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Glucocorticoids mediate circadian timing in peripheral osteoclasts resulting in the circadian expression rhythm of osteoclast-related genes

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ABSTRACT

Circadian rhythms are prevalent in bone metabolism. However, the molecular mechanisms involved are poorly understood. Recently, we suggested that output signals from the suprachiasmatic nucleus (SCN) are transmitted from the master circadian rhythm to peripheral osteoblasts through β-adrenergic and glucocorticoid signaling. In this study, we examined how the master circadian rhythm is transmitted to peripheral osteoclasts and the role of clock gene in osteoclast. Mice were maintained under 12-hour light/dark periods and sacrificed at Zeitgeber times 0, 4, 8, 12, 16 and 20. mRNA was extracted from femur (cancellous bone) and analyzed for the expression of osteoclast-related genes and clock genes. Osteoclast-related genes such as *cathepsin K* (*CTSK*) and *nuclear factor of activated T-cells, cytoplasmic 1* (*NFATc1*) showed circadian rhythmicity like clock genes such as *period 1* (*PER1*), *PER2* and *brain and muscle Arnt-like protein 1* (*BMAL1*). In an *in vitro* study, not β-agonist but glucocorticoid treatment remarkably synchronized clock and osteoclast-related genes in cultured osteoclasts. Chromatin immunoprecipitation (ChIP) assay showed the interaction between BMAL1 proteins and promoter region of *CTSK* and *NFATc1*. To examine whether endogenous glucocorticoids influence the osteoclast circadian rhythms, mice were adrenalectomized (ADX) and maintained under 12-hour light/dark periods at least two weeks before glucocorticoid injection. A glucocorticoid injection restarted the circadian expression of *CTSK* and *NFATc1* in ADX mice. These results suggest that glucocorticoids mediate circadian timing to peripheral osteoclasts and osteoclast clock contributes to the circadian expression of osteoclast-related genes such as *CTSK* and *NFATc1*.

Introduction

The autonomic nervous system and serum levels of hormones show circadian rhythms [1–4]. Histological studies have shown that the circadian rhythm is also prevalent in bone remodeling [5–9]. Biochemical parameters of bone formation and resorption also show circadian rhythms [10–13]. However, the molecular mechanisms of circadian rhythm in bone metabolism are poorly understood.

Circadian rhythms are regulated by molecular clocks such as *circadian locomotor output cycles kaput* (*CLOCK*), *brain and muscle Arnt-like protein 1* (*BMAL1*), *period* (*PER*) and *cryptochrome* (*CRY*), which exist throughout the body and have an autoregulatory feedback loop involving transcriptional and post-translational processes [14–16]. CLOCK protein heterodimerizes with BMAL1 to drive the rhythmic expression of other clock genes (*PER1*, *PER2*, *PER3*, *CRY1*, *CRY2*) through E-box (CACGTGF) elements located in their promoters [17,18]. PER and CRY proteins suppress the induced expression. The peak expressions of these two distinct sets of genes occur in antiphase of one another. Thus, CLOCK and BMAL1 act as positive regulators and PER and CRY as negative ones.

The central clock is located in the suprachiasmatic nucleus (SCN) and is mainly synchronized by light signals through the eye. The output signals from the SCN transmit standard circadian time to peripheral tissue through sympathetic nervous system and humoral routes [19–21]. Recently, we reported that a β-adrenergic agonist, isoprenaline, and a synthetic glucocorticoid, dexamethasone (DEX), synchronized clock genes and osteoblast-related genes such as *type I collagen* and *alkaline phosphatase* in human osteoblasts [22]. Glucocorticoids, which are secreted from the adrenal gland in a circadian rhythm, are used to treat a number of medical disorders [23–25]. However, it remains poorly understood how the master circadian rhythm is transmitted to peripheral osteoclasts.

