Cytokine-induced Prostaglandin E₂ Synthesis and Cyclooxygenase-2 Activity Are Regulated Both by a Nitric Oxide-dependent and -independent Mechanism in Rat Osteoblasts in Vitro*

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Osteoblasts respond to stimulation with interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ) by production of nitric oxide and prostaglandins (PGs). In this study the relationship between nitric oxide and PG synthesis was investigated after cytokine stimulation of cultured rat osteoblasts. IL-1, TNF-α, IFN-γ, and exogenous sodium nitroprusside, a nitric oxide donor, all stimulated PGE₂ production in a dose-dependent manner. PGE₂ production was blocked by L-nitro-arginine methyl ester, an inhibitor of nitric oxide production, after IFN-γ stimulation and was partially blocked after TNF-α stimulation. However, IL-1-induced PGE₂ was unaffected. Similarly, expression of the cyclooxygenase-2 protein was stimulated by cytokines, and IFN-γ-induced expression was again blocked by L-nitro-arginine methyl ester. In contrast, all cytokines induced the cyclooxygenase-2 mRNA expression independently of nitric oxide production, although exogenous sodium nitroprusside was able to induce the cyclooxygenase-2 mRNA in the absence of cytokines. The results show that nitric oxide can induce PG synthesis and cyclooxygenase-2 expression and may regulate cyclooxygenase-2 expression at both transcriptional and post-transcriptional levels. In addition, the data show the existence of both nitric oxide-dependent and -independent pathways of PG synthesis after cytokine stimulation of osteoblasts. The results suggest that nitric oxide may be an important mediator of PG production in inflammatory bone diseases.

The factors that may regulate bone metabolism during inflammation are of considerable importance in understanding the pathogenesis of a number of common inflammatory diseases, including rheumatoid arthritis and osteoarthritis and the periodontal diseases, and may suggest novel therapeutic approaches to control of bone destruction seen in these conditions. The effects of cytokines such as interleukin-1 (IL-1), tumor necrosis factor-alpha (TNF-α), and interferon-γ (IFN-γ) on bone cells have been studied extensively in the past and have complex effects on bone metabolism (for review, see Ref. 1). These cytokines may act directly on osteoblasts to regulate their activity and also regulate osteoclastic bone resorption indirectly via their interaction with osteoblasts. IL-1 is a potent inducer of bone resorption (2, 3) and has a number of different effects on osteoblast metabolism in vitro, including inhibition of bone formation and stimulation or inhibition of proliferation, alkaline phosphatase activity, and collagen synthesis depending on the cell type and the culture conditions used (4–10). TNF-α also stimulates bone resorption and can inhibit makers of osteoblast activity such as alkaline phosphatase and collagen synthesis (10, 11). In contrast, IFN-γ specifically inhibits IL-1-induced bone resorption and can directly inhibit cell proliferation and alkaline phosphatase activity in osteoblast cultures (12, 13).

There is strong evidence that some of the effects of these cytokines on bone cells may be mediated by the induction of prostaglandin synthesis. In particular, inhibition of prostaglandin synthesis can partially reverse the effects of IL-1 on both osteoclastic and osteoblastic activity (4, 8, 14–16). Prostaglandins are potent stimulators of osteoclastic bone resorption and have wide-ranging effects on osteoblast metabolism directly by their interaction with cell surface receptors on the osteoblasts (17). Prostaglandins are produced by the action of cyclooxygenase enzymes on arachidonic acid after its release by the enzyme phospholipase A₂. Two isoforms of cyclooxygenase have been described, namely cyclooxygenase-1 (prostaglandin H synthase-1) and cyclooxygenase-2 (COX-2, prostaglandin H synthase-2). COX-1 is constitutively expressed by many cells, whereas COX-2 expression may be induced by a range of stimuli. For example, IL-1 is a potent inducer of COX-2 expression in a number of cell types, including osteoblasts (18, 19).

Recent studies have demonstrated the production of nitric oxide (NO) by osteoblasts after stimulation with IL-1, TNF-α, and IFN-γ by the induction of expression of the inducible NO synthase enzyme (iNOS) (20–25). Furthermore, these studies have suggested that the production of NO may also mediate some of the effects of these cytokines on bone metabolism. The evidence suggests that NO may both stimulate and inhibit bone

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1 The abbreviations used are: IL, interleukin; COX-2, cyclooxygenase-2; NO, nitric oxide; iNOS, inducible nitric oxide synthase; l-NAME, l-nitro arginine methyl ester; SNP, sodium nitroprusside; TNF, tumor necrosis factor; PG, prostaglandin; PBS, phosphate-buffered saline.
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resorption in a biphasic manner and inhibit osteoblastic activity (21, 22, 24–27). In addition, osteoblastic cells may also produce NO and prostaglandin (PG) after mechanical deformation (28). During acute and chronic inflammatory processes in vivo, both iNOS and COX-2 may be co-expressed in an apparently co-ordinated way (29). Taken together these findings raise the question of the possible relationship between NO and prostaglandin synthesis in the regulation of bone metabolism.

