8-Isoprostaglandin E2 Enhances Receptor-activated NFκB Ligand (RANKL)-dependent Osteoclastic Potential of Marrow Hematopoietic Precursors via the cAMP Pathway*

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Lipid oxidation products promote atherosclerosis and may also affect osteoporosis. We showed previously that oxidized lipids including 8-isoprostaglandin E2 (isoPGE2) inhibit osteoblastic differentiation of preosteoblasts. Since osteoporosis is mediated both by decreased osteoblastic bone formation and by increased osteoclastic bone resorption, we assessed whether oxidized lipids regulate the osteoclastic potential of marrow hematopoietic cells. Treatment of marrow-derived preosteoclasts with isoPGE2 enhanced osteoclastic differentiation as evidenced by increased tartrate-resistant acid phosphatase (TRAP) activity and multinucleation, which were inhibited by calcitonin, and increased numbers of resorption pits. The enhanced osteoclastic differentiation by isoPGE2 was observed whether preosteoclasts were in coculture with stromal cells or in monoculture in the presence of receptor-activated NFκB ligand (RANKL) and macrophage colony-stimulating factor. Receptor antagonist studies suggest that isoPGE2 effects were mediated by prostaglandin receptor subtypes EP2/DP on preosteoclasts and subtype EP1 and thromboxane receptors on stromal/osteoblast cells. The enhanced TRAP activity was also inhibited by cAMP-dependent protein kinase inhibitors, and isoPGE2 elevated intracellular cAMP levels of preosteoclast monocultures. Other oxidized lipids also enhanced these isoPGE2 effects were mediated by prostaglandin receptor subtypes EP2/DP on preosteoclasts and subtype EP1 and thromboxane receptors on stromal/osteoblast cells. The enhanced TRAP activity was also inhibited by cAMP-dependent protein kinase inhibitors, and isoPGE2 elevated intracellular cAMP levels of preosteoclast monocultures. These data suggest that isoPGE2 enhances osteoclastic differentiation of marrow preosteoclasts and that this regulation occurs via the cAMP-dependent protein kinase pathway.

Isoprostanes are chemically stable lipid-oxidation products (1, 2) and were first identified by Morrow et al. (3) as in vivo products of free radical-catalyzed lipid peroxidation independent of cyclooxygenase enzyme. The two most extensively studied isoprostanes produced from arachidonic acid are isoprostanes 8-isoPGF2α (8-isoprostaglandin F2α, isoPGF2α) and 8-isoPGE2 (isoPGE2) (1, 2). Similar to cyclooxygenase-derived prostaglandins, they have biological activity including potent contractile and mitogenic activities in vascular smooth muscle cells (4), modulation of aggregation in platelets (1), and induction of endothelin-1 release in endothelial cells (5). However, the biological effects of isoprostanes and their isomeric prostaglandins appear to vary depending on the tissue systems. In the vascular bed, prostaglandin F2α (PGF2α) (6), isoPGE2, and isoPGF2α have vasoconstrictor effects (7), whereas in the pulmonary bed, prostaglandin E2 (PGE2) has vasodilator effects (8). In porcine small intestine, PGE2, PGF2α, and isoPGE2 induce similar electrical responses, whereas isoPGF2α elicits no response (9).

The receptors and intracellular signaling pathways mediating the biological effects of prostaglandins and isoprostanes also vary among tissue systems. The renal vasoconstricting actions of isoprostanates are mediated by thromboxane receptor (TP), whereas platelet aggregation by isoprostanes may be acting through a unique receptor similar to but distinct from that of TP (1, 10). Ocular hypotensive actions of PGF2α are mediated by the prostaglandin E receptor subtype 1 (EP1) (11), and PGE2-stimulated osteoclast formation (12, 13) and glycosaminoglycan synthesis in human cervical fibroblasts (14) are mediated by the EP4 receptor subtype. In tracheal epithelial cells, PGE2 modulates CAMP levels via the EP4 receptor (15), whereas in human astrogliaoma cells, PGE2 stimulate interleukin-6 production via protein kinase C and p38MAPK pathways (16).

Isoprostanes are present in human tissue, such as atherosclerotic plaque (17, 18), and in the body fluids after oxidant stress (2). In hyperlipidemic patients, increased amounts of esterified isoprostanes have been found in the circulation (2, 19). Vitamin E treatment reduces both atherosclerosis and isoprostane levels in apolipoprotein E (ApoE) knockout mice (20). Isoprostane levels are altered in many diseases putatively associated with oxidative stress, including vascular, cerebral, and pulmonary disorders (2).

