Anabolic action of parathyroid hormone regulated by the β_2-adrenergic receptor

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Parathyroid hormone (PTH), the major calcium-regulating hormone, and norepinephrine (NE), the principal neurotransmitter of sympathetic nerves, regulate bone remodeling by activating distinct cell-surface G protein-coupled receptors in osteoblasts: the parathyroid hormone type 1 receptor (PTHR) and the β_2-adrenergic receptor (β_2AR), respectively. These receptors activate a common cAMP/PKA signal transduction pathway mediated through the stimulatory heterotrimeric G protein. Activation of β_2AR via the sympathetic nervous system decreases bone formation and increases bone resorption. Conversely, daily injection of PTH (1–34), a regimen known as intermittent (i)PTH treatment, increases bone mass through the stimulation of trabecular and cortical bone formation and decreases fracture incidences in severe cases of osteoporosis. Here, we show that iPTH has no osteoanabolic activity in mice lacking the β_2AR. β_2AR deficiency suppressed both iPTH-induced increase in bone formation and resorption. We showed that the lack of β_2AR blocks expression of iPTH-target genes involved in bone formation and resorption that are regulated by the cAMP/PKA pathway. These data implicate an unexpected functional interaction between PTHR and β_2AR, two G protein-coupled receptors from distinct families, which control bone formation and PTH anabolism.

bone anabolism | bone cells

Osteoporosis, a severe bone disorder and one of the most representative age-related diseases in the modern world, reduces bone strength and increases fracture risks associated with morbidity. The N-terminal fragment of the parathyroid hormone [PTH(1–34)], sold under the name Forteo (Lilly), is the only anabolic drug to date that can increase bone mass in humans and animals (1–4). Previous studies showed that PTH(1–34) treatment reduces the risk of fracture by ~65% in osteoporotic patients (5–7). So far, the use of PTH(1–34) has been limited to severe cases of osteoporosis and for a period of only 18 or 24 mo because of the potential risks of osteosarcoma and hypercalcemia, as observed in rat studies (8). To further overcome these limitations, a precise understanding of the physiological, cellular, and molecular bases of the anabolic action of PTH on bone is necessary.

PTH directly acts on bone and kidney, and indirectly on intestine, to regulate calcium ion (Ca^{2+}) levels in the blood and extracellular fluids. In the kidney, PTH induces expression of the 1-α-hydroxylase by acting on proximal tubules, which, in turn, increases the active form of vitamin D (1-α-25 dihydroxy vitamin D_3) involved in calcium reabsorption from the intestine. In bone, PTH acts on osteoblasts (bone-forming cells) to increase bone formation and induces expression of receptor activator of nuclear factor-κB ligand (RANKL), a cytokine that stimulates development and activation of osteoclasts (bone-resorbing cells), which, in turn, enhances calcium release from bone. PTH binds to the PTH receptor type 1 (PTHR), a G protein-coupled receptor (GPCR) predominantly expressed in osteoblasts and in distal and proximal renal tubules among other tissues (e.g., vascular smooth muscles, T lymphocytes). Previous genetic studies showed that the sympathetic tone reduces bone mass by suppressing bone formation and by enhancing bone resorption via activation of the β_2AR expressed in osteoblasts (9, 10). Given that β_2AR and PTHR are both coexpressed in osteoblastic cells (11), we hypothesize that PTH actions on bone might be modulated by the presence of the sympathetic system. Here, we examined this hypothesis by comparing the effects of intermittent injection of PTH (1–34) on bone metabolism in wild-type (WT) mice and in β_2AR-deficient mice (β_2AR-KO). We found that β_2AR deficiency suppressed the osteoanabolic action of PTH by blocking expression of intermittent (i)PTH-target genes involved in osteoblast activity and bone formation. We further found that iPTH treatment-induced increase in bone mass in aged osteoporotic mice is blunted by β_2AR deficiency.

