Targeted overexpression of G protein-coupled receptor kinase-2 in osteoblasts promotes bone loss

Liming Wang, Shiguang Liu, L. Darryl Quarles, and Robert F. Spurney

Division of Nephrology, Department of Medicine, Duke University and Durham Veterans Affairs Medical Centers, Durham, North Carolina

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Wang, Liming, Shiguang Liu, L. Darryl Quarles, and Robert F. Spurney. Targeted overexpression of G protein-coupled receptor kinase-2 in osteoblasts promotes bone loss. Am J Physiol Endocrinol Metab 288: E826–E834, 2005.—To investigate the role of G protein-coupled receptor (GPCR) regulator GRK2 in osteoblasts, using the osteocalcin gene-2 promoter to target expression to osteoblastic cells. Using the parathyroid hormone (PTH) receptor as a model system, we found that overexpression of GRK2 in osteoblasts attenuated PTH-induced cAMP generation by mouse calvaria ex vivo. This decrease in GPCR responsiveness was associated with a reduction in bone mineral density (BMD) in transgenic (TG) mice compared with non-TG littermate controls. The decrease in BMD was most prominent in trabecular-rich lumbar spine and was not observed in cortical bone of the femoral shaft. Quantitative computed tomography indicated that the loss of trabecular bone was due to a decrease in trabecular thickness, with little change in trabecular number. Histomorphometric analyses confirmed the decrease in trabecular bone volume and demonstrated reduced bone remodeling, as evidenced by a decrease in osteoblast numbers and osteoblast-mediated bone formation. Osteoclastic activity also appeared to be reduced because urinary excretion of the osteoclastic marker deoxypyridinoline was decreased in TG mice compared with control animals. Consistent with reduced coupling of osteoblast-mediated bone formation to osteoclastic bone resorption, mRNA levels of both osteoprotegerin and receptor activator of NF-κB ligand were altered in calvaria of TG mice in a pattern that would promote a low rate of bone remodeling. Taken together, these data suggest that enhancing GRK2 activity and consequently reducing GPCR activity in osteoblasts produces a low bone-turnover state that reduces bone mass.

parathyroid hormone; osteoblast; osteoclast; osteocalcin; protein kinase

G PROTEIN-COUPLED RECEPTORS (GPCRs) play a key role in bone growth and remodeling (3, 12, 28, 36, 42). Multiple GPCRs are expressed in osteoblasts (3, 42) and likely regulate the local and systemic effects of hormones. In this regard, agonists of several GPCR systems in osteoblasts promote osteogenesis, including parathyroid hormone (PTH) and E- and F-series prostaglandins as well as agonists of a putative calcium-sensing receptor in osteoblasts (3, 14, 18, 31, 37, 42). Recently, treatment with PTH has been shown to reduce the risk of fractures in patients with osteoporosis (36). The therapeutic effects of these agonists may be limited, however, by the rapid attenuation of GPCR signaling that occurs in the continuous presence of agonist (11, 13, 27, 30, 40). This desensitization is largely mediated by direct phosphorylation of receptor serine and threonine residues by a family of seven enzymes termed G protein-coupled receptor kinases (GRKs) (13, 27, 30, 40). Because of the highly restricted tissue distribution of GRK1, GRK4, and GRK7 (30, 40), either GRK2, GRK3, GRK5, or GRK6 is likely to be the important endogenous regulator of GPCR responsiveness in bone. Recent studies from this laboratory and by others indicate that 1) GRKs are expressed in osteoblasts (5, 16, 47), and 2) GRK2 and/or GRK3 plays a dominant role in regulating responsiveness of GPCR systems in bone (10, 15, 33, 47). These data suggest that GRK2 and/or GRK3 plays an important role in modulating bone growth and remodeling. Indeed, previous studies from this laboratory (47) found that inhibiting GRK2 and GRK3 in osteoblasts enhances bone remodeling and increases bone mass.

To further investigate the role of GRK2 in regulating bone formation in vivo, we used the mouse osteocalcin gene-2 (OG2) promoter (9) to overexpress GRK2 (4, 20, 22) in osteoblastic cells and, in turn, enhance GRK2 activity in osteoblasts. Transgenic (TG) mice demonstrated 1) enhanced levels of GRK2 mRNA in bone, 2) decreased PTH-induced cAMP generation by mouse calvaria ex vivo, and 3) evidence of decreased osteoblastic and osteoclastic activity in vivo. Osteoblast-mediated bone formation appeared to be decreased to a greater extent than osteoclastic activity, however, because the low bone turnover state caused a reduction in bone mineral density (BMD) and bone volume that affected predominantly trabecular bone. Taken together, these data indicate that GRK2 is an important endogenous regulator of GPCR systems in bone and suggest that pharmacological manipulation of GRK activity in bone may be a strategy for modulating bone remodeling.

