

Acute Blood Pressure Effects and Chronic Hypotensive Action of Calcimimetics in Uremic Rats

Tobias Odenwald,* Kumiko Nakagawa,* Charlotte Hadtstein,* Frank Roesch,[†]
Peter Gohlke,[‡] Eberhard Ritz,[§] Franz Schaefer,* Claus Peter Schmitt*

Departments of *Pediatrics and [§]Internal Medicine, University of Heidelberg, Heidelberg; [†]Institute of Nuclear Chemistry, University of Mainz; and [‡]Institute of Pharmacology, University of Kiel, Germany

A previous study in subtotal nephrectomized (SNX) rats suggested beneficial effects of the calcimimetic R-568 beyond the control of mineral metabolism. This study analyzed potential blood pressure (BP)-lowering effects of R-568. Male Sprague-Dawley rats received two-stage subtotal nephrectomy or sham operation. Telemetry devices were inserted into the abdominal aorta, and BP was measured every 5 min. R-568 (20 mg/kg per d) or solvent was infused for 4 wk followed by once-daily subcutaneous injections for 2 wk. Total body sodium was measured by neutron activation analysis. The uremia-induced increase of mean arterial pressure from baseline to day 42 in SNX solvent rats (103 ± 5 to 128 ± 14 mmHg, $P = 0.006$) was attenuated by R-568 (104 ± 5 to 111 ± 8 mmHg; $P < 0.0001$ for difference of slopes). The circadian rhythm was abrogated in SNX rats and not restored by R-568. In sham-operated rats, R-568 had only a minor transient antihypertensive effect. R-568 injection induced a transient rise of mean arterial pressure by 23 ± 4 and 26 ± 10 mmHg in sham and SNX rats but only by 9 ± 3 and 10 ± 5 mmHg in solvent-treated rats ($P < 0.01$ versus baseline and solvent versus R-568). Plasma angiotensin-converting enzyme activity and aldosterone levels were similar; food intake and physical activity did not differ throughout the study. In healthy rats, total body sodium was higher after 14 d of R-568 compared with solvent infusion (37.1 ± 4 versus 32.5 ± 1.4 mmol/kg; $P = 0.01$). The calcimimetic R-568 causes an initial BP increase in sham-operated and uremic rats, which in uremic rats is followed by a marked and sustained antihypertensive effect.

J Am Soc Nephrol 17: 655–662, 2006. doi: 10.1681/ASN.2005090914

Calcimimetics are a major breakthrough in the management of secondary hyperparathyroidism that affect parathyroid hormone (PTH) levels through interaction with the calcium-sensing receptor (CaR). In recent years, it has increasingly been recognized that disturbances of calcium and phosphorus metabolism have an impact on cardiovascular risk and survival in dialysis patients. In this context, it is of interest whether calcimimetics improve cardiovascular risk. A study using cuff-tail plethysmography suggested that intermittent administration of the calcimimetic R-568 lowers systolic blood pressure (SBP) in subtotal nephrectomized (SNX) rats (1).

So far, no consistent effect of calcimimetics on casual BP has been reported in controlled prospective trials in patients who are on dialysis (2–5). However, these studies were not designed to evaluate an impact of the calcimimetic on BP. We believed that it was important to characterize the effects of continuous and intermittent administration of R-568 on BP and heart rate (HR) by measuring the circadian BP profile as well as several determinants that have an impact on BP. To this end, we

compared SNX and sham-operated Sprague-Dawley rats. They received R-568 by continuous infusion in a first period and by daily intermittent subcutaneous injection in a subsequent second period.

Gain-of-function mutations in the CaR reduce NaCl reabsorption in the cortical ascending limb and result in a negative sodium balance (6). Accordingly, continuous administration of the calcimimetic R-568 may induce net sodium losses and thus exert BP-lowering effects. To this end, we determined total body sodium content after R-568 treatment in healthy rats using neutron activation analysis.

Materials and Methods

Animals

Male Sprague-Dawley rats that weighed 180 to 200 g were kept in single cages at constant room temperature (24°C) and humidity (70%) under strict 12-h dark/light cycles. Rats had free access to deionized water and a standard diet (Altromin 1320; Altromin Co., Lage/Lippe, Germany) that contained 2050 MJ/kg, 19% protein, 0.2% sodium, 0.9% calcium, 0.7% phosphorous, 0.2% magnesium, 1% potassium, and 600 IU/kg vitamin D₃. After 7 d to adapt to the environment, rats were assigned to the different experimental groups according to body weight. The animal experimentation and handling were in accordance with the German law for protection of animals.

Experiment I: Effect of R-568 on BP in Sham-Operated and SNX Rats

For subtotal nephrectomy, the right kidney was removed in a first session under anesthesia with ketamine (100 mg/kg body wt) and

Received September 7, 2005. Accepted December 3, 2005.

Published online ahead of print. Publication date available at www.jasn.org.

T.O. and K.N. contributed equally to this work.

Address correspondence to: Dr. Claus Peter Schmitt, Division of Pediatric Nephrology, University Hospital for Pediatric and Adolescent Medicine, Im Neuenheimer Feld 153, D-69120 Heidelberg, Germany. Phone: +49-6221-5639313; Fax: +49-6221-564203; E-mail: claus.peter.schmitt@med.uni-heidelberg.de

xylazine (2 mg/kg body wt). At the time of the first operation, a BP sensor (model PA-C40; Data Science International, Arden Hills, MN) was inserted into the aorta below the level of the renal arteries and the radiofrequency transmitter was fixed intraperitoneally. Signals were sent to a telemetry receiver that was placed under the cage and transmitted to the LabPRO Acquisition System, Version 3.10. BP was measured every 5 min (approximately 12,000 readings/rat). Seven days after uninephrectomy, we selectively removed cortical tissue from the hypertrophied left kidney corresponding to 75% of the weight of the previously excised right kidney. We kept the amount constant between rats, thus achieving similar removal of number of glomeruli. Particular care was taken to preserve adrenals without damage. In sham-operated rats, the kidneys were mobilized in two consecutive sessions.

