Advanced Oxidation Protein Products Inhibit Proliferation and Differentiation of Rat Osteoblast-like Cells *via* NF- κ B Pathway

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Key Words

Advanced oxidation protein products • Osteoblast-like cell proliferation • Differentiation • Reactive oxygen species

Abstract

Background/Aims: Advanced oxidation protein products (AOPPs), a novel marker for oxidative protein damage, regulated cell behavior in many cell types. Although previous reports have revealed that oxidative stress resulted in an increased AOPPs content in cultured osteoblast-like cells, the effects of AOPPs on osteoblast-like cell function remain unclear. This study aimed to investigate the effect of AOPPs on the proliferation and differentiation of rat osteoblast-like (ROB) cells *in vitro*. **Methods:** ROB cells isolated from calvarias of newborn rats were incubated with AOPPs-modified rat serum albumin (AOPPs-RSA) prepared *in vitro* by incubation of RSA with hypochlorous acid. Cell proliferation was evaluated by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay. Alkaline phosphatase activity was measured. Expression of osteocalcin mRNA and protein were examined using reverse transcriptase-polymerase chain reaction and Western blot, respectively. Reactive oxygen species (ROS)

generation was detected by flow cytometry and fluorescent microscope. Phosphorylation of nuclear factor (NF)- κ B p65 subunit was detected by Western blot. **Results:** Exposure of ROB cells to AOPPs-RSA significantly inhibited cell proliferation, decreased alkaline phosphatase activity, down-regulated the expression of osteocalcin mRNA and protein in both a concentration- and time-dependent manner. AOPPs challenge induced ROS generation and NF- κ B p65 phosphorylation, which were inhibited by superoxide dismutase, catalase and NADPH oxidase inhibitors diphenyleneiodonium and apocynin. Furthermore, NF- κ B inhibitor SN50 could reverse the AOPPs-induced inhibition of ROB cell proliferation and differentiation. **Conclusion:** These results suggest that AOPPs can inhibit proliferation and differentiation of ROB cells through ROS-dependent NF- κ B pathway.

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Introduction

An imbalance between peroxidation and antioxidant defense systems results in excessive reactive oxygen species (ROS) generation and oxidative stress, which is associated with oxidative modification of proteins causing structural and functional changes in them [1]. Advanced

oxidation protein products (AOPPs) are the dityrosine-containing and cross-linking protein products and considered as a novel marker of oxidant-mediated protein damage [2, 3]. They are formed during oxidative stress by the reaction between proteins and chlorinated oxidants such as chloramines or hypochlorous acid (HOCl) [2, 3]. Recently, increased levels of AOPPs have been found in the patients with diabetes [4], uremia [2, 3], coronary artery disease [5], chronic inflammatory bowel diseases [6], as well as obesity [7], implying that accumulation of AOPPs may be relevant in a number of pathophysiologic conditions.

In addition to being a marker of oxidative protein damage, AOPPs have shown to play an important role as effector molecules in many biological events. AOPPs are capable of triggering respiratory burst and inflammation by the activation of immune cells such as monocyte [2]. AOPPs have also been reported to induce vascular endothelial dysfunction [8] and to accelerate atherosclerosis by enhancing oxidative stress and expressing inflammatory factor [9]. Furthermore, *in vitro* study has demonstrated that AOPPs stimulate human umbilical vein endothelial cells to generate ROS by activation of NADPH oxidase, which activates mitogen-activated protein kinases and nuclear factor- κ B (NF- κ B), and result in overexpression of adhesion molecules such as ICAM-1 and VCAM-1 [10]. Other studies have provided evidence that AOPPs *in vivo* accelerate renal fibrosis in a remnant kidney model [11] and promote inflammation in diabetic kidney [12]. More recently, Wei et al [13] have reported that AOPPs increase the synthesis of extracellular matrix (ECM) and expression of transforming growth factor- β 1 in cultured rat mesangial cells.

