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iNOS-Derived Nitric Oxide Stimulates Osteoclast Activity and Alveolar Bone Loss in Ligature-Induced Periodontitis in Rats

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Abstract

Background—Inflammatory stimuli activate inducible nitric oxide synthase (iNOS) in a variety of cell types, including osteoclasts (OC) and osteoblasts, resulting in sustained NO production. In this study, we evaluate the alveolar bone loss in rats with periodontitis under long-term iNOS inhibition, and the differentiation and activity of OC from iNOS-knockout (KO) mice in vitro.

Methods—Oral aminoguanidine (an iNOS inhibitor) or water treatment was started 2 weeks before induction of periodontitis. Rats were sacrificed 3, 7, or 14 days after ligature placement, and alveolar bone loss was evaluated. In vitro OC culture experiments were also performed to study the differentiation of freshly isolated bone marrow cells from both iNOS KO and wild-type C57BL/6 mice. OC were counted 6 days later after tartrate-resistant acid phosphatase staining (a marker of osteoclast identity), and bone resorption activity was assessed by counting the number of resorption pits on dentin disks.

Results—Rats with ligature showed progressive and significant alveolar bone loss compared to sham animals, and aminoguanidine treatment significantly inhibited ligature-induced bone loss at 7 and 14 days after the induction. In comparison to bone marrow cells from wild-type mice, cells from iNOS KO mice showed decreased OC growth and the resulting OC covered a smaller culture dish area and generated fewer resorption pit counts.

Conclusion—Our results demonstrate that iNOS inhibition prevents alveolar bone loss in a rat model of ligature-induced periodontitis, thus confirming that iNOS-derived NO plays a crucial role in the pathogenesis of periodontitis, probably by stimulating OC differentiation and activity.

Keywords

Animal experimental use; bone resorption; iNOS enzyme; nitric oxide; osteoclasts; periodontitis

Nitric oxide (NO) is a free radical that plays an important role in the host response to infection. It is synthesized from L-arginine by three NO synthase (NOS) isoforms: 1)

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neuronal NOS; 2) endothelial NOS; and 3) inducible NOS (iNOS). The potential sources of NO in periodontium are inflammatory cells, keratinocytes, fibroblasts, osteoclasts (OC), and blood vessels.¹ Etiologic factors of periodontitis, such as inflammatory cytokines and periodontal pathogens, are also responsible for enhanced iNOS-derived NO production, which is part of the non-specific array of natural defense mechanisms, but can also contribute to the tissue damage observed in periodontal tissues.¹

Low amounts of NO may be essential for normal OC activity.² Bacterially elicited iNOS induction resulting in long-lasting NO production in an attempt to kill plaque bacteria may result in NO-mediated host tissue damage.³

Some in vitro studies show that NO inhibits osteoclastic bone resorption at high concentrations.³⁻⁶ However, another report supports that iNOS-derived NO mediates bone loss, either directly or indirectly, by modulating the production of other proinflammatory cytokines, such as interleukin (IL)-1.⁷ Moreover, pharmacologic inhibition of NO by aminoguanidine (AG) or mercaptoethylguanidine results in decreased bone loss, indicating that NO is a pharmaceutical target in the management of periodontal disease.⁸⁻¹⁰

Gyurko et al.¹¹ showed that iNOS-generated NO is required for normal skeletal and alveolar bone development, and for *Porphyromonas gingivalis* (*Pg*)-induced alveolar bone loss, suggesting that the mechanism by which iNOS performs these functions is by enhancing macrophage colony-stimulating factor and receptor activator of nuclear factor-kappa B ligand (RANKL) induced OC differentiation and bone resorption. However, this source of NO is an important element of the host defense machinery against the periodontal pathogen *Pg*; in fact, neutrophils require a fully functional iNOS enzyme for optimal survival and, in addition, the modulation of superoxide anion effects is likely to be a mechanism by which NO influences neutrophil function.¹¹

As a whole, these observations indicate that iNOS is important for bone resorption and OC differentiation, although it is not quite clear which are the cell types directly targeted by NO and the molecular pathways affected.

