Dehydrocostus lactone inhibits NFATc1 via regulation of IKK, JNK, and Nrf2, thereby attenuating osteoclastogenesis

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INTRODUCTION

Osteoclasts are multinucleated giant cells that resorb bone (1). Excessive osteoclast activity above that of bone-forming osteoblasts leads to an imbalance between bone synthesis and breakdown, resulting in pathological outcomes, such as osteoporosis, rheumatoid arthritis, and periodontitis (2). Thus, the control of osteoclast differentiation has therapeutic implications.

Excessive and hyperactive osteoclast activity causes bone diseases such as osteoporosis and periodontitis. Thus, the regulation of osteoclast differentiation has clinical implications. We recently reported that dehydrocostus lactone (DL) inhibits osteoclast differentiation by regulating a nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1), but the underlying mechanism remains to be elucidated. Here we demonstrated that DL inhibits NFATc1 by regulating nuclear factor-κB (NF-κB), activator protein-1 (AP-1), and nuclear factor-erythroid 2-related factor 2 (Nrf2). DL attenuated IkBα phosphorylation and p65 nuclear translocation as well as decreased the expression of NF-κB target genes and c-Fos. It also inhibited c-Jun N-terminal kinase (JNK) but not p38 or extracellular signal-regulated kinase regulated kinase. The reporter assay revealed that DL inhibits NF-κB and AP-1 activation. In addition, DL reduced reactive oxygen species either by scavenging them or by activating Nrf2. The DL inhibition of NFATc1 expression and osteoclast differentiation was less effective in Nfr2-deficient cells. Collectively, these results suggest that DL regulates NFATc1 by inhibiting NF-κB and AP-1 via down-regulation of IkB kinase and JNK as well as by activating Nfr2, and thereby attenuates osteoclast differentiation. [BMB Reports 2020; 53(4): 218-222]

RESULTS

Osteoclasts are differentiated from bone marrow-derived macrophages (BMM) by macrophage-colony-stimulating factor (M-CSF) and the receptor activator of nuclear factor-κB (NF-κB) ligand (RANKL) (3, 4). Binding of RANKL to its receptor, RANK, attracts TNF receptor-associated factor 6 (TRAF6) to RANK and subsequently activates NF-κB and mitogen-activated protein kinases (MAPKs), including c-Jun N-terminal kinase (JNK), p38, and extracellular signal-regulated kinase (ERK) (5). RANKL also stimulates the expression of c-Fos, a major component of activator protein-1 (AP-1) (6). NF-κB and AP-1 mediate the initial induction of the nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1), a key determinant of osteoclast differentiation, which regulates the expression of osteoclastogenic genes, including tartrate-resistant acid phosphatase (TRAP) and cathepsin K (CTSK) (5).

RANKL signaling induces reactive oxygen species (ROS) production via nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Nox) (7), and the ROS contribute to osteoclast differentiation and bone resorption (8, 9). Antioxidants, Nox inhibitors, and antioxidant enzymes inhibit osteoclast formation and function (10-12), and the loss of nuclear factor-erythroid 2-related factor 2 (Nrf2), a transcriptional regulator of many antioxidant enzymes, promotes osteoclastogenesis (13).

Dehydrocostus lactone (DL) has been reported to possess antioxidant activity (14) and protect osteoblast cell line MC3T3-E1 against oxidative stress and dysfunction (15). Recently, we reported that DL inhibits osteoclast differentiation by regulating NFATc1, and attenuates osteoclast activation by modulating migration and lysosome function (16). However, the mechanism of DL-mediated regulation of RANKL-induced NFATc1 activation has yet to be elucidated. In this study, we demonstrated that DL inhibits NFATc1 via regulation of IKK, JNK, and Nfr2, leading to the attenuation of osteoclast differentiation.

Keywords: AP-1, NFATc1, NF-κB, Nfr2, Osteoclast, ROS

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RESULTS

DL inhibits RANKL-induced NF-κB activation by regulating IKK

In a recent study, it was reported that DL inhibits RANKL-induced osteoclastogenesis by negatively regulating NFATc1 (16). In order to investigate the DL-mediated inhibition of NFATc1, the effect of DL on NF-κB that plays an essential role
in NFATc1 expression was explored. DL strongly inhibited RANKL-induced NF-κB activation as revealed in the luciferase reporter assay (Fig. 1A). DL also inhibited the transcription of NF-κB target genes including superoxide dismutase 2 (Sod2), interleukin-1β (Il1β), and macrophage inflammatory protein 1α (Ccl3), and the expression of SOD2 protein (Fig. 1B, C). In addition, DL decreased the phosphorylation of iκBα and p65 (Fig. 1D), and p65 nuclear translocation (Fig. 1E), indicating that DL inhibits RANKL-induced NF-κB activation via down-regulation of IKK.

