JOURNAL OF BONE AND MINERAL RESEARCH
Volume 17, Number 3, 2002

Evidence That Peroxynitrite Affects Human Osteoblast Proliferation and Differentiation

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ABSTRACT

Peroxynitrite (PN), a nitric oxide ($NO^{\bullet}$)–derived anion, has been associated with $NO^{\bullet}$ damage in various cell types. We examined the effects of adding PN to cultured human osteoblast-like (hOB) cells obtained after hip arthroplasty. Exposure to PN (0.1–0.4 mM) decreased both hOB proliferation and differentiation, measured by [^{3}H]thymidine uptake and alkaline phosphatase production, respectively. Incubation with 3-morpholinosydnonimine (SIN-1; 0.25–1 mM), an $NO^{\bullet}$ and O_2^- donor that leads to PN release, also reduced both hOB proliferation and differentiation. Coincubation with both superoxide dismutase (SOD; 100 U/ml) and catalase (CAT; 50 U/ml), rendering SIN-1 a pure $NO^{\bullet}$ donor, reversed its effects on hOB proliferation and differentiation. However, SIN-1–induced $NO^{\bullet}$ production, measured by nitrite release to the hOB medium, was not altered by cotreatment with SOD and CAT. Expression of nitrotyrosine by hOB, a marker of PN action, was significantly increased after SIN-1 addition, as compared with untreated cells, as revealed by Western blot analysis. Interleukin-1α (IL-1α) and interferon γ (IFN-γ) but not tumor necrosis factor α (TNF-α) also significantly increased nitrotyrosine expression in these cells. These data show that PN is at least partially responsible for osteoblast derangement by $NO^{\bullet}$ and that cytokines released during inflammatory arthropathies can induce PN production in hOB cells. (J Bone Miner Res 2002;17:434–442)

Key words: nitric oxide, peroxynitrite, osteoblasts, osteoporosis, cytokines

INTRODUCTION

NITRIC OXIDE ($NO^{\bullet}$), a gaseous free radical, has been linked to a diverse array of physiological phenomena including vascular tone regulation and neurotransmission[1] as well as to cytotoxic activities such as inhibition of mitochondrial respiration[2] and DNA damage.[3] The identification of the expression of both constitutive and inducible nitric oxide synthase (cNOS and iNOS, respectively) isoforms in both osteoblasts and osteoclasts suggests that $NO^{\bullet}$ is involved in bone metabolism.[4,5] Production of low levels of $NO^{\bullet}$ via cNOS has been assigned a physiological role in bone function, whereas activation of iNOS, mainly under the influence of proinflammatory cytokines, by provoking release of large amounts of $NO^{\bullet}$, would lead to increased bone resorption in certain disease states.[6]

Deleterious effects attributed to $NO^{\bullet}$ may be at least partially linked to the reactive nitrogen species generated after $NO^{\bullet}$ release.[7] If compared with other free radicals, $NO^{\bullet}$ is relatively stable and unreactive toward biological molecules. However, $NO^{\bullet}$ has great reactivity with other free radicals.[8] When produced in large amounts in a microenvironment rich in superoxide (O_2^-), which happens after macrophage triggering in an inflammatory milieu, $NO^{\bullet}$ and O_2^- rapidly combine to produce a strong oxidant, the peroxynitrite (PN) anion ($ONOO^-$). Although the rate constant ($>10^9\ M^{-1}\ s^{-1}$) of this reaction ($O_2^- + NO^{\bullet} \rightarrow ONOO^-$) is similar to the reaction of $NO^{\bullet}$ with other free radicals, the former is assumed to have more pathophysiological relevance, because many cell types are able to re-

The authors have no conflict of interest.

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lease both $NO^{\bullet}$ and O_2^- after being activated.[9] Hence, a potential beneficial effect of $NO^{\bullet}$ to scavenge O_2^-, which might happen in low $NO^{\bullet}$ concentrations, is outweighed during inflammation by the favored kinetics of $ONOO^-$ formation, thus leading to the release of a much powerful and long-lived oxidant.[7]

PN has been shown to affect a diverse array of molecules including proteins, lipids, and nucleic acids. Nitrosylation of tyrosine residues in proteins by PN can significantly affect their function. Formation of 3-nitrotyrosine from PN activity in proteins has been assumed to be a biological marker for PN formation in vivo.[9] In vitro studies have reported cytotoxic activity of PN. Cultures of HL-60 and PC-12 tumor cell lines in the presence of $ONOO^-$ have been shown to induce cell apoptosis.[10,11] Nitrosylation of tyrosine residues and subsequent inactivation of surface receptors in both endothelial and epithelial cells have been reported as evidence of PN derangement of cell function.[12–15] More recently, early loss of P450 activity of cultured rat hepatocytes was associated with $ONOO^-$ production via cNOS activation.[16]

Detection of nitrotyrosine in atherosclerotic plaques,[17] in the serum and synovial fluid of rheumatoid arthritis patients[18] and in rejected human kidney allografts,[19] have been described as evidence of both presence and linkage of PN formation to human disease states. However, appropriate studies focusing on $ONOO^-$ role in bone metabolism and pathologies are still lacking. Considering that production of $NO^{\bullet}$ and O_2^- in the bone microenvironment occurs in both normal and disease states, we aimed to investigate the effect of PN addition to cultured human osteoblasts (hOBs). Because stimulation of bone cells with cytokines has been linked to increased $NO^{\bullet}$ release, we also investigated whether cytokine stimulation of hOBs could be associated with PN formation.

