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Deltamethrin inhibits osteoclast differentiation via regulation of heme oxygenase-1 and NFATc1

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ABSTRACT

Deltamethrin is a widely used pyrethroid pesticide. Although the cytotoxicity of deltamethrin has been reported, especially in neuronal cells, there is no information concerning the effects of deltamethrin on osteoclasts (OCLs). In this study, we showed that deltamethrin inhibited OCL differentiation *in vitro*. The effects of deltamethrin on OCL differentiation by receptor activator of nuclear factor kappa-B ligand (RANKL) were investigated in bone marrow-derived macrophages (BMMs) or the murine monocytic cell line RAW-D. Treatment with deltamethrin inhibited OCL formation and bone resorption and up-regulated expression of heme oxygenase-1 (HO-1), an anti-oxidative stress enzyme. Deltamethrin also decreased the protein levels of nuclear factor of activated T cells cytoplasmic-1 (NFATc1), which is a master regulator for OCL differentiation, and concomitantly reduced the expression levels of Src and cathepsin K, which are transcriptionally regulated by NFATc1. The effects of deltamethrin on intracellular signaling during the OCL differentiation of BMMs indicated that deltamethrin-treated OCLs displayed impaired phosphorylation of extracellular signal-regulated kinase, p38 mitogen-activated protein kinase, Jun N-terminal kinase, and Akt, and slightly delayed phosphorylation of inhibitor of nuclear factor kappa B alpha (IκBα) compared with untreated OCLs. Thus, deltamethrin possibly affects bone metabolism by inhibiting OCL differentiation.

1. Introduction

Bone is a rigid but dynamic organ that is continuously remodeled by a coordinated balance between bone formation by osteoblasts and bone resorption by osteoclasts (OCLs) (Chambers, 2000; Teitelbaum, 2000). OCLs are multinucleated cells that are formed by the fusion of monocyte/macrophage precursor cells. Excessive bone resorption by OCLs is involved in several lytic bone diseases, such as osteoporosis, rheumatoid arthritis, and tumor metastasis (Rodan and Martin, 2000). By contrast, impaired bone resorption by OCLs causes various types of osteopetrosis including pycnodysostosis (Saftig et al., 1998). Therefore, it is speculated that chemicals and drugs that regulate OCLs can contribute to the pathogenesis of bone diseases.

OCL differentiation is regulated by several cytokines such as macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor kappa-B ligand (RANKL). M-CSF is a secreted cytokine that promotes the differentiation of hemopoietic stem cells into macrophages and OCLs (Elford et al., 1987). RANKL, a member of the tumor necrosis factor superfamily, is a key cytokine that regulates osteoclastogenesis and bone resorption (Lacey et al., 1998; Yasuda et al., 1998). The association of RANKL with its receptor, RANK, leads to the activation of the essential signaling pathways for OCL differentiation: nuclear factor kappa B (NF-κB), phosphatidylinositol 3-kinase (PI3K)/Akt, Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (Erk), p38 mitogen-activated protein kinase (MAPK) (Darnay et al., 1999; Matsumoto et al., 2000; Zhang et al., 2001), and nuclear factor of activated T cells cytoplasmic-1 (NFATc1)/calcineurin dependent pathways (Takayanagi et al., 2002), respectively. In addition to these signaling pathways, recent studies have shown that oxidative stresses play important roles in osteoclastogenesis (Ha et al., 2004; Lee et al., 2005). Indeed, the induction of heme oxygenase-1 (HO-1),

Abbreviations: Abs, antibodies; OCLs, osteoclasts; RANK, receptor activator of nuclear factor kappa-B; RANKL, receptor activator of nuclear factor kappa-B ligand; BMMs, bone marrow-derived macrophages; TRAP, tartrate-resistant acid phosphatase; NFATc1, nuclear factor of activated T cells cytoplasmic-1; HO-1, heme oxygenase-1; M-CSF, macrophage colony-stimulating factor; NF-κB, nuclear factor kappa B; JNK, Jun N-terminal kinase; Erk, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; α-MEM, α-minimal essential medium; HMGB1, high mobility group box 1; PI3K, phosphatidylinositol 3-kinase; IκBα, inhibitor of nuclear factor kappa B alpha.

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an anti-oxidative stress enzyme, inhibits osteoclastogenesis (Zwerina et al., 2005). We have recently demonstrated that the RANKL-mediated suppression of HO-1 promoted OCL differentiation and, conversely, the induction of HO-1 inhibited osteoclastogenesis (Sakai et al., 2012). Based on these findings, there is a strong possibility that compounds interfering with OCL differentiation signaling pathways and/or inducing HO-1 have inhibitory effects on OCL differentiation.

Among various candidate compounds, deltamethrin ((*S*) a-cyano-3-phenoxybenzyl-(1*R*)-*cis*-3-(2.2-dibromovinyl)-2,2-dimethyl-cyclopropane carboxylate) has been shown to have at least two different pharmacological effects such as inhibition of calcineurin, a calcium-dependent serine-threonine phosphatase (Enan and Matsumura, 1992); and induction of HO-1 in various neuronal cells (Li et al., 2007). Deltamethrin is a synthetic pyrethroid insecticide used for killing various insects by paralyzing their nervous system (Soderlund et al., 2002), and was believed to be one of the safest classes of pesticides for mammals. Previous studies have, however, reported that deltamethrin specifically inhibits calcineurin in the rat brain (Enan and Matsumura, 1992). In addition, deltamethrin has been shown to up-regulate the expression of HO-1 in rat pheochromocytoma PC12 cells which differentiates into neuronal cells (Li et al., 2007). Given that deltamethrin has similar inhibitory effects on NFATc1/calcineurin and/or inductive effects on HO-1 in OCLs, the compound may inhibit osteoclastogenesis. However, no information is available concerning the effects of deltamethrin on OCLs. In this study, we demonstrate the effects of deltamethrin on OCL differentiation *in vitro*.