**DL inhibits RANKL-induced c-Fos expression and JNK activation**

AP-1 is another important regulator of NFATc1 expression. DL inhibited RANKL-induced expression of c-Fos, a major component of AP-1, at both the mRNA and protein levels (Fig. 2A, B). DL also decreased the phosphorylation of JNK, but not that of ERK and p38 (Fig. 2C). As expected, DL inhibited RANKL-induced AP-1 activation as shown in the luciferase reporter assay (Fig. 2D). These results suggest that DL may inhibit AP-1 via down-regulation of c-Fos expression and JNK activation, leading to the attenuation of NFATc1 expression.

**DL reduces ROS by activating Nrf2 or scavenging them**

DL has antioxidant activity and activates Nrf2 in HepG2 cells (14). Nrf2 regulates ROS via induction of antioxidant enzymes and plays an important role in osteoclast differentiation and bone resorption (13). Therefore, the effect of DL on RANKL-induced ROS production and Nrf2 activation was investigated. When BMMs were incubated with RANKL for two days in the presence of DL, the ROS level was lower than in vehicle-treated cells (Fig. 3A, left panel). In order to explore the possibility that DL directly eliminates ROS as an antioxidant, ROS were measured after incubating BMMs with RANKL or H2O2 for 15 min, which was inadequate to express antioxidant enzymes. DL significantly decreased the RANKL- and H2O2-induced increases in ROS (Fig. 3A, center and right panels). In addition, DL increased the expression of Nrf2 and its target genes at both the mRNA and protein levels, irrespective of the presence of RANKL (Fig. 3B, C, D). These results indicate that DL reduces ROS via activation of Nrf2 or by directly scavenging them.

**DL inhibits osteoclast differentiation by activating Nrf2**

Nrf2 has been known to inhibit osteoclast differentiation (13). In order to investigate whether DL inhibits osteoclast differentiation via Nrf2 activation, the effect of DL on osteoclast differentiation of Nrf2-deficient BMMs was explored. In Nrf2-null cells, RANKL-induced osteoclast differentiation was strongly promoted, and DL was less effective in inhibiting osteoclast formation in Nrf2-deficient BMMs than in wild-type cells (Fig. 4A). The expression of NFATc1 and its target genes at both the mRNA and protein levels was also increased by Nrf2 loss, and the DL inhibition of NFATc1 was much less effective in Nrf2-null cells than in wild-type cells (Fig. 4B, C). Thus, DL may regulate NFATc1 via Nrf2 activation, and thereby inhibit osteoclast differentiation.

**DISCUSSION**

A molecular analysis of the inhibitory effect of DL on RANKL-induced osteoclast differentiation indicated that DL suppressed osteoclastogenesis by inhibiting NFATc1 via regulation of IKK, JNK and Nrf2.
NF-κB and AP-1 regulate initial induction of NFATc1 (5). DL inhibited RANKL-induced activation of NF-κB and AP-1 promoters, indicating that DL may suppress NFATc1 expression by inhibiting NF-κB and AP-1. DL inhibited IκBα phosphorylation and p65 nuclear translocation along with decreased expression of NF-κB target genes. In addition, DL inhibits NF-κB by targeting IκKβ (17). Thus, DL appears to suppress NF-κB-dependent NFATc1 expression by inhibiting IKK activity. DL also inhibited RANKL-induced c-Fos expression and JNK activation. Elk-1 is known to regulate the expression of c-Fos, a major component of AP-1 (18). Elk-1 and c-Fos are downstream target genes of NF-κB (19, 20), assuming that NF-κB regulates AP-1 activity. JNK regulates the phosphorylation of Elk-1 as well as of c-Jun, the other major component of AP-1 (21). Thus, DL inhibits RANKL-induced IKK and JNK activation, leading to the suppression of NFATc1 expression via down-regulation of NF-κB and AP-1, resulting in the inhibition of osteoclast differentiation (Fig. 4D).

