Parathyroid hormone induces bone formation in phosphorylation-deficient PTHR1 knockin mice

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Datta NS, Samra TA, Abou-Samra AB. Parathyroid hormone induces bone formation in phosphorylation-deficient PTHR1 knockin mice. Am J Physiol Endocrinol Metab 302: E1183–E1188, 2012. First published February 14, 2012; doi:10.1152/ajpendo.00380.2011.—Activation of G protein-coupled receptors by agonists leads to receptor phosphorylation, internalization of ligand receptor complexes, and desensitization of hormonal response. The role of parathyroid hormone (PTH) receptor 1, PTHR1, is well characterized and known to regulate cellular responsiveness in vitro. However, the role of PTHR1 phosphorylation in bone formation is yet to be investigated. We have previously demonstrated that impaired internalization and sustained cAMP stimulation of phosphorylation-deficient (PD) PTHR1 leads to exaggerated cAMP response to subcutaneous PTH infusion in a PD knockin mouse model. To understand the physiological role of receptor internalization on PTH bone anabolic action, we examined bone parameters of wild-type (WT) and PD knockin female and male mice following PTH treatment. We found a decrease in total and diaphyseal bone mineral density in female but not in male PD mice compared with WT controls at 3–6 mo of age. This effect was attenuated at older age groups. PTH administration displayed increased bone volume and trabecular thickness in the vertebrae and distal femora of both WT and PD age groups. PTH administration displayed increased bone volume and bone mineral density in female but not in male PD mice compared following PTH treatment. We found a decrease in total and diaphyseal bone mineral density in female but not in male PD mice compared with WT controls at 3–6 mo of age. This effect was attenuated at older age groups. PTH administration displayed increased bone volume and trabecular thickness in the vertebrae and distal femora of both WT and PD mice. These results suggest that PTHR1 phosphorylation does not play a major role in the anabolic action of PTH.

Parathyroid hormone; phosphorylation-deficient parathyroid hormone receptor 1; signaling; bone mass

PARATHYROID HORMONE (PTH) and PTH-related peptide (PTHrP) bind to and activate a common receptor, the PTH/PTHrP receptor or PTHR1. Binding of PTH or PTHrP to the PTHR1 activates several G protein-linked signaling systems and extracellular signal-regulated kinase 1/2 phosphorylation. The activated PTHR1 become phosphorylated on its carboxy-terminal tail and is internalized through a process that involves clathrin and β-arrestin2. The process of PTHR1 phosphorylation is important for internalization of PTHR1 and desensitization of the second messenger system.

We have previously characterized seven serine residues on the PTHR1 carboxy-terminal tail that are subject to agonist-stimulated phosphorylation; these residues occur at positions 489, 491, 492, 493, 495, 501, and 504 (22). We have shown that a mutant PTHR1 bearing seven serine-to-alanine mutations at these sites is defective in agonist-stimulated phosphorylation and internalization (22). The phosphorylation-deficient (PD) PTHR1 shows sustained receptor activity after PTH challenge in vitro (21). Furthermore, we developed a knockin animal model in which we replaced the normal PTHR1 coding gene with a gene coding for the pdPTHR1. The homozygous PD mouse had normal serum calcium levels at the expense of lower PTH concentrations (1), suggesting that a new homeostasis is achieved where calcium is normal and PTH is low, likely because of suppression of PTH secretion from the parathyroid. PTH injection in the PD mice causes sustained elevation of cAMP levels in male mice with little effects on calcium levels (1). However, constant infusion of PTH using a Silastic subcutaneous pump caused more hypercalcemia in PD mice than in wild-type (WT) mice (1). Our studies also suggest that adaptive responses of intracellular signaling pathways in PD mice may be important for maintaining bone homeostasis (6).