In the present study we have investigated the relationship between NO and PG synthesis in osteoblasts after cytokine stimulation. Specifically, the aim of the study was to investigate the hypothesis that NO mediates prostaglandin synthesis and induction of COX-2 expression in osteoblast cultures isolated from fetal rat calvariae after stimulation with the cytokines IL-1, TNF-α, and IFN-γ.

MATERIALS AND METHODS

Cell Cultures—Primary osteoblast-enriched cultures were isolated by sequential enzymatic digestion of neonatal rat calvariae as described previously (30, 31). Briefly, the calvariae were dissected from neonatal Wistar rats and digested with collagenase for periods of 10, 10, 20, and 20 min sequentially. The cells isolated from the final three digests were plated into 75-cm² flasks for 24 h, released by trypsin treatment and pooled, replated at a cell density of 6 × 10⁶ cells/cm², and cultured until confluent for experiments. Cells were cultured in modified Eagle’s medium α (Life Technologies, Inc.) supplemented with 10% fetal bovine serum, penicillin, streptomycin, and fungizone (Life Technologies). For experiments cells were stimulated with recombinant human IL-1β (specific activity, 2.8 × 10⁶ units/mg), recombinant murine IFN-γ (specific activity, 1 × 10⁵ units/mg), and recombinant human TNF-α (specific activity, 1 × 10⁷ units/mg; all obtained from Genzyme Ltd., West Malling, Kent, UK). To block NO production, cultures were treated with the NO donor sodium nitroprusside (SNP), with exogenous PGE2, or with the cyclooxygenase inhibitor indomethacin (all obtained from Sigma).

Prostaglandin and Nitrite Assays—Confluent cultures in 96-well plates were treated for 24 h with cytokines in the presence or absence of the inhibitor of NO production L-NAME at 1 mM concentration. In some experiments cells were stimulated with exogenous PGE2 (Sigma) or the NO donor SNP. Culture medium was then collected and either used immediately or stored for periods of up to 1 week at −20 °C for assay of production of PGE2 by enzyme immunoassay using a commercially available assay kit (Amersham Pharmacia Biotech).

NO production was assayed by measurement of nitrite present in the culture medium using the Griess reaction consisting of 0.2% sulfanilamide and 2% N-(1-naphthyl)ethylenediamine in 1% H₃PO₄ (32). Nitrite measurement was determined by reading absorbance at 590 nm using a 96-well plate reader.

All experiments were carried out in triplicate in a minimum of two independent experiments.

Immunocytochemical Staining for COX-2 and iNOS Enzymes—Confluent cultures grown in 75-cm² flasks were stimulated for 24 h with cytokines either singly or in combination and in the presence or absence of L-NAME (1 mM). Culture medium was decanted, and the cells were immediately fixed with a 1% solution of paraformaldehyde in PBS for 20 min. After several washes in PBS, cell cultures were immunostained by the avidin-biotin-peroxidase complex method. Endogenous peroxidase was blocked by immersing slides in 0.3% hydrogen peroxide in methanol for 30 min, followed by washing in PBS (three washes, 10 min each). After blocking nonspecific binding by incubating in 3% normal goat serum for 20 min, sections were blocked and incubated overnight at 4 °C with rabbit antibodies to murine macrophage iNOS (21) and COX-2 (33), both diluted 1:1000 in PBS containing 0.05% bovine serum albumin and 0.01% sodium azide. Sections were washed in PBS and then successively incubated with biotinylated goat antiserum to rabbit IgG (Vector Laboratories, Burlingame, CA) diluted 1:100 in PBS/bovine serum albumin and avidin-biotin peroxidase complex (Vectorstain, Vector Laboratories), for 30 and 60 min, respectively. Peroxidase activity was revealed using the diaminobenzidine-hydrogen peroxide method.