Five days after the second operation, treatment was started. Sham-operated rats were treated with solvent and R-568 for 2 wk ($n = 4$ per group) and for 6 wk ($n = 4$ per group), respectively, and compared with SNX rats that were treated with solvent and R-568 for 6 wk ($n = 7$ per group). R-568 (Amgen, Thousand Oaks, CA) was dissolved in 45% aqueous cyclodextrin (2-hydroxypropyl- β -cyclodextrin; Sigma-Aldrich Chemie, Steinheim, Germany) and administered *via* osmotic minipumps (Alzet 2002; Alza Corp., Palo Alto, CA). After 2 wk, minipumps were exchanged for a subsequent 2-wk treatment period. This was followed by once-daily subcutaneous injections of the same total amount of R-568 for 2 wk. Food intake and body weight were measured every other day, blood sampling (100 μ l) was performed weekly to measure ionized calcium. Forty-eight hours before rats were killed and 2 h after the last R-568 injection, blood was sampled for hormone analysis; 24 h before the rats were killed, they were transferred into metabolic cages.

Experiment II: Effect of R-568 on Total Body Sodium in Intact Rats

Intact rats were kept either on standard or on high-phosphate diet (Altromin C1049; 1.65% phosphate, 0.24% sodium, 0.95% calcium, 0.07% magnesium, and 0.7% potassium) to induce secondary hyperparathyroidism. They received either solvent (45% cyclodextrin) or R-568 (20 mg/kg per day) *via* osmotic minipumps for 2 wk ($n = 9$ per group). A total of 100 μ l of blood was sampled on day 7. At the end of the study, the minipumps and the gastrointestinal tract were removed and body sodium was determined in the carcass. Meticulous attention was paid to avoid any loss of blood or extracellular fluid to prevent artificial changes of the body sodium content.

Biochemical Measurements

Blood ionized calcium was measured within 30 s after sampling using an ion selective electrode system (Ionometer EH-F; Fresenius, Oberursel, Germany). The results were corrected for pH 7.4; the mean intra- and interassay coefficient of variation was 2.5%. PTH was measured using a rat intact PTH ELISA (Immuntopics Inc., San Clemente, CA), which is specific for rat PTH_{1 to 84}. It has no cross-reactivity with N-terminal 1 to 34 or mid- and C-terminal 39 to 84 fragments. Serum and urine electrolytes, creatinine, and urea were measured using standard laboratory techniques.

Plasma ACE Activity. Angiotensin-converting enzyme (ACE) activity was assayed by a modified fluorometric method using Z-Phe-His-Leu as substrate (7). Plasma (50 μ l) was diluted with phosphate buffer to a volume of 450 μ l. The enzyme reaction was started by adding 50 μ l of a 10-mM substrate solution to the samples and incubated for 30 min at 37°C. The reaction was terminated by transferring 100- μ l aliquots into 1 ml of 0.1 N NaOH. All subsequent steps were continued in the dark. A total of 25 μ l of 2% ortho-phthalaldehyde

solution in DMSO was added to the samples. After 30 min, the reaction was terminated by addition of 1 ml of 0.8 M HCl, and precipitates were spun down by 3000 \times g centrifugation for 3 min. Fluorescence was measured within 60 min. Zero time blank values were subtracted from the corresponding test values. All assays were performed in duplicate. The results are expressed as nmol His-Leu/ml per min.

Plasma Aldosterone. Aldosterone was measured by a specific in-house RIA, using tritiated aldosterone (1,2,4,6, 3 H aldosterone; Amersham Biosciences, Freiburg, Germany) and an antibody, developed as described elsewhere (8). Before RIA, extraction and chromatographic purification were performed to remove cross-reacting steroids. The chromatographic separation of aldosterone was modified from the method previously described (9) using Celite (Celite 545 AW; Sigma Aldrich, Taufkirchen, Germany) as an inert support for partition chromatography. The stationary phase consisted of 30% formamide in water, and the mobile phase consisted of a mixture of ethyl acetate in n-hexane with increasing polarity, eluting aldosterone with 50% ethyl acetate in n-hexane. The standard curve ranged from 1 to 200 pg; sensitivity was 2 pg per tube (1 ng/100 ml). The recovery rate was 104 \pm 8.2% ($n = 12$; mean \pm SD), and intra- and interassay coefficients of variation were 3.5 to 8.5 and 9.6 to 12.2%, respectively.

Neutron Activation Analysis

Body sodium content was measured by neutron activation analysis using the research reactor TRIGA of the Institute of Nuclear Chemistry (Mainz, Germany). The sodium content was determined *via* the $^{23}\text{Na}(n,\gamma)^{24}\text{Na}$ neutron capture process. Rats were irradiated in special polymer capsules for 1 h at 100 kW reactor power in a carousel position, corresponding to a flux of thermal neutrons of 7×10^9 neutrons/cm². The contribution of ^{24}Na activation resulting from those capsules was considered. To simulate the radioactivity resulting from those irradiations, a calibration standard was prepared, according to the reported rat tissue content: 1.64 g/L NaCl, 1.29 g/L NaHCO₃, 1.98 g/L FeSO₄ \times 7H₂O, 4.24 g/L K₂CO₃, and 10.66 g/L NH₄H₂PO₄ (10). Four aliquots of 200 ml of this stock solution were irradiated under identical conditions, yielding a mean experimental error of ^{24}Na activities of 1.68%. This reflects the accuracy of the results of the ^{24}Na determinations in the irradiated rats. After a cooling period of 5 h, rats were positioned at a 100-cm distance to an HPGe detector; an additional 50-mm lead shielding was placed in between. Gamma spectra were recorded for 1 h, and the areas of the two most relevant photo peaks of ^{24}Na ($t_{1/2} = 14.659$ h), namely 1369 keV (100% abundance) and 2754 keV (99.944% abundance), were obtained using the Intertechniq Intergamma software. Both peaks produced similar results. Using the stock solution as calibration source, final data from the 2754 keV peak are given as mmol sodium/g body wt.

