The calcimimetic R-568 increases vitamin D receptor expression in rat parathyroid glands

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Secondary hyperparathyroidism (HPT) is a common complication of chronic kidney disease. In uremic patients, decreased vitamin D levels, hypocalcemia, and phosphorus retention result in increased parathyroid hormone (PTH) secretion and parathyroid gland hyperplasia (26). Traditional treatments for secondary HPT include dietary phosphate restriction and administration of phosphate binders and vitamin D sterols. However, in many patients, secondary HPT is not adequately controlled with these medications (10, 30), and the use of high doses of vitamin D sterols may be associated with an elevated sensitivity of the gland to extracellular calcium. The calcimimetics have been shown to decrease PTH synthesis (18) and secretion (11, 22, 23), reduce parathyroid cell proliferation (9, 27, 29), and correct the histological features of disordered bone turnover (17, 28) in preclinical models.

We previously demonstrated that extracellular calcium regulates vitamin D receptor (VDR) expression by parathyroid cells (13). Since R-568 potentiates the effects of extracellular calcium on the parathyroid gland via the CaSR, it is likely that calcimimetics have the ability to increase VDR expression in parathyroid tissue. However, without translation to active protein, increased VDR mRNA expression is unlikely to have a significant clinical impact. Thus the present study investigated the effect of R-568 on VDR message and protein expression in the parathyroid glands.

Materials and Methods

Animals

Male Wistar rats (250 g body wt) were fed a commercial diet containing 1.1% calcium and 0.8% phosphorus and vitamin D (1 IU/g). All animals received humane care in compliance with the “Principles of Laboratory Animal Care,” formulated by the Spanish National Society for Medical Research and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The experimental protocols were reviewed and approved by the Ethics Committee for Animal Research of the Universidad de Cordoba.

In Vitro Studies

Rat parathyroid glands were obtained as previously described (1). Briefly, rats were anesthetized with thiopental sodium (50 mg/kg ip). Blood was drained by aortic puncture, and within 2 min the parathyroid glands were dissected free of the thyroid gland and removed.

Parathyroid gland culture. Intact rat parathyroid glands were placed inside a nylon basket in individual wells containing 2 ml of buffered (pH 7.4) incubation medium (125 mM NaCl, 5.9 mM KCl, 0.5 mM MgCl2, 1 mM sodium pyruvate, 4 mM glutamine, 12 mM glucose, and 25 mM HEPES with 0.1 IU/ml human insulin, 0.1% BSA, 100 IU/ml penicillin G, and 100 mg/ml streptomycin). A phosphate concentration of 1 mM was achieved by addition of 1.2 Na2HPO4-Na2HPO4. CaCl2 was added as required to achieve 0.4–1.8 mM calcium. All chemical products were obtained from Sigma (St. Louis, MO). The glands were maintained at 37°C with constant rocking and shaking (model AOS-0, SBS).

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In vitro studies of PTH secretion. After extraction, rat parathyroid glands were stabilized in incubation medium at 1.25 mM calcium for 6 h. For study of the secretory response of cultured parathyroid glands in response to changes in extracellular calcium (thereby generating the PTH-calcium curve), the glands were sequentially incubated for 1 h in 0.4–1.8 mM calcium. After stabilization, parathyroid glands were placed for 1 h in incubation medium containing 0.6 or 1 mM calcium and then transferred to wells containing the same calcium concentration (0.6 or 1.0 mM) and increasing concentrations of R-568 (0.001, 0.01, 0.1, and 1 μM). The glands were continuously exposed to each calcimimetic concentration for 1 h. At the end of the incubation period, an aliquot of the medium was frozen at −20°C for PTH measurement.

In vitro studies of VDR and PTH expression. Parathyroid glands were incubated for 6 h at 0.6, 1.0, 1.5, and 1.8 mM calcium, while other glands were incubated in medium containing 0.6 mM calcium and 0.001, 0.01, or 0.1 μM R-568. Additional glands were incubated in medium containing 1 mM calcium and 0.001 or 0.01 μM R-568. At the end of the incubation period, the glands were freeze-dried at −80°C for storage until VDR mRNA and PTH mRNA measurement.

