Dihydroartemisinin represses osteoclastogenesis of bone marrow macrophages through reduced NFATc1 expression and impaired phosphorylation of IκBα

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ABSTRACT

Osteoclasts are multinucleated bone resorbing cells whose differentiation is regulated by several important signaling pathways. Several lines of evidence indicate that dihydroartemisinin (DHA), an anti-malarial drug, inhibits osteoclast differentiation with little cytotoxicity. However, the detailed inhibitory mechanisms of DHA on osteoclastogenesis from native cells remain to be elucidated. In this study, we investigated the effects of DHA on the differentiation of bone marrow-derived macrophages into osteoclasts. DHA inhibited receptor activator of nuclear factor κ-B ligand (RANKL)-induced osteoclast formation and its bone resorbing activity. Mechanistically, DHA treatment markedly abolished phosphorylation of IκBα, and slightly affected a p38 MAPK-dependent pathway. Moreover, DHA treatment induced down-regulation of nuclear factor of activated T cells cytoplasmic-1 (NFATc1), a master regulator for osteoclast differentiation and its target proteins, such as Src and cathepsin K. These results indicate that DHA represses RANKL-induced osteoclastogenesis of bone marrow macrophages through reduced NFATc1 expression and impaired phosphorylation of IκBα.

Osteoclasts are multinucleated giant cells that are predominantly involved in bone resorption (22). Osteoclast differentiation occurs by cell–cell fusion, and is mainly controlled by two essential factors, receptor activator of nuclear factor κ-B ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) (30). When RANKL associates with receptor activator of nuclear factor κ-B (RANK), osteoclast differentiation signaling is transmitted into six key pathways including, nuclear factor of activated T cells cytoplasmic-1 (NFATc1), nuclear factor kappa B (NF-κB), extracellular signal-regulated kinase (Erk), p38 mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K)/Akt, and Jun N-terminal kinase (JNK) (2, 11, 21, 28). In particular, NFATc1 is a master regulator that induces several osteoclast-marker genes such as calcitonin receptor, tartrate resistant acid phosphatase (TRAP), Src, and cathepsin K (9, 14, 21). In addition, osteoclast-specific genes such as c-fms (an M-CSF receptor), c-fos, (a transcription factor), and TRAF6 (an adaptor protein for the RANKL signaling) are known (2, 6, 23). Therefore, discovery of compounds inhibiting osteoclast differentiation may lead to the development of a new therapeutic method for inflammatory or metabolic bone diseases such as arthritis, periodontitis, and osteoporosis.

Dihydroartemisinin (DHA), originally extracted from the traditional Chinese herb Artemisia annua, is a metabolite of artemisinin (27). DHA has been reported to have pharmacological effects such as anti-inflammatory (29), anti-malarial (5), and anti-tumor (4, 24). Recently, several studies have reported the inhibitory effects of DHA against osteoclast differentiation. Zhou et al. (31) have shown that DHA
serves RANKL-induced osteoclast formation using bone marrow-derived macrophages (BMMs) and murine RAW264.7 cells. Feng et al. (4) also investigated signaling pathways of osteoclastogenesis using RAW264.7 cells, although they examined inhibitory effects and cell viability of osteoclasts using BMMs. In addition, Dou et al. (3) have reported that DHA inhibits lipopolysaccharide (LPS)-induced osteoclastogenesis of mouse bone-marrow macrophages through a mitochondria-dependent pathway. However, it is unclear whether DHA affects the 6 key signaling pathways in osteoclast differentiation: NFATc1, NF-κB, PI3K/Akt, JNK, Erk, and p38 MAPK. Therefore, we investigated the effects of DHA on the signaling pathways of osteoclast differentiation using native mouse bone-marrow macrophages.

MATERIALS AND METHODS

Reagents. Dihydroartemisinin (DHA) was purchased from Sigma-Aldrich (Tokyo, Japan). M-CSF was procured from Kyowa Hakko Kogyo (Tokyo, Japan). Recombinant RANKL was prepared as previously described (16). Antibodies (Abs) were purchased as follows: β-actin (Sigma-Aldrich, Tokyo, Japan), Src (Upstate, Lake Placid, NY, USA), c-fms, c-Fos, TRAF6, and NFATc1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), phospho-ERK1/2 (Thr202/Tyr204), phospho-JNK (Thr183/Tyr185), phospho-p38 MAPK (Thr180/Tyr182), IκBα, phospho-IκBα (Ser32), and phospho-Akt (Ser473) (Cell Signaling Technology, Danvers, MA, USA). Cathepsin K antibody was prepared as previously described (8, 19). The Osteo Assay Stripwell Plate was purchased from Corning (Corning, NY, USA). All other reagents were obtained from Sigma-Aldrich.

