Hypertensive effect of calcilytic NPS 2143 administration in rats

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Abstract

Secretion of parathormone (PTH), the main parathyroid hormone, which is under the control of the calcium sensing receptor, might be inhibited by calcimimetics and stimulated by calcilytics. Parathyroid glands also secrete parathyroid hypertensive factor. Recently, it was shown that calcimimetic NPS R-568 induced decreased blood pressure in spontaneously hypertensive rats (SHR) in the presence of parathyroid glands. Therefore, the aim of this study was to determine whether administration of the calcilytic NPS 2143 provoked an increase of mean arterial blood pressure (MAP) in normotensive rats. We used male Wistar rats anaesthetized with thiopental. Clearance experiments were performed and the effect of bolus, 1 mg/kg body weight i.v. of NPS 2143 on MAP in the presence and absence of thyroparathyroidectomy (TPTX) was monitored

continuously. Calcilytic properties of NPS 2143 were confirmed directly by a significant ($P < 0.05$) increase of plasma PTH concentration, and indirectly by a rise of plasma Ca^{2+} concentration and urinary fractional phosphate excretion (FE Pi). NPS 2143 administration markedly ($P < 0.05$) increased MAP, calculated as the difference (Δ) in MAP between sequential measurements and the time of bolus injection of calcilytic. The observed increase of blood pressure in the NPS 2143 group was also significant ($P < 0.05$) compared with the control group. Performance of TPTX prevented the hypertensive effect of NPS 2143. We conclude that NPS 2143 is responsible for increased blood pressure in rats in the presence of parathyroid glands.

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Introduction

The calcium receptor (CaR), located at the surface of parathyroid cells, is sensitive to changes in plasma Ca^{2+} concentration, and plays a key role in the regulation of parathyroid hormone (PTH) secretion (Brown 1991, Brown & MacLeod 2001). Activation of CaR by increased concentration of extracellular Ca^{2+} inhibits PTH secretion, and, in reverse, decrease of extracellular Ca^{2+} raises the PTH secretion by parathyroid cells. Therefore, the discovery of other compounds, in addition to Ca^{2+} , that are able to influence CaR, created the possibility to modulate parathyroid secretion. Development of compounds, called calcimimetics, that activate CaR, enabled the suppression of PTH secretion in rats and humans (Steffey *et al.* 1993, Fox *et al.* 1999, Goodman *et al.* 2000). Calcimimetic compounds have great potential as an innovative medical approach to manage primary hyperparathyroidism (Silverberg *et al.* 1997) and secondary hyperparathyroidism in uraemia (Fox *et al.* 1999, Goodman *et al.* 2000). Moreover, recently synthesized calcilytics possessed properties that inhibit the activity of CaR and thus stimulate PTH secretion (Gowen *et al.* 2000, Nemeth *et al.* 2001). Therefore, calcilytic compounds might potentially be useful as anabolic therapy in osteoporotic patients.

We have recently reported that pharmacological modulation of CaR by calcimimetic NPS R-568 is responsible for decreased blood pressure in hypertensive (SHR) rats with intact parathyroids (not thyroparathyroidectomy (TPTX)), indicating the close relationship between the activation of CaR, the presence of parathyroid glands, and blood pressure (Rybczynska *et al.* 2005). Furthermore, the calcimimetic NPS R-568 caused the marked and sustained antihypertensive effect in uraemic rats (Odenwald *et al.* 2006). Therefore, it might be presumed that the inactivation of CaR by calcilytics leads to increased blood pressure in normotensive rats.

The aim of our study was to compare the effect of the calcilytic NPS 2143 on mean arterial blood pressure (MAP) in normotensive rats in the presence and absence of parathyroid glands.

