Glucocorticoids: Dose-related effects on osteoclast formation and function via reactive oxygen species and autophagy

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A B S T R A C T

Whether glucocorticoids directly enhance or interrupt osteoclastogenesis is still a controversial subject. In this study, we ascertained the dose-dependent positive effects of glucocorticoids on osteoclastogenesis in vivo and in vitro as well as investigated the mechanism in vitro. As the dose of glucocorticoids increased, osteoclastogenesis was stimulated at 0.1 μM, a peak was achieved at 1 μM and a corresponding decrease occurred at 10 μM. Reactive oxygen species (ROS), which play a crucial role in osteoclastogenesis, and autophagy flux activity, a cellular recycling process, were consistently up-regulated along with the dose-dependent effects of the glucocorticoids on osteoclast formation and function. N-acetyl-cysteine (NAC), a ROS scavenger, abrogated the effects of the glucocorticoids on autophagy and osteoclastogenesis. Moreover, 3-methyladenine (3-MA), an autophagy inhibitor, interrupted osteoclastogenesis stimulation by the glucocorticoids. These results implied that with glucocorticoid administration, ROS and autophagy, as a downstream factor of ROS, played vital roles in osteoclast formation and function. 3-MA administration did not enhance ROS accumulation, so that autophagy had no effect on ROS induced by glucocorticoids. Our investigation demonstrated that glucocorticoids had dose-dependent positive effects on osteoclast formation and function via ROS and autophagy. These results provide support for ROS and autophagy as therapeutic targets in glucocorticoid-related bone loss diseases such as glucocorticoid-induced osteoporosis.

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1. Introduction

It is well known that bone homeostasis is a dynamic balance that is maintained by bone formation and bone resorption [1,2]. Osteoclasts, which develop from hematopoietic osteoclast precursors, are the principle cells responsible for bone resorption [3]. Osteoclast precursors are derived from monocytes, which arise in the bone marrow, and differentiate into osteoclasts under influence of M-CSF and RANKL [4,5]. Osteoclast formation and function are increased in osteoporosis [6].

Glucocorticoids remain an effective therapy for many inflammatory/autoimmune disorders [7]. Nevertheless, moderate-to-high doses of glucocorticoids or prolonged glucocorticoid administration have adverse effects, one of the most severe of which is glucocorticoid-induced osteoporosis (GIOP). GIOP is considered to be the most common form of iatrogenic osteoporosis and secondary osteoporosis [8–10], which cause bone loss and fragility fractures in 30–50% of patients receiving long-term glucocorticoid treatment. However, the action of osteoclasts in GIOP is not precisely understood. The inhibitory and stimulatory effects of glucocorticoids on osteoclast formation and function have been investigated. Kim et al. reported that glucocorticoids in vitro prohibited the proliferation of osteoclast precursors in a dose-dependent manner, and high glucocorticoid doses prevented osteoclastogenesis from reorganizing the osteoclast cytoskeleton [11]. However, other studies showed that the number of osteoclasts was usually maintained in the normal range with long-term glucocorticoid therapy [12,13]. And some other studies investigated that the enhancement of osteoclastogenesis with glucocorticoid administration might be attributable to the increase in the life span of the osteoclasts or to the effects on the osteoblast lineage that enhance the expression of receptor activator of nuclear factor κB ligand (RANKL) and macrophage-colony stimulating factor (M-CSF) and increase the RANKL/osteoprotegerin (OPG) ratio [14,15]. In summary, it is still unclear whether glucocorticoids inhibit or stimulate osteoclastogenesis and bone resorptive activity [16]. Now that bisphosphonates, which suppress the activity of osteoclasts, are considered the first-line options for the treatment of GIOP [13], it is necessary to ascertain the actions of osteoclasts in the pathological process.

The generation of intracellular reactive oxygen species (ROS) is increased when RANKL binds to its receptor RANK on the cell surface of osteoclast precursors [17–19]. N-acetyl-cysteine (NAC), which scavenges...
RANKL-induced intracellular ROS, interrupts the differentiation of osteoclast precursors. Therefore, ROS is indispensable to RANKL-stimulated osteoclast differentiation [19–21]. However, the effect of ROS on osteoclast formation and function with glucocorticoid exposure has not yet been reported.

