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Cyclooxygenase-2 gene disruption promotes proliferation of murine calvarial osteoblasts *in vitro*☆

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Abstract

Cyclooxygenase-2 (COX-2) is highly expressed in osteoblasts, and COX-2 produced prostaglandins (PGs) can increase osteoblastic differentiation *in vitro*. The goal of this study was to examine effects of COX-2 expression on calvarial osteoblastic proliferation and apoptosis. Primary osteoblasts (POBs) were cultured from calvariae of COX-2 wild-type (WT) and knockout (KO) mice. POB proliferation was evaluated by ^{3}H-thymidine incorporation and analysis of cell replication and cell cycle distribution by flow cytometry. POB apoptosis was evaluated by annexin and PI staining on flow cytometry. As expected, PGE_2 production and alkaline phosphatase (ALP) activity were increased in WT cultures compared to KO cultures. In contrast, cell numbers were decreased in WT compared to KO cells by day 4 of culture. Proliferation, measured on days 3–7 of culture, was 2-fold greater in KO than in WT POBs and associated with decreased Go/G1 and increased S cell cycle distribution. There was no significant effect of COX-2 genotype on apoptosis under basal culture conditions on day 5 of culture. Cell growth was decreased in KO POBs by the addition of PGE_2 or a protein kinase A agonist and increased in WT POBs by the addition of NS398, a selective COX-2 inhibitor. In contrast, differentiation and cell growth in marrow stromal cell (MSC) cultures, evaluated by ALP and crystal violet staining respectively, were increased in MSCs from WT mice compared to MSCs from KO mice, and exogenous PGE_2 increased cell growth in KO MSC cultures. We conclude that PGs secondary to COX-2 expression decrease osteoblastic proliferation in cultured calvarial cells but increase growth of osteoblastic precursors in MSC cultures.

Keywords: Prostaglandins; Apoptosis; Nonsteroidal anti-inflammatory drugs; Marrow stromal cells; Mitogenesis

Introduction

Cyclooxygenase (COX) is a rate-limiting enzyme in conversion of arachidonic acid (AA) to prostanoids. It converts AA to PGG_2 via a cyclooxygenase reaction and then reduces PGG_2 to PGH_2 in a peroxidase reaction [1]. Nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit the cyclooxygenase activity of COX. There are two isoforms of COX: COX-1, which is constitutively expressed, and COX-2, which is inducible by multiple factors and involved in prostaglandin (PG) production during inflammation and other acute responses [2]. PGE_2 is one of the most abundant PGs produced in bone, and most PGE_2 produced by bone cells is associated with the induction of COX-2 [3].

PGE_2 acts via interactions with a subfamily of G-protein-coupled receptors (GPCRs), called EP1, EP2, EP3, and EP4 [4]. EP1 releases intracellular calcium and EP3 can inhibit cyclic 3,5-adenosine monophosphate (cAMP) formation. EP2 and EP4 can both stimulate cAMP formation, and EP4 may also act via a phosphatidylinositol 3-kinase-dependent pathway and activate extracellular signal-regulated kinases (ERKs) [5]. In addition to initiating different signaling pathways, recent data suggest EP receptors may undergo nuclear compartmentalization [6] or

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heterodimerize with other GCPRs [7], leading to more complex actions.

PGE_2 is a potent stimulator of bone resorption and osteoclastogenesis [8], and both EP2 and EP4 have been implicated in these effects [9–11]. PGE_2 is also anabolic for bone. *In vitro*, exogenous PGE_2 stimulates osteoblastic differentiation in marrow stromal and calvarial osteoblast cultures [12–15]. In agreement with these studies, osteoblastic differentiation is decreased in marrow stromal cell cultures from COX-2 knockout (KO) mice compared to cultures from COX-2 wild-type (WT) mice [16,17]. *In vivo*, administration of PGs in rats, dogs and humans increases cortical and cancellous bone mass [18–21]. PGE_2 given to rats *in vivo* stimulates osteoblastic differentiation in *ex vivo* cultured bone marrow [22]. Both EP2 and EP4 have been implicated in the anabolic effects of PGE_2. Local infusion of PGE_2 adjacent to the femur was shown to produce callus formation in EP1, EP2 or EP3 receptor KO mice but not in EP4 KO mice, and marrow from EP4 KO mice did not mineralize in response to *in vitro* treatment with PGE_2 [11]. EP2 and EP4 agonists have been shown to stimulate bone formation and/or enhance fracture healing *in vivo* [23,24], and bone mechanical properties may be reduced in EP2 and EP4 KO mice [25,26].

Some of the effects of PGs to increase new bone formation could be the result of PG-induced increases in growth of osteoblastic cells, secondary either to increased proliferation or decreased apoptosis. Studies of the effects of PGE_2 on osteoblastic proliferation have been contradictory, showing both increased [27–29] and decreased proliferation [15,30,31], as well as biphasic effects [32,33]. Although COX-2 expression and PGs have been consistently associated with increased resistance to apoptosis and tumor promotion in other tissues [34,35], there have been few studies of the effects of PGE_2 on apoptosis in osteoblasts and these have not produced consistent results. Inhibition of PG production with NSAIDs increased apoptosis in fetal rat calvarial osteoblast cultures [36], but treatment with PGE_2 increased apoptosis in conditionally immortalized human osteoblasts [37]. We found that overexpression of COX-2 increased apoptosis in human osteosarcoma cells, although the effect was PGE_2 independent [38].