Materials and Methods

Clearance experiments

Male Wistar rats, weighing 200–280 g, were purchased from the Animal House of the Polish Academy of Sciences, Warsaw, Poland. All experiments were approved by The Local Ethical Committee on Animal Experiments. The animals

were fed a commercial rodent chow (Purine, Poland) and tap water, available *ad libitum*. Rats were anaesthetized by i.p. injection of thiopental at the dose 40 mg/kg body weight and maintained under anaesthesia by thiopental supplementation during the experiment. The animals were placed on a heated table, and body temperature was maintained between 36 and 37 °C. TPTX by heat cauterization in some groups, and tracheostomy in all experimental groups were performed. Catheters were inserted into the carotid artery for blood sampling and pressure monitoring, into a jugular vein for infusions, and into the bladder for urine collection. Blood pressure was constantly monitored. Glomerular filtration rate (GFR) was measured as [^3H] inulin clearance.

After all surgical procedures, including TPTX, a 2-h recovery period was allowed to establish steady state. During the first hour of this period, rats were infused with 4% albumin in isotonic saline at the rate of 5.6 ml/h. This infusion was then replaced by isotonic saline at the same rate. During the second hour, an i.v. bolus of [^3H] inulin (Amersham), 3 $\mu\text{Ci}/250$ g body weight was given and the infusion of saline supplemented with [^3H] inulin (0.02 $\mu\text{Ci}/\text{min}$) was started and maintained until the end of experiment.

Experimental groups

Six groups of rats were studied according to the following protocols:

Group 1, NPS ($n=10$) After 30-min [^3H] inulin infusion, NPS 2143 (NPS), dissolved in 15% cyclodextrin (Sigma) at a dose of 1 mg/kg body weight through venous catheter, was administered as a 300 μl bolus. The time of administration of NPS was designated as time 0. Blood samples were taken at the midpoint of 10 min urine collection: 5 min before, and at 30, 60 and 90 min after, administration of the tested agent.

Group 2, Control ($n=8$) The procedure with the rats was similar to that in group 1 with the exception that only 15% cyclodextrin was administered i.v.

Group 3, TPTX/NPS ($n=7$) and Group 4, TPTX/control ($n=6$) Before tracheostomy, each rat was thyroparathyroidectomized. All other procedures were the same as in groups 1 and 2, assuming that group 3 corresponds to group 1, and group 4 corresponds to group 2.

In order to measure the effect of NPS 2143 on plasma PTH concentration, two additional groups of rats were included:

Group 5, NPS ($n=10$) After a 2-h recovery period, the NPS 2143, as in group 1, was administered as a bolus. Blood samples (1.5 ml) for assay of PTH concentration were taken 10 min before and at 15, 40 and 60 min after administration of NPS 2143. To prevent excessive blood loss, immediately after

separation of plasma, the sediment of red cells was rinsed with saline and reinfused as the erythrocyte concentrate.

Group 6, Control ($n=5$) The procedure with the rats was similar to that in group 5, with the exception that only 15% cyclodextrin was administered i.v.

Measurements and calculations

Total radioactivities of blood and urine samples were counted on the Liquid Scintillation Counter Wallac 1409 (LKB, Sweden). Phosphate concentrations in plasma and urine were determined according to the adapted method of Fiske & SubbaRow (1925). The plasma pH and ionized calcium [Ca^{2+}] concentration were measured using an AVL 988-4 Ca^{2+}/pH analyser (AVL, Vienna, Austria). Plasma PTH concentration was determined with rPTH radioimmunoassay kit (Pennisula Laboratories, Inc., San Carlos, CA, USA). Values are presented as means $\pm$ S.E.M. Comparisons were made using Student's *t*-test. Significance was designated as $P < 0.05$.

Arterial blood pressure was monitored directly and sampled continuously at 100 Hz, as we described previously (Rybczynska *et al.* 2005), using Biopac Systems, Inc., Model MP 100 (Goleta, CA, USA). The results of blood pressure measurements were elaborated with the help of the ACQKnowledge (Goleta, CA, USA) measurement system that is selected, scaled and filtered to remove accidental signal disturbances. The recorded time domain transient data have been presented as graphs with the help of Matlab Code (MathWorks, Inc., Natic, MA, USA).