MATERIALS AND METHODS

Materials. Rat PTH(1–34) was obtained from Sigma (St. Louis, MO). Pfu DNA polymerase was obtained from Stratagene (La Jolla, CA), and Tag DNA polymerase was obtained from Promega (Madison, WI). All PCR primers were prepared by Operon (Alameda, CA). Cell culture reagents were obtained from Gibco-BRL (Gaithersburg, MD), and [3H]adenine was purchased from New England Nuclear.
(Boston, MA). Dr. Darryl Quarles provided the rat osteosarcoma cell line ROS17/2.8 cells (32). Rabbit polyclonal antibodies to the COOH terminus of GRK2 (2) were a kind gift of Dr. Robert J. Lefkowitz. The cDNA for bovine GRK2 was also a gift of Dr. Robert J. Lefkowitz and was produced as previously described (24, 25, 26) by ligating the GRK2 cDNA to the human β-globulin polyadenylation signal (34). A 1.3-kb fragment of the OG2 promoter was provided by Dr. Gerard Karsenty (9).

Immunoblotting of GRKs. Expression GRK2 was evaluated using previously described techniques (15, 16, 47) with a rabbit polyclonal antibody (2) that recognizes the COOH termini of both GRKs.

Transgene construction and creation of TG mice expressing GRK2. The transgene was constructed in the vector pcDNA 3.1 (Invitrogen, San Diego, CA). To create the transgene, a 1.3-kb fragment of the mouse OG2 promoter was ligated into the unique KpnI/EcoRI site of pcDNA 3.1 vector. The GRK2 cDNA, including the human β-globulin polyadenylation signal, was ligated in the unique EcoRI/Xbal site of pcDNA 3.1, just 3’ to the promoter sequence.

Culture and transfection of ROS17/2.8 with the GRK2 transgene. ROS17/2.8 cells were grown and subcultured as previously described (32). To determine whether our transgene could be expressed by osteoblastic cell lines, we created a construct in which transgene expression was driven solely by the OG2 promoter by removing the CMV promoter sequences in pcDNA 3.1 using the restriction enzymes MluI and HindIII. After the overhangs were filled in with Pfu DNA polymerase, the construct was circularized with T4 DNA ligase. To transfect ROS17/2.8 cells, cells were plated in 60-mm dishes (Costar, Cambridge, MA) and grown to ~80% confluence. Cells were then transfected with the Transfast system (Promega) according to the directions of the manufacturer, using 5 μg of plasmid DNA. After selection in G418 (700 μg/ml concentration) for 2 wk, cells were harvested and transgene expression was evaluated by immunoblotting, as described above.

Creation of TG mice overexpressing GRK2. To create TG mice, the transgene was purified by cesium chloride centrifugation and then linearized by cutting with the restriction enzymes HindIII/Xbal. The linearized transgene was then separated from vector sequences on a 0.8% agarose gel and extracted from the gel using the QIAquick gel extraction kit (Qiagen, Valencia, CA). To remove endotoxins, the transgene was purified by cesium chloride centrifugation and then linearized by cutting with the restriction enzymes MluI and HindIII in the 3’ untranslated region of the bovine β-globulin mRNA (34), with the thermal cycler set at 94°C for 30 s, 50°C for 30 s, and 72°C for 2 min. Thirty cycles of PCR using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers (Clontech Laboratories, Palo Alto, CA) with the thermal cycler set at 94°C for 30 s, 58°C for 30 s, and 72°C for 2 min. PCR products were separated on 1% agarose gels and visualized by staining with ethidium bromide.

Measurement of osteocalcin and PTH levels in blood. At 6 wk of age, mice were anesthetized with ketamine (90 mg/kg) and xylazine (10 mg/kg), and serum was collected using a retroorbital bleeding technique. Serum osteocalcin levels were quantitated using a two-site immunoradiometric assay from Immunotopics (San Clemente, CA) according to the directions of the manufacturer. Serum PTH levels were quantitated with the use of an enzyme-linked immunosorbant assay from Immunotopics according to the directions of the manufacturer.

