Calcimimetic Compound Upregulates Decreased Calcium-Sensing Receptor Expression Level in Parathyroid Glands of Rats with Chronic Renal Insufficiency

MASAHIDE MIZOBUCHI,* IKUJI HATAMURA,† HIROAKI OGATA,‡ FUMIE SAJI,* SUSUMU UDA,§ KAZUHIRO SHIZAKI,* TOSHIUMI SAKAGUCHI,* SHIGEO NEGI,* ERIKO KINUGASA,‡ SHOZO KOSHIKAWA,‖ and TADAO AKIZAWA* *Center of Blood Purification Therapy and †First Department of Pathology, Wakayama Medical University, Wakayama, Japan; ‡Department of Internal Medicine, Showa University Northern Yokohama Hospital, Yokohama, Japan; §Division of Nephrology and Dialysis Center, Kobe University School of Medicine, Kobe, Japan; and ‖Division of Nephrology, Department of Internal Medicine, Showa University Fujigaoka Hospital, Yokohama, Japan

Abstract. The reduced expression level of the calcium-sensing receptor (CaR) is attributed to the hyposensitivity of parathyroid cells to extracellular calcium concentration \([\text{Ca}^{2+}]_o\), which plays a crucial role in the pathogenesis of secondary hyperparathyroidism (SHPT) in patients and rats with chronic renal insufficiency (CRI). Calcimimetic compounds have been demonstrated to improve the decreased sensitivity of CaR to extracellular calcium concentration and to suppress both parathyroid hormone (PTH) oversecretion and parathyroid cell proliferation. However, the effect of calcimetics on the reduced CaR expression level in parathyroid cells in CRI remains unclarified. The aim of this investigation was to examine the effect of the calcimimetic compound NSP R-568 (R-568) on the CaR expression in the parathyroid cells of rats with experimental CRI. Subtotally nephrectomized rats were fed a high-phosphorus diet for 8 (\(n = 12\); Nx-8 group) or 9 wk (\(n = 11\); Nx-9 group) to induce severe SHPT. Another group of uremic rats were fed a high-phosphorus diet for 8 wk and then orally administered R-568 (100 \(\mu\text{mol/kg body wt}\) once a day for 7 d (\(n = 11\); Nx+R-568 group). Sham-operated rats that were fed a standard diet for 9 wk were used as controls (\(n = 8\)). R-568 treatment induced a significant reduction in plasma PTH level with significant decrease in serum calcium and without change in serum phosphorus concentration. Serum 1,25(OH)2D3 level was not affected by R-568 administration. CaR mRNA and protein levels in the Nx-8 and Nx-9 groups significantly decreased compared with those in the controls; however, no significant difference in these parameters was observed between the Nx-8 and Nx-9 groups. In the Nx+R-568 group, CaR mRNA and protein levels significantly increased compared with those in either the Nx-8 or Nx-9 group. R-568 was effective in reducing the number of proliferating cell nuclear antigen–positive cells along with parathyroid gland growth suppression in the Nx+R-568 group compared with that in the Nx-9 group. The results suggest that the calcimimetic compound R-568 upregulates decreased CaR expression, and the upregulation possibly has an enhancement effect on PTH secretion and parathyroid cell hyperplasia through the improved sensitivity of CaR to \([\text{Ca}^{2+}]_o\).

The parathyroid hormone (PTH) plays a critical role in calcium (Ca) homeostasis and bone mineral metabolism. PTH secretion from parathyroid gland cells is closely regulated by several factors, including serum Ca and phosphorus (P) levels. The calcium-sensing receptor (CaR) on the parathyroid cell surface senses extracellular Ca concentration ([Ca\(^{2+}\)]_o) accurately and mediates PTH synthesis and PTH secretion with other factors, including P and vitamin D (1). It has been reported that the expression levels of the CaR gene and CaR protein are reduced in the parathyroid glands of patients with primary or secondary hyperparathyroidism (SHPT), in which PTH is oversecreted despite normal or high [Ca\(^{2+}\)]_o (2–5). The mechanism underlying the regulation of CaR expression remains unknown. However, vitamin D administration (6) or P restriction (7) has been demonstrated to prevent or reverse the decrease in CaR expression level in experimental animal models with renal insufficiency.

