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1-α,25-dihydroxyvitamin D_3 potentiates avian osteoclast activation by increasing the formation of zipper-like structure via Src/Rac1 signaling

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ABSTRACT

Avian bone metabolism diseases affect the development and production of chickens, and many of these diseases can be prevented and controlled by balanced nutrition and hormone medicine. The steroid hormone 1α,25-dihydroxyvitamin D_3 plays a key role in maintaining the balance of avian bone metabolism. Clinically, 1α,25-$(OH)_2D_3$ has been used to treat several bone diseases. Although several previous studies have investigated the effects of 1α,25-$(OH)_2D_3$ on osteoclastogenesis, the mechanisms underpinning osteoclast (OC) activity remain largely unknown. Herein, we used molecular and cell biology approaches to demonstrate that 1α,25-$(OH)_2D_3$ increases avian OC formation and activity, and upregulates bone resorption-related genes. Moreover, 1α,25-$(OH)_2D_3$ regulates the OC cytoskeleton by increasing the formation of zipper-like structure in OC precursor cells to potentiate OC activity via the Src/Rac1 signaling pathway. These findings provide new insight into the role of 1α,25-$(OH)_2D_3$ in OC activity.

1. Introduction

Avian bones undergo continuous remodeling via bone resorption by osteoclasts (OCs) and bone formation by osteoblasts (OBs) [1]. OCs are terminally differentiated multinuclear giant cells derived from monocyte/macrophage lineage cells, which exhibit bone resorption and have a critical role in bone reconstruction. Abnormal formation and activation of OCs is closely associated with the occurrence of metabolic bone disease [2].

1α,25-dihydroxyvitamin D_3 (1α,25-$(OH)_2D_3$) is an active form of vitamin D that performs pleiotropic roles in various biological functions and physiological processes [3]. 1α,25-$(OH)_2D_3$ regulates calcium homeostasis through the vitamin D receptor (VDR). Not only does 1α,25-$(OH)_2D_3$ regulate Ca^{2+} reabsorption in the small intestine and kidneys, it also acts directly on bone cells in skeleton metabolism [4]. 1α,25-$(OH)_2D_3$ can indirectly regulate OC formation and bone resorption by increasing RANKL expression and decreasing OPG expression in OB-lineage cells. Moreover, there is considerable evidence suggesting that 1α,25-$(OH)_2D_3$ can directly influence OC precursor (OCP) cells [5]. Kogawa et al. [6] reported that 1α,25-$(OH)_2D_3$ can increase the number of mature OCs that exhibit bone absorption activity by inducing the differentiation of OCPs. Evidence suggests that 1α,25-$(OH)_2D_3$ regulates OC function both directly and indirectly, but the mechanism by which 1α,25-

Abbreviations: SPF, specific pathogen free; MEM, minimum eagle medium; TRAP, tartrate-resistant acid phosphatase; CaII, carbonic anhydrase 2; Ck, cathepsin K; Mmp-9, matrix metallopeptidase 9; Rac1, Rac family small GTPase 1; RhoA, ras homolog family member A; Cdc42, cell division cycle 42; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; Src, the non-receptor tyrosine kinase Src.

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$(OH)_2D_3$ mediates OC activation remains largely unknown.

The cytoskeleton is involved in various cellular processes, including migration, differentiation, and fusion [7]. Extensive recombination of the actin cytoskeleton is involved in osteoclastogenesis and activation. The cellular mechanics of OC fusion possibly involve a novel actin superstructure, which is termed the "zipper-like structure" because of its morphological similarity with adhesion zippers observed in keratinocytes [8]. Similar structures have been found in the fusion of OCPs during osteoclastogenesis both in the murine macrophage cell line RAW264.7 and in mouse bone marrow macrophages [9]. Another study found that 1,25-$(OH)_2D_3$ regulated cytoskeletal proteins via VDR and the STAT5 signaling pathway [10], while the effects of 1,25-$(OH)_2D_3$ on the OC cytoskeleton are poorly understood. In the present study, we investigated the direct effects of 1,25$(OH)_2D_3$ on cytoskeletal changes during OC activation, and probed the underlying mechanism. The results enhance our understanding of the influence of 1α,25-$(OH)_2D_3$ on OC activation.

2. Materials and methods

2.1. Experimental birds

SPF-grade wild-type Hy-line variety white chicken embryos were purchased from Jiangsu Poultry Institute, and 18-day-old Hy-line variety white chickens were sacrificed by exsanguination. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Research Council. All experiments and procedures on animals were approved by The Animal Care and Use committee of Yangzhou University (approval ID: SYXK (Su) 2007–0005).

