Prostaglandin Production by Human Osteoclasts in Culture

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ABSTRACT. ***Objective***. Prostaglandins (PG) are important mediators of bone metabolism with direct and indirect effects on bone cells. They may have important effects on osteoclasts, but it is not known if these cells can synthesize PG. We used 2 experimental models in order (1) to determine the presence and functionality of cyclooxygenase (COX) and phospholipase A_2 (PLA_2) enzymes in human osteoclasts and (2) to study their role in cell metabolism.

Methods. Experiments were undertaken on authentic human osteoclasts extracted from human fetuses (fhOC) and on human osteoclast-like (hOCL) cells differentiated from peripheral blood mononuclear cells. The presence of COX proteins was determined by immunohistochemistry. COX and PLA_2 enzymatic activity was evaluated at the single-cell level by fluorescence microscopy. An enriched population of hOCL cells was used to evaluate total PG production and the influence of COX activity on bone resorption.

Results. COX-1 was expressed in the cytoplasm and COX-2 was distributed mainly near the nuclear membrane of osteoclasts. These cells showed a high basal level of COX activity that could be inhibited by pretreatment with COX inhibitors. Cytosolic PLA_2 was present in both models. Human osteoclasts actively produced PG, and the COX-1 pathway was implicated in the control of bone resorption.

Conclusion. These results indicate that PG may be important autacoids for the control of osteoclast biology and that the COX-1 pathway is implicated in the inhibition of bone resorption. (First Release June 1 2006; J Rheumatol 2006;33:1320–8)

Key Indexing Terms:
CYCLOOXYGENASES BONE RESORPTION OSTEOCLASTS
PHOSPHOLIPASE A_2 PROSTAGLANDINS

Prostaglandins (PG) are lipid mediators implicated in the control of bone formation[1] and resorption[2,3]. Osteoblasts produce several PG upon stimulation with different biologically relevant agents such as parathyroid hormone (PTH)[4], interleukin 1 (IL-1), tumor necrosis factor-α[5], and bradykinin[6]. PG are also implicated in the regulation of receptor activator of nuclear factor-κB ligand (RANKL) expression[7]; through this pathway they may stimulate bone resorption and osteoclast formation in intact tissue and bone marrow culture[8]. Some actions of PG on mature osteoclasts have also been described: using a culture of purified osteoclasts from neonatal rat bone, Chambers, *et al* showed that PG acted as direct inhibitors of osteoclastic spreading[9]. However, if osteoblasts and osteoclasts were co-cultured, the addition of PG caused a considerable increase in spreading, indicating that crosstalk between cells is implicated in this response. We recently showed, using authentic human osteoclasts isolated from fetal long bones, that prostaglandin E_2 (PGE_2), acting via EP3 and EP4 receptors, disrupts the formation of actin rings[10]. Moreover, prostaglandin E_1 (PGE_1) and PGE_2 were shown to inhibit bone resorption by isolated rat osteoclasts[11]. Osteoclasts have recently been described as secretory cells able to produce factors that can stimulate their own differentiation and activity, such as IL-1, IL-6[12], and osteopontin[13], as well as others that may be involved in the crosstalk between osteoclasts and osteoblasts, such as platelet-derived growth factor BB[14]. Demonstration that osteoclasts produce such autocrine factors represents an important addition to the understanding of the regulation of mature osteoclast activity. It is not known, however, if mature osteoclasts are able to produce PG.

PG are produced by a multistep process that first involves release of arachidonic acid (AA) from membrane phospholipids by phospholipase A_2 (PLA_2). A number of distinct types of PLA_2 have been isolated and characterized. The best

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Supported by grants from The Arthritis Society, the Canadian Institutes for Health Research, and the Fonds de la recherché en santé du Québec.

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Accepted for publication February 26, 2006.

known of these are grouped into 3 broad categories: cytosolic PLA_2 ($cPLA_2$), secretory PLA_2 ($sPLA_2$), and intracellular Ca^{2+}-independent PLA_2 ($iPLA_2$)[15]. Free AA is converted to prostaglandin H_2 (PGH_2) by cyclooxygenase (COX), considered to be the rate-limiting enzyme in PG biosynthesis[16]. Two cyclooxygenase isoenzymes are well known: COX-1, which is present in several cells and tissues in relatively stable levels[17], and COX-2. The latter is usually absent from resting cells, but can be induced by serum, lipopolysaccharide (LPS), phorbol ester, IL-1, and other cytokines in most cells[18] and by parathyroid hormone in osteoblasts[19]; it is decreased by glucocorticoids[20]. Finally, PGH_2 is metabolized by specific synthases into a variety of biologically active prostanoids. Osteoblasts from different species express mainly COX-2 and produce PG[5,21]. It has recently been shown that mouse osteoclast precursor cells express the COX-2 isoenzyme, and that this enzyme is required for osteoclast differentiation[22]. However, it is not known if mature human osteoclasts express COX or PLA_2, or if they can produce PG.

Our objective was to determine if COX and PLA_2 are present and active in human osteoclasts, to evaluate production of PG by these cells, and to elucidate their roles in the control of osteoclast function. Two cell models were used: an *ex vivo* model of authentic fetal human osteoclasts (fhOC) and an *in vitro* model of differentiated human osteoclast-like (hOCL) cells.