In the present study, we investigate the role of iNOS on the degree of alveolar bone loss associated with periodontitis in rats by treating the animals with AG, a selective iNOS inhibitor, and the in vitro differentiation and activity of OCs obtained from iNOS-knockout (KO) mice.

MATERIAL AND METHODS

Animals

Ninety male Wistar rats (6 weeks old, 160 to 180 g) from the Institute of Biomedical Science, University of São Paulo, São Paulo, Brazil, animal facility were used for the ligature-induced periodontitis experiments, and the experimental protocol was approved by the local Ethics Committee for Animal Experimentation.

For the OC culture experiments, we used C57BL/6 mice[§] and iNOS KO mice.^{||} The experimental protocols were approved by the Institutional Animal Care and Use Committee of Boston University, Boston, MA, and were performed according to the standards of the Public Health Service Policy on Humane Care and Use of Laboratory Animals.

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All animals were kept under controlled temperature (22°C to 25°C) and dark/light cycle (12/12 hours) and received standard laboratory chow diet and water ad libitum.

Induction of Periodontitis

Ninety rats were anesthetized with ketamine (80 mg/kg, intraperitoneally[¶]) and xylazine (16 mg/kg, intraperitoneally[#]) and the lower right first molar of each rat received a 3–0 cotton ligature submarginal position to induce periodontitis, as previously described.^{12,13} Thirty animals (sham-operated) had the ligature immediately removed after the procedure and 60 animals were kept with the ligature until the end of each experimental period.

The mandibles were removed and digital x-ray images were obtained at 3, 7, and 14 days after the ligature procedure. Alveolar bone loss was estimated by the distance between the cemento-enamel junction and the height of alveolar bone in mesial root surfaces of lower right first molars with the aid of software.^{**}

Long-Term Inhibition of iNOS

Two weeks before the ligature placement, 30 of the ligatured animals received AG^{††} (800 mg/L, equivalent to ≈ 80 to 110 mg/kg/d¹⁰ dissolved in drinking water¹⁴); control animals received tap water alone. AG treatment continued until the sacrifice of the animals at 3, 7, or 14 days after ligature placement. In this way, for each time period, three groups of 10 animals per group were studied: sham, ligature, and ligature plus AG.

Histologic Analysis

The right hemimandibles collected from rats 14 days after ligature placement were soaked in 10% formalin for 48 hours. Decalcification was carried out in 4.13% EDTA (pH 7.2) at 4°C for ≈ 3 months. Serial paraffin sections of 5 μ m thickness were made on the mesio-distal aspects of the whole first lower right molars and stained with hematoxylin and eosin. Morphologic studies were made on the buccal and lingual gingiva and alveolar bone.

OC Culture

Murine OC cultures were generated as previously described.¹⁵ Briefly, bone marrow cells were flushed from freshly isolated femurs and tibias from 3- to 5-week-old C57BL/6 mice. Bone marrow cells were cultured in 48-well tissue culture plates (5×10^5 cells/ 0.5 mL/well) for tartrate-resistant acid phosphatase (TRAP) staining (a marker of OC identity) and dentin disk resorption. OC differentiation was induced with murine colony-stimulating factor (50 ng/mL^{‡‡}) and soluble RANKL (20 ng/mL^{§§}) in minimum essential medium, alpha modification culture media^{||||} containing 10% heat inactivated fetal bovine serum^{¶¶} and 1% penicillin-streptomycin.^{##} Culture media were replaced every other day. At the end of the incubation period, bone marrow cultures were fixed in 10% formalin and stained for TRAP for 15 minutes. Digital images of TRAP-stained OC were captured with an inverted microscope^{***} and a video camera.^{†††} Images of TRAP-positive cells were digitally filtered

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^{††}Sigma Chemical, St. Louis, MO.

^{‡‡}Peprotech, Rocky Hill, NJ.

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^{||||}Invitrogen, Carlsbad, CA.

^{¶¶}Invitrogen.

^{##}Invitrogen.

^{***}Zeiss Axiovert 200, Carl Zeiss Microimaging, Thornwood, NY.