Isoprostanes have been shown to contribute to a multitude of diseases including atherosclerosis (2, 21) and possibly osteoporosis (22, 23). Growing evidence suggests an age-independent association between these two diseases (for review, see Ref. 24). Recently, we reported that diet-induced hyperlipidemia, which increases tissue deposition of lipid oxidation products, also reduces the numbers of marrow osteoblastic precursors (25) as...
well as bone mineral content and density (26). We also found that among the oxidized lipids, the isoprostane isoPGE2 potently regulates osteoblastic differentiation of osteoprogenitor cells in both artery wall and bone (22). Although the effects of isoprostanes and other lipid oxidation products on vascular and osteoblastic cells have been reported, their effects on osteoclastic differentiation are not known.

Osteoclasts are of hematopoietic origin, and their precursors are present in bone marrow, spleen, and peripheral blood (27). Osteoclasts express tartrate-resistant acid phosphatase (TRAP) and calcitonin receptor, both of which are widely regarded as markers of osteoclast differentiation (27, 28). When they are actively resorbing, osteoclasts are highly polarized and adhere to the bone surface through specialized gasket-like “actin rings,” which are recognized as a marker for fully activated osteoclasts (28). Differentiation of osteoclasts is closely coupled with the function of osteoblasts through a variety of cytokines including macrophage colony-stimulating factor (M-CSF) and receptor activator of NFκB ligand (RANKL) released by the bone-forming cells (29, 30). PGE2, the isomeric prostaglandin of isoPGE2 and an important regulator of local bone metabolism, has been shown to induce osteoclast formation from hematopoietic precursors and to inhibit bone resorption in mature osteoclasts (31). The induction of osteoclast differentiation by PGE2 has been shown to occur directly through osteoblasts (31). It induces the cAMP pathway in osteoblasts, leading to release of cytokines, such as interleukin-1 and interleukin-6, which in turn induce the osteoclast (31, 32).

To investigate whether the loss of bone density associated with hyperlipidemia is due in part to altered osteoclastic activity, we assessed the osteoclastic potential of marrow hematopoietic precursors treated with isoprostanes. Results showed that isoPGE2 induced osteoclastic differentiation and increased resorption pits of marrow preosteoclasts. The results also suggested that the direct effects of isoPGE2 on osteoclasts were mediated by prostaglandin receptor subtype EP2/DP, whereas the indirect effects of isoPGE2 through stromal/osteoblast cells were mediated by EP1/TP receptors. In addition, the intracellular cAMP pathway is involved in mediating isoPGE2-stimulated osteoclast formation. Other oxidized lipids, oxLDL and 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine (oxPAPC), also enhance osteoclast formation. These data suggest that isoPGE2 enhances osteoclastic differentiation of marrow hematopoietic precursor cells via prostaglandin/thromboxane receptor induction of the cAMP pathway.

EXPERIMENTAL PROCEDURES

Materials

Recombinant murine M-CSF and murine RANKL were from R&D Systems Inc. (Minneapolis, MN). 1,25-(OH)2D3 and SC 51089 were from Biomol Research Laboratories (Plymouth Meeting, PA). Osteologic discs for resorption assays were purchased from BD PharMingen (San Diego, CA). The cyclic AMP assay kit was purchased from Amersham Biosciences. Salmon calcitonin, rhodamine-conjugated phallolidin, and dexamethasone were from Sigma. Isoprostanes (both isoPGE2 and isoPGF2α), AH6809, and SQ29548 and U46619 were purchased from Cayman Chemical (Ann Arbor, MI). 3-Isobutyl-1-methylxanthine (IBMX) and PKA-specific inhibitors KT5720 and H89 were from Calbiochem.

Cell Culture

ST2, a murine marrow stromal cell line, was obtained from Riken Cell Bank (Tsukuba, Japan). ST2 cells were maintained in α-minimum Eagle’s medium (Irvine Scientific) supplemented with 10% heat-inactivated fetal bovine serum (HyClone; Logan, UT), sodium pyruvate (1 mM), penicillin (100 units/ml), and streptomycin (100 units/ml). The medium was changed every 3-4 days.

RESULTS

IsoPGE2 Enhances Osteoclastic Differentiation

IsoPGE2 at 0.3 μM enhanced osteoclastic cell formation, as assayed by TRAP activity and cAMP production.