Cell culture. Cell culture was performed as described previously (26). Briefly, BMMs were obtained from femurs and tibias of male C57BL/6 mice, and cultured in α-minimal essential medium (α-MEM) (Wako Pure Chemicals, Osaka, Japan) containing 10% FBS with 100 U/mL of penicillin and 100 μg/mL of streptomycin in the presence of M-CSF (50 ng/mL) at 37°C in 5% CO2. After removal of floating cells, the adhering cells were cultured with new medium containing M-CSF (30 ng/mL) and RANKL (50 ng/mL) for the times indicated. TRAP staining was performed using a previously described method (20, 25). TRAP-positive cells with 3 or more nuclei were regarded as mature osteoclasts.

Bone-resorbing activity. BMMs were seeded onto Osteo Assay Stripwell Plate, cultured with M-CSF and RANKL for 3 days until multinucleated osteoclasts were formed, and then DHA was added at the indicated concentrations. After another 3 days of culture, cells were dissolved in 5% sodium hypochlorite. The resorption area was determined using Image J software (http://rsbweb.nih.gov/ij/). The ratios of the resorbed areas to the total areas were calculated as described previously (16).

Cell viability assay. Cells seeded in 96-well cell culture plates were incubated with the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) for 1 h, after which absorbance at 450 nm was measured with a microplate reader (Bio-Rad iMark™, Hercules, CA, USA).

Western blot analysis. Western blot analysis was performed as described previously (18). Briefly, washed cells were lysed with 50 mM Tris-HCl (pH 8.0), 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, and proteinase inhibitor cocktail. The lysate protein (5 μg) was subjected to SDS-PAGE, and was electroblotted onto a polyvinylidene difluoride membrane. After blocking, the blots were probed with various antibodies overnight at 4°C, washed, incubated with HRP-conjugated secondary antibodies, and finally detected with Luminata Forte (Millipore Corporation, Billerica, MA, USA). The immune-reactive bands were analyzed by LAS4000 mini (Fuji Film, Tokyo, Japan).

Quantitative real-time reverse-transcription-polymerase chain reaction (QRT-PCR) analysis. Total RNA was extracted with TRIzol Reagent (Invitrogen), and QRT-PCR analyses were performed as previously described (17). We normalized the values to those of β-actin. Primers are shown in Table 1.

Statistical analysis. All values were expressed as means ± standard deviations. Tukey-Kramer method was used to identify differences between concentrations when ANOVA indicated that a significant difference (*P < 0.05 or **P < 0.01) existed.

RESULTS

DHA inhibits osteoclastogenesis of BMMs
Artemisinin and its derivatives are a new class of anti-malarials with few side effects. Because artemisinin is barely soluble in both water and oil, it is inadequate for pharmaceuticals. Among the derivatives,
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Dihydroartemisinin (DHA) is a more water-soluble analog of artemisinin with the C-10 lactone group replaced by hemiacetal. Figure 1 shows the structure of DHA. To examine effects of DHA on RANKL-induced osteoclast differentiation, we first performed TRAP staining of multinucleated cells of BMMs. TRAP staining revealed that 0.5 μM DHA almost inhibited the formation of multinuclear osteoclasts, and 1.0 μM DHA completely prevented it (Fig. 2A). The detailed inhibitory profile is shown in Figure 2B. The IC50 value of DHA was 0.35 μM. The cell viability was not decreased, but rather slightly increased upon treatment with 0.5–5 μM DHA (Fig. 2C). These results indicate that DHA prevents osteoclastogenesis without cytotoxicity in vitro in the RANKL-induced culture system.

**Effects of DHA on the bone resorbing activity of osteoclasts**

To further test whether DHA reduces bone resorbing activity of osteoclasts, we conducted a pit formation assay with BMM-derived osteoclasts. As shown in Figure 3A, DHA apparently inhibited bone resorbing activity. The calculated resorption area of DHA-treated osteoclasts was decreased in a dose-dependent manner compared with that of untreated osteoclasts (Fig. 3B). Thus, DHA inhibits bone resorbing activity of osteoclasts.

