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Osteoclastogenic Activity and RANKL Expression Are Inhibited in Osteoblastic Cells Expressing Constitutively Active $G\alpha_{12}$ or Constitutively Active RhoA

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ABSTRACT

$G\alpha_{12}$–RhoA signaling is a parathyroid hormone (PTH)-stimulated pathway that mediates effects in bone and may influence genetic susceptibility to osteoporosis. To further elucidate effects of the pathway in osteoblasts, UMR-106 osteoblastic cells were stably transfected with constitutively active (ca) $G\alpha_{12}$ or caRhoA or dominant negative (dn) RhoA and co-cultured with RAW 264.7 cells to determine effects on hormone-stimulated osteoclastogenesis. Whereas PTH and calcitriol-stimulated osteoclastogenesis in co-cultures with UMR-106 cells expressing pcDNA or dominant negative RhoA, the osteoclastogenic effects of PTH and calcitriol were significantly attenuated when the UMR-106 cells expressed either caRhoA or ca$G\alpha_{12}$. These inhibitory effects were partially reversed by the Rho kinase inhibitor Y27632. None of the constructs affected osteoclastogenesis in untreated co-cultures, and the constructs did not inhibit the osteoclastogenic responses to receptor activator of NFκB ligand (RANKL). To investigate the mechanism of the inhibitory effects of ca$G\alpha_{12}$ and caRhoA, expression of RANKL, osteoprotegerin (OPG), osteopontin (OPN), and intercellular adhesion molecule-1 (ICAM) in response to PTH or calcitriol was examined in the UMR-106 cells. In the cells expressing pcDNA or dnRhoA, PTH and calcitriol increased RANKL mRNA and decreased OPG mRNA, whereas these effects were absent in the cells expressing ca$G\alpha_{12}$ or caRhoA. Basal expression of RANKL and OPG was unaffected by the constructs. The results suggest that $G\alpha_{12}$–RhoA signaling can inhibit hormone-stimulated osteoclastogenesis by effects on expression of RANKL and OPG. Since PTH can stimulate the $G\alpha_{12}$–RhoA pathway, the current findings could represent a homeostatic mechanism for regulating osteoclastogenic action. J. Cell. Biochem. 111: 1531–1536, 2010.

KEY WORDS: OSTEOBLAST; OSTEOCLASTOGENESIS; $G\alpha_{12}$; RHOA; PARATHYROID HORMONE; CALCITRIOL

$G\alpha_{12/13}$ proteins are a subfamily of heterotrimeric G proteins that act as molecular switches to exchange GDP for GTP and by this action transduce effects initiated through seven-transmembrane domain receptors [Kelly et al., 2007] and other effectors [Marty and Ye, 2010]. The $G\alpha_{12/13}$ subfamily is expressed in most tissues [Spicher et al., 1994] and has been shown to be involved in multiple physiological and pathophysiological processes [Worzfeld et al., 2008]. The most extensively studied mediators of $G\alpha_{12/13}$ signaling are the Rho family monomeric G proteins. These ~20 kDa proteins are activated by $G\alpha_{12/13}$ through guanine nucleotide exchange factors, RhoGEFs [Siehler, 2009]. Downstream effectors of Rho family small G proteins include Rho kinase and MAP kinase, as well as other mediators [Bishop and Hall, 2000]. The pathway is best characterized for its effects on the actin cytoskeleton, but other cytoskeletal responses, cell polarity, vesicular trafficking, integrin signaling, membrane transport, cell survival, erythropoesis, lymphocyte development, and cancer progression have been linked to the pathway [DeMali et al., 2003; Heasman and Ridley, 2008; Karlsson et al., 2009; Tybulewicz and Henderson, 2009; Mulloy et al., 2010].