Results

β_2AR Is Necessary for the Osteoanabolic Action of PTH. WT mice subjected to intermittent PTH(1–34) treatment (noted thereafter iPTH) showed an increase in the trabecular content compared with the control (untreated) group (WT) (Fig. 1*A*). In contrast, the trabecular content remained unchanged in mice lacking the β_2AR (β_2AR-KO) after iPTH treatment (β_2AR-KO) (Fig. 1*A*), which expressed the same level of PTHR as WT mice (Fig. S1). Quantification of cancellous bone volume over tissue volume (BV/TV) in long bone showed that iPTH treatment increased bone volume in WT mice but not in β_2AR-KO mice (Fig. 1*B*). We also observed that the capacity of iPTH treatment to increase the thickness of trabecular bone (Tb.Th) was lost in β_2AR-KO mice (Fig. 1*C*). Such an observation was not limited to long bone because we observed that the lack of β_2AR also suppressed iPTH treatment-induced increase in the BV/TV parameter of cancellous bone in vertebral bone (Fig. 1 *D* and *E*).

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The authors declare no conflict of interest.

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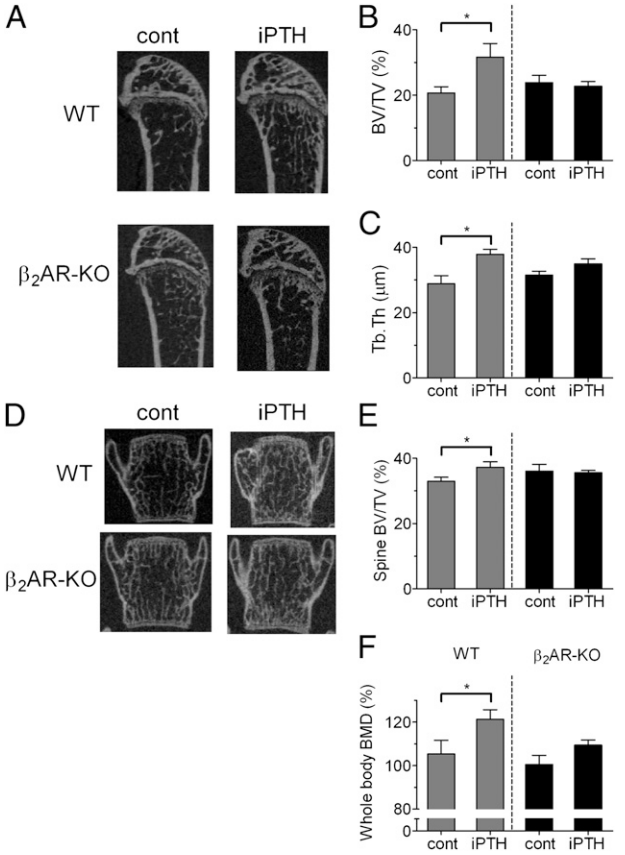

Fig. 1. β_2AR control of PTH-induced bone formation. (*A–C*) 2D micro-CT (μ-CT) images of the distal metaphyses of the femur in WT mice and β_2AR-KO mice treated either with vehicle (cont) or iPTH (*A*) and corresponding values for BV/TV (*B*) and trabecular thickness (*C*). (*D* and *E*) 2D μ-CT images of spine in WT mice and β_2AR-KO mice (*D*) and associated BV/TV parameter obtained from the secondary trabeculae of the fourth lumbar vertebrae (*E*). (*F*) Whole-body BMD was measured before and after iPTH treatment or vehicle (cont), and the ratio of posttreatment per pretreatment values are expressed as percentages. Data are expressed as means ± SD for n = 4–5 (*$P < 0.05$).

