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Protein Kinase C Is a Mediator of the Synthesis and Secretion of Osteoprotegerin in Osteoblast-like Cells

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Osteoprotegerin (OPG) is a member of the TNF receptor superfamily and plays a critical role in the development of osteoclasts from precursor cells. OPG is produced by a variety of cells of mesenchymal origin and has been demonstrated to be present in osteoblasts and osteocytes. However, the mechanisms of regulation of OPG production and secretion are not known. Using a highly specific polyclonal antibody, we demonstrate that OPG is synthesized and secreted by osteoblast-like cells in culture. We further show that phorbol myristate acetate, an activator of protein kinase C, activated the secretion of OPG. Further, the increased secretion of OPG correlated well with a corresponding increase in OPG mRNA abundance. In addition, OPG promoter stably integrated into an osteoblast cell line was activated by phorbol myristate acetate. The increase in OPG expression was blocked by an inhibitor of protein kinase C, although the basal OPG expression was not altered. These results suggest that activation of the protein kinase C pathway may play a critical role in OPG expression.

Bone homeostasis is maintained by a coordinated interaction between cells of mesenchymal origin (osteoblasts) and hemopoietic origin (osteoclasts). Osteoclasts are multinucleated cells that are directly responsible for bone resorption. The formation of osteoclasts is regulated at multiple levels—proliferation, differentiation, and maturation—and requires interaction with osteoblasts/osteoprogenitors (1, 2). Recently, a variety of factors that belong to the tumor-necrosis factor superfamily have been identified and have been implicated in osteoclastogenesis (3–5). These include two membrane proteins namely RANK (receptor activator of NF-κB) and RANK ligand (RANKL), and a 38-kDa secreted protein osteoprotegerin (OPG) (6). RANKL, which is present on cells of mesenchymal origin such as osteoprogenitor cells, binds to its cognate receptor (RANK) that is present on hematopoietic cells, and initiates a series of steps that results in the differentiation of the latter cells into osteoclasts (7, 8). OPG binds to RANKL, preventing its binding to RANK thereby blocking the formation of functional osteoclasts. The ability of OPG to inhibit osteoclast formation is well documented in various *in vitro* and *in vivo* studies (6, 9–11).

OPG production has been demonstrated in preosteoblasts, osteoblasts, osteocytes, and in bone lining cells, *in vivo* and *in vitro* (6, 12). Although a variety of cytokines and hormones have also been shown to influence the expression of OPG, the mechanism of regulation of OPG synthesis is not yet fully understood. Previous studies have suggested that the activation of protein kinase A pathway by agents such as dbcAMP, forskolin, or parathyroid hormone, resulted in a reduction in the synthesis of OPG (12). In this report, we demonstrate that OPG synthesis is stimulated upon activation of protein kinase C (PKC) pathway. Using a human osteoblast-like osteosarcoma cell line (SaOS-2), and an antibody against human recombinant OPG, we show a time-dependent increase in the synthesis and secretion of OPG protein, and a corresponding increase in mRNA abundance in culture. We further show that phorbol myristate acetate (PMA), an activator of PKC, stimulated OPG synthesis in a time and concentration-dependent manner, and that the increase was blocked by bisindolyl maleimide (BIM), an inhibitor of PKC. This increase was due, at least in part to an increase in the transcriptional activation of the OPG gene. These results suggest that PKC activation is likely to play an important role in the expression of OPG.

MATERIALS AND METHODS

Materials. Phorbol-12-myristate-13-acetate (PMA) and bisindolylmaleimide I (BIM) were purchased from Calbiochem (La Jolla, CA). DMEM-F12 (3:1), fetal bovine serum (FBS) was purchased from Life Technologies Ltd. (Gaithersburg, MD). Strepavidin-horse radish

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peroxidase conjugate (streptavidin-HRP) was purchased from Zymed (San Francisco, CA).