In vitro studies of VDR protein in human parathyroid glands. Parathyroid glands obtained from three hemodialysis patients who underwent parathyroidectomy because of refractory hyperparathyroidism were also studied. Permission to perform these studies, in compliance with the ethical principles for medical research involving human subjects set by the World Medical Association Declaration of Helsinki, was obtained from the Ethics Committee of the Hospital Universitario Reina Sofia. Immediately after parathyroidectomy, parathyroid glands were placed at 4°C in RPMI 1640 medium with 1.5 mM calcium; experiments were performed 14–16 h later. Before the experiment, the parathyroid tissue was cut into ~1-mm³ slices, thereby preserving tissue architecture. Ten slices from the same gland were considered representative of the entire gland (7). An equal number of slices from the three to four glands available from each patient were pooled to obtain a sufficient amount of tissue to perform the experiments. The slices were incubated for 6 h in medium with high (1.35 mM) or low (0.6 mM) calcium with or without R-568 (0.1 μM); then the tissue was processed for measurement of VDR protein by Western blot. Histological studies had been conducted in each gland to define whether hyperplasia was diffuse or nodular.

In Vivo Experiments

Rats were randomly allocated into four experimental groups. Control (n = 20) animals received no treatment. The second group of animals (R-568, n = 22) received two injections of R-568 (1 mg/kg) intravenously 6 and 3 h before euthanasia. The third group of animals (calcitriol, n = 22) received an administration of calcitriol (10 pmol ip every 30 min) starting 5.5 h before euthanasia. The final group of animals (n = 22) received both treatments before euthanasia. Since administration of R-568 produced moderate hypocalcemia, an additional group of rats (n = 13) that received EDTA (300 mg/kg im) to decrease serum calcium to the same level observed in rats treated with R-568 was also studied.

Euthanasia was carried out by aortic puncture and exsanguination of the anesthetized (intraperitoneal thiopental sodium) rats. Ionized calcium was measured immediately after blood extraction, and the serum was frozen at −20°C for PTH measurements. The parathyroid glands were removed, freeze-dried, and stored at −80°C for VDR mRNA, PTH mRNA, and VDR protein quantification.

VDR mRNA Measurement: RNA Isolation and RT-PCR

Parathyroid tissue (from the in vitro experiments) at the end of the incubation period and fresh parathyroid tissue (from the in vivo experiments) were freeze-dried in liquid nitrogen and stored at −80°C until RNA isolation. For RNA isolation, 1 ml of phenol-guanidine isothiocyanate solution (Tri-Reagent, Sigma) was added to the glands. The glands were ultrasonicated for 5 min at 4°C to allow for complete cell rupture. Thereafter, total RNA was extracted following a modification of the protocol of Chomczynski and Sacchi (8). Extracted total RNA was dissolved in nuclease-free water (Promega, Madison, WI) and heated for 10 min at 60°C. Total RNA was quantified by spectrophotometry (24). VDR and PTH vs. β-actin were amplified with a RT-PCR kit (Quantitect SYBR green, Qiagen, Hilden, Germany) using specific primers and 100 ng of total RNA per sample. The following primers were used: 5′-ACA GTC TGA GGC CCA AGC TA-3′ (sense) and 5′-TCT CGT AAG TCA GCC TAG GT-3′ (antisense) for VDR, 5′-TCT CCT TAC CCA GGC AGA TG-3′ (sense) and 5′-CAT TGC ATC TCC TCT ACA GA-3′ (antisense) for PTH, and 5′-TGT ACA CAA CTG CGA CTA TAT GGA G-3′ (sense) and 5′-ACA ATG CCA GTG GTA CGA CCA GA-3′ (antisense) for β-actin. DNA amplifications were processed by real-time PCR (LightCycler, Roche, Basel, Switzerland). Data were analyzed using LightCycler 3.5.28 software (Roche).

VDR Protein Extraction and Western Blot Analysis

Rat and human parathyroid tissue was placed in KTED buffer [300 mM KCl, 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 5 mM DTT, and 5 μl of protease inhibitor cocktail (Sigma)] and stored in liquid nitrogen until processing. Protein extraction from rat and human parathyroid tissue was performed as previously described (25). The tissue was homogenized at 4°C in 200 μl of buffer A (pH 7.9, 10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5 mM PMFS, and 1% NP-40), incubated for 15 min on ice, and centrifuged at 13,000 rpm for 1 min at 4°C. The supernatant was removed, and the pellet was resuspended with 100 μl of cold buffer B (pH 7.9, 20 mM HEPES, 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1 mM PMFS). After 15 min of incubation on ice, the extract was centrifuged at 13,000 rpm for 5 min at 4°C, and the supernatant (including nuclear protein) was stored at −80°C until analysis by Western blot. Protein concentrations were quantified by the Bradford method (4), with BSA used as a standard.