Recent findings indicate that the $G\alpha_{12/13}$–RhoA–Rho kinase pathway has important roles in bone. In osteoclasts, RhoA is important for podosome assembly, osteoclast motility, and bone resorption [Chellaiah et al., 2000; Ory et al., 2008]. In C3H10T1/2 murine mesenchymal stem cells, RhoA and Rho kinase regulate fluid-flow-induced osteogenic differentiation, and RhoA activation decreases adipogenic and chondrogenic differentiation [Arnsdorf et al., 2009].

Survival of MC3T3-E1 osteoblastic cells [Yoshida et al., 2009], and the integrity of the actin cytoskeleton [Kazmers et al., 2009] are

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dependent upon the RhoA and Rho kinase signaling. Activation of Rho family small G proteins and their translocation to membrane sites are dependent upon lipid modification [Roberts et al., 2008]. Hence, statins and bisphosphonates, which interfere with the synthesis of isoprenyl lipids, as well as geranylgeranyl transferase inhibitors, can prevent effects mediated through RhoA [Reszka and Rodan, 2004; Liao and Laufs, 2005; Fritz and Kaina, 2006; Russell, 2007; Yuasa et al., 2007]. caRhoA is able to overcome the effects of the bisphosphonate alendronate to degrade the osteoblast cytoskeleton [Kazmers et al., 2009]. In UMR-106 osteoblastic cells, RhoA is crucial for parathyroid hormone (PTH)-stimulated protein kinase Cα (PKCα) translocation to the plasma membrane and is involved in the PTH-mediated stimulation of interleukin-6 expression [Radeff et al., 2004]. Particularly interesting are recent findings that genetic polymorphisms in RhoA and in the Rho GEF, ARHGEF3, show an association with bone mineral density *Z*-score in postmenopausal women [Mullin et al., 2008, 2009]. $G\alpha_{12/13}$ signaling has been less extensively studied in bone, but has been found to stimulate phospholipase D activity through RhoA [Singh et al., 2005].

To further elucidate the roles of $G\alpha_{12}$ and RhoA in osteoblasts, we investigated whether stable expression of mutant forms of the proteins in osteoblastic cells could affect hormone-stimulated osteoclastogenesis. The studies reported here show that expression of $caG\alpha_{12}$ or caRhoA in UMR-106 cells impairs their ability to induce osteoclastogenesis in response to PTH or calcitriol. The effect correlated with effects of $caG\alpha_{12}$ and caRhoA to prevent the stimulation of expression of RANKL mRNA in response to PTH or calcitriol.

METHODS

MATERIALS

RAW 264.7 monocyte–macrophage lineage osteoclast precursor cells and UMR-106 osteoblastic cells were purchased from American Type Culture Collection (Manassas, VA). The G12 construct (G12Q231L) and the control pcDNA were kindly provided by Dr. Tatyana Voyno-Yasenetskaya, University of Illinois. The RhoA dominant negative (RhoA19N) and constitutively active (RhoA63L) constructs were kindly provided by Dr. Said Sebti, University of South Florida. PTH(1-34) was purchased from BaChem (Torrance, CA); human sRANKL was purchased from Peprotech (Rocky Hill, NJ); 1,25-$(OH)_2D_3$ (calcitriol) was a gift from Dr. Milan Uskokovic (Hoffmann-La Roche, Nutley, NJ).

TRANSFECTION

Rat osteoblastic UMR-106 cells were transfected using a CalPhos mammalian transfection kit (Clontech, Mountain View, CA). Briefly, 2 μg of plasmid DNA was added to 12.4 μl 2 M calcium solution, and diluted with deionized water to make 100 μl solution A. Solution A was then added dropwise into 100 μl 2× HEPES-buffered saline (solution B) with slow vortexing to make the transfection solution. After incubation at room temperature for 20 min, the transfection solution was added dropwise to cell culture plates. After 16 h at 37°C, calcium phosphate-containing medium was removed and replaced with 2 ml fresh medium for 24 h. Stable transfectants were selected and maintained with α-MEM + 10% fetal bovine serum containing 600 μM G418.