Measurement of cAMP generation by mouse calvaria. To determine whether the GRK2 transgene affected GPCR responsiveness in our TG animals, we measured PTH-induced cAMP generation by mouse calvaria ex vivo as previously described (47). For the experiments, a symmetric fragment of mouse calvarium containing portions of the occipital, frontal, and parietal bones was isolated aseptically from 6-wk-old mice. Calvaria were cut into right and left halves by dividing the bony fragment along the sagittal suture line. Right and left halves were each divided into three fragments of equal size. Preliminary experiments suggested that this procedure for obtaining portions of mouse calvaria resulted in bony fragments that varied by <15% by weight (average weight 50.7 ± 1.1 mg). Individual bony fragments of calvaria were placed in separate wells of a six-well plastic culture dish (9.5 cm2/well; Costar, Cambridge, MA). For the cAMP measurements, the bony fragments were covered with DMEM containing 1% FCS and 2 μCi/ml [3H]adenine (New England Nuclear, Boston, MA). After 90 min, 100 μM IBMX was added to the medium. Twenty minutes later, calvaria were stimulated with either 100 nM PTH(1–34) or 10 μM forskolin. After 10 min, the medium was aspirated, and the reaction was stopped by adding STOP solution (2.5% perchloric acid containing 100 μM cAMP and 1 μCi of [14C]cAMP) and placing the samples on ice. Generation of cAMP was then measured by the method of Salomon et al. (46) as previously described (15, 47). Data points are the means of triplicate measurements and are expressed as the percent increase above basal cAMP generation.

Expression of osteoprotegrin and receptor activator of NF-κB ligand mRNA. To investigate the effect of the transgene on osteoprotegrin (OPG) and receptor activator of NF-κB ligand (RANKL) mRNA levels in bone, we performed semiquantitative RT-PCR using 2 μg of total cellular RNA isolated from mouse calvaria, as previously described (47). The reverse transcription reaction was performed with Superscript RT (GIBCO) and oligo(dt) primers using RNA prepared with the TRIzol reagent (GIBCO) according to the directions of the manufacturer. PCR for OPG was performed for 30 cycles using Taq DNA polymerase (Promega) and primers previously shown to specifically amplify OPG (47), with the thermal cycle set at 94°C for 30 s, 58°C for 30 s, and 72°C for 2 min. Thirty cycles of PCR were during the linear phase of template amplification in non-TG control mice. Control PCR reactions were performed for 25 cycles with GAPDH primers (Clontech Laboratories), with the thermal cycler set at 94°C for 30 s, 58°C for 30 s, and 72°C for 2 min. PCR products were separated on 1% agarose gels and visualized by staining with ethidium bromide.
Expression of GRK2 mRNA. To determine whether mRNA for GRK2 was overexpressed in TG animals, we treated total cellular RNA from mouse calvaria with RNase-free DNase (GIBCO) and then performed RT-PCR. The reverse transcription reaction was performed with Superscript RT (GIBCO) and oligo(dT) primers, using 2 μg of total cellular RNA prepared with the TRIzol reagent (GIBCO) according to the manufacturer’s recommendations. PCR was performed using the intron-spanning primer pairs encompassing nucleotides 174–197 (GTGAGCTACCTGATGCGATGAGG) and 1058–1041 (AGGCCAAGATGGTATCCTC) of the mouse GRK2 cDNA (20, 22). These portions of the mouse and bovine GRK2 cDNA are identical (4, 20), and therefore the primers are suitable for amplification of endogenous GRK2 mRNA and transgene mRNA. PCR was performed for 30 cycles using Taq DNA polymerase (Promega), with the thermal cycler set at 94°C for 30 s, 55°C for 30 s, and 72°C for 2 min. Thirty cycles of PCR was during the linear phase of template amplification in non-TG control mice. PCR products were separated on 1% agarose gels and visualized by staining with ethidium bromide. For DNA sequencing, the PCR products were cloned into the vector pCR2.1 according to the directions of the manufacturer (Invitrogen). The ligation reactions were used to transform DH5α competent cells according to the directions of the manufacturer (GIBCO). Individual clones containing insert were picked for sequencing.

