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Anabolic Effects of PTH in Cyclooxygenase-2 Knockout Osteoblasts *In Vitro*

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Abstract

PTH is a potent bone anabolic agent *in vivo* but anabolic effects on osteoblast differentiation *in vitro* are difficult to demonstrate. This study examined the role of cyclooxygenase (COX)-2 and prostaglandin (PG) production in the effects of PTH on osteoblast differentiation *in vitro* using marrow stromal cell (MSC) and calvarial osteoblast (COB) cultures from COX-2 knockout (KO) and wild type (WT) mice. Cells were treated with PTH (10 nM) or vehicle throughout culture. Alkaline phosphatase (ALP) and osteocalcin (OCN) mRNA levels were measured at days 14 and 21, respectively, and mineralization at day 21. cAMP concentrations were measured in the presence of a phosphodiesterase inhibitor. PTH did not stimulate differentiation in cultures from WT mice but significantly increased ALP and OCN mRNA expression 6- to 7-fold in KO MSC cultures and 2- to 4-fold in KO COB cultures. PTH also increased mineralization in both KO MSC and COB cultures. Effects in KO cells were mimicked in WT MSC cultures treated with NS-398, an inhibitor of COX-2 activity. PTH increased cAMP concentrations similarly in WT and KO COBs. Differential gene responses to PTH in COX-2 KO COBs relative to WT OBs included greater fold-increases in the cAMP-mediated early response genes, *c-fos* and *Nr4a2*; increased IGF-1 mRNA expression; and decreased mRNA expression of MAP kinase phosphatase-1. PTH inhibited SOST mRNA expression 91% in COX-2 KO MSC cultures compared to 67% in WT cultures. We conclude that endogenous PGs inhibit the anabolic responses to PTH *in vitro*, possibly by desensitizing cAMP pathways.

Keywords

prostaglandin; nonsteroidal anti-inflammatory drugs; sclerostin; IGF-1; MKP-1

INTRODUCTION

Parathyroid hormone (PTH) is a major regulator of calcium homeostasis and bone remodeling. *In vivo*, PTH can exert both anabolic and catabolic effects on bone. Intermittent injections of PTH increase bone mass, while continuous infusion of PTH causes bone loss [1,2]. It has been difficult to demonstrate consistent anabolic effects of PTH in cell culture models used to study osteoblastic differentiation *in vitro*. Addition of fresh PTH with each change of media in these cultures generally inhibits osteoblastic differentiation [3–12]. *In vivo*, the anabolic effects of PTH are thought to be mediated largely via the cAMP pathway [13]. *In vitro*, however, some studies suggest agents that increase cAMP may inhibit osteoblast differentiation [6].

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Prostaglandins (PGs) are local factors whose production in bone is predominantly regulated via the inducible cyclooxygenase-2 (COX-2) [14]. Systemic injection of PGE₂ in rats can increase bone formation and produce substantial increases in bone mass, similar to the effects of PTH [15]. Also similar to PTH, PGE₂ is thought to have its major anabolic effects via the cAMP pathway [16,17]. In contrast to PTH, PGE₂ added continuously to cultures can have anabolic effects, stimulating osteoblastic differentiation in marrow stromal cell (MSC) and primary calvarial osteoblast (COB) cultures [18–20]. The production of endogenous PGs in cultures is stimulated by addition of factors that induce COX-2, such as BMP-2 [21] or strontium ranelate [22], or simply by the addition of fresh serum [23]. Endogenously produced PGs have also been shown to be anabolic *in vitro* [24–26].

PTH is a potent inducer of COX-2 expression and PGE₂ production in osteoblasts [27,28]. The goal of this study was to examine the role of COX-2 in the effects of PTH on osteoblastic differentiation in MSC and COB cultures. We cultured cells from COX-2 wild type (WT) and knockout (KO) mice, treated continuously with PTH, and measured markers of osteoblastic differentiation. PTH was anabolic in cultures from COX-2 KO but not WT mice, an effect that could be mimicked in WT cells by treating with a COX-2 selective inhibitor. We also identified some genes that were differentially regulated by PTH in the COX-2 WT and KO cells.

MATERIALS AND METHODS

Materials

PGE₂ and NS-398 were from Cayman Chemical Company (Ann Arbor, MI). Bovine parathyroid hormone (bPTH; 1–34), 3-isobutyl methyl xanthine (IBMX) and all other chemicals were from Sigma (St. Louis, MO), unless otherwise noted.

Animals

COX-2 KO mice in a C57BL/6, 129SV background were the kind gift of Scott Morham [29]. We backcrossed the mice more than 16 generations into the CD-1 (outbred) background. All animal studies were approved by the Animal Care and Use Committee of the University of Connecticut Health Center.