MATERIALS AND METHODS

Materials

Eagle's modified essential medium (MEM), α-MEM, and fetal bovine serum (FBS) were purchased from Gibco BRL (Rockville, MD, USA). PN, 3-morpholinosydnonimine (SIN-1), nitrotyrosine, mouse antinitrotyrosine monoclonal antibody, nitrotyrosine-bovine serum albumin nitrotyrosine-bovine serum albumine (nitrotyrosine-BSA), L-NMMA, and a nitrite/nitrate detection kit were all purchased from Cayman Chemical Co. (Hornby, Ontario, Canada). Interleukin-1α (IL-1α), tumor necrosis factor α (TNF-α), penicillin/streptomycin, L-ascorbate, amphotericin, trypsin, *p*-nitrophenylphosphate, and 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] were purchased from Sigma (St. Louis, MO, USA). Complete (Mini protease inhibitor cocktail), human interferon γ (IFN-γ) human superoxide dismutase (SOD) and catalase (CAT) were purchased from Boehringer Ingelheim (Mannheim, Germany). [^{3}H]Thymidine, hybond C extra nitrocellulose membranes, horseradish peroxidase–linked rabbit immunoglobulin G (IgG), and ECL-plus enhanced chemiluminescence system were purchased from Amersham Pharmacia Biotech, Inc. (Piscataway, NJ, USA). All other reagents used were of high-grade quality.

Cell culture

Specimens of human trabecular bone were obtained from patients undergoing total hip arthroplasty. In conformity with Québec's civil law, each patient signed an informed consent. HOBs were cultured from these tissues following a previously reported method with minor modifications.[20] Briefly, thin slices of trabecular bone were treated with 2.5 mg/ml of trypsin in phosphate-buffered saline (PBS) for 1 h at 37°C, washed extensively in PBS, and cut into fragments of approximately 2 mm^2. Fragments were seeded in 100-mm culture dishes containing 5 ml MEM supplemented with 10% FBS, 50 U/ml of penicillin, 150 μg/ml of streptomycin, 2.5 μg/ml of amphotericin, 2.2 mg/ml of sodium bicarbonate, and 50 μg/ml of L-ascorbate. The plates were incubated at 37°C in a 5% CO_2 atmosphere. The culture medium was replaced at 3- to 4-day intervals. After reaching confluence (4–5 weeks), cells were trypsinized and subcultured at a ratio of 20,000 cells/cm^2 in 100-mm culture dishes. First-passage cells were grown to confluence, incubated 24 h in MEM containing 0.2% FBS, and used in the experiments. These cells have been shown previously to exhibit phenotypic characteristics of osteoblasts such as response to parathyroid hormone (PTH) and production of alkaline phosphatase and osteocalcin.[20]

PN, SIN-1, SOD, CAT, and cytokine treatments

The original light yellow solution of PN was stored for no more than 1 week on arrival at −80°C in the buffer (NaOH, 0.3N), as supplied by the manufacturer. Immediately before use, PN concentration was checked by measuring absorbance at 302 nm (extinction coefficient; $\epsilon = 1670\ M^{-1}\ cm^{-1}$) using 0.3N of NaOH as blank. Working solutions were made in 5-μl aliquots of 0.3N of NaOH. PN incubations were made in FBS-free α-MEM, during 10 minutes. To maximize the number of cells exposed to $ONOO^-$, the culture dish was inclined and the PN solution was added to the side of the dish followed by a rapid swirl. The aliquots were adjusted using 0.3N of NaOH to achieve the final concentration so that all cells were exposed to the same pH. After these 10-minute incubations, the medium was aspirated and the cells were washed with PBS and reincubated with α-MEM supplemented with 10% FBS, as mentioned previously. Controls for PN included incubation with the 0.3N of NaOH buffer, as well as inactive PN, that consisted of a solution of $ONOO^-$ allowed to decompose in buffer before addition (reverse-order addition) so that the influence of nitrates and nitrites present in the $ONOO^-$ solution could be excluded. The absorbance of this inactive PN solution was checked at 302 nm before addition to the culture medium.

Although it can be considered stable if compared with radicals such as $NO^{\bullet}$ and O_2^-, $ONOO^-$ has a short half-life of <1 s in physiological solutions. Attempts to overcome the obvious difficulties in interpreting data when using authentic PN with both in vivo and in vitro systems include

the use of $ONOO^-$ donors. SIN-1 is considered an $ONOO^-$ donor because its unique ability to chemically release both $NO^\bullet$ and O_2^- leads to $ONOO^-$ generation.[21]

The compounds SIN-1, IL-1α (100 U/ml), TNF-α (100 U/ml), or IFN-γ (10 U/ml) were added directly to the culture medium. Cells were aerated gently after SIN-1 addition to the medium to avoid hypoxia. SOD (50 U/ml), dissolved in PBS, and CAT (100 U/ml) were added to the medium before incubation to give the desired final concentration. Both enzymes always were added before SIN-1.

Cell proliferation

Osteoblast proliferation was studied by measuring [^{3}H]thymidine (2 μCi/ml) incorporation into trichloroacetic acid (TCA)–insoluble material. Briefly, cells were plated at a density of 3×10^4 cells/well in 24-well plates and cultured for 48 h in α-MEM culture medium with antibiotics, supplemented with 10% FBS, in the presence of [^{3}H]thymidine. 48 h later, the plates were washed with PBS and a 10% TCA solution was added to the wells. Incorporated [^{3}H]thymidine was released through washing with 0.2N of NaOH and radioactivity was measured using a β-scintillation counter. Results are expressed as counts per minute of triplicates of three different experiments obtained from different donors.

Measurement of alkaline phosphatase activity

Alkaline phosphatase was used as a marker of osteoblast differentiation and was measured in disrupted cells using *p*-nitrophenyl phosphate as substrate, as described elsewhere.[22] Briefly, cells were seeded at a density of 3×10^4 cells/well in 24-well plates and cultured in the presence of 10 nM of $1,25(OH)_2D_3$ for 72 h with varying treatments. A baseline control for this alkaline phosphatase production consisted of cells cultured in the absence of vitamin D_3. After the incubation period, cells were harvested in 0.4 M of Tris buffer and sonicated, and the alkaline phosphatase activity in the supernatant was measured spectrophotometrically at 405 nm. Values obtained were compared with a standard curve using *p*-nitrophenol as standard. Data were expressed as micromoles of alkaline phosphatase activity per milligram of protein. Protein concentration in the supernatants was assessed using the BioRad method (BioRad, Mississauga, Ontario, Canada).