ROS and macroautophagy (hereinafter referred to as “autophagy”) have intense interactions in many cell types [22,23]. Autophagy is a dynamic catabolic process that delivers cellular components to the lysosome for degradation to retrieve molecules and regain energy to maintain cellular homeostasis [24,25]. Recently, reports have shown that the upregulation of autophagy contributes to osteoclastogenesis in response to hypoxic conditions, oxidative stress and microgravity in vitro [5,26,27]. However, the role of autophagy in osteoclast formation and function with glucocorticoid exposure remains unclear.

To corroborate the alteration of osteoclast formation and function by glucocorticoid administration, we identified the different degrees of positive effects with low-to-high dose glucocorticoids on osteoclastogenesis in vivo and in vitro. Then, we examined the increased levels of ROS accumulation and autophagy flux activity in vitro. We found that NAC, the ROS scavenger, and 3′-methyladenine (3-MA), the autophagy pathway inhibitor, blocked the impact of glucocorticoids on RANKL-induced osteoclastogenesis. Furthermore, autophagy and ROS formed a positive–negative feedback loop in osteoclast precursors. Therefore, our data indicated that glucocorticoids have dose-dependent positive effects on osteoclast formation and function via ROS and autophagy.

2. Materials and methods

2.1. Reagents and antibodies

Antibodies to LC3, p62 and β-actin were purchased from Cell Signaling Technology (Danvers, Massachusetts, USA). RANKL was purchased from California Bioscience (California, USA). Dexamethasone (Dex), NAC, and 3-MA were acquired from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Cell culture

Bone marrow was flushed from the long bones of 6–8 week-old mice (C57BL/6) using α-modified Eagle’s medium. Cells were pelleted at 1500 rpm for 7 min at room temperature and plated in α-modified Eagle’s medium.
Eagle's medium with 10% fetal bovine serum (FBS) supplemented with M-CSF (30 ng/ml) (Peprotech, Rocky Hill, NJ), 100 U/ml penicillin, and 100 mg/ml streptomycin at 5% CO2 and 37 °C for 3 days as reported previously [28].

The RAW264.7 mouse monocyte macrophage cell line was obtained from the American Type Culture Collection (Virginia, USA) and was cultured in RPMI-1640 medium containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin at 5% CO2 and 37 °C.

To investigate the effects of glucocorticoids on osteoclast precursors, bone marrow cells (BM cells) were cultured in α-modified Eagle's medium with 10% fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin for one day, and further incubated with M-CSF for 3 days and then with M-CSF and RANKL (100 ng/ml) and Dex at 0.001 μM, 0.01 μM, 0.1 μM, 1 μM and 10 μM for 7 days. RAW264.7 cells were cultured in α-modified Eagle's medium with 10% fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin for one day, and further incubated with RANKL (100 ng/ml) and Dex at 0.001 μM, 0.01 μM, 0.1 μM, 1 μM and 10 μM for 7 days. NAC (1 mM) and 3-MA (50 μM) were added to the culture system to interfere with the ROS and autophagy flux activity with RANKL addition.

2.3. Animals and glucocorticoid intervention

Thirty 8-week-old C57BL/6J male mice weighing 20 ± 0.2 g were purchased from the Experimental Animal Center of The Fourth Military Medical University (Xi’an, China). The mice were allowed to adapt to the laboratory environment (a well-ventilated controlled room at 20 °C on a 12-h light/dark cycle; the animals were given free access to water and food). The mice were randomly divided into the following three groups (n = 10): control group, Dex (0.5) group and Dex(1) group. There was no significant difference in the initial body weights of the mice among all three groups. The animal groups were treated over 28 days as follows: (1) control group, sham-injected once daily, 5 days/week; (2) Dex(0.5) group, 0.5 mg/kg daily injected dexamethasone subcutaneously (sc), 5 days/week; and (3) Dex(1) group, 1 mg/kg daily dexamethasone injected subcutaneously (sc), 5 days/week. Mice were weighed twice per week to ensure that they did not become excessively catabolic as a result of the high steroid dose and were examined on a daily basis to check for skin sores, diarrhea, and fighting, etc. Mice were killed by an injection of an overdose of pentobarbital, and femurs were removed and cleaned of adherent tissues [29].