PTH activation of PTHR1 in bone has been shown to increase both bone formation and bone resorption (for a review, see Ref. 3). Chronic elevation of PTH, whether caused by a human disease process such as hyperparathyroidism, or experimentally by infusing PTH in animals, stimulates both bone formation and resorption; however, the net effect is a major loss of bone mass. In contrast, when injected daily in humans or animals, the net effect results in increased bone mass (5, 13, 16). Understanding why intermittent (daily) PTH injections favor bone formation over resorption is an important question. The molecular and cellular mechanisms leading to net bone gain in the daily PTH injections regimen are not fully understood. Downregulation of the PTHR1 and desensitization of signaling, due to chronic elevation of PTH, may play an important role in this process. Because we have shown that the PD is deficient in agonist-stimulated internalization and showed sustained activity in vitro and in vivo, we examined whether anabolic effects of PTH are lost in the knockin animal mouse expressing the PD instead of the normal PTHR1 gene. Here we show that PTH administered daily in PD mice is anabolic, and we conclude that phosphorylation of the PTHR1 is not required for the net bone anabolic response to daily PTH administration.

MATERIALS AND METHODS

Mice. We generated a PD knockin mouse model using homologous recombination technology as described (1). All animal protocols were reviewed and approved by Wayne State University’s the Institutional Animal Care and Use Committee for the Use and Care of Animals.

PCR genotyping of PD knockin mice. For routine genotyping, PCR analyses were carried out on DNA extracted from tail biopsies. The sequences of the forward and reverse primers are CCTAAACCT-CCACTGTCCTT and CCTCAAGTTCTGTAGTTACT, respectively, flanking the loxp insertion site. The sizes of the PCR products are 150 and 450 bp for WT and PD alleles, respectively.

PTH administration in vivo. Twelve-week-old WT and PD mice were administered once daily subcutaneous injection of human PTH (1–34) (40 μg/kg; Bachem, Torrance, CA) or vehicle (0.9% sodium chloride) 5 days/wk for a total of 10 wk. Mice were killed 24 h after the last injection, and bone tissues were collected.

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Skeletal phenotyping and micro-computed tomography analysis. Areal bone mineral density (BMD) was measured using dual-energy X-ray absorptometry (DXA by PiXimus) on living animals subjected to local anesthesia every 3 mo (on longitudinal studies) or every 4 wk during anabolic studies. Micro-computed tomography (CT) analyses were performed on the femora and L5 vertebrae of WT and PD mice obtained at the end of the experiment using UCT40 (Scanco Medical, Basserdorf, Switzerland) at the Orthopedic Biomechanics Laboratory, Beth Israel Deaconess Medical Center and Harvard Medical School (Boston, MA).

Primary osteoblast culture. Primary osteoblasts were isolated from calvaria of 5- to 6-wk-old mice by serial digestion (4). Briefly, calvaria were dissected, isolated, and subjected to sequential digestions in collagenase A (2 mg/ml) and trypsin (0.25%) for 20, 40, and 90 min. Cells from the third digest were rinsed, counted, and plated in α-minimal essential medium containing 10% FBS, 100 U/ml penicillin, and 1 μg/ml streptomycin. Primary cultures were used without passage.

von Kossa staining. Primary cells were plated in six-well plates with a density of 2 × 10^5 cells/well, and the medium was replaced two times weekly with osteogenic medium with the addition of ascorbic acid (50 μg/ml) and β-glycerophosphate (10 mM). The culture media were treated with PTH-(1–34) or vehicle control either continuously or intermittently as described (12). The mineralization assays were performed using the von Kossa method. At the end of the culture period (14–21 days), the cells were fixed with 95% ethanol and stained with AgNO3 to detect phosphate deposits in bone nodules (17). The deposits of calcium were shown by the formation of opaque mineralized nodules.

Statistical analysis. The data, means ± SE, were analyzed by one-way ANOVA followed by the Student’s t-test. *P values <0.05 were considered significant.

RESULTS

Body weight, BMD, and bone mineral content in WT and PD mice during aging. We first examined the gross phenotype of the PD mice. No difference was observed in body weight between WT and PD mice from 3 to 12 mo of age (Fig. 1A). The bone phenotype was slightly different between male and...
female PD mice. In female mice, a significant decrease in total bone mineral content (BMC), spine BMD, and femur BMD in PD mice compared with WT controls was observed (Fig. 1A). The total BMD and diaphyseal BMD at 3–6 mo of age were significantly less in PD mice than in control; however, no difference was observed between female PD and WT mice at 9–12 mo of age. We did not observe any difference between PD and WT male mice in these parameters (Fig. 1B). Daily PTH injections significantly increased BMC in female and male mice, and the effects were similar in both genotypes (Fig. 1, C and D).

