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Proinflammatory Agents, IL-8 and IL-10, Upregulate Inducible Nitric Oxide Synthase Expression and Nitric Oxide Production in Avian Osteoclast-Like Cells

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Abstract Nitric oxide synthase (NOS) isoenzymes generate nitric oxide (NO), a sensitive multifunctional intercellular signal molecule. High NO levels are produced by an inducible NOS (iNOS) in activated macrophages in response to proinflammatory agents, many of which also regulate local bone metabolism. NO is a potent inhibitor of osteoclast bone resorption, whereas inhibitors of NOS promote bone resorption both in vitro and in vivo. The possibility that osteoclasts, like macrophages, express a regulated iNOS and produce NO as a potential autocrine signal following inflammatory stimulation was investigated in well-characterized avian marrow-derived osteoclast-like cells. NO production (reflected by medium nitrite levels) was markedly elevated in these cells by the proinflammatory agents lipopolysaccharide (LPS) and the synergistic action of IL-1α, TNFα, and IFNγ. Inhibitors of NOS activity (aminoguanidine, L-NAME) or iNOS induction (dexamethasone, TGFβ) reduced LPS-stimulated nitrite production. LPS also increased the NOS-associated diaphorase activity of these cells and their reactivity with anti-iNOS antibodies. RT-PCR cloning, using avian osteoclast-like cell RNA and human iNOS primers, yielded a novel 900 bp cDNA with high sequence homology (76%) to human, rat, and mouse iNOS genes. In probing osteoclast-like cell RNA with the PCR-derived iNOS cDNA, a 4.8 kb mRNA species was detected whose levels were greatly increased by LPS. Induction of iNOS mRNA by LPS, or by proinflammatory cytokines, occurred prior to the rise of medium nitrite in time course studies and was diminished by dexamethasone. Moreover, osteoclast-like cells demonstrated an upregulation of NO production and iNOS mRNA by IL-8 and IL-10, regulatory mechanism's not previously described. It is concluded that osteoclast-like cells express a novel iNOS that is upregulated by inflammatory mediators, leading to NO production. Therefore, NO may serve as both a paracrine and autocrine signal for modulating osteoclast bone resorption. © 1996 Wiley-Liss, Inc.

Key words: lipopolysaccharide, interleukin-1, tumor necrosis factor, interferon, transforming growth factor β, dexamethasone, RT-PCR, NADPH diaphorase, bone

Bone resorption is a unique function of osteoclasts and is subject to complex regulation by a host of systemic and local signals, including hormones, cytokines, prostaglandins, leukotrienes, and matrix components. Over the past decade, a novel multifunctional signal molecule, the free radical nitric oxide (NO), has been found to act as a sensitive mediator of intercellular communication in such diverse physiological processes as neurotransmission, vascular signaling and vessel tone, blood clotting, immune-activated macrophage cytotoxicity, and long-term memory potentiation [Bredt and Snyder, 1994; Lowenstein et al., 1994, Moncada and Higgs, 1993]. Recently, NO has also been shown to serve as a potent inhibitor of bone resorption by isolated avian or rat osteoclasts as well as in bone organ culture systems [reviewed in Collin-Osdoby et al., 1994]. NO-mediated inhibition of osteoclasts is manifested by a reduction in their cell spread areas on bone (contraction), formation of smaller individual resorption pit sites, and diminished overall amounts of bone resorbed [MacIntyre et al., 1991; Kasten et al., 1994]. Conversely, inhibitors of NO production lead to increased osteoclastic bone resorption both in vitro and in vivo [Kasten et al., 1994; Ralston et al., 1994; Lowik et al., 1994; Brandi et al., 1995]. Further research is needed to understand this and other roles for NO in bone physiology, to discover sites of NO production and

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action, and to elucidate potential interactions of NO with other signaling systems governing homeostatic bone formation and turnover processes.

NO is produced from L-arginine in an oxidative reaction catalyzed by nitric oxide synthase (NOS) isoenzymes of three classes, the neuronal and endothelial constitutive forms and a macrophage inducible form, all of which are highly regulated by diverse mechanisms [Bredt and Snyder, 1994; Lowenstein et al., 1994; Moncada and Higgs, 1993; Knowles and Moncada, 1994; Nathan and Xie, 1994]. Activation of the contitutive NOS isoenzymes by Ca^{++}/calmodulin results in transient local bursts of low levels of NO, whereas NO production by immune activated cells in response to infection, injury, or inflammation is characterized by the prolonged synthesis of very high levels of NO as a consequence of the de novo transcription of an inducible NOS (iNOS). NO levels are typically elevated in various pathological conditions, autoimmune disorders, or inflammatory diseases, such as in rheumatoid and osteoarthritis, diabetes, AIDS, and periodontal disease, and many of these involve localized bone loss [Bredt and Snyder, 1994; Lowenstein et al., 1994; Moncada and Higgs, 1993; Collin-Osdoby et al., 1994; Knowles and Moncada, 1994]. Inflammatory cytokines, such as IL-1, IL-6, IFNγ, and TNFα, and bacterial products like lipopolysaccharide (LPS) are also elevated during infection/inflammation and have been shown to elicit an induction of iNOS expression in macrophages and other cell types. Induction of iNOS by these agents can be inhibited by other typically immune-deactivating cytokines, such as IL-4, IL-8, IL-10, or TGFβ, or by dexamethasone, via transcriptional and/or posttranscriptional mechanisms [Lowenstein et al., 1994; Nathan and Xie, 1994; Schneemann et al., 1993]. The same cytokines that promote iNOS induction also participate in the regulation of bone remodeling under normal or pathological conditions by influencing the recruitment, differentiation, and functional regulation of osteoclasts and osteoblasts. Moreover, IL-1 and TNFα are among those factors well established as mediators of inflammatory bone loss [Gowen, 1992]. Recently, inflammatory cytokines have been shown to promote NO production by bone-forming osteoblasts [Ralston et al., 1994; Lowik et al., 1994; Damoulis and Hauschka, 1994] and to either stimulate or inhibit osteoclast bone resorption [Lowik et al., 1994; Gowen, 1992; Brandi et al., 1995]. Cytokine-stimulated NO production by osteoblasts has been proposed as one mechanism to limit the degree of bone resorption that is promoted by some of the same inflammatory cytokines [Lowik et al., 1994].

Whether osteoclasts produce NO in addition to responding to exogenous sources of this signal is not yet clear. Since NO clearly inhibits osteoclast bone resorptive activity, it might seem unlikely that osteoclasts would also synthesize this free radical. However, several lines of evidence point to this possibility. First, rat [Schmidt et al., 1992] and avian [Kasten et al., 1994] osteoclasts in active bone remodeling regions of tissue sections, as well as isolated avian osteoclasts in the process of resorbing bone slices in culture [Kasten et al., 1994], exhibit marked staining for an NADPH-dependent diaphorase activity, consistent with the possible presence of an NOS activity. In addition, treatment of cell cultures highly enriched for osteoclasts with inhibitors of NOS activity results in a potentiation of their bone resorptive activity [Kasten et al., 1994]. Furthermore, since osteoclasts arise from hematopoietic precursors of the monocyte-macrophage lineage within the bone marrow and, consequently, share certain lineage and functional characteristics with macrophages, it may be that osteoclasts and/or their precursors also express iNOS and produce NO in response to inflammatory cytokines or agents.

