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Effects of Parathyroid Hormone on Wnt Signaling Pathway in Bone

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Abstract The Wnt signaling pathway has recently been demonstrated to play an important role in bone cell function. In previous studies using DNA microarray analyses, we observed a change in some of the molecular components of the canonical Wnt pathway namely, frizzled-1 (FZD-1) and axil, in response to continuous parathyroid hormone (PTH) treatment in rats. In the present study, we further explored other components of the Wnt signaling pathway in rat distal metaphyseal bone in vivo, and rat osteoblastic osteosarcoma cells (UMR 106) in culture. Several Wnt pathway components, including low-density lipoprotein-receptor-related protein 5 (LRP5), LRP6, FZD-1, Dickkopf-1 (Dkk-1), and Kremen-1 (KRM-1), were expressed in bone in vivo and in osteoblasts in vitro. Continuous exposure to PTH (1–38) both in vivo and in vitro upregulated the mRNA expression of LRP6 and FZD-1 and decreased LRP5 and Dkk-1. These effects in UMR 106 cells were associated with an increase in β-catenin as measured by Western blots and resulted in functional activation (three to six-fold) of a downstream Wnt responsive TBE_6-luciferase (TCF/LEF-binding element) reporter gene. Activation of the TBE_6-luciferase reporter gene by PTH (1–38) in UMR 106 cells was inhibited by the protein kinase A (PKA) inhibitor, H89. Activation was mimicked by PTH (1–31), PTH-related protein (1–34), and forskolin, but both PTH (3–34) and (7–34) had no effect. These findings suggest that the effect of PTH on the canonical Wnt signaling pathway occurs at least in part via the cAMP-PKA pathway through the differential regulation of the receptor complex proteins (FZD-1/LRP5 or LRP6) and the antagonist (Dkk-1). Taken together, these results reveal a possible role for the Wnt signaling pathway in PTH actions in bone. J. Cell. Biochem. 95: 1178–1190, 2005.

Key words: PTH; bone; rat; β-catenin; Wnt signaling

Parathyroid hormone (PTH) plays a central role in the regulation of bone and mineral metabolism [Reeve et al., 1980; Slovik et al., 1981; Hock et al., 1989a,b; Riggs and Melton, 1992; Dempster et al., 1993; Oxlund et al., 1993; Sato et al., 1997; Jerome et al., 1999]. PTH actions on bone formation are associated with an increase in osteoblast activity, increased differentiation of osteoblast-precursors, recruitment of lining cells, and increased osteoblast survival [Dobnig and Turner, 1995; Onyia et al., 1995, 1997; Jilka et al., 1999]. Conversely, the resorptive actions of PTH that are mediated through osteoblasts are due to increases in promoting osteoclast differentiation and activity and fibroblast proliferation [Ma et al., 2001; Lotinun et al., 2002]. Important early events in PTH action following binding to the PTH receptor are the interaction of the receptor with G proteins and adenylyl cyclase leading to the production of cAMP and activation of protein kinase A (PKA) [Scott et al., 1992; Rixon et al., 1994]. This leads to the phosphorylation of various enzymes, ion channels, transcription factors, and target proteins. The cAMP/PKA pathway is the most extensively studied and probably predominant PTH signaling pathway in osteoblasts, nevertheless, some of the biological effects of PTH on osteoblasts are not regulated by the cAMP/PKA pathway suggesting that additional mechanisms/pathways may mediate the action of PTH in bone and bone cells [Potts et al., 1995]. PTH can also activate phospholipase C (PLC), via Gq-coupled

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signaling, initiating hydrolysis of phosphatidylinositol bisphosphate (PIP2) to inositol trisphosphate (IP3) and diacylglycerol (DAG) to activate protein kinase C (PKC) [Civitelli et al., 1988; Babich et al., 1991]. Additionally, PTH was recently shown to activate PKC-α via phospholipase D (PLD) [Radeff et al., 2004].

Recently, genetic analysis has implicated the Wnt signaling pathway as an important regulator of bone mass. Wnts are secreted lipid-modified glycoproteins that are involved in cell growth, differentiation, and apoptosis in both embryos and adults [Wodarz and Nusse, 1998; Uusitalo et al., 1999; Westendorf et al., 2004]. Canonical Wnt signaling is transduced by a receptor complex comprised of a member of the family of seven transmembrane domain receptors known as Frizzleds, and the co-receptor lipoprotein-receptor-related proteins 5 or 6 (LRP5/6) [Yang-Snyder et al., 1996; He et al., 1997; Slusarski et al., 1997; Wang et al., 1997; Hsieh et al., 1999a; Pinson et al., 2000; Mao et al., 2001a,b]. The resulting signal leads to the activation of the cytoplasmic protein dishevelled (Dsh), disrupting the protein complex of axin, adenomatous polyposis coli (APC), and glycogen synthase kinase-3β (GSK-3β). As a result, unphosphorylated β-catenin accumulates, translocates to the nucleus, forms a complex with T-cell factor/Lymphoid enhancer factor (TCF/LEF), which then regulates the transcription of Wnt target genes [Wodarz and Nusse, 1998; Uusitalo et al., 1999].

Several classes of secreted factors regulate Wnt action outside of the cell. These include a family of secreted frizzled-related proteins (sFRP) [Moon et al., 1997; Zorn, 1997], Wnt inhibitory factor-1 (WIF-1) [Hsieh et al., 1999b], and Dickkopf proteins [Glinka et al., 1998; Zorn, 2001]. Dickkopf-1 (Dkk-1), a prototypical member of Dkk-related genes, blocks LRP5/6 mediated Wnt/β-catenin signaling by interacting with domains that are distinct from those required for Wnt/Frizzled interaction [Krupnik et al., 1999; Bafico et al., 2001; Semenov et al., 2001; Brott and Sokol, 2002]. Kremen (KRM) is a member of a family of transmembrane proteins (KRM-1 and 2), that function as Dkk receptors and cooperate with Dkk to block Wnt-β-catenin signaling [Davidson et al., 2002; Mao et al., 2002].