Cell cultures

For bone marrow stromal cell (MSC) cultures, marrow was flushed from tibiae and femora of 6–8 week old mice with α -MEM (Invitrogen, Carlsbad, CA). 1×10^6 cells/well were plated in 6 well dishes and cultured up to 21 days. For calvarial osteoblast (COB) cultures, calvariae were dissected from 3–5 neonatal mice and digested with 0.5 mg/ml of collagenase P (Roche Diagnostics, Indianapolis, IN) in a solution of 1 ml trypsin/EDTA and 4 ml PBS at 37° C. Four digests were performed for 10 min and a final digest for 90 min. Digests 2–5 were pooled and plated at 4×10^4 cells/well in 6-well dishes and cultured up to 21 days.

Culture medium was α -MEM with 10% fetal calf serum, 100 U/ml penicillin, 50 μ g/ml streptomycin and 50 μ g/ml phosphoascorbate (Wako Pure Chemical Industry, Osaka, Japan). Cells were cultured in a humidified atmosphere of 5% CO₂ at 37° C. Media were changed every 3–4 days. 10 mM of β -glycerophosphate was added to the medium on day 7 for the duration of the experiment. Vehicle, 0.1% ethanol or 0.1% bovine serum albumin, was added to control cultures.

For the cAMP analysis and the short term gene expression studies, COBs were expanded by one replating and then grown until confluence before being treated with PTH or vehicle. For these experiments, all treatments were pulsed into wells to avoid disturbances associated with medium changes.

Real-time (quantitative) PCR analysis

RNA was extracted with Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's directions. 3–5 µg of total RNA was DNase treated (Ambion, Inc., Austin, TX) and converted to cDNA using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). PCR was performed in 96-well plates using Assays-on-Demand Gene Expression system (Applied Biosystems). Primers were checked for equal efficiency over a range of target gene concentrations. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was the endogenous control. Each sample was amplified in duplicate. The PCR reaction was run in Applied Biosystems ABI Prism 7300 Sequence Detection System instrument utilizing universal thermal cycling parameters. The quantification of target gene in the samples and in the calibrator (pooled cDNA from multiple experiments) and the quantification of GAPDH were determined from standard curves calculated by serially diluting total RNA. The target gene (normalized to GAPDH) was then compared to the calibrator (normalized to GAPDH).

Alizarin red staining

To assess mineralization, cells were washed with PBS, fixed in 100% V/V methanol on ice for 30 min and stained with 40 mM alizarin red-S (Sigma) pH 4.2 for 10 min at room temperature. Dishes were washed with water, air dried and scanned into the computer.

cAMP measurement

Confluent COBs were treated with 0.5 mM of isobutyl methyl xanthine (IBMX) for 30 min prior to adding PTH (10 nM) or PGE₂ (1 µM) for 15 and 60 min. Cells were scraped off in 400 µl of ice-cold ethanol, the ethanolic cell suspension collected in tubes and centrifuged at 1500 g for 10 min at 4 °C. Supernatants were collected and evaporated to dryness using a lyophilizer. cAMP was measured using an enzyme-immunoassay kit (Cayman Chemical, Ann Arbor, MI).

Statistics

Values are reported as the mean ± SEM of 3 wells of cells. Statistical analysis was performed using SigmaStat® version 2.03 (San Rafael, CA). Group differences were examined by two-way ANOVA followed by post-hoc Bonferroni test.

RESULTS

Osteoblastic differentiation in MSC and COB cells

Consistent with previous studies [22,26], there was a trend for the basal expression of osteoblastic markers to be lower in COX-2 KO MSC (Fig. 1A,B) and COB (Fig. 1C,D) cultures compared to WT cultures. There was no effect of PTH on ALP or OCN mRNA expression in either MSC or COB WT cultures (Fig. 1A–D). PTH increased ALP and OCN expression 6.5-fold and 7-fold, respectively, in KO MSC cultures (Fig. 1A,B) and 2.1-fold and 4.4-fold, respectively, in KO COB cultures (Fig. 1C,D). PTH had no effect on mineralization in COX-2 WT MSC or COB cultures but increased mineralization in cultures from COX-2 KO mice (Fig. 1E,F).

We also treated cultures with PGE₂ (1 µM). PGE₂ increased ALP and OCN expression 1.5-fold and 3.1-fold, respectively, in WT MSC cultures and 6.7-fold and 9-fold, respectively, in KO MSC cultures (Fig. 1A,B). In COB cultures, PGE₂ increased ALP and OCN mRNA levels only in KO cultures (5.2-fold and 4.5-fold, respectively) (Fig. 1C,D). PGE₂ also increased mineralization in both MSC and COB KO cultures (Fig. 1E,F).