Effect of PTH on bone parameters of L5 vertebrae in WT and PD male and female mice. Figure 2, A and B, shows bone parameters of L5 vertebrae from female and male WT and PD mice, respectively. Vertebral bone volume/total volume (Bv/Tv) was increased significantly in both WT and PD male (9–12%) or female (23–25%) mice following PTH treatment compared with vehicle control. Vertebral trabecular thickness (TbTh) was only increased in PTH-treated female (6%) PD mice. On the other hand, following PTH administration, both WT and PD male mice showed increased (6–7%) TbTh. Vertebral trabecular number (TbN) was only increased (18–20%) in WT females, and both WT (16%) and PD (22%) females showed a decrease in vertebral trabecular spacing (TbSp) following PTH treatment. Vertebral TbN or vertebral TbSp remained unchanged in male WT or PD animals.

Effect of PTH on bone parameters of distal femora in WT and PD male and female mice. Distal femoral Bv/Tv were higher following PTH administration in both WT and PD female (38–44%) (Fig. 3A) and male (30–32%) (Fig. 3B) mice. Distal femoral TbTh was only increased in male WT (24%) and PD (12%) animals after PTH treatment (Fig. 3B). While TbN was significantly higher (16–18%) in PD female mice compared with WT controls, no change in TbN was observed following PTH treatment either in WT or PD male and female animals. TbSp was only decreased in female PD (12%) and male WT (15%) mice after PTH treatment.

Micro-CT images of distal femora from WT and PD female mice treated with PTH. Micro-CT images displayed an increase in bone mass in distal femora from WT and PD females following PTH administration compared with vehicle-treated controls (Fig. 4).

Effect of impaired PTH1 phosphorylation on mineralization of primary calvarial osteoblasts with continuous or intermittent PTH treatment in vitro. To determine the effects of impaired internalization of PTH1 on osteoblastogenesis and cell maturation, calvarial osteoblasts from 6- to 9-wk-old WT and PD mice were differentiated in the presence or absence of PTH, and mineralized nodule formation was assessed by von Kossa staining. Continuous administration of PTH inhibited mineralization in osteoblast cultures from WT and PD mice to background levels, as previously reported (6). Following intermittent PTH treatment, no change in mineralization was noted in osteoblasts derived from either WT or PD mice (data not shown).

DISCUSSION

Normal skeletal maintenance requires a balance between mature osteoblasts and osteoclasts during the bone remodeling process. PTH, by binding to its receptor PTH1R (13), acts directly on the skeleton and regulates calcium homeostasis.
Because of its therapeutic potential, the anabolic action of PTH has been a focus of investigation in recent years, and much remains unclear about the mechanisms involved in the anabolic action of PTH.

Receptor internalization is a key component of a cell’s response to hormonal stimulation. Several hormonal systems of G protein-coupled receptor (GPCR) internalization and desensitization of the hormonal responses have been charac-

Fig. 3. Effect of PTH on bone parameters of distal femora (DFem) in WT and PD male and female mice. Micro-CT parameters of distal femora of female (A) and male (B) WT and PD mice treated with PTH (40 μg·kg⁻¹·day⁻¹ sc, 5 days/wk). PTH injection started at 12 wk of age, for a total of 10 wk. Data are means ± SE. *P < 0.05 for PTH vs. vehicle. b P < 0.05 for PD vs. WT.

Fig. 4. Micro-CT images of distal femora from WT and PD female mice treated with PTH. Micro-CT images of distal femora of 22-wk-old female WT and PD mice treated with PTH (40 μg·kg⁻¹·day⁻¹ sc, 5 days/wk) or vehicle. PTH injection started at 12 wk of age, for a total of 10 wk.
acterized in vitro; however, the PD mouse model is the only in vivo model for an internalization-deficient GPCR. In this model, we knocked in an internalization-impaired PD mutant at the locus of the normal PTHR1 gene. In these mice, we demonstrated sustained stimulation of pdPTHr1 in vivo that results in sustained elevation of serum cAMP concentrations and exaggerated hypercalcemia after PTH administration compared with WT mice (1). It should be noted that, because of the relatively low receptor density in primary osteoblast cultures, it was not possible to examine PTHR1 internalization in vivo or in primary cells because of a low signal-to-noise ratio using direct quantitative assays. However, with the use of these mice, our recent report suggests that preventing PTHR1 phosphorylation and internalization in the female mice compared with male mice is protective against the consequences of low-calcium diet. In addition, distinct molecular regulation in primary osteoblasts was observed in these mice (6). These studies suggest that phosphorylation may play an important role in the physiology of the PTHR1 in vivo. In this report, we have studied the impact of impaired internalization of PTHR1 on the basal bone parameters and the anabolic action of PTH in vivo.

