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Inflammatory cytokines activate p38 MAPK to induce osteoprotegerin synthesis by MG-63 cells

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Abstract

Inflammatory bone diseases are characterized by the presence of pro-inflammatory cytokines that regulate bone turnover. Osteoprotegerin (OPG) is a soluble osteoblast-derived protein that influences bone resorption by inhibiting osteoclast differentiation and activation. In the present study, we demonstrate that interleukin-1β (IL-1β) and tumor necrosis factor alpha induce OPG mRNA production and OPG secretion by osteoblast-like MG-63 cells. Maximum induction of OPG secretion by either cytokine requires activation of the p38 mitogen activated protein kinase (MAPK) pathway but neither the p42/p44 (ERK) nor the c-Jun N-terminal MAPK pathways. Induction of OPG mRNA by either cytokine is also p38 MAPK dependent. Taken together, these data indicate that cytokine-induced OPG gene expression and protein secretion are differentially regulated by specific MAP kinase signal transduction pathways.

Keywords: Osteoblast; Signal transduction; Osteoprotegerin; MAP kinase; Inflammatory cytokine; IL-1β; TNF-α

Inflammatory cytokines are typically found in elevated concentrations in diseased tissue adjacent to sites of bone resorption. In disease conditions such as arthritis [1] or periodontitis [2–4] and osteoporosis [5], it is postulated that these cytokines play an important role in activating osteoblast–osteoclast interactions that culminate in net bone resorption. Osteoblasts regulate osteoclast activity via cell–cell contact whereby osteoblast cell-surface "receptor activator of nuclear factors κ-B ligand" or RANKL engages osteoclast precursor cell or mature cell receptors termed "receptor activator of nuclear factor κ-B" or RANK. The interaction of RANKL with RANK is the key terminal factor in inducing osteoclast precursor cell differentiation, as well as inducing activation of mature osteoclasts [6,7]. However, osteoblasts also secrete osteoprotegerin (OPG), a bone-protective soluble decoy receptor for RANKL [6,7]. Compelling evidence indicates that the ratio of RANKL/OPG production by osteoblasts is a crucial determinant of osteoclast differentiation and activation [6,8]. In essence, at sites of strong osteoblast-derived OPG secretion, bone resorption will diminish [9,10].

The inflammatory cytokines interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) are strong inducers of mitogen activated protein kinase (MAPK) signal transduction cascades in osteoblasts. Typically, mammalian MAPK pathways include at least three protein kinases that ultimately activate a terminal MAPK which regulates various gene expression-modifying proteins such as transcription factors [11]. Three main MAPK pathways have been extensively investigated and found to have profound effects on gene expression. The three MAPK families/pathways are: (1) the ERK1/2 (extracellular signal related kinase) pathway; (2) the JNK/SAPK (c-Jun NH_2-terminal kinase/stress activated protein kinase) pathway; and (3) the p38 MAPK pathway

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[11–13]. To be activated, ERK, JNK/SAPK, and p38 MAPK must be phosphorylated by dual-specificity kinases on threonine and tyrosine residues within a highly conserved phosphorylation motif T-X-Y where X is E for ERK, P for JNK/SAPK, and G for p38 MAPK. Each of the three pathways demonstrates varying degrees of specificity with regard to downstream effectors.

Early attempts to identify stimulators of OPG production by bone cells led to the discovery that inflammatory cytokines, interleukin-1, and tumor necrosis factor-α, both had such an effect [14,15]. Given that IL-1β and TNF-α activate distinct signal transduction cascades, we sought to investigate the specific contribution of MAPK pathways to cytokine-induced OPG production by osteoblast-like cells.

Materials and methods

Materials. Recombinant IL-1β, TNF-α, OPG enzyme-linked immunosorbent assay (ELISA) antibodies, and recombinant human OPG were purchased from R&D Systems (Minneapolis, MN, USA). SB203580 HCl, PD98059, and JNK inhibitor were purchased from Calbiochem (La Jolla, CA, USA). For ELISA, 2,2′azino-bis-(3-ethylbenzthiazoline-sulfonic acid) (ABTS) and 30% hydrogen peroxide were purchased from Sigma Chemical (St. Louis, MO, USA), and streptavidin–horseradish peroxidase (SA–HRP) was purchased from Pharmingen (San Diego, CA, USA). Total RNA isolation was performed using the RNAEasy kit per manufacturer's instructions (Qiagen, Valencia, CA, USA). Complementary DNA synthesis and semi-quantitative polymerase chain reactions were performed using reagents purchased from Invitrogen/Life Technologies (Carlsbad, CA, USA). Real-time PCRs were performed with reagents purchased from Applied Biosystems (Foster City, CA).