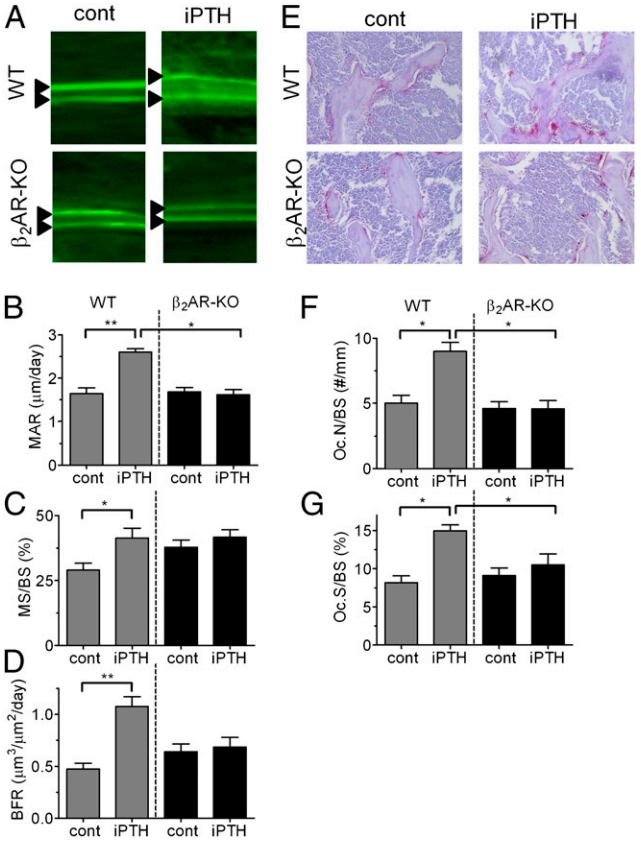

Fig. 2. β_2AR control of PTH-induced increase in the levels of dynamic bone formation and bone resorption parameters. (*A*) Calcein band in the distal metaphyses of the femur were visualized to obtain dynamic histomorphometry parameters in WT mice and β_2AR-KO mice treated either with vehicle or iPTH. (*B–D*) Parameters of osteoblast activity. (*B*) Levels of MAR. (*C*) Lineralizing surface per bone surface (MS/BS). (*D*) Bone formation rate (BFR). (*E*) Secondary trabecular regions of the epiphyses of tibiae were examined for osteoclasts based on TRAP staining in vehicle-treated (cont) and iPTH-treated (iPTH) WT mice and β_2AR-KO mice. (*F* and *G*) Parameters of osteoclast activity. (*F*) Osteoclast number per bone surface (Oc.N/BS). (*G*) Osteoclast surface over bone surface area (Oc.S/BS). Data are expressed as means ± SD of n = 4–5) (*$P < 0.05$; **$P < 0.01$).

Intermittent PTH treatment increased not only the level of morphological parameters but also the level of mineral content in bone measured by dual energy X-ray absorptiometry (DEXA) (Fig. 1*F*). This increase was suppressed in mice lacking the β_2AR (β_2AR-KO) (Fig. 1*F*). These data indicate that the absence of β_2AR suppresses the capacity of intermittent injection of PTH(1–34) to induce structural changes in both long bone and spine associated with an increase in bone mass.

Given that the anabolic effect of iPTH results from a tight balance between bone formation and bone resorption (12), we next examined whether the absence of β_2AR affects the action of iPTH on bone formation. The capacity of iPTH treatment to increase the amount of bone deposited between two timed administrations of calcein labeling, an indicator of bone mineralization in WT mice, was lost in β_2AR-KO (Fig. 2*A*). Quantification of these calcein intervals revealed that the absence of β_2AR suppressed the increase in mineral apposition rate (MAR) by iPTH (Fig. 2*B*), which is an indicator of the activity of individual osteoblasts. Analyses of dynamic parameters of bone formation further indicated that iPTH increased the mineralizing surface over the bone surface (MS/BS), which reflects the quantity of osteoblasts forming the bone matrix in WT mice but not in β_2AR-KO mice. There was a slight elevation in the baseline level in β_2AR-KO in MS/BS. However, iPTH did not increase MS/BS in β_2AR-KO mice (Fig. 2*C*). iPTH treatment increased bone formation rate (BFR), an indicator of the amount of bone formation within a unit time period, in WT mice but not in β_2AR mice (Fig. 2*D*).Therefore, the absence of β_2AR suppressed the increase of bone formation parameters triggered by iPTH treatment.

With respect to bone resorption, iPTH treatment enhanced the number of osteoclasts, which are shown as tartrate-resistant acid phosphatase (TRAP)-positive cells over the bone surface (Oc.N/BS), in WT mice, but not in β_2AR-KO mice (Fig. 2 *E* and *F*). iPTH treatment also increased osteoclast surface over bone surface (Oc.S/BS) in WT mice, which is a rough indicator of osteoclastic activity (Fig. 2 *E* and *G*). In contrast, β_2AR deficiency suppressed this increase (Fig. 2*G*). Thus, these histomorphometry analyses indicated that the lack of β_2AR suppressed the capacity of iPTH treatment to promote osteoclastic number and activity. Taken together, these data indicate that the presence of β_2AR is necessary for the actions of iPTH on both bone formation and bone resorption.