2.4. Assessment of bone micro-architecture and bone mass by micro-computed tomography

The distal femurs were scanned using Explore Locus SP Pre-Clinical Specimen micro-CT (GE Healthcare, USA) with an 8-mm resolution, 50-kV tube voltage and 0.1-mA tube current. The reconstruction and 3D quantitative analyses were conducted using software provided by a desktop micro-CT system (GE Healthcare, USA). Similar settings for scans and analyses were used for all of the samples. In the femur, the scanning regions were confined to the distal metaphysis, extending proximally 2.0 mm from the proximal tip of the primary spongiosa. The following 3D indices in the defined region of interest (ROI) were analyzed: bone mineral density (BMD), structure model index (SMI), trabecular number (Tb.N), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp) and relative bone volume over total volume (BV/TV, %). The operator who conducted the scan analysis was blinded to the procedure associated with the specimens.

2.5. Quantitative real-time PCR

Total cellular RNA was extracted from cells using Trizol reagent. Single strand cDNA synthesis was performed using the Prime Script RT reagent

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**Fig. 2.** Glucocorticoids had dose-related effects that promoted osteoclastogenesis in vivo. A: Tartrate resistant acid phosphatase (TRAP) staining of distal femurs. Arrows indicate areas of TRAP staining positive multinuclear cells. Scale bar: 50 μm. B: TRAP-positive multinuclear (≥ 3) cells were counted. C: Oc.S/BS, OC surface/bone surface (%). Quantification was performed on 4 sections per specimen, and values represent the average of 10 mice per group. *P < 0.05, **P < 0.01, ***P < 0.001 versus the control group. #P < 0.05 and ##P < 0.01.
kit (TaKaRa). The RT-PCR was performed using the CFX96 (BIO-RAD) instrument, and individual PCRs were conducted in 96-well optical reaction plates using SYBR Green-I (TaKaRa) according to the manufacturer’s instructions. Target gene \( (\text{NFATc1}, \text{CTSK, TRAP and RANK}) \) expressions were normalized to the reference gene \( \beta\text{-actin}. \) The \( 2^{-\Delta\Delta Ct} \) method was applied to calculate the relative gene expression. The PCR products were
subjected to a melting curve analysis and a standard curve to confirm the correct amplification. All of the PCR’s were performed in triplicate, and the primers used for PCR are as follows: TRAP (CCAGTGCTCGGCTGGTTT, GCTGAGACGACACACTTCA); NFATc1 (GGCTGCTTCCATTTCTT, GCTTGGACACACCTTCT); RANK (ACATGTCAGGGCAGCATC, GCTCCCTTTT CTATCAGGTTAT); CTSS (ACCACGTCCCTCAAATCC, CGTGGCCTTATAAACA); and β-actin (CATCGGAAAAGCTCTATGGCAAC, ATGGAGGC ACCGATCCACA).

2.6. Osteoclast differentiation assay

Osteoclast formation was measured by quantifying cells positively stained with TRAP. Briefly, cells were fixed with 10% formalin for 10 min and ethanol/acetic acid (1:1) for 1 min, and then stained by using Acid Phosphatase Kit-387-A (Sigma-Aldrich, St. Louis, MO). The osteoclasts (TRAP-positive staining multinuclear cells) in each well were viewed under a light microscope (Leica, Wetzlar, Germany). Meanwhile, the morphological features of the osteoclasts were captured. Analyses of the data were performed using an AutoCAD system. To investigate osteoclast formation in vivo, after fixation for 2 days using 4% paraformaldehyde, the left femurs were transferred to 80% formic acid for decalcification for 10 days, then embedded in paraffin and cut into horizontal sections of 5 μm thickness. For TRAP staining, sections were reactivated in 0.2 M Tris buffer and then stained by using Acid Phosphatase Kit-387-A for 1–2 h at room temperature [30]. For each sample, values represent an average of a minimum of 4 stained sections of equivalent depth.

2.7. Bone resorption assay

Bone slices were kindly provided by Dr. Long Bi (FMMU, Xi’an, China). BM cells were plated onto bone slices and treated for 7 days. The cells were completely removed from the bone slices via ultrasound, and the bone slices were stained with toluidine blue. Photographs of the resorption pits were obtained under a light microscope at 40× magnification, and the areas were measured using Image-J software (Media Cybernetics).