Resorption Assay

Resorption assay discs for resorption assays were purchased from BD PharMingen (San Diego, CA). The cyclic AMP assay kit was purchased from Amersham Biosciences. Salmon calcitonin, rhodamine-conjugated phallolidin, and dexamethasone were from Sigma. Isoprostanes (both isoPGE2 and isoPGF2α), AH6809, and SQ29548 and U46619 were purchased from Cayman Chemical (Ann Arbor, MI). 3-Isobutyl-1-methylxanthine (IBMX) and PKA-specific inhibitors KT5720 and H89 were from Calbiochem.

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Preosteoclast/ST2 Coculture—Bone marrow cells were isolated from 3–6-month-old C57BL/6 mice using protocols established previously by Lacey et al. (33). Briefly, non-adherent cells containing preosteoclasts were plated either in 96-well plates or onto osteologic discs (0.2 × 106 cells/well) in 25 ng/ml murine M-CSF. After 3 days of culture, the adherent cells were treated with control media or 30 μM isoPGE2 (calcitonin or inhibitors, when applicable, in the presence of 125 ng/ml murine M-CSF). After 2 days, medium was removed, and ST2 cells (0.2 × 106 cells/well) in the medium containing 10−5 M 1,25-(OH)2D3 (vitamin D) and 10−5 M dexamethasone together with fresh reagents were added to the wells containing preosteoclasts. After 8 additional days, TRAP activity, actin rings formation, or resorption assays were performed.

Preosteoclast Monoculture—The above non-adherent cells containing preosteoclasts were plated in 96-well plates in the presence of 25 ng/ml murine M-CSF. After 3 days in culture, cells were treated with agents in the presence of 25 ng/ml murine M-CSF and 40 ng/ml murine RANKL. TRAP activity was assayed 6 days after the treatment.

TRAP Staining and Solution Assays

Cells were washed once with PBS and fixed in 10% formalin for 10 min. After washing with PBS, cells were permeabilized with 0.1% Triton X-100 for 1 min, washed once with PBS, and incubated with substrate solution napthol AS-BI phosphate (Sigma) in the presence of 50 mM sodium tartrate at 37 °C for 10 min. A resulting red-stained TRAP activity was visualized by light microscopy.

To quantify TRAP activity, a solution assay adapted from Simonet et al. (34) was performed. Briefly, cells (in 96-well plates) were washed once with PBS and lysed in 80 μl of cold lysis buffer (90 mM citrate buffer, pH 4.8, 0.1% Triton X-100 containing 50 mM sodium tartrate) for 10 min. After lysis, 80 μl of substrate solution (20 mM p-nitrophenyl phosphate in the above lysis buffer) was added and incubated for an additional 3–5 min, and the reaction was stopped by adding 40 μl of 0.5 M NaOH. The optical density was read at a 405-nm wavelength. A standard curve was determined as specified by the manufacturer (Sigma kit no. 387).

Actin Ring Formation Assay

Actin ring formation was visualized by staining with rhodamine-conjugated phallolidin as described previously (28). Briefly, cells were washed once with PBS, fixed in 10% formalin for 10 min, permeated by treatment with 0.1% Triton X-100 for 1 min, and incubated for 40 min with 0.3 mM rhodamine-conjugated phallolidin. The cells were washed with water, and actin rings were visualized under a fluorescence microscope.

Resorption Assay

Resorption assays using osteologic discs were performed as described previously (35). Cells were cultured on the osteologic discs as described above and removed by addition of bleach solution (~6% NaOCl) and agitation for ~5 min. The discs were washed with distilled water and air-dried. The resorption lacunae were visualized and quantified under light microscopy.

