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Nitric Oxide Synthase I Mediates Osteoclast Activity In Vitro and In Vivo

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Abstract Bone resorption is responsible for the morbidity associated with a number of inflammatory diseases such as rheumatoid arthritis, orthopedic implant osteolysis, periodontitis and aural cholesteatoma. Previous studies have established nitric oxide (NO) as a potentially important mediator of bone resorption. NO is a unique intercellular and intracellular signaling molecule involved in many physiologic and pathologic pathways. NO is generated from L-arginine by the enzyme nitric oxide synthase (NOS). There are three known isoforms of NOS with distinct cellular distributions. In this study, we have used mice with targeted deletions in each of these isoforms to establish a role for these enzymes in the regulation of bone resorption in vivo and in vitro. In a murine model of particle induced osteolysis, NOS I-/- mice demonstrated a significantly reduced osteoclast response. In vitro, osteoclasts derived from NOS I-/- mice were larger than wild type controls but demonstrated decreased resorption. Although NOS I has been demonstrated in osteoblasts and osteocytes as a mediator of adaptive bone remodeling, it has not previously been identified in osteoclasts. These results demonstrate a critical role for NOS I in inflammatory bone resorption and osteoclast function in vitro. J. Cell. Biochem. 89: 613–621, 2003.

Key words: osteoclast; osteolysis; inflammation; nitric oxide; cholesteatoma

Localized inflammatory bone resorption causes the morbidity associated with many diseases such as periprosthetic implant osteolysis [Merkel et al., 1999; Haynes et al., 2001], rheumatoid arthritis [Lee and Weinblatt, 2001], periodontitis [von Wowern, 2001], and cholesteatoma [Chole, 1984]. In these diseases, foreign particles or microbial infection causes a chronic inflammatory response that causes the resorption of adjacent bony structures. This bone resorption is the result of the recruitment, development and activation of osteoclasts. Osteoclasts are highly specialized cells with complex regulatory mechanisms. An understanding of the mechanisms underlying osteoclast function is critical to the development of ameliorating and curative therapies for bone eroding diseases.

In cholesteatoma, the accumulation of keratin debris causes a foreign-body type inflammatory response in the middle ear. The expanding mass of keratin and inflammatory cells progressively erodes surrounding bone leading to permanent hearing loss and vestibular dysfunction [Chole, 1997; Chole et al., 2001]. Pro-inflammatory cytokines, growth factors, and non-protein mediators such as prostaglandins, neurotransmitters and nitric oxide (NO) have all been associated with the local inflammatory response leading to bone resorption in cholesteatoma [Jung and Chole, 2002].

NO, a short-lived, neutral, free radical gas, has emerged as an important mediator of bone resorption and has been implicated in osteoclast function under normal and pathologic conditions [Collin-Osdoby et al., 1995]. NO is a multifunctional intracellular and intercellular signaling molecule that mediates vascular tone [Palmer et al., 1987], neurotransmission [Paakkari and Lindsberg, 1995], contractility of smooth muscle [Prast and Philippu, 2001], platelet aggregation [Mellion et al., 1983],

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leukocyte adhesion [De Caterina et al., 1995], and immune system function [Mannick et al., 1994; Wei et al., 1995]. NO is generated from L-arginine by the enzyme nitric oxide synthase (NOS). Currently, three known isoforms of this enzyme have been cloned and characterized, designated NOS I (neuronal, nNOS, bNOS), NOS II (inducible, iNOS) and NOS III (endothelial, eNOS). Although these enzymes have similar catalytic activity, they are encoded by three distinct genes and demonstrate tissue specific distributions, suggesting NO generated by different isoforms may have different functions.