Most PGE₂ produced in marrow or COB cultures is associated with COX-2 expression [21–24,26]. MSC cultures from COX-2 WT mice were treated with PTH or vehicle in the presence

and absence of NS-398 (0.1 μ M), a selective inhibitor of COX-2 activity. Similar to the results observed in COX-2 KO cultures, basal osteoblastic differentiation tended to be decreased in NS-398 treated cultures compared to control cultures (Fig. 2). PTH treatment either decreased or had no effect on ALP and OCN mRNA expression in control cultures but stimulated ALP and OCN mRNA expression 2.2-fold and 3.1-fold, respectively, in NS-398 treated cultures (Fig. 2A,B). PTH also increased mineralization only in NS-398 treated cultures (Fig. 2C).

PTH-stimulated cAMP production and early response gene expression in COX-2 WT and KO COBs

COBs were treated with PTH (1 nM) and PGE₂ (1 μ M) for 15 and 60 min in the presence of IBMX (Fig. 3A,B). There was no difference between basal cAMP concentrations in WT and KO COBs, and PTH increased cAMP concentrations similarly in COX-2 WT and KO COBs in both the experiments. Similar lack of difference between WT and KO responses was seen with lower doses of PTH (0.01–1.0 nM) in a second experiment (data not shown). The PTH-stimulated fold increases in cAMP tended to be higher in KO cells than in WT cells (Fig. 3B) but this was not true in the second experiment (data not shown). A marked increase was observed in cAMP levels (both absolute and relative) in PGE₂-treated COX-2 KO cultures compared to WT cultures.

Also in COBs, we examined short term (1–24 h) effects of PTH on the expression of several early response genes thought to be regulated by PTH via the cAMP pathway and possibly involved in its anabolic effects, including c-fos [30]; Nr4a2 (a member of the Nurr1 orphan nuclear receptor family) [31]; and receptor activity modifying protein-3 (RAMP-3) [32]. The absolute levels of PTH-stimulated gene expression in the KO COBs were either similar to or lower than that in WT COBs (Fig. 3C–E). However, because basal gene expression tended to be lower in KO cells, the PTH-stimulated fold increases were higher in the KO cells. Peak PTH-stimulated fold increases in WT and KO cells were 5.3 and 10.9, respectively, for c-fos; 12.2 and 29.2, respectively, for Nr4a2; and 7.9 and 9.8, respectively, for RAMP3. Another early response gene regulated via the cAMP pathway is mitogen activated protein kinase phosphatase -1 (MKP-1), a dual specificity phosphatase that can dephosphorylate ERK [33]. PTH significantly stimulated MKP-1 mRNA levels in WT but not in KO cells (Fig. 3F).

Expression of other genes potentially mediating anabolic effects in COX-2 WT and KO cells

PTH receptor (PTH1R) mRNA was elevated in control KO MSCs compared to control WT MSCs, but treatment with PTH resulted in similar down regulation of PTH1R mRNA in both WT and KO MSCs (Fig. 4A). Sclerostin, the product of the SOST gene, is an inhibitor of bone formation predominantly expressed in osteocytes *in vivo* [34,35]. At 21 days in MSC cultures, the PTH inhibition of SOST mRNA expression tended to be greater KO cells (–91%) than in WT cells (–67%) (Fig. 4B). We found no COX-1 mRNA compensation for absent COX-2 in COBs (Fig. 4C) or MSCs (data not shown).

In short term cultures of COBs, we examined mRNA expression for Runx2, a transcription factor essential for osteoblastic differentiation [36]. Runx2 mRNA was lower in KO cells, and PTH stimulated Runx2 mRNA expression in both WT and KO COBs similarly (2.2-fold and 1.9-fold, respectively) (Fig. 4D). PTH-stimulated insulin-like growth factor (IGF)-1 has been reported to be necessary for the anabolic effects of PTH [37]. PTH increased IGF-1 mRNA only in KO COBs during the 1–24 h time period (Fig. 4E). Bone morphogenetic protein (BMP)-2 mRNA expression was not regulated by PTH over the 1–24 h time period and was markedly lower in KO COBs than in WT COBs (Fig. 4F). Since the COBs for these short term experiments were grown to confluence before being treated with PTH, the differences in basal expression may reflect differences in the state of differentiation between WT and KO cells at the time of treatment.

DISCUSSION

Despite the anabolic effects of PTH *in vivo*, it has been difficult to demonstrate anabolic effects of PTH *in vitro*. In the present study, PTH was anabolic in the absence of COX-2 expression or activity, suggesting that endogenous PGs inhibit the anabolic effects of PTH *in vitro*. This finding is in contrast to studies with BMP-2 [21] and strontium ranelate [22] where induction of COX-2 enhances their anabolic effects. The anabolic effects of exogenous PGE₂ were also markedly increased in COX-2 KO cultures. Since PTH and PGE₂ are thought to act predominantly via the cAMP pathway, it is possible that endogenous PGE₂ desensitizes cAMP responses to PTH. Both fresh serum [23] and PTH [27,28] can induce COX-2 expression in these cultures and both could be contributing to the inhibitory effects of COX-2 expression. Heterologous desensitization of PTH1R by PGE₂ has been shown in some studies [38,39] but not in others [40]. There was increased PTH1R mRNA expression in COX-2 KO MSC control cultures compared to WT, but treatment with PTH resulted in marked inhibition of PTH1R mRNA in both WT and KO cells.