Bone histomorphometry. Quantitative histomorphometric analyses of trabecular bone in lumbar vertebrae were performed using previously described techniques (47). Mice were given an intraperitoneal injection of tetracycline-HCl (30 μg/g body wt) followed by an injection of calcine (15 μg/gram body wt) on days 3 and 8 before death, respectively. After harvesting, nondecalcified sections were fixed in 70% ethanol, followed by staining with Villaneuva stain, embedding in methylmethacrylate, and sectioning longitudinally at a thickness of 5–10 μm. The 5-μm sections were stained with Goldner’s stain and analyzed under transmitted light, and the 10-μm Villanueva-prestained sections were analyzed under fluorescent light, as previously described (47), using the Osteomeasure digitizing system (Osteometrics, Atlanta, GA). To analyze the effect of the transgene on cortical bone volume, the left tibia was prepared as described above for light microscopy and sectioned transversely at the tibio-fibular junction to standardize the location of the transverse section. Five-micrometer μm sections were stained with Goldner’s stain, and bone area (percentage of tissue area) was measured under transmitted light as previously described (47). The terminology and units used are those recommended by the Histomorphometry Nomenclature Committee of the American Society of Bone and Mineral Research (39).

Bone mineral densitometry (BMD and fat content measurements). BMD was assessed with a Lunar PIXIMUS2 bone densitometer (Lunar, Madison, WI). The instrument was calibrated before scanning sessions using a Phantom with known BMD, according to the manufacturer’s guidelines. For the studies, mice were anesthetized with ketamine (90 mg/kg) and xylazine (10-mg/kg dose) and then placed prone on the PIXImus platform. Whole body BMD was assessed by excluding the skull from the BMD measurement. BMD of lumbar spine was assessed by measuring the BMD of the lumbar vertebrae above the pelvis and below the ribs. Femoral BMD was assessed by measuring the middle one-third of the femoral shaft, avoiding the distal and proximal portions of the femur. Whole body fat content was also recorded in each of the densitometry scans. The individual densitometry measurements varied by 3–5% after repositioning.

Quantitative computed tomography. High-resolution quantitative computed tomography (QCT) was used to evaluate trabecular bone volume and microarchitecture in the distal femur (μCT40; Scanco Medical, Basserdorf, Switzerland). The femur was scanned at 45 kV with conebeam mode and a slice increment of 6 μm. Images from each group were generated at identical threshold. Scanning was started approximately at the growth plate and extended proximally for 350 slices. Morphometric analysis was performed on 150 slices extending proximally, beginning with the slice in which the femoral condyles had fully merged. The cortical bone and trabecular bone were separated manually on each slice by a cursor line. The three-dimensional structure was constructed and analyzed with the internal software of the μCT system. Morphometric parameters included the trabecular bone volume fraction (%), trabecular thickness (mm), trabecular number (no./mm), and trabecular spacing (mm).

Measurement of urinary deoxypyridinoline excretion. Mice were placed in metabolic cages (Hatteras Instrument, Cary, NC), and urine was collected for 24 h. The urine volume was measured before storage at −70°C. Deoxypyridinoline (DPD) excretion was quantitated using the Pyrilynks-D assay kit (Meta Biosystems, Mountain View, CA) according to the recommendations of the manufacturer. Data were expressed as nanomoles DPD per millimole creatinine.

Weights and femur length. Mice were weighed using a ScoutPro digital scale (Pine Brook, NJ) after euthanasia, femurs were harvested and cleaned of adherent soft tissues before measurement of femur length using a digital caliper (Stoelting, Wood Dale, IL).

Statistical analysis. Data are presented as the means ± SE. For comparisons between two groups, statistical significance was assessed by a Student’s t-test using the InStat computer program (GraphPad Software). For comparisons among more than two groups, statistical analysis was performed by analysis of variance followed by the Bonferroni procedure for multiple pairwise comparisons (51).

RESULTS

Creation of a GRK2 transgene. To determine whether modulation of GRK activity affects bone formation in vivo, we used a 1.3-kb fragment of the mouse OG2 promoter (see Fig. 1A) to target expression of GRK2 to osteoblasts (3). Before generating TG mice, we determined whether the transgene was expressed in the rat osteosarcoma cell line ROS17/2.8 cells. The GRK2 construct was designed so that expression of the transgene was driven solely by the OG2 promoter (see MATERIALS METHODS). For the studies, ROS17/2.8 cells were transfected with either empty vector or the GRK2 TG construct. The mammalian expression vector used in the experiments contained a neomycin-resistant cassette that permitted G418 selection. After stable transfectants were selected in G418, expression of GRK2 was investigated by immunoblotting. As shown in Fig. 1B, GRK2 was overexpressed by ROS17/2.8 cells transfected with the GRK2 transgene. With a longer exposure, a band for the endogenous GRK2 was also detected in cells transfected with vector. These data are consistent with the notion that the GRK2 transgene is targeted for expression in osteoblasts and achieves greater levels than endogenous GRK2 expression.