MATERIALS AND METHODS

Materials. Fetal bovine serum (FBS) was purchased from Gibco (distributed by Invitrogen Canada, Inc., Burlington, ON, Canada). Macrophage-colony stimulating factor (M-CSF) was from Peprotech, Inc. (Rocky Hill, NJ, USA). Rhodamine-conjugated phalloidin, Fluo-3, Syto-59, ß-BODIPY®FL C_5-HPC, and carboxylated dichlorodihydrofluorescein (H_2DCFDA) were from Molecular Probes (distributed by Invitrogen Canada). Vectastain ABC kit and DAB substrate were from Vector Laboratories, Inc. (Burlington, ON, Canada). AA, antibodies, NS-398, valeroyl salicylate, and a prostaglandin enzyme immunoassay kit were from Cayman Chemical Co. (Ann Arbor, MI, USA). Ionomycin was from Calbiochem (San Diego, CA, USA). Arachidonyl trifluoromethyl ($AACOF_3$), 7,7-dimethyleicosadienoic acid (DEDA), and haloenol lactone suicide substrate (HELSS) were from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA, USA). DFU (5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methyl-sulphonyl)phenyl-2(5H)-furanone) was kindly provided by Merck Frosst Canada, Ltd. (Kirkland, QC, Canada). Human RANKL-GST fusion protein was produced in *Escherichia coli* strain BL21, purified by affinity chromatography, and its purity was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis; the rhRANKL plasmid was kindly provided by Dr. M. Manolson, University of Toronto. LPS contamination was absent, as confirmed with the use of E-Toxate test (Sigma kit no. ET0200). The RANKL-GST biological activity was tested on RAW 264.7 cells and shown to be comparable, on a molar basis, to commercially available RANKL. Bovine bone was purchased from a local slaughterhouse. All other reagents or products were purchased from Sigma-Aldrich Canada, Ltd. (Oakville, ON, Canada).

Cell culture. Mature osteoclasts were extracted from femurs and tibias of human fetuses (16 to 20 weeks of gestation) as described[10]. The fetuses were obtained from legal abortions after the mother signed an informed consent. Cells were cultured in 48-well plates, empty or containing a devitalized bovine cortical bone slice, or on 25 mm round coverslips in 6-well plates. The cells were allowed to attach for 90 min, then were washed to remove nonadherent cells. Cells were kept in α-modified Eagle's medium (α-MEM) supplemented with 10% FBS and 1% penicillin-streptomycin until their use in experiments, 20 h after isolation. Peripheral blood mononuclear cells (PBMC) were isolated from the blood of healthy donors by Ficoll density gradient and dextran sedimentation. Cells were plated at 1.5×10^6 cells/cm^2 in 48-well plates, empty or containing a bone slice, on 25 mm round coverslips in 6-well plates or in 100 mm Petri dishes coated with collagen type I (1 mg/ml). Cells were maintained in α-MEM supplemented with 10% FBS, 1% penicillin-streptomycin, 60 ng/ml RANKL, and 10 ng/ml M-CSF, and allowed to differentiate for 21 days. NIH/3T3 cells, purchased from American Type Culture Collection (Manassas, VA, USA) were cultured on 25 mm round coverslips in Dulbecco's MEM supplemented with 10% FBS, 2 mM penicillin/streptomycin, 4 mM L-glutamate, and 4.5 g/l glucose. All cells were kept in a 37°C, 5% CO_2, humidified atmosphere until used. All experimental protocols using human cells were approved by the Ethical Review Board of the Centre Hospitalier Universitaire de Sherbrooke.

Characterization of the osteoclastic phenotype. Five osteoclastic markers were studied: presence of tartrate-resistant acid phosphatase (TRAP), formation of F-actin rings, presence of 3 or more nuclei, calcium response to calcitonin, and capacity to resorb bone. Cells seeded on microscope coverslips were washed twice with phosphate buffered saline (PBS), fixed with 3.7% formaldehyde, then permeabilized with 0.1% Triton X-100. The presence of TRAP was determined with the use of the leukocyte-acid-phosphatase kit according to the manufacturer's protocol. The cells were then rinsed twice with PBS and incubated for 30 min at room temperature with 0.3 μM rhodamine-conjugated phalloidin, an F-actin stain, and 0.26 ng/ml bisbenzimide H 133342, a nuclear stain. Coverslips were rinsed in PBS and mounted with Vectashield. Slides were examined using a Nikon Eclipse TE2000-U fluorescence microscope. Cells were examined by brightfield microscopy for the presence of TRAP and by fluorescence for the presence of an actin ring and multiple nuclei. Images were obtained using a Hamamatsu Photonics camera (model C4742-95-12ER) and Simple PCI software. To study the calcium response to calcitonin, cells seeded on coverslips were washed twice with Tyrode's buffer containing 1% bovine serum albumin (BSA) then loaded with 2 μM of the calcium probe Fluo-3 and with 1 μM Syto-59 (nuclear stain) for 45 min. Cells were rinsed and incubated in Tyrode's without BSA for 15 min to allow hydrolysis of the probe. Coverslips were mounted in an open-bottomed chamber to which 1 ml of Tyrode's without BSA was added. Using a scanning confocal microscope (Noran Instruments, Inc., Middleton, WI, USA), a multinucleated cell was localized and the fluorescence level of the Fluo-3 probe was observed. Data were acquired before and after the addition of 1 μM salmon calcitonin.

Although the results presented are from single cells, they are representative of cultures from at least 5 donors (fetuses or adults). For the bone resorption assay, cells differentiated for 21 days on devitalized bovine cortical bone slices were incubated with COX inhibitors or their vehicle for 48 h at 37°C, 10% CO_2. Cells were fixed and stained for the presence of TRAP as described above. Bone slices were stained with 0.2% toluidine blue for 3 min, washed in water, and air dried before being examined by brightfield microscopy. Resorption area was quantified using the image analysis program Simple PCI and the results were expressed as percentage or resorption area compared to untreated control cells from the same donors.

