D-pinitol inhibits RANKL-induced osteoclastogenesis

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ABSTRACT

Numerous studies have indicated that inflammatory cytokines play a major role in osteoclastogenesis, leading to the bone resorption that is frequently associated with osteoporosis. D-pinitol, a 3-methoxy analogue of D-chiroinositol, was identified as an active principle in soy foods and legumes. Here we found that D-pinitol markedly inhibited the receptor activator of nuclear factor kappa B ligand (RANKL)-induced osteoclastic differentiation from bone marrow stromal cells and RAW264.7 macrophage cells. In addition, D-pinitol also reduced RANKL-induced p38 and JNK phosphorylation. Furthermore, RANKL-mediated increase of IKK, IκBα, and p65 phosphorylation and NF-κB-luciferase activity was inhibited by D-pinitol. However, D-pinitol did not affect the proliferation and differentiation of osteoblasts. In addition, D-pinitol also prevented the bone loss inducing by ovariectomy *in vivo*. Our data suggest that D-pinitol inhibits osteoclastogenesis from bone marrow stromal cells and macrophage cells via attenuated of RANKL-induced p38, JNK, and NF-κB activation, which in turn protect bone loss from ovariectomy.

Key Word: D-pinitol; Osteoclasts; Chinese Herb; NF-κB

Running title: D-pinitol inhibits osteoclastogenesis

INTRODUCTION

Osteoporosis ensues from an imbalance bone resorption and bone formation with a net bone loss that may be induced by several conditions, such as hormonal imbalance, diseases, or medications (e.g. corticosteroids or anti-epileptic agents) [1]. Current drugs used to treat osteoporosis include bisphosphonates, calcitonin, and estrogen. These drugs are all bone resorption inhibitors, which maintain bone mass by inhibiting the function of osteoclasts [2]. Since the osteoclasts are responsible for bone resorption, therefore they are one of the main target for treatment of osteoporosis.

Osteoclasts are multinucleated cells formed by the fusion of mononuclear progenitors of the monocyte/macrophage family [3]. *In vitro* maturation of macrophages into osteoclasts requires the presence of stromal cells or their osteoblast progeny [4]. Extensive research in the last few years has indicated that these accessory cells express macrophage colony stimulating factor (M-CSF) and receptor for activation of NF-κB (RANK) ligand (RANKL) that are essential for osteoclastogenesis [5]. RANKL, a member of the TNF superfamily, interacts with the cell surface receptor RANK and in turn recruits TNFR associated factors (TRAF)1, 2, 3, 5, and 6 [6]. The receptor deletion analysis has shown that sequential recruitment of TRAF6 and NF-κB-inducing kinase by RANK leads to NF-κB activation, and recruitment of TRAF2 leads to JNK, p38, and ERK activation [6, 7]. Thus agents that can suppress RANKL signaling can suppress osteoclastogenesis-induced bone loss.

D-pinitol, a 3-methoxy analogue of D-chiroinositol, was identified as an active principle in soy foods and legumes [8]. The mature and dried soybean seeds contain up to 1% D-pinitol. D-pinitol functions as an osmolyte by improving the tolerance to drought stress or heat stress and is involved in reducing the negative effects of osmotic stress and increasing the tolerance of plant tissues to water deficiencies. In addition, D-pinitol has been suggested to possess multifunctional properties, including feeding stimulant, anti-inflammatory, cardioprotective, anti-hyperlipidemic, and creatine retention

promotion properties [9-11]. However, the molecular mechanism of D-pinitol on the osteoclast formation has not been reported. Here, we report that D-pinitol inhibits RANKL-induced osteoclast formation from bone marrow stromal cells and RAW264.7 macrophages. D-pinitol also inhibits the RANKL-induced p38, JNK, and NF-κB activation in macrophages. On the other hand, D-pinitol did not affect the proliferation and differentiation of osteoblasts. Therefore, our data provide evidences that D-pinitol may be an anti-resorption agent for treatment of osteoporosis.