Given that PTHR is expressed in several extraskeletal cell types (proximal and distal tubule cells in kidneys, smooth muscle cells, T lymphocytes), it was unclear whether the suppression of PTH(1–34) osteoanabolism, which was caused by the absence of β_2AR, originated directly from osteoblasts or was a secondary

consequence of a systemic PTH(1–34) action or β_2AR action on extraskeletal tissues. We ruled out this latter possibility by using mice genetically engineered to have increased PTHR signaling activity in osteoblasts only. These mice express a constitutively active PTHR variant (hereafter noted caPTHR) that carries a His/Arg substitution at amino acid 223, which constitutively activates the G_S/cAMP/PKA signaling pathway, and which has been associated with Jansen-type metaphyseal dysplasia (11, 13). Previous studies showed that the osteoblastic expression of caPTHR stimulates an anabolic response in trabecular bone, which is in contrast to the decrease of net bone mass observed after continuous perfusion of PTH (13).We now observed that mice with osteoblast-specific expression of caPTHR increased the levels of trabecular bone content in the presence but not in the absence of β_2AR (caPTHR/β_2AR-KO) (Fig. 3*A*). Quantification of BV/TV indicated that β_2AR deficiency mostly suppressed the capacity of caPTHR to increase bone mass (Fig. 3*B*). As was the case in β_2AR-deficient mice (β_2AR-KO), deletion of β_2AR in caPTHR transgenic mice (caPTHR/β_2AR-KO) blocked increases in mineral apposition rate and BFR triggered by constitutive signaling of caPTHR (Fig. 3*D*). These data, thus, indicate that the absence of β_2AR interferes with PTHR signaling in osteoblasts associated with bone formation.

We next observed that the constitutive activation of PTHR in osteoblasts increased the appearance of osteoclasts (Fig. 3*E*). Quantification of osteoclasts indicated that β_2AR deficiency suppressed the capacity of caPTHR to increase levels of osteoclast number, as well as osteoclast surface (Fig. 3*F*). Thus, the deletion of β_2AR in caPTHR mice recapitulated the loss of the osteoanabolic action of PTH observed in β_2AR-KO mice after iPTH treatment. These data indicate that the β_2AR control of the anabolic action of PTH is likely mediated by a mechanism operating in osteoblasts.

β_2AR Control of PTH-Target Gene Expression. We next determined whether β_2AR deficiency modulated the expression of PTH-target genes involved in bone formation/resorption. To this end, we measured mRNA levels of a series of regulatory proteins in the bone of caPTHR mice and caPTHR/β_2AR-KO mice. Expression levels of alkaline phosphatase (ALP) (a marker of early to late stage osteoblast differentiation), type I collagen α1 (Col1α1) (the most abundant structural protein for bone and connective tissues), bone sialoprotein (BSP) (a specific marker of osteoblasts), RANKL (a critical regulatory factor for osteoclastogenesis), and osteoprotegerin (OPG) (the soluble decoy receptor for RANKL) were increased in caPTHR mice compared with WT mice (Fig. 4*A*). These increases were largely suppressed in caPTHR/β_2AR-

