Bone Morphogenetic Protein 2 Induces Cyclo-oxygenase 2 in Osteoblasts via a Cbfa1 Binding Site: Role in Effects of Bone Morphogenetic Protein 2 In Vitro and In Vivo

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ABSTRACT

We tested the hypothesis that induction of cyclo-oxygenase (COX) 2 mediates some effects of bone morphogenetic protein (BMP) 2 on bone. BMP-2 induced COX-2 mRNA and prostaglandin (PG) production in cultured osteoblasts. BMP-2 increased luciferase activity in calvarial osteoblasts from mice transgenic for a COX-2 promoter-luciferase reporter construct (Pluc) and in MC3T3-E1 cells transfected with Pluc. Deletion analysis identified the −300/−213-bp region of the COX-2 promoter as necessary for BMP-2 stimulation of luciferase activity. Mutation of core-binding factor activity 1 (muCbfa1) consensus sequence (5’-AACCACA-3’) at −267/−261 bp decreased BMP-2 stimulation of luciferase activity by 82%. Binding of nuclear proteins to an oligonucleotide spanning the Cbfa1 site was inhibited or supershifted by specific antibodies to Cbfa1. In cultured osteoblasts from calvariae of COX-2 knockout (−/−) and wild-type (+/+) mice, the absence of COX-2 expression reduced the BMP-2 stimulation of both ALP activity and osteocalcin mRNA expression. In cultured marrow cells flushed from long bones, BMP-2 induced osteoclast formation in cells from COX-2+/+ mice but not in cells from COX-2−/− mice. In vivo, BMP-2 (10 μg/pellet) induced mineralization in pellets of lyophilized collagen implanted in the flanks of mice. Mineralization of pellets, measured by microcomputed tomography (μCT), was decreased by 78% in COX-2−/− mice compared with COX-2+/+ mice. We conclude that BMP-2 transcriptionally induces COX-2 in osteoblasts via a Cbfa1 binding site and that the BMP-2 induction of COX-2 can contribute to effects of BMP-2 on osteoblastic differentiation and osteoclast formation in vitro and to the BMP-2 stimulation of ectopic bone formation in vivo. (J Bone Miner Res 2002;17:1430–1440)

Key words: MC3T3-E1 cells, prostaglandin G/H synthase, prostaglandin E2, COX-2 knockout, promoter regulation

INTRODUCTION

Prostaglandins (PGs) are produced by osteoblasts and are abundant in bone.(1,2) They are complex regulators of bone remodeling that can stimulate both bone formation and bone resorption. The major enzyme regulating the conversion of arachidonic acid released from membrane phospholipids to PGs is PGG/H synthase (PGHS), commonly called cyclo-oxygenase (COX). There are two enzymes for COX encoded by separate genes, COX-1 and COX-2.(2,3) COX-1 is usually expressed constitutively, and COX-2 is induced rapidly and transiently. Both COX-1 and COX-2 are expressed in osteoblastic cells, and COX-2 is the main enzyme regulating the production of PGs in response to various hormones and cytokines.(4)

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Bone morphogenetic proteins (BMPs) belong to the transforming growth factor (TGF) β superfamily. Originally, BMPs were identified by their ability to form ectopic bone. BMP-2 is expressed during murine embryonal skeletogenesis and can stimulate osteoblast differentiation from uncommitted progenitors both in vitro and in vivo. Exogenous BMP-2 can accelerate fracture healing, and BMP-2 is expressed in primitive mesenchymal cells and chondrocytes at the site of callus formation. BMP-2 also can stimulate or enhance the process of osteoclastogenesis by its action on osteoclastic cells. BMPs were identified by their ability to form ectopic bone and osteoblastic cells and chondrocytes in vivo. In this study and for the in vivo studies, rhBMP-2 was purchased from R & D Systems (Minnesota, MN, USA). NS-398 was purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). Phorbol 13-myristate 12-acetate (PMA) and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