Also arguing against PTH receptor desensitization or downregulation is that we did not find any significant differences between PTH-stimulated cAMP concentrations in COX-2 WT and KO cells. However, cAMP measurements at 15 and 60 min of PTH treatment may not reflect the effects of PTH-stimulated COX-2 expression since the PGE₂ produced by PTH induction should take longer than 60 min to reach peak concentrations. In contrast to the PTH, PGE₂-stimulated cAMP concentrations were significantly increased in KO cells, consistent with homologous desensitization of PGE₂ receptors by basal levels of PGE₂ in the culture. These data suggest that if there is desensitization of the PTH stimulated cAMP pathway by PGs, it occurs downstream of PTH1R and cAMP accumulation.

Comparison of the PTH stimulation of early response genes (Nr4a2, RAMP3, and c-fos) thought to be mediated largely via the cAMP pathway suggested that PTH-stimulated fold-increases but not absolute levels were greater in KO COBs than in WT COBs. This observation might suggest greater sensitivity of cAMP responses to PTH in KO cells, but the relevance of fold-increase versus absolute levels for the anabolic effects of PTH is unclear. One early response gene that was clearly differentially regulated was MKP-1. PTH may have some of its actions via the ERK pathway [33,41]. MKP-1 can inactivate ERK, and decreased PTH induction of MKP-1 mRNA expression could be associated with a more sustained induction of ERK activity in KO cells. There was also a trend toward greater PTH inhibition of SOST mRNA expression in COX-2 KO MSCs compared to WT MSCs and increased PTH-stimulated IGF-1 mRNA in COX-2 KO COBs compared to WT COBs. Both of these genes have been implicated in the anabolic effects of PTH [34,35,37].

We have also shown that intermittent PTH (1–34 hPTH, 80 µg/kg, given subcutaneously daily for 22 days) has greater anabolic effects on the skeleton in COX-2 KO mice than in COX-2 WT mice [42]. We believe that this cell culture model will be useful for studying the mechanisms by which PTH has its anabolic effects and also the role of PGs in modulating the effects of PTH.

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Figure 1.

Marrow stromal cell (MSC) and primary calvarial osteoblast (COB) cultures from COX-2 WT and KO mice treated with PTH (10 nM), PGE₂ (1 μ M) or vehicle (Veh). Relative quantification by real time PCR of mRNA of alkaline phosphatase (ALP) on d 14 and osteocalcin (OCN) on day 21 in MSCs (A, B) and COBs (C,D). Alizarin red staining for mineralization on day 21 in (E) MSCs and (F) COBs. Bars are means $\pm$ SEM for n=3. ^aSignificant effect of treatment, p <0.01. ^bSignificant effect of genotype, p <0.01; ^cp <0.05.

Figure 2.

MSC cultures from COX-2 WT mice treated with PTH (10 nM) or vehicle (Veh) in the presence and absence of NS-398 (0.1 μ M). Real time PCR for (A) ALP mRNA on day 14 and (B) OCN mRNA expression on day 21. (C) Alizarin red staining for mineralization on day 21. Bars are means $\pm$ SEM for n=3. ^aSignificant effect of PTH, $p < 0.01$; ^b $p < 0.05$. ^cSignificant effect of NS-398, $p < 0.01$; ^d $p < 0.05$.

Figure 3.

Measurement of intracellular cAMP concentration and early response genes in COX-2 WT and KO COB cultures. Cultures were treated with PTH (10 nM), PGE₂ (1 μ M) or vehicle (V) for the indicated times in the presence of IBMX (0.5 mM). (A) cAMP concentration. (B) Fold increase in PTH- or PGE₂-stimulated cAMP relative to vehicle-treated controls. (C-F) mRNA expression of selected genes in cultures analyzed by real time PCR. Each bar is the mean $\pm$ SEM of n = 3 samples. ^aSignificant effect of treatment, p < 0.01; ^bp < 0.05. ^cSignificant effect of genotype, p < 0.01; ^dp < 0.05.

Figure 4.

Measurement of selected gene responses to PTH in COX-2 WT and KO cultures treated with PTH (10 nM) or vehicle (V) for the indicated times. Real time PCR in MSC cultures (A-B) and in COB cultures (C-F). Each bar is the mean $\pm$ SEM of $n = 3$ samples. ^aSignificant effect of PTH, $p < 0.01$; ^b $p < 0.05$. ^cSignificant effect of genotype, $p < 0.01$.