Requirement of the inducible nitric oxide synthase pathway for IL-1-induced osteoclastic bone resorption


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Nitric oxide has been suggested to be involved in the regulation of bone turnover, especially in pathological conditions characterized by release of bone-resorbing cytokines. The cytokine IL-1 is thought to act as a mediator of periarticular bone loss and tissue damage in inflammatory diseases such as rheumatoid arthritis. IL-1 is a potent stimulator of both osteoclastic bone resorption and expression of inducible nitric oxide synthase (iNOS) in bone cells and other cell types. In this study, we investigated the role that the iNOS pathway plays in mediating the bone-resorbing effects of IL-1 by studying mice with targeted disruption of the iNOS gene. Studies in vitro and in vivo showed that iNOS-deficient mice exhibited profound defects of IL-1-induced osteoclastic bone resorption but responded normally to calciotropic hormones such as 1,25 dihydroxyvitamin D3 and parathyroid hormone. Immunohistochemical studies and electrophoretic mobility shift assays performed on bone marrow cocultures from iNOS-deficient mice showed abnormalities in IL-1-induced nuclear translocation of the p45 component of NFκB and in NFκB-DNA binding, which were reversed by treatment with the NO donor S-nitroso-acetyl penicillamine. These results show that the iNOS pathway is essential for IL-1-induced bone resorption and suggest that the effects of NO may be mediated by modulating IL-1-induced nuclear activation of NFκB in osteoclast precursors.

IL-1 is a pleiotropic cytokine (1) that has potent stimulatory effects on nitric oxide production (2) and bone remodeling (3–5). On binding to its receptor, IL-1 initiates a signaling cascade that culminates in activation of NFκB (6). The importance of this system in normal bone physiology is demonstrated by the fact that transgenic mice that are deficient in the IL-1 receptor are resistant to ovariectomy-induced bone loss (7) and that deficiency of other components of the pathway, such as TRAF6 (8) and the p50/p52 subunits of NFκB (9), leads to osteopetrosis because of failure of osteoclast differentiation. Its importance in the pathogenesis of pathological bone loss is similarly illustrated by the fact that treatment with IL-1 receptor antagonist slows bone erosions in patients with rheumatoid arthritis (10) and that IL-1 inhibitors block the osteoclast-activating activity of bone marrow (BM) cells derived from multiple myeloma patients (11).

Inflammatory conditions such as rheumatoid arthritis, which are characterized by local osteolysis, are associated with activation of inducible nitric oxide synthase (iNOS) (12–14). This fact has led to the suggestion that NO derived from the iNOS pathway may act as a mediator of cytokine-induced effects on bone turnover (15–18). Studies with NO inhibitors and NO donors have indicated that high concentrations of NO inhibit osteoclastic bone resorption in vitro (15, 19, 20), whereas lower concentrations may be essential for normal osteoclast activity (21) and, in some circumstances, may enhance IL-1-induced bone resorption (16). This finding suggests that NO may play a role in mediating some effects of cytokines on bone resorption, but the studies performed so far have been unable to assess the relative importance of NO in relation to other mediators of cytokine action or to determine which isoform is responsible. Finally, it remains possible that some of the responses observed that have been attributed to NO could instead have been mediated by nonspecific inhibition of other metabolic pathways by NOS inhibitors (22). To resolve these issues and to clarify the role that NO derived from the iNOS pathway plays in cytokine-induced bone resorption, we studied the effects of IL-1 on osteoclast formation and bone resorption in transgenic mice with targeted inactivation of the iNOS gene.

Methods

Generation of iNOS-Deficient Mice. The murine iNOS gene was disrupted by introducing a targeted mutation into embryonic stem cells derived from the 129 mouse strain as described (23). The homozygous, heterozygous, and wild-type mice thus generated were backcrossed onto MF1 mice for three generations to generate a colony on a mixed 129 × MF1 background. The phenotype of these mice has previously been extensively characterized (24, 25), and Western blotting has shown that peritoneal macrophages from these mice do not produce iNOS after cytokine stimulation (26). Low levels of nitrite have been detected in peritoneal macrophages stimulated for up to 72 h with bacterial lipopolysaccharide and IFN-γ, however, which seems to be attributable to either cytokine-induced activation or induction of constitutive NOS isoforms (26). A second colony was established in a similar way onto a pure 129 background. Some of the in vitro studies were performed with cells from both strains of mice with similar results (data not shown), whereas all of the in vivo studies were performed on littermates derived from the 129 × MF1 colony, which have an identical genetic background.

