Direct upregulation of parathyroid calcium-sensing receptor and vitamin D receptor by calcimimetics in uremic rats

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opment of secondary hyperparathyroidism, after the second surgery, the mineral content of the diet was changed to calcium (0.6%) and increased phosphorus (1.2%). Sham-operated animals underwent the same procedures without renal manipulation and were maintained on the standard diet.

Experimental design. The experimental design included four experiments, and the two major studies were designed as follows (Fig. 1).

In the long-term, 2-wk treatment protocol (controlled proliferation), rats received the study drugs, AMG 641 (Amgen, Thousand Oaks, CA) and/or calcitriol (Calcijex, Abbot, Madrid, Spain) every 48 h for 13 days, with the last dose being administered 24 h before death (which took place on day 14). The compound AMG 641 (an arylalkylamine with a molecular weight of \~400) is a new type II calcimimetic, still in the research phase, which shows longer duration of effect on CaSR than commercial calcimimetics.

In the short-term treatment study (uncontrolled proliferation), rats received saline solution every 48 h for 13 days and were treated with the calcimimetic AMG 641 and/or calcitriol only once, on day 14, 6 h before death. To ensure an acute effect on parathyroid function, the single dose of both drugs was doubled.

In each experiment, rats were randomly assigned (based on the normal distribution of baseline body weight) into five groups: 1) sham-operated rats receiving vehicle [sham+vehicle, n = 20 (10 long-term, 10 short-term)]; 2) nephrectomized rats receiving vehicle [5/6 Nx+vehicle, n = 20 (10 long-term, 10 short-term)]; 3) nephrectomized rats treated with calcitriol (5/6 Nx+calcitriol); either long-term (80 ng/kg ip every other day, n = 13) or short-term (160 ng/kg ip 6 h before death, n = 22); 4) nephrectomized rats treated with AMG 641 (5/6 Nx+AMG 641) either long-term (1.5 mg/kg sc every other day, n = 24) or short-term (3 mg/kg sc 6 h before death, n = 19); and 5) nephrectomized rats treated with both calcitriol and AMG 641 (5/6 Nx+calcitriol+AMG 641), at the same doses reported above, in the long-term (n = 22) and short-term (n = 20) experiments.

Two additional experiments were carried out to provide a better understanding of the time effect of the calcimimetic AMG 641 on parathyroid cell proliferation and CaSR and VDR expression as shown below.

First, to explore early changes in the cell cycle (% cells in the S phase and cyclin D1 mRNA expression) and CaSR and VDR mRNA expression, two extra groups of nephrectomized rats (n = 5) received vehicle (saline solution) every 48 h for 13 days and were treated with the calcimimetic AMG 641 (3 mg/kg sc) only once, on day 14, 3 h before death.

Second, another experiment was designed to determine CaSR and VDR mRNA expression and cell cycle in uremic rats after 3 days of treatment with the calcimimetic AMG 641 (1.5 mg/kg sc every other day, n = 5) and calcitriol (80 ng/kg ip every other day, n = 5). These rats received vehicle (saline solution) every 48 h for 13 days and were treated with the calcimimetic AMG 641 (1.5 mg/kg sc) twice, on days 14 and 16, and were killed on day 17.

At the end of the experiments rats were killed by aortic puncture and exsanguination under general anesthesia (sodium thiopental ip). Blood was obtained under anaerobic conditions from the aorta for measurement of intact PTH, ionized calcium, phosphorus, and creatinine. Immediately following death, parathyroid glands were removed.

CaSR and VDR mRNA Measurement (RNA Isolation and Real-Time RT-PCR)

