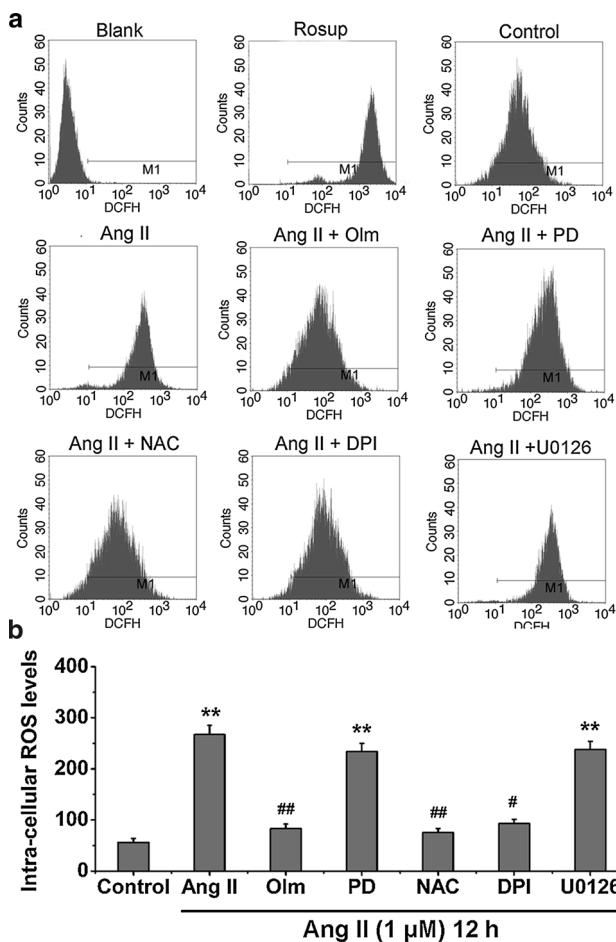


Fig. 3 Effects of Ang II stimulation on reactive oxygen species (ROS) production in osteoblasts. MC3T3-E1 cells were pre-treated in triplicate with, or without, olmesartan (Olm, 10 μM), PD123319 (PD, 10 μM), *N*-acetylcysteine (NAC, 1 mM), a NADPH oxidase inhibitor (diphenyleneiodonium, DPI, 5 μM) or U0126 (50 μM) for 30 min and exposed to Ang II (1 μM) for 12 h. The contents of intracellular ROS were determined by flow cytometry. Data are representative histograms and expressed as the mean ± SD of individual groups from three separate experiments. **a** Flow cytometry analysis of intracellular ROS. **b** The levels of intracellular ROS. * $p < 0.05$ versus the control; # $p < 0.05$ versus the cells treated with Ang II alone

reversely transcribed into cDNA using Revert Aid™ First Strand cDNA Synthesis Kit (Fermentas, Canada). The relative levels of targeted gene mRNA transcripts were determined by Real-time quantitative RT-PCR using SYBR Green 1 PCR master mix (TaKaRa, Japan) in an ABI 7300 sequence detection system (Bio-Rad Laboratories, USA). The sequences of the primers used for the real-time PCR were forward 5'-ATCGCTACATGGCCG TGGTC-3' and reverse 5'-GTAACCTACGCCGCCAG CAT-3' for AT1R (163 bp); forward 5'-AGGATGAAAC AAGCCTTTCAG-3' and reverse 5'-ACCATGAGCCTT CCATCATAG-3' for RANKL (102 bp); forward 5'-GTT TCACACAGTGGTATTTCCGGCG-3' and reverse 5'-GTT

GGTAGGTGGTTGCTTGATGGT-3' for p22^{phox} (344 bp); forward 5'-CATGCCCTCAGGTTGTCTCAT-3' and reverse 5'-CTTGATGATTACATGCGAC-3' for p67^{phox} (173 bp); forward 5'-ATAGACATAGATGCATAATG-3' and reverse 5'-ATAGACTGTTAGCATGTCCC-3' for GAPDH (186 bp). The relative levels of target mRNA transcripts were normalized to that of GAPDH mRNA transcripts and analyzed by $2^{-\Delta\Delta CT}$.

Enzyme-linked immunosorbent assay (ELISA)

The concentrations of RANKL in the supernatants of cultured MC3T3-E1 cells were measured by ELISA using a commercial ELISA kit (Beyotime, Jiangsu, China), according to the manufacturer's instructions. Briefly, samples were analyzed in duplicate and the limitation of detection for RANKL is 10 pg/ml. The concentrations of RANKL were expressed as pg per 10^6 cells.

