Suppression of NF-κB activation blocks osteoclastic bone resorption during estrogen deficiency

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Abstract. Postmenopausal osteoporosis stems from an imbalance in osteoclastic bone resorption with respect to osteoblastic bone formation, a consequence of estrogen deficiency. The nuclear factor-κB (NF-κB) signal transduction pathway is critical for osteoclast formation and resorption, and suppression of NF-κB activation has been shown to block bone resorption in vitro, and to ameliorate inflammatory bone loss in vivo. The use of NF-κB antagonists to blunt the bone loss associated with estrogen deficiency however, has not been previously reported. In this study, we investigated whether pharmacological suppression of NF-κB signaling protects mice against ovariectomy (ovx)-induced bone loss. Ovx mice were treated with the potent NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC) for 4 weeks and bone mineral density (BMD) and indices of bone structure quantitated by μCT, and on

Introduction

Bone homeostasis is achieved by a delicate balance between osteoclastic bone resorption and osteoblastic bone formation. Numerous pathological processes have the capacity to disrupt this equilibrium leading to conditions where the rate of bone resorption outpaces the rate of bone formation leading to osteoporosis. Osteoporosis is a serious worldwide health threat. Postmenopausal osteoporosis is the archetypal osteoporotic condition in women after menopause and accounts for 71% of total fractures (1). The National Osteoporosis Foundation has estimated that one in two women over the age of 50 will have an osteoporosis-related fracture in their remaining lifetimes. Fractures incur monumental health care costs to patients and the community, and can have a serious negative impact on mobility and quality of life. Hip fractures almost always require surgery and the rate of mortality is considerable (2).

The NF-κB signal transduction pathway has long been recognized as critical for osteoclast development and function (3,4) and double knockout of p50 and p52 NF-κB subunits leads to osteoporosis due to a severe defect in osteoclast differentiation in these mice (5). Furthermore, suppression of NF-κB signaling in vitro blocks osteoclast activation (6). Recent studies have demonstrated the utility of pharmacological NF-κB suppression to inhibit osteoclast formation and activity associated with multiple myeloma in vitro (7) and in an animal model of rheumatoid arthritis in vivo (8).

Based on these data we hypothesize that pharmacological suppression of NF-κB in vivo may be an effective approach for ameliorating bone loss induced by estrogen deficiency by suppressing osteoclastic bone resorption.

PDTC is a well characterized NF-κB inhibitor with demonstrated efficacy and good tolerability in numerous in vivo studies of inflammation (9-11). PDTC acts by stabilizing cytosolic IκB-α thus preventing the activation and nuclear translocation of the NF-κB transcription factor (11).

In this study, we injected ovx mice with PDTC and investigated its impact on BMD, physical indices of bone structure using μCT, and on in vivo biochemical markers of bone resorption (CTX). We show that NF-κB blockade by in vivo PDTC treatment significantly protects mice from bone loss following ovx. PDTC treatment alleviated bone loss by suppression of ovx-induced osteoclastic bone resorption. These data suggest that targeting of the NF-κB signal transduction pathway may constitute a novel therapeutic strategy for the amelioration of osteoporosis associated with numerous pathological conditions including postmenopausal osteoporosis.
Materials and methods

Chemicals. Ammonium PDTC and all other reagents were purchased from the Sigma-Aldrich Chemical Corporation (St. Louis, MO), unless otherwise indicated.

Animals. All animal procedures were approved by the Emory University Animal Care and Use Committee and were used in accordance with the NIH Laboratory Guide for the Care and Use of Laboratory Animals.

Female C57BL/6 mice 6 weeks of age were purchased from Charles River Laboratories (Raleigh, NC). Mice were acclimated for 4 weeks in the animal facility before use. Mice were housed in sterile polycarbonate cages with corn cob bedding on static racks and given gamma-irradiated 5V02 phytoestrogen-free mouse chow (Purina Mills, St. Louis, MO), and autoclaved water ad libitum. The animal facility was kept at 23±1°C, with 50% relative humidity and a 12/12 light/dark cycle.

Ovariectomy. Mice (10 weeks of age) were either sham-operated, or ovx. Ovx mice were divided into two groups receiving either vehicle (PBS) or PDTC at a dose of 80 mg/kg intraperitoneally 3 times per week.

Quantitation of BMD. In vivo BMD measurements of femurs (left and right femurs were averaged for each mouse) were made by DXA using a PIXIImus2 bone densitometer (GE Medical Systems) as previously described (12).