We have previously described the in vitro generation of multinucleated osteoclast-like cells from avian bone marrow mononuclear precursors under the influence of osteoblast-derived soluble factors [Collin-Osdoby et al., 1995]. These cells display increased expression of numerous osteoclast phenotypic and functional characteristics including morphological features, tartrate-resistant acid phosphatase (TRAP) activity, calcitonin responsiveness, a series of osteoclast-specific antigenic markers, lysosomal enzyme profiles, and bone resorptive activity [Collin-Osdoby et al., 1995]. In the current studies, we have employed this marrow-derived osteoclast-like cell system to ascertain whether such cells possess an inducible NOS and generate NO. Our results demonstrate the presence of low basal levels of an iNOS protein in osteoclast-like cells. Exposure of these cells to inflammatory agents led to a marked increase in iNOS isoenzyme levels concomitant with elevated NO production. This system also demonstrates the first

reported examples to our knowledge of iNOS induction by IL-8 or IL-10, which may indicate a unique role for iNOS in osteoclasts or osteoclast-like cells. A portion of the avian iNOS gene was cloned by RT-PCR and the regulation of iNOS mRNA by various modulators was investigated. The finding that osteoclast-like cells produce NO in a regulated fashion suggests that osteoclasts, as well as osteoblasts, may synthesize NO in response to inflammatory agents, that NO may function as both a paracrine and autocrine modulator for inhibiting osteoclastic bone resorption activity, and that osteoclast-derived NO may therefore represent an additional mechanism for the regulation of local bone resorption.

MATERIALS AND METHODS

Marrow-Derived Osteoclast-Like Cell Preparation and Culture

Mononuclear cells were isolated from the bone marrow obtained from 15 White Leghorn hatchlings maintained for 28 days on a low calcium diet as described previously [Collin-Osdoby et al., 1995]. All animals were handled in accordance with the institutional Animal Care and Use Committee and standards approved by the NIH Guide for the Care and Use of Experimental Animals. Ficoll-Hypaque separated cells were plated at 7×10^6 cells per well in six-well dishes in α-MEM containing 10% fetal calf serum (FCS) (GIBCO BRL, Gaithersburg, MD) and 1% antibiotic/antimycotic (GIBCO BRL). Fresh medium containing 25% UMR 106-01 cell-free conditioned medium to promote osteoclast-like cell differentiation [Collin-Osdoby et al., 1995] was added to the mononuclear marrow cells every other day starting on the second day of culture and continued until large multinucleated giant cells covered the surface of the dishes (6–8 days). The medium was then changed to phenol red–free α-MEM with 10% FCS and 1% antibiotic/antimycotic, and the various modulators were added to this medium. In the case of multiple modulators, these were added simultaneously to the cells. The modulators used were obtained from the following sources: *E. coli* lipopolysaccharide (LPS) serotype 0111:B4, dexamethasone, sodium nitroprusside (SNP), Nω-nitro-L-arginine-methyl ester (NAME), and aminoguanidine (AG) from Sigma Chemicals (St. Louis, MO), human recombinant cytokines IL-1α, IL-1β, TNFα, IL-8, IL-10, and porcine TGFβ1 from R & D Systems (Minneapolis, MN), and rat recombinant INFγ from GIBCO BRL. Maximal potential contaminating endotoxin levels in the recombinant cytokine preparations were at least 100-fold lower than that required for nitrite induction in these osteoclast-like cells. After 24 h (unless otherwise indicated) the conditioned media were collected, briefly centrifuged, and stored at −20°C. The cells were rinsed with warm Hanks' balanced salt solution (HBSS) three times and either stored at −20°C with 1 ml of lysis solution (0.01% Triton X-100, 1 M NaCl) per well [Collin-Osdoby et al., 1995], fixed for 15 min in 1% formaldehyde/HBSS, or extracted immediately for RNA.

Nitrite Assay

NO production was measured by determination of nitrite levels (as a stable end product of NO) in the culture supernatants from cells using the Greiss reagent in a microplate assay according to the procedure described by Imai et al. [1993]. Nitrite determinations were corrected for basal readings obtained from the feeding medium alone (not incubated with cells). Total cellular protein was analyzed using the Lowry protocol [Lowry et al., 1951] after thawing the frozen cell samples in lysis solution, scrape-harvesting, and freeze-thawing three times [Collin-Osdoby et al., 1995]. Since modulator treatments did not cause detectable differences in cell viability, attachment, or total cell protein yields obtained, all nitrite data was normalized for cellular protein and the results expressed as nanomoles of nitrite per milligram of cellular protein.

Diaphorase Staining

Cytochemical localization of NADPH-dependent nitroblue tetrazolium reduction reflecting potential NOS activity was performed on fixed, permeabilized cells [Kasten et al., 1994]. Following fixation, cells were HBSS-rinsed three times, water-rinsed once, and air-dried. Staining was performed in the dark for 30 min at 37°C as described in Kasten et al. [1994], the cells were rinsed well, and glycerol buffered mounting medium (Becton, Dickenson and Co. Cockeysville, MD) was directly applied for photography and storage at 4°C.

Immunolocalization of NOS

Osteoclast-like cells were fixed, rinsed three times with HBSS, and immediately processed for staining with either of two polyclonal antibod-

ies, designated 586 and 587. These antibodies were raised against the mouse inducible NOS N-terminal region (PAb 587) or C-terminal region (PAb 586) as described in Ellman et al. [1993] and were generously provided to us by Dr. Tom Misko (Monsanto Corp., St. Louis, MO). Following permeabilization of cells by brief treatment with methanol (−20°C, 1 min) or by saponin (0.25%) included during the blocking step, the cells were reacted with the rabbit antiserum (1:50 in block), washed, reacted with a secondary biotinylated goat antirabbit antibody (1:250 in block; GIBCO BRL), washed, reacted with streptavidin β-galactosidase (1:100), and incubated with X-gal according to the procedure described in Collin-Osdoby et al. [1995]. Controls for staining included no primary antibody incubations with LPS-treated or untreated cells, and staining without cell permeabilization, all of which yielded negative results.

RT-PCR

Poly(A) selected RNA was extracted from chicken osteoclast-like cells using the Quick Prep Micro mRNA Purification kit (Pharmacia, Piscataway, NJ), and cDNA was synthesized using a cDNA cycle kit (Invitrogen, San Diego, CA). PCR was performed using the following oligonucleotides: iNOS.F1 and iNOS.R1 (Fig. 5), derived from human hepatocyte iNOS cDNA [Geller et al., 1993] and generously provided by Dr. Charles Rodi (Monsanto Corp., St. Louis, MO), and GDH.F (GATTCTACACACGGACACTTC) and GDH.R (TAGCACCACCCTTCAGATGAG) derived from glyceraldehyde-3-phosphate dehydrogenase (GDH) chicken cDNA [Dugaiczyk et al., 1983]. The PCR products (906 bp from iNOS and 203 bp from GDH) were subcloned into a pCRII vector (TA cloning kit; Invitrogen) and sequenced by the dideoxy chain termination reaction method utilizing the Sequenase kit (USB, Cleveland, OH). The sequence of the 203 bp GDH clone was identical to that previously reported [Dugaiczyk et al., 1983]. Based on the sequence from the 906 bp clone, new oligonucleotides, iNOS.F2 and iNOS.R2 (Fig. 5), were synthesized and used as primers to complete the sequence of this avian cDNA fragment. These oligonucleotides were also used in a RT-PCR reaction to generate a smaller 362 bp product which was subcloned into pCRII and used as a template to generate RNA probes for ribonuclease protection assays (RPA). The sequence of the 362 bp iNOS clone was identical to that contained within the 906 bp fragment. The nucleotide sequence of the 906 bp fragment was compared with published cDNA sequences using computation performed at the NCBI and the BLAST network service. The cloned avian iNOS sequence has been deposited in GenBank and assigned the accession number U34045.