Statistical ANOVAs of MAP were performed for ΔMAP , calculated as the difference in MAP between sequential measurements and time 0 min of the experiment for each group, as we described previously (Rybczynska *et al.* 2005). This allowed for direct comparison of responses to treatment between groups when baselines differed. Data were analysed by ANOVA with repeated measures, using Statistica StatSoft software (StatSoft, Inc., Tulsa, OK, USA), after NPS 2143 or vehicle treatment and in the presence or absence of TPTX. When the effect was significant, *post hoc* comparisons were performed using Bonferroni and Duncan tests. A value of $P < 0.05$ was considered statistically significant.

Synthesis of calcilytic NPS 2143

NPS 2143 was synthesized *via* addition of (*R*)-[1-(2-cyano-3-chlorophenoxy)]-2',3'-epoxypropane (DelMar *et al.* 2003) to 1,1-dimethyl-2-(2-naphthyl)ethylamine obtained from the modified Ritter reaction of the tertiary alcohol and chloroacetonitrile (Jirgensons *et al.* 2000). In this way, the prepared amine was converted into its hydrochloride by a standard procedure. Structure and purity (>99%) of the product was confirmed by ^1H and ^{13}C NMR, and microanalysis (CHN).

The synthesis of NPS 2143 was carried out at the Department of Organic Chemistry, Faculty of Pharmacy, Medical University of Gdansk, Poland.

Results

Effect of synthesized NPS 2143 on plasma Ca^{2+} , urinary phosphate excretion and plasma PTH concentration

In the presence of parathyroid glands, NPS 2143 administration resulted in a significant increase of plasma Ca^{2+} concentration in comparison with the control group as well as with the value before NPS 2143 application (Table 1). In the control group, no changes of plasma Ca^{2+} concentration were observed during the entire experiment. NPS 2143 markedly elevated urinary phosphate excretion, calculated as FE_{Pi} , when compared with the control group and with the time before calcilytic application. However, in the control group, a slight tendency to increased FE_{Pi} was observed. GFR in both control and NPS 2143 groups remained in the physiological range, although a small decrease during the experiment occurred in the control group. There were no changes in GFR in the NPS 2143 group, except for its small increase versus the control group 60 min after calcilytic application. Plasma PTH concentration was significantly increased in the NPS 2143-treated group except 40 min after NPS 2143 injection where significance was not reached. Instead, in the control group the PTH level was decreased. The observed decrease was significant 40 min after vehicle injection (Table 2).

The effect of NPS 2143 administration in the presence of TPTX is shown in Table 3. Removal of parathyroid glands resulted in a significant decrease in plasma Ca^{2+} concentration by approximately 12% in the initial period when compared with control (no TPTX) animals (Table 1). We observed a continued decrease in plasma Ca^{2+} concentration versus the control period during the experiment; however, significance was reached only in NPS 2143-treated rats (Table 3). Performance of TPTX almost completely abolished phosphaturia in control and NPS 2143 groups in comparison with non-thyroparathyroidectomized rats (Table 1). This effect was unchanged throughout the experiment and was not

dependent on NPS 2143 administration. There were no significant differences in GFR between control and NPS 2143 groups.

Blood pressure measurement

The effects of NPS 2143 on MAP in rats are summarized in Fig. 1. NPS 2143 administration resulted in increased MAP in normotensive, Wistar rats in comparison with the control and TPTX groups. The hypertensive effect of NPS 2143 is more sharply expressed when calculated as ΔMAP , the difference in MAP between sequential measurements and time 0 (Fig. 1b). However, baseline values of MAP were stable and remained in the physiological range, and the differences occurred because of the individual disparities between animals. Therefore, the estimation of ΔMAP allowed for direct comparison of the responses to treatment between groups with different baseline values that underlined the significance in growth of blood pressure in the NPS 2143 experimental group compared with control and TPTX rats. Instead, in the control group, a small decrease of MAP was observed. Performance of TPTX also induced a small decrease in blood pressure, not significantly different from the control animals. Administration of the calcilytic in TPTX-NPS 2143 animals did not affect blood pressure significantly in comparison with the TPTX-control group.