Cell culture. MG-63 cells were purchased from American Type Culture Collection (CRL-1427) (Manassas, VA, USA) and cultured in RPMI-1640 supplemented with 50 mg/ml penicillin, 50 mg/ml streptomycin, and 2 mM sodium pyruvate (all from Sigma). Cells were incubated at 37 °C in a 95% air/5% CO_2 atmosphere until 80–90% confluent and passaged by means of 0.2 M trypsin/1 mM EDTA (Sigma) in phosphate-buffered saline (PBS).

Osteoprotegerin ELISA. MG-63 cells were plated in 16 mm plastic wells (24-well plates) at a density of 5×10^4 cells/well and incubated overnight. Media were removed and replaced with serum-free RPMI-1640 for a minimum of 12 h before stimulation with serum-free RPMI-1640 supplemented with interleukin-1β or tumor necrosis factor-α for 8 h. Prior to cytokine exposure, some culture wells were pre-incubated for 60 min with serum-free RPMI 1640 supplemented with various concentrations of the various inhibitors and then stimulated with cytokine for 8 h. For ELISA, 96-well plates were coated with 50 μl of anti-OPG capture antibody (2 μg/ml) (R&D Systems: MAB8051) and incubated overnight at room temperature. After being washed with PBS/Tween (0.5%) and blocked with PBS/10% FBS, 100 μl of experimental supernatants or standards (R&D Systems: 185-OS) was plated and incubated overnight at room temperature. After washing, 100 μl of biotinylated anti-OPG detection antibody (1 μg/ml) (R&D Systems: BAF805) was added and allowed to incubate for 2 h at 37 °C. Plates were washed prior to addition of SA–HRP and incubation at room temperature for 30 min. After further washing, 100 μl of ABTS substrate solution was added and incubated at room temperature for 30–60 min prior to optical density readings at 405 nm. For each experimental culture well, duplicate ELISA readings were obtained. Sample OPG concentrations were interpolated from a linear fit standard curve calculated from known values of OPG ranging from 4000 to 31 pg/ml.

RNA isolation and cDNA preparation. MG-63 cells were plated in 35 mm plastic wells (6-well plates) at a density of $7–8 \times 10^4$ cells/well and incubated overnight. Media were removed and replaced with serum-free RPMI-1640 for a minimum of 12 h before stimulation with serum-free RPMI-1640 supplemented with IL-1β or TNF-α for various time periods. Total RNA was obtained from cell cultures using the RNAEasy kit from Qiagen per manufacturer's instructions. Seven hundred fifty nanograms of total RNA isolate was subjected to DNase I digestion to remove genomic DNA prior to cDNA synthesis using Superscript II (Invitrogen) and anchored oligo(dT) primer in a 20 μl reaction as per enzyme supplier's recommended protocol.

Semi-quantitative reverse transcriptase polymerase chain reaction. In preliminary polymerase chain reaction (PCR) experiments using OPG specific primers and various housekeeping gene primers, we determined that use of the following OPG and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers used in a multiplex PCR yielded band densities within 4% of uniplex reactions with only one set of each primer. OPG: sense 5′-GGGGACCACAATGAACAAGTTG-3′, antisense 5′AGCTTGCACCACTCCAAATCC-3′ (to yield a 408 bp fragment). GAPDH: sense 5′-CCCTTCATTGACCTCAACTAC-3′, anti-sense 5′-TACTCCTTGGAGGCCATGT-3′ (to yield a 902 bp fragment). PCRs were as follows: 1 cycle of 94 °C for 3 min; followed by 5 cycles of 94 °C for 45 s, 62 °C for 45 s, and 72 °C for 45 s; followed by 20 cycles of 94 °C for 45 s, 58 °C for 45 s, and 72 °C for 45 s; and followed by 1 cycle of 72 °C for 7 min. Reactions were conducted in a Progene Thermal Cycler (Techne, Princeton, NJ, USA). PCR products were electrophoresed for 45 min at 100 V on 1.5% agarose/0.5% TAE gels in the presence of ethidium bromide. One microgram of 100 bp DNA ladder was run in parallel as molecular weight markers to verify anticipated band size. Gels were visualized and spot densitometric analysis of bands was performed using the FluorChem8800 imaging system (Alpha Innotech, San Leandro, CA, USA). Fold induction relative to unstimulated cultures was calculated for each condition. All experiments were repeated numerous times (n = at least 3).