Effect of GRK2 overexpression on osteoblast-mediated bone formation in vivo. To determine whether our transgene affected bone formation in vivo, the linearized construct was injected into the pronuclei of one-cell mouse embryos and then surgically reimplemented into pseudopregnant females. Mice were screened for incorporation of the transgene by PCR, using primers specific for the transgene (Fig. 1A). Four independent lines were established from the founder animals, and identical results were obtained using the progeny from these independent founder lines. We first determined tissue-specific expression of our transgene by reverse transcription of total cellular RNA isolated from mouse tissues, followed by PCR using transgene-specific primers (Fig. 1, C and D). As shown in Fig. 1C, a PCR product of the appropriate size was detected in bone from TG mice using GRK2 primers when an RT reaction was performed before PCR. With additional PCR cycles, a small
suggest that GRK2 is overexpressed in bone from the four founder lines.

We next determined whether expression of the transgene was associated with altered responsiveness of GPCRs in bone by measuring PTH-induced cAMP generation by mouse calvaria ex vivo. As shown in Fig. 2B, we found that PTH-induced cAMP generation was significantly decreased in calvaria from TG mice compared with non-TG littermate controls 10 min after the addition of PTH-(1–34). In contrast, direct of activation adenyl cyclase by forskolin increased cAMP generation by mouse calvaria from TG animals and controls to a similar extent (Fig. 2C). Basal cAMP generation was similar in both groups [215 ± 30 dpm (non-TG) vs. 233 ± 31 dpm (TG); P = not significant (NS)]. These data are consistent with the notion that enhancing GRK activity in osteoblasts decreases GPCR responsiveness in bone, and this effect is mediated by inhibiting receptor-effector coupling.

To investigate the phenotype of our TG mice, we first performed BMD measurements on mice at 5–6 wk of age and at 6 mo of age. Progeny from the four founder lines were used for the experiments, and identical results were obtained using these independent founder lines. As shown Fig. 3, there was a statistically significant decrease in both whole body BMD as well as BMD of trabecular-rich lumbar spine in TG mice compared with non-TG littermate controls at 5–6 wk of age. A
Effect of sex on BMD

Table 1. Effect of sex on BMD

<table>
<thead>
<tr>
<th></th>
<th>Non-TG</th>
<th>TG</th>
<th>P Value</th>
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</thead>
<tbody>
<tr>
<td>Whole body BMD, mg/cm²</td>
<td>42.9±0.7</td>
<td>40.3±0.7</td>
<td>0.0221</td>
</tr>
<tr>
<td>Female mice</td>
<td>42.5±0.4</td>
<td>39.8±0.9</td>
<td>0.0097</td>
</tr>
<tr>
<td>Lumbar spine BMD, mg/cm²</td>
<td>47.6±1.0</td>
<td>44.4±0.9</td>
<td>0.0324</td>
</tr>
<tr>
<td>Male mice</td>
<td>48.6±1.3</td>
<td>43.4±1.0</td>
<td>0.0221</td>
</tr>
<tr>
<td>Female mice</td>
<td></td>
<td></td>
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</table>

Bone mineral density (BMD) values are means ± SE; 20 transgenic (TG) mice (11 males, 9 females) and 28 non-TG mice (13 males, 9 females) were studied.

Table 2. Effect of the transgene on mouse femurs

<table>
<thead>
<tr>
<th>BMD of the femoral shaft, mg/cm²</th>
<th>Non-TG</th>
<th>TG</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male and female mice</td>
<td>64.2±0.8</td>
<td>63.2±1.7</td>
<td>NS</td>
</tr>
<tr>
<td>Male mice</td>
<td>67.8±0.8</td>
<td>69.9±2.4</td>
<td>NS</td>
</tr>
<tr>
<td>Female mice</td>
<td>61.2±0.9†</td>
<td>59.1±1.8*</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are means ± SE. For BMD, 20 TG (11 males, 9 females) and 28 non-TG mice (13 males, 9 females) were studied. For fat content, 12-TG (6 males, 6 females) and 12 non-TG mice (6 males, 6 females) were studied. NS, not significant. **P = 0.0033 vs. male mice. †P < 0.0001 vs. male mice. ‡P = 0.019 vs. male mice.