Parathyroid tissue was dry-frozen in liquid nitrogen and stored at \~80°C until RNA isolation. For RNA isolation, 1 ml of phenol-guanidine isothiocyanate solution (Tri-Reagent, Sigma, St. Louis, MO) 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 Chomczynski and Sacchi protocol (7). Extracted total RNA was dissolved in nucleas-free water (Promega, Madison, WI) and heated for 10 min at 60°C. Total RNA was quantified by spectrophotometry. CaSR and VDR expression, normalized to $\beta$-actin, was determined by real-time RT-PCR (QuantiTect SYBR green, Qiagen, Hilden, Germany) using specific primers and 10 ng of total RNA/sample. The following primers were used: CaSR (sense) 5’-TGG AGA GAC AGA TGC GAC TG-3’, (antisense) 5’-TGT CAC CAA GTG GTA CGA CCA GA-3’, VDR (sense) 5’-ACA GTC TGA GGC CCA AGC TA-3’, (antisense) 5’-TCC CTG AAG TCA GCG TAG GT-3’, and $\beta$-actin (sense) 5’-TGC CAC CAA CTG GGA CGA TAT GGA G-3’, (antisense) 5’-ACA ATG CCA GTG GTA CGA CCA GA-3’. cDNA amplifications were processed by real-time PCR (Lightcycler; Roche, Basel, Switzerland). Data were analyzed using Lightcycler 3.5.28 software (Roche).

Assessment of Parathyroid Cell Proliferation

The parathyroid cell cycle was studied by flow cytometry as previously described (28). Briefly, parathyroid tissue was treated with the nonionic detergent Nonidet P-40 (Sigma) and trypsin (Sigma), followed by treatment with a trypsin inhibitor (type II-O, Sigma) to stop the trypsin reaction and RNase to prevent dye binding to double-stranded RNA. In a final step, isolated nuclei were stained with propidium iodide, 20 $\mu$g/ml for 30 min at 37°C in the dark, and stabilized with spermine. The nuclei were immediately acquired by LYSYS II software (Becton-Dickinson) was used for data acquisition and analysis. Cell debris and clumps were excluded from analysis by gating. The cell cycle was analyzed using CELLFIT software (Bec-
ton-Dickinson) with a doublet discrimination module to discriminate cell aggregates. This method measures the percentage of cells in the different phases of the cell cycle: cells in the G0/G1 phase are diploid cells, cells in the S phase show an increase in the synthesis of DNA that precedes cell duplication, and cells in the G2/M phase have doubled the DNA content or are undergoing mitosis. The percentage of cells in the S phase was used as a marker of cell proliferation.

Parathyroid cell proliferation was also studied through quantification of cyclin D1 mRNA expression by real-time RT-PCR (8). The cyclin D1 primers were the following: (sense) 5'-GCA CAA CGC ACT TTC TTG CC-3' and (antisense) 5'-TCT TCC ACT TCC CCT TC-3'.

**Immunohistochemistry**

Fresh tissue was fixed in 10% buffered formalin, embedded in paraffin, and cut into 3-μm sections. The avidin-biotin-peroxidase (ABC) method (Vector, Burlingame, CA) was used for the immunohistochemical examination of CaSR expression in the parathyroid gland. Endogenous peroxidase activity was inhibited by incubation in 3% hydrogen peroxide in methanol for 30 min. After an additional 30-min incubation period in 10% normal goat serum in PBS, pH 7.6, tissue sections were incubated with the primary antibodies against the CaSR overnight at 4°C (Acris Antibodies, Hidenhausen, Germany). After three 10-min rinses in PBS, tissue sections were incubated with the secondary antibody (biotinylated goat anti-rabbit immunoglobulins) for 30 min (Dako, Glostrup, Denmark). After two 10-min rinses in PBS, tissue sections were incubated with ABC diluted in PBS for 1 h. After three 10-min rinses in PBS, tissue sections were incubated for several seconds in Vector NovaRed (Vector), rinsed in tap water, lightly counterstained with Mayer’s hematoxylin, dehydrated, and mounted.