Discussion

The present study demonstrates that i.v. administration of calcilytic NPS 2143, an inhibitor of CaR , increases blood pressure in normotensive rats (Fig. 1). This effect was observed only in the presence of parathyroid glands in rats. Administration of the calcilytic after TPTX did not influence blood pressure. These results are complementary to our previous findings that activation of the CaR by the calcimimetic NPS R-568 generates decreased blood pressure in hypertensive rats with intact parathyroids (Rybczynska *et al.* 2005)

Table 1 Effect of NPS 2143 administration on plasma Ca^{2+} concentration and urinary phosphate excretion in rats. Data are presented as means $\pm$ S.E.M.

	Ca^{2+} (mmol/l)		GFR (ml/min)		FE_{Pi} (%)	
	C (n=8)	NPS (n=10)	C (n=8)	NPS (n=10)	C (n=8)	NPS (n=10)
Time of experiment (min)						
–5	1.21 $\pm$ 0.02	1.25 $\pm$ 0.02	2.73 $\pm$ 0.11	2.84 $\pm$ 0.06	14.2 $\pm$ 1.8	11.1 $\pm$ 1.9
0			NPS 2143, 1 mg/kg body weight			
30	1.22 $\pm$ 0.02	1.37 $\pm$ 0.02* [†]	2.51 $\pm$ 0.12	2.87 $\pm$ 0.17	16.5 $\pm$ 2.1	28.3 $\pm$ 2.1* [†]
60	1.24 $\pm$ 0.02	1.38 $\pm$ 0.03* [†]	2.27 $\pm$ 0.13*	2.83 $\pm$ 0.19 [†]	16.4 $\pm$ 2.1	25.7 $\pm$ 1.3* [†]
90	1.25 $\pm$ 0.01	1.44 $\pm$ 0.03* [†]	2.12 $\pm$ 0.11*	2.32 $\pm$ 0.26	15.8 $\pm$ 2.9	21.5 $\pm$ 1.6* [†]
120	1.25 $\pm$ 0.02	1.42 $\pm$ 0.04* [†]	2.15 $\pm$ 0.19*	2.17 $\pm$ 0.16*	12.3 $\pm$ 1.9	23.4 $\pm$ 3.0* [†]

GFR, glomerular filtration rate; FE_{Pi} , fractional excretion of phosphate; C, control group; NPS, NPS 2143 group. Comparisons were made using Student's *t*-test; * $P < 0.05$ versus –5 min experiment; [†] $P < 0.05$ versus control.

Table 2 Effect of NPS 2143 administration on PTH plasma concentration in rats. Data are presented as means ± s.e.m.

	PTH (pg/ml)	
	Control (n=5)	NPS 2143 (n=10)
Time of experiment (min)		
−5	19.8±5.2	20.7±4.7
0	NPS 2143, 1 mg/kg body weight	
15	10.0±2.2	32.2±0.3*†
40	7.8±1.9*	24.1±2.7†
60	10.2±2.5	32.1±3.0*†

Comparisons were made using Student's *t*-test; **P*<0.05 versus −5 min experiment; †*P*<0.05 versus control.

and indicate the close relationship between parathyroids, activity of CaR, and blood pressure.

It is well established that CaR, belonging to the class III of G-protein-coupled receptors, plays a key role in the mechanism of PTH secretion by parathyroid glands (Brown 1991, Brown & MacLeod 2001). CaR activated physiologically by Ca²⁺ or pharmacologically by calcimimetics, decreases PTH secretion by parathyroids (Steffey *et al.* 1993, Fox *et al.* 1999, Goodman *et al.* 2000). Instead, CaR antagonists, calcilytics, increase the PTH secretion (Gowen *et al.* 2000, Nemeth *et al.* 2001). NPS 2143 was the first reported calcilytic, which, orally applied, induced rapid increase of circulating PTH in ovariectomized rats to an extent that stimulated new bone formation (Gowen *et al.* 2000). A rapid increase in plasma PTH level was also documented after intravenously infused NPS 2143 in normal rats (Nemeth *et al.* 2001).