**Animals**

Mice carrying −371/+70 bp of the 5′-flanking murine COX-2 DNA fused to a luciferase reporter (Pluc) were developed in a CD-1 background by the Transgenic Animal Facility at the University of Connecticut Health Center (UCHC). COX-2 knockout (COX-2−/−) mice were developed at the University of North Carolina. (23) COX-2−/− and wild-type (COX-2+/+) mice were bred by mating +/+ offspring of C57Bl6/+− and 129/sv/+− crosses for reasons discussed previously. (24)

**Osteoblastic cell cultures**

MC3T3-E1 cells were the gift of Dr. Yoshiyuki Hakeda (Meikai University School of Dentistry, Sakado, Saitama, Japan). To make primary osteoblastic cultures, whole calvariae were dissected sequentially with 2 T3 cells. In addition, recently, Cbfal−− calvarial cells failed to generate bone nodules even in the presence of BMP-2. Osteoclastogenesis also was found to be retarded in Cbfal−− mice, presumably because of a requirement of Cbfal for the expression of RANKL, which mediates the interaction between osteoblast and osteoclast lineage required for osteoclast differentiation. (5,13,16) Hence, Cbfal appears essential not only for osteoblasts to become bone-forming cells but also for osteoclasts to become support cells for osteoclast formation.

Several studies have suggested that Cbfal is a downstream factor in the BMP-2 signaling pathway. BMP-2 induced Cbfal mRNA expression in human bone marrow stromal cells (17) and 2T3 cells. (18) In addition, recently, Cbfal has been shown to be a common target of BMP-2 and TGF-β signaling pathways, cooperating with Smads to induce osteoblast-specific gene expression in C2C12 cells. (19)

Because both BMP-2 and PGs can stimulate bone formation and resorption, it is possible that PGs may mediate some actions of BMP-2 on bone. BMP-2 has been shown to induce COX-2 expression in osteoblastic cells. (8) In this study, we examined transcriptional regulation of COX-2 by BMP-2, identified a Cbfal-binding element mediating the BMP-2 induction of COX-2 promoter activity, and showed the relevance of the BMP-2 induction of COX-2 expression for osteoblastic and osteoclastic differentiation in vitro.

**MATERIALS AND METHODS**

**Materials**

Murine COX-2 cDNA and DNA constructs (−371/+70 bp of the COX-2 gene or 5′ deletions of this region fused to a luciferase reporter gene in pXp-2 vector) have been described previously. (20–22) Murine COX-1 cDNA was the gift of Dr. David DeWitt and Dr. William Smith (Michigan State University, East Lansing, MI, USA). Cbfal cDNA was the gift of Dr. Gerad Karsenty (University of Texas Dental Branch, Houston, TX, USA). Murine osteocalcin cDNA was the gift of Dr. John Wozney (Genetics Institute, Cambridge, MA, USA). cDNA for GAPDH was amplified by using an amplimer kit from Clontech (Palo Alto, CA, USA). Recombinant human BMP-2 (rhBMP-2), kindly supplied by Yamanouchi Pharmaceutical Co. (Tokyo, Japan), was used for all in vitro studies except for the study in Table 1. For that study and for the in vivo studies, rhBMP-2 was purchased from R & D Systems (Minnesota, MN, USA). NS-398 was purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). Phorbol 13-myristate 12-acetate (PMA) and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

To measure ALP activity, cultures were washed with PBS and sonicated in 10 mM of Tris–HCl buffer (pH 7.5) containing 0.1% Triton X-100. ALP activity in the lysate was determined by the hydrolysis of p-nitrophenyl phosphate to p-nitrophenol. Absorbance was determined at 405 nm and compared with a p-nitrophenol (Sigma Chemical Co.) standard titration curve. ALP activity was normalized to total proteins measured with a BCA protein assay kit (Pierce Chemical Co., Rockford, IL, USA).
Bone marrow cell cultures

Bone marrow cells (2 × 10⁶ cells/well) were flushed from tibias and femurs of 6- to 8-week-old COX-2+/− and COX-2−/− mice. Cells were cultured in 24-multiwell dishes with α-modified minimum essential medium (α-MEM; Gibco BRL) containing 10% FCS, penicillin (100 U/ml), and streptomycin (50 µg/ml). After 7 days of culture, cells were washed with PBS, fixed with 2.5% glutaraldehyde for 30 minutes, and stained using a leukocyte acid phosphatase A kit (Sigma Chemical Co.). TRAP⁺ multinucleated cells (MNCs) containing more than three nuclei were counted as osteoclast-like cells.