Statistical Analyses

The *t* test was used for group comparison. Even though BP recordings were obtained every 5 min, only the 24-h mean BP was used for calculations of long-term BP effects, resulting in very conservative estimates of *P* values and reducing false-positive results. Data from days of minor interventions (blood sampling) and 48 h after major interventions (implantation, exchange, and removal of minipumps) were excluded from the analysis. Graphs that displayed BP over 6 wk were smoothed with a uniformly weighted moving average spanning 36 data points (equivalent to 3 h). For significance testing of acute BP effects, only one value per rat (averaged from 7 d in sham-operated and 9 d in SNX rats) was used to compare groups.

Results

Experiment 1

At the start of treatment, body weights were similar in sham-operated and uremic rats that were treated with R-568 or solvent, respectively. Subsequent mean daily food intake and body weight gains were not different between treatment groups (Table 1). As expected, mean daily water intake was higher in uremic rats than in controls but did not differ between the R-568-treated and solvent-treated groups. Physical activity, assessed every other day during activity and rest period by the same person (T.O.), was comparable in both groups.

During 4 wk of continuous R-568 infusion, weekly measured ionized calcium was decreased by 0.21 ± 0.05 and 0.19 ± 0.04 mmol/L below baseline in sham-operated and uremic rats but remained stable in solvent-infused controls (-0.02 ± 0.06 and 0.01 ± 0.07 mmol/L). Two hours after subcutaneous R-568 injection, ionized calcium was 0.28 ± 0.08 and 0.32 ± 0.07 mmol/L below baseline but returned to baseline within 24 h in sham-operated and SNX rats. Two hours after subcutaneous solvent injection, ionized calcium was unchanged (0.03 ± 0.03 and -0.03 ± 0.03 mmol/L). PTH was markedly lower with continuous R-568 infusion and 2 h after injection of R-568. No consistent R-568-dependent changes were observed with respect to sodium, potassium, phosphate, creatinine, urea, creatinine clearances, and 24-h urinary sodium excretion as well as plasma ACE activity and aldosterone levels.

Long-Term Effects of R-568 on BP and HR.

Controls. Infusion of R-568 in sham-operated rats induced only small changes in SBP and diastolic BP (DBP) within the first 14 d of treatment (Figure 1). On day 7, SBP/DBP was $5.9 \pm 14/3.3 \pm 8.5$ and on day 11 was $5.1 \pm 11/1.2 \pm 7$ mmHg lower than in solvent-treated sham-operated rats (both NS). In the subgroup that was followed for an additional 4 wk, a similar BP

profile was observed initially, and no difference between the groups was observed from the third week onward. HR was similar in R-568- and solvent-treated rats throughout the study period.

Uremic Rats. In SNX rats, SBP and DBP at baseline (day -3 to -1) was similar in the solvent- and R-568-treated groups ($122 \pm 5/86 \pm 5$ versus $120.4 \pm 4/89 \pm 7$ mmHg; both NS). After SNX, a time-dependent increase in BP was seen in all rats. Over 6 wk, this increase was 0.91 mmHg/d in SBP and 0.82 mmHg/d in DBP for solvent-treated SNX rats, using the above baseline BP as intercepts. With R-568 treatment, this increase was only 0.56 mmHg/d for SBP and 0.27 mmHg/d for DBP (both $P < 0.001$ versus solvent). At the end of the experiment, mean SBP was 16.5 ± 10 and DBP was 17.5 ± 12.6 mmHg lower in R-568-treated SNX rats ($P = 0.01/0.02$; Figure 2). Because of the more pronounced effect on DBP, pulse pressure increased by 0.44 mmHg/d over 6 wk with R-568, whereas it remained unchanged in solvent-treated SNX rats (0.1 mmHg/d; $P < 0.001$ versus R-568). Creatinine clearances correlated neither with intraindividual changes in SBP, DBP, and mean arterial pressure (MAP) nor with BP at the end of the study period.

At baseline (day -3 to -1), HR was 383 ± 47 bpm in the R-568 group and 397 ± 46 bpm in the solvent group (NS). During the first 14 d, HR declined by 15 ± 46 bpm in R-568-treated and by 46 ± 56 bpm in solvent-treated SNX rats ($P < 0.01$). HR remained higher in R-568-treated SNX rats for the subsequent 2 wk and even increased during the last 16 d of the experiment (day 40, 393 ± 46 versus 364 ± 48 ; $P = 0.01$; Figure 3).

Acute Effects of R-568 Injection on BP and HR. A consistent biphasic pattern of BP changes was seen after subcutaneous injection in both sham and SNX rats. The MAP increased sharply after R-568, reaching a peak 36 min after injection of 23 ± 4 mmHg above baseline in sham and 26 ± 10 mmHg in