The calcilytic NPS 2143 applied in our experiment was synthesized in our laboratory; therefore, special attention was given to identify its calcilytic activity, i.e. ability to increase PTH secretion. First, we directly demonstrated the significant increase of plasma PTH concentration after NPS 2143 administration compared with control rats. However, temporary debasement of PTH level was observed in both control and experimental groups; in the light of decreased PTH levels in the control group, the increase of PTH in NPS 2143 rats is evident. Secondly, we considered the significant

increase of plasma Ca²⁺ concentration and fractional phosphate excretion as the supplemental and indirect confirmation of increased PTH secretion after NPS 2143 administration. These findings are consistent with other studies on normal (Nemeth *et al.* 2001) and osteopenic (Gowen *et al.* 2000) rats treated with NPS 2143 and documented that the synthesized NPS 2143 engenders calcilytic properties in our experimental conditions. Additionally, in the absence of PTH, after performance of TPTX, decreased plasma Ca²⁺ concentration and almost complete lack of phosphaturia were observed in rats. Moreover, TPTX procedure prevented the NPS 2143 effect to increase plasma Ca²⁺ and phosphate excretion in rats, indicating that the presence of parathyroids is necessary to demonstrate the calcilytic properties of NPS 2143.

However, several investigations using NPS 2143 (Gowen *et al.* 2000, Nemeth *et al.* 2001) and other calcilytic compounds (Arey *et al.* 2005) have been performed, and changes in blood pressure were not reported in these experiments. Therefore, the hypertensive effect of calcilytic NPS 2143 administration in this study is a new observation. Our data clearly show the significant rise of blood pressure after NPS 2143 injection compared with control animals. The observed slow decrease of blood pressure in control and TPTX animals was a consequence of the duration of the experiment, the surgical procedures and blood sampling in all rats. Therefore, the increase of blood pressure after NPS 2143

Table 3 Effect of NPS 2143 administration on plasma Ca²⁺ concentration and urinary phosphate excretion in TPTX rats. Data are presented as means ± s.e.m.

	Ca ²⁺ (mmol/l)		GFR (ml/min)		FE _{Pi} (%)	
	C (n=6)	NPS (n=7)	C (n=6)	NPS (n=7)	C (n=6)	NPS (n=7)
Time of experiment (min)						
−5	1.10±0.08	1.10±0.02	2.70±0.20	2.69±0.11	0.46±0.20	0.37±0.07
0	NPS 2143, 1 mg/kg body weight					
30	1.10±0.06	1.04±0.02	2.73±0.20	2.49±0.12	0.61±0.20	0.32±0.10
60	1.00±0.06	1.03±0.02*	2.67±0.40	2.48±0.17	0.75±0.50	0.33±0.10
90	1.00±0.07	1.01±0.02*	2.52±0.20	2.68±0.18	0.56±0.56	0.30±0.10
120	1.00±0.05	0.97±0.03*	2.70±0.10	2.72±0.17	1.05±0.60	0.29±0.10

TPTX, thyroparathyroidectomy; GFR, glomerular filtration rate; FE_{Pi}, fractional excretion of phosphate; C, control group; NPS, NPS 2143 group. Comparisons were made using Student's *t*-test; **P*<0.05 versus −5 min experiment.

Figure 1 Effect of NPS 2143 or vehicle (control) administration (a) on MAP and (b) on Δ MAP (calculated as the difference of MAP between sequential measurement and time 0 min of experiment) in rats in the presence and absence of TPTX. Each point represents mean value of MAP or Δ MAP from: $n=10$ (NPS 2143), $n=8$ (control), $n=7$ (TPTX-NPS 2143), $n=6$ (TPTX-control) experiments. Comparisons were made using ANOVA with repeated measures, Bonferoni and Duncan tests; significance $P<0.05$, was found for comparisons: *NPS 2143 versus control; **NPS 2143 versus TPTX-NPS 2143; ***NPS 2143 versus TPTX-control.

administration in comparison with the decrease in the control and TPTX groups underlined the hypertensive effect of the calcilytic. To investigate whether this effect was specifically dependent on the impact of the calcilytic on parathyroid glands, we monitored blood pressure in thyroparathyroidectomized rats. The blood pressure in the TPTX groups was not statistically different from that observed in control animals. Moreover, performance of TPTX procedure induced insensitivity of blood pressure to NPS 2143 administration in rats. These observations suggest that the presence of parathyroids is the necessary condition to induce hypertension after calcilytic administration in rats.