For Western blot analysis, 80 μg of protein were electrophoresed on a 10% SDS-polyacrylamide gel (Invitrogen) and electrophoretically transferred (Transfer Systems, Bio-Rad, Hercules, CA) from the gels onto nitrocellulose membranes (Invitrogen). The following steps were performed with gentle shaking. Membranes were incubated in TTBS-L blocking solution [20 mM Tris-HCl (pH 7.6), 0.2% Tween 20, 150 mM NaCl, and 5% nonfat dry milk (Bio-Rad)] at room temperature for 2 h to block nonspecific binding. Membranes were then washed with TTBS buffer (the same composition as TTBS-L without nonfat dry milk) and incubated for 2 h at room temperature with a 1:500 dilution of a rabbit anti-VDR polyclonal IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The membranes were washed with TTBS buffer and incubated with anti-rabbit IgG conjugated with alkaline phosphatase (AP, 1:1,000 dilution; Santa Cruz Biotechnology) at room temperature for 2 h. The AP Conjugate Substrate kit (Bio-Rad) was used to visualize binding, and the signal was quantified using a densitometric scanner (Gelprinter Plus). Chemicals were obtained from Sigma unless otherwise indicated. β-Actin was used as housekeeping protein to ensure equal loading of the gels.

Biochemical Measurements

Ionized calcium was measured using a selective electrode (model 634, Ciba Corning, Essex, UK), and PTH was quantified according to the vendor’s instructions using the rat-specific immunoradiometric assay (Nichols Institute, San Juan Capistrano, CA).

Statistics

Values are means ± SE. The difference between means for two different groups was determined by t-test; the difference between
The dose-dependent inhibition of PTH secretion by calcium in rat parathyroid glands cultured in vitro is shown in Fig. 1A. Maximal PTH secretion was achieved at 0.6 mM calcium. Increasing calcium concentrations resulted in a progressive decline in PTH values that reached a minimum (~40% of maximal PTH secretion) at 1.5–1.8 mM calcium. Incubation of the parathyroid glands in the presence of 0.001–1 μM R-568 also resulted in a dose-dependent decrease in PTH secretion (Fig. 1B) at 0.6 and 1 mM calcium. R-568 at 0.1–1 μM decreased PTH secretion by 30–40% at 0.6 mM calcium and by 50–60% at 1 mM calcium (Fig. 1B). Therefore, maximal reduction of PTH secretion by high calcium (1.5–1.8 mM) was similar to the inhibition observed with moderately low (1 mM) calcium + 1 μM R-568.

A progressive increase in VDR mRNA was detected when the parathyroid glands were incubated with increasing calcium concentrations. VDR mRNA levels were increased to 178 ± 47% and 330 ± 42% at 1 and 1.5 mM calcium, respectively, compared with 0.6 mM calcium (100 ± 15%, P < 0.05). No further increase in VDR expression was observed at >1.5 mM calcium. Increased VDR expression was detected in glands incubated with 0.6 mM calcium + R-568. The increase was significant at 0.01 μM R-568 (203 ± 38%) vs. calcium alone (100 ± 15%, P = 0.01). No further significant increase in VDR mRNA was detected with higher concentrations of R-568. When parathyroid glands were incubated with 1 mM calcium + 0.001 μM R-568, VDR mRNA levels (306 ± 46%) were significantly (P < 0.05) higher than those of control calcium and similar to those at 1.5 mM calcium. Increasing the dose of R-568 from 0.001 to 1 μM in the presence of 1 mM calcium did not result in a further increase in VDR expression in parathyroid glands (Fig. 2). The effect of calcium and R-568 on PTH mRNA was also evaluated. Compared with the 0.6 mM calcium medium (100%), the media with calcium 1.0 and 1.5 mM significantly (P < 0.05) reduced PTH mRNA to 60 ± 3% and 44 ± 4%, respectively. Addition of 0.01 μM R-568 to the 1.0 mM calcium medium reduced PTH mRNA to 42 ± 5%, which is similar to the PTH mRNA expression observed at 1.5 mM calcium (44 ± 4%).