Real-time quantitative polymerase chain reaction. cDNA quantitation for OPG and an internal reference standard (18S rRNA) was performed using a fluorescence-based real-time detection method (ABI PRISM 7000) Sequence Detection System (TaqMan) (Applied Biosystems). The PCR mixture consisted of 900 nM each of the primers; 250 nM probe; 0.3 U/μl AmpliTaq Gold Polymerase; 200 nM each of dATP, dCTP, dGTP, and dTTP; 3.5 mM $MgCl_2$; and 1× TaqMan buffer A, which contains a reference dye, to a final volume of 25 μl. Cycling conditions were 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The primer/probe set for OPG was purchased from Applied Biosystems (Catalog No. Hs00171068_m1). In addition, parallel reactions were performed for OPG and the 18S rRNA using untranscribed RNA, and using cDNA but with AmpliTaq Gold Polymerase omitted from the PCR. Data were manipulated to calculate the relative ratio of OPG mRNA copies to 18S rRNA copies, i.e., number of OPG mRNA copies for every 18S rRNA copy. All experiments were repeated numerous times ($n = 4–5$) and representative results for one experiment are presented.

Western blotting. MG-63 cells were plated in 35 mm plastic wells (6-well plates) at a density of 3×10^5 cells/well and incubated overnight. Media were removed and replaced with serum-free RPMI-1640 for a minimum of 12 h before stimulation with serum-free RPMI-1640 supplemented with interleukin-1β or tumor necrosis factor-α for different time points before plates were placed on liquid nitrogen for 1 min and lysis buffer (50 mM Hepes, pH 7.8, 1% Triton X-100, 1 mM EDTA, 30 mM sodium pyrophosphate, 1 mM sodium vanadate, 10 mM sodium fluoride, 1 μM phenylmethylsulfonyl fluoride, and 20 ng/ml aprotinin) was added. Thawed lysates were vortexed and centrifuged to pellet detergent-insoluble cell debris. Protein concentrations of lysates were determined using the BCA Protein Assay (Pierce). Volumes of cell lysate were adjusted to a final volume of

200 μl to ensure that equal amounts of total protein were immunoprecipitated with α-human-p38 MAPK antibody (Santa Cruz Labs) for 2 h before addition of protein A–agarose beads (Santa Cruz) for 15–20 min. Immunoprecipitates were washed twice with ice-cold lysis buffer, and twice with 50 mM Hepes, pH 7.8, prior to addition of sample buffer (100 mM DTT) and boiling at 100 °C for 5 min. Samples were divided into two aliquots and separately electrophoresed on 10% SDS–PAGE gels, transferred to nitrocellulose, blocked, and probed with primary antibody to either p38 MAPK or phospho-p38 MAPK (both Santa Cruz) and secondary to rabbit IgG that is linked to horseradish peroxidase (Jackson ImmunoLabs, West Grove, PA). Blots were developed using the ECL detection system (Amersham, Piscataway, NJ).

Statistical analysis. Statistical comparisons of OPG secretion were calculated using analysis of variance. When results indicated an effect of an independent variable (cytokine treatment, inhibitor), Tukey's post hoc test was used to determine differences between group means. A p value of 0.05 was assigned statistical significance.

Results

Il-1β is a stronger inducer of OPG secretion by MG-63 cells than TNF-α

The MG-63 cell line has been previously shown to respond to IL-1β and TNF-α stimulation with increased steady-state levels of OPG mRNA. However, enhanced OPG secretion by this well-characterized cell line in response to cytokine stimulation has not been shown. We cultured cells in serum-free media for 12 h to quiesce MAPK activity, and then stimulated cells with either IL-1β or TNF-α of varying doses for 8 h. In Fig. 1, we observed that both IL-1β and TNF-α-induced OPG secretion and that IL-1β is a significantly more potent inducer than TNF-α since a 20 ng/ml concentration of TNF-α could not induce OPG secretion to the same level as 1 ng/ml IL-1β. We have observed a similar response for IL-6 secretion by this cell line when stimulated with these cytokines (Webb et al. [16] and unpublished data). We attempted to further define the temporal characteristics of OPG induction by IL-1β and TNF-α with a series of time-course and dose-course experiments. Fig. 2 shows that IL-1β induces a stronger response in MG-63 cells in that maximal induction is higher and that induction occurs sooner than in cells stimulated with TNF-α. Specifically, 1 ng/ml of IL-1β stimulates a threefold induction in OPG secretion after 4 h, whereas a 10 ng/ml dose of TNF-α requires 6 h to induce its maximal level of just over a twofold increase. In addition, TNF-α stimulation appears to plateau by