Table 3. Effect of the transgene on body weight and fat content

<table>
<thead>
<tr>
<th></th>
<th>Non-TG</th>
<th>TG</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body wt, g</td>
<td>11.7±0.7</td>
<td>12.2±0.7</td>
<td>NS</td>
</tr>
<tr>
<td>6 wk</td>
<td>26.7±1.0</td>
<td>26.5±1.0</td>
<td>NS</td>
</tr>
<tr>
<td>6 mo</td>
<td>1.9±0.13</td>
<td>1.9±0.10</td>
<td>NS</td>
</tr>
<tr>
<td>Fat content, g</td>
<td>3.5±0.24</td>
<td>3.9±0.35</td>
<td>NS</td>
</tr>
<tr>
<td>6 wk</td>
<td>12.1±3.4</td>
<td>11.6±3.5</td>
<td>NS</td>
</tr>
<tr>
<td>6 mo</td>
<td>12.6±1.6</td>
<td>13.1±1.5</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are means ± SE. For body wt, 23 TG and 23 non-TG mice were studied at 6 wk of age, and 22 TG and 22 non-TG mice were studied at 6 mo of age. For fat content (in g and in % body wt), 20 TG (11 males, 9 females) and 28 non-TG mice (13 males, 9 females) were studied at 5–6 wk of age, and 24 TG and 24 non-TG mice were studied at 6 mo of age.
activity in osteoblasts caused a decrease in trabecular bone volume by reducing osteoblast-mediated bone formation. Effect of the transgene on expression OPG and RANKL mRNA and excretion of DPD. The osteoclast inhibitory factor OPG and the osteoclast differentiation factor RANKL are potent regulators of osteoclast development (49). Both OPG and RANKL are produced by osteoblasts (49), and agonists of several GPCR systems have been shown to modulate their production (29, 6, 38). We therefore determined whether our transgene affected OPG and RANKL mRNA levels. For the experiments, we performed semiquantitative RT-PCR using total cellular RNA isolated from calvaria of TG mice and non-TG littermate controls at 6 wk of age. As shown in Fig. 5A, OPG mRNA levels were increased in TG animals compared with non-TG littermate controls. In contrast, RANKL mRNA levels were decreased compared with those of controls (Fig. 5B). The GAPDH PCR products confirmed that the RT reaction was successful in the animals studied (Fig. 5C). Densitometric analyses indicated that the changes in OPG and RANKL mRNA levels were statistically significant in TG mice compared with non-TG controls (Fig. 5D) This alteration in the pattern of OPG and RANKL production would tend to inhibit osteoclast activation. We therefore measured urinary excretion of DPD, which is a marker of osteoclastic activity (9), in vivo. As shown in Fig. 5E, urinary excretion of DPD was decreased in TG mice compared with non-TG littermate controls. Serum osteocalcin levels tended to be higher in non-TG mice at 6 wk of age (880 ± 121 ng/ml) compared with TG mice (630 ± 38 ng/ml), but this difference did not reach statistical significance (P = 0.0914). These data suggest that the decrease in trabecular bone volume is not a result of increased osteoclastic activity.

**DISCUSSION**

GPCR agonists are important endogenous regulators of bone remodeling (8, 29, 35, 37, 42, 45, 48). Moreover, agonists of

<table>
<thead>
<tr>
<th>Table 4. <em>Trabecular bone structural measurements by QCT</em></th>
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<tr>
<td></td>
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<tr>
<td>BV/TV, %</td>
</tr>
<tr>
<td>Trabecular thickness, μm</td>
</tr>
<tr>
<td>Trabecular no./mm</td>
</tr>
<tr>
<td>Trabecular spacing, μm</td>
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</table>

Values are means ± SE; 7 TG (4 males, 3 females) and 7 non-TG mice (4 males, 3 females) were studied. QCT, quantitative computed tomography; BV, bone volume; TV, tissue volume.