Osteoblast-BM Cocultures. Osteoclast formation was studied by using an adaptation of the BM-osteoblast coculture system (27) as previously described (20). Cocultures of osteoblasts and BM cells were performed in 48-well or 96-well tissue culture plates. In 96-well plates, the osteoblasts and BM cells were plated at 104 cells per well and 2 × 103 cells per well, respectively, in 150 μl of aMEM supplemented with 10% FCS, antibiotics, and 10 nM...
1,25-dihydroxyvitamin D₃. Double the amount of cells and culture medium was used in 48-well cultures. Reagents used in stimulation of the cultures were human recombinant IL-1β (specific activity 5 × 10⁹ units/ml; Boehringer Mannheim), human parathyroid hormone 1–84 (PTH; Sigma); S-nitrosoacetyl penicillamine (SNAP; Alexis Pharmaceuticals, Nottingham, U.K.), and L-n-monomethylarginine (Sigma). The cultures were terminated after 9 days and fixed with 4% buffered formalin/saline (pH 7.4). Osteoclasts were identified by tartrate-resistant acid phosphatase staining and resorption pits by reflected light microscopy as described (20).

**Immunostaining for NFκB.** The effects of IL-1β on nuclear translocation of NFκB were studied in osteoblast-BM cocultures treated with IL-1β or vehicle at day 4. The cultures were fixed with 50% acetone in PBS for 10 min and washed twice in PBS. The cells were incubated for 30 min with an antibody directed against the p65 subunit of NFκB (Santa Cruz Biotechnology), washed three times in PBS, incubated with horseradish peroxidase-conjugated ABC complex (Vectastain ABC HRP elite; Vector Laboratories). After a further three washes, staining was detected by diaminobenzidine according to the manufacturer’s instructions (Vector Laboratories). Cells were scored as positive or negative by an observer who was blinded with regard to the experimental details. Four fields at ×20 magnification were scored per well. The identity of the BM cells in these cultures was determined by their typical morphology and confirmed by staining for the pan-leukocyte marker CD45 with an anti-CD45 polyclonal antibody (Sigma). The identity of osteoblasts was assessed morphologically and confirmed by histochemical staining for alkaline phosphatase by using fast red as the substrate as described (28).

**Electrophoretic Mobility Shift Assays (EMSA).** Osteoblasts (1.5 × 10⁶) and BM cells (15 × 10⁶) were cultured for 5 days in a 9-cm Petri dish in 10 ml of αMEM supplemented with 10% FCS, antibiotics, and 10 nM 1,25-dihydroxyvitamin D₃ before stimulation. EMSA were performed on nuclear protein extracts prepared from these cocultures as described (29), by using double-stranded oligonucleotides (Promega) containing a NFκB consensus sequence (underlined), ATGTGAGGGACTTTC-GGCGCTC, end-labeled with γ-3²P by using T4 polynucleotide kinase (Amersham Pharmacia). Unlabeled oligonucleotides of the same sequence were used in 100-fold excess in the competition experiments. EMSA assays were imaged and quantitated with a Bio-Rad Personal FX molecular imager and QUANTITY ONE software.

**NO Production.** NO production was assessed by measuring the stable end product of NO, nitrite, in culture medium by using the Griess reaction as described previously (17).