RNA Isolation and Northern Blot Analysis

Total RNA was obtained from cultured cells by the method of Chomczynski and Sacchi [1987]. Briefly, the cells in each well of a six-well dish were dissolved in 0.5 ml of the GITC denaturing solution, sonicated using a microtip probe for 10 s at setting 20 (Biosonik model IIA; Bronwill Scientific, Rochester, NY), and incubated for 10 min at 37°C. RNA was extracted twice with phenol at low pH and precipitated twice with 2.5 volumes of ethanol. Northern analysis was performed by separating the RNA samples (10 μg) on a 1.5% agarose/2 M formaldehyde gel, transferring the RNA to a nylon membrane (MSI, Westboro, MA), and hybridizing the RNA blots for 18 h at 42°C with the avian 906 bp iNOS or 203 bp GDH probes labeled by random primer extension (Random Primers DNA Labeling System; GIBCO BRL). After extensive washing, the blots were subjected to autoradiographic detection. Blots were hybridized first with the iNOS probe and then with the GDH probe.

Ribonuclease Protection Assay (RPA)

RNA probes for iNOS and GDH were generated from the 362 bp iNOS and 203 bp GDH PCR clones using the in vitro transcription kit MAXIscript (Ambion, Austin, TX) and purified in a 5% acrylamide/8 M urea gel (0.75 mm). RPA was performed using the RPA II kit (Ambion) by hybridizing total RNA (5 μg) for 18 h at 42°C with both the iNOS and GDH RNA probes (10^5 cpm per probe) and subsequently digesting with RNAse. The protected fragments were separated in a 8% polyacrylamide/8 M urea gel (0.4 mm), detected by autoradiography, and quantified in a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Statistical Analysis

Data are presented as the mean ± standard error of the mean. Significance of differences was analyzed by ANOVA with repeated measures. For biochemical analysis of nitrite as a

function of modulator treatment, two or more wells per condition were each assayed in duplicate, and a minimum of two independent trials were performed for each experiment with similar results. RNA levels (Northerns and RPA) were measured from wells of at least two independent trials. A P value of less than 0.05 was considered significant.

RESULTS

LPS and Inflammatory Cytokines Stimulate Nitrite Production in Avian Osteoclast-Like Cells

Production of NO by osteoclast-like cells was determined by measurement of the levels of nitrite, a stable end product of NO, in the culture medium. Avian bone marrow-derived osteoclast-like cell cultures spontaneously released low levels of nitrite (5–15 nmoles/mg cellular protein in 24 h) that were dramatically elevated twenty- to thirtyfold by LPS treatment of the cells (Fig. 1). Exposure to 0.1 ng/ml LPS was required for induction of nitrite production, and lower LPS concentrations (0.01 pg/ml to 0.01 ng/ml) were ineffective in eliciting this response. Incubation with as little as 1 ng/ml LPS was sufficient to elicit maximal nitrite generation, and higher concentrations of LPS (10 ng/ml to 10 μg/ml) did not further raise the elevated level of nitrite production under these culture conditions. Cytokine treatment of osteoclast-like cells was also capable of eliciting nitrite production. Individually, small (1.5–2-fold) but reproducible and significant increases in nitrite were obtained with either IL-1α (10^{-10} M), IL-1β (10^{-10} M; not shown) or IFNγ (100 U/ml), moderate rises (threefold) were achieved with TNFα (10^{-9} M), and higher nitrite levels (sixfold over control) were obtained by the combined stimulation of these three cytokines for 24 h (Fig. 2A). Any paired combination of these three cytokines yielded nitrite increases equivalent to those of the individual cytokines alone; all three were required for a maximum effect. Moreover, treatment with either IL-8 or IL-10 alone for 24 h caused a dose-dependent increase in nitrite production (Fig. 2B). When any of these cytokines were added together with LPS, little or no further increase in nitrite production was observed over that achieved by LPS alone. Simultaneous administration of either 17-β-estradiol (10^{-8} or 10^{-7} M) or 1,25-dihydroxyvitamin D_3 (10^{-7} M), hormones which, respectively, inhibit or stimulate bone resorption, did not alter the LPS-stimulated rise in nitrite production from osteoclast-like cells.

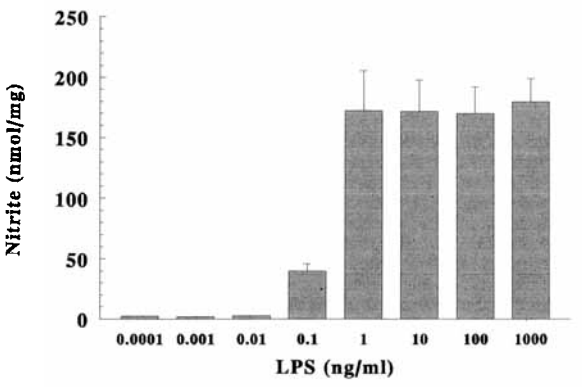

Fig. 1. Nitrite accumulation in the culture medium of osteoclast-like cells as a function of LPS concentration. Avian osteoclast-like cells were incubated for 24 h with the indicated concentrations of LPS, after which the culture medium was assayed for nitrite content as a measure of nitric oxide production. Data was obtained from at least four independent cultures for each LPS concentration. LPS concentrations of 0.1 ng/ml to 10 μg/ml significantly increased nitrite production over control levels ($P \leq 0.001$). In addition, significantly more nitrite was obtained using 1 ng/ml LPS in comparison to 0.1 ng/ml ($P \leq 0.001$) and using 0.1 ng/ml LPS in comparison to 0.01 ng/ml ($P \leq 0.005$). No statistically significant differences in elevated media nitrite levels were observed between cells treated with the higher concentrations of LPS (1 ng/ml to 10 μg/ml LPS) nor in basal nitrite levels for cells treated with the lower concentrations of LPS (0.01 pg/ml to 0.01 ng/ml).

Inhibitors of iNOS Reduce Stimulated Nitrite Production

To test whether the elevated nitrite production by osteoclast-like cells following proinflammatory stimulation was dependent upon an inducible NOS, inhibitors of NOS activity or its expression were evaluated for their effects on these cells (Fig. 3). The general NOS competitive inhibitor L-NAME (500 μM), but not its inactive isomer D-NAME (500 μM), inhibited by approximately 75% the LPS-stimulated nitrite production in osteoclast-like cells. Similarly, AG (500 μM), a selective inhibitor of the inducible form of NOS, inhibited by approximately 55% the LPS-stimulated nitrite production from these cells. Dexamethasone and TGFβ, both of which inhibit the expression of inducible NOS and production of NO in other cell types, also elicited a significant inhibition of the LPS-dependent nitrite accumulation in the culture medium of osteoclast-like cells. Thus, coadministration of dexamethasone (10^{-7} M) or TGFβ (8×10^{-11} M) in conjunction with LPS inhibited the LPS-dependent rise in nitrite production by