It is possible that CaR, through mechanisms similar to those that control PTH secretion, might mediate the secretion of other substances produced by the parathyroid glands. It is well documented that parathyroid glands secrete parathyroid hypertensive factor (PHF), which is confirmed in SHR (Lewanczuk *et al.* 1989, Lewanczuk & Pang 1990, Schluter *et al.* 1993, Benishin *et al.* 1994) and also in patients with primary hyperparathyroidism and hypertension (Schulter *et al.* 1992, Lewanczuk *et al.* 1994). To date, the structure of PHF is not known. There was the proposition that the structure of PHF has a peptide moiety and a lysosomal moiety that both are critical for the biological activity (Benishin *et al.* 1994). The molecular mass of this factor was estimated to be in the range 2.5–3.0 kDa (Benishin *et al.* 1991). Recently, an enzyme immunoassay for detection of PHF in human plasma has been reported (Krylova *et al.* 2003).

It was observed that surgically removing hyperactive parathyroids induced decreased blood pressure in SHR (Mann *et al.* 1987, Onsgaard-Meyer *et al.* 1994, Rybczynska *et al.* 2005) as well as in patients with primary hyperparathyroidism (Diamond *et al.* 1986, Resnik *et al.* 1986, Niederle *et al.* 1987). These findings indicated the connection between parathyroid glands and observed hypertension. Moreover, it was documented recently that pharmacological parathyroidectomy, realized by calcimimetic NPS R-568 administration, resulted in decreased blood pressure in SHR with intact parathyroids (Rybczynska *et al.* 2005). This observation suggests that CaR present on the surface of parathyroid cells may have mediated the mechanisms that influence blood pressure. The possibility should be considered that the hypotensive effect of calcimimetic NPS R-568 in SHR and the hypertensive effect of calcilytic NPS 2143 in normotensive rats are the effects of decreased and increased PHF secretion respectively. This hypothesis is strengthened by the study suggesting the involvement of CaR in the regulation of PHF secretion, indicating an increased PHF secretion by cultured SHR cells in low Ca^{2+} medium (Sutherland & Benishin 2004).

Several investigators have reported that the CaR is expressed in many cardiovascular tissues, including human aortic endothelial cells (Ziegelstein *et al.* 2006), rat cardiomyocytes (Tfelt-Hansen *et al.* 2006) and is also present in rat s.c. small arteries (Ohanian *et al.* 2005). Therefore, the influence of calcilytic on blood pressure is not excluded.

However, in our experimental conditions, such a possibility seems to be unlikely since the blood pressure measurements in control and in TPTX-NPS 2143 treated rats were not statistically different.

Administration of calcilytic NPS 2143 in our study increased the plasma PTH concentration in rats (Table 2). The known effect of the single doses of 1–34 PTH (Pang *et al.* 1980, Saglikes *et al.* 1985, Baksi 1988) or 1–84 PTH (Saglikes *et al.* 1985) is the activity that transiently decreases blood pressure in intact (Pang *et al.* 1980, Saglikes *et al.* 1985) or thyroparathyroidectomized (Baksi 1988) animals. In these experiments, the decrease in blood pressure was observed immediately, within about 1 min, after PTH injection. Moreover, when, after bolus injection, continuous infusion of 1–34 PTH was applied, no changes of blood pressure during the further duration of the experiments on TPTX rats were observed (Rybczynska *et al.* 1990). In our study, increased blood pressure after application of NPS 2143 was observed. Therefore, the possible influence of increased plasma PTH concentration in our study should be taken into consideration as the element that, if anything, might decrease blood pressure in rats. Consequently, the time course of the observed blood pressure changes in rats treated with NPS 2143 might reflect the final effect of secretion by the parathyroids of both PTH and the hypertensive substance, most likely PHF.

In summary, the present study demonstrates that administration of the calcilytic NPS 2143 increases blood pressure in normotensive rats only in the presence of parathyroid glands.

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