Stable and transient transfections

Stable and transient transfections of MC3T3-E1 cells were performed as previously described.(25,26) For stably transfected cultures, colonies (>200) were pooled to minimize effects secondary to variable integration sites. Luciferase activity was measured in soluble cell extracts prepared with a kit from Promega (Madison, WI, USA) using an automatic injection luminometer (Berthold Lumat; Wallac, Inc., Gaithersburg, MD, USA). For each experiment, three wells of a 6-well dish were analyzed per treatment group. Activity was normalized to total protein measured with a kit from Pierce Chemical Co.).

Site-directed mutation

The putative Cbfal-binding sequence 5′-AACCACA-3′ at −267/−261 bp in the COX-2 5′-flanking region was changed to 5′-AATAACA-3′ (mutation of Cbfal [muCbfal]) using the QuickChange Site-Directed Mutagenesis Kit from Stratagene (La Jolla, CA, USA). Mutations of the AP-1 and cAMP response element (CRE) sites have been described previously.(25,26) After introduction of mutations, the COX-2 5′-flanking region was sequenced (Automated Sequencing Facility, UCHC). To avoid the accidental introduction of mutations into regions outside the COX-2 5′-flanking region, the 5′-flanking region was released from the pXp2 plasmid and subcloned into a fresh pXp2 vector.

EMSA

Nuclear extracts were prepared as previously described.(26) For gel electrophoresis, single-stranded oligonucleotides were end-labeled with [γ³²P]-adenosine triphosphate (ATP; New England Nuclear) using T4 kinase (Gibco BRL). The complementary oligonucleotide was annealed, and the resulting double-stranded DNA was purified. Six micrograms of nuclear extract was incubated in 20 µl of binding reaction mixture (10 mM of Tris-HCl, pH 7.5, 1 mM of dithiothreitol [DTT], 1 mM of EDTA, 5% glycerol, and 2 µg of poly dI-dC [Pharmacia and Upjohn, Bridgewater, NJ, USA]) with 50,000 cpm of labeled probe. Five percent nondenaturing acrylamide gel electrophoresis was performed for 2 h and gels were fixed for 30 minutes in 10% methanol and 10% acetic acid. Dried gels were exposed to X-ray film. Competitor (100 M excess) or supershifting antibody (4 µg) was added to the binding mixture 30 minutes before addition of the probe, and incubation continued for 30 minutes. Antibody designed to supershift Cbfal-binding complexes was obtained from Oncogene Research Products (Cambridge, MA, USA).

mRNA extraction and Northern blot analysis

Three wells of cells in 6-well dishes were pooled for RNA extraction using TRI-REAGENT (Molecular Research Center, Inc., Cincinnati, OH, USA). After quantitation at 260 nm, 10 µg of total RNA was run on a 1% agarose–2.2 M of formaldehyde gel and transferred to a nylon membrane (Genescreen; New England Nuclear Research, Boston, MA, USA) by capillary pressure using Turboblotter (Schleicher & Schuell, Keene, NH, USA). After 2 h of prehybridization in 50% formamide solution in rotating cylinders at 42°C, filters were hybridized overnight at 42°C in a similar solution with random primer [³²P]dexoyctidine triphosphat (dCTP; Perkins Elmer Boston, MA, USA)-labeled cDNA probes. Filters were washed once in a 1× SSC and 1% SDS solution at room temperature, once in a 0.1× SSC and 0.1% SDS solution at 65°C, and then three times in the latter solution at room temperature. After washing, filters were exposed to Eastman Kodak Co. XAR-5 film (Eastman Kodak Co., Rochester, NY, USA) at −70°C. X-rays were scanned (ScanJet; Hewlett-Packard Co., Corvallis, OR, USA), and band density was determined using NIH Image 1.62 (National Institutes of Health, Bethesda, MD, USA).