MATERIALS AND METHODS

Materials

D-pinitol was purchased from Wako Chemicals (Osaka, Japan). Anti-mouse and anti-rabbit IgG-conjugated horseradish peroxidase, rabbit polyclonal antibodies specific for p-IKK, IKK, p-IκBα, IκBα, p-p38, p38, p-JNK, JNK, p-p65, and p65 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). JNK and p38 kinase assay kit were purchased from Cell Signaling Technology, Inc., (Danvers, MA, USA). Recombinant human M-CSF and RANKL were purchased from R&D Systems (Minneapolis, MN, USA). pSV-β-galactosidase vector, luciferase assay kit was purchased from Promega (Madison, MA, USA). The NF-κB luciferase plasmid was purchased from Stratagene (La Jolla, CA, USA). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture

Bone marrow cells were prepared by removing from femurs of 6-8 week-old Sprague-Dawley rats and flushing the bone marrow cavity with α-MEM which was supplemented with 20 mM HEPES and 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml). The non-adherent

cells (hematopoietic cells) were collected after 24 h and used as osteoclast precursors. Cells were seeded at a density of 1×10^6 cells/well in 24-well plates in the presence of human recombinant soluble RANKL (50 ng/ml) for 7 days. The culture medium was replaced every 3 days.

Murine RAW264.7 cells (a mouse macrophage cell line obtained from American Type Culture Collection) were grown in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. For differentiation of osteoclasts, RAW264.7 cells (2×10^4, in 24-well plate) were cultured in the presence of RANKL (50 ng/ml) for 5 days. The culture medium was replaced every 3 days.

The human osteoblast-like cell line MG-63 and MC3T3E-1 were purchased from American Type Culture Collection. Cells were cultured in α-MEM supplemented with 10% FBS and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin).

Osteoclast differentiation assay

Osteoclast formation was measured by quantifying cells positively stained by tartrate-resistant acid phosphatase [TRAP (Acid Phosphatase Kit 387-A; Sigma-Aldrich, St. Louis, MO, USA)]. Briefly, the cells were fixed for 30 s and then stained with Naphthol AS-BI phosphate and a tartrate solution for 1 h at 37℃, followed by counterstaining with a hematoxylin solution. Osteoclasts were determined to be TRAP-positive staining multinuclear (>3 nuclei) cells using light microscopy. The total number of TRAP-positive cells and the number of nuclei per TRAP-positive cell in each well were counted. The morphological features of osteoclasts were also photographed [12].

Quantitative real time PCR

Total RNA was extracted from osteoblasts using a TRIzol kit (MDBio Inc., Taipei, Taiwan). Reverse transcription was performed using 2 μg total RNA and an oligo(dT)

primer [13]. Quantitative real-time PCR (qPCR) was carried out using TaqMan® one-step PCR Master Mix (Applied Biosystems, CA). 100 ng of total cDNA were added per 25-µl reaction with sequence-specific primers and Taqman® probes. All target gene primers and probes were purchased commercially, including β-actin as an internal control (Applied Biosystems, CA). qPCR assays were carried out in triplicate on a StepOnePlus sequence detection system. The cycling conditions were 10-min polymerase activation at 95 ℃ followed by 40 cycles at 95 ℃ for 15 s and 60 ℃ for 60 s. The threshold was set above the non-template control background and within the linear phase of target gene amplification to calculate the cycle number at which the transcript was detected (denoted C_T).

Cell proliferation

Cell proliferation was determined by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (MTT) assay [14, 15]. After treatment with D-pinitol for 48 h, cultures were washed with PBS. MTT (0.5 mg/ml) was then added to each well, and the mixture was incubated for 2 h at 37℃. Culture medium was then replaced with an equal volume of dimethyl sulfoxide to dissolve formazan crystals. After shaking at room temperature for 10 min, absorbance was determined at 550 nm using a microplate reader (Bio-Tek, Winooski, VT).

Flow cytometry

Differentiated cells were harvested and washed twice with FACS washing buffer (1% FCS and 0.1% NaN3 in PBS), followed by incubation with monoclonal anti-αvβ3 integrin-FITC antibody at 4℃ for 20 min. After washing with FACS washing buffer three times, the fluorescence of cells was analyzed with a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA) [16].

Western blot analysis

The cellular lysates were prepared as described previously [17, 18]. Proteins were resolved on SDS-PAGE and transferred to Immobilon polyvinyldifluoride (PVDF) membranes. The blots were blocked with 4% BSA for 1 h at room temperature and then probed with rabbit anti-mouse antibodies against IκBα, p-IκBα, p-p38, p-JNK, or JNK (1:1000) for 1 hr at room temperature. After three washes, the blots were subsequently incubated with a donkey anti-rabbit peroxidase-conjugated secondary antibody (1:1000) for 1 hr at room temperature. The blots were visualized by enhanced chemiluminescence using Kodak X-OMAT LS film (Eastman Kodak, Rochester, NY).