Osteoporosis is a systemic skeletal disease characterized by an imbalance between bone formation and resorption resulting in net bone loss enhanced bone fragility and increased susceptibility to fractures [14]. Although the pathophysiologic mechanisms underlying osteoporosis are not well understood, previous studies have shown that oxidative stress is involved in this disease [15, 16]. Furthermore, there is evidence to suggest that bone matrix proteins are particularly susceptible to oxidative damage [17]. More recently, *in vitro* studies have revealed that oxidative stress results in an increased content of AOPPs in cultured mouse MC3T3-E1 osteoblast-like cells [18, 19]. However, to our knowledge, there is no information available in the literatures regarding the effect of AOPPs on osteoblast proliferation, differentiation, and bone formation. In the present study,

we investigated the effect of AOPPs on the proliferation and differentiation of primary cultured rat calvarial osteoblast-like (ROB) cells and investigated whether NF- κ B was involved in the AOPPs actions on the ROB cells.

Materials and Methods

Materials

Rat serum albumin (RSA), collagenase, Limulus Amoebocyte Lysate kit, diphenyleneiodonium (DPI) and apocynin were purchased from Sigma (St Louis, MO, USA). HOCl was purchased from Fluke (Buchs, Switzerland). Fetal bovine serum (FBS), α -modified minimum essential medium (α -MEM) and trypsin were purchased from Gibco BRL (Melbourne, Australia). TRI Reagent® was purchased from Molecular Research Center (Cincinnati, OH, USA). PrimeScript™ RT reagent kit was from Takara Biotechnology (Dalian, China). GoTaq® Green Master Mix was purchased from Promega (Madison, WI, USA). RIPA lysis buffer, superoxide dismutase (SOD) and catalase were purchased from Beyotime (Haimen, China). 2', 7'-dichlorofluorescein diacetate (DCFH-DA) was purchased from Applygen (Beijing, China). Goat anti-osteocalcin antibody and rabbit anti-goat IgG-HRP were purchased from Santa Cruz (Santa Cruz, CA, USA). Rabbit anti- β -actin antibody and goat anti-rabbit IgG-HRP were purchased from Boster (Wuhan, China). Rabbit anti-NF- κ B p65 (C22B4) and Anti-phosphor-NF- κ B p65 (Ser276) antibody were purchased from Cell Signaling (Danvers, MA, USA). SN-50 was purchased from Biomol (Plymouth Meeting, PA). Detoxi-Gel column and ECL kit were purchased from Pierce (Rockford, IL, USA). All other chemicals and reagents were obtained from commercial suppliers and were of analytical grade.

AOPPs-RSA Preparation and Determination

AOPPs-RSA was prepared *in vitro* as described previously [3, 11]. Briefly, RSA solution (100 mg/ml) was incubated with 200 mM HOCl in phosphate buffer Saline (PBS, pH 7.4) for 30 min at 37°C. The reaction was then stopped by overnight dialysis against PBS to remove free HOCl. Control incubation was performed in native RSA dissolved in PBS alone. Samples were passed through a Detoxi-Gel column to remove contaminated endotoxin. Endotoxin levels in AOPPs-RSA and unmodified RSA were measured by Limulus Amoebocyte Lysate kit and were found to be below 0.05 ng/mg protein. AOPPs content was determined in the samples as described previously [2]. Briefly, 200 μ l of sample or chloramine-T was placed in a 96-well plate, followed by 20 μ l of acetic acid. The absorbance at 340 nm was obtained immediately in the microplate reader. AOPPs content in the AOPPs-RSA and unmodified RSA were 52.10 ± 3.60 μ mol/g protein and 0.26 ± 0.15 μ mol/g protein respectively.

Cell culture

The ROB cells from calvarias of newborn (<24 h) Sprague-Dawley rats were isolated using sequential enzymatic digestion as described previously [20]. Briefly, rat calvarias were cleaned

by removing the connective tissue, and cut into small pieces. After washing 3 times in PBS, the bone pieces were gently incubated at 37°C for 30 min with 0.1% collagenase, 0.25% trypsin in calcium-free PBS. This procedure was consecutively repeated 5 times. The first digestion was discarded to avoid fibroblast contamination. Cells obtained during the last 4 digestions were cultured in α -MEM containing 10% FBS and antibiotics (100 mg/ml of penicillin G and 100 IU/ml of streptomycin) at 37°C in a humidified atmosphere with 5% CO₂, and the media were replaced at two-day intervals. After reaching a subconfluent state, the cells were subcultured after trypsinization with 0.25% trypsin/0.02% EDTA. Second passage cells were used for all the experiments [20]. This study was approved prospectively by the authors' institutional Animal Care and Use Committee.