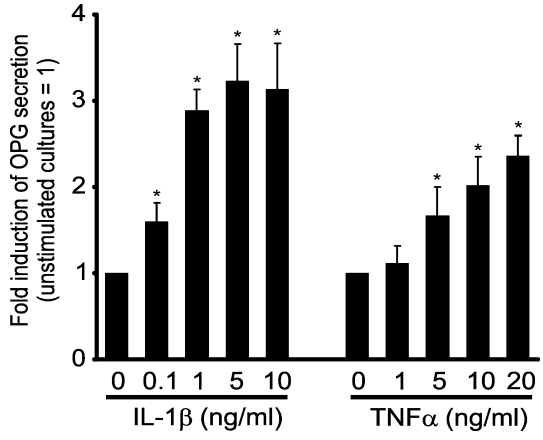

Fig. 1. IL-1β and TNF-α induce OPG secretion in a dose-dependent manner in MG-63 cells. Cells were serum-starved for 12 h and then stimulated with IL-1β or TNFα of varying doses for 8 h. OPG levels secreted into culture supernatant were determined by ELISA ($n = 4$). Experiments were conducted at least three times and data from a single representative experiment are presented. *Statistically significantly different ($p < 0.05$) from unstimulated cultures (=1).

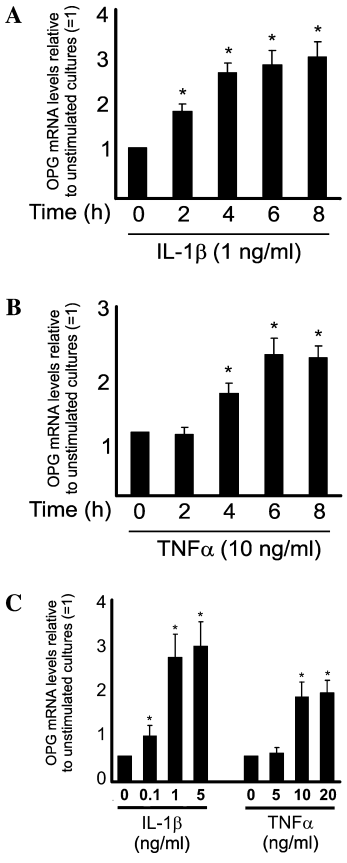

Fig. 2. IL-1β (1 ng/ml) (A) and TNF-α (10 ng/ml) (B) induce OPG gene expression in a time- and dose-dependent manner in MG-63 cells. Cells were serum-starved for 12 h and then stimulated with IL-1β (1 ng/ml) or TNF-α (10 ng/ml) for various time periods and RNA was isolated. Transcribed cDNA was quantified in real-time PCR analysis using a TaqMan 7000. The amount of OPG mRNA was compared to the amount of an internal standard (18S rRNA). Experiments were conducted at least three times to calculate data presented. *Statistically significantly different ($p < 0.05$) from unstimulated cultures (zero hour stimulation = 1). (C) Serum-starved cells were stimulated with different concentrations of IL-1β and TNF-α for 4 h and RNA was isolated. Transcribed cDNA was quantified in real-time PCR as described above. Experiments were conducted at least three times to calculate data presented. *Statistically significantly different ($p < 0.05$) from unstimulated cultures (zero hour stimulation = 1).