Activating mutations in LRP5 have been shown to result in a high bone mass phenotype associated with increased bone formation and no other obvious abnormalities in humans and animals, whereas inactivating mutations in LRP5 result in osteoporosis-pseudoglioma syndrome (OPPG) [Gong et al., 2001; Boyden et al., 2002; Little et al., 2002; Babij et al., 2003; Wesenbeeck et al., 2003]. In mice, targeted disruption of LRP5 leads to a low bone mass phenotype and persistent embryonic eye vascularization due to failure of macrophage-induced endothelial cell apoptosis [Kato et al., 2002]. Biochemical and expression studies have shown the essential role of LRP5 as a Wnt co-receptor with frizzled in mediating canonical Wnt signaling pathway [Akiyama, 2000; Seidensticker and Behrens, 2000; Mao et al., 2001a; Nykjaer and Willnow, 2002].

Previously, in the course of the analysis by microarrays of genes regulated by continuous PTH in vivo an increase in frizzled-1 (FZD-1) and decrease in axil, two components of the Wnt signaling pathway were observed [Onyia et al., 2001]. Given the important roles of both PTH and the Wnt pathway in bone biology, the present study validated the effect of PTH on FZD-1 and demonstrated the regulation of other genes involved in Wnt signaling. To investigate the role of PTH on the canonical Wnt signaling pathway, we examined the effect of PTH (1–38) on the regulation of signaling receptor FZD-1, co-receptor LRP5/6, secreted antagonist Dkk-1, and the Dkk-1 receptor KRM-1, key players in this pathway both in vivo and in vitro. Since stimulation of the canonical Wnt pathway results in the accumulation of β-catenin, we also analyzed the effect of PTH on the levels of β-catenin, and assessed the further downstream Wnt signaling response on a Wnt responsive TBE_6-luciferase reporter gene. We demonstrate that PTH (1–38) treatment results in the differential regulation of the receptor complex, FZD-1/LRP5/6 with an associated decrease in the antagonist Dkk-1. We also show that PTH (1–38) increases the levels of β-catenin and induces the further downstream Wnt signaling response. PTH effects on Wnt responsive TBE_6-luciferase reporter were mimicked by PTH (1–31), PTH-related protein (1–34) [PTHrP (1–34)], and forskolin, activators of cAMP/PKA pathway and unaffected by analogs of PTH (3–34) and (7–34) that do not stimulate cAMP synthesis. The PTH effects were inhibited by H89, an inhibitor of PKA. Our results suggests that PTH actions on canonical Wnt signaling occur at least in part through the

cAMP pathway and could be sustained through altered expression of key components of the Wnt pathway.

MATERIALS AND METHODS

Biochemicals

Synthetic human PTH (1–38), PTH (1–31), PTHrP (1–34), and bovine PTH (3–34) and (7–34) were obtained from Bachem, Torrence, CA. All peptides were prepared in a vehicle of acidified saline containing 2% heat-inactivated rat serum. Forskolin and dibutyryl cAMP were purchased from Sigma Chemical Company (St. Louis, MO). IL-1α obtained from R&D Systems, Inc., (Minneapolis, MN) was resuspended in phosphate buffered saline (PBS) containing 0.1% bovine serum albumin. PKA inhibitor H89 was obtained from Calbiochem (San Diego, CA).

Animals

To explore PTH effects we used parathyroidectomized (PTX) rats which show increased or exaggerated response to exogenous PTH. The increase in sensitivity in this model obviates the challenge of validating moderate but significant changes in animals with intact parathyroids, and reduces the number of animals required for in vivo validation. Female 6-month-old PTX Sprague–Dawley rats used in these studies were obtained from Harlan Sprague–Dawley, Inc., (Indianapolis, IN) 2–3 days post surgery. Rats were maintained under controlled conditions on a daily photoperiod of 12-h light–dark cycle, 50% humidity at 21°C temperature. Rats were fed Purina chow (1% calcium and 0.61% phosphate; PMI Feeds, Inc., St. Louis, MO) and water *ad libitum*. All animal procedures received prior approval by the Lilly Animal Care and Use Committee (Eli Lilly & Co., Indianapolis, IN).

In Vivo Protocols

Rats were weighed and randomized into groups of comparable mean body weight (five rats/group). PTH was prepared in a vehicle of acidified saline containing 2% heat-inactivated rat sera. The rats received continuous subcutaneous infusion of human PTH (1–38) at a dose level of 20 μg/100 g/24 h for 1, 3, 6, and 24 h using an Alzet mini pump (no. 20011, Durect-Corp, Palo Alto, CA). Control rats received an equal amount of the PBS vehicle containing 2% of heat-inactivated serum. Following euthanasia the femora were resected and all connective tissues, including periosteum, were completely removed. The distal epiphysis, including the growth plate, was removed and a subjacent 3 mm wide band of the metaphyseal primary spongiosa was resected and frozen in liquid nitrogen until further mRNA analysis.

Cell Cultures

The rat osteosarcoma cell line, UMR 106 was maintained in DMEM/Ham's F-12 (3:1) (Life Technologies, Inc., Grand Island, NY) containing 10% fetal bovine serum (FBS) (HyClone Laboratories, Inc., Logan, UT), 2 mM glutamine, and 10 mM HEPES buffer (Life Technologies, Inc.). ROS 17/2.8, a rat osteosarcoma cell line was maintained in the growth medium F-12 nutrient mixture (Life Technologies, Inc.) containing 10% FBS and 2 mM glutamine (Life Technologies, Inc.). Primary osteoblast cultures were derived from the rat distal femur metaphysis and diaphysis as previously described [Alvarez et al., 1997; Onyia et al., 1997, 1998]. All cultures were maintained in a humidified 5% CO_2 atmosphere at 37°C. For mRNA analysis, cultures (4-T150 flasks/group) of cells were grown as described above to 80%–90% confluence and then switched to media containing 0.1% FBS overnight. UMR 106 cells were treated with human PTH (1–38) at a concentration of 5×10^{-8}M for 1, 6, 24, and 48 h. The UMR 106 cells were also treated for 1 h with forskolin at a concentration of 10^{-5} or 10^{-6}M, or dibutyryl cAMP (10^{-4}M), or IL-1α (50 ng/ml).