^{†††}Sony DFW-X700, Sony, Tokyo, Japan.

to remove cells with less than three nuclei using an image analysis software.^{†††} OC data are expressed as the percentage of total culture dish area covered by TRAP-stained cells with three or more nuclei (OC covered area).

In Vitro Bone Resorption

Bone marrow cells were cultured, as described previously, in 48-well tissue culture plates containing one 5-mm dentin disk in each well.^{§§§} Fourteen days later, dentin disks were successively washed with 0.2-M ammonium hydroxide and water to remove cells, and then dried. Disks were sputter-coated with gold-palladium and four rectangular images of the disk (total of 14 mm² surface area) were captured with a scanning electron microscope^{|||||} in sequential frames. Resorption pits were counted with a software program.^{¶¶¶} For enumeration, a single pit was counted as any contiguous area of bone resorption, even if it contained more than one scalloped area.

Statistical Analysis

Differences among the treatment groups were analyzed by one-way analysis of variance followed by the Student-Neuman-Keuls test for multiple comparisons. Values of $P < 0.05$ were considered as statistically significant.

RESULTS

Radiographic Evaluation of Bone Loss

Rats with ligature showed progressive and significant alveolar bone loss (after 7 days) compared to the sham group (day 3, 0.22 ± 0.03 versus 0.18 ± 0.02 mm; day 7, 0.48 ± 0.07 versus 0.16 ± 0.02 mm, $P < 0.001$; day 14, 0.63 ± 0.11 versus 0.20 ± 0.03 mm, $P < 0.01$) (Fig. 1). AG treatment significantly inhibited ligature-induced bone loss at days 7 and 14 after the ligature procedure (day 7, 0.18 ± 0.01 versus 0.48 ± 0.07 mm, $P < 0.001$; day 14, 0.18 ± 0.03 versus 0.63 ± 0.11 mm, $P < 0.05$).

Histologic Analysis of Inflammation and Bone Resorption

Gingiva and alveolar bone samples from sham-operated rats presented normal morphology. However, loss of gingival papilla, necrosis of connective tissue, inflammation, and bone resorption in the interproximal and interradicular region was observed in the histologic sections obtained 14 days after the induction of experimental periodontitis. AG treatment resulted in decreased inflammation and necrosis, preservation of connective tissue in the papilla, and less interproximal and interradicular bone resorption compared to ligature-only rats (Fig. 2).

OC Culture and In Vitro Bone Resorption

TRAP-positive cells having three or more nuclei were considered OC and presented as the proportion of the culture dish area covered by OC (OC covered area). OCs from iNOS KO mice covered a significantly lower area of the culture dish compared to the OCs obtained from wild-type (WT) mice ($4 \pm 0.6\%$ versus $8.1 \pm 0.8\%$; $P < 0.01$). To confirm these observations, bone marrow cells from both mouse groups were differentiated on dentin disks for 14 days. At the end of the incubation period, the surfaces of dentin disks were processed for scanning electron microscopy and resorption pits were counted. OCs from iNOS KO

^{†††}Olympus MicroSuite, Olympus America, Melville, NY.

^{§§§}Immunodiagnostic Systems, Boldon, UK.

^{|||||}Philips XL-20, Philips Electronics, Amsterdam, The Netherlands.

^{¶¶¶}Olympus MicroSuite, Olympus America.

mice significantly had fewer resorption pits compared to WT OCs (101.67 ± 17.9 versus 43.75 ± 9.2 pits; $P < 0.01$) (Figs. 3 and 4).

DISCUSSION

Although bacteria and their products can directly cause pathologic effects in periodontal tissues, damage to the periodontium secondary to the destruction of connective tissues (including the alveolar bone) occurs mainly through indirect mechanisms involving the activation of a number of host-mediated processes.^{16,17} In this study, we use the well-established model of ligature-induced periodontitis in rats, an easy, simple, and reliable technique for disease induction,^{18,19} characterized by a progressive accumulation of bacterial biofilm in the ligature and the consequent increase in inflammatory infiltrate, leading to destruction of the periodontal connective and bone tissues.