**In Vitro Studies**

Additional experiments were carried out in vitro to support findings from in vivo experiments which suggest a direct upregulation of CaSR expression by CaSR activation. The experiments were performed in intact rat parathyroid glands from male Wistar rats, weighing 180–200 g, maintained on a normal calcium (0.6%) and phosphorus (0.6%) diet (1). Animals were anesthetized with pentobarbital sodium (50 mg/kg) and, within 2 min after the blood was drained by aortic puncture, the parathyroid glands were dissected free of the thyroid glands, with a dissecting microscope, and removed. Intact rat parathyroid glands were placed in individual wells (5 glands/well) containing 2 ml of incubation medium, resting inside a nylon basket. The glands were maintained at 37°C, with constant rocking and shaking motions (AOS-0, SBS Instruments, Badalona, Spain). Cell viability was >80%. The incubation medium was buffered (pH 7.4) and contained (in mM) 125 NaCl, 5.9 KCl, 0.5 MgCl2, 1 sodium pyruvate, 4 glucose, 12 glucose, and 25 HEPES, as well as 0.1 IU/ml human insulin, 0.1% bovine serum albumin, 100 IU/ml penicillin G, and 100 mg/ml streptomycin. A phosphate concentration of 1 mM was obtained by adding NaH2PO4 and Na2HPO4 in a 1:2 proportion (1). All chemical products were obtained from Sigma. Parathyroid glands were incubated for 6 h in the media described above, to which was added CaCl2 to yield calcium concentrations of 0.8 mM (low-calcium group) and 1.5 mM (high-calcium group). The calcimimetic AMG 641 (1 mM) was added to some of the parathyroid glands incubated in low calcium.

**Biochemical Measurements**

Ionized calcium was measured using a selective electrode (model 634, Ciba Corning, Essex, UK). Intact PTH was quantified according to the vendor’s instructions using the rat-specific IRMA assay (Scantibodies Laboratories, Santee, CA). Creatinine and phosphorus were measured by spectrophotometry (Byosistems, Barcelona, Spain).

**Statistics**

Values are expressed as means ± SE. The difference between means was assessed by ANOVA followed by a Fisher least significant difference test. A correlation study was carried out using the Spearman test. P < 0.05 was considered significant.

**RESULTS**

**Biochemical Parameters**

Blood biochemistry is presented in Table 1. As expected, a significant (P < 0.05 vs. sham) increase in plasma creatinine concentration was detected in all 5/6 Nx groups. Differences in plasma creatinine were found in the long-term treatment study, with lower values recorded in the AMG 641-treated rats (0.99 ± 0.03 mg/dl) and significantly higher values in the calcitriol-treated rats (1.60 ± 0.14 mg/dl) compared with nontreated 5/6 Nx rats (1.20 ± 0.05 mg/dl). Ionized calcium values were decreased in 5/6 Nx rats treated with vehicle in both experiments (0.95 ± 0.02 and 0.93 ± 0.03 mmol/l) and with a calcimimetic (long-term: 0.96 ± 0.01 mmol/l, short-
term: 0.68 ± 0.02 mmol/l) compared with the sham group (1.18 ± 0.01 and 1.14 ± 0.01 mmol/l, P < 0.05). Ionized calcium was also decreased (P < 0.05 vs. sham) in 5/6 Nx rats treated with only one dose of calcitriol in the short-term experiment (0.96 ± 0.02 mmol/l) but not in the long-term experiment (1.12 ± 0.04 mmol/l). Plasma phosphorus was increased in all 5/6 Nx groups (9–16 mg/dl, P < 0.05 vs. sham). Calcitriol exacerbated increases in phosphorus (15.6 ± 1.6 mg/dl, P < 0.05 vs. 5/6 Nx+vehicle) in the long-term study. Nephrectomized rats had increased plasma PTH values (361 ± 35 and 427 ± 31 pg/ml, P < 0.05 vs. sham). Both calcitriol (196 ± 37 pg/ml) and AMG 641 (146 ± 32 pg/ml) reduced PTH levels in the long-term treatment study. However, in the short-term treatment protocol, only AMG 641 decreased PTH (14 ± 4 pg/ml; P < 0.05 vs. vehicle-treated rats, 361 ± 35 pg/ml and calcitriol-treated rats, 242 ± 35 pg/ml).

**Parathyroid Cell Proliferation**

Nephrectomy resulted in parathyroid cell proliferation, as reflected by the significant increase in the percentage of parathyroid cells in S phase compared with the sham group (1.52 ± 0.13 vs. 0.66 ± 0.08%, P < 0.05). In the long-term treatment protocol, calcitriol (0.84 ± 0.15%), AMG 641 (0.88 ± 0.08%), and their combination (0.64 ± 0.09%) significantly (P < 0.01 vs. 5/6 Nx+vehicle) reduced the percentage of parathyroid S phase cells (Fig. 2A). In contrast, proliferation was uncontrolled in the short-term treatment protocols with S phase cell values (1.38–1.48%) that were not significantly different from the 5/6 Nx+vehicle-treated animals (Fig. 2B).

