Implication of Prostaglandin Receptors in the Accumulation of Osteoprotegerin in Human Osteoblast Cultures

RANA SAMADFAM, MAXIME A. GALLANT, MARIE-CLAUDE MIOUSSE, JEAN-LUC PARENT, and ARTUR J. de BRUM-FERNANDES

ABSTRACT. ***Objective.*** Prostaglandins are important mediators in bone metabolism and in pathologies such as rheumatoid arthritis and osteoarthritis. We investigated the roles of cyclooxygenases (COX) and prostaglandin receptors in the accumulation of osteoprotegerin (OPG) in the supernatants of human osteoblasts in culture.

Methods. Three different cellular models were used, the human osteosarcoma cell lines MG-63 and Saos-2, and primary cultures of human osteoblasts. OPG concentrations were determined by ELISA.

Results. RT-PCR analysis showed that, like primary human osteoblasts, MG-63 cells express DP, EP4, FP, IP, and TP receptors, whereas the Saos-2 cells lack IP. Concentration of OPG was highest in MG-63 cell supernatants (36 ± 12.5 ng/ml), followed by human osteoblasts (12.77 ± 2.2 ng/ml) and Saos-2 (3.6 ± 0.76 ng/ml). COX inhibitors did not alter these values. Prostaglandin E_2 and BW 245C (a synthetic DP receptor agonist) decreased OPG in the supernatants of human osteoblasts but not in immortalized cell lines. These effects were concentration-dependent and were inhibited by EP4 and DP receptor antagonists. Fluprostenol, an FP receptor agonist, increased the accumulation of OPG in MG-63 but not in primary human osteoblasts or Saos-2.

Conclusion. Our results show that activation of EP4 or DP receptors decreased the accumulation of OPG in supernatants of osteoblasts in culture, and suggest that these receptors could be interesting pharmacological targets in bone diseases. They also demonstrate important differences between primary osteoblasts and immortalized cell lines, both in the distribution and in the effects mediated by prostaglandin receptors. (J Rheumatol 2006;33:1167–75)

Key Indexing Terms:
OSTEOPROTEGERIN PROSTAGLANDIN OSTEOBLAST CYCLOOXYGENASE

Receptor activator of nuclear factor-κB ligand (RANKL) is a membrane protein expressed by osteoblasts that is essential for the differentiation, activation, and survival of osteoclasts[1,2]. RANKL and RANK are members of the tumor necrosis factor (TNF) protein superfamily[1,3] and the TNF receptor family, respectively[4]. Increases in RANKL expression by osteoblasts induce osteoclastogenesis and bone resorption by mature osteoclasts[1,5]. Osteoclast differentiation inhibitory factor (OCIF) or osteoprotegerin (OPG) is a decoy receptor of RANKL[6-8] that, like RANK, belongs to the TNF receptor superfamily. OPG, by competing with RANK for RANKL, inhibits RANKL osteoclast differentiation and activation and subsequent bone resorption[6,7,9]. OPG knockout mice manifest severe osteoporosis with a markedly increased number of osteoclasts[10]. Conversely, overexpression of OPG in mice results in a profound yet nonlethal osteopetrosis, coincident with a decrease in later stages of osteoclast differentiation similar to that found in animals with RANKL or RANK deficiency[6]. The latter study also showed comparable effects upon administration of recombinant OPG to normal mice.

Prostaglandins (PG) are potent local mediators that produce an astonishing array of biological effects in physiological and pathological conditions. PG are synthesized from arachidonic acid by the action of cyclooxygenases (COX) and specific synthases. The 5 major natural prostanoids, PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2, and thromboxane A_2, produce their biological effects by acting on distinct transmembrane G protein-coupled receptors known as EP, FP, DP, IP, and TP, respectively. The PGE_2 receptors consist of 4 different subtypes, EP1, EP2, EP3, and EP4[11]. Spliced variants of the human TP receptor (referred to as TP alpha and TP beta) have also been identified[12]. IP, DP, EP2, and EP4 receptors usually couple to Gαs and signal through an increased intracellular cAMP level whereas the TP, FP, and EP1 receptors mostly couple to Gαq and induce calcium mobilization. Depending on the splice

From the Division of Rheumatology, Faculty of Medicine, Université de Sherbrooke, Fleurimont, Québec, Canada.

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R. Samadfam, PhD; M.A. Gallant, MSc; M-C. Miousse, MSc; J-L. Parent, PhD, Associate Professor; A.J. de Brum-Fernandes, MD, PhD, Professor, Division of Rheumatology.

Address reprint requests to Dr. A.J. de Brum-Fernandes, Division of Rheumatology, Faculty of Medicine, Université de Sherbrooke, 3001 12th Avenue North, Fleurimont, Québec J1H 5N4, Canada. E-mail: Artur.Fernandes@USherbrooke.ca

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variant of EP3 receptor present, it can signal through Gαi, Gαs, or Gαq[13-16].

PG influence bone formation and resorption. PGE_2 increases bone resorption in bone organ cultures and in animals receiving systemic or local injection of PGE_2[17,18]. *In vitro* PGE_2 promotes osteoblast growth and differentiation: in osteoblast-like cells isolated from young rat calvaria, EP1 agonists stimulate differentiation and inhibit proliferation, but these effects disappear in aged rats[19]. In the same model, however, 11-deoxy PGE_1 (EP2 and EP4 receptor agonist) inhibits differentiation and stimulates proliferation regardless of age[19]. The opposite has been shown in MC3T3-E1 cells, a mouse osteoblastic cell line, where activation of the EP1 receptor stimulated proliferation and decreased alkaline phosphatase expression, while EP2 and EP4 receptors mediated a decrease in proliferation and an increase in differentiation[20,21]. These findings indicate that the effects of PGE_2 may vary according to species and raise the possibility that results obtained from animals may not apply to humans.