Some reports have indicated that a peripheral clock contributes to peripheral tissue differentiation and circadian rhythm [26–30]. Fu et al. [26] reported that a peripheral clock mediates β-adrenergic signaling that regulates bone formation. Shimba et al. [29] reported that *BMAL1*, a master regulator of circadian rhythm, also plays important roles in the regulation of adipose differentiation and lipogenesis in mature adipocytes. However, the role of clock genes on osteoclasts has not been identified.

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Osteoclasts differentiate from macrophages, which require the presence of receptor activator of nuclear factor κB ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) [31,32]. Osteoclasts are characterized by high expression of *tartrate-resistant acid phosphatase* (*TRAP*), *cathepsin K* (*CTSK*), *nuclear factor of activated T-cells, cytoplasmic 1* (*NFATc1*) and *dendritic cell-specific transmembrane protein* (*DC-STAMP*) genes [33,34]. CTSK is the protease involved in the degradation of type I collagen and other noncollagenous proteins [34]. NFATc1 mediates osteoclast cell fusion and differentiation [33]. DC-STAMP is essential for osteoclast cell fusion [35]. Kim et al. [36] reported that DEX suppresses osteoclast precursor proliferation, M-CSF-induced actin ring formation and resorption lacuna formation.

Here, we report that glucocorticoids transmit the circadian rhythm to peripheral osteoclasts and that a peripheral osteoclast clock contributes to the circadian expression of osteoclast-related genes such as *CTSK* and *NFATc1*.

Materials and methods

Cell culture

Cells of the murine macrophage-like cell line RAW264.7 [37] were cultured in α-MEM (Gibco-BRL, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS), 100 IU/ml penicillin and 100 μg/ml streptomycin. In order to differentiate into osteoclasts, RAW264.7 cells were plated at 2.5×10^3 cells/cm^2, incubated for 3 days with 50 ng/ml RANKL (PeproTech, London, UK) and then used for experiments.

Bone marrow cells were collected from male C57BL/6J mice by flushing out femora and tibiae with α-MEM containing penicillin and streptomycin. Bone marrow cells were cultured in α-MEM containing 10% FBS, 100 IU/ml penicillin, 100 μg/ml streptomycin and 50 ng/ml M-CSF (R&D Systems, Minneapolis, MN, USA), and then cultured for 16 h. Nonadherent cells were collected and counted using a flow cytometer (Guava EasyCyte Mini cell cytometer system) with Guava ViaCount fluorescent dye (Guava Technologies, Hayward, CA). These cells were replated at 1.5×10^5 cells/well and incubated with 50 ng/ml M-CSF for 2 days in 48-well plates. The adherent cells were used as bone marrow-derived macrophages. In order to differentiate into osteoclasts, the bone marrow macrophages were cultured for 3 days with 50 ng/ml M-CSF and 50 ng/ml RANKL and then used for experiments.

Treatment with various chemical agents

Osteoclasts derived from RAW264.7 were cultured on 6-well plates in α-MEM with 10% fetal bovine serum and 50 ng/ml RANKL. These cells were incubated with dexamethasone (Sigma Aldrich, St. Louis, USA), isoprenaline (Sigma Aldrich), calcitonin (Bachem Feinchemikalien AG, Torrance, CA, USA) or PTH (Protein Research Foundation, Osaka, Japan). After 2 h of treatment with these agents, the culture medium was replaced with α-MEM containing 0.5% FBS and 50 ng/ml RANKL without washing and cells were collected every 4 h.

Animals

Four-week-old male ddY (Japan SLC Inc., Hamamatsu, Japan) mice were maintained under a 12:12-hour light/dark cycle for at least two weeks and were then sacrificed at Zeitgeber times (ZT) 0, 4, 8, 12, 16 and 20. The mice were acclimated to their cages for 1 week prior to the experiment and treated in accordance with the Guidelines for Animal Experiments of the School of Dentistry, Aichi Gakuin University. Food and water were available *ad libitum*. Five to six mice in each group were housed together under automatically controlled conditions of temperature (23 ± 1 °C) and humidity (50 ± 10%). All mice were sacrificed by exsanguination and the tissues were harvested.