6 h while IL-1β stimulation continues to rise even at 8 h after stimulation began.

p38 MAPK activation is required for maximal IL-1β/TNF-α-induced OPG secretion

We utilized chemical inhibitors of the ERK, p38 MAPK, and JNK pathways to determine whether activation of these pathways resulted in the enhanced OPG secretion observed by MG-63 cells stimulated with either IL-1β or TNF-α. We observed (Fig. 3) that the induction of OPG secretion by 8 h of IL-1β and TNF-α stimulation is significantly decreased if cultures were pre-incubated with SB203580 HCl prior to cytokine stimulation. Whereas IL-1β and TNF-α caused statistically significant induction after 8 h of stimulation, inhibition of p38 MAPK led to a significant inhibition. However, inhibition of the ERK or JNK pathways using specific chemical inhibitors did not affect cytokine-induced OPG secretion. These results corroborate those of Figs. 1 and 2 in that stimulation with IL-1β is a more potent inducer of OPG gene expression and secretion than stimulation with TNF-α. The results strongly suggest that only activation of the p38 MAPK pathway is required for a full OPG secretion response to cytokine stimulation. Inhibition of ERK or JNK activity affected neither basal nor cytokine-induced OPG secretion, whereas p38 MAPK activation was selectively required for maximal cytokine-induced OPG secretion.

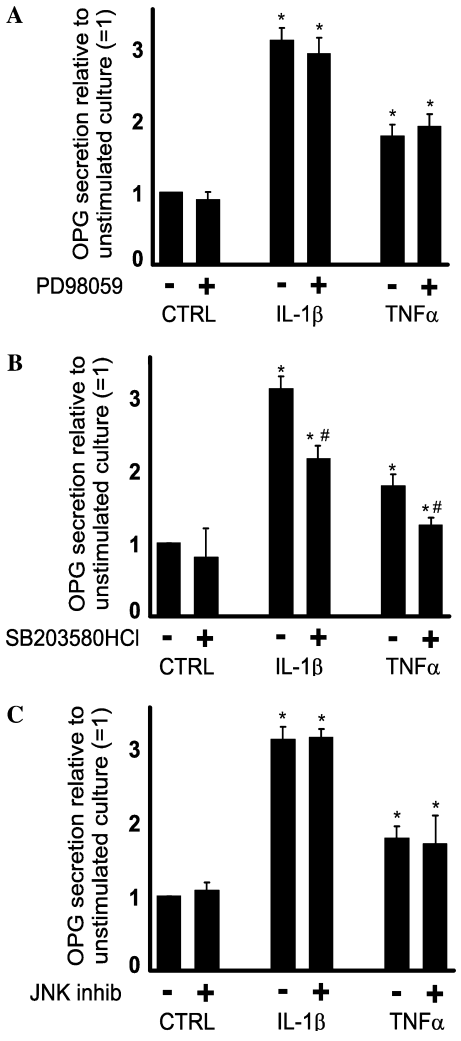

Fig. 3. IL-1β and TNF-α induce OPG secretion in a p38 MAPK-dependent manner in MG-63 cells. Cells were serum-starved for 12 h and then stimulated with IL-1β or TNF-α for 8 h. Some cultures were pre-treated for 1 h with chemical inhibitors of MEK (A: PD98059); (B) p38 MAPK (SB203580 HCl); and (C) JNK (JNK inhib). OPG levels secreted into culture supernatant were determined by ELISA ($n = 4$). Experiments were conducted at least three times and data from a single representative experiment are presented. *Statistically significantly different ($p < 0.05$) from unstimulated cultures (=1); #Statistically significantly different ($p < 0.05$) from respective IL-1β or TNF-α stimulated cultures.

We further confirmed the role of p38 MAPK activation in IL-1β and TNF-α-induced OPG synthesis by verifying the capability of these cytokines to activate p38 MAPK as well as demonstrating that inhibition of p38 MAPK activation diminished OPG gene expression. Figs. 4A and B show that both IL-1β and TNF-α lead to enhanced levels of phospho-p38 MAPK, with induction being earlier and sustained upon IL-1β stimulation compared to TNF-α stimulation. Following on, we utilized both real-time PCR and semi-quantitative multiplex PCR to determine that OPG gene expression was enhanced by both cytokines. Furthermore, induction of OPG secretion by either cytokine was partially blocked if p38 MAPK activation was chemically inhibited by pretreatment of cells with SB203580 HCl.