2.8. Determination of intracellular ROS

The intracellular ROS expression level was determined using an ROS assay kit (Beyotime, China). DCFH-DA can be oxidized by ROS in viable cells to 2′,7′-dichlorofluorescein (DCF), which is highly fluorescent at 530 nm. The cells were washed three times with PBS. DCFH-DA, which was diluted to a final volume of 10 μM, was added, and the cells were cultured for 30 min at 37 °C in the dark. The relative expression of fluorescence was measured using a fluorescence microscope. Additionally, the cells were collected and analyzed with an Accuri C6 flow cytometer (BD Biosciences).

2.9. Transmission electron microscopy

For transmission electron microscopy, RAW264.7 cells were cultured under conditions with administration of the indicated drug. After that, cells were fixed with 2.5% electron-microscopy-grade glutaraldehyde, post-fixed in 1% osmium tetroxide with 0.1% potassium ferrocyanide, dehydrated through a graded series of ethanol (30–90%), and embedded in Epon. Ultrathin sections (65 nm) were cut, stained with 2% uranyl acetate and Reynold’s lead citrate and examined using an H-600 transmission electron microscope (Hitachi).

2.10. Immunofluorescence confocal microscopy for LC3 localization

RAW264.7 cells were seeded into 4-well chamber slides (Lab-Tek, NUNC), fixed with 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100. After blocking with 5% goat serum in PBS for 4 h, cells were incubated with anti-LC3 Ab (CST) in a humidity chamber at 4 °C overnight. The next day, cells were washed and incubated with secondary Alexa Fluor 350-labeled anti-rat Ab. The cells were assessed using an FV1000 Olympus laser scanning confocal microscope.

2.11. Western blot analysis

Total cell lysates were prepared in a buffer containing 20 mM Tris–HCl at pH 7.4, 1% Triton X-100, 1 mM EDTA, 1.5 mM glucocorticoid, 10% glycerol, 150 mM NaCl, 0.1 mM Na2VO4, and 1× protease inhibitor cocktail. The protein content of the samples was measured using a BCA protein assay reagent (Pierce, Rockford, IL). Protein (100 μg) samples were then subjected to SDS-PAGE using 12% Tris–HCl gels, blot transferred onto a PVDF membrane, and immunoblotted with antibodies against LC3-II, p62, and β-actin. The bands were detected using an enhanced chemiluminescence detection system (Pierce, Rockford, IL), and band intensity was quantified by densitometric analysis using the NIH Image-J Program.

2.12. Histological examination by Van Gieson (VG) staining

The femurs of all the mice were collected and fixed in 4% paraformaldehyde for 48 h. After dehydration and embedding, the distal femurs were embedded in polymethyl-methacrylate (PMMA) and processed into 240-mm-thick sections in the coronal plane using a rotation microtome. Subsequently, all of the sections were hand-grounded to a thickness of 20 mm for VG staining, which is used for staining collagen fiber[31].

2.13. Statistical analysis

Data were expressed as the means ± s.e.m. of multiple repeats of the same experiment (n = 3). The data for these measurements were analyzed by one-way analysis of variance (ANOVA) with subsequent post hoc multiple comparisons by Dunnett’s test. Statistically significant values were defined as P < 0.05 [13].

3. Results

3.1. Dex affected osteoclast formation and function in a dose-dependent manner in vivo

To investigate the effects of Dex on osteoclast formation and function in vivo, two doses of Dex (0.5 mg/kg and 1 mg/kg) were administered to the mice. Our data showed that the trabecular bone microarchitecture of the distal femurs from the Dex administration group was deteriorated compared with the sham-injected (control) group (Fig. 1A). Additionally, the more Dex administered, the more disturbed

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**Fig. 3.** Dex stimulated RANKL-induced osteoclast formation and function. BM cells and RAW264.7 cells were treated with RANKL (100 ng/ml) and Dex of different concentrations. A: TRAP staining of BM cells treated with Dex (0.001 μM, 0.01 μM, 0.1 μM, 1 μM and 10 μM) in the presence of RANKL for 7 days. Scale bar: 100 μM. B: Number of TRAP-positive multinuclear cells. C: Average spread area of TRAP-positive multinuclear cells. D: TRAP staining of RAW 264.7 cell treated with Dex (0.001 μM, 0.01 μM, 0.1 μM, 1 μM and 10 μM) in the presence of RANKL for 7 days. Arrows indicate TRAP-positive multinuclear cells. E and F: Effects of Dex (0.1 μM, 1 μM and 10 μM) on the mRNA expression levels of osteoclastogenesis-related genes in RAW264.7 cells by real-time quantitative PCR on day 3 and day 5, respectively. β-actin expression served as control. H and I: BM cells on bone slices were incubated with Dex (0.1 μM, 1 μM and 10 μM) in the presence of RANKL for 7 days. Scale bar: 200 μM. The cells were removed from the bone slices, and the pit area (outlined) was assessed after staining with toluidine blue. All data are presented as the mean ± s.e.m. from three different experiments in duplicate. *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001 versus the RANKL group. 