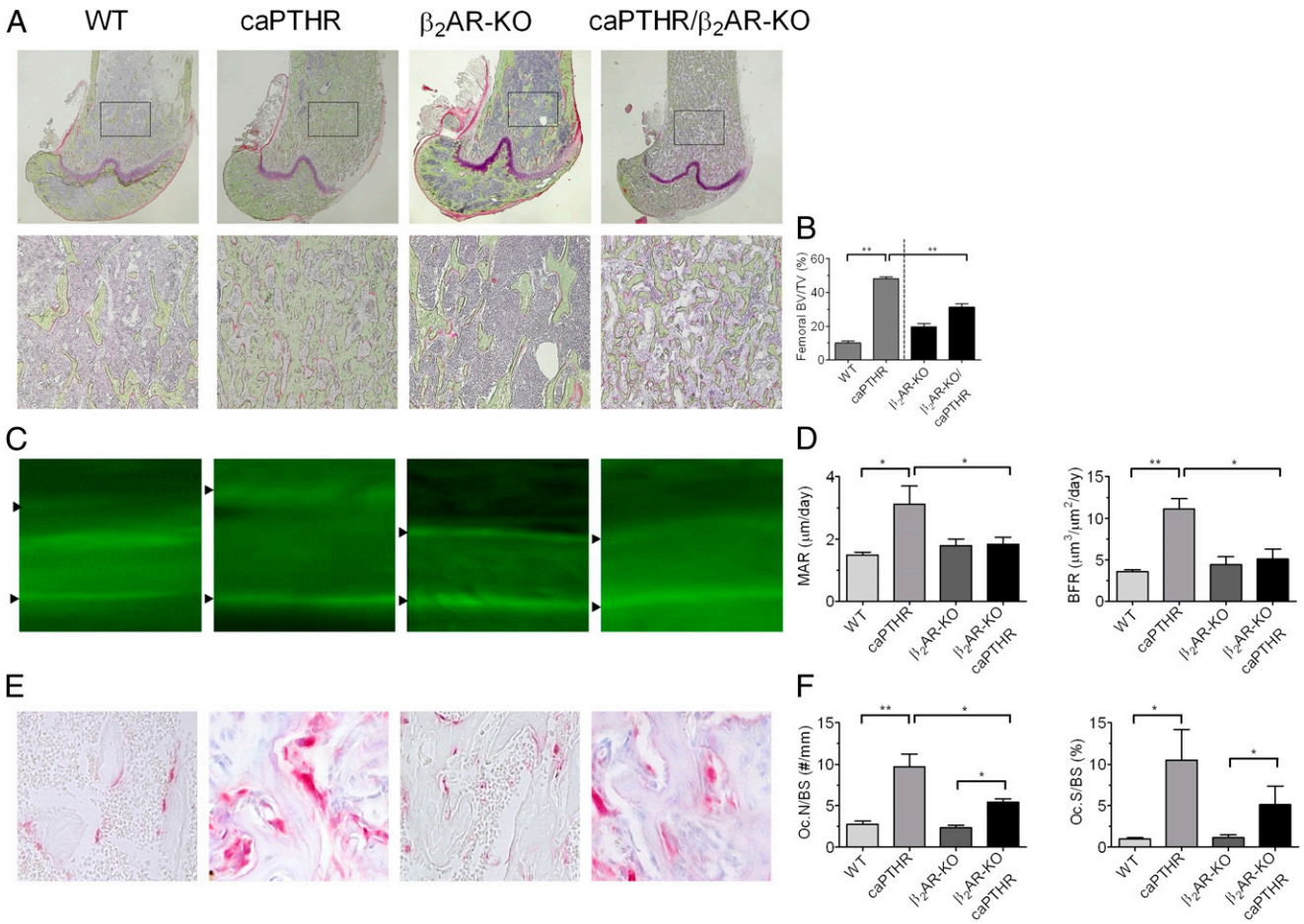

Fig. 3. β_2AR is required for anabolic action of constitutively active PTHR specifically expressed in osteoblasts. (*A*) Upper images represent Masson–Goldner staining in histological sections of WT, caPTHR, β_2AR-KO, and caPTHR/β_2AR-KO mice. Lower images represent enlargement corresponding to sections shown in the upper images. (*B*) BV/TV measured from the secondary trabeculae of the distal metaphyses of the femora. (*C*) Calcein band in the distal metaphyses of the femur were visualized to obtain dynamic histomorphometry parameters in WT, caPTHR, β_2AR-KO, and caPTHR/β_2AR-KO mice. (*D*) MAR and BFR. (*E*) TRAP staining of decalcified sections of the distal metaphyses of the femora. (*F*) Osteoclast number per bone surface (Oc.N/BS) and osteoclast surface per bone surface (Oc.S/BS). Data are expressed as means ± SD (n = 5–6; $^*P < 0.05$; $^{**}P < 0.01$).