Nitrite assay

Total $NO^\bullet$ (nitrate + nitrite) production was assessed indirectly using the release of nitrite to the medium based on the Griess reaction, as described previously,[23] using a commercially available kit. Briefly, cells were seeded at a density of 3×10^4 cells/well in 24-well plates and cultured in the presence of 10 nM of $1,25(OH)_2D_3$ for 72 h, with varying treatments. A baseline control for this experiment consisted of cells cultured in the absence of vitamin D_3. After the incubation period, cells were harvested in 0.4 M Tris buffer and sonicated, and total nitrate/nitrite was assessed using the Griess reaction. Data were expressed as micromoles per liter of nitrite.

Nitrotyrosine detection

Nitrotyrosine detection was used as a marker of PN effect on cell proteins. For the Western blots, cell suspensions of 100-mm culture dishes obtained after different treatments were lysed in the presence of protease inhibitors (Complete, Mini) with Tris-NaCl and NP-40 followed by sonication and centrifugation. The protein concentration on the supernatant was assessed using the BioRad method and compared with a standard curve using the lysis buffer as reference at 620 nm. Samples were run on 9% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE; 50 μg of protein/lane). After migration, proteins were electrophoretically transferred to nitrocellulose membranes. Nonspecific binding sites were blocked overnight in 0.1% blocking buffer Tris-buffered saline (TBS)/Tween containing 5% BSA. Membranes were then probed with 1.5 μg/ml of mouse antinitrotyrosine monoclonal antibody for 1 h at room temperature in 0.1% TBS/Tween 0.1% containing 1% BSA. After five washings with 0.1% TBS/Tween containing 0.1% BSA the membranes were incubated with a horseradish peroxidase–linked anti-mouse polyclonal antibody raised in sheep (1:2000). After five washings with 0.1% TBS/Tween containing 0.1% BSA, the membranes were developed using the ECL detection system. Controls for the antinitrotyrosine antibody specificity included preincubation of the first antibody with nitrotyrosine followed by pH adjustment as well as incubation with just the second antibody. Nitrotyrosine staining was quantitated by scanning densitometry of the Western blot membranes. The means ± SEM of the band intensities of three experiments obtained with the various stimuli were compared with nonstimulated hOB.

Statistics

Data are presented as means ± SEM of triplicate values of at least three experiments, using different donors. To compare means we used analysis of variance (ANOVA), followed by Tukey's test. The value of $p < 0.05$ was considered significant.

RESULTS

PN decreases hOB proliferation and differentiation

Authentic PN addition to hOB culture decreased both basal proliferation (Fig. 1A) and differentiation (Fig. 1B) measured by [^{3}H]thymidine incorporation and vitamin D_3–stimulated alkaline phosphatase production, respectively. At the doses and conditions used, cell viability assessed by trypan blue exclusion was not affected (>95% viable). Addition of vitamin D_3 significantly increased alkaline phosphatase production when compared with baseline values (Fig. 1B).

SIN-1 decreases hOB proliferation

Addition of SIN-1, a $NO^\bullet$ and O_2^- donor, to the hOB culture significantly and dose-dependently decreased prolif-

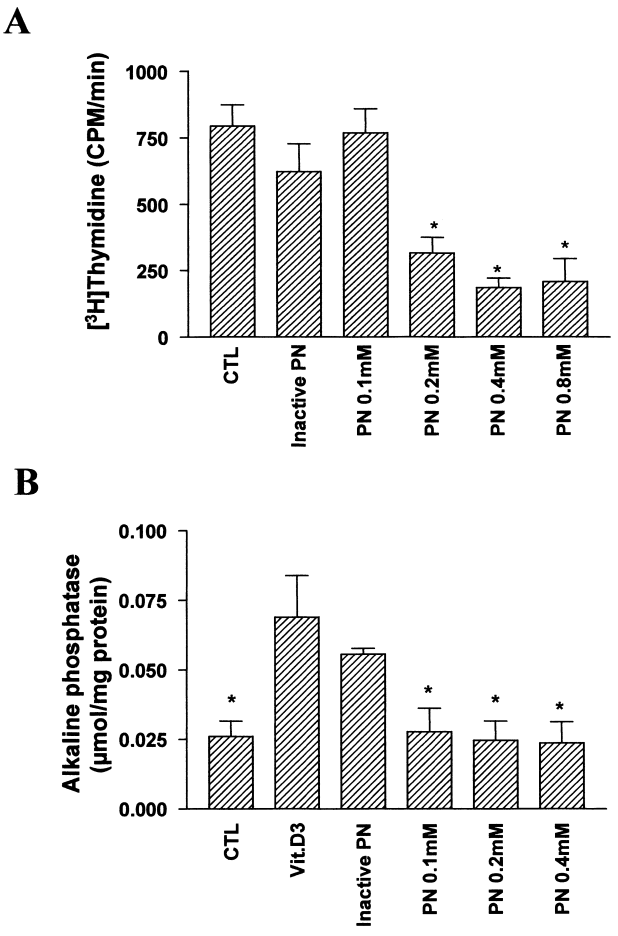

FIG. 1. Effect of addition of PN on the (A) proliferation and (B) 10 nM of vitamin D_3–induced alkaline phosphatase production by cultured hOBs. hOB proliferation was assessed by [^{3}H]thymidine incorporation 48 h after exposure to PN at increasing concentrations. Alkaline phophatase was assessed in the cell lysate using *p*-nitrophenyl phosphate as substrate. A solution of PN allowed to decompose completely before addition to the medium (reverse order addition) was used as inactive PN to exclude the influence of contaminants (nitrate, nitrite, etc.) present in the original PN solution (see text for details). Data are expressed as the mean ± SEM of either [^{3}H]thymidine counts or as micromoles of alkaline phosphatase activity per milligram of protein of three experiments performed with three different donors. Protein concentration was measured in the cell lysates using the Bradford method (panel A, $*p < 0.05$ compared with nonstimulated hOB (CTL), using ANOVA followed by Tukey's test; panel B, $*p < 0.05$ compared with vitamin D_3–stimulated cells, using ANOVA followed by Tukey's test).