NPS R-568 (R-568), a type II CaR agonist, is an allosteric CaR activator (8–11) that can reduce the PTH secretion level in animals (12–15) or patients with primary hyperparathyroidism (16) or SHPT (17). R-568 administration significantly suppresses parathyroid cell hypertrophy and hyperplasia in subtotally nephrectomized rats in 5 d (12). We hypothesized...
that a calcimimetic compound improves the reduced sensitivity of CaR to [Ca\(^{2+}\)] to through not only an allosteric manner but also CaR upregulation in parathyroid cells and suppresses aberrant PTH secretion and parathyroid cell proliferation. In this study, we investigated the reversal effect of R-568 on decreased CaR gene and CaR protein expression levels and analyzed the association between cell proliferation and CaR expression in the parathyroid glands of uremic rats with SHPT.

### Materials and Methods

Male Sprague-Dawley rats that weighed 200 to 250 g (SLC Japan, Tokyo, Japan) were housed in single cages at constant room temperature (25°C) and humidity (75%) under a controlled light/dark cycle. All experimental protocols were approved by the Animal Studies Committee of Wakayama Medical University.

#### Experimental Animal Groups

After a 7-d adaptation period, the rats were randomly allocated into four groups as follows: Sham-operated control rats that were fed a standard diet for 9 wk (controls; \(n = 8\)), subtotal nephrectomized (Nx) rats that were fed a high-phosphorus (HP) diet for 8 wk (Nx-8 group; \(n = 12\)), Nx rats that were fed an HP diet for 9 wk (Nx-9 group; \(n = 11\)), and Nx rats that were fed an HP diet for 8 wk and consecutively treated with 100 μmol/kg body wt per d R-568 for 1 wk (Nx+R-568 group; \(n = 11\)).

The Nx rats were fed an HP diet (1.2% P, 0.4% Ca) to induce severe renal insufficiency and hyperparathyroidism. The control rats were fed a standard diet (0.8% P, 1.1% Ca). Both diets were obtained from Crea Japan (Tokyo, Japan). The rats were provided water ad libitum throughout the experiment.

In subtotal nephrectomy, the right kidney was removed in an initial operation under anesthesia with pentobarbital (100 mg/kg body wt). The right kidney was weighed immediately after excision. Seven days after unilateral nephrectomy, the cortical tissue of the hypertrophied remnant left kidney was removed so that the amount removed corresponded to 75% of the weight of the previously excised right kidney. Care was taken to remove the tissue preferentially from the upper and lower poles without damaging the large arteries. In the sham-operated rats, the kidneys were decapsulated in two consecutive operations.

Twenty-four hours after the second operation, the original diet was started in each group and continued for 8 or 9 wk. After 8 wk, for elucidating the early effect of R-568 on parathyroid cell proliferation and CaR expression, the Nx+R-568 group was treated with R-568 (NPS Pharmaceutical, Salt Lake City, UT) for 1 wk. R-568 was dissolved in 10% aqueous cyclodextrin (2-hydroxypropyl cyclodextrin; Research Biochemical International, Natick, MA) and administered daily by gavage (100 μmol/kg per d) between 8:00 a.m. and 10:00 a.m.

#### Measurements

Blood samples were obtained 2 h after the last administration of R-568. Blood chemistries were measured using standard laboratory methods with an automated multiparametric analyzer (DRI-CHEM 3030; Fuji Film, Tokyo, Japan). Serum PTH level was determined using a rat intact PTH ELISA kit (Immutopics, San Clemente, CA), and 1,25(OH)\(_2\)D3 was quantified by RIA assay (IDS kit, Boldon, UK).

### Tissue Preparation

After 8 or 9 wk, all of the animals were anesthetized with pentobarbital, and both parathyroid glands were quickly removed using microsurgical techniques. Each parathyroid gland was weighed in an electronic microbalance (LA 230S; Sartorius, Tokyo, Japan). One gland was snap-frozen in liquid nitrogen and stored at −80°C for reverse transcriptase–PCR (RT-PCR) analysis. The other gland was fixed in 4% paraformaldehyde.