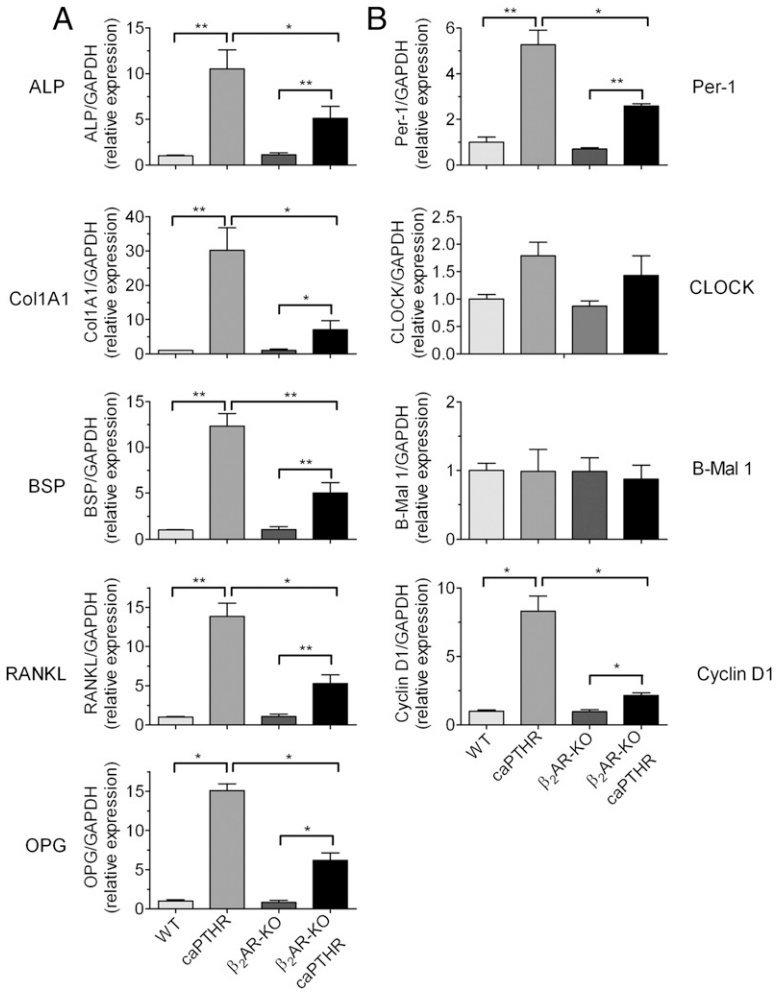

Fig. 4. Expression of PTH target genes regulated by β_2AR. (*A* and *B*) Expression of ALP, Col 1α, BSP, RANKL, OPG (*A*) and Per-1, B-MAL, CLOCK, and cyclin D1 (*B*), all normalized to GAPDH mRNA expression, in bone samples from WT and β_2AR-KO mice. Results are expressed in arbitrary units as means ± SD (n = 5–6/group; $*P < 0.05$; $**P < 0.01$).

KO mice (Fig. 4*A*). We also found that β_2AR deficiency reduced the capacity of intermittent injection of PTH to increase the gene expression of ALP, Col1α1, and Runt-related transcription factor (Runx)2 (Fig. S2). The negative effect of β_2AR deficiency can also take place just 1 h after a single PTH injection, as observed for gene expression of c-fos and RANKL (14–17) (Fig. S3). These increases were reduced in β_2AR-KO mice. These observations indicated that the inhibitory effect of β_2AR deficiency on PTH anabolism depends on the regulation of the expression of genes related to osteoblast activity and bone metabolism.

Because the action of the sympathetic tone in bone is associated with genes involved in the circadian rhythm, we next compared the expression levels of clock genes in WT, β_2AR-KO, caPTHR, and caPTHR/β_2AR-KO mice. We previously observed that caPTHR increases the steady-state levels of Period-1 (Per-1) mRNA expression in vivo (18). We now found that the increase in Per-1 mRNA expression triggered by constitutively active PTHR signaling was reduced in caPTHR/β_2AR-KO mice (Fig. 4*B*). The expression level of B-Mal, another clock gene, was not affected regardless of the presence or absence of either caPTHR or β_2AR, and CLOCK gene expression was not significantly modulated by β_2AR deficiency (Fig. 4*B*). We conclude that the central clock gene transcription system remains intact in β_2AR and caPTHR-β_2AR transgenic mice and that Per-1 is a specific target of PTH signaling in osteoblasts that is modulated by β_2AR in vivo.

