Calcimimetic Compound NPS R-568 Stimulates Calcitonin Secretion But Selectively Targets Parathyroid Gland Ca\textsuperscript{2+} Receptor in Rats

JOHN FOX, STACEY H. LOWE, REBECCA L. CONKLIN, BARBARA A. PETTY, and EDWARD F. NEMETH
NPS Pharmaceuticals, Inc., Salt Lake City, Utah

ABSTRACT

N-(3-[2-Chlorophenyl]propyl)-(R)-α-methyl-3-methoxybenzylamine (NPS R-568) is an orally active compound that activates Ca\textsuperscript{2+} receptors on parathyroid cells and rapidly suppresses plasma levels of parathyroid hormone (PTH) and Ca\textsuperscript{2+} (ED\textsubscript{50} 1 and 10 mg/kg, respectively). We now show that increased calcitonin secretion contributes to NPS R-568-induced hypocalcemia. In parathyroidectomized thyroid-intact rats in which normocalcemia was restored by PTH infusion, NPS R-568 rapidly reduced plasma Ca\textsuperscript{2+} levels, indicating that decreased PTH secretion was not solely responsible for the hypocalcemia seen in normal animals. NPS R-568 decreased plasma Ca\textsuperscript{2+} levels in thyroparathyroidectomized parathyroid-intact rats, but the rate of onset of hypocalcemia was slower than in controls. In contrast, NPS R-568 had no effect on plasma Ca\textsuperscript{2+} levels in PTH-infused, thyroparathyroidectomized rats, providing evidence that increased calcitonin secretion caused the hypocalcemia in PTH-infused parathyroidectomized rats. NPS R-568 rapidly increased plasma calcitonin levels to a peak at 10 to 20 min after oral dosing (ED\textsubscript{50} 40 mg/kg). NPS R-568 did not affect the rate of disappearance of 45Ca from blood, indicating that hypocalcemia resulted from decreased influx of Ca\textsuperscript{2+} into the circulation and not from increased efflux. This suggests that NPS R-568-induced hypocalcemia resulted solely from reduced influx of Ca\textsuperscript{2+} from bone after increased calcitonin and reduced PTH secretion. Thus, NPS R-568 causes hypocalcemia by activating Ca\textsuperscript{2+} receptors on C cells and parathyroid cells; however, NPS R-568 is about 40 times more potent in reducing PTH levels than in increasing calcitonin levels.

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ABBREVIATIONS: PTH, parathyroid hormone; PTX, parathyroidectomized; TX, thyroidectomized; TXPTX, thyroparathyroidectomized.
results in decreased bone turnover, a more physiological means of maintaining plasma Ca\(^{2+}\) levels in PTX rats is to replenish circulating levels of PTH. With this method, we found that NPS R-568 causes a profound hypocalcemic response in PTX rats that results from increased plasma levels of calcitonin.

**Materials and Methods**

**Animals, Diets, and Surgical Procedures.** Male Sprague-Dawley rats (Harlan Sprague-Dawley, Inc., Indianapolis, IN), weighing 250 to 300 g, were used in these studies. They were housed in hanging wire cages for at least 7 days before study and fed a commercial chow (Purina 5001) and tap water ad libitum. All surgical procedures were performed with a combination of ketamine (90 mg/kg) and xylazine (7 mg/kg) injected intramuscularly as anesthetic. Each rat had a blood-sampling catheter implanted chronically in the abdominal aorta via the femoral artery at least 2 days before study. In one study, a venous catheter for the infusion of Ca\(^{2+}\) was also implanted in the inferior vena cava via the femoral vein (Fox, 1990). To determine the dependence of the hypocalcemic effect of NPS R-568 on the presence of parathyroid and/or thyroid glands, rats were subjected to 1) a parathyroidectomy in which the parathyroid glands were exposed and removed by careful dissection leaving the thyroid gland intact, 2) a thyroidectomy in which all thyroid glands were exposed and removed by careful dissection leaving the thyroid gland intact, 3) an Alzet model 2001 osmotic minipump (Alza, Palo Alto, CA) was implanted s.c. The pump infused synthetic rat PTH-(1–34) (Bachem, Torrance, CA), dissolved in 2% cysteine HCl, pH 1.5, at 1 \(\mu\)g/l-day s.c. Three separate experiments were performed in PTX rats with PTH infusion rates of 2.0, 0.8, and 0.6 \(\mu\)g/l-day, which, after 3 to 5 days of infusion, rendered the rats severely and moderately hypercalcemic and normocalcemic, respectively. The PTH infusion rate that rendered the thyroparathyroidectomized (TXPTX) rats normocalcemic was 0.5 \(\mu\)g/day. The rats were studied after PTH had been infused for 5 to 7 days. Each rat received by gavage an oral dose (1.0 ml/200 g b.wt., administered as the hydrochloride salt) or vehicle, a 1.5% aqueous solution of 2-hydroxypropyl-\(\beta\)-cyclodextrin (Research Biochemicals, Natick, MA). Blood samples (0.1 ml) for plasma Ca\(^{2+}\) assay were collected immediately before and for 3 h after dosing.