Isolation of RNA and Northern Blot Analysis

At the end of each treatment, tissue samples were removed from the animals, snap frozen in liquid nitrogen as pooled treated or control groups of five animals/group. Total RNA was isolated from the distal femur metaphyseal primary spongiosa of vehicle or PTH-treated rats or as previously described [Onyia et al., 1995; McClelland et al., 1998] by homogenization in Ultraspec-II™ reagent (Biotecx, Houston, TX) using an LS 10–35 Polytron homogenizer (Brinkmann Instruments, Westbury, NY) as recommended by the manufacturer. RNA was isolated from osteoblast or osteosarcoma cultures by adding Ultraspec-II directly to the culture flasks. The resulting cell lysates were passed several times through a 10-ml pipette before collection. Poly A^+ RNA was isolated from total RNA using Oligotex resin (Qiagen, Santa

Clarita, CA) according to manufacturer's protocol and quantified by spectrophotometry. The absorbance at 260 nm was determined, and the 260/280 nm absorbance ratio was used to ensure RNA quality. Two micrograms of Poly A^+ RNA were denatured in 0.04M 3-(*N*-morpholino) propanesulfonic acid (pH 7.0), 10 mM sodium acetate, 1 mM EDTA, 2.2M formaldehyde, and 50% formamide at 60°C for 10 min; size fractionated by electrophoresis through 1% agarose gels in 2.1M formaldehyde and 1× 3-(*N*-morpholino) propanesulfonic acid; and transferred to nylon membranes (Brightstar-Plus, Ambion, Inc., Austin, TX). The nylon membranes were air-dried, and the samples were cross-linked to the membranes by UV irradiation in a Stratalinker (Stratagene, La Jolla, CA). LRP5, LRP6, Dkk-1, FZD-1, KRM-1, and GAPDH cDNAs were used to generate radioactive probes using the Random Primer DNA labeling kit (Invitrogen, Carlsbad, CA). Twenty-five nanograms of DNA probes were labeled using [α-^{32}P] dCTP (Amersham Pharmacia Biotech). The unincorporated nucleotides were removed by centrifugation through a Centricon-50 column (Amicon, Bedford, MA). Prehybridization and hybridization were carried out at 48°C in NorthernMax buffers (Ambion, Inc.) for 16 h. Following hybridization, membranes were washed for 30 min at room temperature in buffer containing 2× SSC and 0.1% SDS, then for 30 min at 48°C in 0.2× SSC and 0.1% SDS. The membranes were exposed to Biomax MS X-ray film (Eastman Kodak Co.) at −70°C. Autoradiograms were quantitated by scanning laser densitometry (2400 Gel Scan XL, LKB, Piscataway, NJ). Labeled bands were quantitated as densitometric units and normalized to that of GAPDH signals used as internal control. The data were expressed as percent fold-change over untreated controls.

Levels of β-Catenin in Whole Cell Lysates

UMR 106 cultures grown as described were seeded at 2.5×10^6 cells onto T-25 flask in 0.5% serum containing media and allowed to attach overnight at 37°C. The following day, the cells were treated for 1, 3, 6, and 24 h at a concentration of 10^{-8}M PTH (1–38). At the end of treatment, the cells were washed once with PBS. Following PBS wash, the cell pellets were Dounce homogenized using a disposable microcentrifuge pestle in 300 μl freshly prepared lysis buffer containing 10 mM K_2HPO_4, pH 7.2, 1 mM EDTA, 5 mM EGTA, 10 mM $MgCl_2$, 50 mM β-glycerophosphate, 1 mM Na_3VO_4, 2 mM DTT, 1% Triton X-100 protease inhibitors (complete protease inhibitor tablet, Roche, Indianapolis, IN). Lysates were incubated on ice for 30 min and centrifuged at 14,000 rpm for 30 min at 4°C. The supernatant was transferred to a clean tube and the total protein concentration was determined by BCA method (Pierce, Rockford, IL). Five micrograms of total protein in NuPage sample buffer added to a final concentration of 1× was separated on a 10% NuPage gel (Invitrogen, Carlsbad, CA). Following electrophoresis, the protein was transferred to nitrocellulose membrane. The nonspecific binding was blocked using 5% dry milk in PBS containing 0.5% Tween 20 (PBST) for 1 h at room temperature. The blots were incubated, in mouse monoclonal β-catenin antibody (Transduction Labs, San Jose, CA) at 1:1,000 dilution in PBST containing 5% dry milk overnight at 4°C, with gentle agitation. As a control for equal loading of protein anti-MAP kinase ½ (Erk1/2-CT) rabbit polyclonal from Upstate Biotechnology (Waltham, MA) were added at a dilution of 1:5,000 in PBST. Next day, the blots were washed for three times in PBST for 5 min at room temperature and incubated with horse radish peroxidase (HRP) conjugated secondary antibody (NEB anti-mouse and anti-rabbit) at 1:2,000 and 1:5,000 Precision Strep Tactin HRP conjugate (BioRad no. 161-0380) in 5% dry milk in PBST for 1 h at room temperature. Following incubation with the secondary antibody the blots were washed 3× in PBST for 5 min at room temperature. The blots were drained on paper towel and reacted with 1:1 ECL mix (Amersham Pharmacia Biotech, Piscataway, NJ) for 1 min at room temperature wrapped in Saran wrap, and exposed to film.

Stable Transfections With TCF-Reporter Plasmid

The TOPflash TCF reporter plasmid, containing six copies of the TCF/LEF DNA binding site upstream of a TK minimal promoter and a luciferase reporter gene, (TBE_6-luciferase), was obtained from Upstate Biotechnology and stably transfected into UMR 106 cells using Fugene^{TM} 6 reagent (Roche Molecular Biochemicals, Indianapolis, IN) as recommended by the manufacturer. Briefly, 48 h after transfection cells were grown in media containing G418 (1 mg/ml) for selection. Regular medium changes were made at 3–4 day intervals. When cells were 80%–90% confluent, cells were trypsinized, counted by

Coulter Counter, and diluted to <1 cell/well in 96-well plates with fresh culture media. Single colonies were picked, expanded in fresh medium containing 1 mg/ml G418 and confirmed for Wnt-3a responsiveness in reporter gene assays. For reporter gene assays cells were plated at a density of 50,000 cells/well for 16 h, and the experiments were initiated following serum withdrawal for 12–16 h. A Wnt-3a responsive clone was evaluated for responses to PTH, forskolin, analogs of PTH, PTHrP, and PKA inhibitor H89.