CELL CO-CULTURE

For osteoclastogenesis and MTT assays, cells were cultured in 0.2 ml αMEM + 10% fetal bovine serum in 96-well Corning plates (Corning, NY). UMR-106 cells were used between the 18th and 29th passage. RAW 264.7 cells were used between the 13th and 20th passage. UMR-106 and RAW 264.7 cells were seeded at a density of 10^4/ml for UMR-106 cells and 2,500/ml for RAW 264.7 cells, a ratio found to be optimal in preliminary experiments. Treatments (PTH, calcitriol, and RANKL) were added the day after cell seeding. The medium was replaced with fresh treatment-containing medium after 48 h. The total treatment time was 5 days. For RT-PCR, UMR-106 cells expressing the constructs were cultured in six-well plates in 2 ml α-MEM + 10% fetal bovine serum. Treatments (PTH and calcitriol) were for 16 h.

TRAP ASSAY

At the end of the treatment period, medium was removed and cells were fixed by 10-min treatment with 50 μl 10% formalin. After a wash with 100 μl 95% ethanol, wells were dried and incubated for 30 min with 100 μl TRAP substrate (phosphatase substrate, cat #P4744, Sigma–Aldrich, St. Louis, MO), 1.36 mg/ml in buffer consisting of 50 mM Na citrate/10 mM tartrate buffer, pH 4.6. This was then added to 100 μl 0.1 N NaOH, and the absorbance read at 410 nm on a microplate reader (Dynatech, Chantilly, VA).

FORMATION OF MULTINUCLEATED CELLS

Cells were stained with 200 μl TRAP reaction solution, which was prepared by dissolving 5 mg napthol AS-MX phosphate (Sigma–Aldrich) in 0.5 ml *N*,*N*-dimethylformamide. This was mixed with 50 ml of buffer consisting of 100 mM Na acetate, 50 mM Na tartrate, pH 5.0, and 30 mg Fast Violet LB salt (Sigma–Aldrich) was added. The duration of staining was 2–4 h. Cells with three or more nuclei were counted.

RT-PCR

cDNA was synthesized using 1 μg total RNA and a reverse transcription system (Promega, Madison, WI). PCR was performed in a 25-μl reaction solution containing Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA) and cDNA template. After denaturing the template and activating the polymerase at 94°C for 2 min, the PCR reaction was run for 30–40 cycles at: 94°C for 15 s, 55–60°C for 30 s, and 72°C for 60 s. The forward and reverse primers of RANKL were 5′-TCG GGT TCC CAT AAA GTC AG-3′ and 5′-CTT GGG ATT TTG ATG CTG GT-3′. For OPG, forward and reverse primers were 5′-TGG GAA TGA AGA TCC TCC AG-3′ and 5′-GAG GAA GGA AAG GGC CTA TG-3′. For osteopontin, forward and reverse primers were 5′-CTA AGC CTC AGC ATC CTT GG-3′ and 5′-TGT AAT GCG CCT TCT CCT CT-3′. For ICAM, forward and reverse primers were 5′-AGG TAT CCA TCC ATC CCA CA-3′ and 5′-GCC ACA GTT CTC AAA GCA CA-3′. The forward and reverse primers for housekeeping gene Rpl13a were 5′-CCC TCC ACC CTA TGA CAA GA-3′ and 5′-CCT TTT CTT TCC GTT TCT CC-3′.

STATISTICS

The significance of changes was assessed by ANOVA and Bonferroni posttest. A value of $P < 0.05$ was considered significant.