**Table 5. Effect of GRK2 transgene on bone histomorphometry of lumbar spine**

<table>
<thead>
<tr>
<th>Static indexes of bone formation</th>
<th>Non-TG</th>
<th>TG</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trabecular bone volume (BV/TV), %</td>
<td>20.8±1.1</td>
<td>13.4±1.7</td>
<td>=0.0088</td>
</tr>
<tr>
<td>Osteoblast surface/bone surface, %</td>
<td>23.0±1.9</td>
<td>14.1±2.3</td>
<td>=0.0153</td>
</tr>
<tr>
<td>Osteoid surface/bone surface, %</td>
<td>39.8±4.8</td>
<td>37.4±6.2</td>
<td>NS</td>
</tr>
<tr>
<td>Osteoclast surface/bone surface, %</td>
<td>6.6±2.5</td>
<td>5.1±1.5</td>
<td>NS</td>
</tr>
<tr>
<td>Dynamic indexes of bone formation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mineralizing perimeter, μm</td>
<td>51.7±4.0</td>
<td>38.3±3.1</td>
<td>=0.0248</td>
</tr>
<tr>
<td>Mineral apposition rate, μm/day</td>
<td>1.18±0.17</td>
<td>0.96±0.12</td>
<td>NS</td>
</tr>
<tr>
<td>Bone formation rate, μm/μm²day⁻¹</td>
<td>0.64±0.08</td>
<td>0.38±0.06</td>
<td>=0.0355</td>
</tr>
</tbody>
</table>

Values are means ± SE; 7 TG (4 males, 3 females) and 6 non-TG mice (3 males, 3 females) were studied.
several GPCR systems may be useful therapies for regenerating the trabecular skeleton (14, 18, 19, 21, 31, 37, 42, 50). The effects of GPCR agonists as anabolic agents may be modulated by the desensitization of receptor signaling that occurs in the continuous presence of agonist (13, 27, 30, 40). This decrease in receptor responsiveness is largely mediated by direct phosphorylation of GPCR proteins by a family of enzymes termed GRKs (13, 27, 30, 40). Recent studies suggest that GRK2 and/or GRK3 plays a dominant role in regulating responsiveness of GPCRs (13, 27, 30, 40). These findings suggest that GRKs play an important role in regulating GPCR systems in bone, and suggest that manipulation ofGRK activity in bone may be a useful treatment strategy for modulating bone remodeling.

Although both bone formation and bone resorption were reduced in TG mice, the net effect of the transgene was a reduction in bone mass, as evidenced by a decrease in BMD and trabecular bone volume in the TG animals compared with non-TG littermate controls. Although this observation may initially seem paradoxical, bone mass is determined by the rate of bone formation relative to the rate of bone resorption. A decrease in bone mass occurs if the rate of bone formation is less than the rate of bone resorption. With regard to the present study, the data suggest that both bone resorption and bone formation were reduced in the TG mice compared with the non-TG littermate controls. We speculate that the decrease in bone mass in the TG mice is caused by a decrease in osteoblastic activity out of proportion to the decrease in osteoclastic activity. These data suggest that enhancing GRK activity in osteoblasts diminishes the anabolic effects of GPCR systems, leading to bone loss.

Consistent with the BMD data, histomorphometric analysis indicated that trabecular bone volume was decreased in TG mice compared with non-TG littermate controls. In TG animals, the decrease in trabecular bone volume was associated with a diminished osteoblastic surface as well as a decrease in the BFR and mineralizing perimeter. This pattern of histomorphometric findings is consistent with the notion that the GRK2 transgene promotes a decrease in bone mass by decreasing the number of basic multicellular units (BMUs). In contrast to the BFR, however, MAR was not altered by the presence of the transgene. Because the BFR is derived from the MAR and mineralizing perimeter (39), these data suggest that the decrease in bone formation in TG mice is caused, predominantly, by a decrease in the mineralizing perimeter. The transgene, therefore, appears to reduce bone formation in TG mice by decreasing the number of BMUs without altering the activity of individual bone forming units. This decrease in the number of BMUs could be due to either a reduction in activation of new bone formation sites or to a diminished life span of existing sites. The observation that the OG2 promoter drives gene expression in postmitotic mature osteoblasts (3) suggests that osteoblast proliferation or increased recruitment of new osteoblasts was not the dominant factor leading to the decrease in the BFR and osteoblastic surfaces. Studies by Jilka et al. (23) may, however, be relevant with regard to mechanism. These investigators found that GPCR agonists such as PTH are potent inhibitors of osteoblast apoptosis both in vitro and in vivo. Moreover, studies from this laboratory (15) and by other investigators (10, 33) suggest that GRK2 potently inhibits responsiveness of the PTH receptor. Although further studies will be necessary to examine the role of apoptosis in our TG model, it is tempting to speculate that the decrease in osteoblastic surfaces and BFR in the present study may be mediated by promoting osteoblast apoptosis.