2. Materials and methods

2.1. Reagents

Deltamethrin was purchased from Wako Pure Chemicals (Osaka, Japan). M-CSF was purchased from Kyowa Hakko Kogyo (Tokyo, Japan). Recombinant RANKL was prepared as described previously (Hu et al., 2008). Antibodies (Abs) were purchased as follows: β-actin (Cat. No. A5060, rabbit polyclonal Ab, 1:20,000; Sigma–Aldrich, St. Louis, MO, USA), Src (Cat. No. 05-184, mouse monoclonal Ab, 1:1000; Upstate Biotechnology, Lake Placid, NY, USA,), HO-1 (Stressgen, rabbit polyclonal Ab, 1:10,000, Ann Arbor, MI, USA), anti-c-fms (Cat. No. sc-692, rabbit polyclonal Ab, 1:1000), anti-RANK (Cat. No. sc-9072, rabbit polyclonal Ab, 1:1000), and anti-NFATc1 (Cat. No. sc-7294, mouse monoclonal Ab, 1:1000) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Abs specific for phospho-ERK1/2 (Cat. No. 9101S, Thr202/Tyr204, rabbit polyclonal Ab, 1:1000), phospho-JNK (Cat. No. 9751S, Thr183/Tyr185, rabbit polyclonal Ab, 1:1000), phospho-p38 (Cat. No. 9211S, Thr180/Tyr182, rabbit polyclonal Ab, 1:1000), phospho-inhibitor of nuclear factor kappa B alpha (IκBα) (Cat. No. 2859S, Ser32, rabbit polyclonal Ab, 1:1000), and phospho-Akt (Cat. No. 9271S, Ser473, rabbit polyclonal Ab, 1:1000) were purchased from Cell Signaling Technology (Danvers, MA, USA). Cathepsin K Ab was prepared as described previously (Kamiya et al., 1998). The Osteo Assay Plate was purchased from Corning (Corning, New York, NY, USA). All other reagents, including PMSF and the protease inhibitor cocktail, were obtained from Sigma–Aldrich.

2.2. Cell culture

Five-week-old male BALB/c mice were obtained from CLEA Japan, Inc. (Tokyo, Japan), and handled in our facilities under the approved protocols of the Nagasaki University Animal Care Committee. The isolation of bone marrow-derived macrophages (BMMs) was performed as described previously (Sakai et al., 2012). The BMMs were replated in culture plates and incubated in α-minimal essential medium (α-MEM) (Wako Pure Chemicals, Code: 135-15175, bicarbonate buffered with L-glutamine) containing 10% FBS with 100 U/mL of penicillin and 100 μg/mL of streptomycin in the presence of M-CSF (50 ng/mL) and RANKL (50 ng/mL) for 60 h or 72 h until the cells differentiated into multinucleated mature OCLs.

The cells were fixed with 4% paraformaldehyde and stained for tartrate-resistant acid phosphatase (TRAP) activity using a previously described method (Hotokezaka et al., 2002). TRAP-positive red-colored cells with three or more nuclei were considered mature OCLs. Murine monocytic cell line RAW-D cells were kindly provided by Prof. Toshio Kukita (Kyushu University, Japan) and cultured in α-MEM containing 10% FBS with RANKL (50 ng/mL) (Watanabe et al., 2004). For bone resorption pit formation, BMMs were seeded onto Osteo Assay Plates coated with thin calcium phosphate films (Corning, New York, NY, USA) and incubated with M-CSF and RANKL for 5 days until mature OCL resorbed calcium phosphate film. Cells were dissolved in 5% sodium hypochlorite. Images of the resorption pit were taken with a reverse phase microscope (Olympus, Tokyo, Japan). The ratios of the resorbed areas to the total areas were calculated using Image J image-analysis software (http://rsbweb.nih.gov/ij/) as described previously (Narahara et al., 2012).

2.3. 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, and then the absorbance at 450 nm was measured with a microplate reader (Bio-Rad iMark™, Hercules, CA, USA).

2.4. Western blot analysis

BMMs were stimulated with or without RANKL in the presence of M-CSF for the indicated amount of time. Cells were rinsed twice with ice-cold PBS, and lysed in a cell lysis buffer (50 mM Tris–HCl [pH 8.0], 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 1 mM PMSF, and proteinase inhibitor cocktail). The protein concentration of each sample was measured with BCA Protein Assay Reagent (Thermo Pierce, Rockford, IL, USA). Lysate protein (5 μg) was applied to each lane. After SDS–PAGE, proteins were electroblotted onto a polyvinylidene difluoride membrane. The blots were blocked with 5% BSA/TBST for 1 h at room temperature, probed with various Abs overnight at 4 °C, washed, incubated with horseradish peroxidase-conjugated secondary Abs (anti-rabbit IgG, Cat. No. 7074, 1:2000, and anti-mouse IgG, Cat. No. 7076, 1:2000; Cell Signaling Technology), and finally detected with ECL-Plus (GE Healthcare Life Sciences, Tokyo, Japan). The immunoreactive bands were analyzed by LAS1000 (Fuji Photo Film, Tokyo, Japan).

2.5. Statistical analysis

All values were expressed as means ± standard deviations (SD) for three independent experiments. The data were analyzed using Student's *t*-test and differences were considered significant at $^{*}P < 0.05$ or $^{**}P < 0.01$.

3. Results

3.1. Deltamethrin inhibits OCL differentiation by up-regulating HO-1 expression

Fig. 1A shows the structure of deltamethrin. A recent study demonstrated that deltamethrin up-regulated the expression of