#### RT-PCR Analysis

Total RNA from each parathyroid gland was extracted using Trizol according to the manufacturer’s instructions (Life Technologies, Grand Island, NY). One microgram of total RNA was reverse-transcribed to first-strand cDNA using Omniscript reverse transcriptase (Omniscript RT kit; Qiagen, Valencia, CA). The cDNA product was amplified by PCR using primers and antisense primers for CaR and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Table 1), yielding 558-bp and 938-bp fragments, respectively. PCR was initiated by a 10-min incubation at 95°C, followed by 30 cycles of 94°C for 30 s, 60°C for 1 min, 72°C for 2 min, and a final extension step at 70°C for 10 min. A fragment was obtained by reverse transcription of an mRNA from rat parathyroid gland, followed by PCR and subcloning into the TA cloning vector (TOPO TA cloning; Invitrogen, Carlsbad, CA). The identity of the cDNA insert as CaR corresponding to positions 655 to 1212 of the coding region was confirmed by DNA sequence analysis (data not shown). The PCR products were separated by gel electrophoresis (2% agarose). The bands were visualized using ethidium bromide, and image density was measured with NIH image software version 1.63. The density of each target mRNA was normalized to the expression level of GAPDH mRNA particularly, and semiquantitative results were expressed as a ratio of the amount of each RT-PCR product to GAPDH density.

### Immunohistochemistry

Immunohistochemical staining was performed on 4% paraformaldehyde-fixed, paraffin-embedded parathyroid glands using the CaR primary monoclonal antibody, a mouse monoclonal antibody against residues 214 to 235 (ADD) of the human extracellular domain (18), which was a gift of Dr. E.F. Nemeth (NPS Pharmaceutical, Salt Lake City, UT). Briefly, the tissues were deparaffinized in xylene and dehydrated through an ethanol series. Then endogenous peroxidase was inactivated with 0.3% H\(_2\)O\(_2\)-containing methanol for 15 min at room temperature. After washing three times with Tris-buffered saline (TBS), the tissues were blocked with TBS that contained 1% nonfat dry milk for 1 h at room temperature. After blocking, the tissues were incubated with ADD or preimmune IgG overnight at 4°C. For the

### Table 1. Primer sequences

<table>
<thead>
<tr>
<th></th>
<th>Sense Primer</th>
<th>Antisense Primer</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>5′-GCACCTGTACCCAGGGCTGC-3′</td>
<td>5′-ATGAGGTCCCACCCCTGT-3′</td>
<td>938</td>
</tr>
<tr>
<td>CaR</td>
<td>5′-AAGAAGGGGGATATATCCT-3′</td>
<td>5′-TGCAATTGTGCCCACCCAGT-3′</td>
<td>558</td>
</tr>
</tbody>
</table>

\(^a\) GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CaR, calcium-sensing receptor.
incubations, ADD was diluted 1:1000 in TBS that contained 1% nonfat dry milk. After washing with TBS, the samples were incubated with a secondary antibody (containing anti-mouse immunoglobulins) using a commercially available kit (ENVISION System, peroxidase; DAKO, Carpinteria, CA) for 60 min. Finally, after washing with 1% TBS, the immune complexes were visualized with 3,3-diaminobenzidine that contained 0.1% H2O2 in TBS for 3 min.

Six to eight sequential sections of tissue from each parathyroid gland were stained by the above-mentioned methods. These specimens were quantified using image analysis software (NIH image software version 1.63). Images of CaR stained whole tissue sections (at a magnification of ×400) were acquired with an Olympus BX50 microscope coupled to Fuji Digital Camera HC-2500 connected to a Macintosh G3 personal computer and converted to gray scale using graphics software (Adobe PhotoShop version 4.0J, San Jose, CA). The digitized images were analyzed using NIH image software version 1.63. The intensity of CaR was measured with the image software at a fixed threshold. The average optical density per section was calculated by dividing the sum of optical density by the sum of the area. The average of each calculated optical density per section was determined to be the staining value of CaR of the parathyroid glands. The staining values of CaR was expressed relative to that of the normal controls.