RESULTS

In co-cultures of UMR-106 osteoblastic cells and RAW 264.7 osteoclast precursor cells, treatment for 5 days with PTH (30 nM), calcitriol (100 nM), or the combination stimulated osteoclastogenesis as shown by increased TRAP activity (Fig. 1). However, stable transfection of the UMR-106 cells with caGα_{12} or caRhoA prevented the osteoclastogenic effects of PTH and calcitriol. Cells expressing dnRhoA responded similarly to cells expressing the empty vector, with PTH, calcitriol, or the combination eliciting a significant increase in TRAP activity. No stimulation of TRAP activity was detected when the RAW 264.7 cells were cultured in the absence of UMR-106 cells. The responses in co-cultures containing UMR-106 cells transfected with EV were not different from those in co-cultures containing non-transfected UMR-106 cells (data not shown). There was no TRAP activity in UMR-106 cells cultured in the absence of RAW 264.7 cells (data not shown).

Although the 5-day treatment with PTH + calcitriol did not elicit large numbers of osteoclasts, the effects on TRAP were reflected in the numbers of multinucleated osteoclasts formed. Expression of caGα_{12} or caRhoA in the UMR-106 cells largely prevented the formation of multinucleated cells in the co-cultures (Fig. 2).

Since Gα_{12} signaling is often transduced through RhoA, and RhoA effects can be mediated through the downstream kinase, Rho kinase, the effects of the Rho kinase inhibitor Y27632 on TRAP activity in the co-cultures was determined. The inhibitor, at a concentration of 3 μM, significantly reversed the inhibitory effects of caGα_{12} and caRhoA on TRAP activity in co-cultures treated with PTH + calcitriol (Fig. 3). There were no direct effects of Y27632 on TRAP activity in the RAW 264.7 cells under the conditions used.

To determine whether the constructs could influence the response to RANKL, the co-cultures were treated with 30 ng/ml RANKL. As expected, RANKL markedly stimulated TRAP activity in the RAW 264.7 cells in the absence of UMR-106 cells (Fig. 4). Interestingly, the addition of any osteoblastic cells decreased the response of the UMR-106 cells to RANKL, an effect that was observed in most experiments. However, expression of the caGα_{12} or caRhoA constructs failed to alter the response to RANKL, although in the same experiment it produced the usual effect to decrease the response to PTH + calcitriol.

No significant differences in metabolic or proliferative activity, assessed by MTT assay, were observed in UMR-106 cells transfected with the different constructs (Fig. 5). Since the findings indicate that expression of caGα_{12} or caRhoA in osteoblastic cells impairs their ability to activate osteoclast precursor cells in response to the osteoclastogenic ligands PTH and calcitriol, experiments were carried out to determine the effects of the ligands on the expression

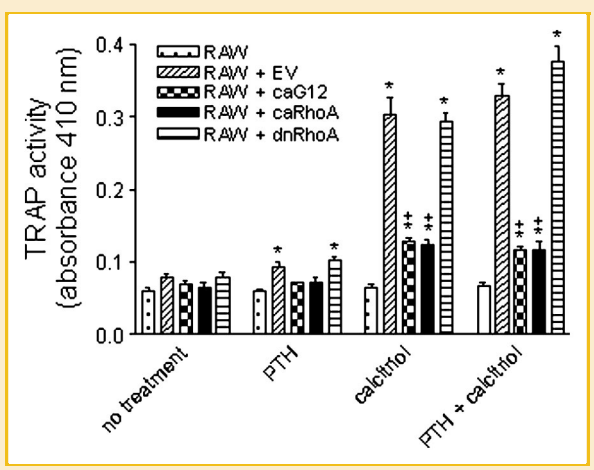

Fig. 1. The osteoclastogenic effects of PTH (30 nM) and calcitriol (100 nM) in UMR-106/RAW 264.7 cell co-cultures as assessed by TRAP activity are markedly attenuated when the UMR-106 cells stably express caGα_{12} or caRhoA constructs. Cells were seeded at a density of 10^4/ml for UMR-106 cells and 2,500/ml for RAW 264.7 cells in 96-well plates and maintained in α-MEM supplemented with 10% fetal bovine serum. Five-day treatments began on the day after seeding. $^*P < 0.05$ versus co-cultures without PTH or calcitriol, $^+P < 0.05$ versus co-cultures containing EV-transfected UMR-106 cells, N = 3.