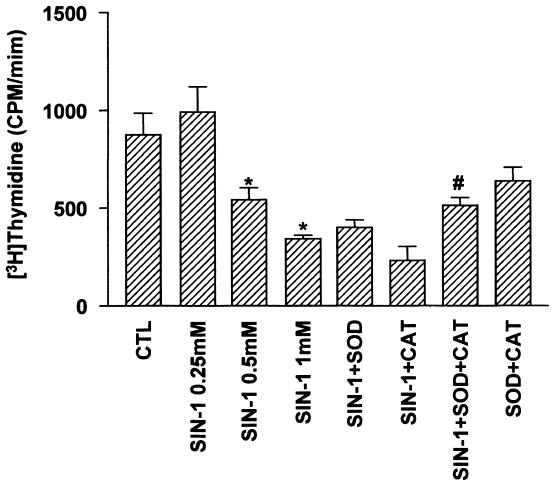

FIG. 2. Effect of addition of different concentrations of SIN-1 isolated or SIN-1 (1 mM) combined with SOD (50 U/ml; SIN-1 + SOD), CAT (100 U/ml; SIN-1 + CAT), or SIN-1 (1 mM) + SOD (50 U/ml) + CAT (100 U/ml; SIN-1 + SOD + CAT) on the proliferation of cultured hOBs. hOB proliferation was assessed by [^{3}H]thymidine incorporation 48 h after exposure to SIN-1. Data are expressed as the mean ± SEM of [^{3}H]thymidine counts of three experiments performed with three different donors ($*p < 0.05$ compared with nonstimulated hOB [CTL] using ANOVA, followed by Tukey's test; $^{\#}p < 0.05$ compared with SIN-1 [1 mM], using ANOVA, followed by Tukey's test).

eration when compared with nonstimulated cells (Fig. 2). SIN-1 (0.5 mM or 1 mM) decreased hOB proliferation to 37% and 63.6% of the untreated cells, respectively, whereas SIN-1 at a concentration of 0.25 mM was ineffective ($p > 0.05$). At the doses and conditions used, cell viability assessed by trypan blue exclusion was not affected (>95% viable cells). Combined treatment with SIN-1 (1 mM) + SOD (50 U/ml) + CAT (100 U/ml), rendering SIN-1 a pure $NO^{\bullet}$ donor, partially but significantly reversed SIN-1 effect on hOB proliferation (Fig. 2). Incubation of SIN-1 with either of the enzymes separately or a combination of SOD + CAT without SIN-1 did not significantly affect hOB proliferation.

SIN-1 decreases hOB differentiation

Addition of SIN-1 to the hOB culture significantly and dose-dependently decreased vitamin D_3–induced alkaline phosphatase production when compared with control (Fig. 3). SIN-1 (1 mM) decreased hOB alkaline phosphatase production to 64% of the control level ($p < 0.01$), whereas SIN-1 at the concentrations of either 0.5 mM or 0.25 mM was ineffective ($p > 0.05$). A combined treatment with SIN-1 (1 mM) + SOD (50 U/ml) + CAT (100 U/ml) but not with either of the enzymes isolated completely reversed SIN-1 effect on hOB differentiation (Fig. 3). Incubation with a combination of SOD + CAT without SIN-1 did not significantly affect the parameter studied (Fig. 3).

Cytokine treatment effect on hOB proliferation

Figure 4 shows that incubation of hOB with IL-1α, TNF-α, or IFN-γ isolated did not significantly alter proliferation. However, when all three cytokines were combined, a reduction of 36% in hOB proliferation was observed, as compared with untreated cells. Addition of 100 μM of L-NMMA, an NOS inhibitor, to the hOB culture arrested proliferation regardless of cytokine presence, suggesting that a certain $NO^{\bullet}$ basal level is needed in order for osteoblasts to proliferate. Cell viability (>95%) assessed by

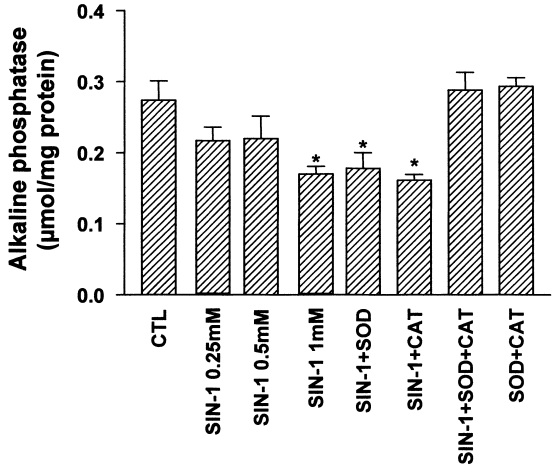

FIG. 3. Effect of addition of different concentrations of SIN-1 isolated or SIN-1 (1 mM) combined with SOD (50 U/ml; SIN-1 + SOD), CAT (100 U/ml; SIN-1 + CAT), or SIN-1 (1 mM) + SOD (50 U/ml) + CAT (100 U/ml; SIN-1 + SOD + CAT) on the 10-nM vitamin D_3–induced alkaline phosphatase production of cultured hOBs. Alkaline phosphatase was assessed in the cell lysate using *p*-nitrophenyl phosphate as substrate. Data are expressed as micromoles of alkaline phosphatase activity per milligram of protein of three experiments performed with three different donors. Protein concentration was measured in the cell lysates using the Bradford method (*$p < 0.05$ compared with nonstimulated hOB [CTL] using ANOVA followed by Tukey's test).