ROS are generated during osteoclastogenesis and are involved in osteoclast differentiation (7-9). DL protected osteoblastic MC3T3 cells against H2O2-induced oxidative stress (15). In the present study, RANKL stimulation increased ROS, which was attenuated by DL. DL also decreased ROS when cells were exposed to H2O2, indicating that DL may directly eliminate ROS. Nrf2 is known to regulate osteoclast differentiation and function by decreasing ROS via induction of antioxidant enzymes, such as sulfiredoxin (Srx) and peroxiredoxin (Prx) (13). DL has been reported to induce nuclear accumulation of Nrf2 and increase the promoter activity of antioxidant response elements in HepG2 cells (14). DL increased the expression of antioxidant enzymes such as Srx and Prx1 via Nrf2 activation in BMMs. In addition, the previous study showed that Nrf2 loss increases NFATc1 expression via up-regulation of MAPKs (especially JNK) and c-Fos expression (13). Furthermore, the inhibitory effects of DL on NFATc1 expression and osteoclast differentiation were markedly reduced by Nrf2 deficiency, suggesting that DL inhibits RANKL-induced NFATc1 expression and osteoclast differentiation by activating Nrf2 (Fig. 4D). NF-κB and JNK are well-known redox-sensitive molecules (22-24). Therefore, it is likely that DL inhibits RANKL-induced osteoclast differentiation via redox regulation of NF-κB and JNK by scavenging ROS or activating Nrf2.

**MATERIALS AND METHODS**

**Reagents and antibodies**

Recombinant mouse RANKL and human M-CSF proteins were purified as previously described (25). A rabbit polyclonal antibody specific for Srx was prepared as previously described (26). We purchased:

- DL from ChemFaces (Wuhan, China).
- A rabbit polyclonal antibody against β-actin and a monoclonal antibody against NAD(P)H dehydrogenase (quinone) 1 (NQO1) from Abcam (Cambridge, MA, USA).
- Rabbit polyclonal antibodies against phosphorylated (p-) IκBα, p-JNK, p-ERK, and p-p38, and a rabbit monoclonal antibody against p-p65 from Cell Signaling Technology (Danvers, MA, USA).
- A goat polyclonal anti-mouse secondary antibody (Alexa Fluor 546 conjugate) from Thermo Fisher Scientific (Waltham, MA, USA).
- Rabbit polyclonal antibodies against IκBα, JNK1, p38, c-Fos and Nrf2, and mouse monoclonal antibodies against...
A rabbit polyclonal antibody against superoxide dismutase 2 (SOD2) from Upstate Biotechnology (Lake Placid, NY, USA);

- A rabbit polyclonal antibody against Prx1 from Young In Frontier (Seoul, Korea).

**Transfection and luciferase reporter assay**
RAW264.7 cells were transfected for 24 h with 0.45 μg of luciferase reporter plasmid and 0.15 μg of pRLSV40 (internal control) in a 24-well plate using Lipofectamine 3000 reagent (Thermo Fisher Scientific) according to the manufacturer’s instructions. A dual luciferase assay (Promega, Fitchburg, WI, USA) was subsequently performed. The activity of firefly luciferase was normalized to that of the Renilla enzyme and was expressed as a fold increase relative to the normalized value of control cells.

**Preparation of BMMs**
BMMs were prepared as osteoclast precursors from the femurs and tibiae of eight-week-old C57BL/6 male mice, as previously described (27); briefly, bone marrow cells were obtained from the bone marrow cavity using α-minimal essential medium (MEM) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin, then were incubated at 37°C for 1 day. Non-adherent cells were collected and incubated in Gey’s solution for 10 min. Following clarification, the cells were washed with phosphate-buffered saline (PBS), fixed with 50% paraformaldehyde, and stained for TRAP using a leukocyte acid phosphatase cytochemistry kit (MilliporeSigma, Burlington, MA, USA) according to the manufacturer’s instructions. TRAP-positive multinucleated cells containing three or more nuclei were counted as osteoclasts under a light microscope (Nikon, Shinagawa, Tokyo, Japan).

**Statistical analysis**
Data are presented as means ± SDs. Statistical significance was assessed by a one-way analysis of variance (ANOVA) using the Prism software version 5.0 (GraphPad, San Diego, CA, USA). A P < 0.05 was considered statistically significant.

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