Adrenalectomy (ADX)

All surgical procedures were performed under pentobarbital sodium (40 mg/kg i.p.) anesthesia. ADX or sham-operated ddY mice were produced by making a single 1-cm dorsal incision. For adrenalectomy, the adrenal glands were bilaterally removed from mice. The ADX mice were given free access to 0.9% NaCl to prevent hyponatremia.

Quantitative real-time polymerase chain reaction (qRT-PCR) analysis

As *in vivo* experiments, total RNA was extracted from SCN, bone marrow and femur (cancellous bone). As *in vitro* experiments, total RNA samples were extracted from the cells. In both cases, RNA was extracted using the SV total RNA isolation kit (Promega, Madison, WI, USA) according to the manufacturer's instructions, and cDNA samples were synthesized using Oligo (dT) primer and Moloney murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA, USA). qRT-PCR experiments were performed using Step One Plus (Applied Biosystems, Foster City, CA, USA), TaqMan MGP probe and Eurogentec qPCR Master Mix Plus (Eurogentec, San Diego, CA, USA). The primers used are listed in Table 1. Each experiment was performed in duplicate, and the results were standardized using Human GAPDH Control Reagents (Applied Biosystems, Foster City, CA, USA).

Laser capture microdissection

At the end of the experiments, the right femur of each mouse was dissected and immediately frozen using liquid nitrogen. Sagittal sections, 7 μm thick, were made as frozen sections in accordance with the manufacturer's instructions and osteoclast stained with tartrate-resistant acid phosphatase (TRAP) for osteoclast. Laser capture microdissection was performed using an LMD6000 (Leica Microsystems, Tokyo, Japan). Osteoclasts were isolated from at least three different mice from each group.

Table 1
Primers used for quantitative real-time PCR.

Gene	Accession no.	Forward primer	Reverse primer
CTSK	NM_007802	ATTGTGACCGTGATAATGTGAACC	ATTGTGACCGTGATAATGTGAACC
NFATc1	NM_198429	CCAAGTCTCTTTCCCCGACATC	AAGCTCGTATGGACCAGAATGTG
TRAP	NM_001102405.1	GGGACAATTTCTACTTCACTGGAG	TCAGAGAACACGTCCTCAAAGG
DC-STAMP	NM_029422	GTTCTTCCTTCCTGTCCTGACC	GGAGATGAGCCGATACAGCGA
RANKL	NM_011613	CCGAGCTGGTGAAGAAATTAGC	CCCAAAGTACGTCGCATCTGG
OPG	NM_008764	CACTCGAACCTCACCACAGAG	TCAATCTCTTCTGGGCTGATCTTC
Type 1 collagen	NM_007742	CAAAGGTCCTCGTGGTGCTG	GGGTCCAGCATTTCCAGAGG
RUNX2	NM_001145920	AAATGCCTCCGCTGTTATGAA	GCTCCGGCCCACAAATCT
PER1	NM_080848	AAAGAAACCTCTGGCTGTTCCTAC	GGAATGTTGCAGCTCTCCAAA
PER2	NM_011066.3	ATGCTCGCCATCCACAAGA	GCGGAATCGAATGGGAGAAT
BMAL1	NM_007489	CCAAGAAAGTATGGACACAGACAAA	GCATTCTTGATCCTTCCTTGGT

*CTSK: *cathepsin K*, NFATc1: *nuclear factor of activated T-cells, cytoplasmic 1*, TRAP: *tartrate-resistant acid phosphatase*, DC-STAMP: *dendritic cell-specific transmembrane protein*, RANKL: *receptor activator of nuclear factor kappa-B ligand*, OPG: *osteoprotegerin*, RUNX2: *runt-related transcription factor 2*, PER1: *period 1*, PER2: *period 2*, BMAL1: *brain and muscle Arnt-like protein 1*.