Proliferating cell nuclear antigen (PCNA) was immunohistochemically stained with a commercial PCNA staining kit (Histostain-Plus mouse kit; Zymed Laboratories, South San Francisco, CA). The number of PCNA-positive nuclei was determined in the entire tissue specimen; thereafter, this number was divided by the total cell number in the same tissue specimen, yielding the number of PCNA-positive nuclei per 1000 parathyroid cells. As in the CaR immunostaining, six to eight sequential sections of tissue from each parathyroid gland were stained, and the average staining value was regarded as the PCNA index.

Statistical Analyses

All results were expressed as mean ± SEM. Statistical differences between the groups were assessed by one-way ANOVA, and a post hoc test (Fisher protected least significant difference) was used for the comparison of groups. P < 0.05 was considered statistically significant.

Results

Body and Parathyroid Weights

At the end of the experiment, the body weight was significantly lower and the weight of the pair of parathyroid glands was significantly higher in the subtotally nephrectomized rats (Nx-8, Nx-9, and Nx+R-568) than in the sham-operated controls. No significant difference in body weight was noted among the nephrectomized rats, that is, the Nx-8, Nx-9 and Nx+R-568 groups. The weight of the pair of parathyroid glands excised was significantly lower in the Nx+R-568 group than in the Nx-9 group (P < 0.01) but not in the Nx-8 (P = 0.48) group (Table 2). This result suggests that R-568 induces parathyroid growth suppression.

Biochemical Data

The serum urea and creatinine levels were significantly higher in the subtotally nephrectomized rats (Nx-8, Nx-9, and Nx+R-568) than in the controls (Table 2). These parameters were not significantly different among the Nx-8, Nx-9, and

<table>
<thead>
<tr>
<th>Table 2. Serum chemistries*</th>
<th>Group</th>
<th>Body Weight (g)</th>
<th>SUN (mg/dl)</th>
<th>Ca (mg/dl)</th>
<th>P (mg/ml)</th>
<th>Cr (mg/dl)</th>
<th>CaR (OD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (n = 8)</td>
<td></td>
<td>359.6 ± 10.4</td>
<td>20.51 ± 0.84</td>
<td>11.56 ± 0.17</td>
<td>7.89 ± 0.32</td>
<td>0.31 ± 0.03</td>
<td>0.06 b</td>
</tr>
<tr>
<td>Nx-8 (n = 12)</td>
<td></td>
<td>433.2 ± 18.4</td>
<td>62.87 ± 4.23</td>
<td>9.03 ± 0.36</td>
<td>15.57 ± 2.27</td>
<td>0.94 ± 0.07</td>
<td>0.18 b,c,d</td>
</tr>
<tr>
<td>Nx-9 (n = 11)</td>
<td></td>
<td>457.0 ± 16.0</td>
<td>74.29 ± 5.73</td>
<td>9.49 ± 0.40</td>
<td>15.53 ± 1.78</td>
<td>0.95 ± 0.06</td>
<td>0.20 b,c,d</td>
</tr>
<tr>
<td>R-568 (n = 11)</td>
<td></td>
<td>457.0 ± 16.0</td>
<td>15.53 ± 1.78</td>
<td>7.73 ± 0.18</td>
<td>12.30 ± 1.18</td>
<td>1.00 ± 0.06</td>
<td>0.06 b,c,d</td>
</tr>
<tr>
<td>ANOVA</td>
<td></td>
<td>P &lt; 0.0001</td>
<td>P &lt; 0.0001</td>
<td>P &lt; 0.0001</td>
<td>P &lt; 0.0001</td>
<td>P &lt; 0.0001</td>
<td></td>
</tr>
</tbody>
</table>

* All data are expressed as mean ± SEM. SUN, serum urea nitrogen; Cr, creatinine; Ca, calcium; P, phosphorus; PTG, parathyroid gland; Nx, nephrectomized; R-568, NPS R-568.