For cell proliferation experiments, ROB cells were plated at a density of 1.0×10^4 cells/well into 96-well plates and cultured for 2 days in 10% FBS- α -MEM. After this period, they were serum-deprived overnight and incubated with control medium, RSA (200 μ g/ml) or AOPPs-RSA (50-200 μ g/ml) for 24h, or with AOPPs-RSA 100 μ g/ml for different periods of time (0, 24, 48, 72 h). For cell differentiation experiments, ROB cells were plated in 10-cm culture dish (2.0×10^5 cells/dish) in 10% FBS- α -MEM. After confluent, cells were serum-deprived overnight and treated with control medium, RSA (200 μ g/ml) or AOPPs-RSA (50-200 μ g/ml) for 24h, or AOPPs-RSA 100 μ g/ml for different periods of time (0, 12, 24, 48, 72 h). For determination of ROS generation, ROB cells were treated with control medium, RSA (200 μ g/ml) or AOPPs-RSA (50-200 μ g/ml) for 60 min, or 100 μ g/ml AOPPs-RSA for 0, 5, 15, 30, 60 and 120 min. For detection of the NF- κ B activation, ROB cells were treated with 100 μ g/ml AOPPs-RSA for 0, 5, 15, 30, 60 and 120 min. Inhibition experiments were carried out by pretreatment for 60 min with SOD (200 U/ml), catalase (100 U/ml), DPI (100 μ M) and apocynin (500 μ M), or SN50 (100 μ g/ml). The effective concentration of AOPPs-RSA, SOD, DPI, apocynin and SN50 were obtained from the literatures.

Cell proliferation assay

Cell proliferation was evaluated by MTT [3-(4,5-dimethylthiazolyl)-2,5-diphenyl tetrazolium bromide] assay as described previously [18]. ROB cells in 96-well plates were washed twice with 0.01 M PBS, then incubated in 200 μ l of FBS-free α -MEM medium supplemented with 20 μ l of 5 mg/ml MTT solution at 37°C for 4 h. After discarding supernatant, the crystals were dissolved by incubation with 150 μ l of dimethyl sulfoxide (DMSO) for 20 min. The plates were shaken gently for 5 min and the absorbance was measured in the microplate reader at 570 nm. Results are expressed in absorbance (OD at 570 nm).

Alkaline phosphatase activity assay

At harvest, the medium was removed and cell monolayer was washed 2 times with PBS. Cell extract was then prepared with RIPA lysis buffer. Alkaline phosphatase (ALP) activity in the cell lysates was assayed at the end of the incubation with 10 mM p-nitrophenyl phosphate in 0.15 M sodium carbonate buffer (pH 10.3) and 1 mM MgCl₂ as described previously [21].

Fig. 1. Effect of AOPPs on proliferation of ROB cells. ROB cells were incubated with control medium, RSA or indicated concentration of AOPPs-RSA for the indicated time. Cell proliferation was evaluated by MTT assay. AOPPs inhibited the ROB cell proliferation in both a concentration- (A) and time- (B) dependent manner. Data were presented as mean $\pm$ SD from triplicate. $P < 0.01$ in A and B, * $P < 0.01$ vs control.

The enzyme activity was normalized against cellular protein concentration and was expressed as U/g protein. Protein concentration was determined using the Bradford method [22].

Reverse transcriptase-polymerase chain reaction

Expression of osteocalcin gene was examined by reverse transcriptase-polymerase chain reaction (RT-PCR). Briefly, Total RNA was extracted using the TRI Reagent® according to the manufacturer's instruction. cDNA was reverse-transcribed from 0.5 μ g of total RNA using PrimeScript™ RT reagent kit. The cDNA (4 μ l) was then amplified by PCR in a 50 μ l reaction mixture using GoTaq® Green Master Mix. Specific oligonucleotide primers were designed on the basis of rat sequences in GenBank as following: osteocalcin: sense 5'-AGT CAC CAA CCA CAG CAT CC-3', and antisense 5'-TTT GTC CCT TCC CTT CTG CC-3'; β -actin: sense 5'-GAC TAC CTC ATG AAG ATC CT-3', and antisense 5'-CCA CAT CTG CTG GAA GGT GG-3'. PCR condition consisted of 35 cycles of