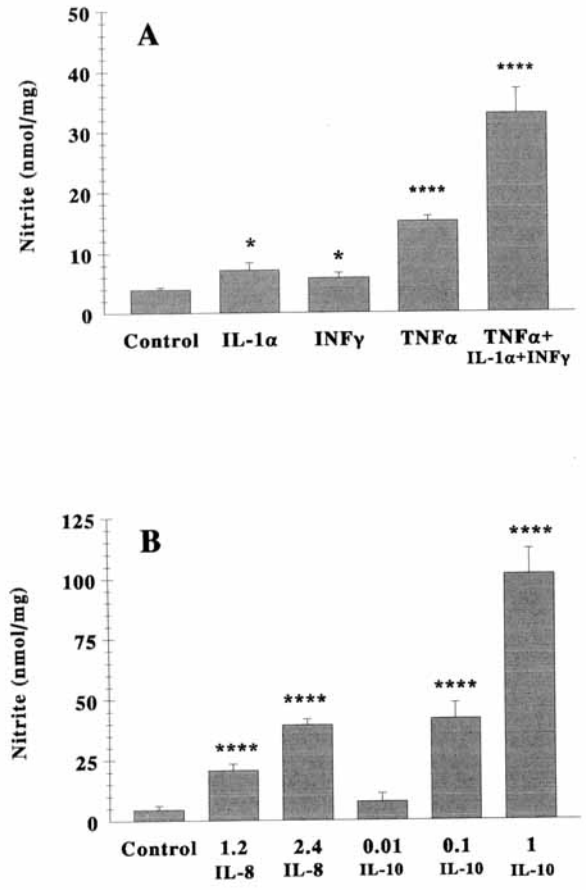

Fig. 2. Cytokine-dependent accumulation of nitrite in the conditioned medium of osteoclast-like cells. Osteoclast-like cells were incubated with cytokines, alone or in combination, for 24 h prior to analysis of the nitrite levels in the conditioned media. **A:** IL-1α (10^{-10} M), IFNγ (100 U/ml), and TNFα (10^{-9} M) elevation of medium nitrite levels. The triple cytokine cocktail contained the same concentrations of each cytokine as was tested individually. Data was obtained from at least four independent cultures for each condition. Significant differences in nitrite levels from control cultures are denoted as follows: $*P \leq 0.05$; $****P \leq 0.001$. The statistical significance between the triple cytokine cocktail and the individual cytokines alone was $P \leq 0.005$ for TNFα and $P \leq 0.001$ for IL-1α and IFNγ. **B:** Dose-dependent elevation of medium nitrite levels for osteoclast-like cells treated with IL-8 or IL-10 for 24 h. Numbers indicate concentration × 10^{-9} M. Significant differences from control: $****P \leq 0.001$.

approximately 35% and 40%, respectively, in comparison with that achieved by LPS alone. Furthermore, L-NAME, AG, dexamethasone, and TGFβ all inhibited basal nitrite production by osteoclast-like cells to a similar degree as in the LPS-stimulated cells. Although IL-8 and IL-10 have been found to dampen cytokine-induced nitrite release in some cell types, neither IL-8 (2.4×10^{-9} M) nor IL-10 (10^{-10} M) coadministered with LPS reduced the LPS-

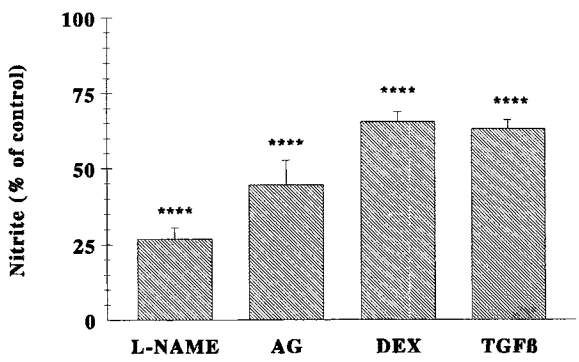

Fig. 3. NOS inhibitors decrease the LPS-induced nitrite production by osteoclast-like cells. Modulators were incubated with osteoclast-like cells for 24 h in the presence of LPS, after which the conditioned medium was assayed for nitrite content. Modulators tested were AG (500 μM), L-NAME (500 μM), dexamethasone (Dex) (10^{-7} M), and TGFβ (8×10^{-11} M). Inhibition was found to be independent of the LPS concentration used (0.01–1 μg/ml). Results are expressed as the percent medium nitrite per milligram of cellular protein in inhibitor plus LPS-treated cultures relative to that of control cultures (receiving LPS alone). Data were obtained from at least six independent cultures. Significant differences from LPS treatment alone: $****P \leq 0.001$. In parallel studies, these inhibitors similarly reduced basal nitrite production by osteoclast-like cells in the absence of LPS by the percent indicated: AG (55%), L-NAME (70%), Dex (35%), and TGFβ (20%).

stimulated nitrite production by these osteoclast-like cells.

LPS Stimulates Diaphorase Activity in Osteoclast-Like Cells

In addition to converting L-arginine to citruilline and NO, NOS isoenzymes also demonstrate an NADPH-dependent diaphorase activity which can be detected cytochemically. To determine whether increased nitrite levels in the media collected from LPS-treated osteoclast-like cells were correlated with increased NADPH diaphorase activity, such cells were stained for NADPH diaphorase activity. Whereas untreated cells displayed only weak diaphorase staining (Fig. 4A), those treated with LPS evidenced substantially stronger diaphorase staining (Fig. 4B). Therefore, LPS treatment of osteoclast-like cells led to both increased diaphorase activity and nitrite production, supporting the premise that LPS induces an NOS activity in these cells.

Immunolocalization of Inducible NOS

The large increases in nitrite produced in response to proinflammatory agents and the inhibition of such induction by AG, dexamethasone, or TGFβ pointed to the likelihood that the