2.2. Cell culture

Bone marrow cells were isolated from chickens as described previously [11] with slight modifications. Briefly, the tibiae and femora were separated from sterilized chickens, the marrow cavity was washed with phosphate-buffered saline (PBS; Thermo Fisher Scientific, Waltham, MA USA), and the medial wall of the marrow cavities were repeatedly scraped. Bone marrow cells were harvested by centrifugation at 200*g* for 5 min at a relatively constant temperature and then purified by Percoll density gradient centrifugation, and resuspended in α-MEM supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA). Cells were transplanted into 12- and 48-well cell culture plates (Corning Inc., Midland, MI, USA) at a concentration of 1×10^6 cells/ml. In the resorption lacuna analysis experiment, bovine cortical bone slices were placed in the wells of 48-well cell culture plates before the cells were transferred. After the cells were incubated for 24 h, the cell culture medium was replaced with fresh medium either with or without 1α,25-$(OH)_2D_3$ (Sigma-Aldrich, Saint Louis, MO, USA), then the cells were incubated for 1, 3, and 5 days.

2.3. Real-time cell proliferation analysis

Cell proliferation was analyzed using an xCELLigence real-time cell analysis (RTCA) system (Roche, Mannheim, Germany), similar to that previously described [12]. The background value was first removed by loading 100 μl of culture medium/well into a 16-well E-plate before starting the test. Changes in the cell activity were monitored by transferring approximately 2×10^5 cells/well in the E-plate and incubating them for 24 h to allow cells to attach and reach the proliferative stage. Then, a range of concentrations of 1α,25-$(OH)_2D_3$ (0, 10^{-10}, 10^{-9}, and 10^{-8} mol/l) were added to the culture medium. Impedance was measured every 15 min from the start of the experiment for 3 days. The normalized cell index related to the treatment time point was obtained by RTCA software.

2.4. Tartrate-resistant acid phosphatase (TRAP) staining and OC counting

Cells were fixed with 4% paraformaldehyde for 20 min. The TRAP staining kit (Sigma-Aldrich) was used according to the manufacturer's instructions. TRAP-positive multinucleate cells (containing more than three nuclei) were counted in ten random fields using inverted phase-contrast microscopy.

2.5. Resorption lacuna analysis

The bovine cortical slices were removed and washed thrice with 0.25 mol/l ammonium hydroxide using an ultrasonic cleaner (for 5 min each time). The bone slices were dehydrated in an ethanol gradient, air-dried, and gilded by ion-plating apparatus (SCD500 Sputter Coater, Bal-Tec Liechtenstein). Osteocommas were observed under an environmental scanning electron microscope (XL30-ESEM, Philips, Netherlands). Finally, the bone resorption pit area was measured using an image analysis system (JD801; Jiangsu JEDA Science-Technology Development, China).

2.6. Cytoskeleton analysis

Cells were incubated with 10 μM Rac1 inhibitor NSC23766 (Sigma-Aldrich) or transfected with 50 nM Src siRNA (or the negative control siRNA), then treated with 10^{-8} mol/l 1α,25-$(OH)_2D_3$ for 3 days. For cytoskeleton staining, cells were fixed onto coverslips with 4% paraformaldehyde for 20 min, then permeabilized with 0.2% (v/v) Triton X-100 for 5 min and blocked with 2% bovine serum albumin (BSA; Thermo Fisher Scientific) for 30 min. Cells were then treated with rhodamine-conjugated phalloidin (Invitrogen; Carlsbad, CA, USA) diluted with 2% BSA in PBS for 60 min at room temperature, and cell nuclei were stained with 5 mg/l 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) for 10 min. Finally, cytoskeletons were observed under a Leica DMI3000B fluorescence microscope (Leica Microsystems GmbH, Wetzlar, Germany).

2.7. RNA interference

A custom small interference RNA (siRNA) targeting chicken Src was designed and synthesized by RiboBio Co., Ltd (Guangzhou, China). After cells were incubated for 24 h, siRNAs were transfected into cells using Lipofectamine 3000 (Invitrogen) according to the manufacturer's protocol. After transfection, cells were cultured with or without 1α,25-$(OH)_2D_3$ for 3 days prior to analysis.

2.8. Western blot analysis

At 3 days after cells were transfected with siRNA, total protein was extracted using RIPA lysis buffer (Thermo Fisher Scientific) and the protein concentration was measured using a BCA Protein Assay Kit (Thermo Fisher Scientific). Samples were denatured, subjected to western blot analysis, and incubated with primary antibodies overnight at 4 °C. The rabbit anti-chicken Src antibody (1:500 dilution; Santa Cruz, CA, USA) and the mouse anti-chicken GAPDH antibody (1:4000 dilution; Thermo Fisher Scientific) were used as internal references. Then, the cells were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:5000) polyclonal antibodies (Cell Signaling Technology, Boston, MA) and HRP-conjugated goat anti-mouse IgG (1:5000) polyclonal antibodies (Santa Cruz) at room temperature for 1 h. The membranes