Table 1. Physical and biochemical findings in the telemetry studies^a

| | Sham-Operated Rats (2 wk, $n = 4$ /group) | | Sham-Operated Rats (6 wk, $n = 4$ /group) | | Uremic Rats (6 wk, $n = 7$ /group) | |
|---|--|-----------------|--|-----------------|---------------------------------------|-----------------|
| | R-568 | Solvent | R-568 | Solvent | R-568 | Solvent |
| Mean daily food intake (g/d) | 19.9 ± 1.0 | 20.5 ± 1.4 | 22.0 ± 1.1 | 23.4 ± 2.1 | 20.0 ± 1.9 | 19.6 ± 1.4 |
| Mean daily body weight gain (g/d) | 3.1 ± 1.1 | 2.4 ± 1 | 1.7 ± 0.6 | 2.0 ± 0.1 | 2.2 ± 0.3 | 2.2 ± 0.6 |
| Mean daily water intake (ml/d) | 35 ± 2 | 34 ± 2 | 34 ± 1 | 29 ± 4 | 67 ± 8 | 65 ± 6 |
| Mean serum Ca ²⁺ (mmol/L) | 1.26 ± 0.06 | 1.45 ± 0.07 | 1.06 ± 0.07 | 1.40 ± 0.04 | 1.02 ± 0.03^b | 1.35 ± 0.02 |
| Serum phosphate (mmol/L) | 1.49 ± 0.30 | 1.90 ± 0.38 | 1.69 ± 0.47 | 1.58 ± 0.23 | 3.30 ± 0.40 | 3.38 ± 0.91 |
| Serum creatinine (mg/dl) | 0.24 ± 0.03 | 0.18 ± 0.02 | 0.23 ± 0.09 | 0.22 ± 0.03 | 1.03 ± 0.26 | 1.36 ± 0.44 |
| Serum urea (mg/dl) | 46 ± 4 | 37 ± 7 | 43 ± 4 | 48 ± 4 | 134 ± 24 | 157 ± 43 |
| Plasma serum iPTH (pg/ml) | 11 ± 3 | 38 ± 32 | 15 ± 5 | 50 ± 22 | 50 ± 29^b | 334 ± 284 |
| ACE activity (nm His-Leu/ml per min) | 259 ± 76 | 238 ± 46 | 201 ± 51 | 172 ± 14 | 279 ± 30 | 257 ± 65 |
| Aldosterone (ng/ml) | 20.3 ± 10.3 | 11.4 ± 3.6 | 30.4 ± 10.9 | 19.1 ± 9.1 | 99 ± 70 | 53 ± 32 |
| 24-h Ccr (μ l/min per 100 g) | ND | ND | 992 ± 370 | 873 ± 151 | 167 ± 93 | 153 ± 40 |

^aMean daily food and water intake, body weight gain, and biochemical findings in all rats after 2 and 6 wk of treatment, respectively. In uremic and healthy rats that were treated for 6 wk, blood was taken 2 h after the last injection of R-568. ACE, angiotensin-converting enzyme; iPTH, intact parathyroid hormone; Ccr, creatinine clearance.

^b $P < 0.05$ in uremic rats, R-568 versus solvent.

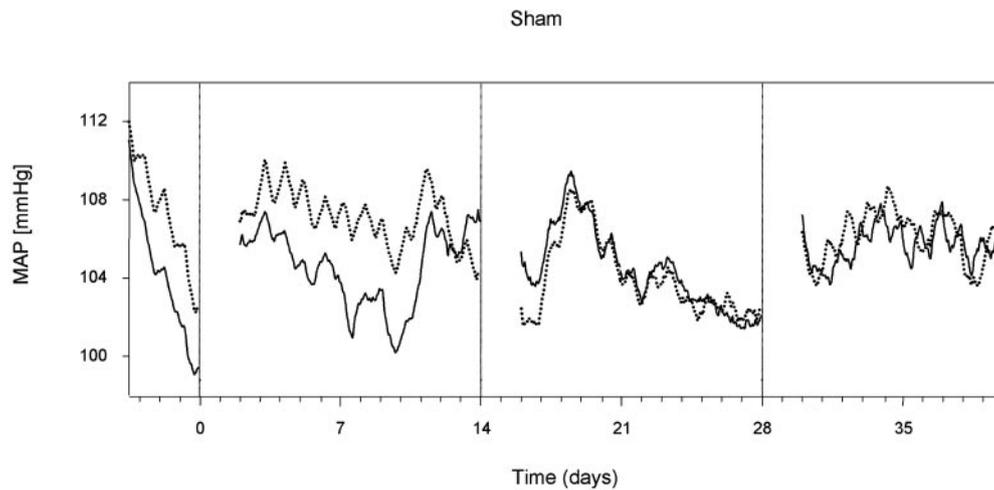


Figure 1. Moving average (180-min intervals) of mean arterial pressure (MAP) in R-568 (solid line) and solvent-treated sham-operated rats (dashed line) over 6 wk.

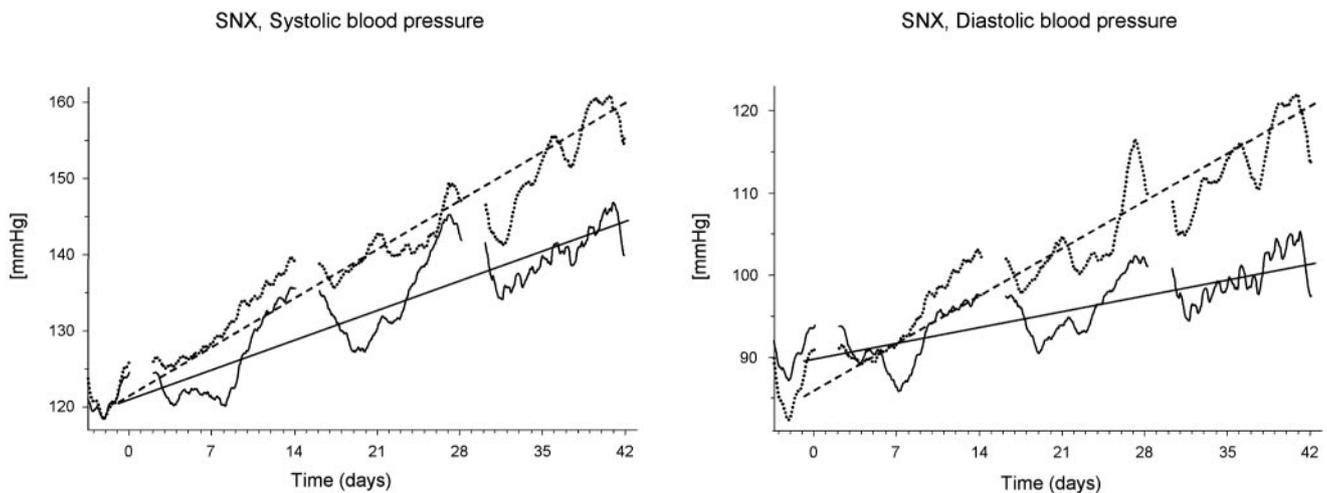


Figure 2. Moving average (180-min intervals) of daily mean systolic blood pressure (left) and diastolic blood pressure (right) in subtotaly nephrectomized (SNX) rats that were treated with R-568 (solid line) or solvent (dashed line).