We observe a decrease in total BMD, BMC of spinal, and femoral and diaphyseal BMD in female mice while no change was noted in male PD animals. It has been shown that the anabolic action of PTH is skeletal site-specific in mice (14). We therefore measured bone parameters in both vertebrae and in distal femora. Our results demonstrate that PTH treatment increases total BMD for both WT and PD male and female mice. The changes in Bv/Tv, TbTh, TbN, or TbSp following PTH treatment in vertebrae or distal femora were similar in male and female WT and PD mice. This further supports our previous observation that internalization-defective PTHR1 mutants are capable to signal, i.e., activate cAMP signaling, and demonstrate that phosphorylation of the seven serine at carboxy-terminal tail motif is dispensable for the anabolic response to PTH. The cellular mechanism of the observed difference in BMD between male and female mice is not clear at the present time since no histomorphometry has been performed. However, our observation emphasized the main finding that phosphorylation of the PTHR1 is not necessary for the anabolic responsiveness to daily PTH administration.

Several intracellular molecules are involved in the process of GPCR phosphorylation, internalization, desensitization, and resensitization. Following agonist activation, GPCRs are recognized and phosphorylated by specific GPCR kinases, leading to binding of β-arrestin molecules (7, 15). With the use of a fluorescent protein-tagged β-arrestin, it was initially shown that PTHr1 endocytosis involves β-arrestins2 (9). Recent studies suggest that β-arrestin biased agonist that selectively activates β-arrestin-dependent signaling leads to PTH-induced trabecular bone formation without a simultaneous increase in bone resorption (10). β-Arrestin 2 knockout mice showed that PTH increases osteoclast number and surface, suggesting β-arrestin’s role in PTH-induced osteoclastogenesis and anabolic effect (2, 11). Transforming growth factor (TGF)-βRII phosphorylates PTHR1, and PTH antagonist reversed osteopetrotic bone phenotype of TGFβRII knockout mice (18). While interaction of PTHR1 with Na/H exchanger regulatory protein has been shown to internalize the receptor independent of receptor activation (20), PTHR1 interacts directly with Disheveled and regulates the β-catenin signaling pathway and osteoclastogenesis (19). Other studies report a functional difference between PTH and PTHrP activation of PTHR1, and sustained cAMP production of the PTH-PTHr1 complex has been suggested for catabolic action of PTH on bone (8, 23).

It is generally accepted that intermittent PTH leads to a transient activation of PTHR1 signaling, which might be required for the anabolic responsiveness, whereas continuous PTH may lead to sustained receptor activation and desensitization of its downstream signaling and less anabolic response. The latter is mediated mostly by receptor phosphorylation. In this study, our model demonstrates that receptor phosphorylation is not required for the anabolic response. The specific role of PTHR1 phosphorylation and internalization is unclear at the present time, and all of these reports imply that the role of PTH and PTHR1 in bone physiology is far more complex than it was originally thought. Numerous signaling pathways appear to have an important regulatory influence on the bone anabolic action of PTH and thus may influence functional adaptation of the skeleton. Based on our studies, we conclude that the sustained increase in intracellular cAMP accumulation in a PD PTHr1 may be required for the molecular regulation in bone or in LLC-PK cells (22), but it does not limit the bone anabolic response of PTH in vivo.

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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: N.S.D. and A.B.A.-S. conception and design of research; N.S.D. and A.B.A.-S. analyzed data; N.S.D. and A.B.A.-S. interpreted results of experiments; N.S.D. and A.B.A.-S. performed experiments; N.S.D. and A.B.A.-S. drafted manuscript; N.S.D. and A.B.A.-S. edited and revised manuscript; N.S.D. approved final version of manuscript; T.A.S. and A.B.A.-S. performed experiments; N.S.D. and A.B.A.-S. approved final version of manuscript.

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