Cells and cell culture. Two osteoblast-like cell lines were used in this study. For the evaluation of OPG synthesis and secretion and for the determination of mRNA abundance, SaOS-2 cells were used. For OPG promoter analysis, UMR106 rat osteosarcoma cells stably transfected with a human OPG promoter, were utilized. Both the cell lines were maintained in growth medium: DMEM/Ham's F12 (3:1) containing 10% FBS, 2 mM glutamine, 20 mM Hepes, and antibiotics (Life Technologies, Inc., Gaithersburg, MD). All cultures were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Experiments were initiated when cells were approximately 80–90% confluent.

OPG promoter activity assay. As previously described, a 5.9 kb fragment of the human OPG promoter fragment, fused with β-Galactosidase (β-gal) reporter gene of the pβgal-Basic reporter vector (Clonetech, Palo Alto, CA) was stably transfected into a rat osteosarcoma cell line, UMR106 cells (13). A second plasmid pRc/CMV (Invitrogen, San Diego, CA) encoding the neomycin gene was cotransfected for selection in G418. The promoter activity was evaluated by measuring β-galactosidase activity by assaying the extracts of cells subjected to various treatments, using the βGal Reporter Gene Assay Kit (Roche Molecular Biochemicals), as recommended by the manufacturer. Assays were done in white, opaque Microlite 2 + 96 well plates (Dynex, Chantilly, VA). Luminescence was measured in a MLX microtiter plate luminometer (Dynex) and light integration measured at 2 s (RLU summed). Results were analyzed using Student's *t* test, and probability (*P*) values of less than 0.05 were considered significant.

SDS–PAGE/Western blot analysis. SaOS-2 cells were grown in COSTAR 6 well culture plates to 80–90% confluence. After overnight serum starvation, the cells were treated with 5 ml of 5 nM PMA in DMEM/F-12 medium + 0.1% FBS for 48 h, and the conditioned media were collected. The cell layer/matrix was washed once with ice-cold PBS, and extracted with 200 μl of ice-cold RIPA buffer containing 1× PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, plus protease inhibitors (0.1 mg/ml PMSF, 1 mM sodium orthovanadate, 45 μg/ml Aprotinin). The extracts were centrifuged for 20 min at 15,000*g*, and the supernatant fraction was collected. Both the conditioned media and cell extracts were dialyzed against H_2O, lyophilized, reconstituted in 100 μl of Novex Tris–glycine SDS sample buffer and 25 μl aliquots were subjected to electrophoresis on a precast 8–16% gradient Tris–glycine polyacrylamide gel (Invitrogen life technologies, Carlsbad, CA) under reducing conditions. For relative molecular weight comparisons, Benchmark prestained protein ladder standards (8.9–178.9 kDa) were included (Life Technologies, Gaithersburg, MD). The proteins were transferred to a 0.45 μ Nitrocellulose membrane (Novex XCell II Blot Module). The membrane was blocked with 5% dry milk in PBS–Tween for 60 min at room temperature, incubated with 1 μg/ml of rabbit anti-human OPG IgG in PBS–Tween containing 1% BSA for 60 min, washed three times with PBS–Tween (5, 15, and 15 min, respectively) and incubated with 1:10000 diluted goat anti-rabbit HRP conjugate in PBS-Tween containing 1% BSA. The proteins were detected using ECL detection reagent (Amersham Pharmacia Biotech Inc., Piscataway, NJ) and visualized on Kodak Blue XB-1 film (Eastman Kodak Company, Rochester, NY).

Quantitative determination of secreted OPG levels. SaOS-2 cells (5×10^4 cells/well) were plated into a 96-well tissue culture plate, and allowed to grow for 4 days in 10% FBS DMEM/F12 in serum. Before the addition of PMA, the medium was removed and the cells were maintained in 100 μl/well of 0.1% FBS DMEM/F12 (3:1) for 16 h. The cells were treated with various concentrations of PMA diluted into the DMEM/F-12 medium + 0.1% FBS for the indicated. For inhibitor studies, cells were first treated with the indicated concentrations of BIM (solubilized in DMSO) for 10 min, before the addition of PMA. After 48 h, the supernatant fractions were collected (100 μl) and the OPG content was determined quantitatively by an OPG ELISA as described previously (13).