P < 0.05 versus controls; P < 0.01 versus Nx-8 group; P < 0.001 versus Nx-9 group (Fisher protected least significant difference).
controls; protein expression level as well as the CaR gene expression level and Nx-9 groups (Figure 2).

nephrectomy (Figure 1). restored the increase in serum PTH level after the subtotal 568 group and controls. One-week administration of R-568 significant difference in serum PTH level between the Nx

R-568 group and controls as well as between the Nx-8 and Nx-9 groups (Figure 3B). The number of PCNA-positive cells per 1000 parathyroid cells in the Nx+R-568 group (2.8 ± 0.4 per 1000 cells) was much lower than that in either the Nx-8 (23.8 ± 5.7 per 1000 cells; \( P < 0.05 \)) or the Nx-9 (24.6 ± 7.1 per 1000 cells; \( P < 0.05 \)) group, indicating an antiproliferative effect of R-568 on the parathyroid cells of uremic rats (Figure 4). However, there was no significant difference in the number of PCNA-positive cells between the Nx+R-568 group and the controls (3.3 ± 1.3 per 1000 cells) as well as between the Nx-8 and Nx-9 groups (Figure 4B).

Discussion

We demonstrated that the decreased expression levels of both CaR mRNA and protein in the hyperplastic parathyroid glands of rats with renal insufficiency are upregulated by

**PTH Concentrations**

The serum PTH level was significantly higher in the Nx-8 (3221.6 ± 343.1 pg/ml) and Nx-9 (3251.2 ± 519.1 pg/ml; \( P < 0.01 \)) groups than in either the controls (84.7 ± 33.3 pg/ml) or the Nx+R-568 group (303.1 ± 105.8 pg/ml). There was no significant difference in serum PTH level between the Nx+R-568 group and controls. One-week administration of R-568 restored the increase in serum PTH level after the subtotal nephrectomy (Figure 1).

**CaR Gene and CaR Protein Expressions in Parathyroid Gland**

Semi-quantitative analysis by RT-PCR revealed an ~70% decrease in CaR mRNA expression level in the Nx-8 (27.8 ± 4.4% of controls; \( P < 0.01 \)) and Nx-9 (25.3 ± 3.7% of controls; \( P < 0.01 \)) groups as compared with either that in the controls or the Nx+R-568 group (97.0 ± 2.0% of controls), respectively. The CaR mRNA levels were comparable between the Nx+R-568 group and controls as well as between the Nx-8 and Nx-9 groups (Figure 2).

Immunohistochemical findings revealed that the CaR protein expression level as well as the CaR gene expression level decreased by ~80% in the Nx-8 (19.8 ± 1.3% of controls; \( P < 0.01 \)) and Nx-9 groups (21.0 ± 2.5% of controls; \( P < 0.01 \)) compared with those in either the controls or the Nx+R-568 group (103.2 ± 4.2% of controls), respectively (Figure 3, A and B). There was no significant difference in CaR protein expression level between the Nx+R-568 group and the controls as well as between the Nx-8 and Nx-9 groups (Figure 3B).

*Figure 1. Effect of daily oral administration (100 μmol/kg body wt) of R-568 for 1 wk on serum parathyroid hormone level in rats with severe secondary hyperparathyroidism (SHPT) at 8 wk after 5/6 nephrectomy. Values are expressed as mean ± SEM (n = 8 to 12). \( P < 0.0001 \) by ANOVA. \(*P < 0.01 \) versus controls; \#P < 0.01 versus R-568 by post hoc test (Fisher protected least significant difference).*

*Figure 2. Effect of daily oral administration (100 μmol/kg body wt) of R-568 for 1 wk on calcium-sensing receptor (CaR) mRNA expression level in rats with severe SHPT at 8 wk after 5/6 nephrectomy. (A) CaR mRNA expression levels analyzed by reverse transcriptase–PCR technique for Nx-8, Nx-9 and Nx+R-568 groups and controls. (B) Quantification of CaR mRNA expression. CaR mRNA expression level was adjusted relative to glyceraldehyde-3-phosphate dehydrogenase mRNA expression level. Values are expressed as mean ± SEM (n = 8 to 12). \( P < 0.0001 \) by ANOVA. \(*P < 0.01 \) versus controls; \#P < 0.01 versus R-568 by post hoc test (Fisher protected least significant difference).*
calcimimetic R-568 administration. The changes in CaR expression level have been assumed to be associated with parathyroid cell proliferation, which is normally vigorous. Many studies have investigated the relationship between CaR expression and parathyroid cell proliferation in pathologic states. Although mice with an inactive CaR mutation exhibit severe hyperparathyroidism with marked parathyroid hyperplasia, the active mutation is associated with hypoparathyroidism (1). Thus, CaR plays a crucial role in the regulation of parathyroid cell proliferation and functions, particularly in PTH synthesis and secretion. CaR has been suggested to play an important role in pathologic parathyroid hyperplastic disorders (7,12,19–26). There are some reports that a decreased CaR level is observed in the hyperplastic parathyroid glands of uremic rats that are fed an HP diet (7,19,20). It was shown that a decreased CaR expression level is observed particularly in a lesion with an increased number of PCNA-positive cells in the hyperplastic parathyroid glands of uremic rats (19).