Immunohistochemistry. Cells seeded in 48-well plates were fixed and incubated in 0.3% H_2O_2 for 20 min, then permeabilized with PBS 1% Triton X-100. The blocking step was performed using PBS containing 10% goat serum and 0.1% BSA for 30 min, then the first antibody (mouse anti-human COX-1 or mouse anti-human COX-2) diluted 1:100 in blocking buffer was added. Cells were incubated overnight at 4°C, then washed. A second blocking step was performed for 30 min before the addition of the secondary antibody, anti-mouse IgG coupled to biotin (dilution 1:100). After 1 h incubation at room temperature, cells were rinsed and treated with the Vectastain peroxidase standard ABC kit and revealed using the peroxidase substrate kit DAB, both according to the manufacturer's protocols. Immunohistochemistry for COX isoenzymes was also performed on paraffin-embedded fetal and normal adult bone tissue samples, as described[23]. Fetal bone specimens were embedded in paraffin by the local pathology service. Normal adult bone tissue samples, paraffin-embedded, were donated by Dr. M. Alini of the Orthopedic Research

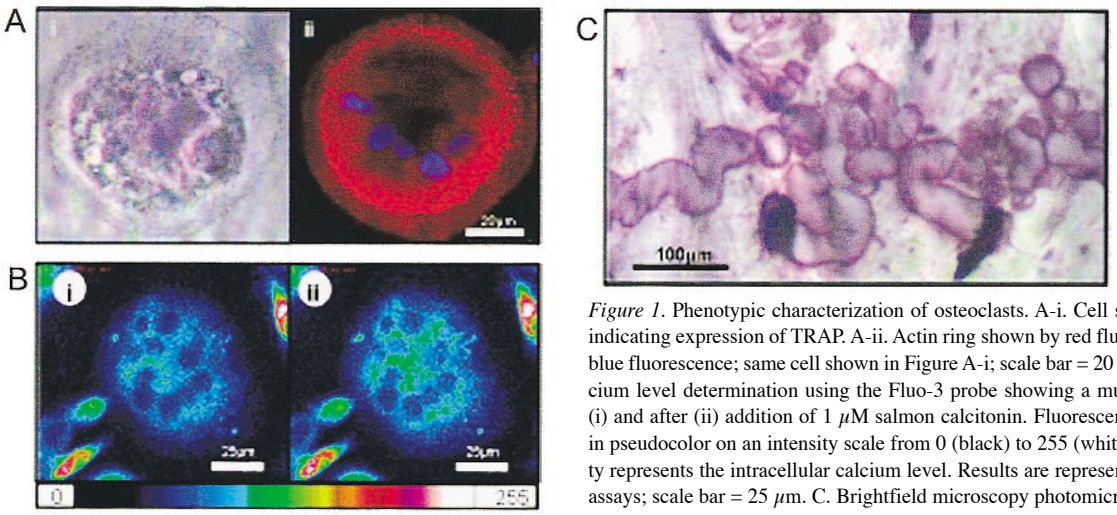

Figure 1. Phenotypic characterization of osteoclasts. A-i. Cell showing purple staining indicating expression of TRAP. A-ii. Actin ring shown by red fluorescence and nuclei by blue fluorescence; same cell shown in Figure A-i; scale bar = 20 μm. B. Intracellular calcium level determination using the Fluo-3 probe showing a multinucleated cell before (i) and after (ii) addition of 1 μM salmon calcitonin. Fluorescence levels are expressed in pseudocolor on an intensity scale from 0 (black) to 255 (white); fluorescence intensity represents the intracellular calcium level. Results are representative of 3 independent assays; scale bar = 25 μm. C. Brightfield microscopy photomicrograph showing resorption pits on a cortical bone slice stained with toluidine blue; scale bar = 100 μm.