cAMP Assay

Non-adherent overnight cultures of marrow preosteoclasts were grown in the presence of murine M-CSF in 6-well plates. After 3 days in culture, cells were treated for 30 min with either control media or 30 μM isoPGE2 in the media supplemented with 1 mM IBMX. After the incubation, cells were washed once with PBS and scraped in PBS containing 4 mM EDTA and 1 mM IBMX. The cells were pelleted and resuspended in the boiling assay buffer (Amersham Biosciences) and sonicated briefly. Cellular proteins were precipitated by boiling for 7 min, and the extract was clarified by centrifugation. The supernatant was assayed for cAMP level using cAMP enzyme immunoassay kit following the manufacturer’s instructions (Amersham Biosciences).
Occlusals were isolated using the techniques and protocols established by Lacey et al. (33) and cocultured with ST2 stromal cells. Treatment of cocultures with 30 μM isoPGE2 enhanced TRAP activity and the number of TRAP-positive multinucleated cells (MNC) (Fig. 1, a and b). TRAP activity was enhanced over a range of isoPGE2 concentrations but not by 30 μM isoPGF2α (Fig. 1c). The TRAP activity and multinucleation induced by isoPGE2 was inhibited by 1 nM calcitonin (Fig. 2, upper and lower). Staining of isoPGE2-induced MNC with rhodamine-conjugated phalloidin revealed actin ring formation (Fig. 3A, arrows). To test whether the TRAP-positive MNC induced by isoPGE2 were able to resorb mineral, marrow preosteoclasts were cocultured with ST2 cells on calcium phosphate-coated (osteologic) discs, which have been validated previously as providing results comparable with the dentin assay (35). At the end of the 8-day culture, the adherent cells were removed from the discs, and the resorption lacunae were viewed under the light microscope. Results showed that isoPGE2 increased the resorption activity as evidenced by the increased number of lacunae (Fig. 3, B and C, arrowheads).

Preosteoclast Monoculture—To examine whether isoPGE2 directly affects preosteoclasts or acts indirectly through stromal

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**Fig. 1. Effects of isoPGE2 on cocultures of preosteoclasts/ST2 cells.**

- **a.** TRAP activity from coculture treated with vehicle alone (control) or 30 μM isoPGE2.
- **b.** Phase contrast photomicrograph (magnification ×40) of TRAP staining showing multinucleated osteoclast-like cells (arrows).
- **c.** TRAP activity of coculture treated with vehicle alone, 30 μM isoPGF2α, and 1, 10, and 30 μM isoPGE2.
cells, marrow preosteoclasts were treated with 1 μM isoPGE2 in the presence of 25 ng/ml M-CSF and 40 ng/ml RANKL, thus eliminating the need for stromal cells. Results showed that isoPGE2 enhanced multinucleation (data not shown) and TRAP activity (control 2.59 ± 0.70, isoPGE2 9.97 ± 1.90). Induction of both TRAP activity and multinucleation was attenuated by 10 μg/ml osteoprotegerin (3.26 ± 0.76), suggesting that the mechanism involves the receptor activator of NFκB.

PKA Pathway Mediates IsoPGE2 Effects—To assess the isoPGE2-mediated intracellular signaling pathway, cocultures of preosteoclasts with ST2 cells were pretreated for 2 h with the PKA inhibitor KT5720 (5 μM), the cyclooxygenase inhibitor indomethacin (1 μM), and the oxygen radical scavenger pyrrolidine dithiocarbamate (10 μM). Results showed that PKA inhibition attenuated isoPGE2-induced TRAP activity, whereas indomethacin had a small effect, and pyrrolidine dithiocarbamate had little or no effect (Fig. 4A). Similarly, KT5720 and H89, another PKA inhibitor, each attenuated isoPGE2-induced TRAP activity in the preosteoclast monocultures (in the absence of ST2) (data not shown). To determine whether the PKA pathway mediates isoPGE2 effects, we measured intracellular cAMP levels in preosteoclast monoculture or stromal ST2 monoculture treated with isoPGE2. Results showed that isoPGE2 caused a 2.6-fold increase in intracellular cAMP preosteoclasts (Fig. 4B) but not in ST2 cells (data not shown).

Receptors Mediating isoPGE2 Effects

To assess whether isoPGE2 acts through a known receptor in mediating osteoclastic potential, we examined the involvement of both prostaglandin and thromboxane receptors, both of which have been shown to mediate specific activities of isoprostanes in different systems (1, 9, 10, 36). First, receptor antagonist studies were performed in monocultures of preosteoclasts. They were pretreated with a prostaglandin receptor antagonist, AH6809 (EP1/EP2/DP antagonist) (14, 15), or the thromboxane receptor antagonist, SQ29548 (TP antagonist) (9, 11, 36), for 2 h followed by cotreatment with isoPGE2, M-CSF (25 ng/ml), and RANKL (40 ng/ml). Results revealed that AH6809, but not SQ29548, attenuated the isoPGE2 response (Fig. 5A). Treatment of preosteoclast monocultures with 5 μM thromboxane receptor agonist U46619 (36) also failed to induce osteoclast-like cell formation (data not shown), suggesting that thromboxane receptor does not mediate isoPGE2 response.
Pretreatment with SC51089 (EP1 antagonist) (11) also did not inhibit isoPGE2 response (data not shown).