**Effect of NPS R-568 in Thyroidectomized (TX) Rats.** Before the experiment and after an overnight fast, plasma Ca\(^{2+}\) levels were measured in each TX rat to ensure that the surgery had not compromised parathyroid gland function. Only rats with a plasma Ca\(^{2+}\) level similar to that of sham-operated controls were studied. NPS R-568 (10 mg/kg) or vehicle (1.5% cyclodextrin) was administered by oral gavage. Blood samples (0.1 ml) were collected before and for 6 h after dosing.

**Time Course and Dose Response to NPS R-568 in Normal Rats.** This study in normal rats tested the effects on plasma calcitonin and Ca\(^{2+}\) levels of a series of oral doses of NPS R-568 (1.0, 3.3, 10, 33, and 100 mg/kg). The 100-mg/kg dose of NPS R-568 was administered in 15% cyclodextrin. The lower doses were prepared by diluting the 100-mg/kg dosing solution with water. Vehicle-dosed rats received 15% cyclodextrin alone. Blood samples (0.8 ml) were collected immediately before and for 4 h after dosing. To prevent excessive blood loss during the experiment, after removal of the plasma sample, the erythrocyte pellet was resuspended in an equal volume of normal rat plasma and reinfected.

**Effects of Prevention of Hypocalcemia on Plasma Calcitonin Response to NPS R-568.** Normal rats received an oral dose of vehicle (15% cyclodextrin) or NPS R-568 (100 mg/kg). In one group of NPS R-568-dosed rats, calcium gluconate was infused i.v. at rates determined empirically to prevent the induced fall in plasma Ca\(^{2+}\) levels. Blood samples (0.8 ml) were collected before and for 6 h after dosing.

**Effect of NPS R-568 on Plasma \(^{45}\)Ca Kinetics in Normal Rats.** After the collection of a basal blood sample (0.4 ml), \(^{45}\)Ca (10 \(\mu\)Ci) was injected via the arterial catheter. Additional blood samples were collected at 1, 2, 2.5, and 3 h after the injection to determine plasma Ca\(^{2+}\) and \(^{45}\)Ca levels. At 3 h, each rat received an oral dose of NPS R-568 (10 or 100 mg/kg) or vehicle (15% cyclodextrin). Additional blood samples were collected for 3 h after dosing.

**Analyses.** Plasma Ca\(^{2+}\) levels were measured immediately on 35 \(\mu\)l of heparinized whole blood with a model 634 Ca\(^{2+}\) analyzer (Ciba Corning, Medford, MA). Ca\(^{2+}\) activity was determined in duplicate on 50 \(\mu\)l of plasma by liquid scintillation counting. Plasma calcitonin levels were determined by radioimmunoassay (Fox, 1988) with goat anti-human calcitonin antiserum G-813, reversed-phase HPLC-purified \(^{125}\)I-labeled human calcitonin, and synthetic rat calcitonin standards. The assay detection limit averaged 20 ± 1 pg/ml in three separate assays, and intra- and interassay coefficients of variation for a rat plasma internal reference standard (68 pg/ml) averaged 15 and 11%, respectively. The assay was validated by testing the effects of hypo- and hypercalcemic conditions on plasma levels of calcitonin. Plasma calcitonin increased from 55 ± 2 to 484 ± 124 pg/ml in normal rats (n = 4) 10 min after calcium gluconate injection (93 \(\mu\)mol i.v. over 2 min), which increased plasma Ca\(^{2+}\) levels by 0.48 ± 0.13 mM. In a separate experiment in four similar rats, calcitonin levels decreased from 59 ± 10 pg/ml to undetectable levels (<19 pg/ml) 10 min after EGTA injection (69 \(\mu\)mol i.v. over 5 min), which decreased plasma Ca\(^{2+}\) levels by 0.41 ± 0.05 mM.