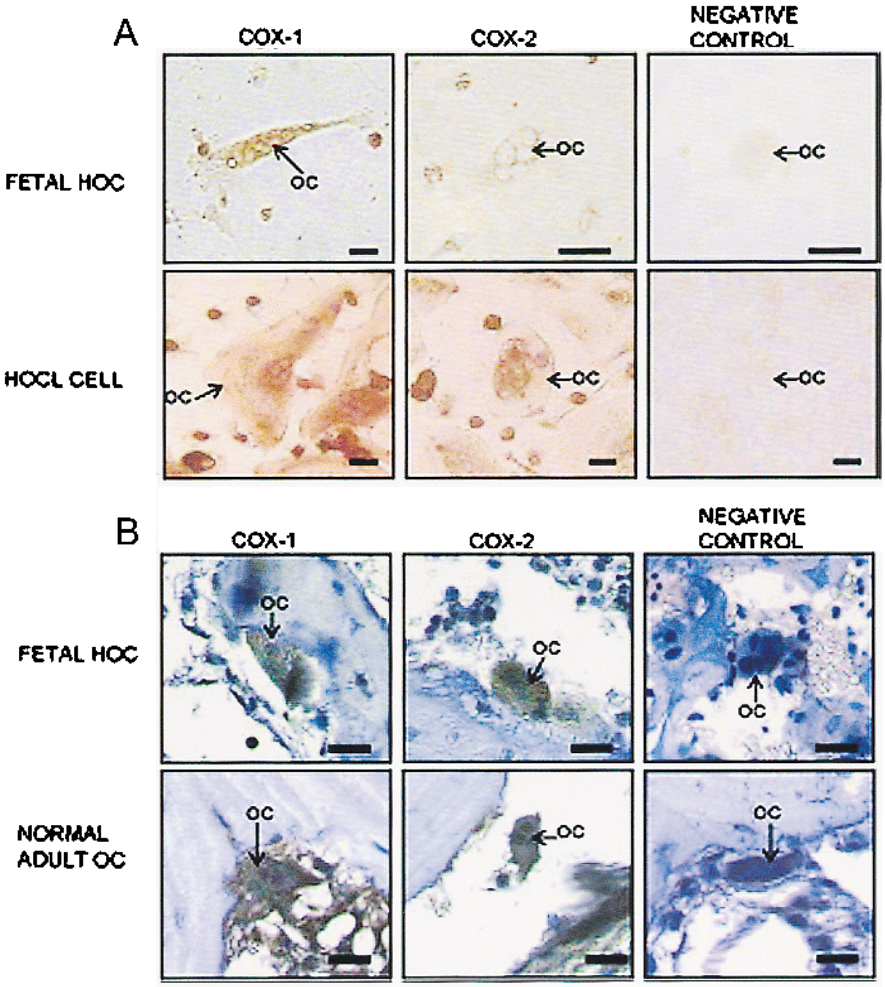

Figure 2. Immunohistochemistry for COX-1 and COX-2. Fetal and *in vitro* differentiated osteoclasts in culture (A) and paraffin-embedded adult and fetal bone tissue samples (B) were treated with mouse anti-hCOX-1, mouse anti-hCOX-2 antibodies, or without the primary antibody (negative control), followed by biotin-conjugated anti-mouse IgG, and revealed using DAB substrate. The results were observed under microscope. Osteoclasts (OC) are indicated by an arrow. Both fetal and *in vitro* differentiated osteoclasts were positive for COX-1 and COX-2, the first with a diffuse distribution and the second with a more intense perinuclear localization. Similar results can be seen in osteoclasts *in situ* in fetal and human normal bone. Results are representative of 3 experiments; scale bar = 20 μm.

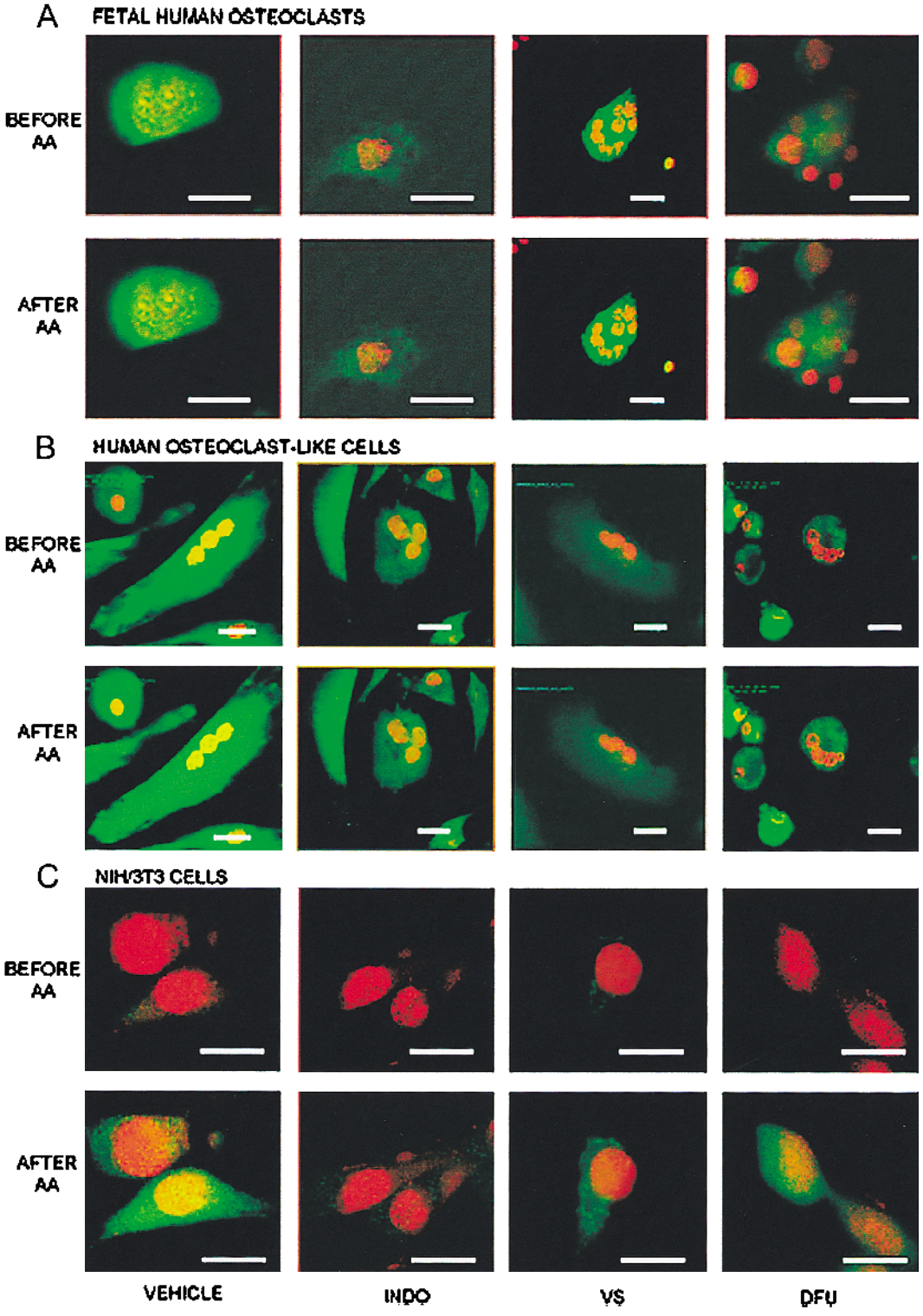

Figure 3. Determination of COX activity in single-cell assays. Confocal microscopy showing multinucleated fetal human osteoclasts (A), *in vitro* differentiated osteoclast-like cells (B), and NIH/3T3 cells (C). The latter were stimulated with 100 U/ml of IL-1α for 3 h. Cells were pretreated with vehicle or with COX inhibitors for 45 min. Nuclei were stained using Syto-59 (red) and COX activity was assessed with H_2DCFDA probe (green). Basal fluorescence level was observed and recorded (first row of each series). Arachidonic acid (AA; 30 μM) was added, and the fluorescence level was observed after 2 min (second row of each series). The first column presents cells treated with the vehicle (DMSO), the second, cells pretreated with 2 μM indomethacin (INDO), a nonspecific COX inhibitor, the third, cells pretreated with 100 μM valeroyl salicylate (VS), a COX-1-specific inhibitor, and the last, cells treated with 22 μM DFU, a COX-2-specific inhibitor. Results are representative of 3 experiments. Scale bar = 25 μm for both fOC and hOCL cells, and 15 μm for NIH/3T3 cells.