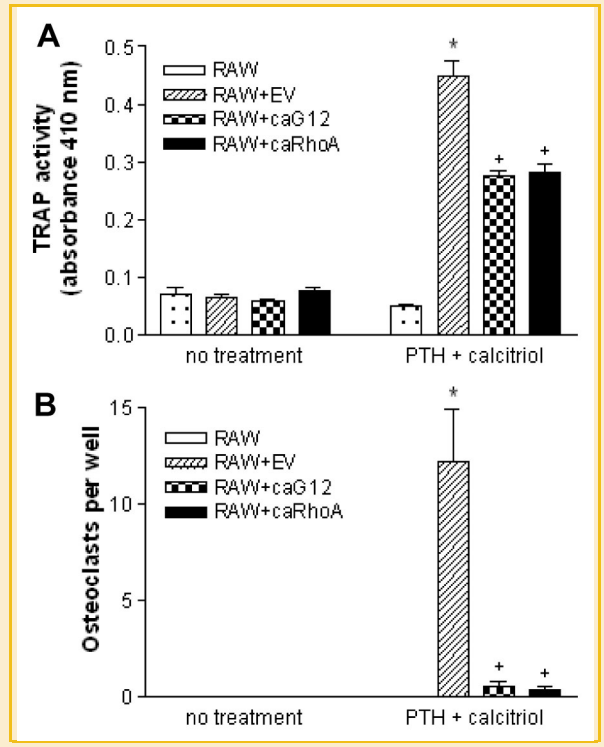

Fig. 2. CaGα_{12} or caRhoA stably expressed in UMR-106 cells prevent the effect of PTH (30 nM) + calcitriol (100 nM) on [A] TRAP activity and [B] multinucleation in UMR-106/RAW 264.7 cell co-cultures. Cells were seeded at a density of 10^4/ml for UMR-106 cells and 2,500/ml for RAW 264.7 cells in 96-well plates and maintained in α-MEM supplemented with 10% fetal bovine serum. Five-day treatments began on the day after seeding. $^*P < 0.05$ versus co-cultures without PTH + calcitriol, $^+P < 0.05$ versus co-cultures containing EV-transfected UMR-106 cells, N = 6.

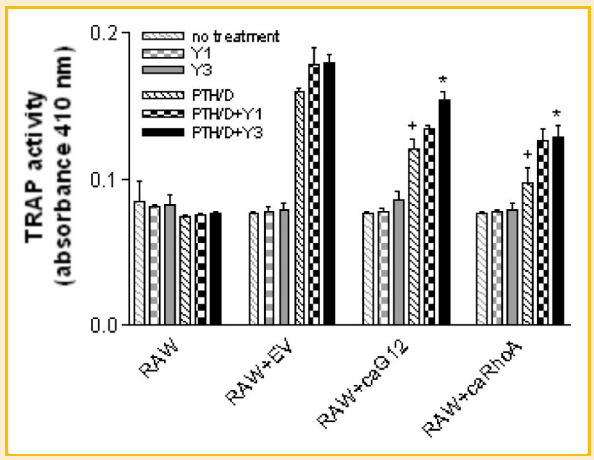

Fig. 3. The Rho kinase inhibitor, Y27632, reverses the inhibitory effects of stable expression of caGα_{12} or caRhoA in UMR-106 cells on PTH (30 nM) + calcitriol (100 nM)-stimulated osteoclastogenesis in co-cultures with RAW 264.7 cells. Cells were seeded at a density of 10^4/ml for UMR-106 cells and 2,500/ml for RAW 264.7 cells in 96-well plates and maintained in α-MEM supplemented with 10% fetal bovine serum. Five-day treatments began on the day after seeding. $^*P < 0.05$ versus no Y27632, $^+P < 0.05$ versus response to PTH + calcitriol in co-cultures containing EV-transfected UMR-106 cells, N = 3.