Northern Blots—Osteoblast total RNA was isolated by the Ultraspec (Biotec Laboratories) isolation method. Total RNA (20 μg) was separated on a 1% agarose-formaldehyde gel, transferred to a nylon mem-

brane (Hybond-N, Amersham Pharmacia Biotech), and immobilized by baking at 80 °C for 4 h. Membranes were prehybridized in ExpressHyb solution (CLONTECH) for 30 min. The membranes were then hybridized for 1 h at 37 °C with continuous shaking with 50 ng/ml 32P-labeled (T4 polynucleotide kinase labeling kit, Promega) murine COX-2 oligonucleotide probe (R & D systems) in fresh ExpressHyb solution. After stringency washes in 3 × SSC, 0.1% SDS and 0.1% SSC, 0.1% SDS, the membranes were exposed to autoradiographic film (Reflection NEF-485, DuPont NEN) for 48 h at −70 °C. Loading of RNA was normalized between lanes by subsequent probing of blots with β-actin.

To determine whether NO affected RNA stability, cultures were treated with actinomycin-D to block new RNA synthesis, and RNA was quantified by Northern analysis after 0, 1.5, and 3 h.

Blots were quantified (Scion Image, Scion Corp.) to determine mean absorbances of mRNA bands (corrected for background).

Western Blots—Cells were grown to confluence in 75-cm² flasks and stimulated for 24 h with cytokines either alone or in combination and in the presence or absence of L-NAME (1 mM). Cells were also incubated with SNP (1 mM). Total cellular proteins were extracted by freeze-thawing the cells in 0.05 M Tris buffer, pH 7.2, containing 5 mM dithiothreitol, 1 μg/ml leupeptin, 10 μg/ml chymostatin, 1 μg/ml pepstatin, 40 μg/ml bestatin, and 50 μg/ml N-α-p-tosyl-l-lysine chloromethyl ke-

FIG. 1. Effects of IL-1β, TNF-α, and IFN-γ with and without 1 mM L-NAME on production of PGE2. Values are mean ± S.D.; n = 3. * , different from control values; #, significant effect of L-NAME; p < 0.05.
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FIG. 2. Effects of combinations of IL-1β (units/ml), TNF-α (ng/ml), and IFN-γ (units/ml) with and without 1 mM L-NAME on production of PGE2. Values are mean ± S.D.; n = 3. * indicates a significant effect of L-NAME, p < 0.05.

FIG. 3. Effects of SNP on PGE2 production. Values are mean ± S.D.; n = 3. * indicates a significant difference from control values; # indicates a significant effect of L-NAME, p < 0.05.

Effects of combinations of IL-1β (units/ml), TNF-α (ng/ml), and IFN-γ (units/ml) with and without 1 mM L-NAME on nitrite accumulation. Values are mean ± S.D.; n = 3. * indicates a significant difference from control values; p < 0.05.

Role of NO in Cytokine-stimulated PGE2 Production

To investigate whether NO is involved in cytokine-stimulated PGE2 production, cells were stimulated with cytokines in the presence or absence of the inhibitor of NO production L-NAME, and PGE2 was determined by enzyme immunoassay. 24-h stimulation of cells with IL-1β, TNF-α, or IFN-γ resulted in a marked, dose-dependent induction of PGE2 release into the culture medium. When normalized for equimolar concentrations of cytokines, IL-1β showed the greatest and IFN-γ the least activity. The addition of L-NAME had no significant effect on IL-1-induced PGE2 production. However, L-NAME consistently blocked IFN-γ-induced PGE2 production. The stimulatory effect of TNF-α on PGE2 production was partially blocked by L-NAME, although all these data were not statistically significant (Fig. 1).

Combinations of cytokines were also tested in further experiments (Fig. 2). Addition of IFN-γ to both TNF-α and to low (10 units/ml) concentrations of IL-1 showed synergistic increases in PGE2 activity, which were largely blocked by L-NAME. However, there was no evidence of additional effects of either TNF-α or IFN-γ when added to higher concentrations of IL-1β (≥100 units/ml), and L-NAME alone did not affect PGE2 production. When the NO donor SNP was added to the cultures, again PGE2 production was induced in a dose-dependent manner (Fig. 3).

Cytokine Stimulation of NO Production

To confirm that cytokines stimulated NO production the accumulation of nitrite, one of the stable end products of NO metabolism, was measured in culture supernatants. TNF-α and IFN-γ produced small but significant increases in nitrite production, and combinations of all cytokines showed strong synergistic increases in nitrite production, which were inhibited by addition of L-NAME (Fig. 4). Addition of indomethacin did not inhibit cytokine-induced nitrite, and addition of exogenous PGE2 did not affect nitrite production (Fig. 5).