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the trabecular bone micro-architecture, as evaluated by a decrease in the following indices: bone mineral density (BMD), relative bone volume over total volume (BV/TV), trabecular thickness (Tb.Th) and trabecular number (Tb.N) (Fig. 1C). In addition, the structure model index (SMI) and trabecular separation (Tb.Sp) displayed significant increases, which were attributed to additional Dex administration (Fig. 1B).

Moreover, VG staining was performed to evaluate the changes in bone micro-architecture. As shown in Fig. 1D, the number of trabeculae decreased, and the direction of trabeculae were disturbed in the Dex groups versus the control group. There was more deterioration with more Dex administration (Fig. 1D). These results were consistent with the micro-CT data (Fig. 1A, B and C).

Moreover, tartrate-resistant acid phosphatase (TRAP) staining was performed to determine whether Dex promoted osteoclastogenesis in vitro (Fig. 2A). In contrast to the control group, the Dex groups showed obvious increases in the number (Fig. 2B) and spread area (Fig. 2C) of mature osteoclasts. Moreover, more Dex led to more significant osteoclastogenesis.

3.2. Dex stimulated RANKL-induced osteoclast formation and function in a dose-dependent manner in vitro

To validate the direct effects of Dex on osteoclast precursors, we assessed osteoclast differentiation and bone resorption without the influence of other cell types in vitro. BM cells were cultured in the presence of RANKL (100 ng/ml) with dexamethasone (Dex) doses of 0.001 μM, 0.01 μM, 0.1 μM, 1 μM and 10 μM for 7 days. After TRAP staining, we observed that the number and average size of TRAP-positive multinuclear (≥3) cells increased in the Dex groups at concentrations of 0.1 μM, 1 μM and 10 μM compared with the control group containing RANKL alone (Fig. 3A). The largest increase was observed in the 1 μM group (Fig. 3B and C). We also utilized the RAW 264.7 cell line, a widely used osteoclast progenitor cell model [27], to verify the effects (Fig. 3D,E). Then, we examined the expression levels of several osteoclastogenesis-related genes, such as receptor activator for nuclear factor-κB (RANK), TRAP, cathepsin K (CTSK), and nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1), by using real-time quantitative PCR and found that the 1 μM group had the most significant increase, which was consistent with TRAP staining. Furthermore, to investigate the effects of Dex on osteoclast function, we assessed resorption pit formation by culturing BM cells on bovine cortical bone slices with RANKL (100 ng/ml) and Dex of different concentrations (0.1 μM, 1 μM and 10 μM) for 7 days and found that all the Dex groups had increased pit areas (outlined) compared with the control group, and the 1 μM group had the most significant increase (Fig. 3H and I). All the data above suggests that in vitro, relatively high concentrations of Dex (0.1 μM, 1 μM and 10 μM) stimulate RANKL-induced osteoclastogenesis and bone resorption, with 1 μM being the peak concentration. Despite these results, low levels of Dex (0.001 μM and 0.01 μM) only induce significant differences in cell size.

3.3. Dex promoted the accumulation of ROS in osteoclast precursors

Because ROS mediates RANKL-induced osteoclastogenesis, it might also play a key role in the mechanisms by which Dex promotes osteoclast precursor differentiation. First, to corroborate the mechanism, the accumulation of intracellular ROS was determined by fluorescence microscopy in osteoclast precursors cultured with RANKL (100 ng/ml) and Dex of different concentrations (0.1 μM, 1 μM and 10 μM). NAC (1 mM) was administered to scavenge intracellular ROS responding to RANKL and 1 μM Dex. The ROS positive cells were bright green spots and were counted to assess the intracellular ROS (Fig. 4A and B). Second, all the cells were collected and analyzed with a flow cytometer to measure the intensity of ROS accumulation (Fig. 4C and D). The data indicates that Dex enhances ROS accumulation in osteoclast precursors, and Dex substantially reduces intracellular ROS levels in osteoclast precursors with Dex exposure. Consistent with the effects of Dex on RANKL-induced osteoclast formation and function, the 1 μM group shows the most notable stimulation.