Laboratory, McGill University, Montreal. All specimens were observed using brightfield microscopy.

Determination of COX activity. COX activity was determined in a single-cell analysis system using H_2DCFDA to assess the oxidative process of COX, as described[24]. Cells seeded on coverslips were pretreated for 30 min with selective COX inhibitors or their vehicle, as well as with 1 μM Syto-59, in Tyrode's containing 1% BSA. The H_2DCFDA probe was added, and incubation proceeded for 15 min. Coverslips were washed and mounted in an open-bottomed chamber to which 1 ml Tyrode's without BSA was added. Multinucleated cells were located and examined with a scanning confocal microscope (Noran Instruments). Digitized images were obtained with Intervision software (Noran Instruments). Images were acquired before and after addition of 30 μM AA. The same procedure was performed with NIH/3T3 cells stimulated with 100 pg/ml of IL-1α for 4 h to increase the expression of COX-2. The results presented are representative of at least 3 experiments from different donors.

Determination of PLA_2 activity. PLA_2 activity was determined in a single-cell analysis system using a method modified from Farber, *et al* and Meshulam, *et al*[25,26]. The fluorescent derivative ß-BODIPY®FL C_5-HPC was combined with phosphatidylserine in a molar ratio of 1:9 in ethanol and desiccated for 4 h. The dried film of lipids was dissolved in PBS 0.1% BSA and sonicated. Cells seeded on round coverslips were incubated for 2 h at 37°C with 60 μg/ml of the BODIPY-labelled liposomes. Prior to incubation with liposomes, cells were treated with different combinations of PLA_2 inhibitors or their vehicle. Cells were washed and mounted in an open-bottomed chamber to which 1 ml of Tyrode's was added. Cells were examined with a BioRad MRC 1024 confocal microscope, and were challenged with 1 μM ionomycin 18 s after the beginning of the recording. The results presented are representative of at least 3 donors.

Evaluation of PG production. Cells differentiated in collagen-coated Petri dishes were detached and enriched following the methods by Akatsu, *et al* and Yu, *et al*[27,28]. Briefly, contaminating adherent cells were removed by treatment with a solution of pronase E (1 mg/ml) and EDTA (5 mM). After washing with PBS, collagen was digested using 0.2% collagenase type 2, the released cells were suspended in Tyrode's solution, layered over a discontinuous FBS gradient (15 ml of 40% FBS overlying 70% FBS) and allowed to settle through the gradient for 40 min at room temperature. Cells from the bottom fraction were collected and plated at 5×10^5 cells per well in 48-well plates and allowed to adhere overnight. Next, cells were treated with COX inhibitors or the vehicle for 24 h in serum-free α-MEM supplemented with 0.5% BSA. The supernatants were collected and screened for total PG production using an enzyme immunoassay kit according to the manufacturer's protocol.

Statistical analysis. Statistical significance was determined using the one-way ANOVA test. Dunnett's multiple comparison test was used to compare groups to their control. Data were considered significant when $p < 0.05$. All statistics were calculated using the GraphPad Prism 4 software.

RESULTS

Characterization of the osteoclastic phenotype. Multinucleated, TRAP-positive cells represented 4.2 ± 0.6% (n = 6) of the cells isolated from human fetuses. These cells have been previously characterized as osteoclasts[10]. Among cells differentiated *in vitro* for 21 days, 15.5 ± 9.6% (n = 6) were TRAP+ and multinucleated (≥ 3 nuclei) and, of these, 26.5 ± 2.2% (n = 7) presented an actin ring (Figure 1A). To further characterize the phenotype of the *in vitro* differentiated multinucleated cells, we studied their response to calcitonin, a hormone known to elevate intracellular calcium levels in osteoclasts[29]. This was done by determining the changes in the levels of intracellular calcium induced by 1 μM salmon calcitonin using the Fluo-3 fluorescent probe. As shown in Figure 1B, salmon calcitonin induced an increase in the intracellular calcium level in hOCL cells. The same result was previously obtained with fhOC[10]. The intensity of fluorescence is expressed in pseudocolor on a scale from 0 (black) to 255 (white), where 255 is the maximum fluorescence. Finally, we showed that *in vitro* differentiated hOCL cells were able to form bone resorption pits (Figure 1C).

COX protein expression. Immunohistochemistry using anti-COX-1 and anti-COX-2 antibodies showed that multinucleated cells in both fhOC and hOCL cultures express both isoenzymes, and that these enzymes have different intracellular localizations. The signal obtained with the COX-1 antibody was mainly cytoplasmic, whereas the signal obtained with the COX-2 antibody was mainly perinuclear (Figure 2A). In the absence of the primary antibodies, no signal for COX-1 or COX-2 could be observed.

Immunohistochemistry was also performed on bone tissue samples. As shown in Figure 2B, both COX-1 and COX-2 are present in osteoclasts from both fetal and normal adult bone tissue, confirming *in situ* the findings *in vitro*. We could not confirm, however, if the intracellular distribution of COX isoenzymes was the same *in situ* as *in vitro*.