SNX rats (both $P = 0.001$ versus baseline). In contrast, solvent injection increased MAP by only 9 ± 3 mmHg in sham-operated and 10 ± 5 mmHg in SNX rats, peaking after 18 min ($P < 0.01$ versus baseline and R-568). In hypertensive SNX rats, an antihypertensive effect of R-568 was seen after 3 h, whereas in normotensive, sham-operated rats, BP returned to baseline within 8 h. HR increased to a similar degree after solvent and R-568 injection in sham-operated rats. In SNX rats, the increase in HR that was induced by R-568 was smaller compared with solvent injection and followed by a transient decline below baseline (Figure 4).

Effects of Uremia and R-568 on Circadian Profile of BP.

In uremic rats, the physiologic decline of BP in the rest period was largely absent. Before any treatment (day -3 to -1), mean decline of MAP during daytime was only 1.7 ± 2.1 mmHg in SNX rats compared with 4.8 ± 2.8 mmHg in sham-operated controls ($P < 0.001$). The attenuated daytime decrease was not

restored by R-568 (mean daytime MAP decline day 8 to 10: R-568 1.2 ± 2.3 mmHg, solvent 1.4 ± 2.9 mmHg).

In sham-operated R-568-treated rats there was no day/night difference during the first 2 wk (days 8 to 10: daytime MAP 100 ± 8 mmHg; nighttime MAP 101 ± 10 mmHg), whereas in solvent-treated controls, daytime dipping persisted (days 8 to 10: daytime MAP 103 ± 7 mmHg; nighttime MAP 108 ± 6 mmHg; $P = 0.017$).

Experiment II

Effect of R-568 on Total Body Sodium Content. To assess a potential effect of R-568 on sodium balance, we measured total body sodium in healthy rats that were fed a normal or a high-phosphate diet to induce secondary hyperparathyroidism. Because initial food intake was less in R-568-treated rats (68 ± 8 versus 81 ± 9 g/kg per d in rats on normal phosphate diet and 50 ± 8 versus 72 ± 8 g/kg per d in rats on a high-phosphate

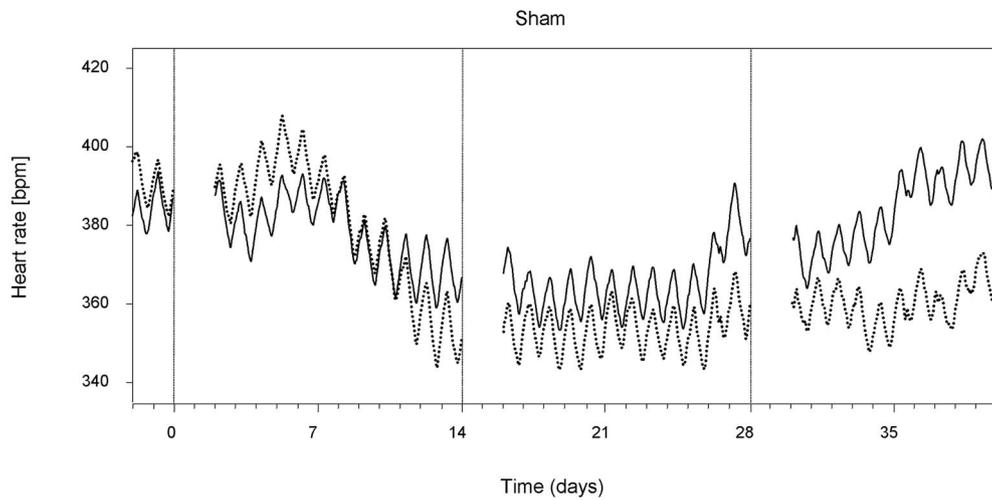


Figure 3. Moving average of heart rate (180-min intervals) in SNX rats treated with R-568 (solid line) or solvent (dashed line).