**Effects of DHA on intracellular signaling and expression levels of marker proteins of osteoclasts**

We next investigated the effects of DHA on RANKL-induced intracellular signaling during osteoclast differentiation of BMMs. Western blotting of phosphorylation of IκBα, p38 MAPK, ERK, JNK, and Akt was performed. DHA treatment considerably inhibited phosphorylation and degradation of IκBα (Fig. 4A). Similarly, DHA caused delayed phosphorylation of p38 MAPK (Fig. 4A). Phosphorylation of ERK in DHA-treated cells was inhibited at 30 min after RANKL stimulation compared with control cells (Fig. 4A). However, phosphorylation of JNK and Akt in DHA-treated cells was slightly enhanced (Fig. 4A). These results indicate that DHA mainly blocks an IκBα-dependent pathway, and partially interferes with p38 and ERK-dependent pathways.

To evaluate the effects of DHA on osteoclast differentiation, we analyzed the expression levels of marker proteins of osteoclasts. The expression of NFATc1 was decreased at 0.5 and 1.0 μM of DHA (Fig. 4B). The expression levels of Src and cathepsin K in BMM-derived osteoclasts were also diminished (Fig. 4B). However, the protein levels of c-fms, TRAF6, and c-fos were comparable between DHA-treated and untreated cells (Fig. 4B). These results

| Primers | Forward | Reverse |
|---------|---------|---------|
| Aco2    | ATCGAGCGGGGAAAGACATAC | TGATGGTACAGCCACCTTAGG |
| Atg5b   | GGTTCATCTCCGCAAGACTA  | AAATCCCTCATCGAAGAGCGA |
| β-actin | ACCAGGATCATATGGAGAC   | GTCAGATGTTCATGAGTATG |
| Bcl6    | AGAGCACATGAAACCCATACAA | GCTCCCAAATGTTACAGCATAG |
| Csk     | CAGTCTTCCACGAGAATGTGAT | AGCACAAGAGAGAGAGAA |
| Ifg8    | GGAAGCCCTTACCTGCTGAC  | AAGGTCCAGGTTGCTTCTAG |
| Mafk    | GGTATAAACGGGTCACAGCAG | CGATTTTCTTCGACCTTCC |
| Nfatc1  | TCACTCTGTCCAAACCAAAA  | TCACCTGTTGCTTTCCT |
| Nqo1    | AAGAGGTTTTAGGTCTCTGGCA| AGCTCTCTATGGGCTGTTAG |
| Ppargc1b| CTCGGAGCAGGTCACACC   | GGGCCAGAGTTCCCTTAG |
| Prdm1   | TGTCATACCCAGCACCCCC  | CTTAGGGTGAAGCTGCTGAC |
| Sdhd    | TGTCAGACCCGGCTTATGTG | GTTCCAGGAGAGATCGAG |
| Uqrc2   | AAGTTTCCCGAAGGTTAAA  | GACCATAGTTTCCAGAGAAG |

**Table 1 Primers for mRNA expression analysis**

![Fig. 1 Structure of DHA.](image)
cathepsin K. indicate that DHA blocks the protein expression of NFATc1 and its regulated proteins, such as Src and
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expression of MafB and Irf8, which are negative regulators of NFATc1 (10, 15). Unexpectedly, both genes were not down-regulated by DHA treatment (Fig. 5). In addition, Bcl6, an anti-osteoclastogenic molecule (12), was slightly —though not significantly— up-regulated. B lymphocyte-induced maturation protein-1 (Blimp1; encoded by Prdm1) has been characterized as a transcriptional repressor of MafB, Irf8,
and Bcl6. Interestingly, DHA significantly down-regulated Prdm1 expression (Fig. 5).

Recent studies reported that RANKL-induced mitochondrial gene expression is required for osteoclastogenesis (7). Specifically, peroxisome proliferator-activated receptor-γ coactivator 1β (PGC-1β; encoded by Ppargc1b) is induced by oxidative stress and promotes osteoclastogenesis via stimulation of mitochondrial biogenesis (7). To explore the effect of DHA on oxidative stress and mitochondrial biogenesis during osteoclastogenesis, we measured the mRNA expression levels of Nqo1 (anti-oxidative stress enzyme), Ppargc1b, and mitochondrial enzymes, such as Aco2, Sdhd, Uqcr, and Atp5b. As shown in Figure 6, Nqo1 expression was markedly up-regulated by DHA treatment at day 1. On the contrary, DHA significantly down-regulated Ppargc1b, Uqcr, and Atp5b at day 1 and day 2, as well as Aco2 and Sdhd at day 1 (Fig. 6). Thus, DHA interferes with the expression of cytoprotective enzymes during oxidative stress and mitochondrial genes of osteoclast differentiation.

