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In Vivo Administration of 1,25-Dihydroxyvitamin D_3 Suppresses the Expression of RANKL mRNA in Bone of Thyroparathyroidectomized Rats Constantly Infused With PTH

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Abstract It is known that pharmacological or toxic doses of vitamin D induce bone resorption both in vivo and in vitro, whereas physiological doses of the vitamin have a protective effect on bone in vivo. To investigate the discrepancies of the dose-dependent effect of vitamin D on bone resorption, we examined the in vivo effect of 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] on the expression of the receptor activator of nuclear factor-κB (NF-κB) ligand (RANKL) and osteoprotegerin (OPG) mRNAs in bone of thyroparathyroidectomized (TPTX) rats infused with or without parathyroid hormone (PTH). Continuous infusion of 50 ng/h of PTH greatly increased the expression of RANKL mRNA in bone of TPTX rats. Expression of OPG mRNA was not altered by PTH infusion. When graded doses of 1,25$(OH)_2D_3$ was daily administered orally for 14 days to normocalcemic TPTX rats constantly infused with PTH, 0.01 and 0.1 μg/kg of 1,25$(OH)_2D_3$ inhibited the PTH-induced RANKL mRNA expression, but 0.5 μg/kg of the vitamin did not inhibit it. Regulator of G protein signaling-2 (RGS-2) gene expression was suppressed by 1,25$(OH)_2D_3$ dose-dependently, but PTH/PTHrP receptor mRNA expression was not altered. Bone morphometric analyses revealed that 1,25$(OH)_2D_3$ suppressed PTH-induced osteoclast number in vivo. These results suggest that pharmacological or toxic doses of 1,25$(OH)_2D_3$ stimulate bone resorption by inducing RANKL, but a certain range of physiological doses of the vitamin inhibit PTH-induced bone resorption, the latter mechanism appeared to be mediated, at least in part, by the suppression of the PTH/PTHrP receptor-mediated signaling. J. Cell. Biochem. 90: 267–277, 2003.

Key words: RANKL; regulators of G-protein signaling; osteoclasts; vitamin D; bone resorption

Bone tissue is remodeled throughout life. Bone-forming osteoblasts and bone-resorbing osteoclasts are primarily involved in this bone remodeling process. To maintain a constant bone mass during adult life, osteoblastic bone formation and osteoclastic bone resorption have to be closely coordinated [Manolagas, 2000]. Thus, increased bone resorption relative to bone formation causes a gradual bone loss, whereas decreased bone resorption relative to bone formation causes an increased bone mass. Since the imbalance between bone formation and resorption is critical for inducing metabolic bone diseases, it is important to understand how formation and function of osteoblasts and osteoclasts are regulated and coordinated.

Of several systemic hormones and local factors affecting bone remodeling during adult life, vitamin D and parathyroid hormone (PTH) may be the most important factors for regulating bone formation and resorption. In fact, PTH has been reported to stimulate both bone formation and resorption [Tam et al., 1982; Uzawa et al., 1995]. Vitamin D was discovered as a nutritional factor to prevent rickets and osteomalacia [McCollum et al., 1922]. However,

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the original notion of vitamin D sharply contrasts with the recent discovery that 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$], the active form of vitamin D_3, induces the differentiation and activation of osteoclasts [Suda et al., 1999].

It is well recognized that osteoclasts are derived from hematopoietic cells of the monocyte–macrophage lineage [Udagawa et al., 1990]. Multinucleated osteoclasts are formed, when spleen cells and osteoblastic cells are cocultured in the presence of bone resorbing hormones or cytokines including 1,25$(OH)_2D_3$ [Takahashi et al., 1988]. The recent findings that 1,25$(OH)_2D_3$ induces the expression of the receptor activator of nuclear factor κB (NF-κB) ligand (RANKL), also known as osteoprotegerin ligand (OPGL) [Lacey et al., 1998], osteoclast differentiation factor (ODF) [Yasuda et al., 1998], and tumor necrosis factor-related activation-induced cytokine (TRANCE) [Wong et al., 1997], have shed light on the molecular mechanism underlying the stimulation of osteoclastogenesis by 1,25$(OH)_2D_3$ [Suda et al., 1999]. RANKL critical for osteoclast formation, activation, and survival is induced primarily in stromal cells and osteoblasts in response to calcitropic hormones such as PTH and 1,25$(OH)_2D_3$. Osteoclast progenitors having RANKL receptor, receptor activator of NF-κB (RANK), binds RANKL by cell-to-cell contact with osteoblasts/stromal cells, then differentiate into osteoclasts. Thomas et al. [2001] showed that basal RANKL mRNA expression did not change during differentiation of murine primary osteoblasts but was significantly enhanced by adding 10^{-8} M 1,25$(OH)_2D_3$ at each step of osteoblast differentiation. Expression of OPG mRNA, a decoy receptor of RANKL, was decreased by 1,25$(OH)_2D_3$ [Hofbauer et al., 1998; Lee et al., 2002]. The RANKL/OPG ratio, an index of osteoclastogenic stimulus, was, therefore, increased by 1,25$(OH)_2D_3$ treatment at all stages of osteoblast differentiation. Kitazawa and Kitazawa [2002] identified a vitamin D responsive element in the 5′-flanking region of the mouse *RANKL* gene. These findings led to the widespread acceptance that 1,25$(OH)_2D_3$ is a bone-resorbing hormone. Indeed, 1,25$(OH)_2D_3$, when administered at pharmacological or toxic doses in vivo, induces hypercalcemia and bone resorption, but physiological doses of 1,25$(OH)_2D_3$ do not induce bone resorption nor hypercalcemia in vivo.