PG may also affect bone resorption by changing the levels of OPG and RANKL expressed by osteoblasts. It has been reported that PGE_2 significantly decreases the expression of OPG in several models[22-24], but the receptors involved in this process have not been studied. The primary objective of our study was to investigate the role of PG receptors in the control of OPG production by human osteoblasts (hOB) and immortalized osteoblastic cell lines. A secondary objective was to determine if there are differences in the distribution of PG receptors between these immortalized cell lines and authentic human osteoblasts, which, as we have reported, present DP, EP4, FP, IP, and TP receptors[25].

MATERIALS AND METHODS

Compound NS-398 and all PG agonists and antagonists were purchased from Cayman Chemicals (Ann Arbor, MI, USA), except for the EP4 antagonist, compound L-161982, which was kindly provided by Merck Frosst Canada. Trizol reagent, poly-d(t) oligonucleotides, DTT, dNTP, and SuperScript reverse transcriptase were obtained from Gibco (Grand Island, NY, USA). DNAase-free RNAase and Taq-polymerase were purchased from Amersham Biosciences (Baie d'Urfé, QC, Canada). Fura-2/AM was obtained from Calbiochem (San Diego, CA, USA). All other reagents unless specified were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

Cell culture. The osteosarcoma cell lines MG-63 and Saos-2 were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (ICN, Montreal, QC, Canada) and 1% penicillin-streptomycin solution. Cells were plated into 6-well plates at a density of 1.6 million cells/well and were stimulated the following day with different PG analogs in the presence of COX inhibitors. After 24 h of stimulation, the supernatants were collected and stored at –80°C. Human osteoblasts were obtained from femoral heads of patients undergoing hip arthroplasty[25]. Informed consent was signed by each patient before surgery, and the research project was approved by the ethics Institutional Review Board of the Centre Hospitalier Universitaire de Sherbrooke. Culture medium was changed twice a week, and cells reached confluence after 4 to 6 weeks. Confluent hOB were passed into 6-well plates (390,000 cells/well) and after 4 days the cells were stimulated as described for the immortalized cell lines.

Sandwich ELISA for OPG. A commercial sandwich ELISA kit (R&D Systems, Cedarlane Laboratories Ltd., Hornby, ON, Canada) was used to determine OPG levels in the supernatants of cell cultures (detection limit 30 pg/ml, interassay variability 10%, intraassay variability 9%). Briefly, 60 µl/well of capture OPG antibody [2 µg/ml, diluted in phosphate buffered saline (PBS)] was transferred into 96-well ELISA high binding, flat bottom plates (Corning, Whitby, ON, Canada) and incubated overnight at room temperature. Each well was then aspirated and washed 3 times with PBS containing 0.05% Tween 20 washing buffer. The plates were blocked by adding 300 µl of PBS containing 1% bovine serum albumin (BSA), 5% sucrose, and 0.05% NaN_3 blocking buffer to each well for 1 h. The reaction medium was removed and each well was again washed 3 times with washing buffer, then the samples or standards of OPG (diluted in PBS) were added in a final volume of 60 µl and incubated at room temperature for 2 h. After washing, the detection antibody (800 ng/ml, diluted in blocking buffer) was added to each well and incubated an additional 2 h. The wells were washed and incubated with 60 µl of streptavidin horseradish peroxidase (diluted 1/200 in PBS) for 35 min. The bound enzyme-protein was assayed using tetramethylbenzidine/H_2O_2 as substrate, and the reaction stopped by addition of 50 µl of 1 M phosphoric acid. The absorbance was measured at 450 nm.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis. RT-PCR analysis was performed as described[25]. Briefly, total RNA was purified from confluent cultures of osteoblastic cells with Trizol reagent following the manufacturer's protocol. Reverse transcription was performed using 5 µg total RNA in 30 µl SuperScript reverse transcriptase buffer containing 50 pmoles oligonucleotide poly-d(t), 6 mM DTT, 0.3 mM dNTP, and 400 U of SuperScript reverse transcriptase. The reaction was performed at 37°C for 1 h and then treated with DNAse free-RNAse for 37 min at 37°C, and heat-inactivated 20 min at 70°C. Amplification of the target sequences was carried out using a commercial kit (Invitrogen, Burlington, ON, Canada). The reactions were performed in a total volume of 30 µl containing the following reagents: 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 1 µM of each primer, cDNA corresponding to 2 µg of RNA, 1 mM dNTP, and 1 U Taq-polymerase. A denaturation step at 95°C for 5 min was performed, then 40 cycles were run as follows: denaturation at 94°C (1 min), annealing at 60°C (1 min), and extension at 72°C (1 min). After completion of the PCR cycles, the last extension time was 5 min at 72°C. The PCR reactions were performed using specific pairs of primers, shown in Table 1.

Intracellular cyclic-AMP determination. Cells were plated in 35 mm Petri dishes for intracellular cAMP determination by measuring the conversion of [^{3}H]ATP to [^{3}H]cAMP using sequential chromatography on Dowex columns (AG 50W-X8 H^+) and Alumina columns, as described[25].

Fluorometric monitoring of intracellular calcium. Cells were plated on 25 mm coverslips and measurement of intracellular calcium variations was done as described[26]. Briefly, cells were washed 3 times with 2 ml of Tyrode's salts containing 0.1% BSA and incubated 40 min in 1 ml of the same buffer supplemented with 3 µM Fura-2/AM at room temperature. The coverslips were then washed twice and incubated for a further 20 min in Tyrode's salts at room temperature to ensure complete hydrolysis of the probe acetoxymethyl ester groups. The stimulation was then applied, and the Fura-2 fluorescence (excitation at 340 nm and 380 nm and emission at 510 nm) was recorded in a Hitachi F-2000 spectrofluorometer.

