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The inhibitory effect of beta-lapachone on RANKL-induced osteoclastogenesis

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ABSTRACT

β-lapachone (β-L) is a substrate of reduced nicotinamide adenine dinucleotide (NADH): quinone oxidoreductase 1 (NQO1). NQO1 reduces quinones to hydroquinones using NADH as an electron donor and consequently increases the intracellular NAD+/NADH ratio. The activation of NQO1 by β-L has beneficial effects on several metabolic syndromes, such as obesity, hypertension, and renal injury. However, the effect of β-L on bone metabolism remains unclear. Here, we show that β-L might be a potent inhibitor of receptor activator of nuclear factor-κB ligand (RANKL)-induced osteoclastogenesis. β-L inhibited osteoclast formation in a dose-dependent manner and also reduced the expression of osteoclast differentiation marker genes, such as tartrate-resistant acid phosphatase (*Acp5* or *TRAP*), cathepsin K (*CtsK*), the d2 isoform of vacuolar ATPase V0 domain (*Atp6v0d2*), osteoclast-associated receptor (*Oscar*), and dendritic cell-specific transmembrane protein (*Dc-stamp*). β-L treatment of RANKL-induced osteoclastogenesis significantly increased the cellular NAD+/NADH ratio and resulted in the activation of 5′ AMP-activated protein kinase (AMPK), a negative regulator of osteoclast differentiation. In addition, β-L treatment led to significant suppression of the expression of peroxisome proliferator-activated receptor gamma (PPARγ) and peroxisome proliferator-activated receptor gamma coactivator 1β (PGC1β), which can stimulate osteoclastogenesis. β-L treatment downregulated c-Fos and nuclear factor of activated T-cells 1 (NFATc1), which are master transcription factors for osteoclastogenesis. Taken together, the results demonstrated that β-L inhibits RANKL-induced osteoclastogenesis and could be considered a potent inhibitor of RANKL-mediated bone diseases, such as postmenopausal osteoporosis, rheumatoid arthritis, and periodontitis.

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1. Introduction

Bone remodeling is maintained by the regulation of two essential cell types, bone resorbing osteoclasts and matrix-forming osteoblasts [1]. Osteoclasts differentiate from hematopoietic macrophage/monocyte lineage cells in the presence of macrophage colony-stimulating factor (M-CSF) and RANKL [1,2]. RANKL binds to receptor activator of nuclear factor NF-κB (RANK), followed by signal transduction to intercellular molecules via TNF receptor associated factor 6 (TRAF6) adaptor molecules [2–4]. Subsequently, the extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), p38, Akt, and NF-κB are activated, leading to upregulation of c-Fos expression. Activated c-Fos then binds to the nuclear factor of activated T-cells c1 (NFATc1) promoter region to induce expression of NFATc1, a master transcription factor in osteoclastogenesis [1,2,5,6]. Induced NFATc1 stimulates osteoclast differentiation, activation, and survival by upregulating the expressions of osteoclast marker genes, such as *Acp5* (*Trap*), *CtsK*, *Atp6v0d2*, *Oscar*, and *Dc-stamp* [7,8].

Peroxisome proliferator-activated receptor γ (PPARγ) plays essential roles in the regulation of cellular differentiation,

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development, and metabolism via the transcription of multiple genes [9,10]. PPARγ coactivator 1-beta (PGC1β) is a transcriptional coactivator that modulates energy metabolism by inducing mitochondrial biogenesis [11,12]. Recent studies demonstrated that PPARγ and PGC1β have essential roles in RANKL-induced osteoclast differentiation and function [13,14]. PGC1β is induced by RANKL-mediated osteoclast differentiation through reactive oxygen species (ROS)-induced activation of the cAMP response element–binding protein (CREB), and PGC1β-deficient mice showed impaired bone resorption associated with an increased number of abnormal osteoclasts [13]. In addition, activation of PPARγ by rosiglitazone and RANKL induced PGC1β expression indirectly via inhibition of β-catenin, which stimulates *c-jun*-induced PGC1β transcription. Consequently, PGC1β induced the expression of PPARγ target genes, such as c-Fos, thus activating osteoclast differentiation [14].

β-Lapachone (3, 4-dihydro-2, 2-dimethyl-2H-naphthol [1,2-b] pyran-5, 6-dione; β-L), originally obtained from bark of the lapacho tree, promotes the NQO1-dependent oxidation of NADH to NAD+, which results in an increase in the intracellular NAD+/NADH ratio. β-L was reported initially as a tumor suppressor because of its cytotoxic activity through NQO1, and is known to induce the deaths of multiple tumor cells by apoptosis, autophagy, and necrosis [15–18]. Recently, other functions of β-L were reported. Treatment of aged mice with β-L prevented age-dependent reduction of motor and cognitive function through potentiation of NQO1 activity, and the induction of cellular NAD + levels by β-L decreased the ototoxicity of cisplatin by modulating PARP-1 and SIRT1 activity [19,20]. In addition, the intracellular NAD+/NADH ratio is an important mediator of energy metabolism and cellular homeostasis [21]. However, the effect of β-L on osteoclastogenesis remains unknown.