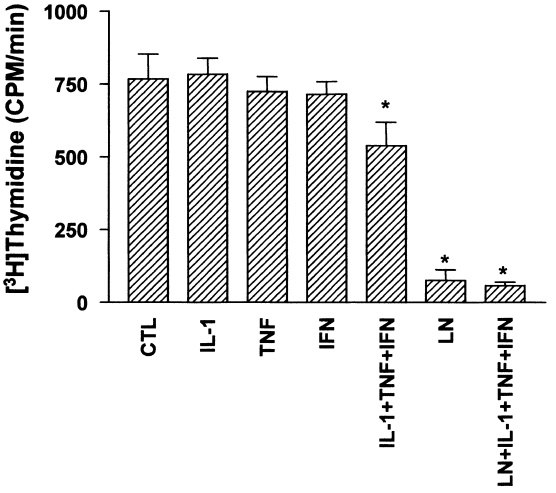

FIG. 4. Effect of addition of IL-1α (100 U/ml), TNF-α (100 U/ml), IFN-γ (10 U/ml), a combination of the three cytokines (IL-1 [100 U/ml] + TNF [100 U/ml] + IFN [10 U/ml]), L-NMMA (LN; 100 μM) or a combination of LN (100 μM) + IL-1 (100 U/ml) + TNF (100U/ml) + IFN (10 U/ml) on the proliferation of cultured hOBs. hOB proliferation was assessed by [^{3}H]thymidine incorporation 48 h after exposure to cytokines. Data are expressed as the mean ± SEM of [^{3}H]thymidine counts of three experiments performed with three different donors (*$p < 0.05$ compared with nonstimulated hOB [CTL] using ANOVA followed by Tukey's test).

trypan blue exclusion was not affected either by cytokine or L-NMMA addition to the culture medium.

Cytokine treatment effect on hOB differentiation

Figure 5 shows that incubation of hOB in the presence of either IL-1α or TNF-α alone did not alter alkaline phosphatase production. However, addition of IFN-γ alone or as a combined treatment with IL-1α + TNF-α greatly enhanced alkaline phosphatase production. Addition of 100 μM of L-NMMA arrested alkaline phosphatase production, regardless of cytokine presence, suggesting that a certain $NO^{\bullet}$ basal level is needed in order for osteoblasts to differentiate.

Nitrite production

Nitrite release to the culture medium was used to evaluate $NO^{\bullet}$ release. Incubation of hOB in the presence of vitamin D_3 slightly but significantly increased nitrite release as compared with control (nonstimulated) cells (Fig. 6). PN, IL-1α, or TNF-α did not alter nitrite release by hOB whereas IFN-γ increased (36%) nitrite release as compared with control (nonstimulated) hOB. Addition of 100 μM of L-NMMA reduced (50%) nitrite levels as compared with nonstimulated cells, regardless of cytokine presence ($p < 0.05$). SIN-1 (1 mM) addition to the culture markedly (around 30 times control level) increased nitrite release, an effect that was not altered by a combined treatment with SIN-1 (1mM) + SOD (50 U/ml) + CAT (100 U/ml). Nitrite production by 1 mM of SIN-1 was 120.1 ± 2.8 μM and SIN-1 (1 mM) + SOD (50 U/ml) + CAT (100 U/ml) produced 122.1 ± 3.4 μM compared with 4.1 ± 0.2 μM for control (nonstimulated) hOBs (data not shown).

SIN-1 induces tyrosine nitration in hOBs

We performed a Western blot analysis in hOB-extracted proteins using an antinitrotyrosine monoclonal antibody. Figure 7A shows the result of a typical experiment. Each treatment was repeated three times using hOB from different donors. SIN-1 addition to hOB culture medium increased tyrosine nitration, a marker of PN action, of hOB proteins, as compared with control (nonstimulated) cells. Exposure of the cells to a combination of SIN-1 (1 mM) + SOD (50 U/ml) + CAT (100 U/ml) reversed the increased tyrosine nitration induced by SIN-1 and thus implied that generation of PN was significantly inhibited by SOD + CAT. Tyrosine nitration by SIN-1 also was inhibited by addition of CAT (100 U/ml), whereas isolated addition of SOD (50 U/ml) to SIN-1 did not alter SIN-1–induced nitrotyrosine formation (Fig. 7B). IFN-γ treatment to hOB induced higher tyrosine nitration as compared with control whereas treatment with a combination of the three cytokines (IL-1α + TNF-α + IFN-γ) did not alter tyrosine nitration. Addition of L-NMMA significantly decreased nitrotyrosine formation when used alone or when combined with cytokines (Fig. 7B).

DISCUSSION

This study provides evidence, for the first time, that PN addition to hOB culture inhibits proliferation and differen-