Statistical analysis. Data are expressed as means ± SEM for triplicates of at least 3 different experiments. Data were analyzed with the repeated measures analysis of variance (ANOVA) followed by Dunnett post-test using Prism 4 software (GraphPad Software, San Diego, CA, USA) and $p < 0.05$ was considered significant.

RESULTS

RT-PCR analysis. RT-PCR analysis was carried out on both MG-63 and Saos-2 cell lines and on primary human osteoblasts to determine the expression of PG receptors at the mRNA level (Figure 1). In MG-63 cells and hOB, bands of the predicted length were found for DP, EP4, FP, IP, and TP. In

Table 1. Prostanoid receptor RT-PCR primer sequences.

	Forward (5'-3')	Reverse (5'-3')
EP1	CTGCTGTTCGTGGCCAGCCTG	GAAGTGGCTGAGGCCGCTGTG
EP2	GTCATGTTCTCGGCCGGGGTG	GCACATCTGTAGCCTTGACCACAG
EP3	GATTGCGGATCGGTGTCCGTG	GGTTGAGGAACACTGGCAGGG
EP4	ATCTTACTCATTGCCACC	TCTATTGCTTTACTGAGCAC
DP	TCTATGCGATGCACCGGCGG	GTCACAGACTGGATTCCATG
FP	GGCATCCTTTCTGCTTTTGG	CCCCACACAGATTTACTGTCC
IP	TGCTCCCTGCCTCTCACGAT	TGGCTTCTGCTTTGGACGAC
TP	GGCCCACAAACATTACCCTGGAG	CAGCTCCTTCTCCGTGGTGCG
GAPDH	GGTGGAGGTCGGAGTCAACG	TCATGAGGTCCACCACCCTG

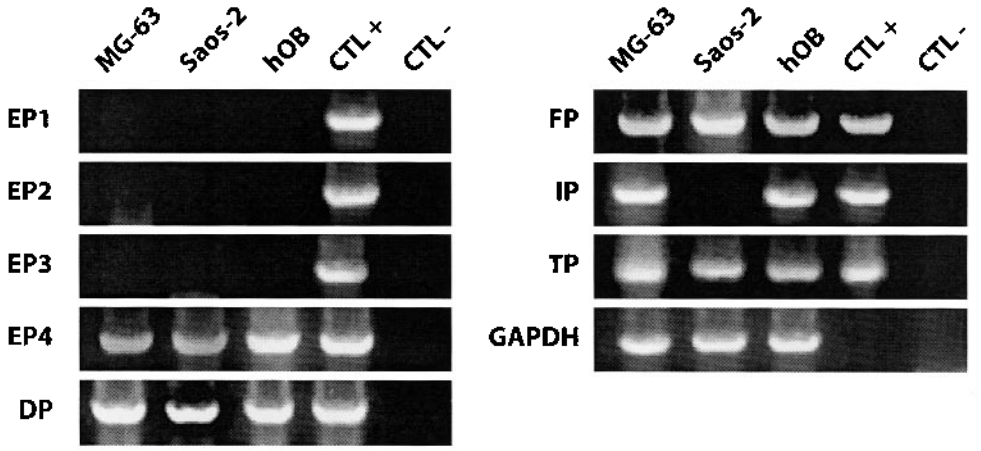

Figure 1. RT-PCR analysis of expression of prostaglandin receptor mRNA in MG-63, Saos-2, and primary human osteoblast (hOB) cell cultures. Positive controls (CTL +) were done using cDNA for each receptor; RNA was omitted in negative controls (CTL –). No positive control was performed for GAPDH.

Saos-2 cells, the same receptors were detected except for IP. We performed amplification of GAPDH as a positive RT-PCR control. Although we did not detect mRNA for the EP1, EP2, and EP3 receptor subtypes, the specific primers used for this experiment were able to amplify their corresponding cDNA at the predicted size.

Effect of COX inhibitors on accumulation of OPG protein in supernatant of hOB, MG-63, and Saos-2 cell lines. Several COX inhibitors were tested for their capacity to modulate OPG expression by human osteoblasts. Figure 2 shows the accumulation of OPG in the supernatants of MG-63, Saos-2, and hOB cells after 24 h. In resting conditions, the concentration of OPG in the supernatants varied widely between the 3 cell models used, being highest in MG-63 cells (36 ± 12.5 ng/ml), followed by primary human osteoblasts (12.77 ± 2.2 ng/ml) and Saos-2 (3.6 ± 0.76 ng/ml) cells. Treatment with the nonspecific COX inhibitor naproxen (10 μM) did not affect the basal concentration of OPG in any of the cell types studied. The results were similar with all the other tested inhibitors: valeroyl salicylate (100 μM), a COX-1 inhibitor, compound NS-398 (1 μM), a COX-2 inhibitor, and diclofenac (10 μM) and indomethacin (1 μM), both nonspecific COX inhibitors.