**Statistical Analyses.** All data are presented as means ± S.E. Plasma calcitonin and Ca\(^{2+}\) levels were initially subjected ANOVA for repeated measures (SuperANOVA; Abacus Concepts, Berkeley, CA). Dunnett’s test was used to determine the significance of differences from vehicle-dosed rats. A t test was used when two means were compared. p < .05 was used to denote a significant difference.

**Results**

**Effect of NPS R-568 in PTH-Infused PTX Rats.** These studies were initially designed to determine whether the failure of NPS R-568 to induce a hypocalcemic response in PTX rats that were either hypocalcemic or rendered acutely normocalcemic by calcium infusion (Fox et al., 1999) occurred because bone turnover was low (a result of the chronic PTH deficiency). In the three experiments, the s.c. infusions of rat PTH-(1–34) rendered the PTX rats severely or moderately hypercalcemic (plasma Ca\(^{2+}\) 2.0 and 1.6 mM, respectively) or normocalcemic (plasma Ca\(^{2+}\) 1.4 mM) (Fig. 1). In the severely hypercalcemic rats, plasma Ca\(^{2+}\) levels tended to decrease more in the animals given NPS R-568 than in vehicle-dosed controls, but the differences were not significant at any time point (Fig. 1A). In contrast, in the moderately hypercalcemic (Fig. 1B) and normocalcemic (Fig. 1C) PTH-infused PTX rats, the administration of NPS R-568 induced a rapid and significant fall in plasma Ca\(^{2+}\). In the moderately hypercalcemic and normocalcemic NPS R-568-dosed rats, plasma Ca\(^{2+}\) was significantly lower than in vehicle-dosed rats.
rats by 30 to 60 min after dosing and reached a nadir at 60 or 90 min before starting to return toward control levels. The maximum decrement in plasma Ca$^{2+}$ levels from basal at 60 or 90 min after dosing was 0.19 ± 0.03 (p < .01) and 0.10 ± 0.01 mM (p < .01) in the moderately hypercalcemic (A), moderate hypercalcemia (B), or normocalcemia (C) rats. Values are means ± S.E.; n = 4 to 6/group. *p < .05, significance of difference from vehicle-dosed rats.

**Effect of NPS R-568 in PTH-Infused TXPTX Rats.** One interpretation of the above experiments would be that PTH infusion, in contrast to calcium infusion, maintains bone turnover rate at near-normal levels in PTX rats. The decrease in plasma Ca$^{2+}$ levels induced by NPS R-568 in these PTH-infused animals could have resulted from direct inhibitory effects of the compound on bone resorption or from indirect effects mediated by increased calcitonin secretion. To test the latter hypothesis, the above experiment was repeated in TXPTX rats in which the source of calcitonin was also removed. The infusion of rat PTH-(1–34) at 0.5 μg/day s.c. rendered the TXPTX rats slightly hypercalcemic (Fig. 2). Basal plasma Ca$^{2+}$ levels averaged 1.46 ± 0.07 mM (normal range, 1.29–1.44 mM). Plasma Ca$^{2+}$ levels decreased progressively throughout the experiment in both vehicle- and NPS R-568-dosed rats. However, no significant differences were observed between the two groups (Fig. 2). These results suggest that increased calcitonin secretion was responsible for the NPS R-568-induced decrease in plasma Ca$^{2+}$ levels in PTH-infused PTX rats.