When parathyroid cell proliferation was studied a very short time after AMG 641 administration (3 h), no changes in either the percentage of cells in S phase [2.93 ± 0.28 vs. 2.54 ± 0.27%, not significant (NS), in vehicle-treated rats] or expression of cyclin D1 (0.86 ± 0.11 vs. 0.77 ± 0.21, NS, in rats receiving vehicle) were found in rats treated with the calcimimetic AMG 641 (Fig. 3A). Conversely, after 3 days of treatment, both AMG 641 (0.74 ± 0.14%) and calcitriol (0.95 ± 0.22%) caused a significant (P < 0.05) decrease in the percentage of cells in S phase compared with the vehicle-treated group (2.54 ± 0.27%) (Fig. 3B).

**CaSR and VDR mRNA Expression**

CaSR mRNA expression (ratio vs. β-actin) was lower (P < 0.05) in nephrectomized (0.89 ± 0.09) than in sham-operated rats (1.38 ± 0.12). A significant (P = 0.01) increase in CaSR mRNA was detected in rats treated with both calcitriol (1.60 ± 0.30) and AMG 641 (1.66 ± 0.25) for 13 days, and there was a further increase when both drugs were administered simultaneously (2.46 ± 0.33, P < 0.05 vs. treatment with either drug alone) (Fig. 4A). However, in the short-term treatment protocol only rats receiving AMG 641 alone (2.01 ± 0.33, P < 0.001 vs. 5/6 Nx) or in combination with calcitriol (1.81 ± 0.20, P < 0.001 vs. 5/6 Nx) increased expression of CaSR mRNA (Fig. 4B). Furthermore, additional studies demonstrated that AMG 641 was able to increase CaSR mRNA expression 3 h after being administered (1.10 ± 0.13, P < 0.05 vs. 0.77 ± 0.04 in 5/6 Nx+vehicle) (Fig. 5A). After 3 days of treatment, both AMG 641 (1.43 ± 0.16) and calcitriol (2.20 ± 0.10) increased (P < 0.05) CaSR mRNA expression compared with the 5/6 Nx+vehicle group (0.77 ± 0.04) (Fig. 5B).

Changes in VDR mRNA paralleled those of CaSR mRNA. Nephrectomy reduced VDR mRNA expression to 0.49 ± 0.10 (P < 0.05 vs. sham, 0.93 ± 0.15). In the long-term treatment, both AMG 641 (0.87 ± 0.14) and calcitriol (0.99 ± 0.12) increased VDR mRNA, and the increase was more accentuated when the drugs were combined (1.49 ± 0.45). In the short-term protocol, only treatment with AMG 641, alone (1.52 ± 0.41, P < 0.01 vs. 5/6 Nx) or combined with calcitriol (1.86 ± 0.24, P < 0.001 vs. 5/6 Nx), increased VDR mRNA (Fig. 6). AMG 641 alone was also able to increase VDR mRNA expression in a very short time (3 h) (Fig. 5A). After 3 days of treatment, both the calcimimetic AMG 641 (1.29 ± 0.20) and calcitriol (1.80 ± 0.38) reversed the decrease in VDR mRNA expression induced by uremia (Fig. 5B).

There was a significant correlation between changes in CaSR mRNA and VDR both in the short-term (R² = 0.770, P < 0.001) and in the long-term protocols (R² = 0.785, P < 0.001).
CaSR Protein Levels

As is shown in Fig. 7, nephrectomized rats receiving vehicle experienced a significantly decrease in CaSR protein immunostaining. Both AMG 641 and calcitriol administration (13 days) prevented the decrease in CaSR protein levels.

In Vitro Studies

In vitro, CaSR expression (ratio vs. β-actin) was increased in parathyroid tissue incubated with low calcium plus AMG 641 and high calcium, compared with the parathyroid glands in low calcium (2.7 ± 0.4 and 3.2 ± 0.5 vs. 1.01 ± 0.3, P < 0.01) (Fig. 8).