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**Fig. 5** Effects of DHA on mRNA expression of osteoclast-related genes during oxidative stress. BMMs were cultured with M-CSF (30 ng/mL) and RANKL (50 ng/mL) for 0–2 days in the absence or presence of DHA (0.5 μM). The mRNA expression was analyzed by real-time quantitative RT-PCR, with the relative levels normalized to β-actin. Values are shown as the average and standard deviation of three independent experiments performed in triplicate. **P < 0.01; *P < 0.05. White bars: mock; Black bars: DHA treatment.
Recent studies have shown that DHA has inhibitory effects on osteoclast differentiation. Zhou et al. have demonstrated that DHA suppresses RANKL-induced osteoclast formation using BMMs and murine RAW264.7 cells (31). Although those authors investigated osteoclastogenesis of BMMs concerning NFATc1, ERK and IκBα, signaling pathway, they failed to investigate p38 MAPK, JNK, and Akt-dependent pathways (31). Feng et al. also investigated the signaling pathways of osteoclastogenesis using BMMs and RAW264.7 cells (4). Although
these authors examined inhibitory effects and cell viability of osteoclasts using native BMMs, they investigated signaling pathways such as p38 MAPK, JNK, ERK, and ixB in the cell line RAW264.7 cells, but not in BMMs. Considering that DHA has anti-tumor effects, it is of importance to examine native cells such as BMMs because the cell line RAW264.7 partially displays characteristics of cancer. Meanwhile, Dou et al. have reported that DHA inhibits LPS-induced osteoclastogenesis of BMMs through a mitochondria-dependent pathway (3). However, those authors did not investigate the major signaling pathways, but did apoptosis mechanisms such as caspase-3, Bax, and Bel-2 (3). Several lines of evidence indicated that RANKL induces 6 key signaling pathways in osteoclast differentiation: NFATc1, NF-kB, PI3K/Akt, JNK, ERK, and p38 MAPK. Therefore, in this study, we investigated these 6 key signaling pathways using mouse BMMs.

Inhibitory action of DHA on the key signaling pathways for osteoclastogenesis is likely to be different from previous studies. Indeed, Feng et al. reported that DHA inhibits osteoclastogenesis of RAW264.7 cells via impaired expression of NFATc1 and decreased phosphorylation of Akt, but not IxBα, p38, ERK, and JNK (4). Zhou et al. have reported reduced expression of NFATc1 and IxBα, but not phosphorylation of ERK (31). The present study shows that DHA mainly inhibits NFATc1 expression and decreases phosphorylation of IxBα. Regarding the inhibitory effects of DHA on the signaling pathways, our findings on NFATc1 and IxBα are consistent with the results reported by Zhou et al. (31). However, those authors did not investigate some signaling pathways including PI3K/Akt, JNK, and p38 MAPK. Our present results indicate that phosphorylation of JNK and Akt in 0.5 μM DHA-treated cells was slightly enhanced. These results suggest that DHA-induced activation of JNK and Akt survive osteoclasts.

Consistent with the results of a previous study (31), our results also indicate that DHA inhibits phosphorylation of IxBα. Because Blimp1 has been reported to have an NFxB binding site in its promoter region and to be up-regulated by activation of NFxB signaling pathway (13), inhibition of NFxB pathway by DHA might cause the down-regulation of Blimp1 expression. Unexpectedly, expression levels of Mafb, Irf8, and Bcl6, which are repressed by Blimp1, were comparable with those in control cells. We do not have any data to explain this observation, however, other cellular processes regulated by Blimp1 might affect osteoclastogenesis. Moreover, NFxB also binds to the promoter region of NFATc1 and up-regulates NFATc1 expression (1). Our results suggest that inhibition of NFxB pathway by DHA also diminishes NFATc1 as well as Blimp1 expression.

Osteoclasts contain abundant mitochondria, and RANKL-induced mitochondrial biogenesis is required for osteoclastogenesis. In this study, we show the enhanced levels of anti-oxidant enzyme (Nqo1) and defects of mitochondria (Ppargc1b, Aco2, Sdhd, Uqrc2, and Atp5b) in DHA treated cells. DHA inhibited osteoclastogenesis via suppression of mitochondrial gene expression even at low concentrations.

In conclusion, DHA strongly inhibited osteoclastogenesis of native BMMs via down-regulation of NFATc1 and impaired phosphorylation of IxBα. Thus, DHA might be a new therapeutic compound for inflammatory or metabolic bone diseases.

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