**Effect of NPS R-568 in TX Rats.** To determine the relative contributions of calcitonin and PTH to the induced decrease in plasma Ca$^{2+}$ levels, NPS R-568 was administered to TX parathyroid gland-intact animals. The TX rats had slightly but significantly higher basal plasma Ca$^{2+}$ levels than the sham-operated controls (1.38 ± 0.02 versus 1.33 ± 0.01 mM; p < .05). Plasma Ca$^{2+}$ levels decreased rapidly after oral administration of NPS R-568 in sham-operated rats and were reduced significantly by 30 min after dosing (Fig. 3). In contrast, plasma Ca$^{2+}$ levels decreased more slowly in the TX rats and were not significantly lower than basal levels until 60 min after NPS R-568 administration. This difference in the rate of onset of hypocalcemia between the sham-operated and TX rats is more obvious when the net changes in plasma Ca$^{2+}$ levels from basal levels are plotted (Fig. 3). At 30 min postdose, the net decrease in plasma Ca$^{2+}$ levels was 0.08 ± 0.02 and 0.03 ± 0.01 mM in the sham-operated and TX rats, respectively (p < .05); by 60 min, the net decreases were 0.12 ± 0.02 and 0.06 ± 0.01 mM, respectively. Thereafter, plasma Ca$^{2+}$ levels tended to return toward control levels more rapidly in sham-operated than in TX rats.

**Plasma Calcitonin and Ca$^{2+}$ Responses to Increasing Doses of NPS R-568 in Normal Rats.** Plasma calcitonin levels increased promptly after the oral administration of NPS R-568, with maximum increases observed by either 10 or 20 min after dosing in all rats studied. The increase in plasma calcitonin levels was dose dependent and did not
plateau at the highest dose (100 mg/kg). NPS R-568 did not significantly affect plasma calcitonin levels at doses below 33 mg/kg. The maximum increases in calcitonin levels were 1.1 ± 0.1-, 1.2 ± 0.1-, 2.1 ± 0.5-, 2.9 ± 0.4- (p < .05), and 10.2 ± 1.6-fold (p < .01), with the 1.0-, 3.3-, 10-, 33-, and 100-mg/kg doses, respectively (Fig. 4). Plasma calcitonin levels declined rapidly from the peak and only remained significantly elevated above control levels for ≤30 min, even in the rats that received the 100-mg/kg dose. Thus, from 60 min after dosing until the end of the experiment, no significant differences in plasma calcitonin levels were observed in any of the groups. Orally administered NPS R-568 thus causes a rapid but transient increase in the plasma levels of calcitonin.

Plasma Ca\textsuperscript{2+} levels decreased rapidly after NPS R-568 administration. The first significant decrease occurred by 20 min in the groups receiving 33 and 100 mg/kg. Lower doses also caused significant decreases in Ca\textsuperscript{2+} at later times (30 min, 10-mg/kg group; 60 min, 3.3-mg/kg group) (Fig. 4). The 1.0-mg/kg dose of NPS R-568 did not significantly affect plasma Ca\textsuperscript{2+} levels at any time point (not shown). A nadir in plasma Ca\textsuperscript{2+} levels occurred in most groups at 60 to 120 min after dosing and, with doses ≥10 mg/kg, remained significantly lower than controls throughout the experiment.

The relationships between the dose of orally administered NPS R-568 and the maximum plasma calcitonin level at either 10 or 20 min and the plasma Ca\textsuperscript{2+} level at 60 min after dosing are shown in Fig. 5. If we take the plasma calcitonin response to the 100-mg/kg dose of NPS R-568 as maximal, then the ED\textsubscript{50} for elevation of plasma calcitonin levels by NPS R-568 is about 40 mg/kg and the ED\textsubscript{50} for reduction of plasma Ca\textsuperscript{2+} levels about 10 mg/kg. The calcitonin value is
probably an underestimation because we were unable to determine whether the calcitonin response at 100 mg/kg was maximal.

Effects of Prevention of Hypocalcemia on Plasma Calcitonin Response to NPS R-568. The hypocalcemic response induced by NPS R-568 might be expected to counteract the effects of this compound on calcitonin secretion. It was therefore of interest to determine the effects of NPS R-568 on plasma calcitonin levels in the absence of any changes in plasma Ca\(^{2+}\) levels. This was achieved by infusion of calcium gluconate, thereby “clamping” plasma Ca\(^{2+}\) levels. Oral administration of NPS R-568 (100 mg/kg) increased plasma levels of calcitonin by 9.5 ± 3.4-fold within 30 min and induced a hypocalcemic response similar to that shown in Fig. 4. Intravenous infusion of calcium gluconate in one group of rats given NPS R-568 prevented the fall in plasma Ca\(^{2+}\) levels and produced a plasma Ca\(^{2+}\) profile similar to the one that occurred in rats receiving vehicle (Fig. 6). Although interanimal variability was large, the plasma calcitonin response was greatly increased in magnitude and duration in rats in which normocalcemia was maintained.