μCT. μCT was performed using a μCT40 scanner, (Scanco Medical, Bassersdorf, Switzerland). Briefly, after careful dissection of muscle tissue, the right femur was fixed in 10% neutral-buffered formalin for 48 h and stored in 70% ethanol at 4°C until analysis. μCT analysis was performed by an operator blinded to the nature of the specimens. Bones were scanned at a resolution of 12 μm. For each sample 50 slices were taken at the identical starting position and covering a total area of 600 μm proximal to the distal metaphyses. Static trabecular measurements were made using a cylindrical core sample that excluded cortical bone, with contouring for all subsequent slices. For visual representation one representative sample from each group was randomly selected for detailed three-dimensional (3D) reconstruction of core images from individual μCT slices.

Biochemical indices of bone resorption. CTx, a sensitive biochemical marker of bone resorption, was measured in mouse serum following an overnight fast, by ‘RatLaps’ a rodent-specific CTx ELISA (Nordic Bioscience Diagnostics A/S, Herlev, Denmark).

Statistical analysis. Statistical significance was determined using GraphPad InStat version 3 software for Windows XP (GraphPad Software, San Diego, CA). Multiple comparisons were performed by One-way ANOVA with Tukey-Kramer post test for parametric data, and Kruskal-Wallis post test for nonparametric data. P≤0.05 was considered statistically significant. Groups were tested for normal distribution using the Kolmogorov-Smirnov test.

Results

Pharmacological suppression of NF-κB signaling by PDTC treatment significantly reduces loss of BMD following ovx.

BMD was quantitated by DXA at the femurs (average of left and right femurs were averaged for each mouse) at baseline (time of surgery) and 4 weeks after surgery. While ovx led to a significant reduction in BMD, administration of PDTC significantly protected mice from ovx-induced bone loss at 4 weeks (Fig. 1).

Suppression of NF-κB signaling by PDTC treatment significantly reduces the decline in indices of bone structure measured by μCT, following ovx. We further verified the DXA findings using μCT analysis of femurs from each group of mice (Table I). The data demonstrate a significant 38% decrease in trabecular bone volume per tissue volume (BV/TV) in vehicle-treated mice following ovx. This decrease resulted from reduced BV as TV was not significantly changed. Decreased BV resulted from a significant 14% decrease in trabecular thickness (Tb.Th.), 18% decrease in trabecular number (Tb.N.), and 43% decrease in trabecular connection density (Conn.D.), with a corresponding 22% increase in trabecular separation (Tb.Sp.). Bone architecture denoted by the structure model index (SMI) was not significantly altered. Finally, volumetric trabecular bone volume BMD (TV.D) was decreased by 28% in ovx mice receiving vehicle. By contrast, ovx mice treated with PDTC were significantly protected from ovx-induced bone loss, and were not significantly different to sham groups. Protection was however observed to be incomplete and varied from ~50 to 70% protection depending on the index examined.
Representative 3D reconstructions for one femur from each group selected at random is show for each group in Fig. 2. 

Biochemical analysis of in vivo bone resorption. To quantitate indices of in vivo bone resorption we measured serum CTx, a specific and sensitive marker. The data revealed a significant 58% elevation in the rate of bone resorption induced by ovx (Fig. 3). PDTC treatment completely prevented the increase in bone resorption four weeks after the ovx.

Discussion

Our data demonstrate that suppression of NF-κB in vivo is an effective strategy for ameliorating osteoclastic bone breakdown under conditions of estrogen deficiency. Although PDTC treatment significantly reduced ovx-induced bone loss and PDTC-treated ovx mice were not statistically significantly different from sham mice for any of the μCT parameters, indices of bone structure nonetheless, fell short of completely returning to sham levels, falling typically between 50 and 70% protection. However, four weeks after PDTC treatment

