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Prostaglandin-mediated inhibition of PTH-stimulated β-catenin signaling in osteoblasts by bone marrow macrophages

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ABSTRACT

Bone marrow macrophages (BMMs), in the presence of cyclooxygenase-2 (Cox2) produced PGE_2, secrete an inhibitory factor in response to Rankl that blocks PTH-stimulated osteoblastic differentiation. This study was to determine if the inhibitory factor also blocks PTH-stimulated Wnt signaling. Primary calvarial osteoblasts (POBs) were co-cultured with conditioned medium (CM) from Rankl-treated wild type (WT) BMMs, which make the inhibitory factor, and Cox2 knockout (KO) BMMs, which do not. PTH induced cAMP production was blocked by WT CM but not by KO CM. In the presence of KO CM, PTH induced phosphorylation at β-catenin serine sites, ser552 and ser675, previously shown to be phosphorylated by protein kinase A (PKA). Phosphorylation was blocked by WT CM and by H89, a PKA inhibitor. PTH did not increase total β-catenin. PTH-stimulated transcription factor/lymphoid enhancer-binding factor response element activity in POBs was blocked by WT CM and by serum amyloid A (SAA), the human recombinant analog of murine Saa3, which has recently been shown to be the inhibitory factor. In POBs cultured with Cox2 KO CM, PTH increased expression of multiple genes associated with the anabolic actions of PTH and decreased expression of Wnt antagonists. This differential regulation of gene expression was not seen in POBs cultured with WT CM. These data highlight the ability of PTH to phosphorylate β-catenin directly *via* PKA and demonstrate the ability of a Cox2-dependent inhibitory factor, secreted by Rankl-stimulated BMMs, to abrogate PTH stimulated β-catenin signaling. Our results suggest that PTH can stimulate a novel negative feedback of its anabolic actions by stimulating Rankl and Cox2 expression.

1. Introduction

Parathyroid hormone (PTH) is a potent regulator of bone homeostasis—able to stimulate both bone formation and resorption by stimulating the differentiation of both osteoblast and osteoclast populations [1,2]. Intermittent therapy with human 1–34 PTH (teriparatide) was the first FDA approved anabolic therapy for osteoporosis [3]. In contrast, continuous PTH administration or elevation has been shown to cause bone loss [2]. PTH acts *via* its receptor PTH1R, a G-protein coupled receptor that is highly expressed by osteoblasts and that activates both $G\alpha_s$ and $G\alpha_q$ signaling pathways [4]. $G\alpha_s$ activates adenylyl cyclase resulting in cAMP production and protein kinase A (PKA) activation, while $G\alpha_q$ leads to activation of protein kinase C (PKC) and release of Ca^{2+}. The anabolic effects of PTH are thought to be mediated *via* $G\alpha_s$ [5,6].

PTH is also known to increase the expression of cyclooxygenase 2 (Cox2), an inducible enzyme responsible for acute production of prostaglandin E_2 (PGE_2), and PGE_2 can also stimulate both bone formation and resorption [7–10]. We observed a Cox2-dependent inhibition of PTH's osteogenic and anabolic actions both *in vitro* and *in vivo* [11–13]. *In vitro*, in bone marrow stromal cell (BMSC) cultures that have precursors for both osteoclast and osteoblast lineage, PTH can stimulate osteoblast differentiation in Cox2 KO BMSCs but not in WT BMSCs [11]. In these studies, PGE_2, produced either by Cox2 in osteoblasts or Cox2 in bone marrow macrophages (BMMs), acted *via* the EP4 receptor to cause BMMs, committed to the osteoclastic lineage by Rankl, to secrete a factor that inhibited the PTH stimulation of osteoblastic differentiation [11]. *In vivo* studies showed that there was a greater anabolic effect of intermittent PTH in Cox2 KO mice compared to WT mice [12]. However, there was a more dramatic effect of Cox2 when PTH was administered continuously. In WT mice, PTH infusion was catabolic, while in Cox2 KO mice it was markedly anabolic [13]. Cox2 is a rapidly and transiently inducible gene, and PGE_2 is rapidly metabolized *in vivo* [7]. Thus, with intermittently injected PTH, PGE_2 is expected to be increased only transiently. With the continuous infusion of PTH, both Cox2 and PGE_2 were expected to be persistently elevated and, indeed, Cox2 mRNA in bone

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and PGE_2 in serum were found to be elevated after 12 days of infusion [13].