Northern blot analysis of OPG mRNA. The details have been previously described (12). SaOS-2 cells grown to 80–90% confluence in T150 flasks (0.7×10^7 cells) were transferred to DMEM/F12 (3:1) medium containing 0.1% FBS for 12–16 h and were then treated with PMA (1.5 nM) for the indicated periods of time. Total RNA was extracted using Ultraspec-II reagent (Biotecx, Houston, TX), and poly(A)$^+$ RNA was isolated using Oligotex resin (Qiagen, Santa Clarita, CA) and quantified by spectrophotometry. OPG and GAPDH cDNAs (25 ng each) were used to generate radioactive probes using the Random Primer DNA labeling kit (Life Technologies, Gaithersburg, MD). [α-^{32}P]dCTP (Amersham Pharmacia Biotech Inc., Piscataway, NJ), and the free nucleotides were removed by centrifugation through a Centricon-50 column (Amicon, Beverly, MA). OPG mRNA expression was analyzed by northern blot. The poly(A)$^+$ RNA was loaded (2 μg/lane) and GAPDH was used as a control for RNA integrity and to normalize for variations in loading and transfer efficiency. Prehybridization and hybridization were carried out at 48°C in NorthernMax buffers (Ambion, Inc., Austin, TX). After hybridization, the membranes were washed for 30 min at room temperature in buffer containing 2× SSC and 0.1% SDS, and then for 30 min at 48°C in 0.2× SSC and exposed to Biomax MS X-ray film (Eastman Kodak Company, Rochester, NY) at −70°C. Autoradiograms were quantitated by laser densitometry (LKB 2400 Gel Scan XL, Piscataway, NJ).

Cell viability. The XTT based colorimetric cell proliferation kit II (Roche Molecular Biochemicals, Indianapolis, IN) was used for quantification of cell viability. The kit was used according to manufacturer's instruction. The absorbance of the samples was measured using a microtiter plate reader at a wavelength of 490 nm.

RESULTS

Effect of PMA on OPG Synthesis/Secretion in SaOS-2 Osteoblastic Cells

Initially, we evaluated the effects of PMA on OPG synthesis and secretion. SaOS-2 cells were treated with various concentrations of PMA (0–100 nM) for various time intervals (4–48 h), and the amount of OPG secreted into the medium was determined by an ELISA. Treatment with PMA resulted in increased OPG synthesis and secretion in a concentration- and time-dependent manner (Fig. 1). OPG was detectable in the medium of cells treated for 16 h with 5 nM PMA. At each time point, a concentration-dependent increase in OPG secretion was observed. A robust secretion was detected at 48 h with 5 nM PMA. These results suggest that PMA, an activator of PKC, stimulated the synthesis of OPG by SaOS-2.

Western Blot Analysis of OPG

To establish that the OPG synthesized and secreted into the medium of SaOS-2 cells was the intact molecule, conditioned media and the extracts of cells treated with PMA were fractionated on a SDS–PAGE with and without reduction, transferred to a nitrocellulose membrane and evaluated by Western blot. In confirmation of previous results, OPG from the media of cells, under non-reducing conditions, displayed the

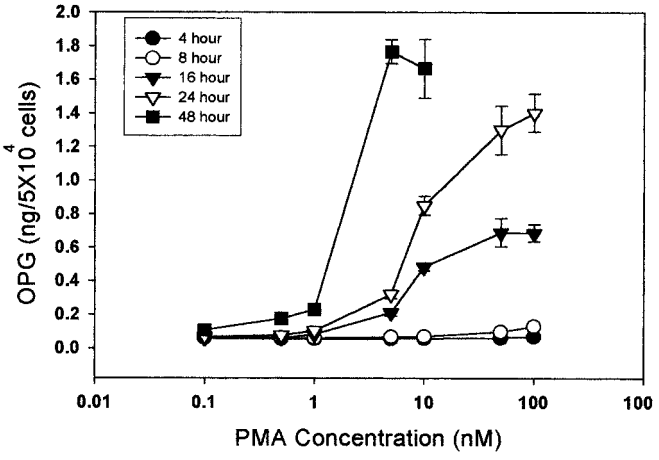

FIG. 1. Effect of PMA on OPG secretion. SaOS-2 cells (5×10^4 cells/well), plated into 96-well tissue culture plates, were incubated with various concentrations of PMA (0–100 nM) and for various time intervals (4–48 h). The OPG secreted into the culture medium at each time point was determined by an ELISA, using recombinant human OPG as a standard.