COX activity. Determination of COX activity in single cells was performed on fhOC, hOCL cells, and on NIH/3T3 cells, used as positive controls. NIH/3T3 cells were prestimulated with 100 U/ml of IL-1α, a condition where both COX-1 and COX-2 are present[24]. The cells were double-labelled, the nuclei being stained in red with Syto-59 and H_2DCFDA fluorescence in green. Figures 3A and 3B show that multinucleated cells from both models pretreated with the vehicle had a higher basal fluorescence level than NIH/3T3 cells. Challenge with 30 μM AA did not significantly increase the signal in fhOC or in hOCL cells, but an increase in fluorescence was seen in NIH/3T3 cells. Cells pretreated with 2 μM indomethacin, a concentration that totally inhibits both COX-1 and COX-2 activities[30], suppressed basal fluorescence, and no response to AA was detected in any of the 3 cell types. To determine the contribution of COX-1 to the observed fluorescence, the cells were pretreated with 100 μM valeroyl salicylate, a selective COX-1 inhibitor[31]. Valeroyl salicylate-pretreated cells showed a weaker basal fluorescence than untreated cells, and the addition of AA did not increase the COX signal in fhOC or in hOCL, but significantly increased the signal in NIH/3T3 cells. The contribution of COX-2 to the observed fluorescence was studied by pretreating the cells with 22 μM DFU, a selective COX-2 inhibitor[32]. DFU-pretreated cells showed basal fluorescence weaker than vehicle-treated cells, and challenge with AA slightly increased the signal in the 3 cell types studied. Even though single-cell results are presented, they are representative of the experiments in cells from at least 3 donors.

Determination of PLA_2 activity. In order to determine which PLA_2 enzyme is active in both models, PLA activity at the sin-

gle-cell level was studied using the bis-BODIPY®FL C_5-HPC lipid probe. When this probe is incorporated into the cell membrane, the proximity of the BODIPY® fluorophores on adjacent acyl chains results in self-quenching. This is alleviated by release of a labeled acyl chain by PLA_2, resulting in an increase in fluorescence intensity. PLA activity can thus be monitored by confocal microscopy. Ionomycin was used to increase free intracellular calcium in order to activate the 2 calcium-dependent PLA_2 isoforms. As shown in Figure 4A, there is an increase in the intensity of the fluorescent signal following stimulation with ionomycin. This peak of fluorescence was abolished by a combined pretreatment of the cells with $AACOF_3$, an inhibitor of $cPLA_2$[33] and $iPLA_2$[34], DEDA, a $sPLA_2$ inhibitor[35], and with HELSS, an $iPLA_2$ inhibitor[34] (Figure 4B). Treatment with $AACOF_3$ alone abolished the response to ionomycin stimulation (Figure 4C), but neither HELSS nor DEDA separately (data not shown) or combined inhibited the increase in fluorescence (Figure 4D), showing that $cPLA_2$ is the main isoform in these cells under these experimental conditions. Results are representative of both fhOC and hOCL cells.

PG production assay. In order to determine if human OCL cells are actively producing PG, an enriched population of *in vitro* differentiated osteoclasts was used. Cells from the bottom fraction of the FBS gradient were mostly (82.5 ± 2.2%) TRAP-positive and multinucleated (≥ 3 nuclei). PG production was measured as total PG accumulation in the cell culture media and corrected for total cell protein. In basal conditions, human osteoclasts in culture produced 37.2 ± 11.3 ng of PG per mg of protein. This production was significantly inhibited by 10 μM naproxen (nonspecific COX inhibitor), 100 μM valeroyl salicylate (COX-1 inhibitor), and 10 μM NS-398 (COX-2 inhibitor)[36] to 9.03 ± 3.7, 5.21 ± 1.7, and 9.25 ± 3.2 ng/mg protein, respectively (Figure 5), and the differences between these different agents were not significant. These results are represented as a percentage of the control in Figure 5.

Effect of COX inhibition on bone resorption. Human OCL cells differentiated on bovine cortical bone discs were used to assess the effect of the inhibition of COX enzymes on bone resorption (Figure 6). Treatment of cells with 10 μM naproxen, a nonspecific COX inhibitor, increased the total resorption area when compared to vehicle (DMSO)-treated control cells. Similar results were obtained when cells were treated with a different nonspecific COX inhibitor, indomethacin (results not shown). Inhibition of COX-1 with 100 μM valeroyl salicylate also increased the total resorption area, mimicking the effect of naproxen. Treatment of cells with 10 μM NS-398, a COX-2 inhibitor, however, had no significant effect on bone resorption. Treatment with DFU, another COX-2 inhibitor, also did not significantly affect bone resorption (results not shown).