Fig. 2. Effect of AOPPs on differentiation of ROB cells. ROB cells were treated with control medium, RSA or indicated concentration of AOPPs-RSA for the indicated time. AOPPs-RSA treatment decreased the ALP activity (A&B) and the expression of osteocalcin mRNA (C&D) and protein (E&F) in ROB cells in both concentration- and time-dependent manner. Data were presented as mean $\pm$ SD from triplicate. $P<0.01$ in A, B, C, D, E and F. * $P<0.01$ vs control.

denaturing at 94°C for 30s, annealing at 59°C for osteocalcin, 55°C for β -actin for 30s, and extension at 72 C for 30s. PCR products (osteocalcin: 328bp; β -actin: 510 bp) were electrophoresed in a 1.5% agarose gel containing ethidium bromide. The experimental bands were visualized and analyzed using Eel Documentation and Analysis System (Uvitec, Cambridge, UK). Relative expression of osteocalcin was calculated using β -actin mRNA expression as an internal control.

Western blot analysis

For immunodetection of osteocalcin, NF- κ B p65 and phosphor NF- κ B p65 protein, ROB cells were washed 3 times with ice-cold PBS, then lysed with RIPA lysis buffer. The protein-containing supernatant was obtained by a centrifugation at

4°C, 12000 rpm for 30 minutes. Protein concentration was determined according to Bradford's method. Proteins (40 μ g) were loaded per lane onto a tricine-SDS-PAGE for osteocalcin (5.5 kDa) or 10% SDS-PAGE for β -actin (43 kDa), NF- κ B p65 (65 kDa) and phosphor NF- κ B p65 (80 kDa), and electrotransferred subsequently to a PVDF membrane. After blocking with 5% skim milk in TBST, the membrane were immunoblotted with Goat anti-osteocalcin, rabbit anti- β -actin, rabbit anti-NF- κ B p65 and rabbit anti-phosphor NF- κ B p65 antibodies (1:1000) overnight at 4°C. The membrane were washed 3 times with TBST and followed by incubation with anti-goat or anti-rabbit horseradish peroxidase-conjugated secondary IgG (1:3000) at room temperature for 1 hour. The immunoreactive bands were visualized using an ECL kit.

Fig. 3. Effect of AOPPs on ROS generation in ROB cells. ROB cells were incubated with control medium, RSA or indicated concentration of AOPPs-RSA for the indicated time, and then treated with DCFH-DA for 30 min, ROS generation was measured by flow cytometry evaluation of DCF fluorescence. AOPPs challenge triggered intracellular ROS generation in both a concentration- (A) and time- (B) dependent manner. Data were presented as mean $\pm$ SD from triplicate. $P < 0.05$ in A and B, * $P < 0.05$ vs control.

Determination of intracellular ROS generation

The level of intracellular ROS was assessed by flow cytometry with the probe DCFH-DA, which can be oxidized to the fluorescent 2',7'-dichlorofluorescein (DCF) in the presence of ROS, as described previously [23]. Briefly, ROB cells were incubated in 10 μ M DCFH-DA for 30 min in the darkness followed by AOPPs treatment as described above. DCF fluorescence was measured on a FACSCalibur flow cytometry (Becton Dickinson, Franklin Lakes, NJ). Data were normalized to the control values.

In some experiments, intracellular ROS generation was visualized using a fluorescent microscope (Olympus IX71, Tokyo, Japan).

Statistical Analyses

Results were expressed as mean $\pm$ standard deviation (SD). Statistical differences between means for different groups were evaluated using one-way ANOVA or Welch ANOVA (equal variances not assumed). Multiple comparisons were performed using the LSD method or Dunnett's T3 method. Dunnett procedure was used for comparing several treatments with a control. Two-tailed P value < 0.05 was considered as statistical significant. Statistical analyses were performed using SPSS 13.0 software. All experiments were repeated three times with triplicate samples.