Transfection and reporter gene assay

Cells were co-transfected with 0.8 μg κB-luciferase plasmid, 0.4 μg β-galactosidase expression vector. The cells were grown to 80% confluent in 12 well plates and were transfected on the following day by Lipofectamine 2000 (LF2000; Invitrogen, Carlsbad, CA). DNA and LF2000 were premixed for 20 min and then applied to the cells. DMEM containing 20% FBS was added 4 h later. After 24 h transfection, the cells were then incubated with the indicated agents. After further 24 h's incubation, the media were removed, and cells were washed once with cold PBS. To prepare lysates, 100 μl reporter lysis buffer (Promega, Madison, WI) was added to each well, and cells were scraped from dishes. The supernatant was collected after centrifugation at 13,000 rpm for 2 min. Aliquots of cell lysates (20 μl) containing equal amounts of protein (20–30 μg) were placed into wells of an opaque black 96-well microplate. An equal volume of luciferase substrate was added to all samples, and luminescence was measured in a microplate luminometer. The value of luciferase activity was normalized to transfection efficiency monitored by the co-transfected β−galactosidase expression vector [19].

Ovariectomy-induced osteoporosis

Female ICR mice (4 week-old; 22~28 g) were used for this study. Mice were ovariectomized bilaterally under trichloroacetaldehyde (100 mg/kg) anesthesia and control mice were sham-operated (Sham) for comparison, after 2 days the mice were treated with various concentrations of D-pinitol by orally feeding them every 2 days for 4 weeks. Bone mineral density and bone mineral content of full-body were determined by dual-energy x-ray absorptiometer (DEXA; XR-26; Norland, Fort Atkinson, WI) after treated with D-pinitol using a mode for small subjects as described previously [12, 20] . All protocols complied with institutional guidelines and were approved by Animal Care Committee of China Medical University.

Statistics

The values given are means ± S.E.M. The significance of difference between the experimental groups and control was assessed by Student's *t* test. The difference is significant if the *p* value is <0.05.

RESULTS

D-pinitol inhibits osteoclastogenesis from bone marrow stromal cells and macrophages

Osteoclasts are specialized monocyte/macrophage family members that differentiate from bone marrow hematopoietic precursors [3]. Cultures of osteoclast precursors in the presence of RANKL (50 ng/ml) for 7 days induced the formation of large mature osteoclasts with multi-nuclei characterized by the acquisition of mature phenotypic markers, such as TRAP (Fig 1A). D-pinitol markedly inhibited the differentiation of osteoclast in a dose-dependent manner (Fig. 1A&B). The stimulating effect on osteoclast differentiation was also observed in murine RAW264.7 macrophages. Culturing for 5

days in RAW264.7 cells, D-pinitol inhibited the formation of TRAP-positive cells (Fig. 1C). In addition, treatment of bone marrow stromal cells or RAW264.7 macrophages for 3 days with D-pinitol (3 or 10 μM) did not affect cell viability, which was assessed by MTT assay (data not shown). To the functional characterization, we examined whether αvβ3 integrin was expressed in the differentiated osteoclast [21]. The undifferentiated RAW264.7 macrophages expressed lower levels of αvβ3 integrin (Fig. 1D). Incubation of cells with RANKL for 5 days increased the expression of αvβ3 integrin (Fig. 1D). On the other hand, treatment with D-pinitol inhibited the expression of αvβ3 integrin (Fig. 1D). These data suggest that D-pinitol inhibits osteoclastogenesis from bone marrow stromal cells and macrophages.

D-pinitol inhibits the RANKL-induced p38 and JNK activation

p38 and JNK have been implicated in the mediation of RANKL-regulated osteoclastogenesis [22]. Stimulation by RANKL markedly induced the phosphorylation of p38 and JNK (Fig. 2A&B). RANKL-induced increase in p38 and JNK phosphorylation were inhibited by the pretreatment of cells with D-pinitol (Fig. 2A&B). In addition, D-pinitol also reduced RANKL-induced p38 and JNK kinase activity by determining phosphorylation of one of its substrates ATF-21 and c-Jun, respectively (Fig. 2C&D). However, D-pinitol did not affect the basal level of p38 and JNK kinase activity (Supplementary Fig. S1).

D-pinitol inhibits RANKL-induced NF-κB activation

Activation of transcription factor NF-κB is also involved in osteoclast differentiation [23]. Activation of NF-κB by most agents requires phosphorylation of IKK, IκBα, and p65. Treatment of cells with D-pinitol reduced RANKL-induced IKK, IκBα, and p65 phosphorylation (Fig. 3A). However, D-pinitol did not affect the basal level of p65 phosphorylation (Supplementary Fig. S1). In addition, D-pinitol also inhibited

RANKL-increased κB-luciferase activity (Fig. 3B). These results suggest that p38, JNK, and NF-κB signaling pathways are necessary for D-pinitol-reduced osteoclastogenesis.