The inhibitory factor produced by the BMMs has recently been shown to be serum amyloid A3 (Saa3) [14]. Saa3 was found to be produced by the BMMs in a Cox2-dependent manner only when stimulated with Rankl, which drives these cells towards the osteoclast lineage. Saa3 was produced by Rankl-stimulated BMMs prior to the appearance of tartrate resistant acid phosphatase positive (TRAP+) multinucleated osteoclast-like cells, and Saa3 was produced only by the flow-sorted osteoclast precursor population, suggesting that Saa3 was produced by the preosteoclasts [14].

cAMP-dependent PKA has been shown to cause the phosphorylation of β-catenin at two distinct sites: serine 552 (Ser552) and serine 675 (Ser675) [15,16]. These sites have been linked to increases in β-catenin mediated transcriptional activity. Traditionally, β-catenin has been linked with osteoblast differentiation *via* the canonical Wnt pathway, in which Wnt agonists act *via* Lrp5/6 and Frizzled receptors/co-receptors on the β-catenin-destruction-complex to prevent the ubiquitination and proteolysis of β-catenin [17]. The cAMP/PKA pathway provides for a novel Wnt-independent mechanism in which β-catenin proteins are acted upon directly to increase their signaling efficacy. PTH signaling has recently been shown to phosphorylate β-catenin at Ser552 in a cAMP-dependent manner and to increase the downstream transcriptional activity of β-catenin *via* this pathway [18].

The goal of the current study was to investigate the effects of the Cox2-dependent inhibitor on PTH-stimulated β-catenin signaling. We cultured POBs with conditioned medium (CM) from Rankl-treated WT BMMs, which produce the inhibitor, and Cox2 KO BMMs, which do not. Using this model we demonstrated that PTH stimulation of (1) phosphorylation of β-catenin at Ser552/675, (2) β-catenin transcriptional activity and (3) expression of genes thought to mediate osteoblast differentiation were blocked by the Cox2/PGE_2–dependent inhibitor produced by Rankl-stimulated BMMs.

2. Materials and methods

2.1. Materials

Bovine PTH (bPTH; 1–34) was obtained from Sigma-Aldrich (St. Louis, MO). Forskolin (cAMP agonist), H-89 (PKA inhibitor) and GF109203X (PKC inhibitor) were obtained from Enzo Life Sciences (Farmingdale, NY). Human recombinant SAA (Apo-SAA), which corresponds to human Apo-SAA1α, except for the presence of an N-terminal methionine and two substituted residues present in Apo-SAA2β, was purchased from PeproTech (Rocky Hill, NJ). Antibody for Actin C-11 (sc-1615) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for phospho-β-catenin Ser552 (#9566), phospho-β-catenin Ser675 (#9567), phospho-β-catenin Ser33/37/Thr41 (#9561), and amino terminal β-catenin (#9581) were obtained from Cell Signaling Technology (Danvers, MA).

2.2. Animals

Mice that produce non-functional Cox2 protein, due to disruption of *Ptgs2* in a C57BL/6, 129SV background, which we call Cox2 KO mice, were the gift of Scott Morham [19]. Cox2 KO mice were backcrossed into the outbred CD-1 background [12]. Following 20 generations of backcrossing, the Cox2 KO mice no longer developed either renal failure or female infertility [12]. Maintenance colonies heterozygous for the Cox2 gene disruption were refreshed twice a year with WT mice from Jackson Laboratory (Bar Harbor, ME) to prevent genetic drift. Mice are genotyped as described previously, and experimental mice are bred by WT × WT or KO × KO mating [12]. Animal studies were performed in compliance with protocols approved by the Animal Care and Use Committee of UConn Health.