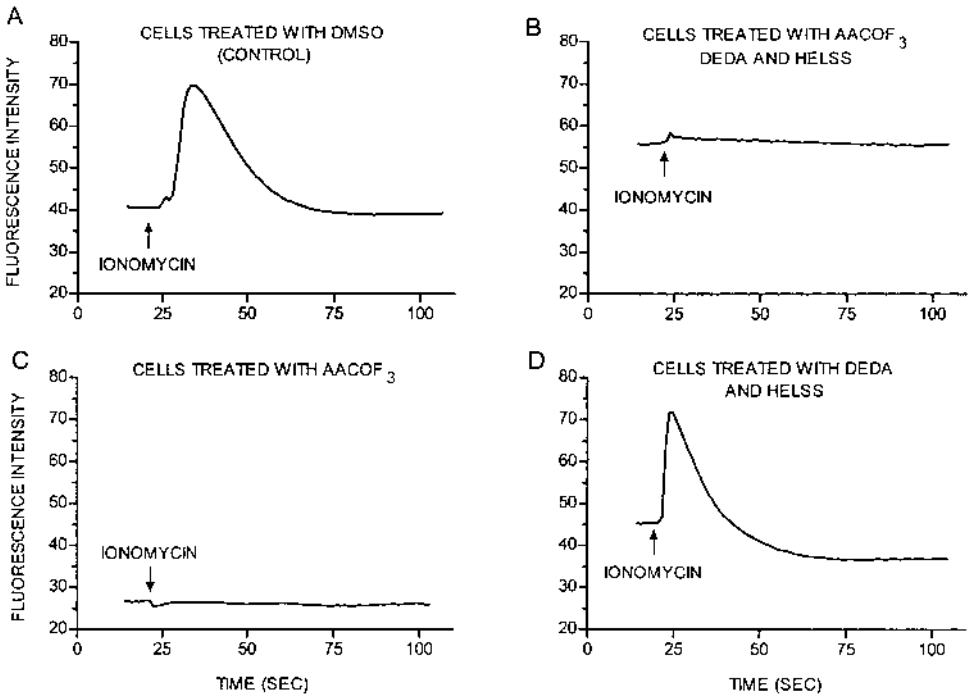

Figure 4. Determination of PLA_2 activity in single-cell assays. Human OCL cells were treated with (A) DMSO (control); (B) $AACOF_3$ ($cPLA_2$ and $iPLA_2$ inhibitor), DEDA ($sPLA_2$ inhibitor), and HELSS ($iPLA_2$ inhibitor); (C) $AACOF_3$ alone; or (D) DEDA and HELSS. Effect of inhibitors on PLA_2 activity is measured by intensity of emitted fluorescence and corrected for photobleaching. Addition of ionomycin is indicated by an arrow. Results are from osteoclasts from a single donor and represent 3 independent experiments.

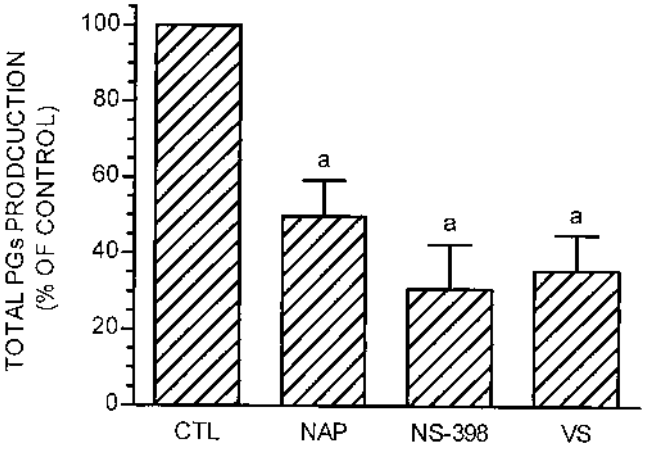

Figure 5. Prostaglandin production by human osteoclasts. Enriched hOCL cells were assayed for total PG production in basal conditions and following treatment with COX inhibitors. Results were corrected for total protein content and are represented as a percentage of the control (CTL). NAP: naproxen; VS: valeroyl salicylate. Results are representative of 5 independent experiments. a: $p < 0.01$, compared to the control.

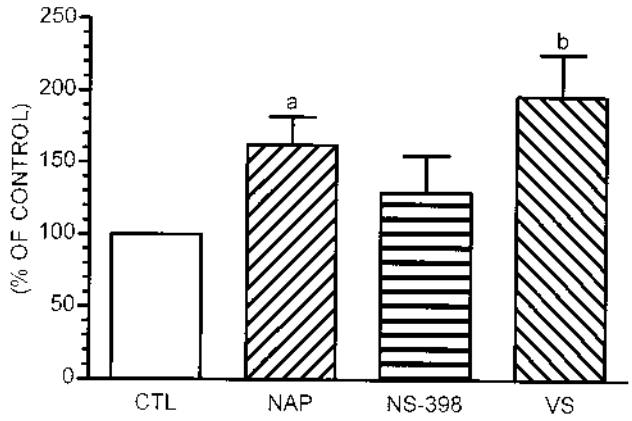

Figure 6. Effect of COX inhibitors on bone resorption. PBMC differentiated for 21 days on bovine cortical bone discs were treated with either COX inhibitors or their vehicle (CTL) for 48 h. Resorption pits were stained with toluidine blue and visualized with a brightfield microscope. NAP: naproxen; VS: valeroyl salicylate. Results are means ± SE of 5 independent experiments. a: $p < 0.05$; b: $p < 0.01$, compared to the control.

DISCUSSION

The main objective of our study was to determine if human osteoclasts can produce prostaglandin. To set up a model of authentic human osteoclasts, we isolated cells from human fetuses because of the high level of bone remodeling and, consequently, the high number of osteoclasts in the tissue. Although in some pathological conditions, such as osteoporosis and Paget's disease, high numbers of osteoclasts may be found and probably isolated, we avoided these sources because the cells may not be representative of normal cells.

We applied the procedure described by Chambers, *et al* to extract osteoclasts from rabbit bones[37] to human fetal long bones. This method allowed us to extract TRAP+, multinucleated cells that respond to calcitonin and form actin rings and bone resorption pits. The presence of these markers confirms that the multinucleated cells found in our preparation are functional human osteoclasts. It is possible that fetal osteoclasts differ from their adult counterparts since differences due to development and cell activity may occur. On the other hand, studies in our laboratory have clearly shown that the culture procedure used to study fetal human mature osteoclasts does not alter the distribution of the PGE_2 receptors compared to *in situ* fetal osteoclasts[23].

We have demonstrated that human osteoclasts in culture express EP3 and EP4 but not EP1 and EP2 receptors[10], a distribution corresponding to that found in human adult osteoporotic and Pagetic tissue[23]. For the study of PG receptors, isolated fetal human osteoclasts are thus a representative model of *in situ* fetal osteoclasts and of *in situ* osteoclasts from adult patients with osteoporosis and Paget's disease. Although it is not possible to determine if this model is also representative of normal *in vivo* human adult osteoclasts, it seems reasonable to assume that it is the closest model available. Unfortunately, the study of normal adult osteoclasts is hampered by several limitations such as the source of cells, the short *in vitro* osteoclast lifespan, the heterogeneity of cell types present in culture, and the small number of osteoclasts obtained. This last limitation, which also applies to fetal human cells, stymied our efforts to evaluate the implication of COX isoenzymes in bone resorption. We thus completed our studies using a model of *in vitro* differentiated human osteoclast-like cells.