Expression of NOS and COX-2 after Cytokine Stimulation

Intracellular Expression of Enzymes—COX-2 and iNOS enzyme expression in osteoblasts after stimulation was determined by immunohistochemistry on cultured cells. Cells stimulated with IL-1, TNF-α, or IFN-γ showed strong immunoreactivity for both iNOS and COX-2 in ~10% of all cells. A combination of all three cytokines together increased the number of cells stained to ~60%. Staining for iNOS was not affected if the cytokine-stimulated cells were additionally incubated with L-NAME. Immunoreactivity for COX-2, however, was inhibited by the presence of L-NAME, such that staining seen in cells incubated with IFN-γ and L-NAME was comparatively much weaker than that seen with the cytokine alone. L-NAME had no effect on the immunoreactivity of cells stimulated with IL-1 (Fig. 6).

Total Expression of Enzymes—Total COX-2 and iNOS en-
zyme expression was quantified by Western blots. The rabbit antibodies to iNOS and COX-2 reacted with protein bands corresponding to ~130 and 70 kDa, respectively, in homogenates of osteoblasts stimulated with IL-1β, TNF-α, or IFN-γ, either alone or in combination (Fig. 7). These bands correspond to the known molecular masses of rodent iNOS and COX-2. The antibodies did not recognize any protein bands in control, unstimulated cells. The presence of L-NAME had no effect on cytokine-stimulated expression of iNOS, nor did it affect IL-1β-stimulated expression of COX-2. However, the signal produced for COX-2 protein in cells stimulated with IFN-γ in the presence of L-NAME was consistently much weaker than that seen with the cytokine alone. The expression of COX-2 by osteoblasts was also stimulated in a dose-dependent manner by the NO donor SNP. The reduced expression of COX-2 seen in cells stimulated by IFN-γ in the presence of L-NAME was partially abolished if the cells were additionally incubated with SNP for the last 12 h of incubation. Analysis of the absorbancies of the COX-2 protein bands revealed a 2–3-fold reduction in the amount of protein expressed by cells stimulated by IFN-γ in the presence of L-NAME compared with the cytokine alone. Furthermore, densitometric analysis of COX-2 protein in cells stimulated with IFN-γ in the presence of L-NAME and increasing concentrations of SNP revealed a gradual recovery of COX-2 protein expression (Fig. 7).

Expression of COX-2 mRNA—The effects of NO on expression of COX-2 mRNA were determined by Northern blots. Osteoblast cultures exposed to IL-1β, TNF-α, or IFN-γ for 0, 6, and 15 h showed induction of a single band of ~4.4 kb corresponding to the expected size of COX-2 mRNA (Fig. 8). The cytokine-stimulated induction of COX-2 mRNA was unaffected by the presence of L-NAME for all cytokines tested. However, exogenous SNP stimulated the expression of COX-2 in a concentration-dependent manner. In cultures treated with actinomycin D there was a small decline in RNA levels by 3 h after...
both IL-1 and IFN-γ treatment. However, addition of l-NNA did not affect the decline in RNA levels (Fig. 9).

DISCUSSION

The mechanisms of action of cytokines in modulating tissue responses during inflammation have been extensively studied and may be useful targets for therapeutic control of tissue damage occurring during chronic inflammatory diseases. Cytokines such as IL-1, TNF-α, and IFN-γ stimulate both PG and NO synthesis in osteoblasts by regulation of the inducible isoforms of the cyclooxygenase (COX-2) and nitric oxide synthase (iNOS) enzymes, respectively. Inhibition of PG synthesis by indomethacin may abrogate the effects of, for example, IL-1 in regulating osteoblast metabolism and osteoclastic bone resorption (8, 14, 15), and inhibition of NO production by l-arginine analogues has been shown to abrogate both stimulatory and inhibitory effects of IL-1, TNF-α, and IFN-γ on both osteoblast and osteoclast activities (21, 22, 24–27). These data suggest the importance of both PG and NO production as mediators of the actions of these cytokines in regulating bone metabolism during inflammatory disease processes.

Previous studies have shown that NO may increase PG synthesis by activation of the COX-2 protein (34–38). NO is known to react with iron-containing enzymes, resulting in their activation, and in this respect the heme-containing COX-2 enzyme is a potential target for the direct action of NO. Conversely, production of PG has been reported to increase or decrease NO production in a biphasic manner in murine macrophages and mesangial cells (39, 40).