Results

AOPPs inhibited ROB cell proliferation

To determine whether AOPPs stimulation effects ROB cell proliferation, we measured the proliferative response of ROB cells to AOPPs using MTT assay. As shown in Fig. 1A, inhibition effects were observed in proliferation among cells exposed to 50-200 μ g/ml of AOPPs-RSA for 24 h in comparison with the control medium and unmodified RSA. Cell proliferation was markedly inhibited for cells treated with 100 μ g/ml of AOPPs-RSA for 24-72h (Fig. 1B). The data showed that AOPPs inhibited ROB cell proliferation in both a concentration- and time-dependent manner.

AOPPs inhibited ROB cell differentiation

The ALP activity and osteocalcin expression were considered as the key markers of osteoblastic differentiation [24, 25]. To investigate the potential impact of AOPPs on ROB cell differentiation, ROB cells were treated with indicated concentration of AOPPs-RSA for indicated time periods, we then examined the effects of AOPPs on the ALP activity and expression of osteocalcin mRNA and protein. As shown in Fig. 2, the exposure of ROB cells to AOPPs-RSA resulted in a significant decrease in ALP activity (Fig. 2A&B) and the expression of osteocalcin mRNA (Fig. 2C&D) and protein (Fig. 2E&F). The effects of AOPPs-induced inhibition of ROB cell differentiation were both concentration- and time-dependent. By contrast, ALP activity and osteocalcin expression had no significant change in cells cultured with unmodified RSA.

AOPPs stimulated ROS generation in ROB cells

To determine whether AOPPs stimulation increases ROS generation in ROB cells, DCF fluorescence has

Fig. 4. Visualization of AOPPs-induced ROS generation in ROB cells. ROB cells were treated with control medium, RSA or AOPPs-RSA (100 $\mu\text{g/ml}$) for 60min. ROS generation was visualized by a fluorescent microscopy with DCFH-DA. Images represented a random field after different stimulation (Scale bar =100 μm).

been used to implicate ROS generation. As shown in Fig. 3, the incubation of ROB cells with AOPPs-RSA induced a concentration-dependent (Fig. 3A) and time-dependent (Fig. 3B) increase in ROS generation, as detected by fluorescence measurements, while no increase of DCF fluorescence was present with unmodified RSA. Moreover, AOPPs-triggered ROS generation was observed by a fluorescence microscopy with DCFH-DA (Fig. 4).

To evaluate whether ROS were the contributing radicals, cells were pre-incubated with SOD (a superoxide scavenger) and catalase (a hydrogen peroxide scavenger). As shown in Fig. 5, the addition of SOD and catalase significantly decreased DCF intensity in AOPPs-treated cells.

To ascertain whether NADPH oxidase was the source of ROS generation, the experiment was repeated with the NADPH oxidase inhibitors DPI and apocynin. Fig. 5 depicts that the level of ROS in ROB cells in the presence of DPI and apocynin were significantly decreased, compared with those of AOPPs-treated group. These results suggested that AOPPs-triggered ROS generation in ROB cells at least partially resulted from cellular NADPH oxidase activity.

AOPPs activated NF- κ B through ROS generation

To gain insight into the molecular mechanism underlying the AOPPs actions on ROB cells, we investigated whether AOPPs triggers NF- κ B activation in our system. As shown in Fig. 6, the phosphorylation of NF- κ B p65 subunit was increased markedly from 5 to 120 min after AOPPs challenge. The data suggested that AOPPs can induce NF- κ B activation in ROB cells.

There was evidence that NF- κ B is a redox-sensitive transcription factor [26]. Therefore, we investigated whether ROS mediates AOPPs-induced NF- κ B activation, by treating cells with SOD, catalase, DPI and apocynin. Fig. 7 showed that NF- κ B p65 phosphorylation was prevented in the presence of SOD, catalase, DPI and apocynin. These experiments suggested that AOPPs-

Fig. 5. Effect of pre-incubation of ROB cells with SOD, catalase, DPI and apocynin on AOPPs-induced ROS generation. ROB cells were incubated with AOPPs-RSA in the presence or absence of SOD, catalase, DPI, apocynin at indicated times after DCFH-DA treatment. ROS generation was evaluated by flow cytometry measuring DCF fluorescence. Data were presented as mean $\pm$ SD from triplicate. * P <0.001 vs AOPPs group.