In this study, we demonstrated the inhibitory effect of β-L on RANKL-mediated osteoclastogenesis. β-L inhibited c-Fos and NFATc1 expression via regulation of the AMPK/PPARγ/PGC1β axis.

2. Materials and methods

2.1. Experimental animals

C57BL/6J mice were purchased from Orient Bio Inc. (Seongnam, Korea) and were maintained in Wonkwang University School of Medicine (Iksan, Korea). All mouse experiments were performed using protocols approved by the Animal Care and Use Committee of Wonkwang University.

2.2. Reagents

β-L was purchased from Sigma Aldrich (St Louis, MO, USA). Recombinant human M-CSF and soluble recombinant mouse RANKL were supplied by Dr. T Kim (KIOM, Daejeon, Korea). All cell culture media, fetal bovine serum (FBS), and supplements were purchased from Hyclone (Rockford, IL, USA). Antibodies against p-ERK, p-JNK, p-p38, and p-IκBα were purchased from Cell signaling Technology (Danvers, MA, USA). Anti-p-AMPK, anti-AMPK, anti-NFATc1, anti-c-Fos, anti-PGC1β, and anti-PPARγ antibodies were purchased from Santa Cruz Biotechnology (Dallas, Texas, USA). The anti-actin antibody was bought from Sigma (Sigma, Korea).

2.3. Cell viability

Cell viability assays were performed using the EZ-Cytox Enhanced Cell viability assay kit (Itsbio, Seoul, Korea), following the manufacturer's instruction. Bone marrow cells (BMs) were collected from the tibia and femora of 6 to 8-week-old C57BL/6J mice by flushing the marrow space with PBS. The cells were plated on 100-mm culture dishes, and cultured in α-minimal essential medium (α-MEM) containing 10% heat-inactivated FBS with M-CSF (30 ng/ml). After 3 days, the adherent cells were used as bone marrow-derived monocytes/macrophages (BMMs), osteoclast precursor cells. BMMs were plated at a density of 1×10^4 cells/well in 96-well plates, and cultured with diverse concentrations of β-L in the presence of M-CSF (30 ng/mL) for 4 days. Cells were incubated with the EZ-Cytox reagent for 4 h at 37 °C. After incubation, the optical density was measured using an ELISA reader (Sunrise, Tecan, Switzerland) at 450 nm.

2.4. Osteoclast formation and pit formation

BMMs were cultured with diverse concentrations of β-L in the presence of M-CSF (30 ng/mL) and RANKL (100 ng/ml) for 4 days. Every 3 days, the culture medium was replaced with fresh α-MEM containing M-CSF and RANKL. After aspiration of the medium, cells were fixed with 10% formalin for 10 min at room temperature, followed by TRAP staining. Cells that were positive for TRAP activity, and contained three or more nuclei, were scored as mature osteoclasts. Total TRAP activity was measured at an absorbance of 405 nm, as described previously [22]. For the bone resorption activity assay, pit formation was measured according to manufacturer's direction. Briefly, BMMs were cultured on a bone resorption assay plate 24 (COSMO BIO, Tokyo, Japan), with (1.5 μM β-L) or without (dimethyl sulfoxide vehicle), in the presence of M-CSF (30 ng/mL) and RANKL (100 ng/mL) for 7 days. The cells were then cleared using bleach, and the resorption pit area was analyzed using Image J (NIH 1.62).

2.5. NQO1 activity and NAD+/NADH ratio assay

BMMs were cultured with or without β-L (1.5 μM) in the presence of M-CSF (50 ng/mL) and RANKL (100 ng/mL) for the indicated times. The NQO1 activity was assayed by using an Abcam NQO1 activity assay kit (Abcam, Cambridge, MA, USA) and the intracellular NAD+/NADH ratio was determined using the Enzychrom NAD+/NADH assay kit (Bioassay Systems, CA, USA) according to the manufacturer's protocols. The details of these experiments are described in supplementary information.

2.6. Reverse transcription PCR (RT-PCR) and quantitative real-time PCR (qPCR)

BMMs were cultured with M-CSF (30 ng/ml) and RANKL (100 ng/ml) in the presence or absence of β-L for 4 days. Total RNA of the cells was harvested using the Trizol reagent (Invitrogen, USA). Total RNA (1 μg) was reverse transcribed to cDNA using random hexamers in a 20-μl volume using the Maxima reverse transcriptase (Thermo scientific). The RT-PCR for NQO1 was performed as previously described, with small modifications [23]. In addition, the mRNA levels for osteoclast marker genes were quantified by qPCR with a veriQuest SYBR Green qPCR Master Mix (USB products, USA) in the StepOnePlus™ system (Applied Biosystems, CA, USA). To control for variation in mRNA concentrations, all results were normalized to the mRNA level of the *Gapdh*. The primers used in this study are listed in supplementary information (S1 Table).

2.7. Western blotting analysis

BMMs were stimulated with or without β-L (1.5 μM) in the presence of M-CSF (30 ng/ml) and RANKL (100 ng/ml) for the indicated times. In some experiment, BMMs were pretreated with

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