Luciferase Assays

Cell extracts were assayed for luciferase activity using the luciferase reporter gene assay kit (Roche Biochemicals, Indianapolis, IN) as recommended by the manufacturer. Luminiscence was measured in a Dynatech MLX Luminometer, and light integration was measured at 5 s as summed relative luminescence.

Statistical Analysis

Results were analyzed by student's *t*-test, and probability (*P*) values of less than 0.05 were considered statistically significant.

RESULTS

Components of Wnt Signaling Pathway Are Expressed in Bone In Vivo and Osteoblasts In Vitro

The expression of different components of the Wnt signaling pathway was examined by Northern analysis of RNA obtained from distal metaphyseal and diaphyseal rat femur and from cultured primary stromal and metaphyseal osteoblasts and osteoblastic osteosarcoma cell lines (Fig. 1). Expression of mRNA for FZD-1 was barely discernible in bone or in osteoblastic cell lines but was readily evident in primary osteoblasts. In contrast, mRNA for the antagonist Dkk-1 was abundant in bone and osteoblastic cell lines, but exhibited low to undetectable levels in primary osteoblasts. The co-receptors (LRP5 and 6) and KRM-1 were also expressed in bone, primary osteoblasts, and osteoblastic cell lines. These results suggest a negative correlation in the expression of FZD-1, a signaling receptor and the antagonist Dkk-1.

Temporal Regulation of Components of the Wnt Signaling Pathway by Continuous PTH in Rats and UMR 106 Cells

To investigate if PTH modulates the key players of the Wnt signaling pathway as a function of time, we studied the effects of continuous PTH (1–38) treatment in vivo in rats and also in cultured UMR 106 cells. PTX animals, infused continuously with PTH or vehicle, were sacrificed at various time intervals (1, 3, 6, or 24 h) and the distal femoral metaphysis analyzed for the expression of mRNA for different components of the Wnt signaling pathway. As illustrated in Figure 2A, mRNA for FZD-1, LRP6, and KRM-1 showed increases by 6 and 24 h treatment while levels of LRP5 and Dkk-1 decreased in a time-dependent manner. In vitro time course effects of PTH were also examined in UMR 106 cells. As shown in Figure 2B, upregulation of FZD-1, LRP6, KRM-1 and downregulation of LRP5 and Dkk-1 were observed in UMR 106 cells, similar to the in vivo observations. Quantitative analyses of these results in rat bone and UMR 106 cells are shown in Figure 2C–F. The levels of mRNA for LRP6 were increased by two to five-fold, those of

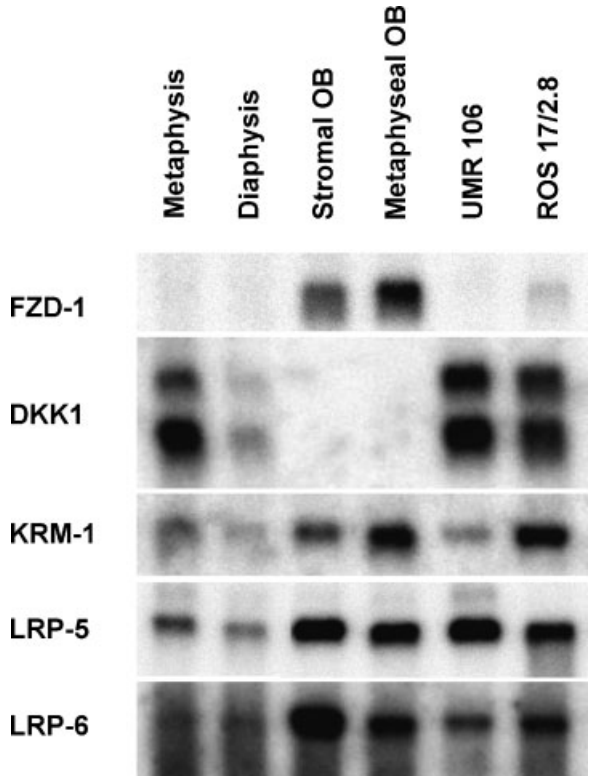

Fig. 1. Expression of components of the Wnt signaling pathway in bone and bone cells. Two micrograms of poly A^+ RNA from distal femoral metaphyseal, or diaphyseal, rat bone; primary cultures of osteoblasts derived from diaphyseal (stromal OB) and metaphyseal (metaphyseal OB) femur bone of rat; cultured UMR 106 and ROS 17/2.8 rat osteoblastic osteosarcoma cells were analyzed by Northern blot.

FZD-1 by 5 to 12-fold and of KRM-1 by 1.5 to 6-fold (Fig. 2C,E) while those of LRP5 decreased by 2.6 to 5-fold and of Dkk-1 by five to six-fold relative to control (Fig. 2D,F). Strikingly, mRNA for Dkk-1 in UMR 106 cells in response to PTH treatment was decreased at two successive time points (1 and 6 h) and thereafter reverted back to near control. These results suggest that PTH actions on bone and bone cells could be influenced in part by the differential expression of the key components of Wnt signaling pathway.

Forskolin/cAMP Mimic PTH Effects on Dkk-1 and KRM-1

Given the rapid decrease in mRNA for Dkk-1 in response to PTH in UMR 106 cells, we speculated that this effect might be mediated via cAMP, a key-signaling mediator of PTH action. UMR 106 cells were treated for 1 h with indicated concentrations of forskolin or dibutyryl cAMP. As shown in Figure 3, both forskolin, a direct activator of adenylate cyclase and dibutyryl cAMP (a stable analog of cAMP) substantially decreased Dkk-1 expression in a manner similar to PTH. In contrast, IL-1α, a stimulatory ligand of these cells, acting independently of cAMP had no effect on Dkk-1 expression (Fig. 3). These results suggest that PTH actions on Wnt signaling might in part be mediated through the cAMP pathway. Although forskolin treatment mimicked the effects of PTH on Dkk-1 expression, there was no discernible effect on expression of either LRP5 or LRP6. This might reflect the fact that the effect of PTH on LRP5 or LRP6 is much less than that on Dkk-1 (Fig. 2).