2.3. Cell culture

All cell cultures were grown in humidified incubation conditions of 5% CO_2 at 37 °C. Basic medium was 10% heat inactivated fetal calf serum (HIFCS), 100 U/mL penicillin, and 50 μg/mL streptomycin in α-MEM (Invitrogen, Carlsbad, CA). Osteoblast differentiation medium was basic media supplemented with 50 μg/mL phosphoascorbate. Treatment vehicles were the following: 0.001 N hydrochloric acid-acidified 0.1% bovine serum albumin (BSA) in 1× phosphate buffered saline (PBS) for PTH; 0.1% BSA in 1× PBS for osteoprotegerin (OPG), macrophage colony stimulating factor (M-CSF), and receptor activator of nuclear factor κ-B ligand (Rankl); and dimethyl sulfoxide (DMSO) for isobutyl methyl xanthine (IBMX), H-89, GF109203X, and forskolin.

2.4. Primary osteoblasts (POBs)

POBs were harvested from calvariae of neonatal mice. Sutures were removed and the calvariae were minced, washed multiple times with 1× PBS, and subsequently digested with 0.5 mg/mL collagenase P (Roche Diagnostics, Indianapolis, IN) solubilized in 1 mL trypsin/EDTA and 4 mL PBS at 37 °C. Four 10 min digests were performed followed by a fifth and final digest for 90 min. After each digest the reaction was halted by the addition of 10% HIFCS. Cells from digests 2–5 were collected, filtered through a Nitex membrane (Millipore, Bedford, MA), and plated at a density of 50,000 cells/well in 6-well cell culture plates in differentiation medium. Medium was changed every three days. We used only freshly plated cells for all experiments.

2.5. Culture of conditioned medium (CM) with POBs

CM was collected from BMM cultures, centrifuged for 5 min at 800 rpm at 4 °C to remove debris and frozen for later use. BMMs were cultured following the Faccio protocol: http://www.orthoresearch.wustl.edu/content/Laboratories/2978/Roberta-Faccio/Faccio-Lab/Protocols.aspx. BMMs were obtained from 8 week old mice. In brief, nucleated bone marrow cells were plated in 150 mm petri dishes (Fisher Scientific, Pittsburgh, PA) at a density of 1×10^7 cells/dish in basic medium, supplemented with 100 ng/mL M-CSF. Cultures were expanded twice for three days each. Following expansion, BMMs were re-plated in 12-well cell culture plates at a density of 6×10^4 cells/well in basic medium and treated with M-CSF and Rankl (both at 30 ng/mL). CM was collected after 3 days of culture, a day before tartrate resistant acid phosphatase positive multinucleated cells formed in the BMM cultures. CM was added to Cox2 KO POBs 2 h before agonist treatment at a concentration of 3 parts CM to 1 part differentiation medium. The only exception was for the study of differentiation, where treatments with agonists and CM were begun at the time of plating and continued for the entire 14 day period. Unless noted otherwise, all cultures were treated with 50 ng/mL OPG to prevent Rankl in the CM or PTH-stimulated Rankl in the POBs from inducing any remaining hematopoietic cells in the POB cultures from becoming osteoclasts and making more of the inhibitory factor [11].

2.6. Intracellular cAMP measurement

On day five of culture, Cox2 KO POBs were treated with 3 parts WT or KO CM and 1 part differentiation medium for 2 h, followed by 0.5 mM IBMX for 45 min, and PTH (10 nM) and FSK (10 μM) for 15 min. For extraction, 400 μL of ice-cold ethanol was added to each well and the cultures were detached from the plate by scraping and collected in 1.5 mL centrifuge tubes. Samples were then centrifuged at 1500 ×*g* for 10 min at 4 °C. The supernatant was collected and lyophilized and cAMP concentration was determined using an enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI).