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Altered vitamin D metabolism in the kidney of the spontaneously hypertensive rat

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A decrease in plasma Ca^{2+} and increases in plasma immunoreactive parathyroid hormone (PTH) have been reported in spontaneously hypertensive (SH) rats as compared with normotensive Wistar–Kyoto (WKy) rats. These changes should lead to a higher plasma $1,25(OH)_2D$ (1,25-dihydroxycholecalciferol/1,25-dihydroxyergocalciferol) concentration in SH rat if the kidney responds appropriately. Plasma $1,25(OH)_2D$, however, has been reported to be normal in SH rats, suggesting possible impairments of vitamin D metabolism in this animal model of hypertension. To test this possibility, we studied the effect of PTH on renal production of $1,25(OH)_2D$ in SH rats before (4 weeks of age) and after (12 weeks of age) the onset of hypertension. Basal serum levels of $1,25(OH)_2D$ were normal in SH rats at both ages. At 4 weeks of age, the rise in serum $1,25(OH)_2D$ after PTH injection (50 units subcutaneously every 2 h; four times) was also normal in SH rats. By contrast, at 12 weeks of age, the rise in serum $1,25(OH)_2D$ was approximately one-half of that in WKy rats, despite the similar rises in serum Ca^{2+} levels in both groups by PTH injection. The attenuated rise in serum $1,25(OH)_2D$ in SH rats was consistent with the impaired response of renal 1-hydroxylase (25-hydroxycholecalciferol 1α-hydroxylase) activity to PTH. Basal $1,25(OH)_2D$ production by the kidney in SH rat was higher than that in WKy rats both at 4 and 12 weeks of age. These data suggest that, in SH rats: (1) serum $1,25(OH)_2D$ is inappropriately low in relation to the elevated PTH and this may be due, at least in part, to the impaired responsiveness to PTH of renal 1-hydroxylase and to the enhanced metabolism of $1,25(OH)_2D$, and (2) elevated PTH or other agents may stimulate the 1-hydroxylase in the kidney even before the onset of hypertension.

INTRODUCTION

Accumulating evidence suggests that calcium homoeostasis has a significant role in the development and maintenance of hypertension. Dietary calcium intake is lower in patients with hypertension than in normotensive individuals (Belizan & Villar, 1980). In epidemiological studies, low dietary calcium has been associated with hypertension (Neri *et al.*, 1972; Masironi *et al.*, 1976). Low calcium intake increases blood pressure in WKy rats (McCarron, 1982*a*), which are controls for SH rats developed from this strain. High calcium intake lowers blood presure in SH rats (Ayachi, 1979; McCarron *et al.*, 1981; Chen *et al.*, 1982; Schneiderman *et al.*, 1984) as well as in WKy rats (Ayachi, 1979; Schneiderman *et al.*, 1984).

Total serum calcium is similar in patients with essential hypertension and in normotensive controls, but serum ionized calcium (Ca^{2+}) is decreased in hypertensive patients (McCarron, 1982*b*). This is also true in SH rats as compared with WKy rats; under comparable feeding conditions, serum Ca^{2+} is lower in SH rats than in WKy rats (McCarron *et al.*, 1981; Wright *et al.*, 1980; Wright & Rankin, 1982). Serum immunoreactive PTH is elevated in patients with essential hypertension (McCarron *et al.*, 1980) and in SH rats (McCarron *et al.*, 1981). Intestinal Ca^{2+} absorption is decreased (Schedl *et al.*, 1984), unchanged (Stern *et al.*, 1984) or less responsive to exogenous $1,25(OH)_2D_3$ (Toraason & Wright, 1981) in SH rats. Plasma $25(OH)D_3$ is lower in patients with hypertension treated with hydrochlorothiazide (Kokot *et al.*, 1981) and higher in SH rats than in WKy rats (Schedl *et al.*, 1984), whereas plasma $1,25(OH)_2D_3$ is reported to be normal in SH rats at 12 weeks of age (Schedl *et al.*, 1984; Stern *et al.*, 1984).

These findings suggest abnormalities in calcium homoeostasis in this animal model of hypertension, particularly in vitamin D metabolism, since increased plasma PTH levels seem to fail to raise $1,25(OH)_2D$ above normal values. To detect possible defects in the animal model of essential hypertension, we studied the responsiveness to PTH of renal 1-hydroxylase and serum concentration of $1,25(OH)_2D$ in SH and WKy rats.

EXPERIMENTAL

Animals

Male SH and WKy rats were purchased from Charles River Breeding Laboratories (Wilmington, MA, U.S.A.) and fed a Purina rat chow *ad libitum*. Rats, at 4 and 12 weeks of age, were used in this study. Animals, at each age, were divided into two groups. A group of rats was given a subcutaneous injection of PTH (Beckman

Abbreviations used: $1,25(OH)_2D_3$, 1,25-dihydroxycholecalciferol; $25(OH)D_3$, 25-hydroxycholecalciferol; 1-hydroxylase, 25-hydroxycholecalciferol 1α-hydroxylase; PTH, parathyroid hormone (parathyrin); SH, spontaneously hypertensive; WKy, Wistar–Kyoto; PTX, parathyroidectomy; $1,25(OH)_2D$ and 25(OH)D refer to a mixture of the corresponding metabolite of cholecalciferol and ergocalciferol.

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Instrument Company, Palo Alto, CA, U.S.A.) every 2 h (four times). The control group received saline (0.9% NaCl) as a vehicle. At 2 h after the last injection, rats were exsanguinated by aortic puncture and sera were collected for the determination of calcium and $1,25(OH)_2D$. Kidneys were excised for the assay of the $1,25(OH)_2D$ production. Serum calcium concentration was measured by the orthocresolphthalein complexone method (Connerty & Briggs, 1966). Blood pressure was monitored with an electrosphygmomanometer (Narco Biosystem, Houston, TX, U.S.A.) on the day before the experiment.

To examine renal function, creatinine clearance was measured in another set of experiments. Animals were anaesthetized with sodium pentobarbital (40 mg/kg, intraperitoneally) and were infused via a femoral arterial catheter at a rate of 1 ml/h with a solution composed of 5 mM-$CaCl_2$, 5 mM-$MgCl_2$, 20 mM-NaCl, 2.5 mM-KCl and 4% (w/v) glucose, starting at 5 h. Blood samples were obtained by a catheter inserted into the femoral vein. Bladder was also catheterized and urine was collected from 6 to 8 h. Endogenous creatinine concentrations in urine and serum were determined by the method of Bonsnes & Taussky (1945).

Serum $1,25(OH)_2D$ assay

The serum concentration of $1,25(OH)_2D$ was determined by the method of Reinhardt *et al.* (1984), with bovine thymic receptor for $1,25(OH)_2D$ (see below).

Production of $1,25(OH)_2D$ by the kidney

Mitochondria were prepared from the kidney, and incubations were performed by the method of Vieth & Fraser (1979). The kidneys were placed in an ice-cold homogenization medium (250 mM-sucrose/10 mM-Hepes/10 mM-KCl, adjusted with NaOH to pH 7.42 at room temperature) and cortical tissues were obtained after removing capsules, papilla, ureter and medulla. The tissues were homogenized in 10 vol. (w/v) of ice-cold homogenization medium with a motor-driven Potter–Elvehjem homogenizer. The homogenate was centrifuged at 4000 *g* and 4 °C for 40 s, and the supernatant was centrifuged at 9000 *g* and 4 °C for 20 min. The pellet was suspended in 10–15 vol. of ice-cold incubation medium (125 mM-KCl/20 mM-Hepes/10 mM-L-malic acid/2 mM-$MgSO_3$/1 mM-dithiothreitol/0.25 mM-EDTA, adjusted to pH 7.42 at room temperature; the final concentration of mitochondrial protein was 2.5–5 mg/ml. Each incubation sample consisted of a 1.0 ml portion of mitochondrial suspension. Samples, in 25 ml Erlenmeyer flasks, were placed in a Dubnoff incubator at 25 °C, shaking at 100 cycles/min. At 3 min, 5 μg of $25(OH)D_3$ (kindly provided by the Upjohn Co., Kalamazoo, MI, U.S.A.) in 20 μl of ethanol were added. After substrate addition, the flask was gassed for 1 min with a direct flow of O_2/CO_2 (19:1) at a rate of 0.5 litre/min. Immediately after gassing, the flask was sealed with a rubber stopper, incubated at 25 °C for 15 min and the reaction was stopped by the addition of 3.75 ml of methanol/chloroform (2:1, v/v).

