The Calcimimetic Compound NPS R-568 Suppresses Parathyroid Cell Proliferation in Rats with Renal Insufficiency

Control of Parathyroid Cell Growth Via a Calcium Receptor

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Abstract

Parathyroid (PT) cell hyperplasia is a common consequence of chronic renal insufficiency (CRI). NPS R-568 is a phenylalkylamine compound that acts as an agonist (calcimimetic) at the cell surface calcium receptor (CaR). To test the hypothesis that the CaR plays a role in PT hyperplasia in CRI, we tested the effect of NPS R-568 on PT cell proliferation in rats with renal insufficiency. Rats were subjected to 5/6 nephrectomy and then infused intraperitoneally with 5-bromodeoxyuridine (BrdU) to label S-phase cells. Two groups of nephrectomized rats received NPS R-568 by gavage twice daily for 4 d (1.5 and 15 mg/kg body wt). On day 5, the number of BrdU-positive PT cells of vehicle-treated nephrectomized rats was 2.6-fold greater than that of the sham-operated control. Low and high doses of NPS R-568 reduced the number of BrdU-positive PT cells by 20 and 50%, respectively. No changes in staining, however, were observed in ileal epithelial cells (CaR-negative) or in thyroidal C-cells (CaR-positive). Furthermore, the effect of NPS R-568 could not be explained by changes in serum 1,25(OH)_{2}D_{3} or phosphorus. These results indicate that NPS R-568 suppresses PT cell proliferation in rats with renal insufficiency, and lend support to the linkage between the CaR and PT hyperplasia in CRI. (J. Clin. Invest. 1997;100:2977–2983.) Key words: calcium receptor • calcimimetics • parathyroid hormone • hyperplasia • chronic renal insufficiency

Introduction

Parathyroid (PT) cells secrete PTH and play a central role in maintaining circulating levels of ionized calcium (Ca^{2+}; 1).

Proliferation of PT cells governs the growth of the PT glands, and thereby exerts a long-term effect on the potential ability to synthesize PTH (2). In normal adults, very few PT cells proliferate, and growth of the PT glands is controlled at low levels (2). In contrast, abnormally increased PT cell proliferation (hyperplasia) is a common trait of hyperparathyroidism secondary to chronic renal insufficiency (CRI; 3, 4). Management of PT hyperplasia is of great clinical importance because malignancy of secondary hyperparathyroidism depends on the progression of PT hyperplasia (2–4). Although not precisely defined, the onset of PT hyperplasia due to CRI involves several factors such as 1,25-dihydroxyvitamin D_{3} (1,25(OH)_{2}D_{3}) deficiency, phosphorus (Pi) retention, and hypocalcemia (5–7).

It has been proposed for a long time that hypocalcemia plays a pivotal role in secondary hyperparathyroidism development and PT cell hyperplasia (8). Extracellular Ca^{2+} (Ca^{2+}_{o}) serves as a physiological regulator of PTH secretion (9, 10). PT cells sense small changes in extracellular Ca^{2+} (Ca^{2+}_{o}) by means of the cell-surface Ca^{2+}_{o} receptor (CaR) that recognizes Ca^{2+}_{o} as its physiological ligand (9, 10). The CaR gene was cloned first from bovine PT cells, and sequence analysis demonstrated that the CaR is a G-protein–coupled receptor (GPCR) sharing limited homology with metabotropic glutamate receptors (11, 12). Two recent studies have demonstrated that CaR expression is downregulated at both mRNA and protein levels in the hyperplastic PT glands of patients with CRI (13, 14), suggesting linkage between this receptor and PT hyperplasia.

We hypothesized that the CaR might regulate PT cell proliferation and play an important role in PT hyperplasia observed in CRI. This hypothesis in turn indicates that Ca^{2+}_{o} controls PT cell growth by CaR-mediated mechanisms. The effect of Ca^{2+}_{o} on PT cell growth, however, still remains controversial (2, 15). For example, the results from in vitro experiments studying the effects of low ambient concentrations of Ca^{2+}_{o} on PT cell growth are contradictory; some authors observed increased cell proliferation (16, 17) whereas others showed no effect on cell growth (18, 19). Likewise, in vivo there is no agreement as to whether PT cell growth is stimulated by hypocalcemia (6) or not (20). Furthermore, little is known about the effect of hypercalcemia on PT cell growth because it is difficult to produce hypercalcemia without affecting general cellular functions for a long enough time to evaluate cell proliferation (15, 21).