presence of two bands of 112 and 56 kDa (Fig. 2, lane 6). Upon reduction, the OPG from the conditioned media of control (Fig. 2, lane 1) or PMA-treated cells (lane 2) migrated as a 56-kDa protein. This band comigrated with purified rhOPG (lane 3). The conditioned media of PMA-treated cells contained a much greater amount of the 56-kDa band (lane 2) than the control cell (lane 1). The extracts of cell layer/matrix contained a band of 80 kDa, that is recognized by the antibody to the 56-kDa protein (lanes 4 and 5). However, PMA-treatment resulted in only a very increase in the level of the 80-kDa band. Collectively, these results confirm a robust secretion of OPG (56-kDa band) in response to PMA treatment and that the newly secreted OPG in PMA-treated cell conditioned media is intact.

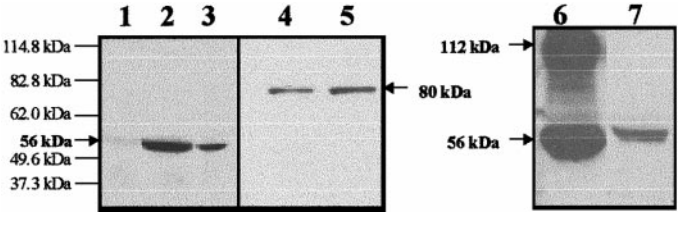

FIG. 2. Western blot analysis of secreted OPG. The conditioned media (lanes 1, 2, 6, and 7) and cell extracts (lanes 4 and 5) of SaOS-2 cells (24 well costar plates) treated for 48 h with or without PMA (5 nM) were evaluated by Western blot, using rabbit anti-hOPG IgG (1 μg/ml) and goat anti-rabbit HRP-conjugated second antibody (1: 10,000). The proteins were detected with ECL reagent and captured on Kodak XB-1 film. Lanes (1–5 and 7) are samples after reduction with dithiothreotol. Lane 6 is a sample under nonreducing conditions (lanes 1 and 4, control; lanes 2, 5, 6, and 7, 5.0 nM PMA; lane 3, rhOPG standard).

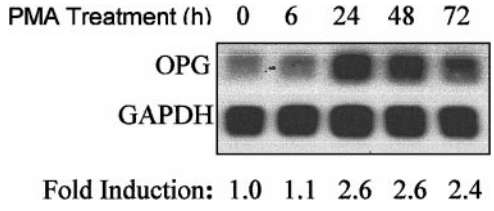

FIG. 3. Northern blot analysis of OPG mRNA. SaOS-2 cells grown in T150 flasks were treated with 1.5 nM PMA for the indicated times and poly(A)$^+$ RNA was isolated as described and was evaluated by Northern blot using cDNAs for hOPG or GAPDH. The intensity of the bands was quantitated by laser densitometry of the autoradiograms. The OPG values were expressed as fold induction, relative to GAPDH levels at each time point. Lane 1, control; lane 2, 6 h; lane 3, 24 h; lane 4, 48 h; lane 5, 72 h.

Effect of PMA on OPG mRNA Abundance

In order to evaluate whether PMA stimulation of OPG production is associated with increased mRNA expression, SaOS-2 cells were treated with PMA (1.5 nM) for various time intervals, and the poly(A)$^+$ RNA was evaluated by Northern blot using a cDNA for hOPG. The results (Fig. 3) show that PMA treatment resulted in a time-dependent increase in OPG mRNA levels. OPG mRNA levels increased 2.6-fold after a 24 h PMA treatment, and remained elevated at least until 72 h post-PMA treatment. These results establish that the PMA-mediated increase in OPG production is associated with a corresponding increase in mRNA abundance.

Effect of PMA on OPG Promoter

In order to further establish whether the PMA effects involve transcriptional regulation of OPG, we utilized a UMR 106 cell line that has been stably transfected with hOPG promoter (5.9 kb)/β-gal construct. Cells were treated with PMA (0–1000 nM) for 8 or 24 h and the β-gal activity was determined. The results (Fig. 4) demonstrate concentration-dependent increase in OPG promoter activity in response to PMA, and the peak level of stimulation was approximately twofold. These results further establish that the PMA stimulation of OPG production involves at least in part, the transcriptional regulation of OPG promoter.

Effect of PKC Inhibitors on PMA-Induced OPG Secretion and OPG Promoter Activation

To further validate the involvement of PKC in OPG production, OPG secretion was evaluated in the presence of a PKC inhibitor, bisindolylmaleimide I (BIM). SaOS-2 cells or UMR 106 cells, stably transfected with OPG promoter, were treated with PMA (10 or 100 nM, respectively) in the presence of various concentrations of BIM, and OPG secretion and OPG promoter activity were determined. The results (Figs. 5A and 5B) estab-

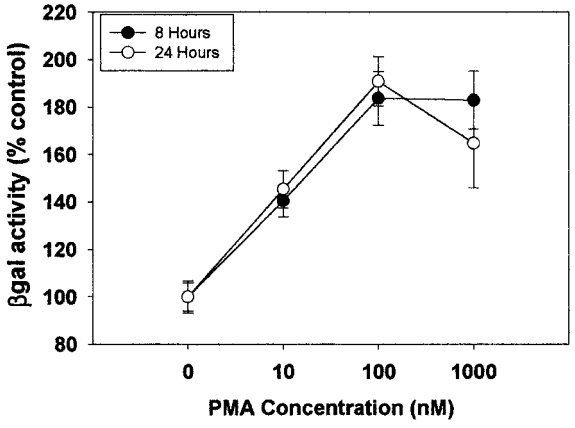

FIG. 4. Activation of OPG promoter by PMA. UMR106 cells containing a stably integrated hOPG promoter/βgal construct was incubated with PMA (0–1000 nM) for 8 and 24 h. The βgal activity was determined as described.

lish that BIM was able to block the PMA-stimulated OPG protein secretion, as well as OPG promoter activity, without affecting cell viability. BIM (1–3000 nM) alone did not change the basal OPG secretion by SaOS-2 cells or OPG promoter activity.

DISCUSSION

OPG is a key inhibitor of osteoclastogenesis and is produced by osteoblasts, osteocytes, and osteoprogenitor cells. OPG blocks the interaction of RANKL with its receptor RANK, which is required for the differentiation, maturation, and survival of osteoclasts. Thus, the mechanisms that regulate the production of OPG would also play a key role in the maintenance of bone homeostasis. In this report, we have evaluated whether PKC plays a role in OPG production. Our results suggest that the activation of PKC leads to increased OPG transcription, and protein synthesis and secretion.

OPG production was evaluated by quantitative determination of secreted protein by an ELISA, determination of mRNA abundance, and measurement of OPG promoter activity. At all levels of analysis, PMA, an activator of the PKC pathway, was able to stimulate an increase in OPG expression. Collectively, these results establish a role for protein kinase C-mediated activation of OPG expression.