Discussion

Disturbances in the ratio of OPG to RANKL are considered to be pivotal causes for a number of osteolytic disorders including glucocorticoid-induced osteoporosis, hyperparathyroidism, rheumatoid arthritis, myeloma bone disease, osteolytic bone metastases, and humoral hypercalcemia of malignancy [5]. Induction of OPG gene expression and OPG secretion by inflammatory cytokines may seem counter-intuitive to the general assumption that IL-1β and TNF-α are bone resorption-promoting proteins and attention has focused on the role of these cytokines in nuclear factor-κ B (NF-κB)-dependent effects on osteoclast apoptosis and activation. However, the results of this and previous studies suggest that these cytokines activate pathways in osteoblasts that are bone protective as OPG secretion and gene expression is clearly enhanced in MG-63 cells or primary bone marrow stromal cells [14,15]. Nevertheless, it also appears that activation of OPG synthesis by inflammatory cytokines may be relatively short-lived and if so, any beneficial effects of OPG on bone resorption are

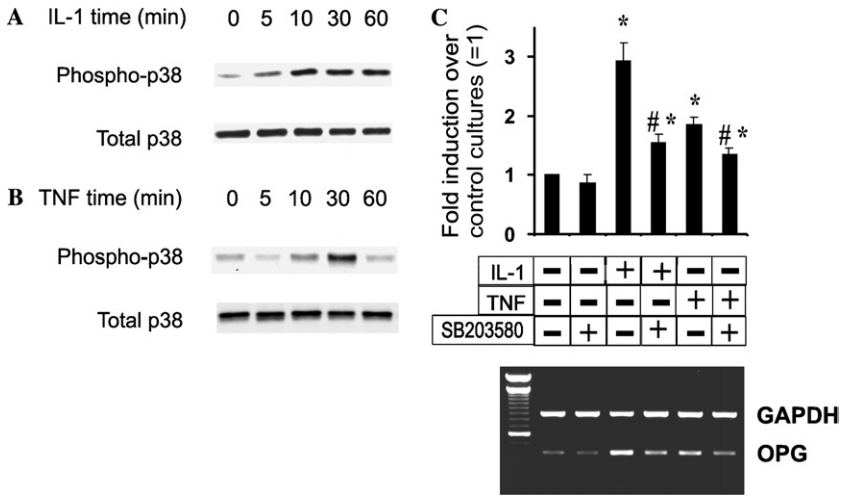

Fig. 4. IL-1β and TNF-α activate p38 MAPK in MG-63 cells. Cells were serum-starved for 12 h and then stimulated with IL-1β (1 ng/ml) (A) or TNF-α (10 ng/ml) (B) for different time points. Cell lysates were probed for p38 MAPK and activated p38 (phospho)-MAPK pathway using indirect Western blotting. (C) IL-1β and TNF-α induce OPG gene expression in a p38 MAPK-dependent manner in MG-63 cells. Cells were serum-starved for 12 h and then stimulated with IL-1β or TNF-α for 4 h. Some cultures were pre-treated for 1 h with chemical inhibitor of p38 MAPK. After RNA isolation, transcribed cDNA was assessed either by real-time PCR (upper palette) or semi-quantitative PCR (lower palette) as described above. *Statistically significantly different ($p < 0.05$) from unstimulated cultures (zero hour stimulation = 1). #Statistically significantly different ($p < 0.05$) from respective IL-1β or TNF-α stimulated cultures.

outlasted by sustained RANKL production. Ultimately, net bone resorption results as other pro-resorptive IL-1β and TNF-α-dependent effects dominate the local osseo-environment.

In the present study, activation of a specific MAPK cascade is necessary for maximal induction of OPG gene expression and secretion. Inhibition of the p38 MAPK pathway only, and not inhibition of the ERK or JNK pathways, produced a muted response to IL-β and TNF-α, despite both ERK and JNK pathway activation by these cytokines (data not shown). The specificity of the p38 MAPK involvement parallels previous reports. PDGF-induced OPG secretion by vascular smooth muscle cells and p38 MAPK activation was necessary for maximal induction of OPG secretion by PDGF whereas ERK inhibition affected both basal and PDGF-induced OPG secretion to a similar degree [17]. In addition, induction of OPG secretion by bone morphogenetic protein-4 (BMP-4) from a mouse bone marrow-derived stromal cell line was also specifically dependent upon p38 MAPK activity and not ERK activation [18]. Taken together, the data suggest that activation of the p38 MAPK pathway plays a strong role in OPG synthesis and that elements considered to be traditionally both bone formative (PDGF, BMP-4) and resorptive (IL-1β and TNF-α) utilize this MAPK pathway to induce OPG production.

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