PTH activates bone formation, in part, by stimulating osteoblast proliferation. Expression of caPTHR in osteoblasts enhanced the expression level of cyclin D1, a regulatory protein involved in the cell cycle of early osteoclasts (Fig. 4*B*). In contrast, β_2AR deficiency suppressed this enhancement. Overall, these data indicate the existence of a functional interaction between PTHR and β_2AR signaling in osteoblasts that allows the anabolic action of PTH via up-regulation of genes involved in cell proliferation and that is also necessary for bone formation.

β_2AR Control of iPTH-Induced Bone Formation in an Osteoporosis Model. Next, we tested the anabolic action of iPTH in a mouse model for osteoporosis. To this end, we used >50-wk old mice that showed a significant reduction in bone mass compared with younger 10 wk-old mice. Trabecular patterns of these aged mice were sparse (Fig. 5*A* vs. Fig. 1*A*: WT, control), and iPTH treatment produced additional trabecular bone (Fig. 5*A*, WT, iPTH). Aged mice that lack β_2AR showed more trabecular bone in the distal femur but did not show an increase in bone after iPTH treatment (Fig. 5*A*). Quantification of trabecular BV/TV indicated that baseline levels were lower in aged mice (~5%) than in younger adult mice (~20%) (Fig. 5*B* vs. Fig. 1*B*: WT, control). iPTH treatment was effective and significantly increased BV/TV levels in aged WT mice under condition of low bone mass (Fig. 5*B*, WT). The absence of β_2AR increased bone volume levels by

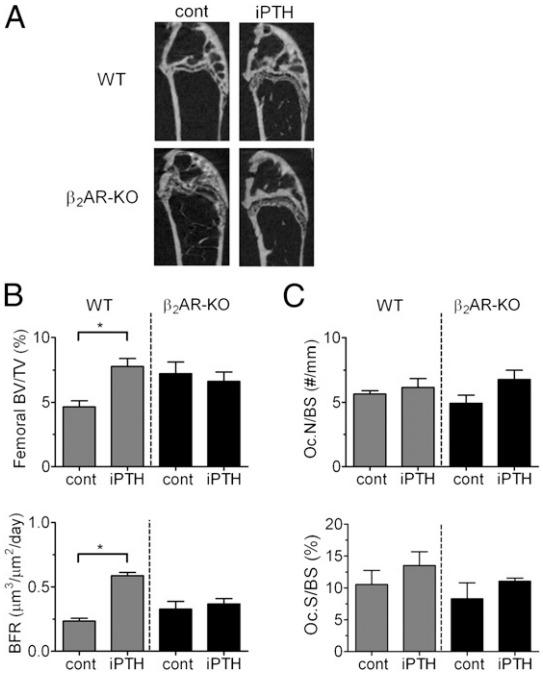

Fig. 5. Effect of β_2AR deficiency in aged mice. Aged mice of >50 wk were subjected to iPTH or vehicle (cont) injection for 4 wk. (*A*) 2D μ-CT images of the distal end of femur in WT mice and β_2AR-KO mice treated either with vehicle (cont) or iPTH . (*B*) BV/TV and BFR. (*C*) Osteoclast number per bone surface (Oc.N/BS) and osteoclast surface per bone surface (Oc.S/BS). Data are expressed as means ± SD of $n = 4–5$ ($*P < 0.05$).

2–3%. In contrast to WT, iPTH-induced increase in bone volume (BV/TV) was not observed in β_2AR-KOmice (Fig. 5 *A* and *B)*. The higher BV/TV level in β_2AR-KO compared with WT aged mice at baseline, presumably resulting from reduced age-related bone resorption, might hide some of the changes that could be caused by iPTH treatment. Despite the uncertainty in differentiating the effect of iPTH and β_2AR deficiency in aged mice, the data indicate, without ambiguity, that the absence of β_2AR blocks PTH-induced increases in BFR (Fig. 5*B*).