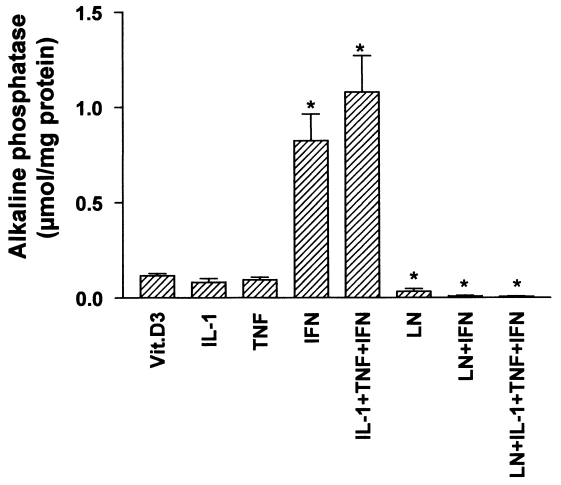

FIG. 5. Effect of addition of IL-1α (100 U/ml), TNF-α (100 U/ml), IFN-γ (10 U/ml), a combination of the three cytokines (IL-1 [100 U/ml] + TNF [100 U/ml] + IFN [10 U/ml]), L-NMMA (LN; 100 μM), or a combination of LN (100 μM) + IL-1 (100 U/ml) + TNF (100 U/ml) + IFN (10 U/ml) on the 10 nM of vitamin D_3–induced alkaline phosphatase production by cultured hOBs. Alkaline phosphatase was assessed in the cell lysate using *p*-nitrophenyl phosphate as substrate. Data are expressed as micromoles of alkaline phosphatase activity per milligram of protein of three experiments performed with three different donors. Protein concentration was measured in the cell lysates using the Bradford method (*$p < 0.05$ compared with vitamin D_3–stimulated cells, using ANOVA followed by Tukey's test).

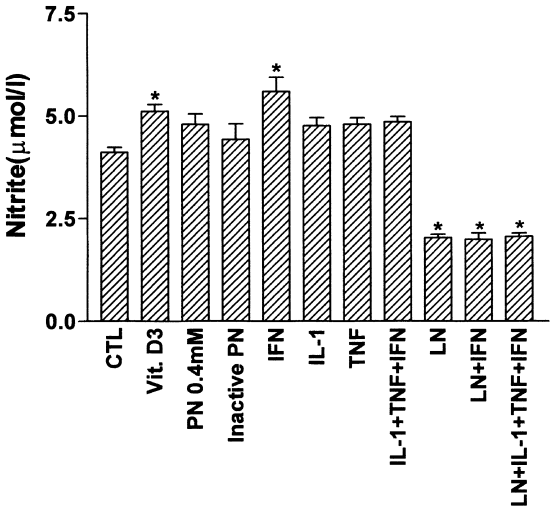

FIG. 6. Effect of addition of 0.4 mM PN, inactive PN, IL-1α 100 (U/ml), TNF-α (100 U/ml), IFN-γ (10 U/ml), a combination of the three cytokines (IL-1 [100 U/ml] + TNF [100 U/ml] + IFN [10 U/ml]), L-NMMA (LN; 100 μM), or a combination of LN (100 μM) + IL-1 (100 U/ml) + TNF (100 U/ml) + IFN (10 U/ml) or LN (100 μM) + IFN (10 U/ml) on the nitrite production of cultured hOBs. Nitrite production was assessed in the cell supernatant using the Griess reaction. Data are expressed as micromoles of three experiments performed with three different donors (*$p < 0.05$ compared with nonstimulated hOB [CTL] using ANOVA followed by Tukey's test).

tiation of these cells. It also shows that IFN-γ addition to cultured hOB leads to 3-nitrotyrosine formation, suggesting that these cells are able, under cytokine stimulation, to generate PN.

As mentioned previously, technical difficulties in working with PN pose a serious problem in exploring the mechanisms of action of this anion. Because of the short half-life of PN, especially in physiological solutions, addition of authentic PN to cell culture medium leads to irregular exposition of the cells. Incubation in alkaline solutions by increasing the half-life of PN is a strategy to homogenize this exposition. However, in our system, use of alkaline buffers severely affected cell viability. Our alternative was to add PN to the α-MEM FBS-free medium in an attempt to increase PN availability to the cells.

We found that addition of 0.2–0.8 mM of authentic PN to hOB significantly affected both proliferation and differentiation of the cells. However, a typical dose-response curve was not obtained. Although 0.2 mM of PN arrested hOB proliferation, a concentration of PN as low as 0.1 mM was enough to significantly decrease vitamin D_3–induced alkaline phosphatase production by these cells. At the concentrations studied, hOB viability was not affected by PN treatment. The fact that a decomposed PN solution was ineffective argues against the possibility that contaminants (hydrogen peroxide, nitrite, etc.) present in the PN solution were involved in the response attributed to PN.[24]

Although the PN concentrations that we used were similar to previous studies using other cell types, comparison of our data with the literature is difficult because there are no previous studies focusing on PN effects on osteoblasts. Further, most studies have used transformed cell lines and PN was added to alkaline buffers, a strategy that we could not use with hOB.

Pharmacologic manipulation using $NO^{\bullet}$ donors that can generate PN is an alternative to bypass the difficulties when working with authentic PN. SIN-1 has the unique ability to chemically generate both $NO^{\bullet}$ and O_2^- and, thus, leads to PN production. In this study, incubation of hOB in the presence of SIN-1 dose-dependently decreased both proliferation and differentiation. The SIN-1 effect on hOB proliferation was more pronounced because either 0.5-mM or 1-mM solutions were equally effective in inhibiting proliferation, whereas differentiation was inhibited only with the 1-mM SIN-1 solution. These were reproducible and sustained effects that did not affect cell viability. Coincubation with SOD and CAT but not with either of these scavengers alone did significantly reverse the SIN-1 effect on hOB proliferation. Moreover, vitamin D_3–induced alkaline phosphatase production was restored completely when cells were cotreated with SIN-1 + SOD + CAT, thus confirming that the effect observed was caused by the action of PN and not by $NO^{\bullet}$. This strategy has been used already in a previous study showing that incubation of normal human keratinocytes in the presence of 1 mM of SIN-1 inhibited their growth, an effect that was reversed by a combined treatment with SIN-1 + SOD + CAT.[25] It has been shown that adding only SOD to SIN-1 does not inhibit PN generation, as could be expected. In addition to the unfavored kinetics of O_2^- scavenging by SOD as compared with the faster $NO^{\bullet}$ and O_2^- reaction, the presence of SOD progres-