To investigate the discrepancies of the dose-dependent effect of 1,25$(OH)_2D_3$ on bone resorption and the relation of bone-resorbing effects of PTH and vitamin D, we examined the in vivo effect of 1,25$(OH)_2D_3$ on the expression of RANKL and OPG mRNAs in bone of thyroparathyroidectomized (TPTX) rats. When graded doses of 1,25$(OH)_2D_3$ were daily administered orally for 14 days to normocalcemic TPTX rats constantly infused with PTH, 0.5 μg/kg of 1,25$(OH)_2D_3$ further increased the PTH-induced bone resorption, but 0.01 and 0.1 μg/kg of the vitamin rather inhibited RANKL mRNA expression in bone and urinary levels of deoxypyridinoline (D-Pyr/Cr) induced by PTH. We report here that a certain range of physiological doses of 1,25$(OH)_2D_3$ have the capacity to inhibit PTH-induced bone resorption.

MATERIALS AND METHODS

Chemicals

All chemicals used in this study were of analytical grade. Rat PTH (1–34) was purchased from Peninsula Laboratories, Inc. (Belmont, CA). 1,25$(OH)_2D_3$ was purchased from Wako Pure Chemicals (Osaka, Japan). Hybond N^+ membrane was purchased from Amersham International plc (Bucks, UK). Calcein was obtained from Dojin (Kumamoto, Japan). TRIzol reagent was obtained from Gibco-BRL (Rockville, MD). [γ-^{32}P]-ATP was obtained from Du Pont-New-England Nuclear (Boston, MA). T4-Polynucleotide kinase was obtained from Life Technologies (Tokyo, Japan).

Animals

In Experiment 1, male weanling rats (Sprague–Dawley strain) were maintained on a synthetic diet containing either 0.03% calcium and 0.6% phosphorus (low Ca diet) or 0.6% calcium and 0.6% phosphorus (adequate Ca diet) (Teklad, Madison, WI), for 1–3 weeks. Both diets contained 2 U of vitamin D_3 per gram of diet. In Experiment 2, 8-week-old male Sprague–Dawley rats were maintained on a standard diet containing 1.2% calcium and 0.9% phosphorus. For making TPTX rats, the glandular tissues were removed by blunt dissection under light ether anesthesia. All TPTX rats were administered 4 μg of L-thyroxine (Sigma, St. Louis, MO) every other day. After measuring the serum calcium levels on the second day after

the surgery to assess the validity of the surgery, rats were constantly infused for 2 weeks with either vehicle (2% cysteine–HCl in 0.9% saline) or rat PTH (1–34) at 50 ng/h (flow rate; 0.5 μl/h) using an osmotic mini-pump (model 2002; Alza Corporation, Palo Alto, CA). $1,25(OH)_2D_3$ (0.01, 0.1 and 0.5 μg/kg bw), dissolved in PBS containing 0.2% ethanol and 0.01% Tween-20, was daily administered orally for 2 weeks. We defined the words "physiological doses of $1,25(OH)_2D_3$" as the doses of maintaining normocalcemia, and "pharmacological or toxic doses of $1,25(OH)_2D_3$" as the doses of producing hypercalcemia in TPTX rats constantly infused with 50 ng/h of PTH. All procedures were approved by and followed the guidelines of the Animal Care Committee of Showa University.

Measurement of Serum Levels of Calcium, PTH, and $1,25(OH)_2D_3$, and Urinary D-Pyr Excretion

Serum levels of calcium and phosphorus were measured with an autoanalyzer (model AU-550; Olympus, Tokyo, Japan). The serum concentration of PTH was measured by radioimmunoassay with the rat PTH IRMA Kit (Immutopics, Inc., San Clemente, CA). Serum levels of $1,25(OH)_2D_3$ were measured by a radioreceptor assay using purified calf thymus vitamin D receptors (VDR, Yamasa Co., Chiba, Japan) [Iida et al., 1995]. Urinary creatinine (Cr) was measured with an autoanalyzer. Urinary D-Pyr was measured using a PYRILINKS-D assay kit (Metra Biosystems, Mountain View, CA) [Fujimoto et al., 1995], and the data were corrected for urinary Cr concentrations (D-Pyr/Cr).

Polymerase Chain Reaction (PCR) and Southern Blotting

At autopsy, femora were dissected, and all connective tissues, bone marrow, and trabecular bone were removed. Total RNA was extracted from femoral cortical bone using the TRIzol reagent according to the manufacturer's instructions. Trabecular bone was not used as a bone sample, since they contained much more bone marrow cells. Five micrograms of total RNA obtained from each sample were reverse transcribed to synthesize cDNA using Superscript II RT (Life Technologies) and random hexamer primer. One microliter of the resulting cDNA solution was subjected to PCR using gene-specific primers.

For RANKL mRNA amplification, the sense primer was 5′-cgctctgttcctgtactttcgagcg-3′ (defined by bases 195–219 of cDNA) and the antisense primer was 5′-tcgtgctccctcctttcatcaggtt-3′ (defined by bases 759–783 of cDNA). For cathepsin K mRNA amplification, the sense primer was 5′-tgtctgaaaagagcatagacaacag-3′ (defined by bases 3–27 of cDNA) and the antisense primer was 5′-atagttctcagacacacagtccaca-3′ (defined by bases 543–567 of cDNA). For OPG mRNA amplification, the sense primer was 5′-agtttatttaacagactgccaccag-3′ (defined by bases 1955–1979 of cDNA) and the antisense primer was 5′-gtaataagagggcgcatagtcagta-3′ (defined by bases 2,196–2,220 of cDNA). For PTH/PTH-related protein (PTHrP) receptor 1 (PTH/PTHrP-R1) mRNA amplification, the sense primer was 5′-gatgtctttaccaaagaggaacaga-3′ (defined by bases 160–184 of cDNA) and the antisense primer was 5′-gattgaagtcataaatgtaatcggg-3′ (defined by bases 366–490 of cDNA). For the regulator of G protein signaling-2 (RGS-2) mRNA amplification, the sense primer was 5′-aaagcaaggaaaatctataccgact-3′ (defined by bases 411–435 of cDNA) and the antisense primer was 5′-atcaatagttctgagcgatgttttc-3′ (defined by bases 770–794 of cDNA). RT-PCR of β-actin was served as an internal standard. The expression of a housekeeping *β-actin* gene detected by PCR was used for quantifying the sample RNA. The β-actin sense primer was 5′-tcctagcaccatgaagatc-3′ which corresponds to bases 2,845–2,863 and the antisense primer was 5′-aaacgcagctcagtaacag-3′ which corresponds to bases 3,140–3,158 of the rat *β-actin* gene.