DISCUSSION

The objective of this study was to test the hypothesis that calcimimetics have a direct effect on CaSR and VDR expression and that the regulation of these receptors by calcimimetics does not require a prior overt change in the parathyroid cell cycle. Our results demonstrate that the calcimimetic AMG 641 elicits a rapid increase in CaSR mRNA and VDR mRNA in uremic rat parathyroid glands. CaSR activation may also produce parallel changes in the expression of proteins that inhibit the cell cycle.

In the course of secondary HPT, parathyroid glands become hyperplastic to increase their secretory capacity (12). Hyperplasia is basically achieved by increasing parathyroid cell proliferation, although hypertrophy and a decrease in apoptosis may also play a minor role (27). Downregulation of parathyroid CaSR and VDR is a common feature of hyperplastic parathyroid glands. As a consequence, parathyroid cells become relatively insensitive to the inhibitory action of extracellular calcium and calcitriol (5).

The primary treatment goal for secondary HPT is to reduce circulating serum PTH levels and associated disorders in mineral metabolism. Phosphorus restriction, vitamin D sterols, and calcimimetics are the main therapeutic measures used to control secondary HPT. Calcimimetics are allosteric modulators of the CaSR which lower the CaSR activation threshold to extracellular calcium. Thus, when a calcimimetic is present, the parathyroid glands sense a level of calcium which is higher than the actual extracellular calcium. Obviously, calcium and calcimimetics are more effective in suppressing PTH if CaSR is not downregulated. In the same manner, downregulation of VDR may hinder the therapeutic benefit of vitamin D derivatives. Thus increasing CaSR and VDR expression in hyper-

![Fig. 3. A: parathyroid cell proliferation (% of cells in S-phase and cyclin D1 mRNA expression) in control group (sham+vehicle, n = 5) and in nephrectomized rats treated with either vehicle (5/6 Nx+vehicle, n = 5) or calcimimetic AMG 641, 3 mg/kg sc 3 h before death (5/6 Nx+AMG 3h, n = 5). B: parathyroid cell proliferation (% of cells in S phase) after 3 days of treatment. Sham+vehicle, sham-operated rats receiving vehicle (n = 5); 5/6 Nx+vehicle, nephrectomized rats receiving vehicle (n = 5); 5/6 Nx+calcitriol 3d, nephrectomized rats treated with calcitriol (80 ng/kg ip every other day, n = 5); 5/6 Nx+AMG 641 3d, nephrectomized rats treated with calcimimetic AMG 641 (1.5 mg/kg sc every other day, n = 5).](http://ajprenal.physiology.org/)

![Fig. 4. Parathyroid calcium-sensing receptor (CaSR) expression. A: CaSR expression (CaSR mRNA/β-actin) in the long-term treatment protocol. B: CaSR expression (CaSR mRNA/β-actin) in the short-term treatment protocol, as described in the legend of Fig. 2.](http://ajprenal.physiology.org/)
plastic glands would likely result in increased therapeutic efficacy of the respective drugs in patients with secondary HPT. Our results showing that the calcimimetic AMG 641 controlled parathyroid cell proliferation and increased CaSR expression in the parathyroid glands of uremic rats with chronic treatment confirm previous findings (13). However, since parathyroid cell hyperplasia precedes downregulation of CaSR in the uremic rat model (23), in previous studies it was not possible to differentiate whether the effect on CaSR was secondary to the control of parathyroid cell proliferation. In the short-term studies reported here (3 and 6 h) in which parathyroid cell proliferation was still increased, the calcimimetic AMG 641 induced an increase in CaSR mRNA. These results demonstrate that AMG 641 increases CaSR expression without a previous control of hyperplasia. The direct upregulatory effect of AMG 641 on CaSR mRNA was corroborated in vitro. In these studies, AMG 641 restored CaSR expression when incorporated into the media with low extracellular calcium (similar to the calcium levels in vehicle-treated nephrectomized rats). Activation of the CaSR normalized the percentage of parathyroid cells in the S phase as soon as day 3 of treatment. It is likely that CaSR-dependent intracellular signaling may induce more rapid changes (possibly posttranscriptional) in the expression of proteins that regulate the cell cycle (9, 10).