Fig. 6. Effect of AOPPs on the NF- κ B activation in ROB cells. ROB cells were treated with 100 $\mu\text{g/ml}$ of AOPPs-RSA for indicated time. The phosphorylation of NF- κ B p65 subunit was detected by Western blot analysis. AOPPs-RSA treatment significantly increased phosphorylation of NF- κ B p65. Data were shown as mean $\pm$ SD from triplicate. * P <0.05 vs control.

Fig. 7. Effect of pre-incubation of ROB cells with SOD, catalase, DPI and apocynin on AOPPs-induced NF-κB p65 phosphorylation. ROB cells were pre-treated with SOD, catalase, DPI or apocynin for 60 min. AOPPs-RSA (100 μg/ml) was then added for 60 min. NF-κB p65 phosphorylation was detected by Western blot analysis. Addition of SOD, catalase, DPI or apocynin blocked AOPPs-induced phosphorylation of NF-κB p65. Data were presented as mean ± SD from triplicate. *P<0.001 vs AOPPs group.

induced NF-κB activation was dependent on ROS generation.

NF-κB inhibitor SN50 reversed AOPPs-induced inhibition of ROB cell proliferation and differentiation

To further explore whether NF-κB activation was linked to AOPPs-induced inhibition of ROB cell proliferation and differentiation, ROB cells were pre-treated with NF-κB specific inhibitor SN50. Proliferation and differentiation of ROB cells were detected by MTT assay, ALP activity assay and Western blotting for osteocalcin protein expression. As shown in Fig. 8, the NF-κB inhibitor SN50 abolished AOPPs-induced decrease of cell proliferation (Fig. 8A), ALP activity (Fig. 8B) and osteocalcin expression (Fig. 8C). These results suggested that NF-κB activation was required for AOPPs-induced inhibition of ROB cell proliferation and differentiation.

Fig. 8. Effects of the NF-κB inhibitor SN50 on AOPPs-induced inhibition of ROB cell proliferation and differentiation. ROB cells were pretreated with or without NF-κB specific inhibitor SN50 (100 μg/ml) for 60 min followed by exposure to 100 μg/ml AOPPs-RSA for 24h. SN50 pretreatment abolished AOPPs-induced reduction of cell proliferation (A), ALP activity (B) and osteocalcin protein expression (C). Data were shown as mean ± SD from triplicate. *P<0.01 in A, B and C.

Discussion

The formation and accumulation of AOPPs were well documented in many pathological conditions [2-7]. Increasing evidence showed AOPPs may be important

factor modulating numerous biological activities [8-13]. Therefore, there is an urgent need to understand its effects on cellular, tissue and organ under physiological and pathological conditions. The present study demonstrated that AOPPs-RSA significantly inhibited the proliferation and differentiation of cultured ROB cells through the activation of NF- κ B pathway. To the best of our knowledge, this is the first reported of the potential effects of AOPPs on osteoblasts-like cells.

It is well known that the processes of osteoblastic proliferation and differentiation are characterized by three sequential periods: proliferation, ECM maturation and mineralization [27]. We found that ROB cells in the control medium showed a high proliferation capacity, a moderate to high ALP activity, and synthesis of bone matrix protein such as osteocalcin. These findings were consistent with the previous studies which demonstrated that primary ROB cells isolated by sequential enzyme digestion can differentiate spontaneously toward the mature osteoblast and show various features of osteoblasts [25, 28]. Therefore, this *in vitro* cell culture system is a suitable model for the investigation of the osteoblast proliferation and differentiation.

Osteoblast proliferation is a key feature of skeletal development and is essential for maintaining homeostasis during bone remodeling [29]. To investigate the effect of AOPPs on ROB cell proliferation, we measured the metabolic activity of ROB cells using the MTT assay, which reflects a linear relationship between the number of live cells and the amount of formazan produced from tetrazolium salt MTT [30]. Results of the MTT assay suggested AOPPs-RSA treatment was significant associated with a decrease in the OD values at 570 nm compared with the control (Fig. 1). The data indicated that AOPPs can induce impairment in osteoblastic proliferation, which may further effect on the subsequent process of osteoblastic development [31].