In the presence of periodontitis, iNOS expression has been found in rat macrophages, lymphocytes, and neutrophils, and in human macrophages, endothelial cells, and polymorphonuclear cells.^{8,20,21} Similarly to other inflammatory situations, iNOS-derived NO contributes to the amplification and progression of inflammatory cell infiltration and plasma extravasation,⁸ despite evidence in favor of the need of iNOS for an appropriate host defense against *Pg* infection in mice.²² In addition, iNOS-derived NO can stimulate apical migration of the junctional epithelium and the destruction of collagen fibers mediated by matrix metalloproteinases (MMPs).^{22,23} Interestingly, NO not only can upregulate MMP-1 activity in osteoblasts present in regions of bone resorption,²³ and activate neutrophil procollagenase (MMP-8) via the derived reactive intermediates peroxynitrite anion and nitrogen dioxide,²⁴ but also can downregulate the synthesis of tissue inhibitors of MMP.³ Furthermore, augmented levels of mRNA for both iNOS and MMP-9 have been found in gingiva from rats with periodontitis.²⁵

In this study, we show that the oral administration of AG to rats can inhibit the progression of ligature-induced alveolar bone loss (Fig. 1), and also prevent gingival papilla loss, connective tissue necrosis, inflammation, and bone resorption in the interproximal and interraderic region (Fig. 2). These results are in agreement with previous studies that show that the administration of either selective^{8,10,26} or non-selective^{26,27} NOS inhibitors can prevent bone loss secondary to periodontitis in rodents.

The results shown in this study probably reflect, at least in part, the ability of NO to regulate bone loss secondary to the activation of cytokines, such as IL-1 and interferon (IFN)- γ . For example, IL-1 acts primarily by increasing NO production by inducing iNOS expression in osteoblasts, and promoting the activation of the nuclear factor-kappa B (NF- κ B) in OC precursor cells, leading to differentiation. Furthermore, the presence of iNOS-derived NO is critical for the maintenance of NF- κ B expression, binding to the DNA and the resulting OC activity, because in IL-1 β -stimulated OCs from iNOS-KO mice, NF- κ B activation occurred only transiently and resulted in abnormal NF- κ B-DNA binding and failure in OC formation.⁷ However, both inhibitory and stimulatory effects have been shown for IFN- γ on OC formation from cultured human bone marrow cells.^{28,29} Actually, it seems that it is the time at which pre-OC cells are exposed to IFN- γ that determines the effects of this cytokine on bone metabolism. In fact, IFN- γ rapidly induces NO production in peripheral macrophages and inhibits the RANKL-mediated osteoclastogenic response in these cells. However, after macrophages are exposed to RANKL (as is the case in the bone microenvironment) and differentiation of cells to OCs takes place, IFN- γ -induced NO production and antiosteoclastogenesis are inhibited.³⁰ Because both IL-1 β and IFN- γ can trigger the induction of iNOS expression and activity, the subsequent production of NO

during long time periods may contribute to the dysregulation of the inflammatory response during periodontitis and the associated tissue destruction.³¹