The identity of the secreted OPG protein was established by Western blot analysis of OPG secreted by SaOS-2 cells, using an antibody raised against purified recombinant human OPG. The estimated relative molecular weight under reducing conditions is 56 kDa and is comparable to that of authentic recombinant hOPG (Fig. 2). In addition, the conditioned media of nonreduced samples revealed the presence of a dimeric form of OPG (112 kDa). Previous studies have suggested the monomeric molecular weight to be approximately 60 kDa under reducing conditions and 53 kDa under nonreducing conditions (4, 14, 15). Although there are slight differences in the reported molecular weights, these differences are only minor and could be due to differences in cell type or the gel electrophoresis conditions. There are differences in gel electrophoresis conditions. For example, we have utilized an 8–16% running gel, where as other studies have utilized a 7% running gel (15). The monomeric form (56 kDa) is biologically active in blocking RANK-mediated osteoclastogenesis (Galvin, personal communication). Interestingly, the molecular weight (81 kDa) of the cell matrix/layer form (Fig. 2) was greater than that of the conditioned media samples, and has not been previously described. The reason for the difference in molecular weight between the secreted- and the cell-associated OPG is not clear. The polyclonal antibody raised against the secreted form (56 kDa) recognizes both the forms. There could be differences in primary sequence as a consequence of failure to cleave some

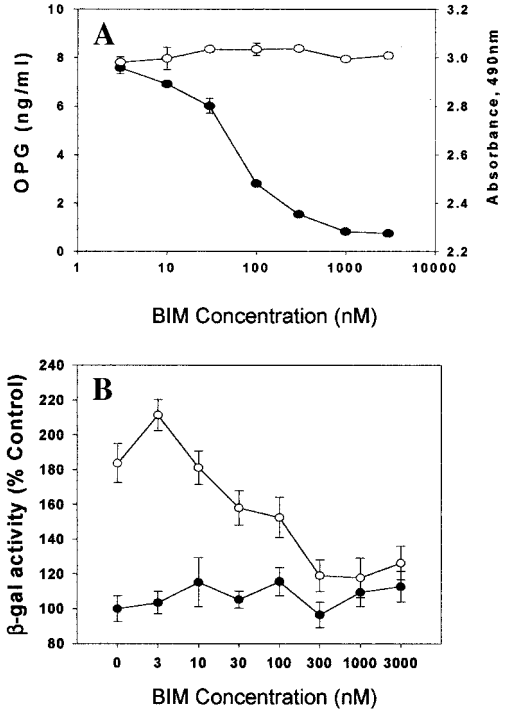

FIG. 5. The effect of BIM on OPG secretion (A) and OPG promoter activity (B). Cells were pretreated for 10 min with the indicated concentrations of BIM, prior to the addition of PMA. SaOS-2 cells were treated for 48 h, with 10 nM PMA, while UMR 106 cells containing hOPG were incubated for 8 h with 100 nM PMA. At the end of the incubation periods, media from SaOS-2 cells were assayed for OPG levels, while the UMR 106 cells were assayed for promoter activity. The cell viability was determined in both the cell cultures using an XTT assay, but the results are shown only for SaOS-2 cells. No change in cell viability was observed in UMR 106 cells. (A) OPG secretion in SaOS-2 cells (solid circle, OPG levels; open circle, absorbance for XTT assay). (B) OPG promoter activity in UMR 106 cells.

sequence leading to its retention as a membrane-bound form. The differences could be due to extensive post-translational processing of the cell-associated form. It also could be due to covalent association with other components of matrix such as heparan sulfate proteoglycan, as previous immuno-histochemical studies have demonstrated the presence of OPG in the extracellular matrix of bone (12). However, further studies are needed to establish the differences between the two forms of OPG.

OPG production has been shown to be influenced by a variety of mediators. For example, Cbfa-1/RUNX-2, a transcription factor that facilitates the expression of osteoblast phenotype contributes to OPG expression (13). In contrast, PTH, IBMX, and other agents that increase intracellular cAMP accumulation and activate the PKA pathway, reduced the expression of OPG (16, 17). Our results indicate that an agent or factors that stimulate the PKC pathway would increase OPG production. However, PKC activation does not appear to play a role in basal secretion of OPG, as the inhibitor of PKC activation (BIM) was not effective in blocking the basal OPG production. Several growth factors and cytokines that are known to be activators of PKC are present in the bone cell environment. Although we do not know which of these growth factors regulate OPG production, our results suggest that PK-C activating agents could play a critical role in OPG production and osteoclastogenesis, thereby influencing bone cell homeostasis.

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