Effect of exogenous PGE_2 on accumulation of OPG. PGE_2 (1 μM) induced a marked and significant ($p < 0.01$) decrease in OPG accumulation in hOB, but not in the immortalized cell lines (Figure 3). To determine the EP receptor subtype involved in the response to PGE_2, we tested the effect of more specific EP receptor agonists on the accumulation of OPG after 24 h of stimulation. We used several agonists to mimic the effect of PGE_2 on each of the EP receptors: 11-deoxy PGE_1 as an EP2 and EP4 agonist, butaprost as an EP2 agonist, 17-phenyl trinor PGE_2 as an EP1 and EP3 agonist, and sul-

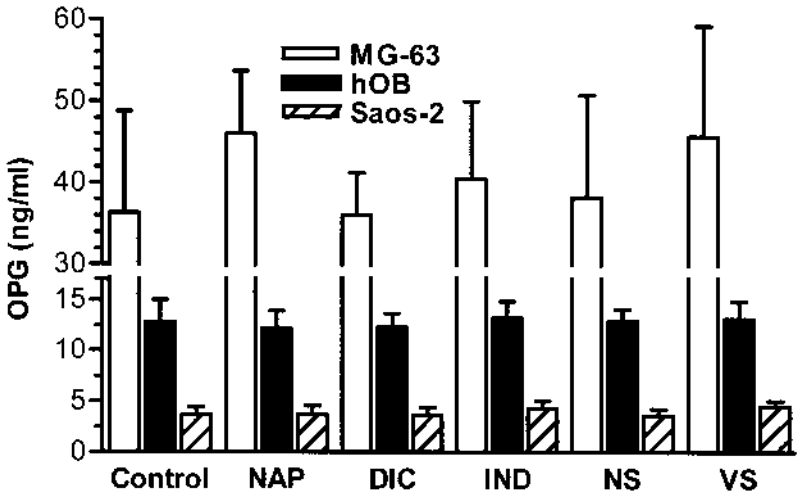

Figure 2. Effects of COX inhibition on OPG accumulation in human osteoblasts (hOB). COX inhibitors were tested for accumulation of OPG in supernatants of hOB, MG-63, and Saos-2 cells for 24 h of stimulation: naproxen 10 μM (NAP), diclofenac 10 μM (DIC), indomethacin 1 μM (IND), NS-398 1 μM (NS), valeroyl salicylate 100 μM (VS). Data shown as mean ± SEM (n = 4).

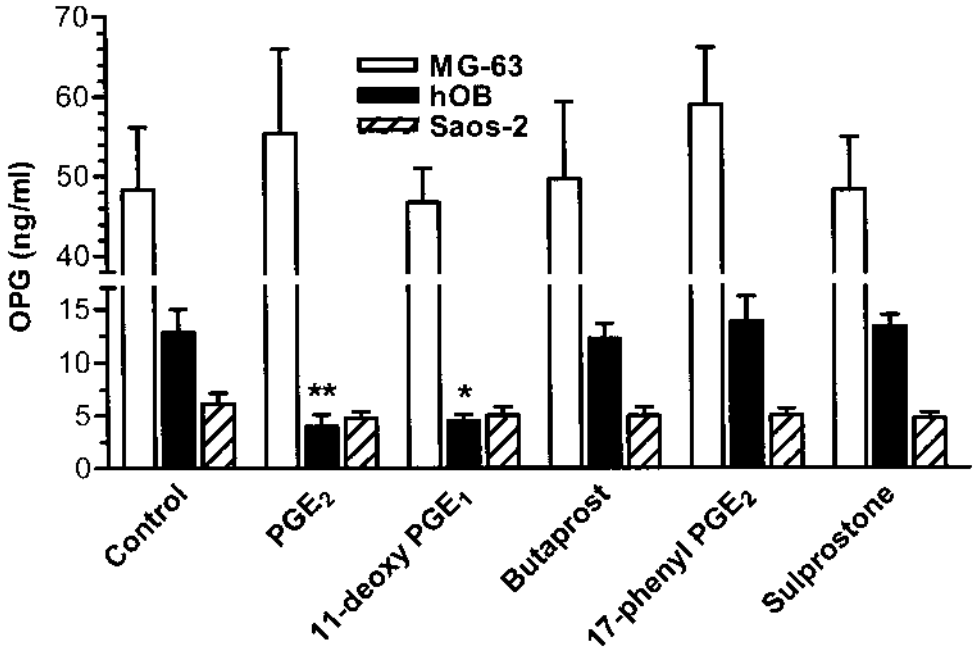

Figure 3. Effects of EP receptor stimulation on OPG accumulation in human osteoblasts (hOB). EP receptor agonists (1 μM) were tested for accumulation of OPG in supernatants of hOB, MG-63, and Saos-2 cells for 24 h of stimulation; naproxen (10 μM) was present in all groups. Data are mean ± SEM (n = 4). *p < 0.05 compared to control; **p < 0.01 vs control.

prostone as an EP3 agonist. Of all the EP receptor agonists tested (at 1 μM), only 11-deoxy PGE_1 significantly decreased by 65% the concentration of OPG in the supernatant of hOB ($p < 0.05$). Butaprost, a specific EP2 receptor agonist, had no effect, strongly suggesting that the response observed was mediated only by the EP4 receptor (Figure 3). The effects of PGE_2 and 11-deoxy PGE_1 on the concentration of OPG in hOB were dose-dependent, as shown in Figure 4A, with EC_{50} of 49 nM for PGE_2 and 17 nM for 11-deoxy PGE_1. To confirm the involvement of EP4 in 11-deoxy PGE_1-induced OPG reduction, hOB were stimulated in the presence of a specific EP4 antagonist, compound L-161982 (Figure 4B). This antagonist dose-dependently reversed the OPG decrease triggered by 11-deoxy PGE_1 (1 μM) and completely blocked it at a concentration of 1 μM ($p < 0.01$) with an IC_{50} of 0.33 μM.