**In Vivo Experiments.** We studied the effects of IL-1 on bone resorption in wild-type and iNOS-deficient mice in vivo by using an adaptation of the method described by Boyce et al. (5). Four-day-old mice were injected three times a day over the period of 4 days with 10 μl of recombinant murine IL-1α (5 mg/ml; Calbiochem) or vehicle (saline) on 3 consecutive days. The mice were killed 4 days after the last injection, and the calvarial bones were fixed in 4% buffered formalin/saline (pH 7.4). Undecalcified calvarial bones were embedded in glycolmethacrylate, and 3-μm sections were prepared on a microtome (Jung, Heidelberg, Germany). Sections were stained with von Kossa reagent, and histochemical staining was performed for tartrate-resistant acid phosphatase, followed by counterstaining with light green. Histomorphometric analysis was performed according to standard nomenclature (30), using a Leitz Q500 MC image analysis system.

**Statistics.** Results were analyzed by using analysis of variance, followed by Dunnet’s post test with SPSS for Windows, unless otherwise stated.

**Results**

**iNOS-Knockout (KO) Mice Show Defects in IL-1-Induced Osteoclast Formation and Bone Resorption in Vitro.** IL-1β (10 units/ml) failed to stimulate osteoclast formation (Fig. 1a) or bone resorption (Fig. 1b) in cocultures prepared with osteoblasts and BM cells from iNOS-deficient mice. This failure appeared to be caused by a deficiency of osteoblast-derived NO, because the response to IL-1β was normal in cocultures of wild-type (WT) osteoblasts and iNOS KO BM cells but was defective when WT BM cells were cultured with KO osteoblasts (Fig. 1a and b). The amount of NO produced by cocultures of WT osteoblasts and KO BM was similar to that in WT/WT cocultures and was significantly greater than that produced by cocultures where the BM cells were derived from WT and osteoblasts from KO animals (Fig. 1c), indicating that osteoblasts are the main source of NO in this system. Expression of the bone resorption data in terms of the area of resorption produced per osteoclast showed no significant difference between the groups, indicating that the main effect of IL-1 was on osteoclast formation rather than on osteoclast activity (Fig. 1d). The pharmacological NO donor SNAP did not stimulate osteoclast formation or bone resorption when administered alone (data not shown), but reversed l-n-monomethylarginine-mediated inhibition of IL-1-induced bone resorption in WT cocultures (Fig. 2). These data indicate that although NO is necessary for IL-1-induced bone resorption, it does not stimulate bone resorption in the absence of IL-1. The defective osteoclast formation and bone resorption observed in cocultures prepared...
from KO animals was relatively specific for IL-1 because osteoclasts formed normally in response to 1,25 dihydroxyvitamin D₃ (Fig. 1a and b, without IL-1). Furthermore, stimulation of 1,25 dihydroxyvitamin D₃-treated cultures with 40 nM PTH resulted in a 2.5-fold increase in osteoclast numbers and bone resorption as compared with controls in both WT and KO cultures (Table 1).

**iNOS-Deficient Mice Exhibit Defects in IL-1-Induced Bone Resorption in Vivo.** The effects of IL-1 on bone resorption *in vivo* were studied by injecting IL-1α over the calvarial bones of KO and WT control mice. There was no difference in the histological appearance of calvarial bones of WT and iNOS-deficient animals that had been injected with vehicle (Fig. 3A and B). Osteoclastic bone resorption clearly was increased in response to IL-1α injections in WT mice (Fig. 3C) but not in KO mice (Fig. 3D). The defective response to IL-1α in KO mice was confirmed by quantitative histomorphometric analysis of the calvarial bones (Fig. 4). These data showed a substantial increase in both osteoclast numbers and eroded surfaces in WT animals as compared with no increase in osteoclast numbers and only a marginal increase in eroded surfaces in KO mice.