Synthetic compounds referred to as calcimimetics, acting as specific agonists at the CaR, have been developed (22, 23). Upon binding to the CaR, calcimimetics enhance the sensitivity of the CaR to Ca^{2+}_{o} in an allostERIC fashion and exert a suppressive effect on PTH secretion (22, 23). Of these calcimimetics, NPS R-568, a phenylalkylamine derivative of the most potent calcimimetics, has been subjected to clinical trials, and has been shown to produce a dose-dependent decrease in the circulating levels of PTH in both normal women (24) and patients with primary hyperparathyroidism (25). In this study, to
test the hypothesis that the CaR plays a role in PT hyperplasia development. We evaluated the effect of NPS R-568 on PT cell growth in rats with renal insufficiency. Our results demonstrate that oral administration of NPS R-568 clearly inhibits PT cell proliferation, which is markedly accelerated in rats with renal insufficiency.

Methods

Chemicals. NPS R-568, N-[3-[2-chlorophenyl]-propyl]-R-α-methyl-3-methoxybenzylamine HCl, referred to as KN9568 in Japan, was synthesized by OREAD Laboratories, Inc. (Lawrence, KS). Cyclo- dextrin (2-hydroxycetyl-β-cycloextrin) was obtained from Research Biochemicals International (Natick, MA) and 5-bromo-2′-deoxyuridine (BrdU) was from Sigma Chemical Co. (St. Louis, MO).

Experimental protocols. 10-wk-old male Sprague-Dawley rats weighing 310–350 g were purchased from Charles River Japan (Tokyo, Japan) and fed a diet containing 11.1% P, 1.2% Ca, 25% protein, and 250 IU/100 g vitamin D3 (CLEA Japan, Tokyo, Japan). Rats were allowed free access to food and water. After acclimatization for 10 d, three groups of rats were subjected to 5/6 nephrectomy under ether anesthesia by removing the right kidney and ligating branches of the left renal artery in a one-step procedure. Another group of rats underwent sham operation and served as a normal control. Each group was assigned six animals. Two groups of 5/6 nephrectomized rats received NPS R-568 by gavage twice daily for 4 d at doses of 1.5 and 15 mg free base/kg body wt, respectively. One group of 5/6 nephrectomized rats and the sham-operated rats were given vehicle (a 10% aqueous solution of cyclodextrin). Administration was carried out at 1000–1700 and 2100–2200 h on the day of the operation, and at 0900–1000 h and 1800–1900 h on three subsequent days. 4 d after 5/6 nephrectomy, animals were killed by aortic puncture under ether anesthesia and subjected to necropsy (see Fig. 1). At the time of necropsy, one animal treated with NPS R-568 at 1.5 mg/kg body wt was excluded from the study because of severe uremia.

Infusion of BrdU. To evaluate cell proliferation, animals were intraperitoneally infused with BrdU, a thymidine analog, for 4 d (see Fig. 1). Alzet osmotic pumps with a speed of 10.51±0.48 (SD) μl/h (Model 2ML1; Alza Corp., Palo Alto, CA) were filled with 25 mg/ml BrdU in PBS, incubated in PBS at 37°C overnight for conditioning, and implanted in the abdominal cavity at the time of 5/6 nephrectomy or the sham operation. BrdU was released at a dose of 6 mg/animal/d.

Serum biochemistry. Blood was collected via the tail artery to evaluate creatinine, blood urea nitrogen during the course of the experiment, and at necropsy. Serum was separated at 3°C overnight. The bound primary antibody was detected by the avidin–biotin complex immunoperoxidase method (Vector Laboratories, Inc., Burlingame, CA). Slides were developed using the Vector-Red system. The specificity for cyclin D1 was verified by preabsorbing the primary antibodies with 10-fold excess human cyclin D1 and BrdU appeared as brown staining in the nucleus.

Detection of apoptosis. To identify apoptosis, nuclear DNA fragment was detected in situ using the ApopTag system (Oncor Inc., Gaithersburg, MD). Slides were digested with 20 μg/ml proteinase K (Boehringer Mannheim, Mannheim, Germany) in PBS at room temperature for 15 min and incubated with 3% hydrogen peroxide/methanol to block endogenous peroxidase for 5 min. Slides were rehydrated with 100% ethanol for 1 min at 37°C with terminal deoxynucleotidyl transferase (TdT) to label exposed 3′-OH DNA ends with digoxigenin-tagged deoxyuridine (26). Digoxigenin-labeled DNA was detected by the immunoperoxidase method. Slides were developed with 3,3′-diaminobenzidine, and the nuclei of apoptotic cells were stained brown.