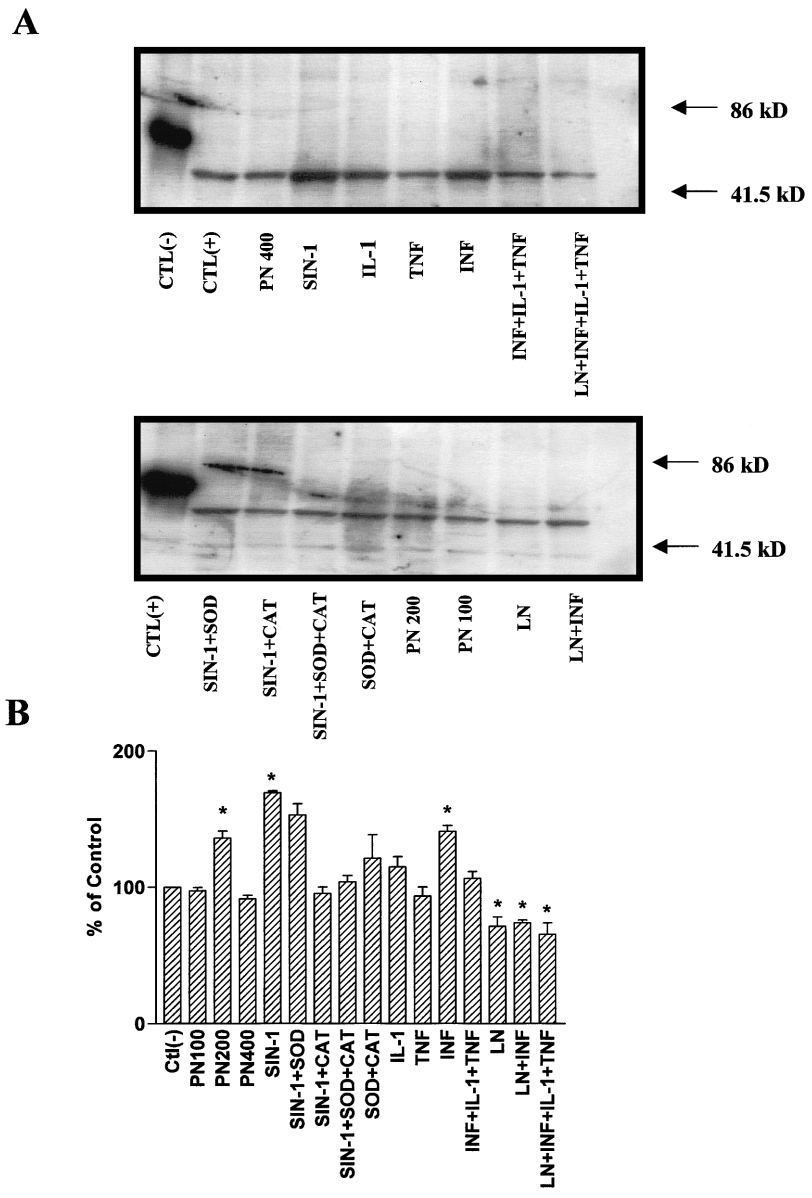

FIG. 7. (A) Results of a typical experiment to analyze nitrotyrosine presence in cultured hOBs after exposure to different treatments, assessed in hOB lysates. hOBs were cultured in α-MEM supplemented with 0.2% FBS and either of the following treatments was added to the medium: PN (400, 200, or 100 μM), SIN-1 (1 mM) with or without SOD (50 U/ml; SIN-1 + SOD) or CAT (100 U/ml; SIN-1 + CAT), IL-1α (100 U/ml), TNF-α (100 U/ml), IFN-γ (10 U/ml), a combination of the three cytokines (IL-1 [100 U/ml] + TNF [100 U/ml] + IFN [10 U/ml]), L-NMMA (LN; 100 μM), or a combination of LN (100 μM) + IL-1 (100 U/ml) + TNF (100 U/ml) + IFN (10 U/ml) or LN (100 μM) + IFN (10 U/ml). Positive control (CTL+) means nitrotyrosine-BSA and negative control (CTL−) means nonstimulated hOB. The PN and SIN-1 incubations were done in α-MEM FBS-free medium. Nitrotyrosine expression in hOB proteins obtained after cell lysis was analyzed by Western blot (see Material and Methods section for details). (B) Quantitative analysis of nitrotyrosine staining after scanning the membranes and analyzing the mean ± SEM of the band intensities. Results are expressed as percentage of the nonstimulated hOB. One hundred percent was set as the intensity of the negative control (CTL−) cells (*$p < 0.05$ using ANOVA followed by Tukey's test).

sively increases NO concentration that will outcompete SOD for O_2^-. Also, the combination of hydrogen peroxide and PN can be more harmful to the cells than either of them alone.[26] Further, SOD leads to H_2O_2 formation, which, coupled to SOD, has been shown recently to be able to generate PN probably through increasing $NO^\bullet$ breakdown.[27] These facts explain why SIN-1 effects on hOBs were reversed only when cells were cotreated with SIN-1 + SOD + CAT. As expected, SIN-1 greatly increased nitrite release (30 times the level in nonstimulated cells), and that effect was not altered by cotreatment with SOD + CAT. It should be pointed out that, in this case, $NO^\bullet$ release was not caused by hOB stimulation. Rather, it was a chemical reaction provoked by SIN-1 addition to the cells.

To our knowledge, there are no previous data exploring the effects of SIN-1, as a source of PN, on hOB. Although a previous study reported that SIN-1 (0.1–10 mM) decreased both hOB proliferation and differentiation, the SIN-1 effects in that study were attributed to $NO^\bullet$ generation without mention to the possible role of other reactive species as final mediators of the SIN-1 effects.[28] In another study, treatment with two different $NO^\bullet$ donors, *S*-nitroso-acetyl-penicillamine (SNAP) and *S*-nitroso-glutathione (GSNO) at concentrations ranging from 0.1 to 1 mM induced apoptosis of mouse osteoblasts, an effect that was associated with increased $NO^\bullet$ levels.[29] Our data add to these previous studies by providing evidence that SIN-1 effects on normal hOB proliferation and differentiation are caused by PN rather than $NO^\bullet$ release.