Further analysis of dynamic parameters of bone metabolism indicated that the BFR was reduced by ∼50% in old WT mice compared with young WT (10-wk-old) mice (0.2 vs. 0.4 $\mu m^3/\mu m^2$ per d; Fig. 5*B* vs. Fig. 2*D*). iPTH injection enhanced BFR levels approximately twofold in WT (Fig. 5*B*). β_2AR deficiency in these aged mice slightly elevated the baseline levels of BFR. In contrast to WT, β_2AR deficiency failed to respond to iPTH treatment with regard to BFR (Fig. 5*B*). Bone resorption parameters showed that the osteoclast number and the osteoclast surface were similar in old and young mice (Fig. 5*C*). In contrast to bone formation parameters, osteoclastic parameters in aged mice were not significantly altered by iPTH injection regardless of the presence or absence of β_2AR (Fig. 5*C*). These data indicated that in a mouse model for osteoporosis, β_2AR is required for iPTH-induced increase in bone mass, and this is mainly based on β_2AR requirement for iPTH activation of bone formation.

Discussion

Given that the sympathetic tone via the β_2AR has been reported to exert negative effects on bone mass by activating bone resorption and suppressing bone formation (10, 19), we initially predicted that mice lacking the β_2AR would improve the anabolic effect of iPTH treatment. Surprisingly, our results indicate the opposite. They support the unexpected feature that β_2AR deficiency suppresses the bone-anabolic effect mediated by iPTH (Figs. 1 and 2). Our in vivo studies further show that β_2AR deficiency in mice expressing a constitutively active PTHR selectively in osteoblasts suppresses bone formation. This impaired capacity to promote bone formation is accompanied by a reduced expression of several PTH-target genes encoding proteins involved in bone matrix formation (ALP, Col1α1, and BSP), in osteoblast proliferation (cyclin D1), and in the regulation of circadian protein expression (Per-1) in β_2AR-deficient caPTHR transgenic mice. The reduction in iPTH-induced increases of gene expression in β_2AR-KO mice compared with WT mice further supports the conclusion that PTHR signaling in osteoblasts requires the presence of β_2AR for its anabolic action in bone, at least in part, through the regulation of genes encoding proteins necessary for the osteoblastic function. However, additional factors may also be involved in the lack of PTH anabolism seen in β_2AR-KO mice. Among them are the levels of CART (cocaine amphetamine regulated transcript), a neuropeptide in the brain and in the circulation that is a major inhibitor of osteoclastogenesis by suppressing RANKL expression, which could be different in WT and β_2AR-KO and, thus, be one of the possible mechanisms whereby bone resorption is blunted in the β_2AR-KO mice, as we observed in our experiments. Other factors might also originate from osteoclasts, given that the BFR in caPTHR/β_2AR-KO mice remains unchanged compared with β_2AR-KO mice.

In summary, our studies demonstrate that the β_2AR plays a critical role in the osteoanabolic action of PTH by controlling expression of PTH-target genes involved in osteoblast activation and bone formation.

Materials and Methods

Adrb2-*deficient mice.* Breeding pairs of *Adrb2* heterozygous (*Adrb2*$^{+/-}$) with a genetic background of C57/BL6 were used to generate *Adrb2* WT and homozygous mutant (*Adrb2*$^{-/-}$) mice for this study (21).

Adrb2-*deficient Col1α1-caPTHR transgenic mice.* *Adrb2*$^{-/-}$ mice (noted as β_2AR-KO mice) with a genetic background of C57/BL6 and α1(I) collagen-caPTHR transgenic mice with a genetic background of FVB/N were used (11).

In vivo PTH administration. Ten-week-old (young adult mouse model) or 54-wk-old (aging mouse model) female mice were given s.c. injections of vehicle (saline) or hPTH (1–34) (Bachem) at a concentration of 80 μg/kg body weight for 5 d/wk over 4 wk. For some experiments, 4-wk-old mice were used.

Other methods are described in *SI Materials and Methods*.

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