Effect of BW 245C, a DP receptor agonist, on accumulation of OPG. The DP receptor agonist BW 245C (1 μM) in the presence of naproxen (10 μM) significantly decreased ($p < 0.05$) the concentration of OPG in the supernatants of hOB by 55% (Figure 5A), but had no effect on immortalized cell lines

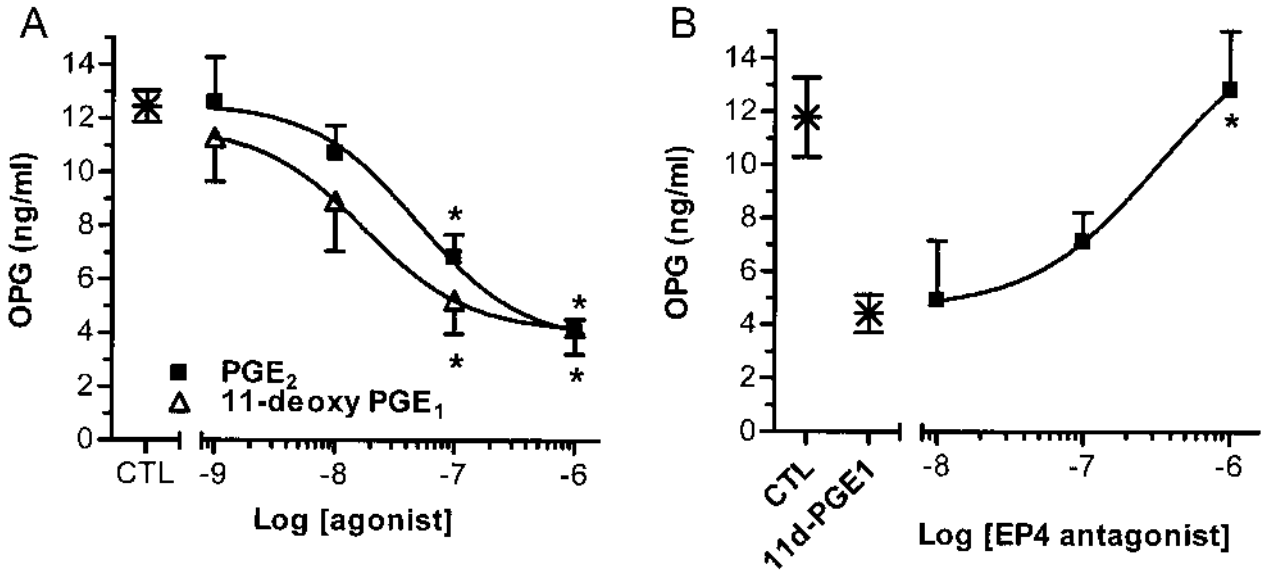

Figure 4. Effect of PGE_2 and 11-deoxy PGE_1 on accumulation of OPG in human osteoblasts. A. PGE_2 and 11-deoxy PGE_1 dose-dependently decreased OPG accumulation, and this effect was reversed by increasing concentrations of the EP4 antagonist L-161982 (B). Data are shown as mean ± SEM (n = 4). *p < 0.01 compared to control for A; p < 0.01 vs 11d-PGE_1 for B.

(data not shown). The decrease in OPG triggered by BW 245C was shown to be dose-dependent. To further confirm the implication of the DP receptor in the response to BW 245C, we used a DP antagonist, BW A868C, to specifically block this receptor. At 1 μM, the antagonist completely reversed the decrease seen with BW 245C stimulation ($p < 0.05$; Figure 5B), while a lower concentration was not sufficient for a significant inhibition.

Effect of fluprostenol, an FP receptor agonist, on the accumulation of OPG. Fluprostenol significantly increased the concentration of OPG in a dose-dependent manner in the supernatants of MG-63 cells, but not in those of hOB or Saos-2 (Figure 6A). This effect was completely blocked by the FP receptor antagonist $PGF_{2\alpha}$ dimethyl amine (20 μM; Figure 6B). The antagonist alone at the same concentration had no effect on the accumulation of OPG, and lower concentrations of $PGF_{2\alpha}$ dimethyl amine had no effect on the accumulation of OPG (data not shown).

Effect of IP and TP receptor agonists on accumulation of OPG. Carbaprostacyclin, an IP receptor agonist, and U-46619, a TP receptor agonist, both at 1 μM concentration, had no effect on the production of OPG in supernatants of any cell type tested (hOB, MG-63, Saos-2; data not shown).

Intracellular second messenger determination. As only the FP receptor in MG-63 influenced OPG expression, we sought to determine if the PG receptors found at the mRNA level in

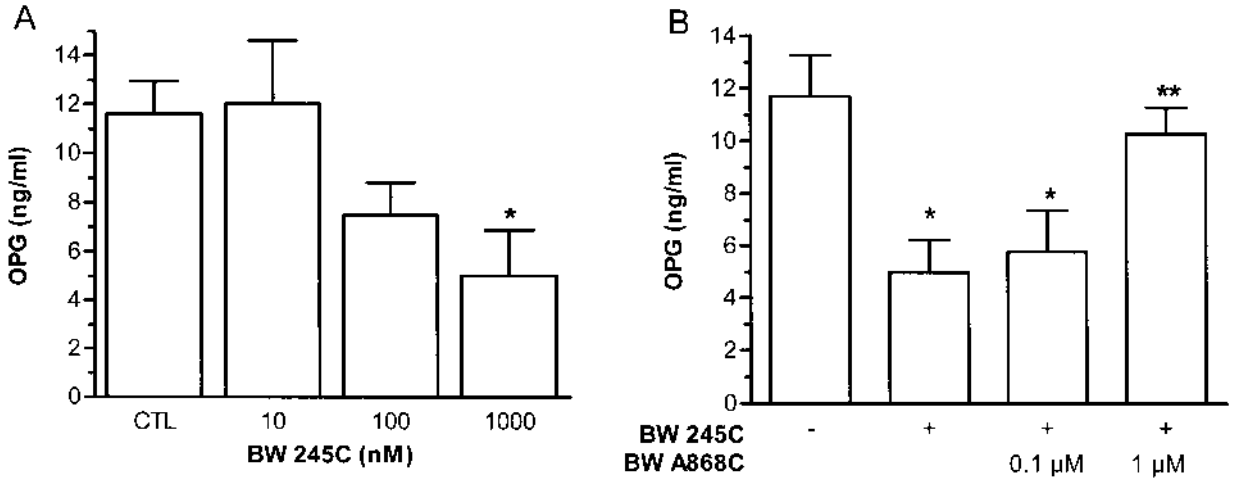

Figure 5. Implication of the DP receptor in OPG modulation. A. Effect of DP receptor stimulation by increasing doses of BW 245C on accumulation of OPG in human osteoblasts (hOB) and immortalized cells. B. hOB were stimulated with BW 245C (1 μM) alone or in the presence of increasing concentrations of the DP antagonist BW A868C, and OPG was measured as described. Data are shown as mean ± SEM (n = 4). *p < 0.05 compared to control; **p < 0.05 vs BW 245C alone.