Effect of NPS R-568 on Plasma \(^{45}\)Ca Kinetics. Plasma \(^{45}\)Ca activity decreased progressively and at identical rates in the three groups during the 3 h after \(^{45}\)Ca injection, whereas plasma Ca\(^{2+}\) levels did not change (Fig. 7). After oral administration of NPS R-568 at 3 h after the \(^{45}\)Ca injection, a rapid, dose-dependent decrease in plasma Ca\(^{2+}\) levels occurred. Plasma Ca\(^{2+}\) levels were significantly (p < 0.01) decreased below control by 30 min after both the 10- and 100-mg/kg doses of NPS R-568 and were significantly (p < 0.01) lower throughout the remainder of the experiment. The maximum decreases in plasma Ca\(^{2+}\) levels were 0.14 ± 0.03 and 0.29 ± 0.02 mM after the 10- and 100-mg/kg doses, respectively. In contrast to the marked decreases in plasma Ca\(^{2+}\) levels seen after NPS R-568 administration, the rate of decrease in plasma \(^{45}\)Ca activity did not change after either dose of NPS R-568 (Fig. 7).

Discussion

The results of these studies reveal that the calcimimetic compound NPS R-568 induces hypocalcemia in normal rats not only by inhibiting PTH secretion but also by stimulating the secretion of calcitonin. In a previous study (Fox et al., 1999), we showed that, at doses ≥10 mg/kg, the initial rate of onset of hypocalcemia was more rapid than at lower doses, despite similar suppression of PTH levels. The studies reported here provide compelling evidence that increased secretion of calcitonin is primarily, if not solely, responsible for the more rapid fall in plasma Ca\(^{2+}\) levels at the higher doses of NPS R-568. Thus, we have shown that 1) NPS R-568 induced a slower rate of decrease in plasma Ca\(^{2+}\) levels in TX rats in which the endogenous source of calcitonin but not of PTH was removed, 2) a substantial hypocalcemic response also occurred when NPS R-568 was administered to PTH-infused PTX rats, and 3) NPS R-568 had no effect on plasma Ca\(^{2+}\) levels in PTH-infused TXPTX rats. Moreover, the failure of NPS R-568 to induce a hypocalcemic effect in TXPTX rats in which normocalcemia was restored by PTH infusion also provides strong evidence that NPS R-568 does not act directly in bone to inhibit resorption.
These studies have therefore demonstrated that NPS R-568 activates the Ca\(^{2+}\) receptor on C cells in vivo in addition to its similar activity at the Ca\(^{2+}\) receptor on parathyroid cells (Fox et al., 1999; Nemeth et al., 1998). However, these studies have also revealed that the potency of NPS R-568 to reduce the plasma levels of PTH is considerably greater than its ability to increase calcitonin levels. Oral doses of NPS R-568 of 10 mg/kg were required before an increase in calcitonin levels could be detected, whereas earlier studies (Fox et al., 1999) had shown that the ED\(_{50}\) for reducing the plasma levels of PTH was about 1 mg/kg. The estimated ED\(_{50}\) for the elevation of plasma calcitonin levels was about 40 mg/kg, although this may be an underestimate, because the maximum calcitonin levels achieved with the 100-mg/kg dose of NPS R-568 were somewhat less than occurred when calcium was used to stimulate secretion.

The mechanism responsible for the differing secretory responses of C cells and parathyroid cells to NPS R-568 is uncertain. The nucleotide sequence of the coding region of the Ca\(^{2+}\) receptor is identical in parathyroid cells and C cells, and Western blot analysis has shown that the expressed proteins are similar in molecular size (Garrett et al., 1995b). Thus, any differences in posttranslational modifications in the two cell types, such as degree of glycosylation, if present, are likely to be subtle. Other possible explanations include differing receptor densities in the two cell types, because down-regulation of the Ca\(^{2+}\) receptor in parathyroid cells is associated with an impaired sensitivity to Ca\(^{2+}\) (Mithal et al., 1995); however, immunocytochemistry has not revealed any gross quantitative differences in expression levels in parathyroid cells and C cells (our unpublished data). Alternatively, the different postreceptor mechanisms linking the Ca\(^{2+}\) receptor to hormone secretion may be coupled with different efficiencies in the two cell types (Nemeth, 1990; Kifor et al., 1997).