The in vivo effect of R-568 on VDR is depicted in Fig. 3. A significant increase in VDR mRNA (168 ± 9%) was detected in rats treated with R-568 (1 mg/kg iv) 6 and 3 h before death compared with the untreated control group (100 ± 17%, P < 0.001). The increase in VDR mRNA induced by R-568 was similar to the elevation in VDR mRNA induced by calcitriol (10 pmol/kg ip every 30 min starting 5.5 h before death; 198 ± 16%, P < 0.001 vs. control). The simultaneous administration of R-568 and calcitriol potentiated the individual effects of each drug. Accordingly, a marked increment in VDR mRNA (306 ± 51%) was detected in rats treated with R-568 + calcitriol. VDR mRNA levels were significantly (P < 0.05) higher in rats treated with R-568 + calcitriol than in rats treated with R-568 or calcitriol. A similar trend was detected in VDR protein, which was increased in the R-568- and calcitriol-treated rats. However, the synergistic effect of both treatments was not evident at the protein level (Fig. 3). Since administration of R-568 decreased serum ionized calcium, additional experiments were performed in a separate group of rats in which a similar degree of hypocalcemia was induced by injection of EDTA. As shown in Fig. 4, a marked increase in VDR protein was observed in rats treated with R-568 compared with EDTA-treated hypocalcemic rats. Treatment with R-568 also influenced PTH mRNA levels, which decreased by one-half (51 ± 13%, P < 0.05 vs. control). Calcitriol, however, did not modify PTH mRNA levels (89 ± 17%). Interestingly, R-568 + calcitriol also reduced PTH mRNA (34 ± 11%, P < 0.05 vs. control; Fig. 3).

VDR protein levels in human parathyroid glands from hemodialysis patients are shown in Fig. 5. Histological examination of these glands indicated diffuse hyperplasia in patient 1 and nodular hyperplasia in patients 2 and 3. After incubation at high (1.35 mM) and low (0.6 mM) calcium + R-568 (0.1 μM), VDR protein levels were increased in glands from patient 1. However, VDR protein levels were relatively low in glands from patients 2 and 3 and did not change in response to increased calcium or R-568.

**DISCUSSION**

The present study investigated the influence of the calcimimetic R-568 on VDR expression in parathyroid cells. Our results showed that R-568 increased VDR mRNA and protein in rat parathyroid tissue in vitro and in vivo, as well as VDR protein in a human parathyroid gland with diffuse hyperplasia in vitro.

Extracellular calcium has been reported to regulate VDR expression in parathyroid tissue and to increase VDR.

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**Fig. 1.** Secretion of parathyroid hormone (PTH) by parathyroid tissue in vitro. PTH secretion was inhibited by extracellular calcium (A) and by increasing doses of R-568 at 0.6 mM (●) and 1 mM (□) extracellular calcium (B). Each data point represents mean ± SE of 12 experiments. *P < 0.05 compared with 1 mM Ca. All values are different from tissue in the absence of R-568.
protein in parathyroid cells independently of calcitriol (13). Moreover, the downregulation of VDR during hypocalcemia has been proposed as a mechanism that prevents the feedback of calcitriol on the parathyroid glands (13). However, any effect of extracellular calcium on parathyroid cell function should be elicited by the interaction of calcium with the CaSR. In the presence of the allosteric CaSR modulator R-568 and calcium, the CaSR senses an extracellular calcium level greater than is sensed with calcium alone (23). Thus it was expected that parathyroid cell activity in the presence of R-568 would mimic that of elevated extracellular calcium. Our results demonstrate that R-568 has a dose-dependent stimulatory effect on VDR mRNA expression. Such an effect was evident in vitro, even at low (0.6–1 mM) calcium levels, and could be modulated by the extracellular calcium concentration. Moreover, at a relatively low (1 mM) calcium concentration, the addition of 0.001 μM R-568 to the culture medium resulted in stimulation of VDR mRNA similar to the maximal stimulation achieved during hypercalcemia (1.5–1.8 mM calcium).

Previous work has shown rapid downregulation of the CaSR in primary cultures of isolated parathyroid cells (5). The mechanism of action of the calcimimetics is focused on the CaSR, and alterations in its expression may have an impact on the efficacy of the drug. Accordingly, in studies of this nature, the interpretation of the association between the CaSR and the VDR and, ultimately, the association between serum calcium levels and vitamin D activity may be unduly biased if this is not taken into account. However, here, as previously reported (12, 23), R-568 caused a dose-dependent inhibition of PTH secretion, providing evidence that the cultured intact parathyroid tissue remained functional throughout the experimental period.