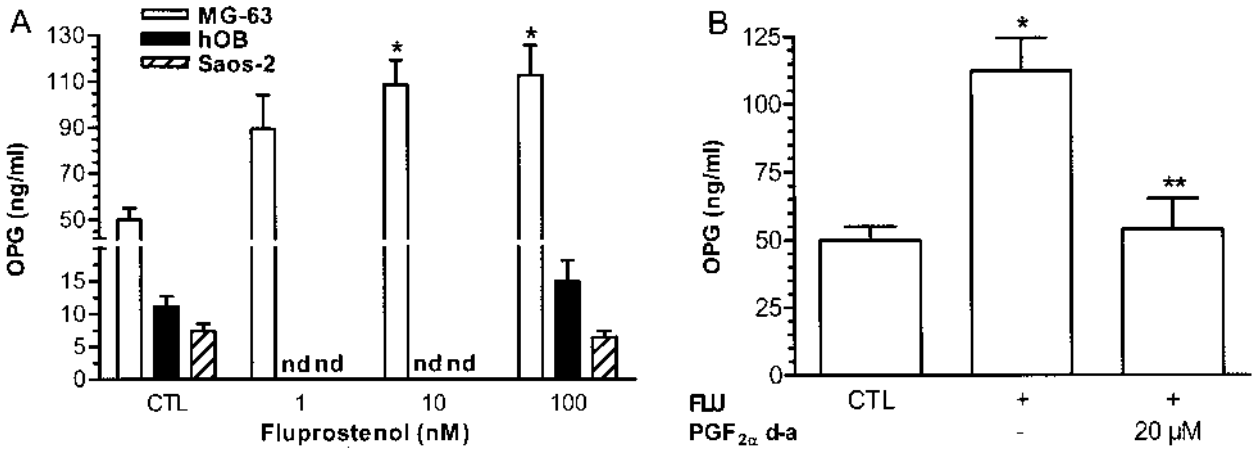

Figure 6. FP receptor-mediated OPG increase. A. The 3 osteoblast models were stimulated with fluprostenol (FLU), an agonist of FP receptor, for modulation of OPG accumulation. B. Effect of FP antagonist $PGF_{2\alpha}$ dimethyl amine ($PGF_{2\alpha}$ d-a) on increased accumulation of OPG induced by FLU (100 nM) in MG-63 cell line. Data are shown as mean ± SEM (n = 3). *p < 0.01 compared to control; **p < 0.01 vs FLU alone. nd: not determined.

MG-63 and Saos-2 cells were functional. These cells were treated with different agonists and their effects on intracellular levels of second messengers were determined. PGE_2 induced an elevation of the intracellular cAMP (icAMP) in both MG-63 and Saos-2 cell lines (Figures 7A, 7B) with EC_{50} of 0.38 μM and 1.76 μM, respectively. Carbaprostacyclin, an IP receptor agonist, induced a small but significant dose-response increase of icAMP in the MG-63 cell line with an EC_{50} of 1.12 μM (Figure 7A), but not in Saos-2 cell line (Figure 7B). Compound BW 245C, a selective DP agonist, did not induce significant changes in icAMP in either cell line. Fluprostenol, a selective FP receptor agonist, induced an elevation of the intracellular calcium concentration in MG-63 cells (Figure 8A) and in Saos-2 cells (Figure 8B). U-46619, a selective TP receptor agonist, also induced elevation of the intracellular calcium level in both cell lines (Figures 8C, 8D). These results confirm that receptors found at the mRNA level were functional, except for the DP receptor in MG-63 cells.

DISCUSSION

The OPG/RANK/RANKL system plays an important role in the pathophysiology of bone, and has the potential to be an important direct or indirect pharmacological target for the treatment of bone diseases. Understanding the mechanisms implicated in the control of expression of these proteins is necessary to achieve this goal. PG are important mediators in the control of bone metabolism and are implicated in the expression and/or accumulation of these factors. Our objective was thus to determine the role of endogenous and exogenous PG in the accumulation of OPG in the supernatants of primary human osteoblasts and immortalized osteoblastic cell lines in culture.

Using the primary human osteoblast culture technique described here, we previously showed that the cells retain the osteoblastic phenotype during at least the first 2 passages, as seen by increases in intracellular cAMP upon parathyroid hormone stimulation, expression of alkaline phosphatase and osteocalcin, and upregulation of these indicators following treatment with $1,25(OH)_2$ vitamin D_3[27]. Moreover, more than 99% of the cell population was positive for osteocalcin mRNA as shown by *in situ* hybridization[28].