**BM Cells from iNOS-Deficient Mice Exhibit Abnormalities of IL-1-Induced NFκB Activation.** To investigate the mechanisms responsible for the defect in osteoclast formation in KO mice, we studied the effects of IL-1 on nuclear translocation of NFκB in BM cocultures from WT and KO mice by quantitative immunohistochemical staining (Fig. 5). Nuclear staining for the p65 component of NFκB was detected in 90% of BM cells and virtually 100% of osteoblast-like cells within 30 min of exposure to IL-1 (10 units/ml) in cocultures from both WT and KO animals. Osteoblast nuclear staining for NFκB-p65 disappeared rapidly in both WT and KO cultures and was absent 24 h after stimulation. In BM cells, however, nuclear staining for NFκB persisted in about 90% of cells for up to 24 h. Nuclear staining was lost more rapidly from BM cells in KO cultures. By 6 h, less than 20% of cells were positive (P < 0.05 from WT), and by 24 h,

Table 1. Effect of PTH on osteoclast formation and bone resorption in iNOS-deficient and WT mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Treatment</th>
<th>Osteoclasts per well</th>
<th>Resorption, mm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>Vehicle</td>
<td>78.7 ± 29.2</td>
<td>0.23 ± 0.07</td>
</tr>
<tr>
<td>WT</td>
<td>PTH</td>
<td>211.0 ± 80.9**</td>
<td>0.68 ± 0.12***</td>
</tr>
<tr>
<td>KO</td>
<td>Vehicle</td>
<td>86.5 ± 38.7</td>
<td>0.32 ± 0.11</td>
</tr>
<tr>
<td>KO</td>
<td>PTH</td>
<td>196.8 ± 65.8**</td>
<td>0.74 ± 0.21***</td>
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Cocultures were performed on dentine as described. PTH (4 × 10⁻⁸ M) or vehicle was added on day 7, and the cultures were terminated on day 10. Values are expressed as mean ± SD. **, P < 0.01; ###, P < 0.001; from vehicle; n = 6.

**Fig. 2.** Effect of NOS inhibitor and NO donor on IL-1-stimulated bone resorption in WT BM cocultures. (a) Resorption surface area. (b) Number of osteoclasts per dentine slice. Recombinant human IL-1β (10 units/ml), the NOS inhibitor N-monomethyl arginine (NMMA; 1 mM), or vehicle was added at day 7. The NO donor SNAP (5 μM) was added on days 7, 8, and 9, and the cultures were terminated on day 10. *, P < 0.05 from vehicle; **, P < 0.01; ###, P < 0.001; n = 6. The results shown are of a representative experiment of three performed.

**Fig. 3.** Effect of IL-1 on bone resorption in WT and iNOS KO mice *in vivo*. (A) WT, vehicle control. (B) iNOS KO, vehicle control. (C) WT, injected with IL-1α. (D) iNOS KO, injected with IL-1α. Arrowheads in A–D indicate tartrate-resistant acid phosphatase stain (red) and resorption surfaces.
Histomorphometric analysis of calvarial bones in WT and iNOS KO mice. OC/BS, number of osteoclasts per mm of bone surface; %ES, percentage of eroded surface; OC/mm², number of osteoclasts per mm² of tissue. All results are expressed as percentage of vehicle control from the same litter. Mice from two different litters were used in each group. *< P < 0.05 from vehicle; **< P < 0.01; ***< P < 0.001. WT saline, n = 7; WT IL-1, n = 8; iNOS KO saline, n = 6; iNOS KO IL-1, n = 7.

Discussion
Previous studies have indicated that NO may play a role in mediating the effects of IL-1 on bone resorption in vitro (15, 16), but they have been unable to assess its role in vivo, to determine the relative importance that NO plays in relation to other possible mediators of cytokine action, or to define which isoform is responsible. Finally, it remains possible that some of

NFκB-DNA binding had returned to control levels in the iNOS KO cultures. Summated data from three experiments showed that the stimulation of NFκB-DNA binding after 2 h was 1.87-fold in WT cultures as compared with 1.64-fold in iNOS KO. Corresponding figures for the 24 h time point were 1.77-fold for WT and 1.02-fold for iNOS KO (< P < 0.01). Further studies showed that NO alone was capable of causing NFκB activation because NFκB-DNA binding was enhanced in both WT and iNOS cocultures by addition of the NO donor SNAP in the absence of IL-1.