Calcitriol administration to uremic rats increased parathyroid CaSR mRNA in the long-term but not in the short-term study. Thus the effect of calcitriol on CaSR mRNA seems to be more related to the control of parathyroid hyperplasia. This contention is supported by the longitudinal studies in which calcitriol was able to upregulate CaSR mRNA after 3 days of treatment, when parathyroid hyperplasia was already controlled. Since in the long-term studies calcitriol induced hypercalcemia, it is also possible that the actions of calcitriol were partially mediated by the elevated serum calcium levels. The effects of calcitriol cannot be separated from those attributed to its calcitropic effect in the long-term studies (2). Although the effect of calcium on parathyroid cell proliferation is controversial, it is apparent that activation of CaSR leads to a reduction in proliferation (25). CaSR mRNA levels have been reported to increase 16 h after intraperitoneal administration of high doses of calcitriol to normal rats. The presence of functional vitamin D-response elements in the CaSR gene has been proposed as the mechanism by which calcitriol increases CaSR mRNA.

Fig. 5. A: parathyroid CaSR (CaSR mRNA/β-actin) expression and vitamin D receptor (VDR; VDR mRNA/β-actin) expression a very short time (3 h) after treatment with calcimimetic, as described in the legend of Fig. 3A. B: parathyroid CaSR (CaSR mRNA/β-actin) and VDR (VDR mRNA/β-actin) expression after 3 days of treatment, as labeled in the legend of Fig. 3B.

Fig. 6. A: parathyroid VDR (VDR mRNA/β-actin) expression in the long-term treatment protocol. B: VDR (VDR mRNA/β-actin) expression in the short-term treatment protocol, as described in Fig. 2.
In agreement with previous reports (21), chronic administration of a calcimimetic or calcitriol prevented decreases in CaSR protein in the parathyroid glands of uremic rats. By contrast, no changes were found in the short-term studies with any treatment (data not shown). Protein expression may not necessarily parallel mRNA expression because of a temporal effect (it is possible that treatment with the calcimimetic produces an immediate effect on the mRNA but that the change in protein expression occurs later). On the other hand, the early changes in CaSR mRNA after treatment with calcimimetics may reflect a posttranscriptional effect of the calcimimetic, as has been reported with PTH mRNA (19).

In normal rats, VDR has been shown to be regulated by extracellular calcium (16) and by calcimimetics (24). To our knowledge, this is the first report of VDR upregulation by calcimimetics in uremic rats.

The effect of AMG 641 on CaSR and VDR appears to be independent of the ability of the calcimimetic to control serum phosphorus levels because 1) in the short-term experiment, rats treated with AMG 641 and with calcitriol had similar phosphorus levels but only rats treated with AMG 641 increased both receptors; 2) in the long-term experiment, AMG 641 potentiated the effect of calcitriol even though these rats had elevated phosphorus levels; and 3) the effect of AMG 641 on CaSR was also confirmed in vitro, and in these experiments extracellular phosphorus was unchanged.

The results of this study highlight the importance of CaSR signaling not only in PTH synthesis and secretion but also in regulation of two main receptors of the parathyroid cell (CaSR and VDR). Further studies will be necessary to elucidate the molecular pathways involved in the regulation of CaSR and VDR expression by CaSR signaling.

It is interesting to note that calcimimetics upregulate CaSR and VDR earlier than calcitriol. The difference may be related to the fact that calcitriol acts through nuclear receptors; in addition, the resulting genomic effect may also affect cell proliferation. The results of the present study suggest that calcimimetics are able to “sensitize” hyperplastic parathyroid cells to its main regulators (calcium and calcitriol) through increased expression of the respective receptors (CaSR and VDR).
VDR). These effects are observed quickly, before any decrease in the percentage of cells in S phase is evident. In addition, these data highlight the synergistic effect of calcimimetics and vitamin D derivatives on the treatment of secondary HPT. It is also important to note that our results have been obtained in a rat model of parathyroid hyperplasia which does not develop nodularity. In human patients with advanced nodular parathyroid hyperplasia, in which CaSR and VDR levels are even further downregulated, calcimimetics may not stimulate these effects on CaSR and VDR expression to the same degree as observed here. Thus, in clinical practice, the beneficial effects of calcimimetics may be more apparent in patients in the early stages of secondary HPT.

In conclusion, the results of this study support the hypothesis that calcimimetics directly increase CaSR and VDR expression by hyperplastic parathyroid glands.

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DISCLOSURES

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