Statistics. Data are expressed as means ± SEM. The statistical significance of differences between groups was determined using Dunnnett’s multiple comparison test, and the correlation coefficients between pairs of parameters were obtained using Pearson’s correlation analysis. P < 0.05 was taken to indicate statistical significance.
Results

There were no significant differences in body weight between NPS R-568–treated animals and the vehicle-treated controls during the course of the experiment (Fig. 1). Uremia was produced by 5/6 nephrectomy, as evidenced by increased serum creatinine and BUN levels, while these indices were not significantly affected by treatment with NPS R-568 (Table I). In the 5/6 nephrectomized animals, the circulating levels of 1,25(OH)2D3 were 50% lower than those in the sham-operated controls. NPS R-568 did not significantly change serum 1,25(OH)2D3 levels (Table I). In contrast, serum PTH levels were reduced by NPS R-568 in a dose-dependent manner (Fig. 2A).

The reduction in serum PTH levels was associated with decreased serum Ca levels (B) and increased Pi levels (C). Blood was collected sequentially 1 and 14 h after the last gavage of NPS R-568. Results are shown as means±SEM. #P < 0.05, ##P < 0.01 vs. the Sham control; and *P < 0.05, **P < 0.01 vs. NX controls at each time point.

Table I. Effects of NPS R-568 on Serum Biochemical Indices in 5/6 Nephrectomized Rats

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Creatinine</th>
<th>BUN</th>
<th>Na+</th>
<th>K+</th>
<th>Cl-</th>
<th>1,25(OH)2D3</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>mg/dl</td>
<td>mg/dl</td>
<td>mEq/liter</td>
<td>mEq/liter</td>
<td>mEq/liter</td>
<td>pg/ml</td>
</tr>
<tr>
<td>Sham</td>
<td>6</td>
<td>0.29±0.01</td>
<td>15.9±0.9</td>
<td>147.7±0.6</td>
<td>4.77±0.21</td>
<td>109±0.4</td>
<td>54.3±12.8</td>
</tr>
<tr>
<td>NX</td>
<td>6</td>
<td>0.91±0.06</td>
<td>40.3±2.9</td>
<td>147.0±0.9</td>
<td>5.25±0.19</td>
<td>105±0.9*</td>
<td>25.9±2.6*</td>
</tr>
<tr>
<td>568 low</td>
<td>5</td>
<td>1.07±0.08</td>
<td>53.5±6.5*</td>
<td>148.4±2.3</td>
<td>5.51±0.30</td>
<td>105±1.7*</td>
<td>20.9±3.3*</td>
</tr>
<tr>
<td>568 high</td>
<td>6</td>
<td>1.16±0.12</td>
<td>49.3±5.5*</td>
<td>147.1±0.6</td>
<td>5.33±0.32</td>
<td>104±1.1*</td>
<td>31.1±3.9</td>
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Sham, sham-operated control; NX, 5/6 nephrectomized controls treated with vehicle; 568 low, 5/6 nephrectomized animals treated with NPS R-568 at 1.5 mg/kg body wt; 568 high, 5/6 nephrectomized animals treated with NPS R-568 at 15 mg/kg body wt. *P < 0.05; †P < 0.01 vs. the sham control.

Figure 1. Effects of NPS R-568 on body weight changes. Rats were subjected to 5/6 nephrectomy or sham operation on day 1, and to necropsy on day 5. The sham-operated (Sham) and nephrectomized controls (NX) were treated with vehicle, and two groups of nephrectomized animals received NPS R-568 twice daily at 1.5 and 15 mg/kg body wt (568 low and 568 high, respectively). NPS R-568 was administered p.o. for 4 d (solid bar), and BrdU was infused intraperitoneally throughout the experiment (hatched bar). Results are expressed as means±SEM (five or six animals per group as shown in Table I).

Figure 2. Serum PTH levels reduced by NPS R-568 (A) associated with decreased serum Ca levels (B) and increased Pi levels (C). Blood was collected sequentially 1 and 14 h after the last gavage of NPS R-568. Results are shown as means±SEM. #P < 0.05, ##P < 0.01 vs. the Sham control; and *P < 0.05, **P < 0.01 vs. NX controls at each time point.
increased serum Ca and increased serum Pi levels (Fig. 2). Administration of NPS R-568, however, did not affect circulating levels of the electrolytes Na\(^+\), K\(^+\), and Cl\(^-\) (Table I).