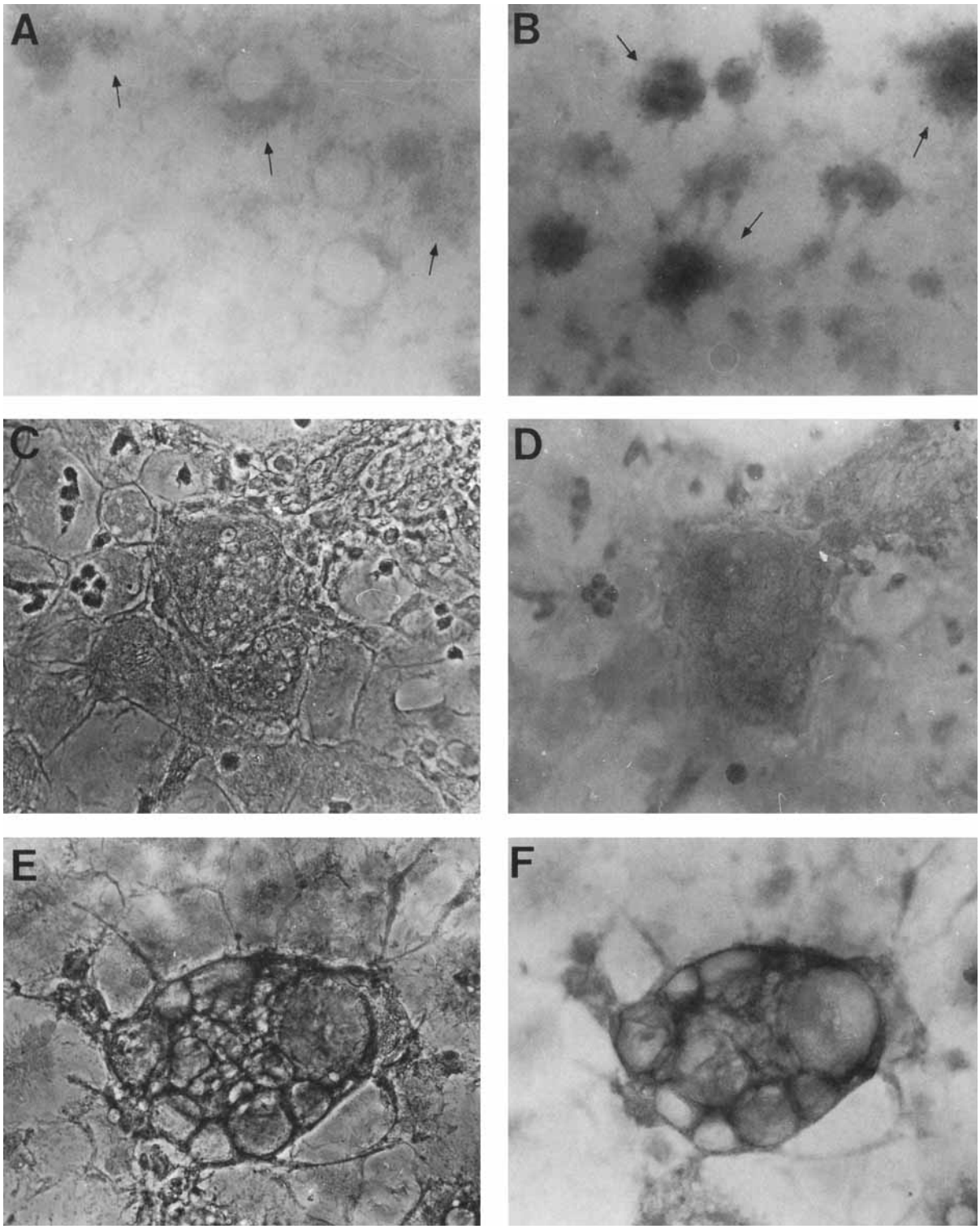

Fig. 4. NOS-associated diaphorase staining and immunolocalization of iNOS in osteoclast-like cells. **A,B:** NADPH-dependent diaphorase activity was evaluated by staining control (A) and LPS (1 μg/ml, 24 h)-treated (B) osteoclast-like cells (*arrows*) after their fixation and permeabilization (see Materials and Methods). Increased staining was observed for the multinucleated osteoclast-like cells in LPS-treated cultures in comparison with untreated cells. ×272. **C,D:** Untreated osteoclast-like cells were immunostained with an anti-iNOS antibody, PAb 587, and the same field photographed under phase contrast (C) to visualize cells and bright field (D) microscopy to better observe immunoreactivity. ×680. **E,F:** LPS-stimulated osteoclast-like cells were similarly stained with PAb 587 and analyzed by phase contrast (E) and bright field (F) microscopy. Staining was significantly greater in the LPS-treated cells. ×680.

osteoclast-like cells expressed an inducible NOS. Since induction of iNOS activity is typically achieved via an upregulation in the expression of iNOS message and protein levels, differences in iNOS protein levels were analyzed in control vs. LPS-stimulated osteoclast-like cells using two antibodies directed against iNOS. Immunocytochemical staining revealed an enhanced reactivity of LPS-stimulated cells (Fig. 4E,F) over untreated cells (Fig. 4C,D) with antibodies either to the N-terminal (PAb 587) (Fig. 4) or C-terminal (PAb 586) (not shown) domain regions of iNOS. Therefore, LPS stimulation of NOS activity correlated with elevated levels of iNOS protein in the osteoclast-like cells.

RT-PCR Cloning and Northern Analysis of Avian iNOS From Osteoclast-Like Cells

RT-PCR was performed to determine whether osteoclast-like cells express iNOS mRNA. Complementary DNA was generated from uninduced osteoclast-like cell mRNA and used as a template for a PCR reaction with oligonucleotides (iNOS.F1 and iNOS.R1) that derived from regions of the human iNOS (nucleotides 520–541 and 1424–1402) which were perfectly conserved in the mouse and rat genes but mismatched for similar regions of endothelial or neuronal constitutive NOS genes [Geller et al., 1993]. A single amplicon product of 906 bp was obtained and fully sequenced (Fig. 5). The results revealed a novel sequence having the highest homology (76% nucleotide and 80% amino acid identity) to the corresponding regions of the human hepatocyte iNOS, the mouse and rat macrophage iNOS, and the rat smooth muscle iNOS genes [Geller et al., 1993; Xie et al., 1992; Nunokawa et al., 1993; Wood et al., 1993] over other sequences deposited in the gene banks. Less homology was observed in comparisons with constitutive NOS isoenzymes. Table I summarizes these findings.

To further establish that iNOS message was present in the osteoclast-like cells, total RNA was isolated from cells cultured for 24 h in the presence or absence of LPS (1 μg/ml) and analyzed in a Northern blot using the 906 bp PCR clone as a probe for avian iNOS. A 4.8 kb transcript was strongly evident in LPS-treated cells which was only weakly detectable in unstimu-

CGAAACAACA GGAACCTACC ATCTGACAAA GGATGAACTG ATCTTTGCTG CCAAACAGGC
iNOS.F1
CTGGAGGAAT GCTCCAAGAT GTATTGGGAG AATCCAGTGG TCCAATCTAC AGGTATTTGA

TGCACGTGAT TGTAAAACAG CCAAGGAAAT GTTTGAATAT ATCTGTCGCC ATATTCAGTA

TGCCACAAAC AATGGAAATA TAAGATCAGC CATCACCATT TTCCCTCAGA GGACTGATGG

GAAACATGAT TTCCGTGTTT GGAACAGCCA GCTCATCCGA TATGCTGGAT ATCAAATGCC
iNOS.F2
AGATGGGTCT GTCATAGGAG ACCCTGCAAG TGTGGAGTTC ACAAAGTTGT GCATTGAGCT

TGGGTGGAAG CCGAAATATG GCCGCTTTGA TGTAGTTCCA CTCATTCTCC AAGCAAACGG

CCAAGATCCA GAAATATTTG AATACCCGCC AGAGATTATC CTTGAAGTGC CAATGGAGCA

TCCAAAATAT GAGTGGTTTA AGGAGTTGGA TCTGAAGTGG TATGCTCTGC CTGCTGTTGC

CAACATGCTC CTTGAGGTGG GAGGTCTGGA ATTTACTGCG TGTCCTTTCA ACGGCTGGTA

CATGGGAACA GAGATTGGAG TGCGAGACTT CTGTGATGTA CAGCGGTACA ATATCCTGAA
iNOS.R2
GGAGGTTGGA AGAAGAATGG GACTGGAAAC CAACAAACTT GCATCATTGT GGAAGGACCG

AGCTGTTGTA GAGATAAATG TGGCTGTGCT TCATAGCTTC CAGAAACAAA ATG TTACTAT

AATGGATCAT CACTCAGCTG CTGAGTCCTT CATGAAATAC ATGCAGAATG AGTACCGTGT

GCGAGGAGGC TGCCCAGCTG ATTGGGTGTG GATTGTACCT CCTATGTCTG GGAGCATCAC
iNOS.R1
CCCCGT

Fig. 5. Nucleotide sequence of the iNOS clone obtained from osteoclast-like cells. A 906 bp clone was generated by RT-PCR from avian osteoclast-like cells using iNOS primers and sequenced by the dideoxy chain termination reaction procedure. The oligonucleotides used in the PCR to obtain the iNOS fragment are underlined here and described in Materials and Methods.