Western blot analysis

The cells were harvested, washed with ice-cold PBS, and lysed in RIPA buffer (Thermo Scientific, USA). After denaturation at 95 °C for 5 min and centrifugation, the cell lysates (20 μl/lane) were separated on 10 % SDS-polyacrylamide gels and transferred onto PVF membranes. The membranes were blocked with 5 % fat-free milk and incubated with anti-AT1R, anti-p22-phox, anti-p67-phox, anti-ERK1/2, anti-phospho-ERK1/2, and anti-β-actin, respectively. After being washed, the bound antibodies were detected with HRP-conjugated second antibodies and visualized using the enhanced chemiluminescence kit (NEN Life Science Products, USA). The relative levels of each target protein to the control β-actin were determined by densitometric scanning and analyzed using Quantity One software (Bio-Rad Laboratories, USA).

Flow cytometry and fluorescent microscopy

The impact of Ang II on the ROS production was determined by flow cytometry analysis and fluorescent microscopy. Briefly, MC3T3-E1 cells were pre-treated with olmesartan, PD123319, DPI, NAC or U0126 for 30 min, washed twice with PBS, and exposed to Ang II (10^{-6} M) for 12 h. After being washed with FCS-free medium, the cells were continually cultured in α-MEM containing DCFH-DA (10 μM) for 30 min. Rosup (50 mg/L) was used as a control of ROS inducer. The cells were then collected, washed twice with serum-free medium, and resuspended in 500 μl PBS for further analysis. The levels of intracellular ROS were detected by flow cytometry or

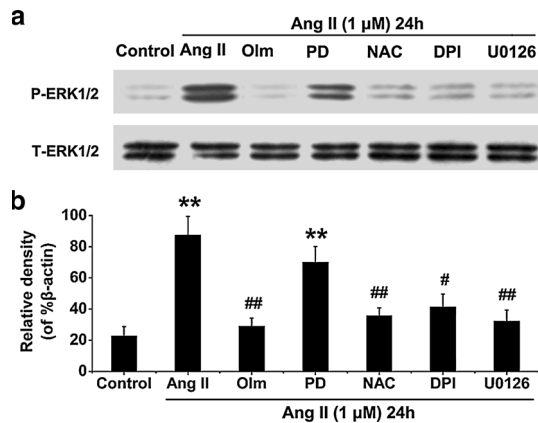


Fig. 4 Effect of Ang II treatment on the ERK1/2 activation in osteoblasts. MC3T3-E1 cells were pre-treated in triplicate with, or without, olmesartan (Olm, 10 μ M), PD123319 (PD, 10 μ M), *N*-acetylcysteine (NAC, 1 mM), diphenylethiodonium (DPI, 5 μ M) or U0126 (50 μ M) for 30 min and exposed to Ang II (1 μ M) for 24 h. The relative levels of ERK1/2 phosphorylation were determined by Western blot assays. The data are representative images and expressed as the means \pm SD of individual groups from three separate experiments. **a** Western blot analysis of the relative levels of ERK1/2 phosphorylation using anti-phospho-ERK1/2 and anti-ERK1/2 (total). **b** Quantification of phospho-ERK1/2. * $p < 0.05$ versus the control; # $p < 0.05$ versus the cells treated with Ang II alone

examined under a fluorescent microscope (BX50, Olympus Co. Ltd., Tokyo, Japan).

NADPH oxidase activity assay

After treatment, the cells were harvested and lysed in a phosphate buffer (50 mM monobasic potassium phosphate [pH 7.0], 1 mM EGTA, 150 mM sucrose, and protease inhibitor cocktail), followed by brief sonication on ice. After centrifugation, the concentrations of proteins in cell lysates were measured with the Bradford protein assay kit (Beyotime, Jiangsu, China). NADPH oxidase activity in individual cell lysate samples was analyzed using a lucigenin-enhanced chemiluminescence assay kit (Genmed Scientifics, USA), according to the manufacturer's instructions. The data were expressed as fold increase in NADPH oxidase activity of treated cells over that of control cells.

Statistical analysis

All statistical analysis was performed. All measurement data were expressed as the means \pm SD. The difference among groups was analyzed using one-way analysis of variance or the Kruskal–Wallis test using SPSS 17.0 (SPSS Inc., Chicago, IL, USA). A p -value of <0.05 was considered statistically significant.