Addition of 100 μM of L-NMMA arrested hOB proliferation and differentiation and significantly reduced nitrite levels, as compared with nonstimulated cells. This is in accordance with recent data showing that endothelial (e)NOS knockout mice display bone abnormalities and also

that osteoblasts derived from these animals display reduced growth rate in vitro and are less differentiated, as compared with wild-type mice.[30,31] These data suggest an important anabolic effect of $NO^{\bullet}$ produced via cNOS in hOB metabolism.

Although our results with PN and SIN-1 pointed to an important role for this oxidant (or other reactive nitrating species) in hOB metabolism, the pathophysiological relevance of these data are not straightforward. It has been suggested that increased bone resorption during inflammatory arthropathies may be associated with cytokine-induced $NO^{\bullet}$ release by osteoblasts. Hence, we sought for a possible association between $NO^{\bullet}$ and/or PN production by hOB under cytokine stimulation and cytokine effects on proliferation and differentiation of these cells.

Our data have shown that isolated addition of IL-1α, TNF-α, or IFN-γ did not modify hOB proliferation whereas a combined treatment with the three cytokines significantly decreased it. A previous study showed that treatment with IFN-γ significantly decreased hOB proliferation, whereas treatment with TNF-α increased it.[32] However, culture conditions in that study were not specified, thus limiting comparison to our results. In a more recent study using hOB, addition of either IL-1β or IFN-γ but not of TNF-α to the culture medium slightly decreased osteoblast proliferation, whereas treatment with a combination of these three cytokines arrested it.[4] The discrepancy between these results[4] and ours could be caused by methodological reasons such as the density of the cells plated (1×10^5 cells/well in 96-well plates in the mentioned study and 3×10^4 cells/well in 24-well plates in our study). Additionally, our osteoblasts were obtained from patients with severe osteoarthritis, whereas normal osteoblasts were used in that study.[4]

Our data also showed that neither IL-1 nor TNF-α altered vitamin D_3–induced alkaline phosphatase production by hOB, as compared with nonstimulated cells. Incubation with IFN-γ greatly increased it, whereas a combination of the three cytokines did not add to the IFN-γ effect alone. Few studies have investigated the effects of cytokines on osteoblast functions. Using human cells, a previous study obtained data similar to ours, showing a great increase in alkaline phosphatase production by IFN-γ whereas TNF-α decreased it.[32]

The effect of cytokines on $NO^{\bullet}$ release by hOB was evaluated by measuring the amount of nitrite released into the culture medium. Nitrite levels were significantly higher in vitamin D_3–stimulated cells compared with nonstimulated cells. Incubation with either IL-1α or TNF-α increased ~22% nitrite levels that did not reach statistical significance. Addition of IFN-γ significantly increased up to 32.2% nitrite release compared with nonstimulated cells. Addition of a combination of the three cytokines did not alter $NO^{\bullet}$ production. Thus, the decrease in hOB proliferation observed with the combined cytokine treatment as well as the great enhancement of alkaline phosphatase production induced by IFN-γ could not be ascribed directly to alteration of $NO^{\bullet}$ production by these cytokines. As a whole, although these data indicate that cytokines affect hOB metabolism, the exact mechanism implicated in this effect still demands investigation.

Finally, we aimed to show PN release after stimulation of hOB with cytokines and SIN-1. Immune identification of nitrotyrosine in the cell extracts was chosen as a fingerprint of PN presence. Authentic PN addition had at most a slight effect on increased nitrotyrosine expression. In addition, 0.2 mM of PN induced higher nitrotyrosine levels compared with 0.4 mM of PN, thus reinforcing the possibility that difficulties in handling PN accounting for irregular cell exposition might explain these data. As expected, SIN-1 greatly increased (70%) nitrotyrosine expression compared with nonstimulated cells. This increase was inhibited when SIN-1 was coadministered with SOD + CAT. These data confirm our results obtained on hOB proliferation and differentiation showing that the effect of SIN-1 was associated with PN release, as shown by tyrosine nitrosylation. The fact that coincubation with SOD + CAT inhibited SIN-1–induced nitrotyrosine increase suggests that PN release rather than other reactive nitrating species was responsible for SIN-1 effects on hOB proliferation and differentiation. L-NMMA significantly decreased nitrotyrosine formation when used alone or when combined with cytokines. IL-1α or TNF-α did not modify nitrotyrosine expression whereas IFN-γ increased it up to 44% compared with nonstimulated cells, thus showing that this proinflammatory cytokine is able to induce PN production by hOB.

Curiously, we observed that nitration of tyrosine residues in hOB appeared to be restricted to a specific polypeptide. Apparently, PN displays specificity regarding protein nitration in the cells.[33] For instance, it was shown that specific manganese SOD nitration by PN is involved in the mechanisms of chronic rejection of human renal allografts.[19] Presently, we are trying to characterize the polypeptide that was targeted at nitration in hOB after exposure to PN.

In summary, this work shows that either authentic PN addition to cultured hOB cells or its indirect release through SIN-1 affects both hOB proliferation and differentiation. Blockade of $ONOO^-$ release in spite of persistent $NO^{\bullet}$ generation can efficiently inhibit $ONOO^-$ effect on hOB. Thus, these data provide evidence that PN may be a final mediator of $NO^{\bullet}$-induced deleterious effects on hOB metabolism. It also shows that hOBs are able to produce PN after IFN-γ stimulation.

ACKNOWLEDGMENTS

This work was supported by grants from the Medical Research Council of Canada and from the Arthritis Society. Francisco Airton Castro da Rocha is a recipient of a postdoctoral scholarship from CNPq–Brazil.

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Received in original form February 27, 2001; in revised form September 26, 2001; accepted October 29, 2001.