PGE₂ assay

Medium was removed from cultured cells and PGE₂ accumulation was measured by radioimmunoassay as described previously.(26) The lower limit of detection of this assay is 0.1 nM. One hundred microliters of unmodified medium was assayed for each sample.

In vivo mineralization

A solution of 0.4 ml of rat type I collagen (BD Biosciences, Bedford, MA, USA) with BMP-2 (10 µg) or vehicle PBS with 0.1% BSA was freeze-dried and formed into pellets. Pellets were implanted subcutaneously in the flanks of 7-week-old COX-2−/− and COX-2+/− mice under general anesthesia. Mice were killed and pellets were removed 3 weeks later. Each mouse carried one BMP-2 pellet and one vehicle pellet in opposing flanks. Mineralized bone volume in the pellets was analyzed by microcomputed tomography (µCT; microCT20; Scanco Medical AG, Zurich, Switzerland). All animal protocols were approved by the Animal Care and Use Committee at UCHC.

Statistical analysis

Means of groups were compared by ANOVA (SigmaStat, San Rafael, CA, USA). The significance of differences was determined by post hoc testing using the Bonferroni method.
RESULTS

BMP-2 induction of COX-2 and PGE₂ production

BMP-2 (100 ng/ml) rapidly induced both COX-2 mRNA and PGE₂ production in primary calvarial osteoblastic cell cultures derived from COX-2⁺/⁺ mice (Fig. 1A). Both COX-1 mRNA and Cbfa1 mRNA were expressed constitutively and not increased by BMP-2. COX-2 expression and PGE₂ production are induced in calvarial osteoblasts by addition of fresh serum at the time of medium changes (Pilbeam, data not shown). In calvarial osteoblastic cells cultured for 14 days, treatment with BMP-2 (100 ng/ml) increased medium PGE₂ accumulation by 45% in cells derived from COX-2⁺/⁺ mice relative to cells from COX-2⁻/⁻ mice (Fig. 1B).

BMP-2 induction of luciferase activity

In calvarial osteoblasts from neonatal mice transgenic for −371/+70 bp of the murine COX-2 promoter fused to a luciferase reporter (Pluc), BMP-2 induced luciferase activity in a dose-related manner with peak effects at 7 h (Figs. 2A and 2B). In MC3T3-E1 cells stably transfected with Pluc, BMP-2 dose-dependently increased luciferase activity with peak effects at 3 h (Figs. 2C and 2D). The delayed peak response in primary cells compared with MC3T3-E1 cells has been observed for all COX-2 agonists we have studied (Pilbeam, data not shown). For both primary cells and MC3T3-E1 cells, the maximal stimulation of luciferase activity by BMP-2 at 100 ng/ml was fourfold.

Identification of the cis-acting site(s) mediating response to BMP-2

To determine regions within the 371-bp 5′-flanking region that mediated the response to BMP-2, MC3T3-E1 cells were stably transfected with a series of 5′-deletion constructs and treated with BMP-2 (100 ng/ml) for 3 h (Fig. 3A). There was no reduction in BMP-2–stimulated luciferase activity with deletion to −300/+70 bp but BMP-2 stimulation was abrogated by deletion to −213/+70 bp. Hence, site(s) mediating the BMP-2 stimulation of COX-2 promoter activity lay between −300 and −213 bp. Although distant from the −150/−40 bp region previously shown to contain several important cis-acting elements regulating COX-2 activity, including the AP-1 and CRE sites (25,26,27), Examination of the DNA sequence identified a Cbfa1 consensus sequence (5′-AACCACA-3′) at −267/−261 bp (Fig. 4B). MC3T3-E1 cells were transiently transfected with either the wild-type Pluc construct or the Pluc construct carrying a 2-bp muCbfa1 and treated with BMP-2 (100 ng/ml) for 3 h (Fig. 4A). In cells carrying muCbfa1, the BMP-2 stimulation of luciferase activity was reduced 82% and was not significantly different from the untreated control.