Fig. 4. Histomorphometric analysis of calvarial bones in WT and iNOS KO mice. OC/BS, number of osteoclasts per mm of bone surface; %ES, percentage of eroded surface; OC/mm², number of osteoclasts per mm² of tissue. All results are expressed as percentage of vehicle control from the same litter. Mice from two different litters were used in each group. *< P < 0.05 from vehicle; **< P < 0.01; ***< P < 0.001. WT saline, n = 7; WT IL-1, n = 8; iNOS KO saline, n = 6; iNOS KO IL-1, n = 7.

Fig. 5. IL-1-stimulated activation of NFκB is abnormal in iNOS KO BM cells. Nuclei were scored as positive or negative for NFκB staining in five fields from each well at ≥20 magnification. (a) BM cells. (b) Osteoblasts. Differences between groups were analyzed by using Student's t test. *< P < 0.05; **< P < 0.01; ***< P < 0.001, all from WT at the same time point. Values shown are means ± SD for three replicates at each time point.

Fig. 6. Histochecmical staining for NFκB nuclear translocation in cocultures. Filled arrows indicate staining of BM cell nuclei, and open arrows indicate staining of osteoblast nuclei. (A) iNOS KO coculture, unstimulated. (B) iNOS KO coculture, 2h after stimulation with 10 units/ml IL-1. (C) WT coculture, 24 h after stimulation with 10 units/ml IL-1. (D) iNOS KO coculture, 24 h after stimulation with 10 units/ml IL-1. (E) Negative control showing absence of staining when cells were incubated with FCS instead of anti-p65.

Fig. 7. IL-1-stimulated NFκB-DNA binding is abnormal in BM cocultures prepared from iNOS-deficient mice. (A) Lane 1, iNOS KO, unstimulated; lane 2, iNOS KO, 2 h of IL-1β; lane 3, iNOS KO, 24 h of IL-1β; lane 4, WT, unstimulated; lane 5, WT, 2 h of IL-1β; lane 6, WT, 24 h of IL-1β; lane 7, WT, 2 h of IL-1β; lane 8, WT, 2 h of IL-1β and unlabeled competitor probe; lane 9, WT, 4 h of 30 µM SNAP; lane 10, iNOS KO, 4 h of 30 µM SNAP. (B) Quantification of EMSA. Data are displayed as mean ± SD of three experiments. **< P < 0.01.
the responses observed that previously have been attributed to NO could have been mediated by nonspecific effects of NOS inhibitors on other metabolic pathways (22). The data presented here are important in that they demonstrate unequivocally that NO derived from the iNOS pathway plays an essential role in mediating the effects of IL-1 on osteoclast formation and bone resorption both in vitro and in vivo. BM cocultures prepared from mice with iNOS deficiency did not show the expected stimulation of osteoclast formation or bone resorption in vitro on stimulation with IL-1, and injections of IL-1 over the calvarial bones of iNOS-deficient mice similarly failed to stimulate bone resorption in vivo. Coculture studies demonstrated that the effects of IL-1 on osteoclast formation and bone resorption were crucially dependent on osteoblast-derived NO. Thus, cocultures of WT osteoblasts and knockout BM cells responded to IL-1 normally, whereas the stimulatory effects of IL-1 on NO production were blunted and those effects on osteoclast formation and bone resorption were absent in cocultures of knockout osteoblasts and WT BM cells. These data indicate that osteoblasts are the main source of NO in the coculture system and that osteoblast-derived NO acts in a paracrine fashion on cells within the BM compartment to promote IL-1-induced osteoclast formation and bone resorption. It is of interest that the requirement for NO was relatively specific for IL-1-induced bone resorption; the NO donor SNAP did not stimulate osteoclast formation or bone resorption in the absence of IL-1, and osteoclasts formed normally in KO cultures stimulated with 1,25 dihydroxyvitamin D3 and PTH. These findings are in broad agreement with our previous work, which has shown that NOS inhibitors suppress bone resorption induced by IL-1 and tumor necrosis factor and that the NO donor SNAP acts to enhance the bone resorptive effects of IL-1, but not PTH, in the mouse calvarial bone resorption assay (16).