4 d after 5/6 nephrectomy, the number of BrdU-positive PT cells in the vehicle-treated 5/6 nephrectomized animals was 2.6-fold greater than that of the sham-operated control (Figs. 3 and 4). NPS R-568 clearly reduced the number of BrdU-positive PT cells by 20% at a low dose (1.5 mg/kg body wt), and by 50% at a high dose (15 mg/kg body wt), indicating an antiproliferative effect on PT cells (Figs. 3 and 4). In contrast, NPS R-568 did not change the number of BrdU-positive cells in other tissues. In the ileum, almost 100% of epithelial cells were BrdU-positive, and were not affected by NPS R-568 treatment (data not shown). In addition, double immunostaining for calcitonin and BrdU (Fig. 3 D) showed that the number of BrdU-positive C cells was not affected by 5/6 nephrectomy or NPS R-568 treatment (Fig. 4). This result was further confirmed by plotting the number of BrdU-positive cells against serum creatinine levels. In PT cells, but not in C cells, the number of BrdU-positive cells was correlated with serum creatinine levels (r = 0.879, P < 0.01 for pooled normal and uremic controls), while this relationship was abolished by high dose of NPS R-568 (Fig. 5).

It was reported that cyclin D1 might serve as a regulator of Ca\(^{2+}\)-dependent PT cell proliferation (28). To test this hy-

Figure 3. Immunohistochemistry for BrdU. The parathyroid glands of Sham (A), NX (B), and NPS R-568 at a high dose (C) and the thyroid gland of NPS R-568 at a low dose (D). BrdU is seen as brown staining in the nucleus, and (D) calcitonin appears as red staining in the cytoplasm. Arrows indicate double-positive cells. 400×, original (A–C) and 1,000× (D).

Figure 4. Effects of NPS R-568 on the proliferation of parathyroid and thyroid C cells. Results are shown as mean±SEM. **P < 0.01 vs. the Sham control, and ***P < 0.01 vs. NX controls. Black bars, parathyroid cells; hatched bars, thyroid C cells.
Calcium Receptor and Parathyroid Cell Growth

Pohypothesis, we next evaluated cyclin D1 expression by immuno-

staining using two independent anti–cyclin D1 antibodies (H-295

and C-20). In the cytoplasm, the staining intensity for cyclin

D1 in PT cells was stronger than that in thyroid follicular cells,

but was not apparently changed by 5/6 nephrectomy or NPS

R-568 treatment; the two antibodies gave similar results in the

thyroid–parathyroid tissue (data not shown). It should be

noted, however, that the cell volume of PT cells was increased

by 37% after 5/6 nephrectomy, i.e., hypertrophy was induced,

whereas NPS R-568 reduced PT cell volume in a dose-depen-
dent manner (Fig. 6). Hence, the total amount of cyclin D1 ex-

pressed in PT cells might be affected by the changes in cell

volume.

The speed of cell proliferation is generally balanced by the

rate of cell death. Thus, we examined nuclear DNA fragmen-
tation, a hallmark of apoptosis, in situ. We detected no apopto-
sis, however, in the PT glands of any sham-operated, 5/6 ne-
phrectomized, or NPS R-568–treated animals, even at the high
dose of 15 mg/kg body wt (Fig. 7A). On the other hand, apop-
tosis was observed in epithelial cells in the ileum as reported
previously (Fig. 7B; 26).

Discussion

There has been as yet no consensus as to whether PT cell pro-
liferation is regulated by Ca$^{2+}$ (2, 15). Previous studies, in-
dicating that PT hyperplasia begins within 48 to 72 h in nephrec-
tomized rats (29–31), prompted us to perform our experiments
within 5 d after 5/6 nephrectomy. The main finding of this
study was that the calcimimetic compound NPS R-568, which
acts as an agonist at the CaR (22, 23), inhibited PT cell prolif-
eration in rats with renal insufficiency. The animal model used
here is an approximation of human polyclonal PT cell expan-
sion in CRI (2, 3). Our results lend support to the linkage be-
tween the CaR and development of PT hyperplasia in CRI.
Several lines of evidence support the hypothesis that the CaR plays a critical role in regulating PT cell proliferation. First, loss-of-function mutations in the CaR, which cause the diseases familial benign hypocalciuric hypercalcemia and neonatal severe hyperparathyroidism, are generally associated with PT hyperplasia (32). Second, this observation was confirmed by the PT hyperplasia seen in mice nullizygous for the CaR, a genetic model for neonatal severe hyperparathyroidism (33). Third, CaR expression is downregulated at both mRNA and protein levels in the hyperplastic PT glands in patients with CRI (13, 14), suggesting a role for this receptor in the development of PT hyperplasia.