Ritter et al. (20) demonstrated that parathyroid cell proliferation progressed in 2 d, whereas CaR expression level decreased in a few days after the development of parathyroid cell
proliferation in subtotally nephrectomized rats with an HP diet. Their study suggested that parathyroid cell proliferation precedes the reduced level of CaR expression in parathyroid cells. They also reported that parathyroid cell proliferation is inhibited and that the reduced CaR expression level is restored by switching to a low-P diet (LP) in uremic rats. An LP diet could induce both the regression of parathyroid hyperplasia and the restoration of CaR expression. It is interesting that as compared with the regression of parathyroid hyperplasia, it took longer to restore CaR expression by P restriction (7). The stop of para-

Figure 4. Effect of daily oral administration (100 μmol/kg body wt) of R-568 for 1 wk on parathyroid cell proliferation in rats with severe SHPT at 8 wk after 5/6 nephrectomy. (A) Immunohistochemistry of proliferating cell nuclear antigen (PCNA) in rat parathyroid glands of Nx-8, Nx-9, and Nx + R-568 groups and controls. (B) Immunohistochemical quantification of PCNA reported per 1000 parathyroid cells. Values are expressed as mean ± SEM (n = 8 to 12). *P < 0.05 versus controls; #P < 0.05 versus R-568 by post hoc test (Fisher protected least significant difference). Magnification, ×100 in A.
thyroid cell proliferation was assumed to precede CaR restoration. These findings suggest that P might act as a regulator in not only parathyroid cell proliferation but also CaR expression in uremic milieu. It has been reported that vitamin D and a high-Ca diet inhibit parathyroid overgrowth along with the induction of the cell-cycle inhibitor p21 and the prevention of TGF-α overexpression (27). The study also indicates that vitamin D and a Ca load diet, as well as P restriction, have an inhibitory effect on parathyroid hyperplasia.

On the basis that calcemic action (CaR activation) suppresses parathyroid growth (27,28), we investigated the effect of calcemic action by a calcimimetic compound on the CaR expression of uremic rats that were fed an HP diet. It was reported that R-568 reduces PTH level and prevents parathyroid cell proliferation in rats with renal insufficiency. The antiproliferative effect of R-568 on parathyroid cells was shown at a very early stage, within 5 d after subtotal nephrectomy (12). However, the early effect of R-568 on the reduced CaR expression level of parathyroid cells in chronic renal insufficiency remains unclarified. In the present study, in agreement with previous studies (12–14), R-568 inhibited parathyroid cell proliferation rapidly, that is, within 1 wk. Moreover, we initially reported that R-568 upregulates the reduced CaR expression level in the hyperplastic parathyroid glands of uremic rats with SHPT.

In our study, extensive parathyroid cell proliferation was observed and a decreased CaR expression level was also observed in the subtotally nephrectomized rats that were fed the HP diet. No difference in serum P level after R-568 administration was found between the rats in our experiment. Consequently, it was impossible to evaluate the effect of changes in P metabolism upon R-568 treatment on parathyroid cell proliferation and CaR expression. However, P retention plays an important role in the pathogenesis of SHPT, including PTH oversecretion and parathyroid cell proliferation. It is assumed that P plays an important role in the abnormal CaR expression of parathyroid cells in uremia.

In a previous study, hyperphosphatemia developed after R-568 administration in uremic rats (12). The rapid decrease in PTH secretion by R-568 suppressed renal P excretion, and serum P level increased. In this experiment model, severe osteitis fibrosa developed, in which the level of Ca and P efflux from bone tissues increased. After cessation of SHPT, the levels of Ca and P influx into bone tissues rapidly increased along with bone formation. Serum P concentration may depend on the balance between decreased phosphaturia and increased level of P influx to bones. Thus, both were comparable in this experiment model.