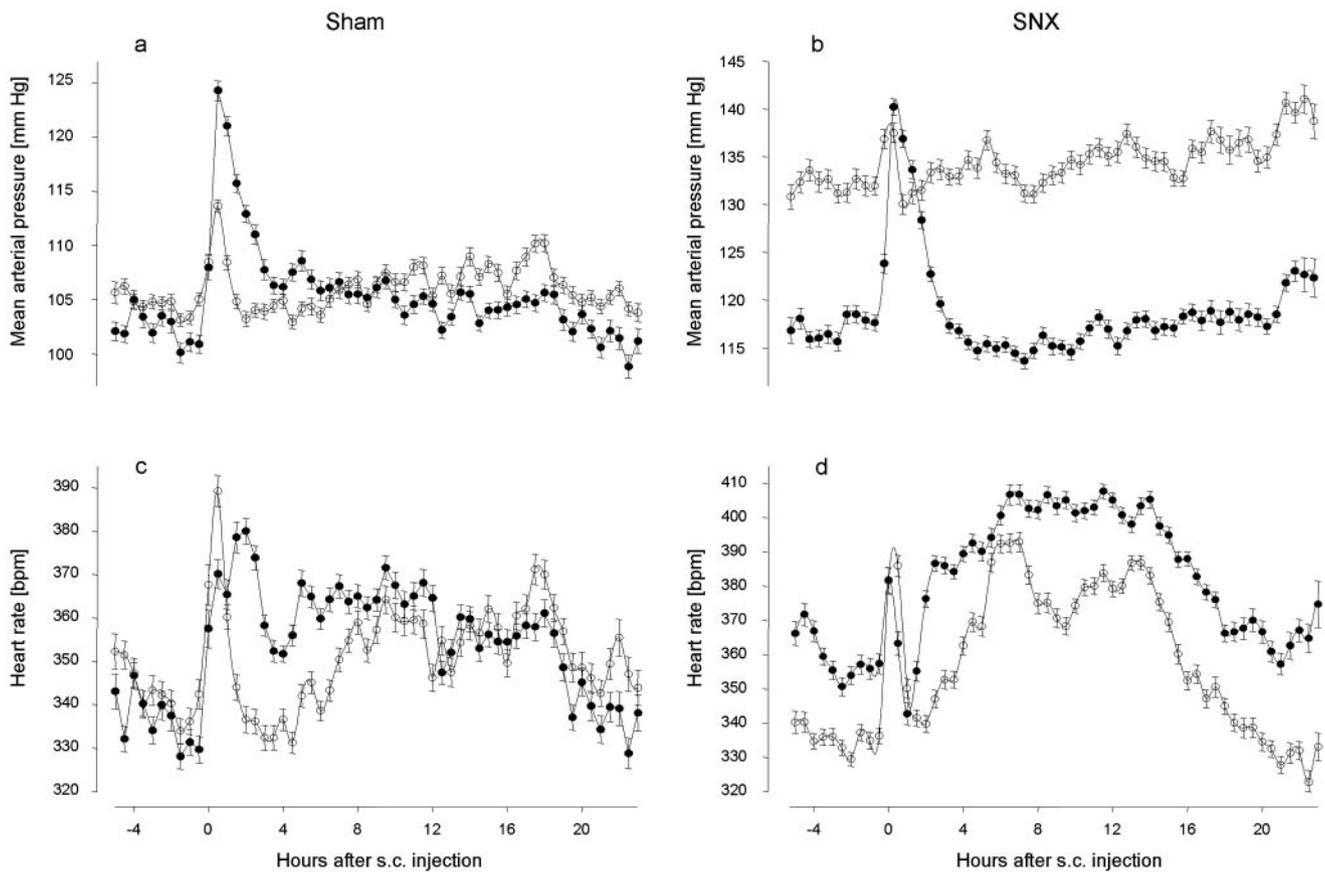


Figure 4. Changes of mean arterial pressure (a and b) and heart rate (c and d) in sham-operated (left) and SNX rats (right) after injection of R-568 (●) or solvent (○) on days with no other interventions (7 d in sham-operated and 9 d in SNX rats). Mean values \pm SE for 30-min intervals are given.

diet; $P < 0.05$), pair feeding was performed from the fourth day onward, resulting in identical food intake subsequently. Water intake was similar in rats that were on a normal phosphate diet, significantly increased in rats that were on high-phosphate diet, and partially reduced by R-568 treatment (Table 2). On day 7,

sodium, potassium, and pH were similar between groups, whereas ionized calcium was strongly reduced by R-568. Tail-cuff plethysmography revealed a lower SBP in R-568-treated rats that were on a normal phosphate diet after 7 d but similar BP after 14 d. In rats that were on high-phosphate diet, how-

Table 2. Effect of R-568 on total body sodium^a

| | R-568 (0.7% P) | Solvent (0.7% P) | P | R-568 (1.7% P) | Solvent (1.7% P) | P |
|--|-------------------|---------------------|--------|-------------------|---------------------|--------|
| Daily food intake (g/kg per d) | 64 ± 4 | 67 ± 3 | 0.04 | 54 ± 4 | 59 ± 3 | 0.01 |
| Daily water intake days 1 to 14 (ml/kg per d) | 96 ± 14 | 92 ± 15 | NS | 114 ± 15 | 131 ± 16 | 0.04 |
| Serum Na ⁺ day 7 (mmol/L) | 139 ± 2 | 138 ± 2 | NS | 134 ± 2 | 135 ± 1 | NS |
| Serum Ca ²⁺ , day 7 (mmol/L, pH adjusted) | 1.11 ± 0.08 | 1.38 ± 0.07 | <0.001 | 1.05 ± 0.04 | 1.33 ± 0.05 | <0.001 |
| SBP day 7 (mmHg) | 126 ± 8 | 143 ± 12 | 0.004 | 122 ± 17 | 142 ± 12 | 0.01 |
| SBP day 14 (mmHg) | 129 ± 13 | 138 ± 7 | NS | 124 ± 14 | 139 ± 6 | 0.02 |
| Total body sodium, day 14 (mmol/kg) | 37.1 ± 4.0 | 32.5 ± 1.4 | 0.01 | 38.9 ± 4.0 | 40.7 ± 3.8 | NS |

^aBiochemical findings, BP measured by tail-cuff plethysmography, and total body sodium content measured by neutron activation analysis in rats that were fed a normal- (0.7%) and a high-phosphate diet (1.7% phosphate), *n* = 9/group. SBP, systolic blood pressure.

ever, the difference in BP persisted after 14 d. Total body sodium content was not lower in R-568–treated rats that were on normal phosphate diet but was even 10% higher compared with solvent-treated controls. In rats that were on high-phosphate diet, body sodium content was not changed by R-568 treatment. Dietary sodium intake did not correlate with total body sodium content in rats that were fed a normal and a high-phosphate diet, respectively.

Discussion

The salient feature of our study is the clear documentation of a consistent and persistent BP-lowering effect of the calcimimetic R-568 in uremic rats. The antihypertensive effect was independent of the mode of R-568 administration, continuous or once daily, and persisted even when R-568 was administered by subcutaneous injection, although this caused an initial increase in BP and HR. There were no changes in plasma ACE activity, plasma aldosterone levels, food intake, and sodium balance to explain the changes in BP. Although not assessed quantitatively, regular inspection of locomotor activity and sleep-wake cycles, which might have accounted for BP differences, did not indicate any changes and could not explain the BP changes either.

An antihypertensive effect of calcimimetic agents in rats was first suggested by Ogata *et al.* (1), who reported a reduction in SBP by tail plethysmography in uremic rats that were treated with intermittent R-568. Our study confirms this finding using the gold standard of telemetry and adds a number of important details.