The study results are in agreement with previous investigations from this laboratory (47) that examined the osseous effects of inhibiting GRK activity in osteoblasts using TG technologies. In these previous studies, TG mice demonstrated enhanced bone remodeling, as evidenced by an increase in osteoblast-mediated bone formation, as well as enhanced osteoclastic activity, as evidenced by increased urinary DPD excretion. The net effect of the transgene in this previous study, however, was anabolic, because both BMD and trabecular bone volume were increased in TG mice compared with non-TG littermate controls. Thus inhibition of osteoblast GRK activity increased bone mass, whereas, in the present study, enhancing GRK2 activity in osteoblasts decreased bone volume. Taken together, these data provide compelling evidence that modulating GRK activity in osteoblasts has potent effects on bone mass.

GRKs phosphorylate GPCR proteins at serine and threonine residues and, in turn, promote binding of a second group of protein cofactors termed arrestins, which interfere with receptor-effector coupling, presumably through steric mechanisms (30). In the present study, we measured cAMP generation by mouse calvaria ex vivo after treatment with either PTH (1–34) or the direct adenyl cyclase activator forskolin. We found that GPCR-stimulated cAMP generation was attenuated by the presence of the GRK2 transgene without altering cAMP generation after direct activation of adenyl cyclase by forskolin. These data suggest that the ability of adenyl cyclase to generate cAMP is intact; the reduction in PTH-induced cAMP generation is, therefore, caused by a decrease in the ability of the PTH receptor to couple to its effector systems (adenyl cyclase). This pattern of attenuated GPCR responsiveness is mechanistically consistent with the known actions of the GRKs (30).

While a major action of GRKs is to phosphorylate GPCR proteins, GRK2 also has effects that are not directly related to receptor phosphorylation (27). For example, GRK2 has domains that show significant homology to proteins termed regulators of G protein signaling (RGS proteins). These RGS proteins enhance GTPase activity of G protein α-subunits and, in turn, decrease the duration of G protein α-subunit activation (27). RGS domains in GRK2 weakly enhance GTPase activity of Goq subunits but significantly impair the ability of Goq-coupled receptors to stimulate phospholipase C, apparently by sequestering Goq subunits and preventing coupling to their effector systems (27). Although the role of Goq-coupled signaling cascades in regulating bone mass are not known with certainty, studies using the PTH receptor system suggest that the anabolic effects of PTH in vivo are mediated predominantly by adenyl cyclase-dependent pathways (1); Goq-coupled...
coupled pathways have little effect on PTH-induced bone formation in vivo (1). In contrast, activation of the \( \text{G}_{\alpha}\)-coupled receptor for prostaglandin \( \text{F}_2\alpha \) (7) potently stimulates new bone formation (41). In some instances, therefore, inhibition of \( \text{G}_{\alpha}\) signaling by GRK2 might favor a reduction in bone mass. Thus it is possible that the effect of the transgene in the present study is, in part, mediated by mechanisms for inhibiting GPCR signaling not directly related to GPCR phosphorylation.

Normally, osteoblastic activity is closely coupled to the activity of osteoclasts. This coupling is due, at least in part, to production of the regulatory factors produced by osteoblasts (49), including the osteoclast inhibitory factor OPG and the osteoclast differentiation factor RANKL (29, 38). In the present studies, we found that inhibition of osteoblastic activity by overexpression of GRK2 in osteoblasts was associated with enhanced mRNA levels of OPG and decreased mRNA levels of RANKL. This pattern of OPG and RANKL production would promote a low rate of bone remodeling. Indeed, excetration of the osteoclast activity marker, DPD, was decreased in urine of TG mice compared with control animals. Despite this increase in DPD excetration, we did not find a decrease in osteoclast perimeter in TG mice by bone histomorphometry. The inability to detect a difference in osteoclast number between TG and non-TG animals may, in part, be related to the sensitivity of the methodology. Alternatively, there may be little change in the actual number of osteoclasts, but the activity of individual osteoclasts is reduced. Taken together, the alterations in OPG and RANKL mRNA levels in conjunction with decreased urinary DPD excetration suggest that the transgene promotes reduced coupling of osteoblast-mediated bone formation to bone resorption by osteoclasts.

In summary, we found that overexpression of the potent GPCR regulator GRK2 in osteoblasts produced a low bone turnover state that reduced bone mass, ostensibly by reducing osteoblast-mediated bone formation out of proportion to osteoclast-mediated bone resorption. These studies, taken together with previous work from this laboratory (47), suggest that GRKs play a key role in regulating bone formation. We speculate that modulating GRK activity in bone may be a useful strategy for altering bone mass.

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Present addresses: S. Lui and D. Quarles are currently affiliated with the Kansas University Medical Center, Kansas City, KS 66160.

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