It has been demonstrated that the hyperphosphatemic state disturbs Ca\(^{2+}\) sensing in parathyroid cells (29). However, calcimimetic agents are effective for SHPT in uremic animals despite the persistence of hyperphosphatemia (12–14). The inhibitory action of R-568 may be superior to the precipitative action of P on PTH secretion and parathyroid cell proliferation. In clinical studies, treatment with a calcimimetic compound in hemodialysis patients with SHPT reduced serum P level as well as PTH level (30,31).

The effect of vitamin D on CaR remains unclear. Brown et al. (6) demonstrated that rat parathyroid CaR mRNA and protein levels are lower in vitamin D–deficient rats than in normal rats and that CaR mRNA could be upregulated by 1,25(OH)\(_2\)D\(_3\) administration. However, Rogers et al. (32) reported that there is no effect of 1,25(OH)\(_2\)D\(_3\) on parathyroid CaR mRNA expression. Recently, it was reported that the human CaR gene has two transcriptional starting sites in its promoter, in which vitamin D response elements were identified (33). This study strongly suggests that 1,25(OH)\(_2\)D\(_3\) regulates CaR expression. Ca, P, and vitamin D play an important role in the regulation of CaR expression in parathyroid cells. However, the principal regulator of CaR expression has not yet been determined. Previously, a decrease in serum 1,25(OH)\(_2\)D\(_3\) level and an increase in serum P level were observed after R-568 administration in subtotally nephrectomized rats (12–14). In the present study, R-568 treatment did not affect serum 1,25(OH)\(_2\)D\(_3\) level in uremic rats. CaR upregulation was independent of vitamin D action.

The precise mechanism underlying CaR upregulation by R-568 remains unknown. Further studies are necessary to elucidate the mechanism of the CaR decrease after parathyroid cell proliferation in uremia. It is presumed that parathyroid cell proliferation itself is implicated in CaR downregulation, which exacerbates parathyroid growth. R-568 could break the vicious cycle of hyperparathyroidism. Thus, parathyroid cell growth/hyperfunction can be inhibited by improvement in Ca\(^{2+}\) sensitivity; in addition, it can restore the CaR downregulation, which brings about further improvement of hyperparathyroidism.

It is reported that R-568 suppresses the increase in hyperplastic parathyroid cell number and volume in uremic rats (13,14). Although we did not examine these parameters, they may mediate the suppression of parathyroid gland growth. We used the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) method to determine apoptosis induction; however, no significant difference in the number of TUNEL-positive cells by R-568 administration was noted in this study (data not shown). It is also not clarified whether R-568 administration to uremic rats directly or indirectly upregulates CaR expression in parathyroid cells. This point should also be investigated further.

In summary, we have demonstrated that the calcimimetic compound R-568 upregulates the decreased CaR expression level of parathyroid cells in uremic rats, without a significant change in serum P or 1,25(OH)\(_2\)D\(_3\) level. The accurate mechanism of upregulated CaR expression by R-568 remains unknown. Further studies are necessary to elucidate whether this favorable effect is a direct or indirect action by the calcimimetic compound. R-568 could ameliorate SHPT in uremic rats through at least two mechanisms. First, the compound can terminate PTH secretion through its allosteric action against CaR, by which reduced extracellular Ca\(^{2+}\) sensitivity is restored. Second, the reduced CaR expression level could be upregulated by R-568 treatment in uremic milieu, and such upregulation further improves Ca\(^{2+}\) sensitivity of parathyroid cells, additively or synergistically. Consequently, R-568 exerts...
its antiproliferative action on parathyroid cells through several mechanisms.

Acknowledgments

We are grateful to Dr. Edward F. Nemeth (NPS Pharmaceuticals, Salt Lake City, UT) for generously providing the calcimimetic compound NPS R-568 and the CaR primary monoclonal antibody (ADD). We also thank Dr. Michihito Wada and Dr. Nobuo Nagano (Pharmaceutical Research Laboratory, Kirin Brewery Co., Gunma, Japan) for helpful suggestions.

References