To determine if the mutation was specific for the response to BMP-2, we treated cells with PMA (1 μM), which stimulates COX-2 promoter activity through the AP-1 and CRE sites (26). PMA-induced luciferase activity was not decreased in cells carrying the muCbfa1 promoter construct (Fig. 4A). Although other transactivating sites in the pro-

FIG. 1. BMP-2 induction of COX-2 mRNA and PGE₂ in calvarial osteoblastic cultures. Primary osteoblastic cultures were prepared from calvariae of COX-2⁺/⁺ and COX-2⁻/⁻ mice. Cells were treated with vehicle (control) or BMP-2 (100 ng/ml) for the indicated periods. (A) Cells were cultured for 6 days and serum-deprived for 24 h before being treated. Steady-state mRNA levels of COX-2 were measured by Northern analysis and compared with mRNA levels of COX-1 and Cbfa1. mRNA levels for GAPDH are shown to assess loading. (B) Cells were cultured with 10% FCS for 2 weeks. Media were removed for measurement of PGE₂ levels and fresh media were added on days 3, 7, and 10. Medium PGE₂ values were measured by radioimmunoassay (RIA) and cumulative levels were calculated. Data are means ± SEM for n = 6 samples. aSignificant difference from control group; p < 0.01.
moter might not be sufficient to permit BMP-2 stimulation of promoter activity in the absence of the Cbfa1 site, they might, nevertheless, be necessary for BMP-2 stimulation of activity. However, 2-bp mutations of the AP-1 and CRE sites, which were previously shown to inhibit PMA stimulation of luciferase activity, did not reduce BMP-2–stimulated luciferase activity (Fig. 4B).

Binding to the Cbfa1 site on EMSA

Nuclear extracts were prepared from MC3T3-E1 cells treated for 1 h with or without BMP-2 (100 ng/ml) and run on gel with a 32P-labeled oligonucleotide spanning the Cbfa1 site (−278/−253 bp of the COX-2 5′-flanking sequence). Binding to this probe was constitutive (Fig. 5A, lanes 2 and 3, arrows a–c). All bands were competed by the unlabeled probe (Fig. 5A; lanes 4 and 5). Only the upper band (Fig. 5B, arrow a) was competed by the unlabeled probe containing the muCbfa1 sequence (Fig. 5B; lanes 4 and 5), indicating that protein binding was unaffected by the mutation. Proteins in the lower two bands (Fig. 5B, arrows b and c) could only bind the intact Cbfa1 site. The lower bands were inhibited by an antibody specific for Cbfa1 as shown in Fig. 6C and a light supershifted band was evident (Fig. 5C, lanes 4 and 5, arrow d). Nonspecific immunoglobulin G (IgG) antibody did not inhibit the binding (Fig. 5B, lanes 5 and 6).

Role of COX-2 in BMP-2–induced osteoblastic cell differentiation

Primary calvarial osteoblastic cells derived from COX-2+/+ and COX-2−/− mice were cultured for 14 days with and without BMP-2 (100 ng/ml). ALP activity was similar in COX-2−/− and COX-2+/+ control cultures, but treatment with BMP-2 stimulated ALP activity only in COX-2+/+ cultures (Fig. 6A). In this experiment, treatment with PGE 2 (1 μM) alone did not significantly increase ALP activity in either COX-2+/+ or COX-2−/− cultures. However, treatment with both PGE 2 and BMP-2 increased ALP activity in COX-2+/+ cultures to the same level as seen in COX-2+/+ cultures. Treatment of COX-2+/+ cultures with NS-398 (0.1 μM), a selective inhibitor of COX-2 activity in osteoblasts at this dose, did not significantly inhibit ALP activity in control
cultures but inhibited the fold stimulation of ALP activity by BMP-2 by 38% (Fig. 6B). In addition, BMP-2 stimulated osteocalcin mRNA expression at day 14 of culture in primary calvarial cells derived from COX-2+/H11001/H11001 mice but not in COX-2-/H11002/H11002 cells (Fig. 6C).