The PCR conditions were as follows: RANKL: 30 cycles at 94°C for 30 s, at 58°C for 45 s, and at 72°C for 1 min; OPG, PTH/PTHrP-R1, and RGS-2: 24 cycles at 94°C for 30 s, at 58°C for 45 s, and at 72°C for 1 min; cathepsin-K and β-actin: 20 cycles at 94°C for 30 s, at 58°C for 45 s, and at 72°C for 1 min in a DNA thermal cycler (GeneAmp PCR System 9700, Perkin-Elmer Corp., Norwalk, CT). The PCR products were separated by electrophoresis on 2% agarose gel. For Southern blot analysis of PCR products, gels were denatured, neutralized, and blotted onto a nitrocellulose membrane (Hybond N^+ membrane, Amersham). The specific oligonucleotide probe was labeled with [γ-^{32}P]-ATP at the 5′-end. Prehybridization, hybridization, and washing were the same as previously described [Iida et al., 1993]. The relative amount of the blot was determined using a Bio-Image analyzer BAS2000 (Fuji Film, Tokyo, Japan).

Bone Histomorphometry

All rats were subcutaneously injected with calcein (8 mg/kg bw) on the fifth and second days before sacrifice. Their femura were dissected, stained by the Villanueva's method and embedded in methylmethacrylate without decalcification. Sections of femura, 20 μm thick, were obtained by grinding on moistened carborundum paper, and prepared for measurement. Histomorphometry was performed under a microscope using an image-analyzing computer (Osteoplan II, Carl Zeiss, Thornwood, NY). The histomorphometric parameters were named and defined according to the nomenclature proposed by the report from the American Society for Bone and Mineral Research (ASBMR) Committee [Parfitt et al., 1987].

Statistical Analysis

The statistical significance of the differences between the experimental group and the control group was determined using Student's *t*-test.

RESULTS

Effect of Low Ca Feeding on the Expression of RANKL and OPG mRNAs in Bone

First, we examined the amplification efficiency of cDNAs after several cycles of PCR. One microliter of cDNA was PCR-amplified and the products were quantified by Southern hybridization. An exponential increment (1.9^n; n = number of cycles) of the PCR product was observed during cycles 26–34 for RANKL cDNA, cycles 20–28 for OPG, PTH/PTHrP-R1 and RGS-2, cycles 16–24 for cathepsin K and β-actin. As expected, rats maintained on a low Ca diet for 1 week showed much stronger expression of RANKL mRNA (fourfold to fivefold) and cathepsin K mRNA (twofold) in the cortical bone than those maintained on an adequate calcium diet (Fig. 1A,B). Further enhancement of RANKL and cathepsin K mRNA expression was not observed at 2 and 3 weeks of the feeding period. Expression of OPG mRNA in the cortical bone did not change appreciably during 1–3 weeks.

In the group of rats maintained on a low Ca diet for 1–3 weeks (low Ca group), serum levels of calcium were significantly decreased in a time-dependent manner (7.5 ± 0.3 mg/dl at 3 weeks), compared with those in the adequate

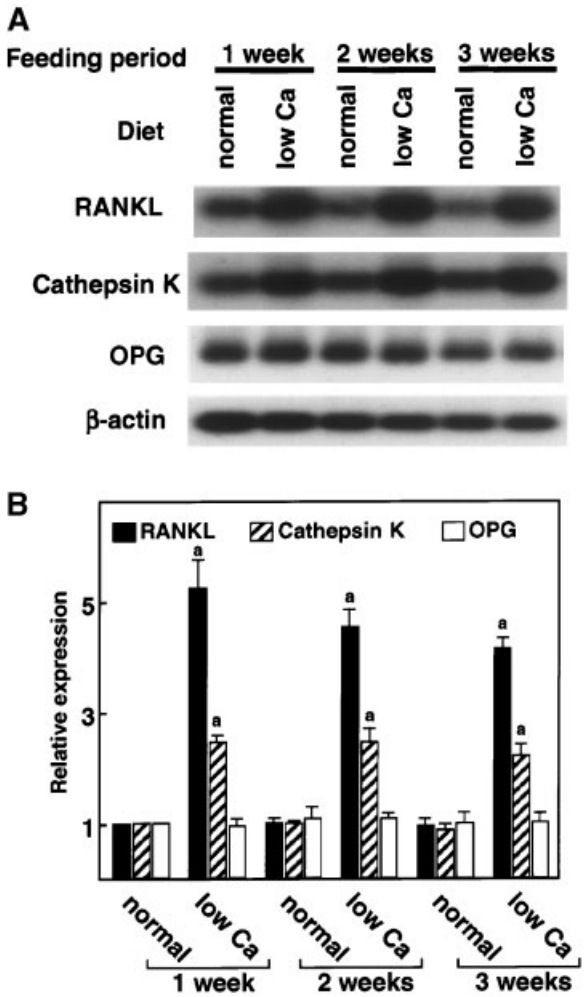

Fig. 1. Effects of low calcium feeding on the expression of receptor activator of nuclear factor-κB (NF-κB) ligand (RANKL), cathepsin K, and osteoprotegerin (OPG) mRNAs in the cortical bone of rats. Male weanling rats were fed either a vitamin D-replete low calcium (0.03% Ca and 0.6% P) diet or an adequate calcium (1.2% Ca and 0.6% P) diet for 1–3 weeks. Southern blots of RT-PCR of RANKL, cathepsin K, and OPG mRNAs in the cortical bone (**A**). Relative quantification of RT-PCR products of RANKL, cathepsin K, and OPG mRNAs in the cortical bone of rats (**B**). For relative quantification, the value from the cortical bone of sham rats administered the vehicle was used as the standard (ratio 1). Solid bar, RANKL; hatched bar, cathepsin K; and open bar, OPG. Values are expressed as the means ± SE of four rats. a, $P < 0.01$ (significantly different from rats fed the adequate calcium diet).