An emerging consensus suggests that NO has a biphasic effect on resorption [Calabrese, 2001], with inhibition at high concentrations of NO [Ralston et al., 1995; Damoulis and Hauschka, 1997; Armour et al., 1999; Collin-Osdoby et al., 2000] and stimulation or potentiation at lower concentrations [Brandi et al., 1995; Chae et al., 1997; Collin-Osdoby et al., 2000; van't Hof et al., 2000]. NOS I and NOS III are low output enzymes (nanomolar) regulated by the calcium/calmodulin system, while NOS II is a high output enzyme (micromolar) induced by various cytokines and stress factors. Thus, the inhibition of osteoclast resorption at high concentrations may reflect NOS II activity under inflammatory conditions while the stimulation or potentiation observed with lower concentrations of NO may be generated by NOS I and NOS III.

Previous studies demonstrated that cells express one or more NOS isoforms. NOS III has been demonstrated in stromal cells, osteoblasts, osteocytes and osteoclasts [Brandi et al., 1995; Helfrich et al., 1997; Hukkanen et al., 1999]. NOS II has been observed in osteoblasts and osteoclasts [Ralston et al., 1994; Fox and Chow, 1998]. NOS I has been demonstrated in osteoblasts and osteocytes [Pitsillides et al., 1995; Hilbig et al., 2001]. However, other studies were unable to detect NOS I or NOS II in osteoblasts or osteoclasts [Helfrich et al., 1997; Fox and Chow, 1998]. Because of the heterogeneity of methods and animals used in these studies, the role of individual isoforms in osteoclast function remains unclear.

This study examines the role of the NOS isoforms individually both in vivo and in vitro by utilizing mice with targeted deletions in NOS I, II or III. The in vivo model examines the role of NOS in localized inflammatory bone resorption [Chole et al., 2001]. The in vitro assays examine the role of each NOS isoform in osteoclast development and activity.

MATERIALS AND METHODS

Materials

The GST tagged receptor activator of NFκB (GST-RANKL) construct was a gift from Dr. Steve Teitelbaum and Dr. F. Patrick Ross (Department of Pathology, Washington University, St. Louis, MO). RANKL was produced from high expressing BL21 cells (Stratagene, La Jolla, CA) containing a Glutathione-S-Transferase-RANKL construct [McHugh et al., 2000]. BL21 cells were grown in LB supplemented with 100 μg/mL TurboAmp (Stratagene) at 30°C in a shaking incubator at 350 rpm to $OD_{600} = 0.5$ and lysed under non-denaturing conditions. GST-RANKL was purified over a glutathione-agarose column (Sigma, St. Louis, MO) followed by ion-exchange chromatography (BioRad, Hercules, CA) and dialysis. Minimal essential media and mCSF were obtained from Sigma Aldrich. Cetacean dentine was obtained from the Department of Vertebrate Zoology at the Smithsonian Institution, Washington DC, and the Vertebrate Museum at Humbolt State University, Humbolt, CA, in cooperation with the National Marine Fisheries Service. Knockout mice, generated on C57Bl/6 backgrounds, were obtained from The Jackson Laboratory (Bar Harbor, ME). Wild type littermates or age-matched controls of the appropriate background were used for comparison in all experiments.

In Vivo Studies

This study was performed in accordance with the PHS Policy on Humane Care and Use of Laboratory Animals, the NIH Guide for the Care and Use of Laboratory Animals, and the protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of Washington University in St. Louis.

Following a procedure modified from Merkel et al. [1999] and previously described by Chole et al. [2001], keratin particles were ground in a mortar and pestle. Consistent with prior studies investigating particle-induced osteolysis [Green et al., 1998], the majority of the particles used in these experiments had a diameter of less than 10 μm. For these studies, five 6 week old mice were included in each group (NOS I-/-, NOS II-/-, NOS III-/-) and age-matched C57Bl mice were used as wild type

controls (WT). Mice were anesthetized with a mixture of ketamine (87 mg/kg) and xylazine (13 mg/kg). An incision was made through the skin over the dorsum of the head, the skin was retracted laterally and the periosteum removed from the parietal bone. Approximately 10–12 mg of keratin particles were placed onto the exposed bone and the incision closed with sterile wound clips. After 6 days, animals were euthanized with pentobarbital, the calvaria removed and placed in fixative (4% paraformaldehyde and 0.05% glutaraldehyde in Sorensen's phosphate buffer) for 2 days. Specimens were then decalcified in 0.35 M tetrasodium EDTA in PBS, dehydrated in graded solutions of acetone, and embedded in araldite-epon resin. Three 1.0 μm thick sections, at 100 μm intervals, were obtained from each specimen and stained with toluidine blue and basic fuchsin. Osteoclast number was counted based on standardized criteria (adjacent to bone and exhibit three of the following: a distinct ruffled border, granular cytoplasm, multiple nuclei, or loss of the lamina limitans on underlying bone).