3.4. Dex-induced ROS led to autophagy in osteoclast precursors

Because autophagy plays roles in up-regulated osteoclastogenesis under many conditions, we hypothesized that autophagy might also enhance RANKL-induced osteoclast precursor differentiation with Dex exposure. To probe autophagy flux activity, we measured the expression and recruitment of microtubule-associated protein light chain 3 (LC3), an autophagy marker, by confocal microscopy and found that the Dex groups possessed many more LC3-positive cells (Fig. 4E and F). Moreover, autophagosomes/autolysosomes were monitored by transmission electron microscopy. The number of autophagic structures in the Dex groups significantly exceeded that in the control group (Fig. 4G). To assess the intensity of autophagy flux activity, degradation of p62/SQSTM1, the substrate of autophagy [32], and lipidation of LC3 (conversion of LC3-I into LC3-II) were analyzed by western blot. Dex led to down-regulated p62 and up-regulated LC3-II which signifies stronger autophagy flux activity (Fig. 4H). The alteration of Dex-induced autophagy was consistent with ROS and osteoclastogenesis as shown previously. In addition, scavenging ROS by NAC prevented autophagy flux activity in osteoclast precursors (Fig. 4E, F, G and H). These data demonstrate that Dex enhances autophagy flux activity via ROS in osteoclast precursors with RANKL present.

3.5. Functional role of ROS and autophagy in Dex-stimulated RANKL-induced osteoclast formation and function

To further investigate the involvement of ROS and autophagy in the promotion of osteoclast precursor differentiation with Dex exposure, NAC or 3-methyladenine (3-MA) was added to the culture system (RANKL and 1 μM Dex) to scavenge ROS or interrupt autophagy flux activity. The actions of Dex on osteoclast formation and function were blocked with NAC and 3-MA (50 μM) administration. The number and size of TRAP-positive multinuclear BM cells (Fig. 5A, B and C) were reduced as were the number of TRAP-positive multinuclear RAW264.7 cells (Fig. 5D and E). The decreased areas of bone resorption pits (Fig. 5F and G) were consistent with the alterations shown with TRAP staining. Additionally, expression of osteoclastogenesis-related genes was down-regulated (Fig. 5H). These data reveal that ROS and autophagy play critical roles in Dex-stimulated RANKL-induced osteoclast formation and function.

3.6. Autophagy had no effects on ROS

The data above demonstrate that Dex-induced ROS leads to autophagy in osteoclast precursors. However, the crosstalk between ROS and autophagy remains obscure in osteoclastogenesis. Thus, 3-MA was utilized to block the autophagy pathway and resulted in the up-regulation of ROS accumulation with no significance (Fig. 6A, B, C and D), which implied that ROS-induced autophagy had no effects on ROS.

4. Discussion

With glucocorticoids widely used to treat immunological diseases, such as rheumatoid arthritis and Crohn’s disease, adverse effects such as GIOP are increasingly obvious [33]. To the best of our knowledge, glucocorticoids influence all elements involved in bone remodeling in GIOP, including stromal/osteoblastic cells, osteoclasts and their interaction [10]. Previous investigations have placed an emphasis on stromal/osteoblastic cells and their effects on osteoclastogenesis [34–36].
However, the alterations to osteoclastogenesis remain obscure. The divergent effects of Dex on osteoclasts are always attributed to the difference in cell origin, methodology and criteria [16]. In particular, the mouse strain, age and gender, type of glucocorticoids, method and time of administration, and many other factors lead to this difference. For instance, Kim et al. reported that glucocorticoids inhibited the