The clinical significance of these findings obtained in the rat is uncertain because the physiological importance of calcitonin in the regulation of plasma Ca\(^{2+}\) levels in humans has been questioned (Austin and Heath, 1981; Deftos, 1996). However, these studies have provided strong support for the concept that calcitonin is a potent Ca\(^{2+}\)-regulating hormone in the rat that indeed appears to be exclusively sensitive to the hypocalcemic actions of calcitonin; a 2-fold increase in plasma levels of calcitonin induced by a 10-mg/kg dose of NPS R-568 was associated with a maximal rate of decrease in plasma Ca\(^{2+}\) levels. Although the plasma calcitonin response and the magnitude and duration of the hypocalcemia were greater at higher doses, the initial rate of decrease of plasma Ca\(^{2+}\) was the same at doses of NPS R-568 ≥10 mg/kg (Fox et al., 1999). Moreover, in studies in PTX rats that were rendered normocalcemic or mildly hypercalcemic by PTH infusion, the 10-mg/kg dose of NPS R-568 was sufficient to cause a marked hypocalcemic response. The failure of NPS R-568 to induce a significant decrease in plasma Ca\(^{2+}\) in the severely hypercalcemic PTH-infused PTX rats most likely occurs because calcitonin secretion was already maximally stimulated.

Finally, these studies also provided further evidence that the kidneys play little, if any, direct role in the hypocalcemia induced by NPS R-568. We showed (Fox et al., 1999) previously that acute total nephrectomy affected neither the rate of onset nor the magnitude of the hypocalcemia after NPS R-568 administration. In this study, we showed that NPS R-568, even at doses as high as 100 mg/kg, had no detectable effect on the rate of disappearance of \(^{45}\)Ca from the circulation. Thus, the substantial hypocalcemia induced by NPS R-568 appeared to be caused solely by a decreased influx of Ca\(^{2+}\) into the circulation. Although the Ca\(^{2+}\) receptor is expressed in the kidneys, particularly in cells of the thick ascending limb, which also appear to respond to changes in extracellular Ca\(^{2+}\) and have been hypothesized to regulate Ca\(^{2+}\) reabsorption (Ricardi et al., 1995; Wang et al., 1996; Brown and Hebert, 1997), these in vivo experiments have not provided any evidence that NPS R-568 at the doses tested is also acting as an agonist at those receptors. Whereas both PTH and calcitonin play roles in the regulation of Ca\(^{2+}\) reabsorption in the kidney (Breslau, 1996), and NPS R-568-induced changes in the secretion of these two hormones would be expected to result in corresponding changes in urinary Ca\(^{2+}\) excretion, these studies have provided no evidence that any induced changes in renal Ca\(^{2+}\) handling are contributing significantly to the regulation of plasma Ca\(^{2+}\) levels in the rat within the time frame of these experiments.

In conclusion, we propose that the following mechanism is predominantly, if not exclusively, responsible for the observed hypocalcemia when NPS R-568 is administered to normal rats: First, increased circulating levels of calcitonin directly lead to a rapid decrease in osteoclastic activity and a reduced efflux of Ca\(^{2+}\) from bone. Second, decreased PTH levels also result in decreased osteoclastic activity, a process that occurs more slowly and indirectly most likely via a reduction in osteoblastic activity. Although no comparable data are available in humans, the similar time course of changes in plasma levels of PTH and Ca\(^{2+}\) after oral administration of NPS R-568 to patients with primary hyperparathyroidism (Silverberg et al., 1997) suggests that a similar mechanism may be operating in humans.

References

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Send reprint requests to: John Fox, Ph.D., NPS Pharmaceuticals, Inc., 420 Chipeta Way, Salt Lake City, UT 84108. E-mail: jfox@npsp.com