In Vitro Studies

Osteoclast cultures were generated using a protocol adapted from McHugh et al. [2000]. Bone marrow cells were obtained from dissected femurs and tibias of 3–5 week-old mice. Cells were suspended in α-MEM containing 10% fetal calf serum (α-10 MEM) and cultured overnight at 37°C in 5% CO_2. Non-adherent cells were gently washed from the plate, re-suspended, and the monocyte fraction isolated by Ficoll-Hypaque centrifugation. Cells were plated in 48 well plastic tissue culture plates (200,000 cells/mL) and maintained with the addition of mCSF (10 ng/ml) and RANKL (30 ng/mL) at 37°C in 5% CO_2. Media with fresh RANKL and mCSF was changed every other day. Cells were fixed and stained for tartrate-resistant acid phosphatase (TRAP) using a leukocyte acid phosphatase staining kit (Sigma) and the number of TRAP-positive osteoclasts were manually scored under microscopic examination. TRAP-positive cells with three or more nuclei were counted in three low power fields. Nuclei, in a total of 100 osteoclasts in random high power fields, were counted in four individual wells per experimental group. To verify the absence of stromal cells in these cultures, RANKL expression was examined by RT-PCR with the following forward and reverse primers: TCCCATCGGGTTCCCATAAA and TCGGAGCTTGAAAAATCCCC (accession no. AF019048).

Osteoclast activity assay. For resorption studies, cells were plated directly onto dentine slices. Dentine was cut into 500-μm thick slices using a Buehler Isomet low speed saw (Irvine, CA), sterilized in 70% ethanol overnight, washed in α-MEM and soaked in α-10 MEM for 1 h prior to use. Cells were cultured for 7 days with mCSF and RANKL. In order to obtain osteoclast number, the dentine slices were then fixed and TRAP-stained. TRAP-positive cells in 2 low power fields were counted.

Pits on the surface of dentine slices were visualized by staining with a solution containing 1% toluidine blue and 1% sodium tetraborate. Dentine slices were photographed with a digital camera, and image analysis was performed using SigmaScan® Pro version 5.0 (SPSS Science, Chicago, IL). Resorption pits on the surface of the dentine slices were photographed at low power and the pits were counted and traced manually to determine area.

For cross-sectional area measurements, the dentine slices were decalcified in 0.35 M ethylenediamine tetraacetic acid (EDTA) for 3 days with changes of solution each day. Dentine slices were then dehydrated in graded solutions of acetone and embedded in epon-araldite resin (Electron Microscopy Sciences, Fort Washington, PA) and sectioned on a microtome. A total of 6 sections (1.0 μm) were collected at 100 μm intervals and stained with toluidine blue and basic fuchsian. Sections were photographed with a digital camera, and the image analysis performed using SigmaScan® Pro. Resorption pit cross sectional areas were manually traced to determine area.

RT-PCR

Total RNA was isolated from osteoclast cultures using TRIZOL Reagent (GIBCO, Rockville, MD) following the manufacturer's protocol with the addition of glycogen to a final concentration of 250 μg/mL. RNA concentration was determined by absorption 260 nm. Isolated total RNA (1.0 μg) was digested with DNAse I and used to synthesize cDNA using the 1st Strand cDNA Synthesis Kit for RT-PCR (Roche Biochemicals), following manufacturer's protocol. Primers were synthesized at the Protein and Nucleic Acid Chemical Laboratory facilities at Washington University School of Medicine. PCR products are separated on a 2.0% agarose

gel at 90 mV for 1.5 h, stained with ethidium bromide and visualized under ultraviolet light. Forward and reverse primer sequences used for these studies were as follows: Control 18s (accession no. 56974) CTTTCGAGGCCGTGTAAT and AGCCCAACTACGAGCTTT, NOS I (accession no. D14552) TCTTGGCTTGGAGGTCTT and CAATGCCCCTGAGAACTT, NOS II (accession no. M87039) GGTGGTGACAAGCACATT and CGGACATCAAAGGTCTCA, NOS III (accession no. U53142) CCGGGACTTCATCAATCAGT and CACTGTGATGGCTGAACGAA.