Osteoblast proliferation was regulated by various local and systemic factors including oxidative stress [32]. It has been reported recently that oxidative stress by 2-deoxy-D-ribose caused not only decrease of cellular viability, but also an increased content of AOPPs in MC3T3-E1 osteoblast-like cells [18, 19]. However, the consequence of AOPP increase remains unclear. Our study clearly showed that AOPPs-RSA treatment in ROB cells resulted in the decrease of cell proliferation. Based on the previous reports and our findings, we speculated that AOPPs, a marker of oxidative protein damage, may further participate in the oxidative damage in osteoblast seen during oxidative stress.

Enhanced ALP activity, an early marker for osteoblast differentiation, and osteocalcin expression, a late marker for osteoblast differentiation, represent key events during the differentiation process of osteoblasts [33]. Level of ALP activity and osteocalcin expression have been used as a relative measure of differentiation stages of osteoblasts [24, 25]. In the present study, AOPPs-RSA challenge resulted in decrease of ALP activity in time- and concentration-dependent manner. The inhibitory effect of AOPPs-RSA on ROB cell differentiation was further confirmed by significant inhibition of the expression of osteocalcin gene and protein. However, exposure of ROB cells to unmodified RSA did not significant changed the ALP activity and osteocalcin expression. Taken together, these results indicated that AOPPs-RSA, not RSA, inhibit ROB cell differentiation.

ROS, mainly including superoxide, hydrogen peroxide and hydroxyl radicals, are generated as a result of normal cellular metabolism in most cell types. However, excess intracellular ROS generation in response to various stimuli could result in oxidative stress. The present study showed that AOPPs stimulation induces the increased ROS generation in ROB cells as assessed by DCF fluorescence, which was further demonstrated by the ROS-scavenging experiments in which the addition of SOD and catalase to the culture prevented the increase in DCF fluorescence after AOPPs stimulation. Furthermore, we also demonstrated that ROS contributed to the AOPPs-induced NF- κ B activation. Taken together, these findings suggested that of ROS was involved in signaling downstream of AOPPs stimulation.

In mammalian cells, ROS originate from diverse subcellular origins including mitochondria, NADPH oxidases, arachidonic acid pathway enzymes lipoyxygenase and cyclooxygenase, cytochrome p450s, xanthine oxidase, NO synthase, peroxidases, and other hemoproteins [34]. Increasing evidence has shown that NADPH oxidase is the predominant ROS-producing enzyme in many cell types [35-37]. In our experiments, the treatment of ROB cells for 60 min with the NADPH oxidase inhibitors DPI and apocynin prevented the increased levels of ROS caused by AOPPs, in accordance with an involvement NADPH oxidase in AOPPs-triggered ROS generation as previously reported [10, 13].

NF- κ B has been identified as a transcription factor, which was associated with cell adaptation to stress, immunity, inflammation, proliferation, apoptosis and differentiation induced by different stimuli [38]. Our results indicated that AOPPs induced the activation of NF- κ B, as assessed by p65 subunit phosphorylation. It has

recently been reported that AOPPs induced the p65 nucleus translocation in endothelial cells through NADPH oxidase-dependent ROS generation [10]. In our model, the ROS scavengers (i.e., SOD and catalase) and NADPH oxidase inhibitors (i.e., DPI and apocynin) inhibited AOPPs-triggered p65 phosphorylation, suggesting that the mechanism involved in AOPPs actions over NF- κ B in ROB cells was through ROS generation.

NF- κ B, a p50/p65 heterodimer, is maintained in an inactive form in the cytoplasm by association with an inhibitor κ B α (I κ B α) [39]. NF- κ B activity is regulated by the I κ B α degradation and nuclear translocation of NF- κ B complexes [40]. It was previously demonstrated that SN50 can inhibit the NF- κ B nuclear translocation, causing the inhibition of NF- κ B activation [41]. In our experiments, NF- κ B inhibitor SN50 significantly suppressed the AOPPs-induced reduction of cell proliferation, ALP

activity and osteocalcin expression. These results suggested that NF- κ B activation is involved in AOPPs-induced inhibition of ROB cell proliferation and differentiation.

In conclusion, this study demonstrates for the first time that AOPPs inhibit the proliferation and differentiation of ROB cells *in vitro*, which is mediated by ROS-dependent NF- κ B activation. The findings will be helpful in better understanding the effect of AOPPs on bone disease such as osteoporosis and may provide new targets for intervention.

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