Effects of NO on PGE2 Synthesis—The data from the present study suggest that NO may regulate PG synthesis at a number of different levels of control and furthermore suggest the existence of both NO-dependent and -independent pathways of PG synthesis in osteoblasts after cytokine stimulation. This conclusion is reached by the finding that 24-h stimulation of cells...
with IL-1β, TNF-α, or IFN-γ resulted in a dose-dependent increase in PGE₂ production, the major PG type synthesized by osteoblasts. The suggestion of the existence of both NO-dependent and -independent pathways is supported by our findings that IL-1β-induced PGE₂ synthesis was independent of NO synthesis, as judged by the lack of effect of added l-NAME, whereas l-NAME almost totally inhibited IFN-γ-stimulated PGE₂ synthesis. The effects of l-NAME on TNF-α-stimulated PGE₂ synthesis were less conclusive, consistently producing a partial inhibitory effect, but overall this was found not to be statistically significant. Combinations of TNF-α and IFN-γ showed a synergistic effect on PGE₂ production, which was largely reversed by l-NAME. In addition, exogenous NO provided by treatment with the NO donor SNP also stimulated PGE₂ production. IL-1β was the most potent inducer of PGE₂ production of the cytokines tested. In support of its important role in stimulating PGE₂ synthesis, l-NAME did not inhibit synthesis of PG in combinations of cytokines that included IL-1β, and in these experiments addition of both TNF-α and IFN-γ did not significantly increase PG production over IL-1β stimulation alone.

**Regulation of COX-2 Protein Expression by NO after Cytokine Stimulation**—Investigation of COX-2 protein expression by immunohistochemistry and Western blot analysis gave results consistent with the data for PGE₂ production. First, all cytokines and exogenous SNP induced COX-2 protein expression after 24-h stimulation. Addition of l-NAME did not affect IL-1β-induced COX-2 expression but markedly inhibited IFN-γ-induced protein expression, and the effect of l-NAME was reversible by addition of SNP for the final 12 h of stimulation. In other experiments we found that COX-1 expression was not regulated by cytokines or NO donors (data not shown).

**Regulation of COX-2 mRNA Expression by NO after Cytokine Stimulation**—In contrast to the findings of both PGE₂ and COX-2 protein expression, the data suggest that NO was not required for cytokine-induced COX-2 mRNA expression. Measurement of steady state levels of mRNA for COX-2 by Northern blot demonstrated the induction of COX-2 mRNA by each of the cytokines tested, but this was not affected by addition of l-NAME. Despite this, NO was also able to induce COX-2 mRNA expression, as shown by the observation that exogenous SNP also induced COX-2 mRNA. Taken together these data suggest that although NO can induce COX-2 expression, cytokine-in-
duced COX-2 mRNA was independent of NO production.

Mechanisms of NO Regulation of COX-2 Enzyme Activity—Although the effects of NO on the activation of COX-2 enzyme were not directly examined in these studies, it is possible that at least part of the effect of NO on PGE$_2$ production by IFN-γ (and TNF-α) could be attributable to enzyme activation. However, COX-2 activity was not further activated by NO in osteoblast cultures already exposed to IL-1β. In addition to effects of NO on COX-2 enzyme activation, which have been previously described (35, 36), the present data demonstrate the regulation of PG synthesis by NO at both the transcriptional and post-transcriptional levels. First, exogenous SNP increased COX-2 mRNA levels in a dose-dependent manner. Second, although NO was not required for cytokine-induced COX-2 mRNA expression and PG synthesis, as seen in the current studies with the use of IFN-γ, suggesting that relatively low levels of NO are able to regulate PG synthesis, as seen in the current studies with the use of combinations of single cytokines, particularly IFN-γ.

In summary, the results of the study presented here demonstrate that NO can induce PG synthesis and can regulate COX-2 expression both at the levels of gene transcription and by post-transcriptional mechanisms in cultured osteoblastic cells. The data further demonstrate that although IL-1β-induced PGE$_2$ production was independent of NO production, IFN-γ-induced COX-2 protein expression, and PGE$_2$ production was dependent on its ability to induce NO production. Although IL-1 shows a greater potency in inducing PGE$_2$ production when compared with IFN-γ, these results suggest that NO may play a significant role in PG-mediated bone resorption in inflammatory bone disease processes. In addition, the ability of NO to produce PGE$_2$ may be an important mechanism in regulating bone metabolism in other circumstances, such as in mediating responses to mechanical loading of bone.

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