Ca group (10.8 ± 0.2 mg/dl at 3 weeks). In contrast, the serum levels of $1,25(OH)_2D_3$ and PTH were markedly increased compared with those in the adequate calcium group ($1,25(OH)_2D_3$, 1034.6 ± 47.3 vs. 148.4 ± 7.8 pg/ml; PTH, 237.3 ± 10.5 vs. 20.3 ± 0.2 pg/ml at 3 weeks).

Effect of $1,25(OH)_2D_3$ Administration on the Expression of RANKL, OPG, and Cathepsin K mRNAs in the Cortical Bone of Hypocalcemic TPTX Rats

Table I shows serum levels of calcium, phosphorus, PTH, and $1,25(OH)_2D_3$ and urinary excretion of D-Pyr after PTH and/or $1,25(OH)_2D_3$ treatment in TPTX rats. TPTX greatly decreased the serum levels of calcium and $1,25(OH)_2D_3$, but increased the serum phosphorus. After hypocalcemia was confirmed on day 2 after the surgery, PTH (50 ng/h) or vehicle was constantly infused at a flow rate of 0.5 μl/h for 2 weeks. Daily administration of $1,25(OH)_2D_3$ to TPTX rats dose-dependently increased the serum concentration of calcium without PTH infusion. Administration of $1,25(OH)_2D_3$ at 0.5 μg/kg/day for 2 weeks corrected serum calcium to normocalcemia, but significantly increased the serum $1,25(OH)_2D_3$ levels in TPTX rats, which was much higher than that of sham rats without PTH infusion. Daily administration of 0.01 and 0.1 μg/kg bw of $1,25(OH)_2D_3$ for 2 weeks had no effect on the serum levels of calcium in TPTX rats infused with PTH. However, 0.5 μg/kg bw of $1,25(OH)_2D_3$ for 2 weeks induced severe hypercalcemia in TPTX rats infused with PTH, and it also induced much higher serum levels of $1,25(OH)_2D_3$ (Table I).

Urinary excretion of D-Pyr (D-Pyr/Cr) was significantly decreased after TPTX without PTH and $1,25(OH)_2D_3$ treatment. $1,25(OH)_2D_3$ treatment increased D-Pyr/Cr at 0.5 μg/kg bw, but had no influence at 0.01 and 0.1 μg/kg without PTH infusion. In TPTX rats treated with PTH alone, D-Pyr/Cr was elevated to a level slightly higher than the control level. Treatment with $1,25(OH)_2D_3$ at 0.5 μg/kg bw further enhanced D-Pyr/Cr, but 0.01 and 0.1 μg/kg of $1,25(OH)_2D_3$ rather decreased PTH-induced increment of D-Pyr/Cr (Table I).

TPTX greatly reduced the expression of RANKL and cathepsin K mRNAs, but not of OPG mRNA in the cortical bone (Fig. 2A,B). Administration of 0.01 and 0.1 μg/kg/day of $1,25(OH)_2D_3$ for 2 weeks had no effect on the expression of all the genes examined, but 0.5 μg/kg/day of $1,25(OH)_2D_3$ markedly increased the expression of RANKL and cathepsin K mRNAs in the cortical bone of TPTX rats (Fig. 2B).

Effect of $1,25(OH)_2D_3$ Administration on the Expression of RANKL, OPG, and Cathepsin K mRNAs in the Cortical Bone of Normocalcemic TPTX Rats Infused With PTH

To determine whether the suppression of endogenous PTH is a prerequisite for the skeletal action of $1,25(OH)_2D_3$, the effects of the vitamin on the expression of RANKL, OPG, and cathepsin K mRNAs in bone were examined in TPTX rats, of which serum calcium was maintained nearly normocalcemic by constantly infusing PTH with an osmotic minipump. These animals with a constant PTH level (20.1 ± 2.3 pg/ml) were daily treated with 0.01, 0.1, or 0.5 μg/kg bw of $1,25(OH)_2D_3$, and gene expression was examined on day 14. Infusion of

TABLE I. Serum Levels of Calcium, Phosphorus, and $1,25(OH)_2D_3$, and Urinary D-Pyr Excretion in TPTX Rats Treated With PTH and/or $1,25(OH)_2D_3$

	Treatment		Serum levels			Urinary level
Operation	PTH (ng/h)	$1,25(OH)_2D_3$ (μg/kg bw)	Ca (mg/dl)	P (mg/dl)	$1,25(OH)_2D_3$ (pg/ml)	D-Pyr/Cr (nM/mM)
Sham	0	0	$9.6 \pm 0.3^{**}$	$7.5 \pm 0.2^{**}$	$67.6 \pm 7.9^{**}$	75.1 ± 2.2
		0	$5.1 \pm 0.2^{*}$	$10.5 \pm 0.4^{*}$	$29.8 \pm 2.3^{*}$	$54.2 \pm 3.0^{*}$
		0.01	$6.9 \pm 0.5^{*}$	$10.0 \pm 0.5^{*}$	42.5 ± 5.7	$59.8 \pm 2.7^{*}$
TPTX	0	0.1	$7.9 \pm 0.8^{**}$	9.2 ± 0.6	45.1 ± 8.6	$59.6 \pm 3.3^{*}$
		0.5	$10.3 \pm 0.7^{**}$	$6.9 \pm 0.6^{**}$	105.9 ± 11.5	$91.7 \pm 4.9^{**}$
		0	$10.4 \pm 1.1^{**}$	6.8 ± 1.2	79.4 ± 16.5	$84.8 \pm 2.2^{**}$
		0.01	$11.1 \pm 0.9^{**}$	7.4 ± 1.0	73.4 ± 15.3	$71.8 \pm 1.2^{**,***}$
TPTX	50	0.1	$11.2 \pm 0.6^{**}$	$6.5 \pm 0.8^{**}$	84.2 ± 9.8	$66.5 \pm 1.8^{**,***}$
		0.5	$14.6 \pm 1.2^{*,**}$	$3.5 \pm 0.4^{*,**}$	133.5 ± 21.3	$110.2 \pm 3.7^{*,**,***}$

$1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; D-Pyr, deoxypyridinoline; TPTX, thyroparathyroidectomized; PTH, parathyroid hormone. Eight-week-old rats were either sham-operated or TPTX under light ether anesthesia. PTH (50 ng/h) or vehicle was infused at a flow rate of 0.5 μl/h for 2 weeks. Graded doses of $1,25(OH)_2D_3$ were orally administered for 2 weeks. Rats were killed 12 h after the final administration of $1,25(OH)_2D_3$. Values are expressed as the means ± SE of at least five rats.