Statistical Analysis

Statistical analysis was performed using SigmaStat® (SPSS Science). One-way ANOVA was used for all analyses with the power of the performed tests at $\alpha = 0.05$. Multiple comparisons were evaluated where predicated with α adjusted by Bonferroni correction.

RESULTS

NOS I-/- mice have reduced osteoclast response in a model of inflammatory bone resorption.

Keratin particles, like PMMA particles, induced a foreign-body inflammatory response leading to bone resorption when placed on mouse calvaria [Merkel et al., 1999 no. 894] [Iino et al., 1990; Wright et al., 1996; Chole et al., 2001] and may reflect the mechanism of osteoclast activation in cholesteatoma-induced bone resorption. The osteolytic response of wild type and knock out mice was quantified by counting the number of osteoclasts in histologic sections (Fig. 1). NOS I-/- mice had a significantly attenuated osteoclast response when compared to wild type mice (27% of control). Osteoclast response in NOS II-/- and NOS III-/- mice were not significantly different from wild type.

Osteoclasts generated from NOS I-/- mice demonstrate increased size and decreased resorption in vitro.

To determine if the observed in vivo result was due to the loss of NOS in the osteoclast itself, osteoclasts were generated from NOS I, II, and III knock out mice. The effect of NOS deletion on osteoclast development in vitro was determined by comparing the osteoclast number and area. Surprisingly, NOS I-/- osteoclasts were visibly much larger than wild type cells while NOS II-/- and NOS III-/- osteoclasts were not qualitatively different (Fig. 2B). Quantitative analysis of osteoclasts generated from these knock out mice demonstrated that the number of NOS I-/- osteoclasts were similar to controls but the total area occupied increased to 338% of control, demonstrating that individual osteoclasts were larger. NOS I-/- osteoclasts also had increased number of nuclei (274% of control, representing 21.3 ± 2.2 nuclei per osteoclast), suggesting that enhanced osteoclast size was due to enhanced fusion (Fig. 2A). The number and area of osteoclasts generated from NOS II-/- and NOS III-/- were not statistically different from wild type. No RANKL expression was detected in these cultures by RT-PCR, suggesting that these effects were not mediated by stromal cells (data not shown).

To verify this in vitro phenotype, NOS I-/- osteoclasts were also plated on dentine and cell areas were similarly quantified (Fig. 2C). On dentine, there were significantly more osteoclasts (221% of control) as well as an increase in total area occupied (244% of control). Interestingly, NOS I-/- osteoclasts were more numerous when plated on dentine but not as large compared to NOS I-/- osteoclasts on plastic.

When osteoclast resorption on dentine was analyzed, pit number was not significantly different in any of the knockouts compared to wild type (Fig. 3). However, a decrease in resorption was observed in NOS I-/- osteoclasts but not from NOS II-/- or NOS III-/- osteoclasts, compared to wild type controls (Fig. 3). Concomitantly, nitrite measurements from NOS I-/- osteoclasts demonstrated significantly reduced levels compared to wild type osteoclasts (data not shown). Basal nitrite levels in wild type osteoclasts cultures were 3.7 ± 0.6 μM while NOS I-/- osteoclasts generated 2.5 ± 0.5 μM nitrite ($P = 0.006$). Consistent with these results, RT-PCR of unstimulated osteoclast cultures demonstrate expression of NOS I but not NOS II or NOS III (Fig. 4).