of the osteoblast cytokines RANKL and OPG, the former being a principal stimulator of osteoclastogenesis, and the latter a decoy receptor for RANKL that prevents its effects on the receptor RANK on the preosteoclast target cell. PTH, calcitriol, or the combination increased RANKL mRNA and decreased OPG mRNA in UMR-106 cells expressing pcDNA or dominant negative RhoA (Fig. 6). However, in cells expressing caGα_{12} or caRhoA, these responses were prevented or even slightly reversed. In addition to RANKL and

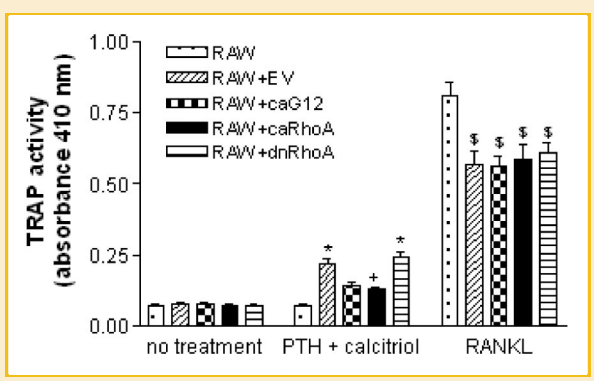

Fig. 4. Stable expression of caGα_{12} or caRhoA in UMR-106 cells fails to differentially attenuate responses to RANKL (30 ng/ml) in UMR-106/RAW 264.7 cell co-cultures. In the same experiment, caGα_{12} and caRhoA attenuated the response to PTH (30 nM) + calcitriol (100 nM). Cells were seeded at a density of 10^4/ml for UMR-106 cells and 2,500/ml for RAW 264.7 cells in 96-well plates and maintained in α-MEM supplemented with 10% fetal bovine serum. Five-day treatments began on the day after seeding. $^*P < 0.05$ versus co-cultures without PTH + calcitriol, $^+P < 0.05$ versus co-cultures containing UMR-106 cells stably expressing dnRhoA or EV, $^\$P < 0.05$ versus effect of RANKL in RAW 264.7 cells alone, N = 6.

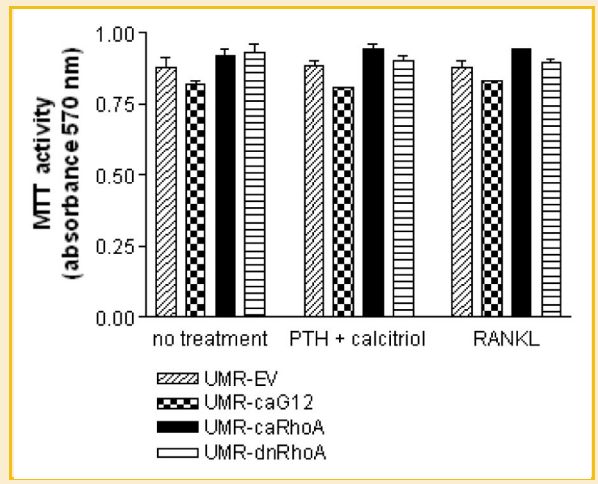

Fig. 5. Stable expression of caGα_{12}, caRhoA, or dnRhoA in UMR-106 cells do not affect cell viability, as assessed by MTT assay. UMR-106 cells were seeded at a density of 10^4/ml in 96-well plates and maintained in α-MEM supplemented with 10% fetal bovine serum. Five-day treatments began on the day after seeding, N = 3.