Because cells differentiated *in vitro* have been shown to respond differently to IL-1[38], suggesting that the behavior of *in vitro* differentiated cells may differ from that of osteoclasts *in vivo* and *ex vivo*, we compared the results of both models. As presented in the Results section and discussed below, TRAP+ multinucleated cells from both models share all the characteristics studied.

The heterogeneous properties of our preparations precluded the use of methods such as Northern and Western blots because these would reflect the variety of cell types in culture. Most of our analyses were thus limited to studies at the single-cell level. We initially showed, using specific antibodies for each COX isoenzyme, that the multinucleated cells from both models express both COX-1 and COX-2 proteins, and that this expression does not differ from that of *in situ* osteoclasts. COX-1 was expressed mostly in the cytoplasm and COX-2 showed a mainly perinuclear localization. This difference in the distribution of both COX isoenzymes has been described in mononuclear NIH/3T3, HUVEC, and BAEC[24] and may be interpreted as an indication that prostanoids formed via COX-2 operate at the nuclear level. It is interesting that the EP3 and EP4 receptors are not only present at the plasma membrane of human osteoclasts, but also show a strong perinuclear distribution[10]. Recent studies have shown the presence of functional nuclear EP3 and EP4 receptors in neonatal porcine brain

and adult rat liver[39]. This perinuclear distribution of both COX-2 and EP receptors might suggest that PG produced by COX-2 could act in an autocrine fashion via nuclear PG receptors, a hypothesis that remains to be explored.

To confirm the presence of the COX isoenzymes in human osteoclasts and to determine if these enzymes are active we used a fluorescent probe capable of assessing the COX-mediated oxidative process in the transformation of prostaglandin G_2 into prostaglandin H_2. Interestingly, in basal conditions and without the addition of exogenous AA, both osteoclast models presented a very high fluorescence when compared to IL-1-stimulated NIH/3T3 cells, which express both COX-1 and COX-2. Although part of this basal fluorescence in osteoclasts could be due to other oxidative processes, as indicated by its incomplete inhibition by indomethacin, the decrease in the basal fluorescence in the presence of indomethacin, valeroyl salicylate, and DFU indicates that both COX-1 and -2 are active in these experimental conditions. These results also strongly suggest that a PLA_2 is active and is feeding the cyclooxygenase with endogenous free AA. The very small increase in fluorescence upon addition of AA in these cells when compared to NIH/3T3 cell also suggests a strong basal PLA_2 and COX activity in osteoclasts. We cannot exclude the possibility that this high activity may be due to experimental manipulation, but the fact that stimulated NIH/3T3 cells in conditions where both COX-1 and COX-2 are expressed[24] did not show a high basal activity level in the same experimental conditions would argue against this hypothesis.

The high basal COX activity seen in our models suggests the presence of PLA_2 activity. Indeed, our results clearly show that $cPLA_2$ is present in both models and that its activity can be augmented by an increase in intracellular calcium. It is interesting that $cPLA_2$ appears to be implicated in collagen-induced arthritis and in PGE_2-mediated bone resorption in mice[40]. Further, vitamin D3 and parathyroid hormone, 2 calcitropic hormones, activate $cPLA_2$ in rat enterocytes[41], but it remains to be determined if these or other hormones, such as calcitonin, influence the activity of $cPLA_2$ in human osteoclasts.

High basal COX activity and readily activated $cPLA_2$ indicate that human osteoclasts actively produce PG. Using an enriched population of *in vitro* differentiated TRAP+ multinucleated cells, we showed significant production of PG after 24 h incubation without any stimulus; moreover, PG production could be inhibited by specific and nonspecific COX inhibitors. These results demonstrate that both COX-1 and COX-2 contribute to the production of PG in basal conditions. Although these results must be interpreted with caution, considering the heterogeneous character of the purified population of osteoclast-like cells, they support the existence of spontaneous activity of both COX-1 and COX-2 in the experimental model studied.

To determine the role of the endogenous PG on the control of bone resorption we used specific and nonspecific COX inhibitors. Inhibition of COX-1 increased resorption pit formation by osteoclasts, whereas inhibition of COX-2 had no effect, although it was as effective as the COX-1 inhibitor in decreasing PG synthesis. These results indicate that PG may inhibit bone resorption by osteoclasts and suggest a segregated function of each COX isoenzyme in these cells. The hypothesis that different COX isoenzymes may be linked to different effects in a single cell type is also supported by another study demonstrating that the influence of COX-1 on the activity of rat chondrocytes differs from that of COX-2[42]. Although the molecular mechanisms implicated in this difference are unknown, it is possible to speculate about the existence of functional couplings between COX isoenzymes and prostanoid synthases or specific receptors. Recent work by Kudo, *et al* has revealed that cytosolic PGE_2 synthase (PGES)[43] preferentially couples to COX-1, whereas membrane-associated PGES[44], thromboxane synthase, and PGI_2 synthase[45] couple mainly to COX-2. Whether similar mechanisms can explain the predominant role of COX-1 products in the inhibition of osteoclast bone resorption found in our study remains to be determined. It is also very significant that COX-2 is the most important isoenzyme in human osteoblasts[4,5]; given the role of osteoblasts in orchestrating osteoclast development and function, it is possible that in a pluricellular system containing both cell types the final effect of specific inhibition of COX-1 or COX-2 may differ from the one we describe because of the added interference in osteoblast-driven osteoclast control.

Our study shows that human osteoclasts express COX-1, COX-2, and $cPLA_2$, and strongly suggests that active PG production occurs in these cells in nonstimulated conditions. The prostaglandins produced mainly through the COX-1 pathway, which remain to be identified, seem particularly implicated in the inhibition of bone resorption.

ACKNOWLEDGMENT

We thank Dr. Morris Manolson for his generous donation of the rhRANKL plasmid.

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