DISCUSSION

NOS I was first discovered in central nervous system neurons but has been subsequently shown in peripheral and myenteric neurons [Prast and Philippu, 2001], as well as skeletal, cardiac and smooth muscle [Stamler and Meissner, 2001]. The constitutive expression of NOS I in osteoclasts and the effects of its deletion on bone resorption both in vitro and in vivo was an exciting and unexpected finding. In other tissues, NOS I functions at specialized

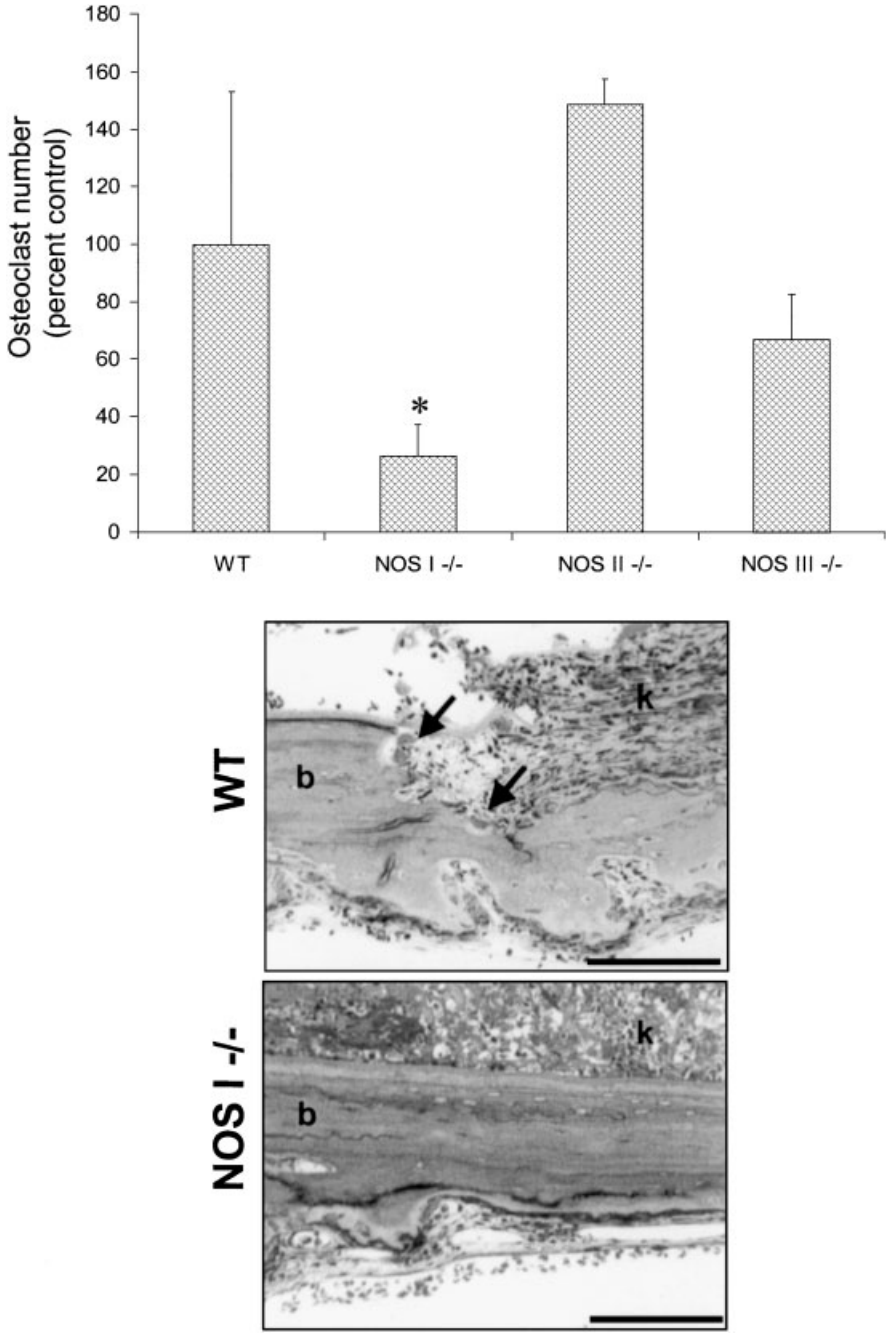

Fig. 1. In vivo model of inflammatory bone resorption. Keratin was implanted on the calvaria of wild type and NOS knock out mice. After 6 days, calvaria were embedded, sectioned and stained. Osteoclasts were counted in three different sections and averaged. Results are shown as a percent of wild type controls. Osteoclast numbers were decreased in NOS I-/- mice while NOS II-/- and NOS III-/- mice were not different from wild type (n = 5). * indicates $P < 0.05$. Photomicrographs of calvaria demonstrate reduced numbers of osteoclasts (arrow) in NOS I-/- (**bottom panel**) compared to wild type (**top panel**). Keratin particles (k) adjacent to bone (b) were evident. Bars represent 100 μm.

cell-cell or cell-extracellular matrix junctions where, through protein-protein interactions at the plasma membrane, it participates in signal transduction [Brenman et al., 1996b; Jaffrey et al., 1998]. Also potentially relevant to osteoclast biology is the finding that NOS I plays a role in muscle cell fusion [Blottner and Luck, 1998; Lee et al., 1994]. During osteoclast development, mononuclear osteoclast precursors fuse to form multinucleated cells, in a process