TABLE I. Comparison of the Avian iNOS cDNA Sequence With Reported cDNA Sequences Corresponding to Various NOS Genes*

Reported NOS cDNA	% identity	Accession number
Human hepatocyte iNOS	76	L09210, D26525
Human chondrocyte iNOS	76	X73029, U05810
Human tumor cell iNOS	76	L24553
Mouse macrophage iNOS	75	M87039, M84373, M92649
Rat smooth muscle iNOS	75	D14051, X76881
Rat macrophage iNOS	75	L12562
Rat hepatocyte iNOS	75	D12520
Rat astrocyte iNOS	75	U03699
Human brain cNOS	71	D16408, L02881
Rat brain cNOS	71	X59949
Mouse brain cNOS	70	D14552
Human endothelial (aortic) cNOS	66	M89952, M99057, M95674
Human endothelial (umbilical) cNOS	64	M93718, L26914

*Sequence comparisons were done using computation performed at the NCBI and the BLAST network service. Percent identity of the avian NOS cDNA to the reported genes and their accession numbers are indicated.

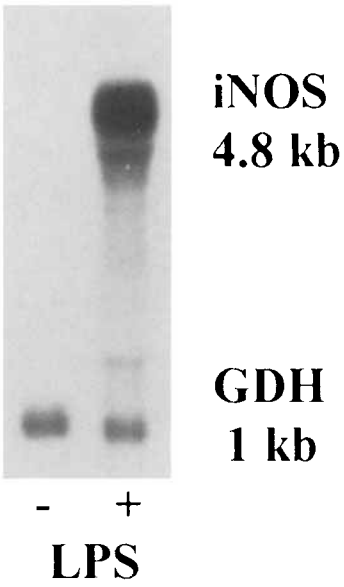

Fig. 6. Northern analysis of iNOS mRNA in avian osteoclast-like cells. Total RNA (10 μg) from cells incubated for 24 h in the absence (−) or presence (+) of 1 μg/ml LPS was analyzed in a Northern blot with the 906 bp iNOS clone. A 4.8 kb message was strongly detected in the LPS-treated cells. GDH (203 bp of the avian glyceraldehyde-3-P dehydrogenase cDNA) was used as a control and was not increased by LPS treatment.

lated osteoclast-like cells, although GDH mRNA was detected at similar levels in both the LPS-treated and untreated osteoclast-like cells (Fig. 6).

LPS and Proinflammatory Cytokines Induce the Expression of iNOS mRNA in Avian Osteoclast-Like Cells, and Dexamethasone Inhibits Induction

RNAse protection assays were used to determine the steady-state levels of iNOS mRNA in osteoclast-like cells as a function of LPS concentration. Stimulation with various concentrations of LPS resulted in a dose-dependent increase of the iNOS mRNA levels, indicating that the NO production stimulated by LPS was a consequence of induced iNOS gene expression (Fig. 7A,B). These studies also demonstrated that relatively lower levels of iNOS mRNA induction by 0.1 μg/ml LPS were sufficient to elicit near maximal nitrite production under these culture conditions (Fig. 7B). Furthermore, dexamethasone (10^{-7} M), which reduced both basal and LPS-dependent nitrite accumulation in the culture medium, inhibited by approximately 50% the LPS-mediated induction of iNOS mRNA in the osteoclast-like cells (Fig. 7B). Unstimulated cells had sufficiently low levels of iNOS mRNA that no effect of dexamethasone on basal iNOS mRNA levels was apparent.

Time course experiments showed that the steady-state levels of iNOS mRNA were already significantly elevated after 2 h of incubation with LPS (1 μg/ml) and reached a maximum level between 4 and 8 h of LPS treatment, after which they dropped considerably by 24 h of treatment (Fig. 8). Similarly, the maximum steady-state levels of iNOS mRNA in cells stimulated with the combination of cytokines IL-1α (10^{-10} M), INFγ (100 U/ml), and TNFα (10^{-9} M), although not as high as those elicited by LPS, were reached by 8 h of treatment and decreased to near basal levels by 24 h of treatment (Fig. 8). Induction of iNOS mRNA by either LPS or cytokines was followed by a proportional rise in the nitrite levels of the culture media, with maximal rates of 15 and 5 nmoles/h for LPS- or cytokine-treated cells, respectively, occuring between 8 and 12 h of culture following the addition of modulators (Fig. 8). Further

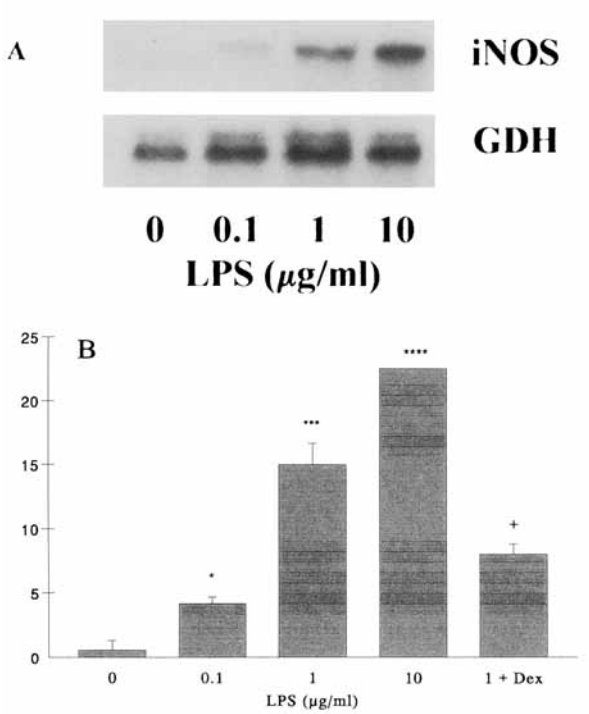

Fig. 7. Dose-dependent induction of iNOS message by LPS in osteoclast-like cells and inhibition of iNOS mRNA induction by dexamethasone. Osteoclast-like cells were treated for 24 h with the indicated concentrations of LPS or with a combination of LPS (1 μg/ml) and dexamethasone (Dex) (10^{-7} M). Total RNA (5 μg) was analyzed by ribonuclease protection assay (RPA) using the 362 bp PCR avian iNOS clone and the 203 bp PCR avian GDH clone. **A:** Protected bands from a representative experiment visualized by autoradiography. **B:** Protected bands from two independent experiments were quantified in a PhosphorImager and the results expressed as iNOS mRNA/GDH mRNA × 100. Significant differences from control cultures are denoted as follows: $^{*}P \leq 0.05$; $^{***}P \leq 0.005$; $^{****}P \leq 0.001$; significant differences from 1 μg/ml LPS-treated cultures: $+P \leq 0.05$. The corresponding μM nitrite accumulated in the culture medium of these experiments was 1.05, 112.05, 117.83, 120.4, and 78.6 for 0, 0.1, 1, and 10 μg/ml LPS and 1 μg/ml LPS + 10^{-7} M Dex, respectively.

studies demonstrated that treatment of osteoclast-like cells with either IL-8 (5×10^{-9} M) or IL-10 (10^{-9} M) for 8 h similarly led to increased expression of iNOS mRNA in parallel with elevated nitrite accumulation in the media (Table II).