Second, the receptor antagonist studies were repeated in cocultures of preosteoclasts with ST2 stromal cells. TRAP activity assay revealed that in the presence of ST2 stromal cells, all three antagonists (AH6809, SC51089, and SQ29548) attenuated the isoPGE2 response (Fig. 5B). The effects of these antagonists on PGE2-induced osteoclastogenesis also paralleled those on isoPGE2-induced osteoclastogenesis for both monoculture and coculture (data not shown).

**Effects of Other Oxidized Lipids**

To assess whether other oxidized lipids affect the formation of osteoclasts, preosteoclast monocultures were treated with isoPGE2 (1 μM), oxLDL (1 μg/ml), or oxPAPC (1 μg/ml), all three antagonists (AH6809, SC51089, and SQ29548) attenuated the isoPGE2 response (Fig. 5B). The effects of these antagonists on PGE2-induced osteoclastogenesis also paralleled those on isoPGE2-induced osteoclastogenesis for both monoculture and coculture (data not shown).

**DISCUSSION**

In the present study, we investigated the effects of lipid oxidation products on osteoclastic differentiation of marrow hematopoietic precursor cells. Results showed that isoPGE2 enhances in vitro osteoclastic potential and activity based on TRAP activity, multinucleation, actin ring formation, and functional resorptive activity. Results also indicate that isoPGE2 directly affects preosteoclasts based on the finding that isoPGE2 enhanced osteoclastic differentiation in the preosteoclast monoculture lacking a stromal feeder layer.

IsoPGE2 may have the same or different effects as compared with other isoprostanes depending on tissue systems. In the present system, unlike isoPGE2, the isoprostane, isoPGF2α, was not osteoclastogenic. The two isoprostanes also have different effects in osteoblastic differentiation (22) and in electrical responses of the small intestine (9). However, in other tissues, these two isoprostanes have the same effects (1, 7). IsoPGE2 may also have the same or different effects as compared with its isomeric prostaglandin PGE2. Similar findings of PGE2 effects on osteoclastogenesis have been reported (12, 13, 31, 32, 37). In other systems, isoPGE2 has opposite effects to those of PGE2 (7, 8, 10). One explanation, as Morrow and Roberts (10) suggested, is that these differential effects may depend on which part of the molecule is active, the ring structure or the stereochemistry of the side chains.

The receptor(s) by which isoprostanes exert their biological effects also varies with tissue systems. In the present study, prostanoid-receptor antagonist treatments revealed that isoPGE2 as well as PGE2 may be acting directly on preosteoclasts through EP2/DP receptors but not EP1 or TP. In contrast, in cocultures of preosteoclasts with stromal/osteoblast cells, both EP and TP receptor antagonists inhibited the isoPGE2 response, suggesting that isoPGE2 is enhancing osteoclastic differentiation in part by acting on osteoblasts through EP1 and TP receptors. These results are consistent with other reports suggesting a possible involvement of EP receptor sub-
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...types in isoprostane signaling (9, 36). The EP4 receptor subtype has also been shown to be involved in mediating PGE2 effects in osteoclast formation (12, 13).

The results also indicate that osteoclastic differentiation induced by isoPGE2 is independent of production of reactive oxygen species and minimally dependent on prostaglandin synthesis based on results with oxygen radical scavengers and cyclooxygenase inhibitors. However, PKA inhibition clearly attenuated osteoclastic differentiation, suggesting that this pathway mediates the effects of isoPGE2. This was further supported by evidence of cAMP elevation in isoPGE2-treated preosteoclast mononucleates. These findings are in agreement with reports by Lacey et al. (33) and Wani et al. (37) showing that cAMP analogs induce osteoclastogenesis in non-adherent bone marrow hematopoietic precursor cells. PGE2 also acts through cAMP; however, this effect is in osteoblasts, which in turn affect osteoclastogenesis (13, 31, 32).

We recently reported that a high fat diet is associated with lowered bone mineral density and content in a mouse strain susceptible to the effects of lipid oxidation product (26). We have also found that a high fat diet reduces the number of marrow osteoclast precursor cells (25) and that lipid oxidation...
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