PTH Increases the Levels of β-Catenin Protein in UMR 106 Cells and Activates Downstream TBE_6-Luciferase Reporter Gene Activity

Wnt signaling through the canonical pathway involves the activation of a receptor complex comprised of members of the frizzled and

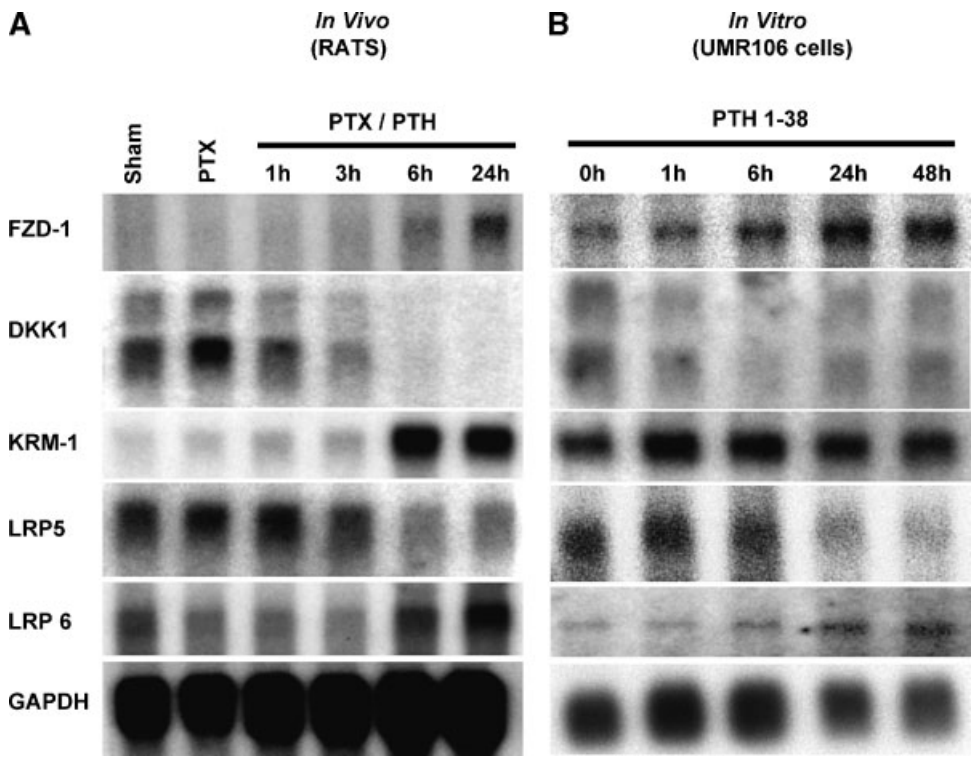

Fig. 2. Time course effects of continuous infusion of parathyroid hormone (PTH) on the expression and regulation of Wnt signaling pathway in rat distal femoral metaphysis (**A**) and UMR 106 cells (**B**). Female, 6 month parathyroidectomized (PTX) Sprague–Dawley rats were infused with human PTH (1–38) (20 μg/100 g/24 h) for 1, 3, 6, and 24 h using an Alzet minipump. UMR 106 cells were treated with hPTH (1–38) (5×10^{-8}M) for 0, 1, 6, 24, and 48 h. Poly A^+ RNA was isolated at the end of the treatment and the mRNA expression levels were determined by Northern analysis. (2 μg of Poly A^+ RNA/lane). GAPDH was used as an internal control. Graphical representation of the time course effects of continuous infusion of PTH on gene expression changes involved in Wnt signaling pathway in rats (C&D) and UMR 106 cells (E&F). The mRNA levels were determined by Northern analysis and normalized to GAPDH used as internal control. The changes in gene expression are expressed as percent change over PTX control rats or untreated control in UMR 106 cells.

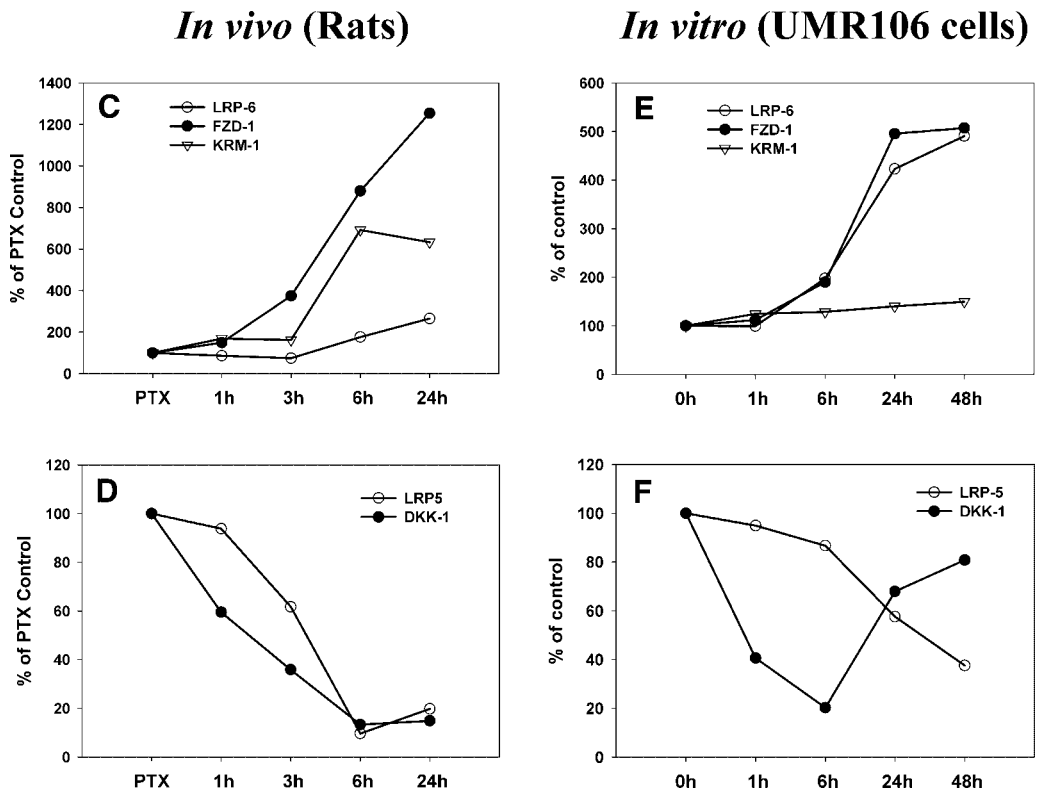

Fig. 2. (*Continued*)