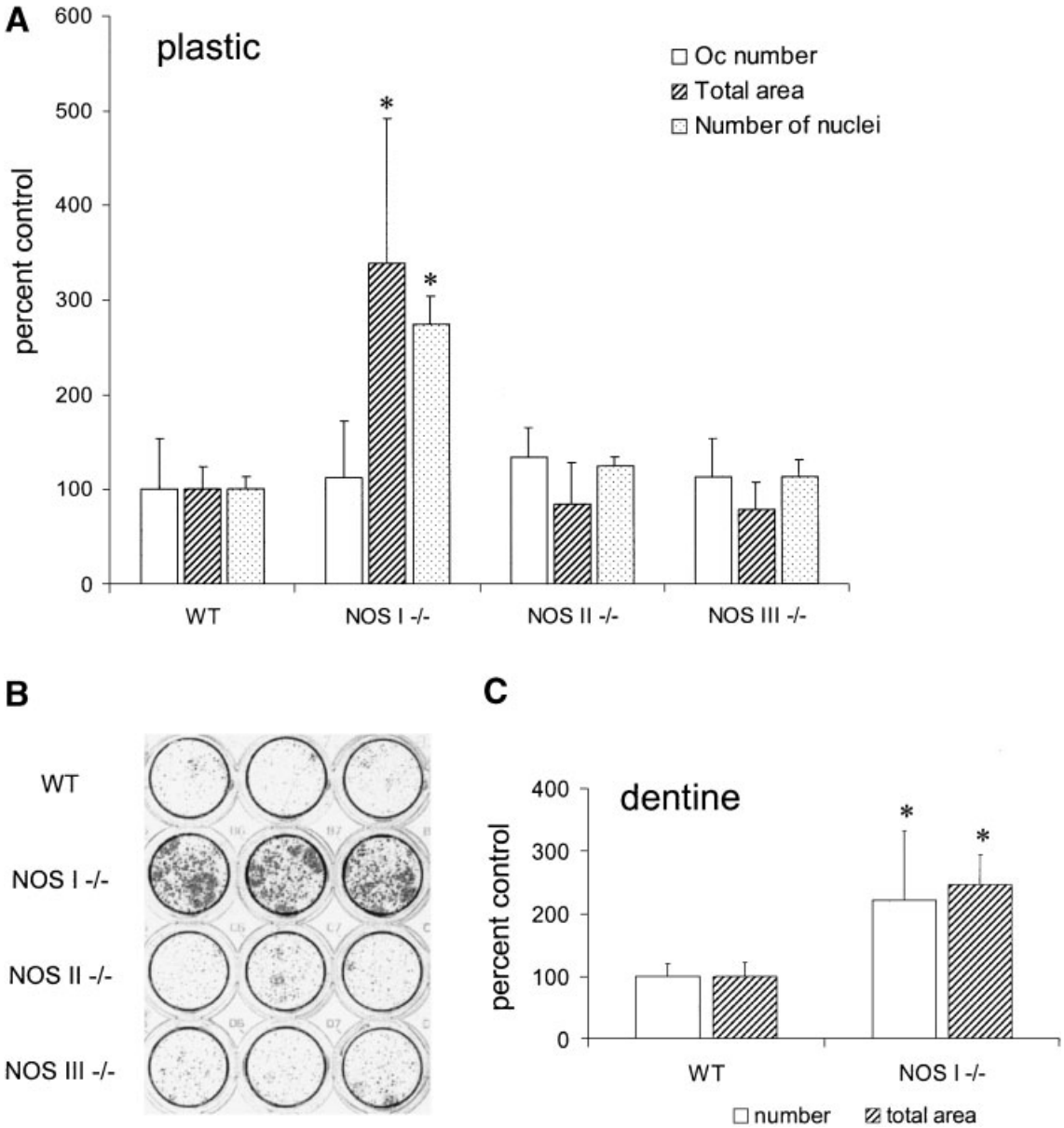

Fig. 2. In vitro osteoclast development. Bone marrow monocytes from wild type, NOS I-/-, NOS II-/-, and NOS III-/- mice were plated at 200,000 cells/mL in 48 well plates either directly onto plastic (**A** and **B**) or onto dentine slices (**C**). Cells were maintained for 7 days with mCSF and RANKL, fixed and TRAP stained. Osteoclast numbers were counted and areas traced manually in three high power fields per well and averaged. (A) Quantitative analysis of osteoclast number and size demonstrated the total osteoclast area was increased compared to wild type when plated on plastic ($n = 8$). Number of nuclei was also increased. (B) Photograph of a 48 well plate shows that NOS I-/- osteoclasts have greatly increased spread area compared to either wild type, NOS II-/- or NOS III-/- osteoclasts. (C) When plated on dentine, osteoclast number, area and area per osteoclast were increased compared to wild type ($n = 6$). Results are the means $\pm$ standard deviations. * indicates $P < 0.05$ and ** indicates $P < 0.001$.

that may be similar to the multi-nucleation of muscle cells.

In our study, NOS I deletion increased the size and number of osteoclasts, in vitro, (Fig. 2) but these larger osteoclasts demonstrated reduced resorption (Fig. 3). One possible interpretation of these in vitro findings is that NOS I may regulate the switch from osteoclast