Measurement of BP by means of tail plethysmography is difficult to standardize and may easily result in imprecise data. Accordingly, in nonuremic R-568–treated rats, we measured greater BP differences with tail-cuff plethysmography as compared with telemetric BP measurements, suggesting a transient BP-lowering effect of R-568 after 1 wk but no longer after 2 wk. In intact rats that were fed a high-phosphate diet to induce hyperparathyroidism, the antihypertensive effect persisted after 2 wk. This is in line with the persistent antihypertensive effect of R-568 observed in uremic hyperparathyroid rats. Whether the transient effect in nonuremic rats reflects the in-

tervention of compensatory mechanisms or less parathyroid activity compared with uremic rats is unclear.

Neutron activation analysis allows for a highly precise determination of total body sodium. In contrast, measurement of urinary sodium output at the end of the study does not necessarily reflect cumulative renal sodium losses, because sodium depletion may already occur within the first days of R-568 treatment and thus escape later analysis, when a new steady state may already have been obtained. Repeated 24-h urine collections in metabolic cages are not well tolerated by the rats and may vitiate the sodium balance by unmeasured sodium losses, *e.g.*, from bleeding food pads. Analysis of total body sodium by neutron activation analysis circumvents these potential artifacts. Surprisingly, it did not reveal a negative sodium balance in healthy rats that were fed a standard diet and were treated with R-568, as one may have expected given that gain-of-function mutations in the CaR reduce NaCl reabsorption in the cortical ascending limb and induce natriuresis (6). To the contrary, body sodium content was significantly increased within 14 d of R-568 infusion, although food intake and thus sodium intake was, if anything, lower with R-568. A compensatory activation of the renin-angiotensin and aldosterone system in response to the BP decline may have escaped the single-point measurements of plasma ACE activity and aldosterone levels.

In SNX rats, R-568 persistently reduced the progressive increase in BP that was observed in the solvent-treated SNX controls. HR declined within the first 10 d in both groups but remained higher in R-568 rats during the subsequent 2 wk and even increased further in R-568–treated rats after that. This is compatible with a compensatory increase in HR in response to a decrease of BP induced by vasodilation of resistance arteries. The CaR is expressed in a wide variety of rat small arteries, including renal, mesenteric, cerebral, and subcutaneous vessels (11–13). It has been localized in adventitial perivascular nerves. Activation of the CaR by a progressive increase in ambient calcium results in a U-shaped response with constriction at medium calcium concentrations and marked vasodilation at high and low calcium concentrations. The known agonists magnesium and aminoglycosides induce dose-dependent vascular

relaxation (11); the neuronally released vasodilator substance is a cannabinoid receptor agonist (14). Accordingly, R-568 may result in reduced peripheral vascular resistance in the rat model, either directly *via* activation of the CaR or indirectly by the decrease of ionized calcium to subnormal levels.

Alternatively, the antihypertensive effect of R-568 may be mediated *via* suppression of PTH plasma levels. Infusion of PTH significantly increases BP in rats (15) and humans (16,17). Conversely, in normotensive and spontaneously hypertensive eucalcemic rats, parathyroidectomy reduces BP to a similar degree as the calcimimetic agent (1,18). The acute injection of R-568 reduces BP within 50 min in spontaneously hypertensive but not in normotensive rats (19). Clinical studies with calcimimetic agents have not been designed to evaluate effects on BP, and none of the published trials has reported any impact on BP (2–5).

Meanwhile, >600 ESRD patients have been treated with the calcimimetic cinacalcet HCl in phase 3 studies. All together, lower SBP and DBP values were recorded with cinacalcet (144.4 ± 2.4 and 78.6 ± 1.5 versus 138.6 ± 2.4 and 76.9 ± 1.5 mmHg after 1 yr; personal communication, U. Fraass, Amgen, March 2005). Still, these findings may be confounded by factors that influence BP, including fluid and salt intake, antihypertensive medication, and dialysis regimen.

The striking acute increase in BP after subcutaneous injection of R-568 is more difficult to explain. Presumably, it is not an artifact of differences in pain perception, because the HR increased to the same degree in sham-operated and to a smaller degree in SNX rats that were treated with R-568. The brisk and transient increase in BP was twice as high with R-568 compared with solvent injection. In view of the expression of the CaR in different central nervous system structures (20,21), central effects cannot be excluded. The parallel striking increase in BP and HR argues in favor of central effects mediated by R-568, whereas the subsequent reduction in HR below baseline that was observed in uremic rats would be consistent with a hypothetical overshooting baroreceptor-mediated counterregulation. Whether the acute R-568 effects are also related to differences in PTH and ionized calcium concentration between groups is unknown. It is interesting that some patients do not experience an immediate decline in PTH plasma levels but a transient increase before a sustained reduction, suggesting an initial antagonistic effect of calcimimetics (2,22).

So far, no data have been published on circadian BP rhythm in uremic rats. Analogous to the findings in uremic patients (23), circadian BP rhythm was absent in SNX rats. It could be restored neither by R-568 infusion nor by subcutaneous administration during the daytime.

Conclusion

Our studies clearly demonstrate a persistent antihypertensive action of the calcimimetic agent R-568 in uremic rats. In healthy rats, R-568 does not exert a major hypotensive action; total body sodium is not reduced. On the basis of these experimental observations, it will be of interest to investigate whether similar BP lowering occurs in uremic patients as well.

Acknowledgments

The studies were supported by Amgen (Thousand Oaks, CA).

We are grateful to M. Jennewein and S. Zauner for assistance with neutron activation analysis. We are thankful to H. Ehmke for constructive comments.