*$P < 0.01$ (significantly different from sham rats).

**$P < 0.01$ (significantly different from TPTX rats given the vehicle).

***$P < 0.01$ (significantly different from TPTX rats given the PTH alone).

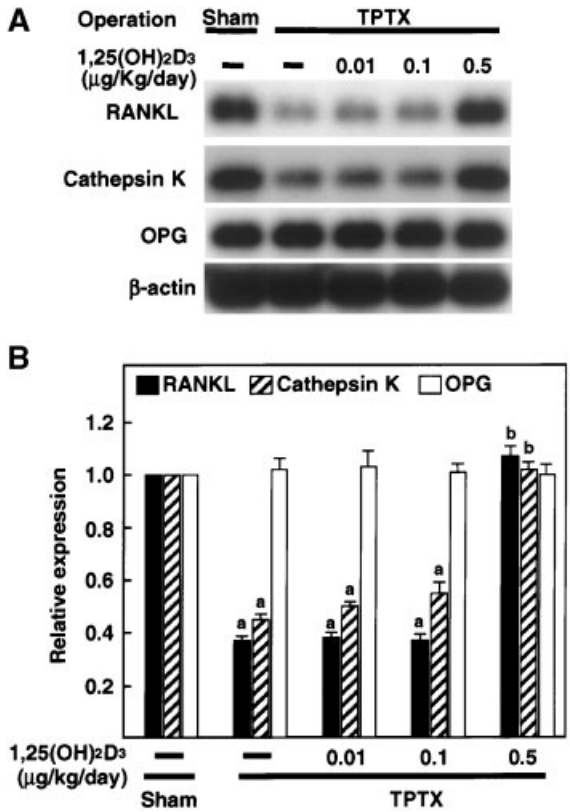

Fig. 2. Effect of $1,25(OH)_2D_3$ on the expression of RANKL, cathepsin K, and OPG mRNAs in the cortical bone of thyroparathyroidectomized (TPTX) rats. Eight-week-old rats were either sham operated or TPTX under light ether anesthesia. Two days after the operation, $1,25(OH)_2D_3$ or vehicle was daily administered orally for 2 weeks at a dose of 0.01, 0.1, or 0.5 μg/kg bw. Twelve hours after the final administration of $1,25(OH)_2D_3$, total RNA was prepared from the cortical bone of each rat. **A**: Southern blots of RANKL, cathepsin K, and OPG mRNAs in the cortical bone of TPTX rats. **B**: Relative expression of RANKL (solid bar), cathepsin K (hatched bar), and OPG (open bar). For relative quantification, the value from the cortical bone of sham rats administered the vehicle was used as the standard (ratio 1). Values are expressed as the means ± SE of four rats. a, $P < 0.01$ (significantly different from sham rats). b, $P < 0.01$ (significantly different from TPTX rats given the vehicle).

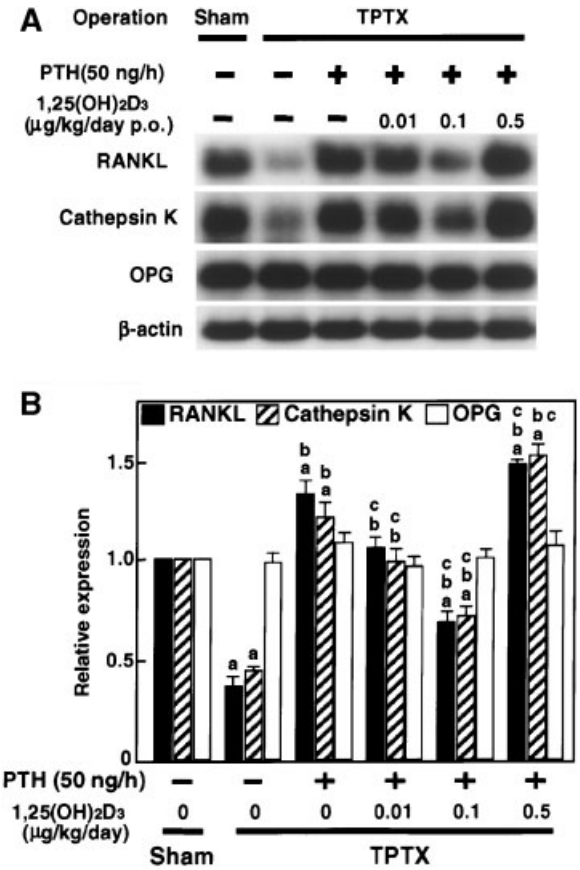

Fig. 3. In vivo effects of parathyroid hormone (PTH) and $1,25(OH)_2D_3$ on the expression of RANKL, cathepsin K, and OPG mRNAs in the cortical bone of TPTX rats. Eight-week-old rats were either sham operated or TPTX under light ether anesthesia. Two days after the operation, vehicle (2% cysteine–HCl in 0.9% saline) or rat PTH (1–34) at 50 ng/h (flow rate, 0.5 μl/h) was infused for 2 weeks using an osmotic mini-pump. $1,25(OH)_2D_3$ was daily administered orally for 2 weeks at a dose of 0.01, 0.1, or 0.5 μg/kg bw. Twelve hours after the final administration of $1,25(OH)_2D_3$, total RNA was prepared from cortical bone of each rat. **A**: Southern blots of RANKL, cathepsin K, and OPG mRNAs in the cortical bone of TPTX rats. **B**: Relative expression of RANKL (solid bar), cathepsin K (hatched bar), and OPG (open bar). For relative quantification, the value from the cortical bone of sham rats administered the vehicle was used as the standard (ratio 1). Values are expressed as the means ± SE of four rats. a, $P < 0.01$ (significantly different from sham rats). b, $P < 0.01$ (significantly different from TPTX rats given the vehicle). c, $P < 0.01$ (significantly different from TPTX rats given the PTH alone).