We previously characterized the PG receptors expressed on human osteoblasts in culture and demonstrated that they

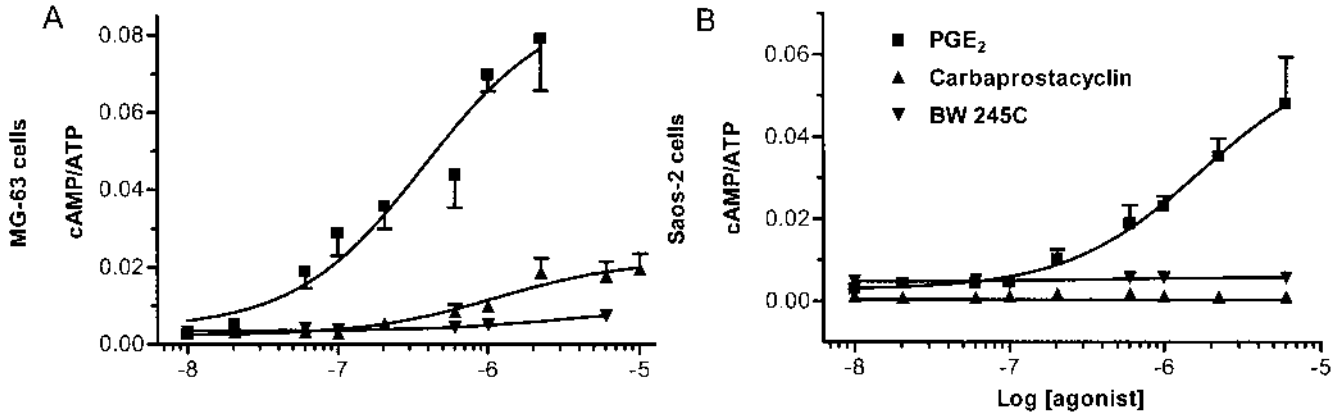

Figure 7. Modulation of cAMP in human osteoblast cell lines. MG-63 (A) and Saos-2 (B) were stimulated with increasing concentrations of PGE_2, carbaprostacyclin, or BW 245C, and intracellular cAMP concentrations were measured (n = 3).

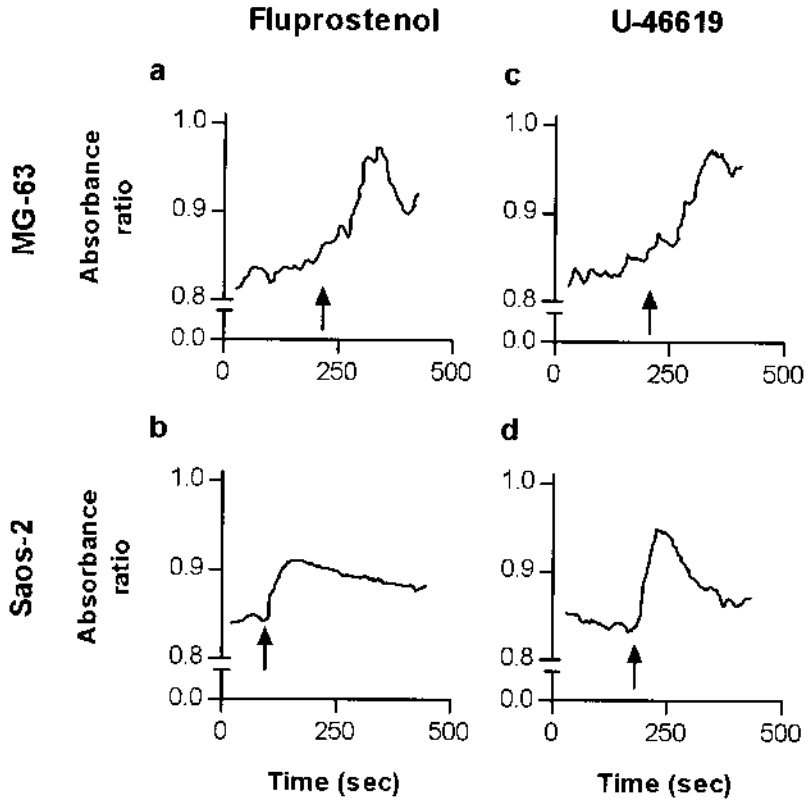

Figure 8. Calcium fluxes in osteoblast cell lines. Cells were stimulated with the FP receptor agonist fluprostenol or the TP receptor agonist U-46619 to modulate intracellular calcium levels in MG-63 (a, c) and Saos-2 cells (b, d). Data are representative of 3 independent experiments.

express DP, EP4, FP, IP, and TP receptors[25]. To determine if distribution of PG receptors in the immortalized cell lines differed from that described for hOB, we used RT-PCR assays. RT-PCR analysis indicated that, like hOB, MG-63 cells express the DP, EP4, FP, IP, and TP receptors, whereas Saos-2 cells express DP, EP4, FP, and TP but lack the IP receptor.

To determine if endogenous PG could affect accumulation of OPG in the supernatants of hOB, MG-63, and Saos-2 cultures, we incubated the cells in the presence of COX-1 or COX-2-specific inhibitors, or one of 3 nonspecific inhibitors (diclofenac, naproxen, or indomethacin). None of these interventions changed the accumulation of OPG, suggesting that in the models and experimental conditions we used, endogenously produced PG do not participate in the control of OPG production by osteoblasts. The lack of effect of COX inhibitors in the system could also be explained by the absence of endogenous PG in the conditions we used. Indeed, our previous findings showed that COX-1 and COX-2 are not expressed in confluent and nonstimulated human osteoblasts, and that the levels of endogenous PGE_2 in the supernatants were undetectable in these conditions[27,28]. However, one cannot exclude the possibility that in conditions where osteoblasts or other cells are actively producing PG, such as upon stimulation with hormones and cytokines, these lipid mediators could influence OPG production in a complex physiological system. Similarly, in a more complex system than the one used in this study, O'Brien, *et al* showed that COX blockade by indomethacin enhanced OPG production in mouse calvaria[29], suggesting that PG produced by osteoblasts or other cell types present in bone may be implicated in this effect. Although our results could be interpreted as a lack of effect of PG on the variable studied, our data clearly show that exogenous PG can affect OPG accumulation.