LRP protein families, stabilization of β-catenin and stimulation of TCF/LEF-mediated transcription factors. To determine if the activation of canonical Wnt signaling pathway by PTH led to an increase in β-catenin, we performed Western analysis using monoclonal anti-β-catenin on whole cell lysates from UMR 106 cells treated with PTH. The increase in β-catenin levels in UMR 106 cells treated with 10^{-8}M PTH was marginal (20%–50% increase) but was observed as early as 1 h and maintained throughout the time course studied (Fig. 4). This increase in β-catenin levels by PTH treatment is consistent with the possibility that PTH may activate canonical Wnt signaling pathway in UMR 106 cells.

Evidence of activation of Wnt signaling was obtained by using PTH to treat UMR 106 cells stably transfected with TBE_6-luciferase reporter plasmid, TOPFLASH (Fig. 5). PTH stimulated the TBE_6-luciferase reporter gene activity in a dose-dependent manner at concentrations ranging from 10^{-12} to 10^{-7}M. A six- and threefold increase in the reporter gene activity was observed at 8 and 24 h as compared to the untreated control following treatment with PTH in UMR 106 cells.

Effects of Forskolin, PTH Analogs and PTHrP on TBE_6-Luciferase Reporter Gene Activity in UMR 106 Cells

Distinctive and discernible biological activities are associated with various domains of the natural PTH molecule and are indicative of specific intracellular signals. As is the case with the native PTH (1–84) molecule, both PTH (1–38) and PTHrP (1–34), activate both the cAMP/PKA and PLC/PKC pathways. However, PTH (1–31) has been suggested to activate exclusively through the cAMP/PKA pathway (Jouishomme et al., 1994). Conversely, PTH (3–34) and PTH (7–34) do not stimulate the cAMP/PKA pathway. We therefore evaluated the effects of forskolin (a direct activator of adenylate cyclase) and used these PTH analogs and PTHrP on downstream Wnt response in UMR 106 cells stably transfected with TBE_6-luciferase reporter gene. Figure 6A shows that forskolin stimulates the TBE_6-luciferase reporter expression in a dose-dependent manner at

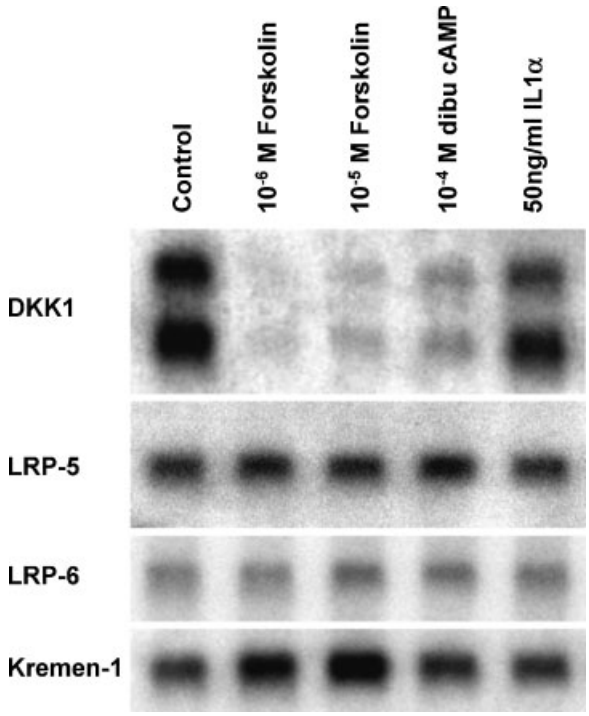

Fig. 3. Expression and regulation of key components of Wnt signaling pathway in bone cells in vitro. Cells were treated with forskolin or dibutyryl cAMP or IL-1α at indicated concentrations for 1 h. Poly A^+ RNA was isolated at the end of the treatment and the expression levels of Dickkopf-1 (Dkk-1), lipoprotein-receptor-related proteins 5 (LRP5), LRP6, and Kremen-1 (KRM-1) were determined by Northern analysis (2 μg of Poly A^+ RNA/lane).

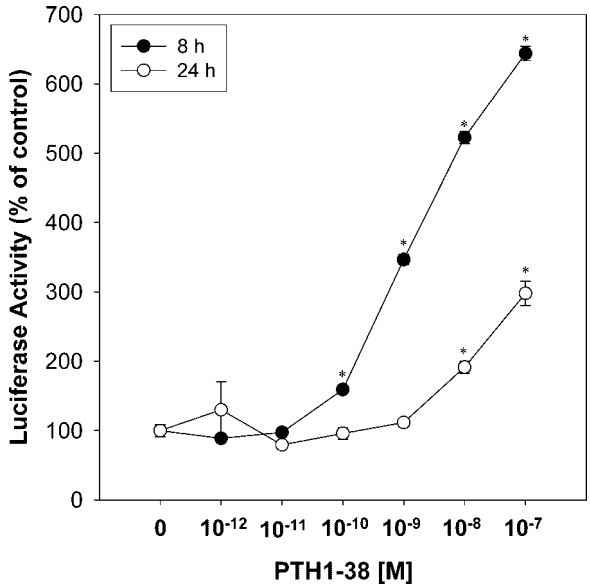

Fig. 5. PTH stimulates a Wnt responsive reporter in a dose-dependent manner after both 8 and 24 h treatment in UMR 106 cells. The TBE_6-luciferase reporter plasmid (TCF/LEF binding element fused to the luciferase gene) was stably transfected into UMR 106 cells and treated with hPTH (1–38) at concentrations ranging from 10^{-12} to 10^{-7}M for 8 and 24 h. At the end of the treatment the cells lysates were assayed for luciferase activity. The changes in TBE_6-luciferase reporter gene activity are expressed as percent change over control activity (serum-free control with no PTH treatment), $^*P < 0.05$ as compared to untreated controls. The results are expressed as mean ± SEM of eight separate treatments.

concentrations ranging from 10^{-8} to 10^{-4}M at both 8 and 24 h following treatment. As shown in Figure 6B, PTH analogs [PTH (1–31) and PTH (1–38)] and PTHrP (1–34) stimulated TBE_6-luciferase reporter gene expression both at 8 and 24 h. On the other hand, both PTH (3–34) and PTH (7–34) showed no discernible effect on the luciferase reporter expression (Fig. 6B). Together these results indicate that the PTH activation through the cAMP/PKA pathway is the likely route of PTH/PTHrP stimulation of Wnt signaling.