PTH markedly increased the expression of RANKL and cathepsin K mRNAs in the cortical bone of TPTX rats (Fig. 3A,B). Under the experimental conditions that endogenous PTH is not secreted and that the serum level of exogenous PTH is constant, daily administration of 0.01 and 0.1 μg/kg bw of $1,25(OH)_2D_3$ suppressed the PTH-induced expression of RANKL and cathepsin K mRNAs in bone of TPTX rats. However, a higher dose (0.5 μg/kg bw) of $1,25(OH)_2D_3$ did not inhibit the expression of RANKL and cathepsin K mRNAs in the cortical bone of TPTX rats constantly infused with PTH. These results suggest that a certain range of physiological dose levels of $1,25(OH)_2D_3$ suppresses RANKL mRNA expression in bone, independent of the suppression of PTH secretion. To confirm the protective effects of $1,25(OH)_2D_3$ on bone in vivo, urinary excretion of D-Pyr was measured. Continuous infusion of PTH into TPTX rats caused a

significant increase in the urinary D-Pyr excretion, which was suppressed by the daily administration of 0.01 or 0.1 μg/kg bw of $1,25(OH)_2D_3$ (Table I). A higher dose (0.5 μg/kg bw) of $1,25(OH)_2D_3$ greatly increased urinary excretion of D-Pyr with and without PTH infusion (Table I).

Potential Mechanism of the Inhibition of Bone Resorption by $1,25(OH)_2D_3$, Independent of the Suppression of PTH Secretion

The initial step mediating the transduction of the PTH signals involves binding of PTH to its G protein-coupled receptor (PTH/PTHrP receptor) and activation of adenylate cyclase and phospholipase C pathways [Abou-Samra et al., 1992; Huang et al., 1996; Civitelli et al., 1998]. Regulators of G protein signaling (RGS) proteins are GTPase-activating proteins which function to accelerate the rate of intrinsic GTP hydrolysis by Gα and thereby shorten the duration of G protein activation [Koelle, 1997; Berman and Gilman, 1998; Zerangue and Jan, 1998]. Recently, a member of the RGS gene family, RGS-2, has been reported to be rapidly and selectively up-regulated in bone in response to PTH [Miles et al., 2000]. The increased RGS-2 expression is one of the earliest events influencing PTH signaling [Miles et al., 2000]. To determine whether $1,25(OH)_2D_3$ modulates PTH signaling in bone, we next examined the effect of daily administration of $1,25(OH)_2D_3$ on the expression of PTH/PTHrP receptor and RGS-2 mRNAs in bone of normocalcemic TPTX rats constantly infused with PTH. TPTX significantly decreased the expression of RGS-2 mRNA, but not of PTH/PTHrP receptor 1 [PTH/PTHrP-R1] mRNA. Constant infusion of PTH caused an increase in the expression of RGS-2 mRNA in bone of TPTX rats. Daily administration of $1,25(OH)_2D_3$ dose-dependently suppressed the expression of RGS-2 mRNA induced by PTH in TPTX rats (Fig. 4A,B). Treatment with $1,25(OH)_2D_3$ did not alter the expression of PTH/PTHrP-R1 mRNA in bone. These results suggest that $1,25(OH)_2D_3$ inhibits PTH signaling in bone.

Histological Indices of Cortical Bone

The assessment of bone turnover in femoral cortical bone after sequential calcein labeling revealed that the parameters of bone formation such as mineral apposition rate (MAR), mineralizing surface (MS/BS), bone formation rate

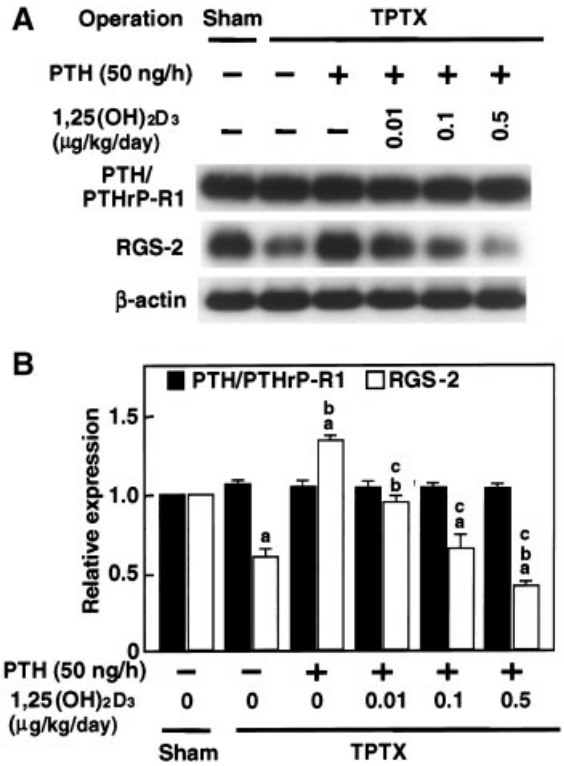

Fig. 4. In vivo effects of PTH and $1,25(OH)_2D_3$ on the expression of PTH/PTHrP-receptor (PTH/PTHrP-R1) and regulators of G protein signaling (RGS-2) mRNAs in the cortical bone of TPTX rats. Eight-week-old rats were either sham operated or TPTX under light ether anesthesia. Two days after the operation, vehicle or PTH (50 ng/h) was infused at a flow rate of 0.5 μl/h for 2 weeks. $1,25(OH)_2D_3$ was daily administered orally for 2 weeks at a dose of 0.01, 0.1, or 0.5 μg/kg bw. **A**: Southern blots of PTH/PTHrP receptor 1 (PTH/PTHrP-R1) and RGS-2 mRNAs in the cortical bone of TPTX rats. **B**: Relative expression of PTH/PTHrP-R1 (solid bar), and RGS-2 (open bar). For relative quantification, the value from the cortical bone of sham rats administered the vehicle was used as the standard (ratio 1). Values are expressed as the means ± SE of four rats. a, $P < 0.01$ (significantly different from sham rats). b, $P < 0.01$ (significantly different from TPTX rats given vehicle). c, $P < 0.01$ (significantly different from TPTX rats given the PTH alone).