development to activation, simultaneously reducing proliferation and enhancing resorption. Thus, the loss of NOS I would result in enhanced development (increased number and size) and reduced activity which was seen in our results. Similarly, in muscle, NOS activity was shown to

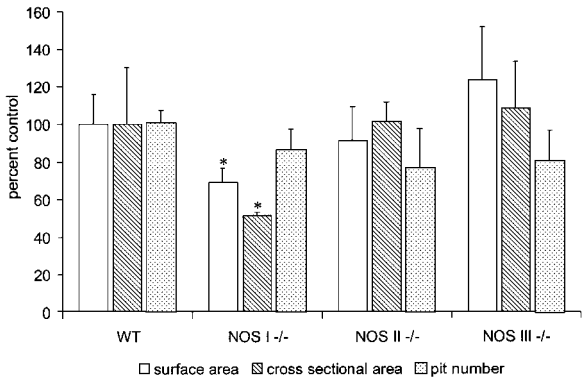

Fig. 3. In vitro osteoclast resorption. Bone marrow monocytes from wild type, NOS I-/-, NOS II-/-, and NOS III-/- mice were plated at 200,000 cells/mL in 48 well plates onto dentine slices. Cells were maintained for 7 days with mCSF and RANKL. Dentine slices were sonicated to remove cells, stained with toluidine blue and surface resorption pits were digitally photographed. Resorption pits were manually counted and traced to determine number and area. Dentine slices were then embedded and sectioned. Six sections from each dentine slice were photographed and pits were manually traced to determine cross sectional areas. Results are shown as a percent of wild type control. Surface and cross sectional area measurements of resorption pits demonstrate NOS I-/- osteoclasts have a small but significant decrease in resorptive activity compared to wild type osteoclasts. Resorption pits of NOS II-/- and NOS III-/- osteoclasts were not different from wild type ($n = 6$). * indicates $P < 0.05$.

coincide temporally with mononuclear myoblasts fusion to form myotubes [Lee et al., 1994].