D-pinitol did not affect the proliferation and differentiation of osteoblasts

We next determined the effect of D-pinitol on the cell proliferation of MG-63 and MC3T3-E1 osteoblastic cells by MTT assay. As shown in Fig. 4A, D-pinitol did not exhibit significant effects on cell growth after 48 h of treatment in either cell line (BMP-2-induced cell growth was used for positive control). Differentiated osteoblasts exhibit elevated alkaline phosphatase (ALP), bone morphogenetic protein-2 (BMP)-2, and osteopontin (OPN) activity [12]. Treatment of osteoblasts with D-pinitol did not affect ALP, BMP-2, and OPN mRNA expression (Fig. 4B-D) (BMP-2-induced ALP, BMP-2, and OPN expression were used for positive control).

Inhibition of bone loss by D-pinitol in ovariectomized mice

To examine the effect of D-pinitol on bone loss, female mice were induced osteoporosis by ovariectomy. Ovariectomized mice showed a decreased bone mineral density and bone mineral content of total body. Treatment with D-pinitol (10-50 mg/kg) for 4 weeks inhibited the loss of bone mineral density and bone mineral content in a dose-dependent manner (Fig. 5A&B). Blood concentration of C-terminal telopeptide of collagen can reflect the osteoclastic activity. As shown in Fig. 5C, D-pinitol also inhibited the increase of osteoclast activity by ovariectomy.

DISCUSSION

D-pinitol has various biological activities such as anti-hyperlipidemic, antioxidant, cardioprotective, and anti-inflammatory functions [9-11]. Numerous studies have indicated that inflammatory cytokines play a major role in osteoclastogenesis, leading to

the bone resorption that is frequently associated with osteoporosis [24]. Our current study showed that D-pinitol inhibited osteoclastogenesis from bone marrow stromal cells and macrophages. The RANKL-induced p38, JNK, and NF-κB activation was attenuated by D-pinitol. D-pinitol also prevented ovariectomized-induced the bone loss *in vivo*.

To evaluate the effects of D-pinitol on the formation of osteoclasts, we used a bone marrow culture system from healthy rats and murine macrophages to generate osteoclasts with *in vitro* RANKL stimulation. We showed in this study for the first time that D-pinitol can inhibit the formation of osteoclast. MAPK family members are proline-directed serine/threonine kinases that play important roles in cell growth, differentiation, and apoptosis [24]. In the present study, we used a homogenous clonal population of murine monocytic RAW 264.7 cells to clarify the effects of D-pinitol on the signaling pathways in osteoclast progenitor cells. We found that D-pinitol strongly inhibited the phosphorylation of p38 and JNK in RAW 264.7 cells stimulated with RANKL. In addition, D-pinitol also reduced RANKL-induced p38 and JNK activity. These findings suggest that the inhibitory effect of D-pinitol on osteoclast differentiation into mature osteoclasts may be related to the responsible for the regulation of activation of p38 and JNK. In addition, we also demonstrated that D-pinitol inhibited RANKL-induced IKK, IκBα and p65 phosphorylation and NF-κB activation in RAW 264.7 cells. These data support the hypothesis that D-pinitol inhibits NF-κB activity in macrophages, resulting in inhibition of osteoclasts formation. On the other hand, we also found that p38 and JNK inhibitor reversed the D-pinitol-reduced NF-κB luciferase activity (data not shown). These data suggested that p38 and JNK are upstream molecules in D-pinitol-mediated NF-κB activation. Therefore, D-pinitol reduced RANKL-induced osteoclastogenesis through p38/JNK and NF-κB pathway.

Bone is a complex tissue composed of several cell types which are continuously undergoing a process of renewal and repair. When resorption and formation of bone are not coordinated and bone breakdown overrides bone building, osteoporosis results.

Current drugs used to treat osteoporosis include bisphosphonates, calcitonin and estrogen. These are all anti-resorption medications, which maintain bone mass by inhibiting the function of osteoclasts [1]. Their effects in increasing or recovering bone mass is relatively small, certainly no more than 2% per year [2]. It is desirable, therefore, to have satisfactory bone-building agents, such as teriparatide, that would stimulate new bone formation and correct the imbalance of trabecular microarchitecture that is characteristic of established osteoporosis [25]. Since new bone formation is primarily a function of the osteoblast, agents that act by either increasing the proliferation of cells of the osteoblastic lineage or inducing differentiation of the osteoblasts can enhance bone formation [25, 26]. Unfortunately we found that D-pinitol did not affect the proliferation of two osteoblast cell lines. D-pinitol also did not affect the differentiation marker including ALP, BMP-2, and OPN of osteoblasts. These data suggested the anti-resorption effect but not bone formation activity of D-pinitol.