Fig. 4. Dex promoted the accumulation of ROS and autophagy flux activity in osteoclast precursors. RAW264.7 cells were treated with RANKL (100 ng/ml) and Dex (0.1 μM, 1 μM and 10 μM). Particularly, NAC (1 mM) was administered with RANKL and 1 μM Dex to scavenge ROS. The cells were treated for 10 min before ROS was assayed and 6 h before autophagy was measured. A: Assessment of the accumulation of intracellular ROS by fluorescence microscopy in different groups. Scale bar: 100 μm. B: Number of ROS positive cells viewed as bright green spots. C: Analysis of ROS accumulation intensity of osteoclast precursors in different groups by flow cytometer. D indicates dexamethasone administration. D: Intensity of ROS accumulation shown as the mean FL1-A value. E: Measurement of LC3 recruitment after immunostaining by confocal microscopy. Scale bar: 20 μm. F: Number of LC3 positive cells. G: Autophagosomes/autolysosomes were monitored by transmission electron microscopy and number of autophagic structures. Arrows indicate autophagic structures. Scale bar: 0.2 μm. H: Expression levels of p62 and LC3 by Western blot. β-actin served as control. All data are presented as the mean ± s.e.m. from three different experiments in duplicate. *P < 0.05, **P < 0.01, ***P < 0.001 versus the RANKL group. △△△P < 0.001. △△△△P < 0.001.
bone-degrading capacity of osteoclasts. However, with a higher dose and longer duration of RANKL and Dex administration, we obtained different results in our study. First, we ascertained that glucocorticoids promoted osteoclastogenesis in vivo in a dose-related manner, with dexamethasone as the glucocorticoid because it is commonly used. Then, to investigate the mechanism of the dose-dependent effects, it is necessary to establish a specific culture system to investigate the direct action of glucocorticoids on osteoclast precursors. In this study, we cultured BM cells and the RAW264.7 cell line, which contains pure monocyte/macrophage cells, as the osteoclast precursors with a definite concentration of RANKL but without any stromal/osteoblastic cells in vitro. Dexamethasone was administered in different concentrations.

Fig. 5. Reduction of ROS and inhibition of autophagy blocked Dex-stimulated osteoclast formation and function. NAC was utilized to scavenge intracellular ROS, and 3-MA was used to interrupt autophagy flux activity. BM cells and RAW264.7 cells were treated with NAC (1 mM) or 3-MA (50 μM) together with RANKL (100 ng/ml) and Dex (1 μM). A: TRAP staining of BM cells treated with NAC (1 mM) or 3-MA (50 μM) together with RANKL (100 ng/ml) and Dex (1 μM) for 7 days. Scale bar: 100 μm. B: Number of TRAP-positive multinuclear cells. C: Average spread area of TRAP-positive multinuclear cells. D: TRAP staining of RAW 264.7 cell treated with NAC (1 mM) or 3-MA (50 μM) together with RANKL (100 ng/ml) and Dex (1 μM). Arrows indicate TRAP-positive multinuclear cells. Scale bar: 100 μm. E: Number of TRAP-positive multinuclear cells. F and G: BM cells were removed from the bone slices after 7 days, and the pit area (outlined) was determined by staining with toluidine blue. Scale bar: 200 μm. H: The mRNA expression levels of osteoclastogenesis-related genes in RAW264.7 cells by real-time quantitative PCR on day 3. β-actin expression served as control. All data are presented as the mean ± s.e.m. from three different experiments in duplicate. *P < 0.05; **P < 0.01; ***P < 0.001.
Our data showed that the glucocorticoid up-regulated osteoclastogenesis at high doses while low doses had no effects. In addition, there was a peak concentration. Excessive amounts of glucocorticoid led to relatively decreased osteoclast formation and function. Our results above sustain the conclusion that bisphosphonates should be used as the first-line treatment for GIOP.

To understand the decreases associated with more glucocorticoid exposure, we investigated the mechanism of action in an in vitro study. ROS refers to molecules containing highly reactive free oxygen radicals, including superoxide anion ($O_2^-$), the hydroxyl radical (OH$^-$), and stable non-radical oxidants such as hydrogen peroxide (H$_2$O$_2$) [22]. As ROS play a vital role in osteoclastogenesis [19], we monitored the intracellular ROS accumulation in the high-dose Dex groups and found that the enhancement of ROS accumulation was synchronized with the up-regulation of osteoclast formation and function, which could be blocked by the ROS scavenger NAC. Our results affirm that ROS mediate the effects of glucocorticoids on osteoclast precursors. Increased intracellular ROS accumulation causes autophagy in many cell types [37]. In this study, ROS led to autophagy in osteoclast precursors with glucocorticoid exposure, and the alteration in autophagic activity was synchronized with ROS accumulation and osteoclastogenesis, which were blocked by treating with NAC. 3-MA, an inhibitor of autophagy flux activity, was administered and interrupted the effects of glucocorticoids on osteoclast formation and function. Thus, autophagy mediates the effects of glucocorticoids on osteoclast precursors, acting as a downstream factor of ROS.