(BFR/BS), in femoral cortical bone were decreased by TPTX (Table II). The parameter of bone resorption (osteoclast number: N. Oc/B. Pm) was also decreased by TPTX (Table II). Infusion of PTH significantly increased the all parameters of bone formation and resorption examined in TPTX rats (Table II). Daily oral administration of 0.1 μg/kg bw of $1,25(OH)_2D_3$ significantly inhibited the PTH-induced increases of N. Oc/B. Pm. (Table II).

DISCUSSION

$1,25(OH)_2D_3$ plays a pivotal role in calcium and bone homeostasis through its binding to

TABLE II. Morphometric Parameters of Cortical Bone of the Femur

	MAR (μm/day)	MS/BS (%)	BFR ($\mu m^3/cm^3$/year)	N. Oc/B. Pm (number/10 mm)
Sham	4.70 ± 0.49**	25.53 ± 2.50**	40.55 ± 6.61**	196.0 ± 10.5**
TPTX	1.84 ± 0.31*,***	5.88 ± 2.49*,***	5.51 ± 1.03*,***	79.7 ± 13.8*,***
TPTX + PTH	4.73 ± 0.74**	25.53 ± 2.53**	26.20 ± 4.43**	233.0 ± 22.6**
TPTX + PTH + $1,25(OH)_2D_3$	2.09 ± 0.28*	18.21 ± 4.05	14.71 ± 1.86*,**	138.5 ± 11.7*,***

Rats were constantly infused for 2 weeks with vehicle (2% cysteine–HCl in 0.9% saline) or rat PTH (1–34) at 50 ng/h (flow rate, 0.5 μl/h) using an osmotic mini-pump. $1,25(OH)_2D_3$, (0.1 μg/kg bw) dissolved in PBS containing 0.2% ethanol and 0.01% Tween-20, was daily administered orally for 2 weeks. Mineral apposition rate (MAR), mineralizing surface (MS/BS), bone formation rate (BFR/BS), and number of osteoclasts (N. Oc/B. Pm) were measured by the methods of Parfitt et al. [1987]. Values are expressed as the means ± SE of four rats.
*$P < 0.01$ (significantly different from sham rats).
**$P < 0.01$ (significantly different from TPTX rats given the vehicle).
***$P < 0.01$ (significantly different from TPTX rats given the PTH alone).

nuclear VDR in the target organs, including the intestine, bone, kidney, and parathyroid glands [Reichel et al., 1989]. It is widely accepted that administration of $1,25(OH)_2D_3$ exerts an anabolic effect in bone in vitamin D deficiency [Baylink et al., 1970; Tanaka and DeLuca, 1971]. Under these circumstances, $1,25(OH)_2D_3$ appears to stimulate calcium and phosphate absorption in the intestine, thereby increasing serum calcium and phosphate levels, and stimulating bone formation [Weinstein et al., 1984]. Simultaneously, endogenous PTH levels are suppressed by two independent mechanisms of vitamin D involving VDR and calcium-sensing receptors in the parathyroid cells [Brown, 1993], which then play an important role in the inhibition of bone resorption.

Nevertheless, the clear evidence for the action of vitamin D in the mobilization of bone calcium was first reported by Carttar et al. [1950]. They noted that administration of toxic doses of vitamin D resulted in bone calcium mobilization as measured by the elevation of serum calcium levels. Their results were subjected to the criticism that the effect they used may be attributable to the toxicity of the vitamin. Subsequently, Carlsson and Lindquist [1955] reported that pharmacological doses of vitamin D (2.5 μg/rat) also induced bone calcium mobilization under experimental conditions in which no intestinal absorption of calcium could occur. The latter observation was confirmed by many research laboratories including ours [Suda et al., 2003]. However, the mode of action of vitamin D on bone resorption was not known until the recent discovery of RANKL.

$1,25(OH)_2D_3$ appears to affect RANKL induction and bone resorption in two different ways: one is the stimulating effect of $1,25(OH)_2D_3$ through RANKL induction in osteoblasts/stromal cells, and the other the inhibitory effect on PTH-induced RANKL induction by suppressing its signal transduction. The stimulating effect of $1,25(OH)_2D_3$ occurred in TPTX rats as well, but the minimal dose level of the vitamin to induce RANKL mRNA expression was 0.5 μg/kg bw (Fig. 2). Lower doses (0.01 and 0.1 μg/kg bw) of the vitamin showed only a marginal effect on induction of RANKL mRNA expression (Fig. 2). It is known that $1,25(OH)_2D_3$ induces RANKL mRNA expression in vitro as well, but the concentration of the vitamin to induce RANKL mRNA in vitro is 10^{-8} M, which is about 100-fold higher than its normal plasma levels. In contrast, the inhibitory effect of $1,25(OH)_2D_3$ on PTH-induced RANKL mRNA expression is initiated at much lower doses (0.01 and 0.1 μg/kg bw) of the vitamin and it occurred at a dose-dependent manner (Figs. 3 and 4). Thus, it is likely that pharmacological or toxic doses of $1,25(OH)_2D_3$ have direct stimulating effects on RANKL induction and bone resorption, whereas physiological doses of the vitamin rather inhibit bone resorption by inhibiting PTH signaling.