PGE_2 is thought to be the most important PG in bone. To test the effect of EP receptor activation on accumulation of OPG in the supernatant of human osteoblasts and immortalized cell lines, we used natural and synthetic agonists. PGE_2, which acts on all EP receptors, decreased the expression of OPG in human osteoblasts but not in MG-63 or Saos-2 cells. Of all the specific agonists tested, only an EP2 and EP4 agonist (11-deoxy PGE_1) mimicked the effect of PGE_2 in a concentration-dependent fashion. Butaprost, a specific EP2 agonist, as well as the EP1 and EP3 agonists, failed to do so in concentrations known to elicit a maximal response of their specific receptors, strongly suggesting that the effect of PGE_2 was driven by EP4 receptor activation. This was confirmed using a specific EP4 receptor antagonist, compound L-161982, which concentration-dependently reversed the OPG reduction seen with the EP4 agonist. These results confirm the expression of EP4 as the only PGE_2 receptor in these cells[25].

Other studies have shown that PGE_2 decreases the expression of OPG by osteoblasts and/or bone marrow stromal cells[22-24] and calvaria[29], possibly through activation of the PKA pathway[30], which is consistent with the EP4-cAMP signaling pathway. In addition, EP4 antagonists were shown to significantly reduce the osteoclastogenesis induced by PGE_2 in different animal models[31-33]. Although the latter effect was associated with a reduction in the production of RANKL[31,34], a concomitant effect on OPG cannot be excluded. It is thus possible that the interaction of PGE_2 with the EP4 receptor in hOB may both increase RANKL and decrease OPG, having a major positive effect on osteoclastogenesis and making antagonism of the EP4 receptor an attractive pharmacological target to reduce bone resorption.

Our study shows that activation of the DP receptor, like that of the EP4 receptor, leads to an inhibition of OPG production. This effect was not observed in MG-63 and Saos-2 cultures even if DP was shown to be present in these cells. The use of a specific DP antagonist, compound BW A868C, confirmed that this action was due to interaction with the DP receptor. We recently showed that PGD_2 and BW 245C have the same effect on OPG accumulation in hOB, but observed no contribution for the second PGD_2 receptor, CRTH2, in this process[35]. As for the EP4 receptor, activation of the DP receptor also increases intracellular cAMP through activation of a Gαs protein. The cAMP pathway is known to be implicated in OPG downregulation[22,36]. Although hOB in culture present functional IP receptors under experimental conditions[25], activation of the IP receptor, which also increases intracellular cAMP, had no effect on the production of OPG. Thus, it is possible that other second messengers may be implicated in the inhibitory effect of EP4 and DP activation on OPG production by hOB, but this hypothesis should be confirmed by further studies. As for IP, specific activation of FP and TP receptors had no effect on the accumulation of OPG in the supernatants of hOB, even though these receptors were shown to be present and functional in hOB under the same experimental conditions used in this study[25]. In MG-63 cells, however, activation of the FP receptor significantly increased the accumulation of OPG. Our results clearly show that immortalized cell lines and hOB do not respond similarly to exogenous PG. These differences could be explained by different expression levels of PG receptors in hOB and immortalized cell lines or differences in activation of intracellular signalling pathways.

To investigate the functionality of the PG receptors found in MG-63 and Saos-2 cell lines that did not trigger changes in OPG, we challenged those cells with selective agonists for each receptor subtype detected by RT-PCR and determined their effects on icAMP or calcium. Upon stimulation with PGE_2, both cell lines showed a dose-dependent increase of icAMP levels similar to that found in human osteoblasts[25], confirming activation of the EP4 receptor. Stimulation of the IP receptor increased the icAMP in MG-63 cells but not in Saos-2 cell lines, confirming the absence of the IP receptor in the latter. Although the mRNA of the DP receptor was detected in the 2 immortalized cell lines, stimulation with BW 245C, a DP agonist, did not increase icAMP. These results suggest that the DP receptor may have a low expression level, or may be coupled to other G proteins. Even though activation of FP and TP receptors was able to increase intracellular calcium in MG-63 and Saos-2 cells (as we observed) and in human osteoblasts[25], only FP activation increased OPG concentration in the supernatants of MG-63, again suggesting that coupling of these receptors or processing of the second messenger pathways may differ in these cells compared to authentic hOB.

Our study clearly shows the important activities of prostaglandins in the control of osteoblast-osteoclast crosstalk by the OPG axis. Studies have demonstrated the benefits of COX-2 inhibition on disease progress and pain perception in pathologies such as osteoarthritis and rheumatoid arthritis[37–40]. Unfortunately, despite the efficacy of these COX-2 inhibitors, the risk of thrombotic events restricts their use. Based on our findings and those of others, the PG pathway seems to be relevant for the control of bone metabolism and disease. Targeting this pathway downstream of COX enzymes remains interesting, as it would allow action against more specific targets, such as the receptors or specific PG synthases.

Our results first indicate that activation of EP4 or DP receptors leads to decreased accumulation of OPG in the supernatants of human osteoblasts in culture. Consequently, targeting EP4 or DP receptors with antagonists might rescue basal OPG levels and decrease osteoclastogenesis and activation of mature osteoclasts, thus decreasing bone resorption. The more limited distribution of the DP receptor makes it a more interesting pharmacological target than the EP4 receptor because of the lower risk of interfering with other physiological systems and causing side effects. Second, differences in the distribution and function of the PG receptors in human osteoblasts and the immortalized cells we studied suggest that caution should be exerted in interpretation of studies using MG-63 and Saos-2 cells.

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