Fig. 6. Autophagy had no effect on ROS. RAW264.7 cells were treated with 3-MA (50 μM) together with RANKL (100 ng/ml) and Dex (1 μM). The cells were treated for 10 min before ROS was assayed. A: Accumulation of intracellular ROS by fluorescence microscopy. Scale bar: 100 μm. B: Viewed as bright green spots, ROS positive cells were counted. P = 0.0952. C: Osteoclast precursors were collected and analyzed with flow cytometer. R indicates RANKL treatment. D indicates dexamethasone administration. D: Mean FL1-A was presented to show the intensity of ROS accumulation. P = 0.0689. All data are presented as the mean ± s.e.m. from three different experiments in duplicate. E: The model of glucocorticoid-regulated osteoclastogenesis.
For autophagosome membrane formation, critical autophagy proteins (ATGs) are required in addition to the insertion of lipidated microtubule-associated protein light chain 3 (LC3) [38,39]. Autophagy acts as a cellular housekeeper by recycling proteins and organelles, and by managing nutrient supply under starvation conditions, immune responses and antigen presentation [40,41]. Furthermore, autophagy affects cell differentiation. Mounting studies have indicated that autophagy has influence on the development and differentiation of multiple cell types, such as chondrocytes, lymphocytes, and adipocytes [42,43]. Additionally, autophagy plays a part in bone homeostasis [44], as evidenced by the fact that the induction of autophagy down-regulates osteoclast precursor differentiation and bone absorptive activity in vivo. It is unknown whether the direct effects on osteoclasts or disrupted angiogenesis are responsible for these effects [45]. Recent studies have demonstrated that autophagic flux activity mediates osteostegenesis by degrading certain proteins involved in the process, such as p62/SQSTM1 and TNFR receptor-associated receptor 3 (TRAF3) [6]. Autophagy proteins, such as LC3 and Atg5, have been shown to be important factors for generating the osteoclast ruffled border [46]. However, previous findings have indicated that autophagy proteins such as LC3 and beclin-1 may be involved in osteoclast formation and function via non-autophagic pathways [47,48]. In our study, autophagosomal/autolysosome formation and autophagic marker LC3 were observed. These data imply that autophagy flux activity takes part in osteoclast formation and function. The detailed mechanisms by which autophagy modulates osteostegenesis remain to be investigated.

In tumorigenesis, the field in which autophagy is predominantly studied, ROS induces autophagy while inhibiting positive regulators, and autophagy modulates ROS in turn [22,23]. In our study, autophagy flux activity failed to reduce intracellular ROS accumulation. We speculate that ROS may not have been abundant enough to destroy organelles in Dex-treated osteoclast precursors. Autophagy, which acted as a downstream factor of ROS, contributed to osteostegenesis instead of survival. So autophagy had no effect on ROS in our investigation.

According to our study, we speculate that autophagy may be a potential therapeutic target for GIOP in addition to ROS. Developing drugs targeting autophagy might shed light on the treatment of GIOP. However, one key point is that autophagy occurs in many cell types including bone marrow stem cells, osteoblasts and osteocytes. It is impossible for an autophagy inhibitor to identify osteoclast precursors and mature osteoclasts specifically, and this inability prevents us from ascertaining the functional role of autophagy in osteostegenesis in vivo.

In conclusion, our results show that glucocorticoids have dose-dependent effects on RANKL-induced osteoclast formation and function resulting from the up-regulation of ROS accumulation and autophagy flux activity. As more glucocorticoids are administered, there is a significant enhancement in osteoclast formation and function until a threshold is reached. Exposure to excessive amounts of glucocorticoids leads to a reduction in ROS accumulation and autophagy flux activity, resulting in a relative reduction in osteostegenesis. The detailed mechanism of action by which autophagy modulates osteostegenesis-related molecules needs to be further investigated.

Author contributions
Jun Shi, Long Wang, Hongyang Zhang and Qiang Jie performed all of the experiments. Jian Liu, Liu Yang and Zhuojing Luo supervised the experiments. All authors were involved in planning the experiments, analyzing the experimental data and/or commenting on the paper. Jun Shi wrote the paper.

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