References

- Ogata H, Ritz E, Odoni G, Amann K, Orth SR: Beneficial effects of calcimimetics on progression of renal failure and cardiovascular risk factors. *J Am Soc Nephrol* 14: 959–967, 2003
- Goodman WG, Hladik GA, Turner SA, Blaisdell PW, Goodkin DA, Liu W, Barri YM, Cohen RM, Coburn JW: The calcimimetic agent AMG 073 lowers plasma parathyroid hormone levels in hemodialysis patients with secondary hyperparathyroidism. *J Am Soc Nephrol* 13: 1017–1024, 2002
- Lindberg JS, Moe SM, Goodman WG, Coburn JW, Sprague SM, Liu W, Blaisdell PW, Brenner RM, Turner SA, Martin KJ: The calcimimetic AMG 073 reduces parathyroid hormone and calcium \times phosphorus in secondary hyperparathyroidism. *Kidney Int* 63: 248–254, 2003
- Block GA, Martin KJ, de Francisco AL, Turner SA, Avram MM, Suranyi MG, Hercz G, Cunningham J, Abu-Alfa AK, Messa P, Coyne DW, Locatelli F, Cohen RM, Evenepoel P, Moe SM, Fournier A, Braun J, McCary LC, Zani VJ, Olson KA, Druke TB, Goodman WG: Cinacalcet for secondary hyperparathyroidism in patients receiving hemodialysis. *N Engl J Med* 350: 1516–1525, 2004
- Lindberg JS, Culleton B, Wong G, Borah MF, Clark RV, Shapiro WB, Roger SD, Husserl FE, Klassen PS, Guo MD, Albizem MB, Coburn JW: Cinacalcet HCl, an oral calcimimetic agent for the treatment of secondary hyperparathyroidism in hemodialysis and peritoneal dialysis: A randomized, double-blind, multicenter study. *J Am Soc Nephrol* 16: 800–807, 2005
- Vargas-Poussou R, Huang C, Hulin P, Houillier P, Jeunemaitre X, Paillard M, Planelles G, Dechaux M, Miller RT, Antignac C: Functional characterization of a calcium-sensing receptor mutation in severe autosomal dominant hypocalcemia with a Bartter-like syndrome. *J Am Soc Nephrol* 13: 2259–2266, 2002
- Unger T, Gohlke P: ACE inhibition in the tissues. *Clin Physiol Biochem* 9: 89–93, 1992
- Vecsei P, Abdelhamid S, Mittelstadt GV, Lichtwald K, Haack D, Lewicka S: Aldosterone metabolites and possible aldosterone precursors in hypertension. *J Steroid Biochem* 19: 345–351, 1983
- Abraham GE, Buster JE, Lucas LA, Corrales PC, Teller RC: Chromatographic separation of steroid hormones for use in radioimmunoassays. *Anal Lett* 5: 509–517, 1972
- Yasumura S, Jones K, Spanne P, Schidlovsky G, Wielopolski L, Ren X, Glaros D, Xatzikonstantinou Y: In vivo animal models of body composition in aging. *J Nutr* 123: 459–464, 1993
- Ohanian J, Gatfield KM, Ward DT, Ohanian V: Evidence for a functional calcium-sensing receptor that modulates myogenic tone in rat subcutaneous small arteries. *Am J Physiol Heart Circ Physiol* 288: H1756–H1762, 2005
- Bukoski RD, Bian K, Wang Y, Mupanomunda M: Perivascular sensory nerve Ca²⁺ receptor and Ca²⁺-induced re-

- laxation of isolated arteries. *Hypertension* 30: 1431–1439, 1997
13. Wang Y, Bukoski RD: Distribution of the perivascular nerve Ca²⁺ receptor in rat arteries. *Br J Pharmacol* 125: 1397–1404, 1998
 14. Ishioka N, Bukoski RD: A role for N-arachidonylethanolamine (anandamide) as the mediator of sensory nerve-dependent Ca²⁺-induced relaxation. *J Pharmacol Exp Ther* 289: 245–250, 1999
 15. Iseki K, Massry SG, Campese VM: Effects of hypercalcemia and parathyroid hormone on blood pressure in normal and renal-failure rats. *Am J Physiol* 250: F924–F929, 1986
 16. Hulter HN, Melby JC, Peterson JC, Cooke CR: Chronic continuous PTH infusion results in hypertension in normal subjects. *J Clin Hypertens* 2: 360–370, 1986
 17. Fliser D, Franek E, Fode P, Stefanski A, Schmitt CP, Lyons M, Ritz E: Subacute infusion of physiological doses of parathyroid hormone raises blood pressure in humans. *Nephrol Dial Transplant* 12: 933–938, 1997
 18. Mann JF, Wiecek A, Bommer J, Ganten U, Ritz E: Effects of parathyroidectomy on blood pressure in spontaneously hypertensive rats. *Nephron* 45: 46–52, 1987
 19. Rybczynska A, Boblewski K, Lehmann A, Orlewska C, Foks H, Drewnowska K, Hoppe A: Calcimimetic NPS R-568 induces hypotensive effect in spontaneously hypertensive rats. *Am J Hypertens* 18: 364–371, 2005
 20. Ye C, Kanazirska M, Quinn S, Brown EM, Vassilev PM: Modulation by polycationic Ca(2+)-sensing receptor agonists of nonselective cation channels in rat hippocampal neurons. *Biochem Biophys Res Commun* 224: 271–280, 1996
 21. Rogers KV, Dunn CK, Hebert SC, Brown EM: Localization of calcium receptor mRNA in the adult rat central nervous system by in situ hybridization. *Brain Res* 744: 47–56, 1997
 22. Rothe HM, Sun WY, Shapiro WB, Mayer G, Matalon A: Response to a single dose of cinacalcet in relation to calcium-sensing receptor gene polymorphism ARG990GLY [Abstract]. *Nephron* 10: 14, 2005
 23. Farmer CK, Goldsmith DJ, Cox J, Dallyn P, Kingswood JC, Sharpstone P: An investigation of the effect of advancing uraemia, renal replacement therapy and renal transplantation on blood pressure diurnal variability. *Nephrol Dial Transplant* 12: 2301–2307, 1997