**Role of COX-2 in BMP-2–induced osteoclast-like cell formation**

To investigate osteoclast formation, we cultured bone marrow cells from COX-2+/H11001/H11001 and COX-2-/H11002/H11002 mice and counted the number of TRAP+ MNCs formed after 7 days of culture with or without BMP-2 (100 ng/ml). BMP-2 stimulated osteoclast formation in marrow from COX-2+/+ mice but not in marrow from COX-2-/- mice (Figs. 7A and 7B). The TRAP+ MNCs formed in response to BMP-2 were able to resorb pits on dentine slices (data not shown). In a similar experiment, PGE2 (1 μM) added to BMP-2–treated cultures synergistically enhanced the formation of TRAP+ MNCs in both COX-2+/+ and COX-2-/- cultures and reversed the difference between COX-2+/+ and COX-2-/- cultures (Table 1).
Role of COX-2 in BMP-2–induced mineralization in vivo

Collagen pellets containing BMP-2 (10 μg/pellet) implanted in the flanks of 7-week-old mice for 3 weeks showed markedly increased mineralized volume, measured by μCT analysis, compared with pellets containing vehicle (Fig. 8). BMP-2–induced mineralization was reduced by 78% in pellets implanted in COX-2−/− mice relative to pellets implanted in COX-2+/+ mice.

DISCUSSION

Cbfa1 is an essential transcription factor for osteoblast differentiation and can regulate the expression of many genes associated with the development of bone matrix such as osteocalcin, type I collagen, and osteopontin.12,30–32 Recent studies have shown that parathyroid hormone (PTH) stimulates expression of MMP-13 via a Cbfa1-binding site.33–35 Our study shows that BMP-2 also can induce COX-2 expression and PGE2 production in osteoblastic...
BMP-2 study has shown that the ERK pathway may be involved in Cbfa1 mRNA or binding of Cbfa1 on EMSA. (30) Considered to act via Cbfa1 did not increase expression of Cbfa1 by the MAPK (ERK) pathway (37) and another study has shown activation and phosphorylation signaling pathways that might lead to this activation, although one study has shown activation and phosphorylation of Cbfa1 to enhance Cbfa1 transactivation. (19,40,41) It also is known about the role of endogenous PGs, but marrow interactions with Smads (11) Other Smad DNA-binding partners may be active on their own, requiring input from different signaling pathways and only having their activity modulated by interactions with Smads (11) A number of studies have shown that BMP-2–activated Smads can form complexes with Cbfa1 to enhance Cbfa1 transactivation (19,40,41) It also is possible that interaction with Smads could relieve an autoinhibitory conformation of Cbfa1.

Exogenous PGs can be anabolic for bone. In vitro, PGE_2 stimulates the formation and differentiation of osteoblastic colonies. (42,43) In vivo, PGs given to rats produce substantial increases in bone mass (44) and enhance osteoblastic differentiation in explanted marrow stromal cells. (45) Less is known about the role of endogenous PGs, but marrow stromal cells cultured from COX-2^{+/+} mice show decreased osteoblastic differentiation compared with cells from COX-2^{−/−} mice and this decrease can be reversed by adding back PGE_2 (46) Small decreases in trabecular bone volume and parameters of bone formation in COX-2^{−/−} mice compared with COX-2^{+/+} mice suggest that a deficiency of endogenous PGs may decrease bone formation more than resorption. (46) Endogenous PGs also appear to play a role in acutely stimulated bone formation in vivo. Studies have shown that periorientive treatment with nonsteroidal anti-