In our study, OCs from iNOS KO mice showed diminished capacity for differentiation and activity, as evidenced by both the lower number of OCs in culture and the fewer resorption pits produced on dentin disks (Figs. 3 and 4) compared to the WT-derived cells. In terms of the direct effects of NO on OCs in vitro, previously published data are sometimes contradictory, thus making difficult the proposal of a unique mechanism without considering the experimental details. For example, Holliday et al.³² showed that the NO-donor sodium nitroprusside at low concentrations (1 μ M) reduces OC formation and activity in murine bone marrow culture. However, van't Hof and Ralston⁶ observed that in osteoblast–OC coculture, high levels of NO produced by osteoblasts can inhibit bone resorption by two mechanisms: induction of apoptosis of OC progenitors present in the bone marrow cell population, and inhibition of the resorptive activity of mature OCs. Using either pure NO (at 30 μ M) or the NO-releasing agents 3-morpholino-sydnonimin-1 and sodium nitroprusside (both at 250 μ M), MacIntyre et al.⁴ observed a marked decrease in cell spread area and bone resorption pit number. Collin-Osdoby et al.³³ showed that NOS inhibition by AG favored OC formation and promoted OC bone resorption using the chick chorioallantoic membrane model of OC development. Fukada et al.³⁴ reported that iNOS-KO mice developed greater inflammatory cell recruitment, osteolytic lesions, and more TRAP-positive OCs than WT mice in a model of bone loss secondary to pulp exposure followed by bacterial inoculation. In addition, these observations correlated with increased expression of NF- κ B, stromal-cell-derived factor-1 α (SDF-1 α /CXCL12), and reduced expression of osteoprotegerin in the osteolytic lesions,³⁴ thus evidencing that NO deficiency is associated with imbalanced bone-resorption-modulating factors. However, Brandi et al.² proposed that intermediate levels of NO stimulate OC activity, whereas either the absence or the excess of NO represses OC activation in isolated rat bone marrow cells. Based on these results and the conditions under which our supportive experiments were performed, we suggest that the effects of NO on bone metabolism are highly dependent on the available amounts of this mediator. Whereas in low quantities NO may support osteoblast bone formation and OC-mediated bone remodeling (under both basal and cytokine-induced conditions),^{2,7,35–37} the augmented biosynthesis of NO (or the administration of NO-donating compounds) can inhibit, in a concentration-dependent manner, OC formation and bone resorption. Consequently, NO can prevent the in vitro bone loss observed secondary to isolated rat⁴ or avian OCs,³⁸ or in bone cell cultures,^{5,37,39} and the in vivo bone resorption in rats,⁴⁰ as it happens to occur during severe inflammation or in estrogen-deficient animals.^{41–44} Conversely, iNOS deficiency or pharmacologic inhibition of NO can accelerate OC formation and bone resorption in vivo and in vitro, decrease normal bone mass, exacerbate bone destruction in arthritis or osteoporosis models, and interfere with normal fracture healing.^{43,45–48}

It must be noted that both pharmacologic and KO animal studies have limitations. In pharmacologic studies, for example, the specificity of the NOS inhibitors,⁴⁹ or even the actual chemical identity (mainly related to the redox state) of the donated NO from the heterogeneous group of compounds generically known as “NO-donors”⁵⁰ continues to be an issue of debate. However, in the studies with KO mice (lacking each of the NOS isoforms), it is well demonstrated that NO from other isoforms could compensate for gene deletion.^{33,51} In this way, additional studies are mandatory to fully elucidate the roles of endogenous NO derived from the various NOS sources on bone metabolism.⁵²

CONCLUSIONS

Using both pharmacologic and gene-KO approaches, the present study shows that iNOS-derived NO is involved in periodontitis-induced alveolar bone loss by enhancing OC

differentiation and activity. These observations reinforce the potential therapeutic value of specific iNOS-inhibitor compounds to be used for the prevention of inflammatory bone diseases, including periodontal disease.

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Figure 1.

Chronic treatment with the iNOS inhibitor AG inhibits alveolar bone loss induced by the placement of a subgingival cotton ligature around the lower first molar. **A)** Measurement of the distance from the cemento-enamel junction (CEJ) to the alveolar bone crest was performed from digital x-ray images of the jaws ($n = 10$). * $P < 0.001$ and † $P < 0.01$ versus sham group and * $P < 0.001$ and † $P < 0.05$ versus ligature group.

B) Representative x-ray images.

Figure 2. Histologic sections of the interproximal and interradicular region of right lower first molar of rats stained with hematoxylin and eosin. Representative histologic images of each experimental group at 14 days after ligature placement (original magnification $\times 10$). *S* = sham; *L* = ligature; *L+AG* = ligature + AG.

Figure 3.

OC stained for TRAP after 7 days of culture. **A)** Representative images (original magnification $\times 4$). **B)** Percentage of total culture dish area covered by TRAP-positive OCs with three or more nuclei. * $P < 0.01$ versus WT; $n = 6$.

Figure 4. In vitro bone resorption. **A)** Representative images (original magnification $\times 50$). **B)** Resorption pits counted on scanning electron microscopy; $n = 12$. * $P < 0.001$ versus control and $^{\dagger}P < 0.01$ versus WT.