Consistent with these findings, we have demonstrated that the calcimimetic compound NPS R-568 that specifically acts on the CaR can inhibit PT cell proliferation in rats with renal insufficiency. The possibility that the antiproliferative action was mediated by alleviation of 1,25(OH)2D3 deficiency or Pi retention was excluded since 1,25(OH)2D3 levels were not affected by treatment with NPS R-568, and the Pi level of NPS R-568-treated animals was rather greater than that of the vehicle-treated controls. The increase in Pi level is thought to be ascribed to reduced PTH levels (1). Moreover, the possibility that the action was mediated by renal functions was also ruled out because the antiproliferative effect of NPS R-568 was independent of serum creatinine levels. In contrast, PTH secretion and PT hypertrophy were clearly inhibited by NPS R-568. Therefore, we concluded that NPS R-568 principally, if not solely, exerted its suppressive effect on PT cells by directly acting on the CaR.

This conclusion was further supported by the tissue-specific antiproliferative effect of NPS R-568. Intestinal epithelial cells (CaR-negative) and thyroidal C-cells (CaR-positive) were not affected, suggesting that NPS R-568 has no general cytotoxic action and exerts a cell type–specific antiproliferative effect on PT cells. This specificity contrasts with the finding that injected 1,25(OH)2D3 inhibits cell proliferation in a variety of cell types, including intestinal mucosa (5). It should be noted, however, that the cell type–specific antiproliferative effect of NPS R-568 is not simply due to CaR-positive or CaR-negative of the cell. The CaR is not only expressed on PT cells, but is also expressed on thyroid C-cells (34, 35). These two types of cells, however, show opposite regulation by Ca2+-sensing, for their hormone release, i.e., suppression for PTH versus stimulation for calcitonin (10, 23). It has been proposed that GPCRs might regulate cell proliferation via divergent cascades depending on the types of cells used. For example, p21waf1-dependent MAP kinase is activated by GPCRs through cell type–specific tyrosine kinases, e.g., the epidermal growth factor receptor in Rat-1 fibroblasts (36), Lyn and Syk in avian B cells (37), and Src coupled to Pyk2 in PC12 cells (38). Therefore, it is feasible that the antiproliferative action of NPS R-568 is closely related to growth mechanisms specific to PT cells.

It remains unclear, however, which cascade(s) lie between the CaR and PT cell proliferation. Cyclin D1 was reported to be upregulated at mRNA levels by low concentrations of Ca2+, in a rat PT cell line, and possibly to serve as a regulator of Ca2+-dependent PT cell proliferation (28). It seems unlikely, however, that cyclin D1 operates directly downstream of the CaR in rats treated with NPS R-568 because no apparent difference in cyclin D1 expression was observed between NPS R-568–treated and vehicle-treated animals by immunostaining for cyclin D1. This result is in concert with a finding that no overexpression of cyclin D1 was observed in the PT glands of patients with hyperparathyroidism secondary to CRI (4). We are currently investigating the molecular mechanisms involved in CaR-regulated PT cell proliferation.

Our results may be of clinical relevance for intervention in PT hyperplasia. The importance to control PT hyperplasia stems from the findings that very few PT cells undergo apoptotic cell death under physiological conditions and in response to 1,25(OH)2D3 treatment or low Pi diet (2, 6, 15, 21). These findings suggest that PT hyperplasia is an irreversible process (6). Consistent with these findings, administration of NPS R-568 did not induce PT cell apoptosis in 5/6 nephrectomized rats. This result accounts for our previous observations that PT hyperplasia was almost completely prevented, but not reversed, by treating 5/6 nephrectomized rats with NPS R-568 by various regimens (unpublished personal data; 39, 40). Therefore, our results suggest that the CaR is a potential target for prevention of PT hyperplasia.

In summary, we have demonstrated that the calcimimetic compound NPS R-568 inhibits PT cell proliferation in rats with renal insufficiency principally by directly acting on the CaR. The critical piece of human data in relation to this result is that the CaR is downregulated in hyperplastic PT glands in patients with CRI (13, 14). Our results lend support to the hypothesis linking the CaR with PT hyperplasia development. Finally, the results of this study suggest that the CaR is a potentially useful target for therapeutic agents to prevent PT hyperplasia.

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