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**TABLE 1. EFFECT OF EXOGENOUS PGE_2 (1 μM) ON THE BMP-2 (500 ng/ml) INDUCTION OF OSTEOCLAST FORMATION IN COX-2^{+/+} AND COX-2^{−/−} MARROW CULTURES**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>COX-2^{+/+}</th>
<th>COX-2^{−/−}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PGE_2</td>
<td>10–12</td>
<td>64.2 ± 6.0</td>
<td>46.3 ± 4.5*</td>
</tr>
<tr>
<td>BMP-2</td>
<td>6</td>
<td>11.2 ± 2.3</td>
<td>0</td>
</tr>
<tr>
<td>+PGE_2</td>
<td>6</td>
<td>137.8 ± 21.2*</td>
<td>158.3 ± 15.2*</td>
</tr>
</tbody>
</table>

Data are means and SEM for n wells.
* Significant difference relative to treatment with single agent, _p_ < 0.01; † significant effect of genotype, _p_ < 0.05.
inflammatory drugs (NSAIDs) can prevent heterotrophic ossification after hip surgery, suggesting that this problem is caused by PG-mediated bone formation. In addition, NSAIDs can inhibit fracture repair and spinal fusion. This study suggests that the effects of BMP-2 on osteoblastic differentiation are enhanced by BMP-2-induced COX-2 expression and PG production and that BMP-2-stimulated PG production also may be important for the effects of BMP-2 on ectopic bone formation and fracture healing.

The substrate for COX-2, arachidonic acid, also can be metabolized via lipoxygenase to leukotrienes and hydroxyeicosatetraenoic acids (HETEs). In COX-2−/− mice, more arachidonic acid may be available for metabolism via the lipoxygenase pathway than in COX-2+/+ mice. Several studies have suggested that the products of the lipoxygenase pathway can inhibit bone formation. In one study, leukotriene B4 and 5-HETE blocked the BMP-2 action on ectopic bone formation and fracture healing.

Hence, it is possible that an increase in the products of the lipoxygenase pathway caused by COX-2 depletion might be involved in the decreased osteoblastic differentiation response to BMP-2 in COX-2−/− cultures and the decreased ectopic calcification response to BMP-2 in COX-2−/− mice.

Marrow cultures from some strains of mice, such as C57Bl/6 or C57Bl/6 × 129 (the background of our COX-2−/− and COX-2+/+ mice), produce osteoclasts in response to multiple agonists without the need for additional treatment with RANKL or macrophage colony-stimulating factor (M-CSF) or the need for coculture with osteoblasts (e.g., see the study by Okada et al.). BMP-2 alone was able to stimulate osteoclast formation in COX-2−/− marrow cultures but not in COX-2−/− marrow cultures, indicating a dependence of BMP-2-stimulated osteoclastogenesis on COX-2 expression. PGs, which are themselves potent stimulators of resorption, can enhance osteoclastogenesis stimulated by many agonists. Similarly, PGE2 added back to the marrow cultures synergistically enhanced the BMP-2 stimulation of osteoclastogenesis to equal levels in COX-2−/− and COX-2+/+ cultures. Other studies have also implicated BMP-2 in osteoclast formation. BMP-2 stimulated osteoclast-like cell formation and bone-resorbing activity in mouse bone cell cultures. Antagonism of BMP-2 and BMP-4 action by treatment with noggin inhibited both osteoblast and osteoclast formation in mouse bone marrow cultures. A role for COX-2 in the BMP-2 induction of osteoclast formation was suggested by a study showing that BMP-2 induced COX-2 expression and enhanced IL-1α-induced osteoclastogenesis in ddY marrow stromal cells.

In summary, we have shown that BMP-2 induced COX-2 in osteoblasts transcriptionally via a Cbfa1 DNA-binding site and that the induction of COX-2 may play a role in the effects of BMP-2 on bone metabolism both in vitro and in vivo.

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