Prostaglandins also have been suggested to act as mediators of IL-1-induced effects on bone resorption in rats (31), and other workers have shown that cytokines, including IL-1, activate both the iNOS pathway and prostaglandin E2 production in rat osteoblasts and that NO can itself activate prostaglandin E production (32). Our data do not exclude a role for prostaglandins as mediators of IL-1-induced bone resorption, but rather indicate that NO plays a predominant role, at least under the experimental conditions described in this paper. This finding is in keeping with the work of previous investigators who found that the stimulatory effects of IL-1 on bone resorption in mice could not be blocked by indomethacin either in vitro (4) or in vivo (5).

Although our studies demonstrate that NO derived from the iNOS pathway is essential for IL-1-induced bone resorption, the NO donor SNAP did not increase bone resorption in the absence of IL-1 stimulation. This result indicates that NO probably acts by enhancing or potentiating the effects of signaling pathways induced by IL-1 in bone cells to enhance osteoclast differentiation. We investigated this possibility by studying the effects of IL-1 on nuclear translocation of the p65 component of NFκB and on NFκB-DNA binding in BM cocultures from WT and iNOS-deficient mice. NFκB was chosen for these studies because it is the terminal component of the IL-1 signaling cascade (33) and because previous work had shown that NFκB is essential for osteoclast differentiation (9, 34). Normally, NFκB is held in an inactive state by the IκB inhibitory proteins. When cells are exposed to stimuli that activate the NFκB pathway, IκB proteins become phosphorylated, inducing proteasome-mediated degradation of IκB and allowing nuclear translocation of NFκB (35, 36). Quantitative immunohistochemical staining showed no differences in the kinetics of nuclear translocation of the p65 component of NFκB in osteoblasts from WT and KO cultures. However, studies of BM cells in these cultures showed marked differences; nuclear staining of NFκB-p65 was sustained for up to 24 h in 90% of cells from WT cultures, but only 20% of nuclei remained positive in knockout cultures at 6 h, and less than 2% were positive at 24 h. EMSA showed that these differences in p65 nuclear translocation were accompanied by markedly reduced NFκB-DNA binding in the iNOS KO cultures after 24 h, which indicates that an intact iNOS pathway is required to sustain NFκB activation in BM cells from which osteoclasts are derived. The abnormalities of nuclear translocation of NFκB were restricted to cells in the BM compartment and could be reversed by the NO donor SNAP, which was able to directly stimulate NFκB-DNA binding in the iNOS KO cocultures. These data are consistent with the findings of other workers who have shown that NO can stimulate NFκB-DNA binding in T cells (37), endothelial cells (38), macrophages (39), and neurons (40). Because NFκB plays an essential role in osteoclast differentiation (9, 34), the rapid attenuation of IL-1-induced NFκB nuclear translocation in BM cells would be predicted to impair osteoclast formation and bone resorption, as was observed in the present study. Recent work has shown that the receptor activator of NFκB (RANK) ligand/osteoprotegerin pathway plays an essential role in osteoclast differentiation and activation (41–43), and that many bone-resorbing factors, including IL-1, up-regulate expression of RANK ligand in osteoblasts, suggesting that these substances may regulate osteoclast differentiation and activity by modulating the RANK ligand/RANK pathway (44). Major defects of this pathway in iNOS-deficient mice can be excluded by the fact that 1,25 dihydroxyvitamin D3 and PTH stimulated osteoclast formation and bone resorption normally. Further work is necessary to determine whether the defects of IL-1-induced bone resorption and NFκB activation that we observed in iNOS-deficient mice are mediated by direct effects on the NFκB pathway or by changes in expression of RANK ligand, osteoprotegerin, RANK, and other components of the RANK signaling pathway.

In conclusion, the results presented in this paper indicate that the iNOS pathway plays an essential role in mediating the effects of IL-1 on bone resorption by an NFκB-dependent mechanism. These findings suggest that pharmacological inhibitors of this pathway could be of value in preventing bone loss in diseases associated with cytokine activation, such as rheumatoid arthritis.

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