OPG mRNA, osteopontin (OPN) and intercellular adhesion molecule-1 (ICAM) mRNA expression were examined. OPN has been found to antagonize some effects of PTH [Ono et al., 2008] and ICAM is regulated by RhoA and Rho kinase [Anwar et al., 2004] and is involved in cell–cell interactions mediating PTH-stimulated osteoclast differentiation and bone resorption [Okada et al., 2002]. If these genes were altered by the constructs, they could potentially mediate the observed effects. OPN expression was slightly increased by caGα_{12}, an observation that we had noted previously in microarray studies [Wang et al., 2008]. However, OPN expression in response to PTH, calcitriol, and the combination was not markedly different in cells expressing the constitutively active constructs and in cells expressing the empty vector, although it was decreased in cells expressing dnRhoA. Also, there were no differences in the expression of ICAM among the treatments or with the different constructs.

DISCUSSION

Osteoblast-stimulated osteoclastogenesis is an important component of bone remodeling as well as a potential limitation in the therapeutic use of PTH to build bone. Excessive calcitriol can also stimulate osteoclastogenesis. Factors that modulate this osteoclastogenic effect are important for understanding bone physiology and could lead to new therapeutic agents that lack this limitation. The current studies reveal that in co-cultures of UMR-106 osteoblastic cells and RAW 264.7 osteoclast precursor cells, expression of caGα_{12} or caRhoA in the UMR-106 cells impairs the ability of PTH or calcitriol to stimulate the differentiation of the RAW 264.7 cells into cells producing TRAP (Figs. 1–4) and having the ability to form multinucleated osteoclasts (Fig. 2). The cells expressing caGα_{12} or caRhoA also had impaired ability to express RANKL in response to PTH or calcitriol (Fig. 6). The small decrease in expression of OPG in