These results clearly demonstrate a role for NOS I in osteoclast function. Comparison of average osteoclast area (total area divided by osteoclast number) of cells on plastic and dentine (Fig. 2) demonstrated a dramatic increase in average osteoclast size on plastic (over 600%) but only a modest increase in NOS I-/- average osteoclast size on dentine (70% increase). These results may reflect the loss of extracellular signals on plastic that act to limit osteoclast size, suggesting NOS I may mediate extracellular signals, cell-cell fusion or motility.

In our study, we found that NOS I-/- mice showed an attenuated response in an in vivo model of inflammatory bone resorption (Fig. 1). A caveat to these findings is the potential presence of alternate NOS I splice variants in the knock out. The knock out strategy used by Huang et al. [1993] deleted portions of the first expressed exon, exon 2, that contains the translation start site for full-length nNOS [Huang et al., 1993]. However, it has been shown that there are alternate translation start sites resulting in tissue specific RNA splice variants [Silvagno et al., 1996; Brenman et al., 1997; Wang et al., 1997]. In neurons, about 5% of NOS activity is attributed to the alternate splice forms nNOSβ and nNOSγ [Brenman et al., 1996a]. Both of these splice variants lack exon 2 and are present in the mice used in this study [Huang et al., 1993]. However, since the splice variants are present in the wild type mice as well, they may not contribute to the phenotype seen. Alternately, the phenotype in the knock out may be the result of some functional redundancy or compensation. Preliminary studies in our laboratory indicate that osteoclasts may have one or more alternate splice variants but further investigation is necessary to identify and characterize these potentially novel splice variants.

These studies demonstrate a role for NOS I in inflammatory bone resorption as well as osteoclast development and activity, in vitro. In an in vivo murine model of inflammatory bone resorption, NOS I-/- mice demonstrated a significantly attenuated osteoclast response compared to wild type control, suggesting a

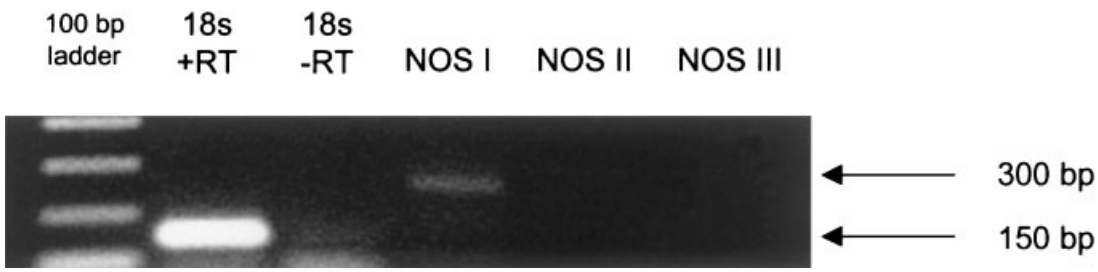

Fig. 4. NOS isoform expression in osteoclasts. Lanes 1 and 2 are positive and negative RT controls using 18s rRNA primers (150 base pairs). Lanes 3, 4, and 5 demonstrate the expression of NOS I, II, and III, respectively (300 base pairs). Basal NOS I expression was seen in unstimulated control cultures and NOS II expression was inducible by a cocktail of cytokines (IL-1β, TNFα, IFNγ). NOS III was not expressed in osteoclasts. Bands were purified and identities confirmed by sequencing.

defect in osteoclast development or activity. In vitro, the loss of NOS I resulted in larger but less active osteoclasts, which may explain the in vivo phenotype. NOS I-/- osteoclasts demonstrated abnormal development and activity in vitro that may affect their survival in vivo.

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