<table>
<thead>
<tr>
<th>Morphometric indices</th>
<th>Sham</th>
<th>Ovx + Vehicle</th>
<th>Ovx + PDTC</th>
<th>Δ Sham vs. Ovx</th>
<th>Δ Sham vs. Ovx + PDTC</th>
</tr>
</thead>
<tbody>
<tr>
<td>TV (mm³)</td>
<td>1.6±0.1</td>
<td>1.6±0.1</td>
<td>1.70±0.1</td>
<td>0.0</td>
<td>6.3</td>
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<tr>
<td>BV (mm³)</td>
<td>0.10±0.01</td>
<td>0.06±0.02a</td>
<td>0.09±0.02b</td>
<td>-40.0</td>
<td>-10.0</td>
</tr>
<tr>
<td>BV/TV (%)</td>
<td>6.0±0.5</td>
<td>3.7±1.1a</td>
<td>5.1±0.8b</td>
<td>-38.3</td>
<td>-15.0</td>
</tr>
<tr>
<td>Tb.Th. (μm)</td>
<td>45.5±4.6</td>
<td>39.3±4.1a</td>
<td>42.2±2.4</td>
<td>-13.6</td>
<td>-7.3</td>
</tr>
<tr>
<td>Tb.N./mm</td>
<td>3.3±0.2</td>
<td>2.7±0.2b</td>
<td>3.1±0.3</td>
<td>-18.2</td>
<td>-6.1</td>
</tr>
<tr>
<td>Tb.Sp. (μm)</td>
<td>310.7±17.7</td>
<td>377.7±25.3a</td>
<td>337.8±34.2b</td>
<td>21.6</td>
<td>8.7</td>
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<tr>
<td>SMI</td>
<td>2.8±0.2</td>
<td>2.8±0.3</td>
<td>2.8±0.2</td>
<td>0.0</td>
<td>0.0</td>
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<tr>
<td>Conn.D./mm³</td>
<td>62.6±13.4</td>
<td>35.9±12.3a</td>
<td>55.2±14.9b</td>
<td>-42.7</td>
<td>-11.8</td>
</tr>
<tr>
<td>TV.D (mg HA/cm³)</td>
<td>134.9±18.4</td>
<td>96.9±31.6a</td>
<td>111.8±26.05</td>
<td>-28.2</td>
<td>-17.1</td>
</tr>
</tbody>
</table>

Table 1. μCT morphometric analysis of bone structure in sham-operated mice, ovx mice, and ovx mice receiving PDTC administration.

Trabecular indices of bone structure were computed from μCT scans. Data are presented as Mean ± SD of 6 mice per group. Percentage change between Sham and Ovx + Vehicle and between Sham and Ovx + PDTC are shown in the last two columns. aP≤0.05 vs. Sham; bP≤0.05 vs. Ovx + Vehicle, One-way ANOVA with Tukey-Kramer post test: There were no statistically significant differences between Sham and Ovx + PDTC for any of the indices.

Figure 2. 3D μCT reconstruction of femurs. One representative femur from each group (sham-operated, ovx receiving vehicle and ovx receiving PDTC) was reconstructed from individual μCT scans at the distal metaphysis.

Figure 3. Biochemical markers of in vivo bone resorption. Four weeks after surgery CTxs, a marker of bone resorption, was quantitated in the serum of each mouse, n=9-17 mice/group. Data are presented as mean ± SEM. aP≤0.05; One-way ANOVA; Kruskal-Wallis post test.

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CTx levels indicated that bone resorption had completely returned to sham levels. These data suggest that the dose and/or frequency of administration of PDTC used in this particular experiment may have been inadequate to completely stall the initial wave of osteoclastic bone loss, but was ultimately effective in restoring osteoclastic activity to sham levels by 4 weeks of treatment, thus preventing bone loss during the later stages of the study.

In addition, at the dose of PDTC used bone resorption returned to sham levels by 4 weeks of treatment, but did not completely abrogate bone resorption. Maintaining a basal rate of bone resorption is advantageous for remodeling of trabecular bone structure to achieve an optimal mechanical strength, and consequently the maintenance of a basal osteoclastic activity is desirable. It remains unknown as to whether PDTC administration in excess of 4 weeks would ultimately lead to a more pronounced suppression of osteoclastic activity.

At the relatively low dose of 80 mg/kg, 3 times per week as used in this experiment, no ill effects were observed in the mice. A preliminary screening by a veterinary pathologist (D.L.D.) for toxic effects of PDTC breakdown in the liver and kidney, by examination of tissue sections stained with hematoxylin and eosin 4 weeks after ovx, revealed no evidence of PDTC-induced organ damage (data not shown). Nonetheless, we cannot exclude long-term toxic effects of PDTC breakdown products, or PDTC-induced toxicity or impaired function of other organs and tissues following long-term administration. Exposure to high doses of PDTC for extended periods (58 weeks) have been associated with myelin breakdown products, or PDTC-induced toxicity or impaired function of other organs and tissues following long-term administration. We thank Xiaoying Yang for excellent technical assistance and kidney, by examination of tissue sections stained with hematoxylin and eosin 4 weeks after ovx, revealed no evidence of PDTC-induced organ damage (data not shown).

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Acknowledgements

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We thank Xiaoying Yang for excellent technical assistance with the μCT.

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