More recently, Shevde et al. [2002] reported that 2-methylene-19-nor-(20S)-$1,25(OH)_2D_3$ (2MD), a potent analog of $1,25(OH)_2D_3$, induces bone formation and resorption both in vivo and in vitro. They showed that 2MD was 100 times more potent than $1,25(OH)_2D_3$ in inducing RANKL mRNA expression in osteoblasts in vitro, a process essential to osteoclast formation. Surprisingly, 2MD at concentrations as low as 10^{-12} M caused a significant increase in bone nodule formation in primary cultures of osteoblasts in vitro, whereas in vitro osteoclast formation was stimulated at a concentration of 10^{-10} M 2MD. These results suggest that vitamin D compounds are somehow involved in bone formation as well at much less dose levels

than those to induce bone resorption. The mechanism of the action of vitamin D compounds to stimulate bone formation has to be elucidated in future.

Continuous infusion of PTH in vivo also leads to an increase in bone resorption, resulting in a net loss of bone mass. It was suggested that PTH acts on osteoblasts or bone marrow stromal cells to stimulate osteoclast differentiation and activation. The recent discovery of RANKL, RANK, and OPG has established the molecular mechanism of the action of PTH on bone resorption. PTH induces RANKL expression in osteoblasts/stromal cells [Suda et al., 1999]. Ma et al. [2001] reported that continuous infusion or a single subcutaneous injection of PTH (>1 μg/100 g bw) greatly induced a rapid and sustained increase in RANKL mRNA expression and decreased in OPG mRNA expression in osteoblasts of parathyroidectomized rat bone. In the present study, we also showed that constant infusion of PTH at a dose of 50 ng/h for 2 weeks markedly increased the expression of RANKL and cathepsin K mRNAs in the cortical bone of TPTX rats. The same dose of PTH had no effect on the expression of OPG mRNA. These results provide further evidence that the regulation of these molecules by PTH and $1,25(OH)_2D_3$ is indeed involved in bone resorption in vivo as well.

In vivo experiments and observations in animals and patients have suggested that PTH is required for bone-resorbing effects of vitamin D, and vice versa [Rasmussen et al., 1963; Arnaud et al., 1966; Gerblich et al., 1977]. In contrast, either agent alone is capable of inducing bone resorption in vitro [Suda et al., 1999]. The discrepancy between the in vivo and in vitro results could raise the possibility that the in vitro systems were not completely devoid of vitamin D or PTH. Alternatively, the requirement of both PTH and vitamin D in vivo could be due to the changes in serum calcium and phosphate or other factors. In the present study, we showed that the daily administration of 0.01 or 0.1 μg/kg bw of $1,25(OH)_2D_3$ is capable of suppressing bone resorption through inhibiting RANKL mRNA expression in normocalcemic TPTX rats constantly infused with PTH (Fig. 3). The results support the previous finding that 22-oxa-$1,25(OH)_2D_3$, a synthetic analog of $1,25(OH)_2D_3$, has the potential not only to suppress PTH production [Funahashi et al., 1998] but also to counteract the action of PTHrP in bone [Endo et al., 2000].

PTH controls cell differentiation and function through its specific G protein-coupled receptors (PTH/PTHrP-R1) in two principal target organs, kidney and bone [Lee et al., 1995; Segre et al., 1995]. Binding of PTH to the receptors leads to the activation of adenylate cyclase and phospholipase C pathways that result in the accumulation of multiple signal transducers including cAMP, inositol triphosphate, intracellular calcium, and the activation of both protein kinase A (PKA) and protein kinase C (PKC) [Abou-Samra et al., 1992; Huang et al., 1996; Civitelli et al., 1998]. Miles et al. [2000] reported that the rapid regulation of RGS-2 by PTH was specific to bone, but not to other target tissues of PTH, such as brain, kidney, heart, liver, and spleen. RGS proteins are GTPase activating proteins, which function to accelerate the rate of intrinsic GTP hydrolysis by Gα and thereby limit the duration of G protein activation. The role of intracellular cAMP in regulating RGS-2 has been suggested. Pepperl et al. [1998] demonstrated that RGS-2 mRNA, but not RGS-4 and RGS-7 mRNAs, was strongly induced by cAMP in phenochromocytoma cells. Tseng and Zhang [1998] showed that glucose-dependent insulinotropic peptide (GIP), a potent stimulator of intracellular cAMP levels, induced a small, but significant, increase in RGS-2 mRNA in GIP-treated PTC3 cells. Taken together, these findings suggest that RGS-2 mRNA may be induced by the changes in the intracellular cAMP levels. It is suggested that the agonist-stimulated cAMP production induces RGS-2 expression. In the present study, we showed that RGS-2 gene expression markedly increased by PTH infusion and its expression was dose-dependently suppressed by the treatment with $1,25(OH)_2D_3$. These results suggest that $1,25(OH)_2D_3$ inhibits PTH-mediated G-protein signaling.

Kondo et al. [2002] showed that cAMP/PKA signaling via the PTH/PTHrP-R1 is the primary mechanism for controlling RANKL-dependent osteoclastogenesis. The cAMP responsiveness to PTH involving membrane receptors is notably modified by several steroids which involve nuclear receptors (e.g., glucocorticoids and $1,25(OH)_2D_3$) [Rizzoli and Fleisch, 1986]. In bone cells, vitamin D metabolites are also known to attenuate PTH activation of adenylate cyclase without affecting receptor population. Wong et al. [1977] reported that $1,25(OH)_2D_3$ suppressed the production of PTH-responsive

cAMP in isolated osteoblast-like cells. Catherwood [1985] also reported that $1,25(OH)_2D_3$ suppressed the linkage between PTH receptors and G protein in ROS 17/2.8 cells. According to the report of Kubota et al. [1985], treatment with $1,25(OH)_2D_3$ also suppressed PTH-dependent cAMP production by UMR106-06 cells. These in vitro findings support our concept that a certain range of dose levels of $1,25(OH)_2D_3$ inhibit PTH-induced bone resorption, which appears to be mediated by the suppression of the PTH-induced G protein signaling in vivo.

In conclusion, daily administration of 0.01 or 0.1 μg/kg bw of $1,25(OH)_2D_3$ into TPTX rats constantly infused with PTH suppresses bone resorption in vivo. The inhibitory action appears at least in part to be due to the inhibition of PTH-mediated G-protein signaling. Further studies are needed for a better understanding of the relation between PTH and vitamin D in vivo.

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