In previous in vivo work (13), we observed that a 6-h period was sufficient to demonstrate an effect of calcium concentration on VDR mRNA expression. To ensure optimal pharmacokinetics, R-568 was administered at the start of, and halfway through, the experimental period. We used a diet containing higher-than-normal calcium and phosphorus concentrations (1.1% calcium-0.8% phosphorus vs. 0.9% calcium-0.6% phosphorus) compared to a low-phosphorus diet (0.2% phosphorus).
phosphorus in the normal diet) that was intended to result in slightly elevated serum calcium levels.

In vivo, administration of R-568 resulted in an increase in VDR mRNA and protein expression to a degree equivalent to that observed with the administration of calcitriol. Interestingly, the combination of R-568 and calcitriol potentiated the individual effects of each drug on parathyroid gland VDR mRNA, but not protein, expression, suggesting that the VDR translation system may have already reached its maximal capacity.

Activation of the CaSR by calcimimetics also reduced PTH mRNA in vitro and in vivo. Previous reports showed that the regulation of PTH mRNA by calcium is posttranscriptional and dependent on AUF activity (19). Conversely, treatment with calcitriol alone did not decrease PTH mRNA in vivo. Since calcitriol regulates PTH mRNA through a genomic effect, it is likely that significant changes in PTH mRNA may not be detected for <6 h. This contention is supported by preliminary data from our laboratory which shows that treatment with calcitriol (240 ng/rat ip) significantly \((P < 0.02)\) reduced PTH mRNA 12 h (from 100% to 58 ± 9%) and 24 h (from 100% to 12 ± 3%) after treatment.

The ability of the calcimimetics to increase VDR protein expression may have potentially significant ramifications on clinical use of vitamin D sterols in the treatment of secondary HPT. Any increase in VDR would facilitate the inhibitory feedback of vitamin D on the parathyroid glands and would assist in optimization of the positive action of the pharmacological administration of calcitriol or other vitamin D analogs. Hence, although cinacalcet HCl works as effectively with or without concomitant vitamin D (3), the combined use of vitamin D sterols and calcimimetics may permit a reduction in the dose of vitamin D, allowing greater reductions in \(\text{Ca} \times \text{P}\) and, potentially, a decreased risk of associated negative sequelae, such as vascular calcifications (15, 16, 21). However, the results of our experiments with human parathyroid tissue indicate that the stimulatory effects of calcimimetics on the VDR are likely to be restricted to hyperplastic glands with moderate (diffuse) hyperplasia. The lack of effects of R-568 on VDR expression by hyperplastic glands with severe (nodular) hyperplasia may be related to low CaSR expression, which has been reported to occur in nodular compared with nonnodular parathyroid tissue (14). Previous work has demonstrated an ability of the calcimimetics to slow the rate of parathyroid gland proliferation (9), and this, together with the work presented in this report, suggests that earlier treatment with calcimimetics would allow for improved efficacy of calcimimetic and vitamin D therapy. These data, in association with clinical results describing the negative sequelae associated with elevated PTH, calcium, and phosphorus levels in patients with chronic kidney disease...

Fig. 4. Western blot of parathyroid gland VDR. Rats (26 glands per group) were treated with R-568 (1 mg/kg iv 6 and 3 h before euthanasia), and a comparable degree of hypocalcemia was induced by EDTA administration (300 mg/kg im). Results are representative of observations from 3 experiments.

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Fig. 5. VDR protein content of parathyroid glands \((n = 10)\) from hemodialysis patients with secondary hyperparathyroidism incubated at low (0.6 mM) and high (1.35 mM) calcium with or without R-568. Values are reported as optical density of Western blot bands. Histological examination of parathyroid glands revealed diffuse hyperplasia in patient 1 \((n = 4)\) and nodular hyperplasia in patients 2 \((n = 3)\) and 3 \((n = 3)\).
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(2, 31), as well as data demonstrating that biochemical target attainment is improved with earlier treatment (20), stress the importance of starting calcimimetic treatment in dialysis patients early in the course of the disease.

In conclusion, the present study shows that the calcimimetic R-568 exerts a stimulatory effect on VDR expression in the parathyroid glands of study models, thereby supporting the further examination of vitamin D-calcimimetic combinations in clinical settings.

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