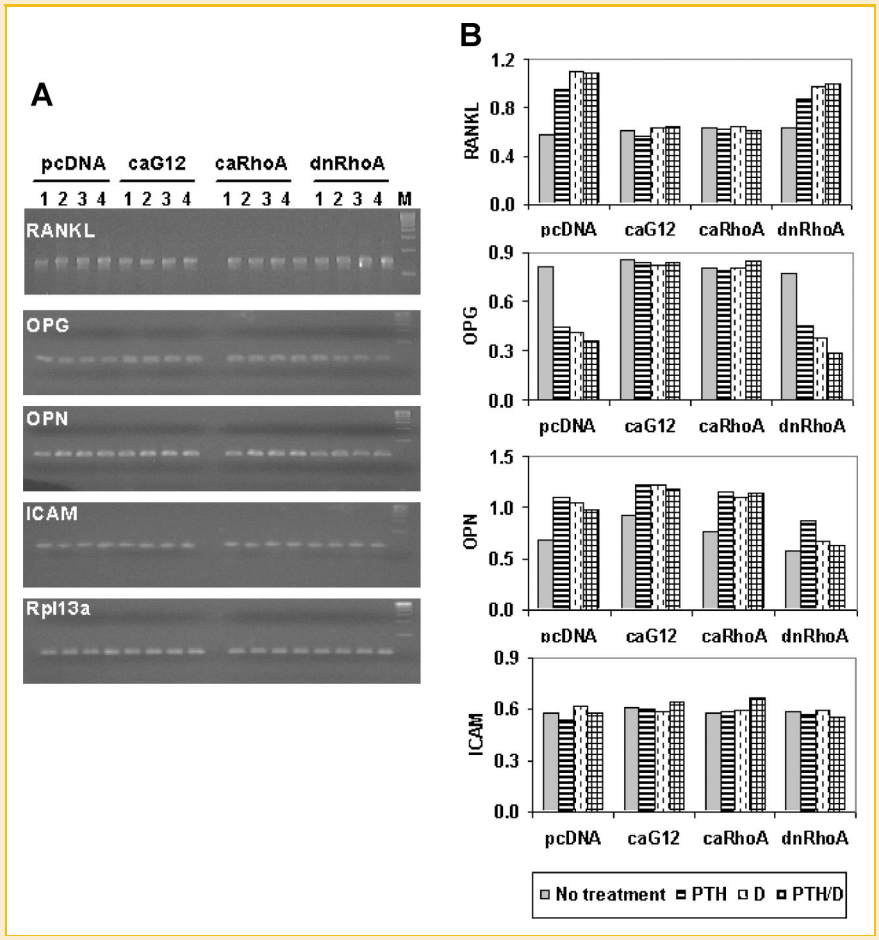

Fig. 6. Constitutively active G12 and RhoA constructs attenuate the effects of PTH, calcitriol, and the combination to increase RANKL and decrease OPG expression in UMR-106 cells, but do not influence PTH, calcitriol, or PTH + calcitriol responses to OPN or ICAM. A: RT-PCR of effects of treatments on RANKL, OPG, OPN, and ICAM. 1. control; 2. PTH (P); 3. calcitriol (D); 4. PTH + calcitriol (P + D); M. 100 bp molecular ruler. B: The relative expression levels of genes were analyzed and normalized against the housekeeping gene Rpl13a. The bands shown in (A) were scanned and quantified for the analysis.

response to PTH or calcitriol was not seen in the cells transfected with caGα_{12} or caRhoA. However, it seemed unlikely that this was an important factor in the response, since the cells expressing the constitutively active constructs were not different from empty vector or dnRhoA-expressing cells in their response to RANKL. Thus, the effect appears to be mediated at the level of RANKL production rather than by interference with the response to RANKL. The results demonstrate that the Gα_{12}–RhoA signaling pathway in osteoblastic cells is able to modulate hormone-stimulated osteoclastogenesis.

Several years ago, Wang et al. [2002] described experiments on the effects of geranylgeranoic acid on bone, which are pertinent to the current findings, since geranylgeranoic acid could conceivably serve as a geranylgeranyl group donor and activate RhoA. They had investigated the effects of geranylgeranoic acid as a retinoid, a class of compounds that they had found to induce osteoblast differentiation and to inhibit osteoclast formation [Park et al., 1997; Ishimi et al., 1999]. Wang et al. found that geranylgeranoic acid inhibited osteoclast formation induced by calcitriol in co-cultures of mouse bone marrow cells and primary osteoblasts, an effect similar to what we observed in the co-cultures of RAW 264.7 cells with UMR-106 osteoblatic cells treated with caGα_{12} or caRhoA. They also observed that when ST2 stromal cells were treated with geranylgeranoic acid, the effects of calcitriol or prostaglandin E2 to decrease OPG mRNA were antagonized, similar to what we observed in the UMR-106 cells expressing caGα_{12} or caRhoA. They observed that geranylgeranoic acid inhibited the effect of RANKL in cultures of bone marrow macrophages. This is not necessarily inconsistent with our results showing that the constructs did not affect the response to RANKL, since the studies in macrophages would represent an effect of geranylgeranoic acid directly on the osteoclast precursor, which our experiments were designed to circumvent. Even Y-27632, added directly to the cultures, failed to have significant effects unless osteoblastic cells were present in addition to the RAW cells. Wang et al. [2002] also found that geranylgeranoic acid increased bone mineral density when administered in vivo to SAMP6 senescence-accelerated mice. It is possible that a similar effect might be seen with activation of Gα12 or RhoA in osteoblasts.

The processes downstream of RhoA that lead to the observed effects on RANKL and on osteoclastogenesis remains to be determined. However, the effects could have physiological

implications. Although both PTH and calcitriol can have effects leading to bone formation, prolonged continuous treatment with PTH or exposure to high concentrations of calcitriol can result in accelerated resorption and bone loss, likely through stimulation of RANKL expression. Since PTH can activate RhoA [Radeff et al., 2004] possibly through $G\alpha_{12}$ [Singh et al., 2005], although conceivably through other pathways, the current studies suggest the presence of a homeostatic mechanism by which stimuli that lead to excess resorption could be modulated, resulting in the protection and preservation of bone.

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