DISCUSSION

Proinflammatory Agents Induce iNOS in Osteoclast-Like Cells

We have demonstrated in the current study that proinflammatory agents promote the production of NO from avian osteoclast-like cells, that IL-8 and IL-10 also upregulate NO production from these cells, and that their response to these agents is associated with an elevated expression of the mRNA and protein levels for an inducible nitric oxide synthase (NOS). Bacterial LPS was the most potent of the proinflammatory modulators tested, and near maximal levels of NO induction were already achieved by LPS concentrations below those typically reported to induce NO production in other cell types [Lowik et al., 1994; Schneemann et al., 1993; Damoulis and Hauschka, 1994; Punjabi et al., 1992; Stadler et al., 1991; Nussler and Billiar, 1993]. Thus, as little as 0.1 ng/ml LPS was effective in generating NO from osteoclast-like cells, and maximal nitrite production was obtained with LPS concentrations of at or above 1 ng/ml. Whether the high sensitivity to LPS demonstrated by osteoclast-like cells is due to 1) higher levels of the developmentally upregulated cell surface LPS receptor CD14 [Martin et al., 1994], 2) more effective intracellular signal transduction mechanisms, perhaps involving tyrosine kinases of the src family as reported for monocytes [Geng et al., 1994], 3) enhanced iNOS gene expression mediated by the interactions of transcription factors (eg. NFκB) with the LPS-responsive element of the iNOS gene promoter [Bredt and Snyder, 1994; Nathan and Xie, 1994], or other potential mechanisms will require further re-

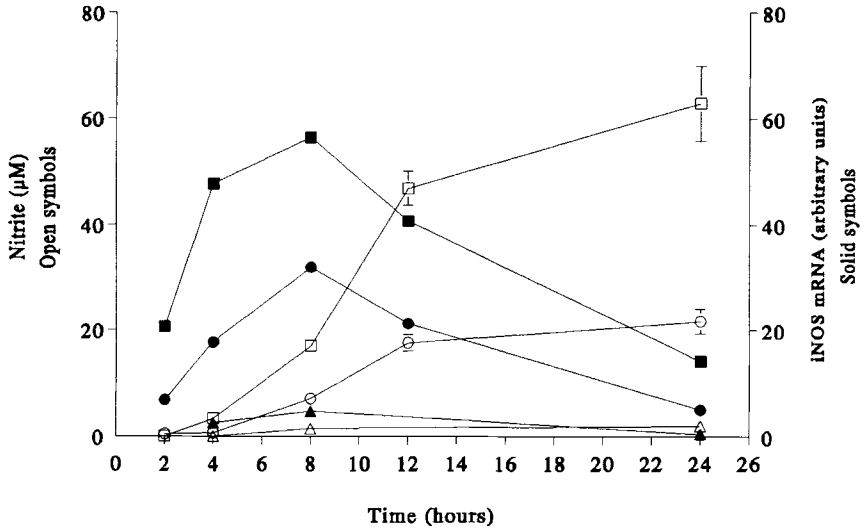

Fig. 8. Time-course analysis of nitrite and iNOS mRNA accumulation in osteoclast-like cells treated with either LPS or cytokines. Osteoclast-like cells were incubated for 2, 4, 8, 12, or 24 h in the absence (*triangular symbols*) of modulators, in the presence of LPS (1 μg/ml) (*square symbols*), or in the presence of a combination of the cytokines (*circular symbols*) IL-1α (10^{-10} M), IFNγ (100 U/ml), and TNFα (10^{-9} M). Nitrite levels accumulated in the conditioned medium from time 0 to the indicated times are depicted (*open symbols*). Corresponding iNOS mRNA levels present in the cells were determined by RPA analysis as in Fig. 7, quantified in a PhosphorImager, and expressed as iNOS mRNA/GDH mRNA × 100 (*solid symbols*). Data were obtained from two independent cultures.

TABLE II. Induction of iNOS mRNA and Nitrite Accumulation in Osteoclast-Like Cells by IL-8, IL-10, or LPS Treatment*

Treatment	iNOS mRNA	μM nitrite
IL-8 (5×10^{-9} M)	31.8	12.6 ± 0.2
IL-10 (10^{-9} M)	105.6	31.1 ± 0.1
LPS (1 μg/ml)	104.6	32.0 ± 1.4
Control	8.2	3.8 ± 0.4

*Osteoclast-like cells were treated for 8 h with IL-8, IL-10, or LPS. Steady-states levels of iNOS mRNA were determined by RPA analysis, quantified in a PhosphorImager, and expressed as iNOS mRNA/GDH mRNA × 100 as in Fig. 7. Nitrite accumulated in the culture media of the same experiments is indicated as μM nitrite.

search. Neither $1,25(OH)_2$ vitamin D_3 nor 17-β-estradiol altered the LPS-induced NO production in the osteoclast-like cell populations employed here. Similarly, Lowik et al. [1994] have reported that the systemic bone-active hormones PTH and vitamin D_3 did not affect the upregulated production of nitrite by rat osteoblasts. These findings are consistent with the prevailing view that NO functions in local regulatory mechanisms. Competitive inhibitors of NOS activity, including the iNOS selective inhibitor AG [Corbett et al., 1992], effectively reduced both the basal and LPS-induced NO production by these cells. These results correlate well with our previous findings that L-NAME or AG potentiated bone resorption activity by isolated avian osteoclasts cultured on bone in vitro as well as exacerbated bone mineral density loss in sham or ovariectomized rats in vivo [Kasten et al., 1994]. Osteoclasts may release low levels of NO which dampen their maximal resorptive activity, and modulation of their autocrine NO production by agents that either increase NO production or inhibit NO basal levels could impact dramatically on localized bone resorption and overall bone integrity.

The above results suggested that osteoclast-like cells contained an inducible NOS responsive to inflammatory stimuli, and this premise was substantiated by a series of further biochemical, immunological, and molecular studies. In addition to elevated nitrite production, osteoclast-like cells stimulated by LPS exhibited increased NADPH diaphorase activity staining, consistent with a putative increase in NOS activity. Similarly, substantial NADPH diaphorase activity has been observed associated with resorbing osteoclasts either in sections of bone or isolated and cultured on bone slices [Kasten et al., 1994; Schmidt et al., 1992]. LPS-treated osteoclast-like cells also displayed greater reactivity with

antibodies to the N-terminal or C-terminal regions of iNOS, suggesting that their increased NOS activity was a consequence of greater iNOS protein levels. Confirmation of the presence of an iNOS in the osteoclast-like cells was obtained from RT-PCR cloning of a novel 906 bp fragment that demonstrated high sequence homology with the 5′ region of iNOS genes from other sources. Furthermore, when used as a probe in Northern analysis of osteoclast-like cells, this clone hybridized to an mRNA species of 4.8 kb, similar to the size of iNOS messages (4–5 kb) reported for other cell types such as rat renal mesangial cells [Kuntz et al., 1994], murine macrophages [Knowles and Moncada, 1994; Lyons et al., 1992], and human chondrocytes [Knowles and Moncada, 1994; Palmer et al., 1993]. Our preliminary studies have demonstrated no cross-hybridization of this probe with RNA from either chicken brain tissue or chicken aortic endothelial cells, further establishing its specificity for the inducible form of NOS over the constitutive forms of the enzyme. The LPS dose-dependent increase in steady-state levels of the 4.8 kb mRNA in osteoclast-like cells, its inhibition by dexamethasone, and appearance of this mRNA in time course experiments prior to the rise of nitrite in the culture medium indicate that inflammatory stimulation of osteoclast-like cells leads to the production of high levels of NO as a consequence of an upregulation of iNOS.