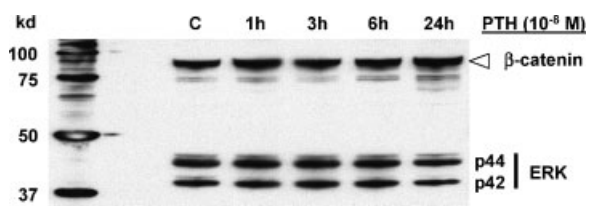

Fig. 4. Western blot analysis of β-catenin from whole cell lysates obtained from UMR 106 cells treated with human PTH (1–38) at different time points. Cells were treated for 1, 3, 6, and 24 h at a concentration of 10^{-8}M. At the end of treatment the cells were lysed in lysis buffer as described in materials and methods. The blots were immunostained using anti-β-catenin monoclonal antibody at 1:1,000 dilution. Anti-MAP kinase Erk 1 and 2 (p42 and p44) was used as internal control.

Effect of PTH on the TBE_6-Luciferase Reporter Gene Activity in UMR 106 Cells in the Presence of PKA Inhibitor H89

Further evidence for the involvement of the cAMP/PKA pathway, was obtained by measuring the TCF/LEF mediated luciferase reporter expression, in the presence of H89, an inhibitor of PKA in UMR 106 cells stably transfected with TBE_6-luciferase reporter gene. Cells were treated with PTH (1–38) at a concentration of 5×10^{-8}M for 8 h in the presence or absence of 1.0 μM H89 and measured for luciferase reporter gene activity. As shown in Figure 7, the PTH-increased TCF-reporter gene activity in UMR 106 cells stably transfected with TBE_6-luciferase

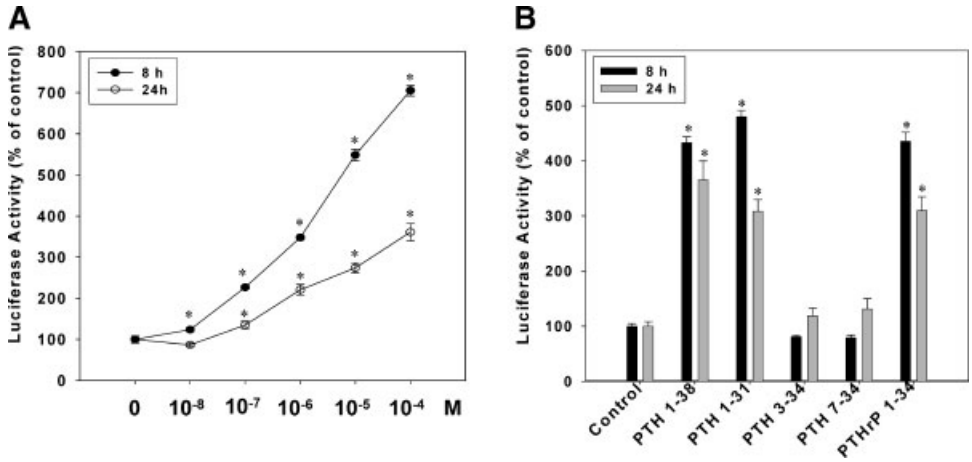

Fig. 6. Effects of forskolin, PTH analogs, and PTHrP on UMR106 cells stably transfected with TBE_6-luciferase reporter plasmid, TOPflash. Cells were treated with forskolin at a concentration of 10^{-8}–10^{-4}M (**A**), various analogs of PTH and PTHrP at a concentration of 5×10^{-8}M (**B**) and assayed for luciferase activity at 8 and 24 h. The change in luciferase activity is expressed as the percent increase over untreated control cells, $^*P < 0.05$ as compared to untreated controls. The results represent the mean ± SEM of eight separate treatments.

reporter gene was decreased in the presence of PKA inhibitor H89. These results also imply a role for cAMP/PKA pathway in PTH action on the Wnt signaling pathway.

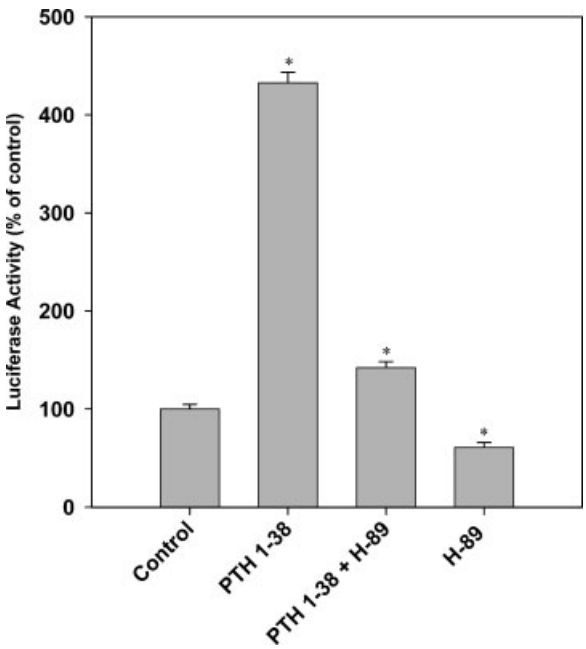

Fig. 7. Effect of PTH on the TBE_6-luciferase reporter gene activity in stably transfected UMR 106 cells in the presence or absence of protein kinase A (PKA) inhibitor H89. Cells were treated with PTH (1–38) at a concentration of 5×10^{-8}M for 8 h in the presence of PKA inhibitor H89 at 1.0 μM concentration and measured for luciferase reporter gene activity. The change in luciferase activity is expressed as the percent increase over untreated control cells, $^*P < 0.05$ as compared to untreated controls. The results are expressed as mean ± SEM of eight separate treatments.