In conclusion, the present study demonstrated that D-pinitol inhibits the osteoclastogenesis from bone marrow stromal cells and macrophages. D-pinitol also attenuated the RANKL-induced p38, JNK, and NF-κB activation. Therefore, D-pinitol may show beneficial effects in reducing the osteoclast formation and activity to promote bone health in osteoporosis therapy.

ACKNOWLEDGMENTS

This work was supported by grants from National Science Council of Taiwan (NSC100-2320-B-039-032; NSC100-2320-B-039-028-MY3).

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FIGURE LEGENDS

Fig. 1 Inhibition of osteoclast differentiation by D-pinitol.

Osteoclast precursors isolated from long bones of adult male rats were plated on a 24-well plate at 1×10^6 cells/well and cultured in the presence of RANKL (50 ng/ml) for 7 days. Following TRAP staining, the cells with more than 3 nuclei were counted (A). Compared with RANKL, D-pinitol treatment markedly inhibited the differentiation of osteoclast. The quantitative data are shown in B. RAW264.7 cells were seeded at 2×10^4 and incubated for 5 day with RANKL (50 ng/ml) without or with D-pinitol. Treatment with D-pinitol inhibited osteoclastogenesis in a concentration-dependent manner *: $p<0.05$ as compared with RANKL-treated group. (C). RAW264.7 cells were treated with RANKL (50 ng/ml) without or with D-pinitol for 5 days. After incubation, cells were treated with anti-αvβ3 integrin antibody and analyzed by flow cytometry *: $p<0.05$ as compared with RANKL-treated group. (D). Results are expressed as the mean ± S.E.M. of four independent experiments. *: $p<0.05$ as compared with control group. #: $p < 0.05$ as compared with RANKL-treated control group

Fig. 2 D-pinitol inhibits the RANKL-induced p38 and JNK activation.

RAW264.7 cells were pretreated with D-pinitol for 30 min followed by stimulation with RANKL (50 ng/ml) for 120 min, and the p38 and JNK phosphorylation was determined by Western blotting (A&B). RAW264.7 cells were pretreated with D-pinitol for 30 min followed by stimulation with RANKL (50 ng/ml) for 120 min, and the p38 and JNK kinase activity was determined by p38 and JNK kinase assay kit (C&D).

Fig. 3 D-pinitol inhibits RANKL-induced NF-κB activation.

RAW264.7 cells were pretreated with D-pinitol for 30 min followed by

stimulation with RANKL (50 ng/ml) for 120 min, and the IKK, IκBα and p65 phosphorylation was determined by Western blotting (A). Cells were transfected with κB-luciferase expression vector and then pretreated with D-pinitol for 30 min before incubation with RANKL (50 ng/ml) for 24 hr. Luciferase activity was then assayed. Results are expressed as the mean ± S.E.M. (B). Results are expressed as the mean ± S.E.M. of four independent experiments. *: $p<0.05$ as compared with control group. #: $p < 0.05$ as compared with RANKL-treated control group.

Fig. 4 D-pinitol did not affect the cell proliferation and differentiation of osteoblasts.

(A) MG-63 or MC3T3-E1 cells were treated with D-pinitol or BMP-2 (10 ng/ml) for 2 days and cell proliferation were evaluated by the MTT assay. (B-D) MG-63 or MC3T3-E1 cells were treated with D-pinitol or BMP-2 (10 ng/ml) for 24 h, the ALP, BMP-2, and OPN mRNA expression was examined by qPCR. Data are presented as mean ± S.E. (n=4).

Fig. 5 Inhibition of ovariectomy-induced a decrease of bone mineral density and bone mineral content by D-pinitol.

Female ICR mice were given a sham operation or were ovariectomized. Mice that underwent ovariectomized were treated with the indicated concentrations of D-pinitol (10, 30, 50 mg/kg) by oral feeding. Total body bone mineral density (A), bone mineral content (B), and serum C-terminal telopeptides of type-1 collagen (C) were determined 4 weeks after surgery. Data are presented as mean ± S.E. (n=8-10 mice/group). *: $p<0.05$ as compared with sham. #: $p < 0.05$ as compared with ovariectomized- group.