The inflammatory cytokines IL-1α, IFNγ, and especially TNFα caused a modest NO induction when employed either individually or in paired combinations, whereas a significantly higher rise in NO production was obtained when all three were administered simultaneously to osteoclast-like cells. The synergistic effect of these cytokines on osteoclast-like cell NO production agrees well with reports for other cell types, such as fibroblasts, chondrocytes, or osteoblasts [Lowik et al., 1994; Stadler et al., 1991; Nussler and Billiar, 1993; Riancho et al., 1995; Werner-Felmayer et al., 1990]. Unlike some systems, however, little or no further upregulation of NO production was obtained when such cytokines were combined with LPS treatment; this may reflect the already high induction levels of NO generated by avian osteoclast-like cells due to LPS alone. It has been proposed that the NO produced during inflammation or other pathological conditions may serve a protective role against otherwise unopposed bone resorption and that IFNγ functions in this regard as a key regulatory braking factor to suppress osteoclast-mediated bone resorption [Lowik et al., 1994]. Since the synergistic action of IL-1α, TNFα, and IFNγ was necessary for significantly elevating osteoclast-like cell NO production, IFNγ may limit the stimulation of osteoclast resorption by IL-1 α and TNFα, at least in part, by increasing NO production to levels sufficiently high for inhibiting osteoclast activity. The cytokines IL-1α, TNFα, and IFNγ, which together elevate osteoblast NO production, also decrease osteoblast cell proliferation [Ralston et al., 1994; Damoulis and Hauschka, 1994; Riancho et al., 1995] and bone formation [Gowen et al., 1992]. Therefore, local rises in NO may lead to inhibition of both the formative and resorptive phases of the normal cyclical bone remodeling sequence.

Recently, the osteoclast proton pump, which is essential for the acidification and dissolution of bone within the sealed resorption lacuna underlying active osteoclasts, has been shown to be unusually sensitive to nitrate but not other oxyanions [Chatterjee et al., 1993]. Inhibition of the osteoclast proton pump by nitrate exhibited an IC_{50} of 100 μM, a level significantly below that necessary to inhibit related ion pumps involved elsewhere in the cell (e.g., endocytic or secretory pathways). Since constitutive NOS activity and basal NO production by iNOS-containing cells lead to nitrite concentrations well below this inhibitory IC_{50} value, inhibition of this specialized proton pump may occur in response to particular physiological conditions that substantially raise NO levels. In the current study, nitrite concentrations approaching this IC_{50} value ($\sim$60 μM) were obtained with LPS treatment (0.001–10 μg/ml LPS), and somewhat lower nitrite levels (10–15 μM) were generated by the IL-1α + TNFα + IFNγ cytokine cocktail. Thus, autocrine production of NO by osteoclasts might result in an intercrine inhibition of their resorptive activity, and blocking proton pump activity may be one mechanism by which NO inhibits osteoclasts.

IL-8 and IL-10 Induce iNOS in Osteoclast-Like Cells

The antiinflammatory cytokines IL-8 and IL-10 have been shown to interfere with the production of NO induced by inflammatory agents in a number of cell types [Schneeman et al., 1993; Nussler and Billiar, 1993; Geng et al., 1994; McCall et al., 1992; Cuna et al., 1992].

However, neither IL-8 nor IL-10 modified the LPS-induced levels of NO release from osteoclast-like cells. Instead, we show here for the first time a dose-dependent induction of NO production and increased iNOS mRNA expression by both IL-8 and IL-10. These novel NO induction mechanisms in osteoclast-like cells are consistent with the overall immune-dampening and antiinflammatory roles typically ascribed to IL-8 and IL-10, since increased NO levels inhibit osteoclast function. Such observations more generally suggest that these two cytokines may participate in the regulation of local inflammatory states through bidirectional modulation of NO production in various cell types. Thus, IL-8 and IL-10 may depress osteoclast, monocyte, macrophage, and neutrophil activities by different means: through elevating NO levels which inhibit bone resorptive activity in osteoclasts and by reducing NO levels which are needed for the cytotoxic actions of monocytes, macrophages, and neutrophils. Regarding IL-10, this cytokine has recently been shown to inhibit the osteogenic commitment of mouse bone marrow cells by a mechanism involving TGFβ [Van Vlasselaer et al., 1994], but its effects on osteoclasts are not known. However, protein tyrosine kinases of the src family have been implicated in the deactivation of monocytes by IL-10 [Geng et al., 1994] and might therefore also mediate IL-10 actions in osteoclasts. If so, IL-10 might serve as an important modulator of osteoclast activity since c-src has an essential role in osteoclast function: mice deficient in c-src exhibit osteopetrosis [Soriano et al., 1991], and c-src is required for integrin-induced tyrosine phosphorylation in osteoclasts [Baron et al., 1993].

Like IL-10, IL-8 alone was capable of eliciting substantial nitrite production and iNOS mRNA expression in osteoclast-like cells. Next to IL-1, IL-8 is considered a cytokine of key importance in regulating inflammation with a wide range of both proinflammatory and antiinflammatory effects, the latter being generally more prominent [McCall et al., 1992]. IL-8, like IL-1 and IL-10, promotes superoxide production in various cell types such as macrophages and neutrophils [Baggiolini and Clark-Lewis, 1992; Wozniak et al., 1993] and superoxides have been shown to stimulate osteoclast bone resorption [Garrett et al., 1990]. However, whereas IL-1 stimulates resorption [Gowen, 1992], IL-8 may inhibit osteoclast-mediated bone resorption [Fuller et al., 1995]. One possible explanation for the inhibition of osteoclast resorption by IL-8 may be that IL-8 elicits a greater production of NO than superoxide in these cells. Moreover, NO and superoxide may readily combine chemically to form the highly reactive and longer-lived free radical peroxynitrite [Beckman and Crow, 1993]. Therefore, scavenging superoxide radicals by this mechanism could interfere with osteoclast resorptive activity [Collin-Osdoby et al., 1994; Damoulis and Hauschka, 1994; Garrett et al., 1990].

Dexamethasone and TGFβ Inhibit iNOS Induction in Osteoclast-Like Cells

Dexamethasone antagonized the LPS-stimulated induction of NO and depressed the iNOS mRNA steady-state levels in osteoclast-like cells, analogous to the decreases in nitrite and/or iNOS mRNA induction reported for chondrocytes, osteoblasts, and macrophages [Ralston et al., 1994; Nathan and Xie, 1994; Damoulis and Hauschka, 1994; Nussler and Billiar, 1993; Palmer et al., 1993]. Glucocorticoids, such as dexamethasone, typically cause a generalized osteopenia in patients as a result of both increased bone resorption and decreased bone formation processes [Luckert and Raisz, 1990]. Based on findings reported here, glucocorticoids might counteract high NO production by osteoclasts during inflammatory stimulation and thereby favor greater bone resorption, contributing to generalized osteopenia. Like dexamethasone, TGBβ caused a reduction in the elevated NO production elicited by LPS treatment of osteoclast-like cells, consistent with findings that TGFβ inhibits iNOS induction in macrophages, bone marrow cells, endothelial cells, and osteoblasts [Lowenstein et al., 1994; Lowik et al., 1994; Nathan and Xie, 1994; Schneeman et al., 1993; Punjabi et al., 1992]. Overall, TGFβ has been reported to inhibit osteoclast formation, differentiation, and bone resorptive activity [Gowen, 1992]. Furthermore, osteoclasts themselves may produce TGFβ, which, together with the latent TGFβ present within the bone matrix that can be activated by resorbing osteoclasts, could serve as a negative signal for osteoclasts to cease resorption [Gowen, 1992]. Both dexamethasone and TGFβ are thought to inhibit iNOS mRNA steady-state levels, with TGFβ decreasing both the stability and translation of iNOS mRNA [Nathan and Xie, 1994].

We conclude that osteoclast-like cells express an iNOS that is regulated by inflammatory

agents, including IL-8 and IL-10. Further investigations will be needed to fully understand NO production by osteoclasts, its modulation by bone-acting hormones or local regulatory factors, and the overall paracrine and autocrine actions of NO in bone physiology.

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