NSAIDs have been used to study the role of PGs in many systems, but they can clearly have potent effects unrelated to their inhibition of COX activity [31,36,39]. We used primary calvarial cells from COX-2 WT and KO mice to study the role of endogenous COX-2 in the growth of osteoblasts *in vitro*. Osteoblastic growth was increased in KO cells relative to WT cells and the increased growth was due to increased proliferation, not decreased apoptosis. Differences between WT and KO cells were eliminated by the addition of NS398, a selective inhibitor of COX-2 activity, to WT cells or by the addition of PGE_2 to KO cells and, hence, appear to be PG mediated.

Materials and methods

Materials

PGE_2 and NS398, a selective inhibitor of COX-2 activity, were purchased from Cayman Chemical (Ann Arbor, MI). Culture media were purchased from Gibco-BRL (Grand Island, NY) unless otherwise specified. Other reagents were purchased from Sigma (St. Louis, MO).

Animals

The COX-2 KO mice used in this study were developed at the University of North Carolina in a C57Bl/6, 129SV background [40,41]. COX-2 WT and KO mice were bred for experiments by mating mice heterozygous for the disrupted COX-2 allele in a mixed C57Bl/6,129SV background. Mice were genotyped as described previously [8]. Mice were sacrificed at 5–8 weeks of age. All animal protocols were approved by the Animal Care and Use Committee of the University of Connecticut Health Center, Farmington, CT.

Primary calvarial osteoblast cultures

Calvariae were dissected from 2–5 mice, washed with PBS and sequentially digested with 0.5 mg/ml of crude collagenase P (Roche Molecular Biochemical, Indianapolis, IN) in a solution of 1 ml trypsin/EDTA and 4 ml PBS at 37 °C with gentle rocking. Five digests were performed, all for 10 min except the last one, which was for 90 min. After each digest, released cells were collected, the reaction stopped with 10% FCS, and the solution filtered through a Nitex membrane (Millipore Corp., Bedford, MA) to ensure a single cell suspension. Digests 2–5 were pooled and plated, grown to confluence, and replated at 5000 cells/cm^2 before use. Cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2, in phenol red-free DMEM with 10% heat-inactivated fetal calf serum (FCS), and 100 U/ml penicillin, 50 μg/ml streptomycin. PGE_2, NS398 and vehicle (EtOH, 0.1%) were added at the beginning of the cultures. For differentiation studies, 50 μg/ml of L-ascorbic acid phosphate (Wako Pure Chemical Industries, Japan) and 10 μM beta-glycerophosphate (Sigma) were added at the first medium change.

For alkaline phosphatase (ALP) staining, cultures were fixed with citrate, acetone and formaldehyde for 1 min and stained for ALP using a kit from Sigma according to the manufacturer's instructions. For Von Kossa staining, cultures were fixed with formaldehyde and washed with 0.1 M cacodylate buffer (pH 7.4). Cells are incubated sequentially with saturated $LiCO_3$ (20 min), 5% $AgNO_3$ (60 min under lamp), and 5% Na thiosulfate (5 min), with H_2O washes between incubations.

To examine cell number, cells were rinsed with PBS, suspended with trypsin–EDTA (0.25%), centrifuged, and resuspended in 500 μl of culture medium. An aliquot of 100 μl of cell suspension was counted with Coulter Counter (Coulter Corporation, Miami, FL), lower limit set at 8.0 μm. Triplicate wells were counted for each experimental group.

Marrow stromal cell (MSC) cultures

Marrow from both tibiae and femurs of a single mouse was flushed with a total of 1 ml of α-minimum essential medium (α-MEM). Marrow cells were plated at 1×10^6 cells/well in 6-well plates in α-MEM containing 10% FCS and 50 μg/ml L-ascorbic acid phosphate (Wako Pure Chemical Industries). Cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 in air with medium changes twice a week. PGE_2 (1 μM) or vehicle (EtOH, 0.1%) was added at the beginning of the cultures and at each medium change. To stain for ALP, cultures were fixed with citrate, acetone and formaldehyde for 1 min and stained using a kit from Sigma according to the manufacturer's instructions. To estimate the area covered by all cells, cultures were counterstained with crystal violet according to the manufacturer's protocol (Sigma). To calculate the area of crystal violet or alkaline phosphatase staining, dishes were scanned with a Hewlett Packard ScanJet and stained areas manually circumscribed and quantified using NIH Image 1.6.

ALP activity

Cultures were lysed in 10 mM Tris solution (pH 7.5) supplemented with 0.1% Triton X-100. Supernatants were incubated with an alkaline buffer (pH 10.5) containing 5 mM of *p*-nitrophenol phosphate as substrate and 2 mM $MgCl_2$. Absorbance was determined at 405 nM and compared with a *p*-nitrophenol standard titration curve (Sigma). ALP activity was normalized to