DISCUSSION

The present study was undertaken to further evaluate gene array analysis data, which revealed that continuous PTH regulated the expression levels of components of the canonical Wnt signaling pathway in rat bone [Onyia et al., 2001]. We have examined the expression of different components of the Wnt signaling pathway in rat bone in vivo and in UMR 106 cells, and show how different components of the Wnt signaling pathway were modulated as a function of time by PTH treatments. Finally, we have determined the ability of PTH to increase the levels of β-catenin protein, and have shown functional activation of the downstream Wnt pathway as assessed by the TCF/LEF binding element fused to the luciferase gene in UMR 106 cells. The molecular mechanisms of PTH action on bone are not well understood. In this study, we provide evidence that key molecular components of the Wnt signaling pathway are regulated by PTH and may play a role in the bone response to PTH.

Our results show that key components of the Wnt signaling pathway are expressed in adult bone and bone cells, but additional work will be necessary to establish what the key components of this system are in bone at different stages of development. This is the challenge that presents itself in the many biological systems in which Wnt signaling plays an important role [Akiyama, 2000]. The interesting finding of a negative correlation in the basal expression

levels of FZD-1, a signaling receptor and the antagonist Dkk-1 in bone and bone cells could reflect regulatory roles for FZD-1 and Dkk-1 in Wnt signaling in bone. Wnt ligands initiate signaling by interacting with a cell membrane receptor complex composed of the frizzled family of seven transmembrane domain proteins and co-receptor, a single pass transmembrane protein either LRP5 or LRP6 [Yang-Snyder et al., 1996; He et al., 1997; Slusarski et al., 1997; Wang et al., 1997; Hsieh et al., 1999a; Pinson et al., 2000; Seidensticker and Behrens, 2000; Mao et al., 2001a,b; Nykjaer and Willnow, 2002]. FZD-1 is a G protein-linked receptor that signals through the β-catenin-LEF/TCF pathway in response to Wnt-1, 2, 3, 3a, 8, and 8b [Westendorf et al., 2004]. Our data shows that PTH induced a considerable increase in the expression of mRNA for FZD-1 by 24 h in rat metaphyseal bone. In contrast, PTH downregulated the levels of the LRP5 antagonist Dkk-1, in a time-dependent manner with virtually undetectable levels by 24 h, consistent with possible involvement of FZD-1 and Dkk-1 in the activation of the Wnt pathway by PTH. It has been shown that Dkk-1 interacts directly with LRP5/6 to inhibit Wnt signaling [Bafico et al., 2001]. Dkk-1 also interacts with another class of receptors, the transmembrane proteins, KRM-1 and 2, to synergistically inhibit LRP6 [Davidson et al., 2002; Mao et al., 2002]. In our study, we show that PTH treatment differentially regulates the expression of Dkk-1 and KRM-1 in rat bones. Since Dkk-1 and KRM are required equally for Wnt inhibition, the decreased levels of Dkk-1 upon continuous exposure to PTH might result in continuous signaling of the Wnt pathway and might be occurring through LRP6 and FZD-1, as evident from their increased expression levels in bone and bone cells in the current study. Though PTH increases the expression levels of KRM-1, it requires its partner Dkk-1 for any inhibitory action [Mao et al., 2002].

Here we show that forskolin, a direct activator of adenylyl cyclase increases the TBE_6-luciferase reporter gene expression in a manner similar to that obtained with PTH (1–38) and PTHrP (1–34) that have complete biological activity in terms of activating cAMP/PKA pathway. On the other hand, there was no stimulation of the TCF-reporter gene with PTH analogs, PTH (3–34) and PTH (7–34), which do not induce cAMP. The data support the view that PTH effects on Wnt signaling are mediated at least in part through the cAMP/PKA pathway. The role of the PKA pathway on Wnt signaling by PTH is further substantiated when we used a specific PKA inhibitor H89 and observed an inhibition of further downstream Wnt response as determined by TCF-reporter activity. These results confirm and extend previous studies demonstrating cross talk between cAMP/PKA and the Wnt pathway [Fang et al., 2000; Li et al., 2000; Tanji et al., 2002; Ni et al., 2003; Yam et al., 2003; Chen et al., 2005]. It has been shown that phosphorylation of GSK-3, a downstream component of the Wnt signaling pathway by cAMP-dependent PKA leads to inhibition of GSK-3 thus resulting in the stimulation of the Wnt pathway [Fang et al., 2000; Li et al., 2000]. Furthermore, the phosphorylation of the cAMP response element-binding protein (CREB) by GSK-3β is required for the increased transcriptional activity of CREB in response to PTH [Tyson et al., 2002].

Bone is a dynamic tissue that is subject to the balanced processes of bone formation and bone resorption that are regulated by a complex network of systemic hormones and local factors. The administration of amino-terminal fragments of PTH in vivo has either an anabolic or catabolic effect on bone. Whether PTH acts in a catabolic or anabolic mode depends on the dose and method of administration of PTH, that is, intermittent or continuous [Hock and Gera, 1992; Frolik et al., 2003]. In summary, we have demonstrated that some key components of the Wnt signaling pathway including signaling receptors (FZD-1), co-receptors LRP5 and 6, antagonists Dkk-1, KRM-1 are expressed in rodent bone and bone cells. The differential regulation of receptor complex, FZD-1, LRP5, or LRP6 accompanied by downregulation of the antagonist Dkk-1 by continuous infusion of PTH results in the stimulation of canonical Wnt signaling pathway both in vitro and in vivo. The alteration of mRNA expression of different components of the Wnt pathway by PTH is associated with an increase in β-catenin protein and functional activation of the downstream Wnt reporter gene. Taken together, our data suggest that the Wnt pathway is a target of PTH and the action(s) of PTH activity in bone could be mediated at least in part by the activity of the Wnt signaling pathway. Although this is the first report connecting PTH action with the Wnt pathway in bone, further investigations are

needed to explore the effect of intermittent PTH administration to ascertain the exact role of PTH and Wnt signaling pathway in the skeletal system.

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