Results

Ang II induces RANKL expression in osteoblasts

First, we examined the effect of treatment with Ang II on RANKL expression in osteoblasts. Osteoblasts were treated with various concentrations of Ang II (1 nM–1 μ M), and the levels of RANKL mRNA transcript and protein were determined by quantitative RT-PCR and ELISA. Clearly, treatment with different concentrations of Ang II induced RANKL expression in a dose-dependent manner (Fig. 1a, b). Next, osteoblasts were stimulated with Ang II (1 μ M) for 6–36 h. The results indicated that Ang II induced RANKL expression in osteoblasts as early as 6 h post stimulation and the levels of RANKL mRNA transcripts gradually increased up to 24 h after stimulation (Fig. 1c). Similarly, treatment with Ang II for varying periods induced RANKL expression in a time-dependent manner (Fig. 1d). However, pre-treatment with olmesartan (an AT1R blocker), but not with PD123319 (the AT2R antagonist) almost completely abrogated Ang II-induced RANKL expression in osteoblasts, suggesting that Ang II, through its AT1R, but not AT2R, stimulated RANKL expression in osteoblasts [8]. In addition, pre-treatment with NAC (a scavenger of free radicals), or U0126 (MEK1/2 inhibitor) also blocked the Ang II-induced RANKL expression in osteoblasts (Fig. 1e, f), implying that the ROS and ERK pathways might be important for Ang II-induced RANKL expression in osteoblasts.

Ang II enhances AT1R and NOX p22^{phox} and p67^{phox} expression and activity in osteoblasts

To understand the potential mechanisms underlying the action of Ang II, we tested the impact of Ang II on AT1R expression in osteoblasts. MC3T3-E1 cells were pre-treated with, or without, olmesartan, an AT1R blocker, and then exposed to Ang II (10^{-6} M) for 24 h, followed by measuring the relative levels of AT1R mRNA transcripts and protein by quantitative RT-PCR and Western blot, respectively. We found that treatment with Ang II increased the relative levels of AT1R mRNA transcripts, which were dramatically attenuated by pre-treatment with olmesartan (Fig. 2a). A similar pattern of AT1R protein was detected in different treatment groups (Fig. 2b).

Next, the relative levels of NOX p22^{phox} and p67^{phox} expression, two main subunits of the NOX and the activity of NOX were examined in different treatment groups. The results showed that the relative levels of p22^{phox} and p67^{phox} mRNA transcripts in the Ang II-treated cells significantly increased by 2.2 and 2.6 folds, respectively, as compared to those in the control cells (Fig. 2c), which was attenuated by pre-treatment with olmesartan, but not with

PD123319. Similar patterns of p22^{phox} and p67^{phox} proteins were detected in the different treatment groups (Fig. 2d). Furthermore, analyses of NOX activity indicated that the levels of NOX activity in the Ang II-treated cells were significantly higher than that in the control cells. Following pre-treatment with olmesartan, but not with PD123319, dramatically mitigated Ang II-enhanced NOX activity in osteoblasts (Fig. 2e). These data indicated that Ang II enhanced AT1R expression and through its AT1R enhanced NOX expression and activity in osteoblasts.

Ang II through AT1R induces ROS production in osteoblasts

Ang II induces ROS production in many types of cells [15, 16, 18]. However, little is known about the effects of Ang II on ROS levels in osteoblasts. MC3T3-E1 cells were pre-treated with, or without, olmesartan, PD123319, NAC, DPI, or U0126 and then exposed to Ang II (1 μ M) for 12 h, following by analysis of the levels of ROS using flow cytometry analysis. We found that treatment with Ang II significantly increased the contents of intracellular ROS, which was almost completely blocked by pre-treatment with olmesartan, NAC or DPI (Fig. 3a and b). However, pre-treatment with PD123319 or U0126 failed to significantly affect the Ang II-enhanced ROS production in osteoblasts *in vitro*. Similarly, immunofluorescent assays indicated that Ang II enhanced ROS production, which was blocked by pre-treatment with olmesartan, NAC or DPI in osteoblasts (Supplemental Figure). These data suggest that Ang II through the AT1R enhanced NOX activity and ROS production, contributing to enhanced RANKL expression in osteoblasts.

ROS are involved in Ang II-induced ERK1/2 activation in osteoblasts

To determine whether ROS is involved in Ang II-induced ERK1/2 activation in osteoblasts, the relative levels of ERK1/2 phosphorylation were examined in the different groups of cells by Western blot assays. As shown in Fig. 4, treatment with Ang II significantly increased the relative levels of ERK1/2 phosphorylation, which was blocked by pre-treatment with olmesartan, NAC, DPI, or U0126, but not with PD123319. These data suggest that Ang II, through its AT1R, increased ROS production that may be crucial for activating the ERK pathway in osteoblasts.

Discussion

In the present study, we demonstrate that Ang II-induced ROS generation in MC3T3-E1 cells depends on NOX.

ROS are also required for Ang II-induced activation of ERK1/2. AT1R inhibitor (olmesartan), scavenger of free radicals (NAC), NOX inhibitor (DPI) and MEK inhibitor (U0126) all significantly abolish Ang II-induced RANKL formation, indicating that the NOX/ROS/ERK signaling regulates Ang II-induced RANKL expression in osteoblasts.

The RAS is associated with bone metabolism and the activation of RAS induces bone metabolism disorder characterized by suppressing osteoblastic activity and promoting osteoclastic activity [10, 11, 14]. Hatton et al. [19] indicated that Ang II was a potent stimulator of osteoclastic bone resorption. However, osteoclast itself is not the direct target of Ang II in the process of osteoclast activation. Previous studies clearly demonstrated that Ang II indirectly promoted the activation of osteoclasts for bone resorption via up-regulation of RANKL in osteoblasts [8, 14]. In agreement with this, we found that Ang II (1 μ M) induced RANKL mRNA and protein expression in osteoblasts.

Ang II can bind to AT1R and AT2R and both receptors are expressed in osteoblasts [10]. Shimizu et al. [8] found that Ang II directly induced RANKL expression in osteoblasts through the activation of AT1R, while Asaba et al. [14] found that Ang II induced RANKL expression via AT2R. In this study, we found that pre-treatment with olmesartan, a selective AT1R antagonist, dramatically inhibited Ang II-induced RANKL expression in osteoblast. These indicated that Ang II through its AT1R induced RANKL expression in osteoblast, consistent with a previous report [8]. It is possible that Ang II induces RANKL expression by binding to the different receptors which may depend on the cell types.

Previous studies have shown that Ang II can induce ROS production in renal glomerular endothelial cells and proximal tubular cells, cardiac myoblasts [15, 16, 20]. We found that Ang II stimulated ROS production in osteoblasts, extending previous findings and suggest that Ang II may through its receptors promote ROS production in many types of cells. A previous study has shown that ROS can induce RANKL expression in osteoblasts [17]. We found that pre-treatment with NAC inhibited Ang II-induced RANKL expression in osteoblastic cells. These indicate that ROS positively regulates Ang II-induced RANKL expression in osteoblastic cells.

It is well known that NOX is responsible for ROS production in mammalian cells [15, 16, 21]. Chen et al. [22] have shown that NOX are highly expressed in osteoblasts. In the current study, we have found that Ang II up-regulated the p22^{phox} and p67^{phox} expression (two major subunits of the NOX) and increased the NOX activity in osteoblasts. Pre-treatment with DPI, a specific inhibitor of NOX, mitigated Ang II-induced ROS in osteoblasts.

Therefore, Ang II induced ROS production in osteoblasts by enhancing NOX expression. In addition, we found that pre-treatment with U0126, an inhibitor of the ERK pathway abrogated the effect of Ang II on RANKL expression in osteoblasts, supporting the notion that the ERK pathway, is involved in the Ang II-induced RANKL expression [8]. Interestingly, we found that pre-treatment with U0126 failed to modulate Ang II-induced ROS production in osteoblasts while pre-treatment with NAC or DPI attenuated Ang II-induced ERK1/2 phosphorylation in osteoblasts. These data suggest that ROS production may be at upstream of the ERK pathway, similar to a previous study [20]. Therefore, Ang II may induce ROS production by enhancing NOX expression and in turn activating the ERK pathway, leading to RANKL expression in osteoblasts.

In summary, we have studied molecular signaling cascades in osteoblasts by which Ang II induced RANKL in osteoblastic cells. Our data indicated that Ang II up-regulated NOX expression and stimulated ROS production, which activated the ERK pathway, leading to inducing RANKL expression. Therefore, our data may provide new insights in the molecular mechanisms underlying Ang II-induced RANKL expression in osteoblasts. Given that RANKL expression by osteoblasts is important for osteoclastogenesis, our findings may aid in the design of new therapies, such as antioxidants, for patients with aberrant osteoclast activity, including osteoporosis. We are interested in further investigating other function of these pathways in regulating bone metabolism.

Conflict of interest The authors declare no conflicts of interest.

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