Determination of $1,25(OH)_2D$

Lipid extraction was performed by the method of Bligh & Dyer (1959). The extract was dried under an N_2 gas stream and resolved in n-hexane/methanol/chloroform (9:1:1, by vol.), and then subjected to Sephadex LH-20 column chromatography. The column was eluted with the same solvent at a rate of 0.6 ml/min, and fractions (3 ml each) were collected. Fractions (nos. 63–74) containing $1,25(OH)_2D$ were pooled, dried under an N_2 gas stream and resolved in n-hexane and propan-2-ol (9:1, v/v), and subjected to h.p.l.c. (μPorasil; column size 0.39 cm × 30 cm). The column was eluted with the same solvent and $1,25(OH)_2D$ fractions were collected. This $1,25(OH)_2D$ fraction was applied to another h.p.l.c. system (μBondapak C_{18}) and eluted with water/methanol (7:3, v/v). The $1,25(OH)_2D$ fractions were collected and extracted again by the method of Bligh & Dyer (1959). The extracted $1,25(OH)_2D$ was dried under an N_2 stream and stored at −70 °C until the time of assay. $1,25(OH)_2D$ was determined by the method of Reinhardt *et al.* (1984), with bovine thymic receptor. In short, standard solutions of $1,25(OH)_2D_3$ (generously given by Dr. Uskokovic of Hoffman–La Roche, Nuttley, NJ, U.S.A.) (1–64, and 800 pg for non-specific binding determination) and samples (in 30 μl of ethanol) were added to 12 mm × 75 mm glass tubes on ice. Freeze-dried receptor was solubilized and diluted in a buffer containing 50 mM-Tris/HCl, 500 mM-KCl, 5 mM-dithiothreitol, 10 mM-Na_2MoO_4 and 1.5 mM-EDTA, pH 7.5, and then 450 μl of receptor solution (approx. 0.7 mg of protein/tube) was added to the standards and samples on ice, followed by vortex-mixing. The samples and standards were incubated in a 25 °C water bath for 45 min with gentle shaking. At the end of 45 min the tubes were transferred to an ice bath and allowed to cool for 5 min, and then each tube received 5000 c.p.m. of [26,27-*methyl*-^{3}H]$1,25(OH)_2D_3$ (160 Ci/mmol) (Amersham, Arlington Heights, IL, U.S.A.) in 25 μl of ethanol. The tubes were vortex-mixed and the incubation was continued for 15 min in a 25 °C water bath with shaking. The tubes were allowed to cool for 5 min in an ice bath, and 200 μl of dextran-coated charcoal suspension were added to each tube, followed by vortex-mixing. The tube contents were vortex-mixed again after 10 min and, after 2 min of charcoal treatment, bound and free hormones were separated by centrifugation at 2000 *g* for 10 min. The supernatant containing bound hormones was decanted into a scintillation vial and radioactivity was determined in a liquid-scintillation counter (Beckman; model 8100) (counting efficiency approx. 40%).

Protein determination

The mitochondrial protein concentration was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Calculations

The $1,25(OH)_2D$ value (pg/tube) was calculated by using a logit–log plot of the data. The $1,25(OH)_2D$ concentration of serum (pg/ml) or $1,25(OH)_2D$ production by tissues (fmol/mg of protein) were obtained after correcting for recovery (approx. 50%) and sample volume.

Statistical analysis

Results are given as ± S.E.M. and statistical analysis was performed by Student's *t* test.

Table 1. Body weights and blood pressures of WKy and SH rats

Blood pressure was measured the day before the experiment by using a electrosphygmomanometer, and body weight was measured before the start of the experiment. Abbreviation used: n.s., not significant.

Group	Age (weeks)	No. of animals	Blood pressure (mean ± S.E.M.) (mm Hg)	Body weight (g) (mean ± S.E.M.)
WKy	4	8	83 ± 5	58 ± 6
SH	4	8	92 ± 7	47 ± 4
Significance (WKy versus SH) . . .			n.s.	$P < 0.05$
WKy	12	8	121 ± 6	214 ± 6
SH	12	8	175 ± 5	260 ± 7
Significance (WKy versus SH) . . .			$P < 0.001$	$P < 0.001$

Table 2. Effect of PTH on the serum concentrations of calcium and $1,25(OH)_2D$

Rats received subcutaneous injections of either PTH or vehicle at 0, 2, 4 and 6 h. Blood samples were collected at 8 h. Abbreviation used: n.s., not significant.

Group	Age (weeks)	No. of animals	$1,25(OH)_2D$ (pg/ml) (mean ± S.E.M.)		Calcium (mg/100 ml) (mean ± S.E.M.)	
			Control	PTH	Control	PTH
WKy	4	4	135.0 ± 8.3	483.7 ± 80.0	9.5 ± 0.3	12.5 ± 0.3
SH	4	4	123.7 ± 18.4	517.8 ± 62.6	10.0 ± 0.3	13.0 ± 0.4
Significance (WKy versus SH) . .			n.s.	n.s.	n.s.	n.s.
WKy	12	4	50.1 ± 8.3	217.6 ± 14.8	9.7 ± 0.2	12.5 ± 0.4
SH	12	4	37.4 ± 4.2	105.3 ± 12.4	10.2 ± 0.3	12.5 ± 0.3
Significance (WKy versus SH) . .			n.s.	$P < 0.001$	n.s.	n.s.

RESULTS

Table 1 lists the body weights and blood pressures of WKy and SH rats used in the present study. At 12 weeks of age the systolic blood pressure of SH rats was significantly elevated. At 4 weeks of age, on the other hand, the blood pressure of SH rats remained normal.

Serum total calcium concentrations of SH rats were in the same range as those of WKy rats at either age (Table 2). There was no significant difference in serum $1,25(OH)_2D$ between SH and WKy rats at either age, although the concentration in SH rats tended to be lower than that in age-matched WKy rats (Table 2). When rats were treated with PTH, however, a marked difference in serum $1,25(OH)_2D$ of SH rats with age was observed. At 4 weeks of age, serum $1,25(OH)_2D$ increased 4-fold in SH rats, which is similar to the value obtained for WKy rats. By contrast, at 12 weeks of age, the increase in serum $1,25(OH)_2D$ in SH rats was approximately one-half that in WKy rats (Table 2). The serum 25(OH)D levels were in the same range for both SH and WKy rats (28.3 ± 0.7 ng/ml and 26.6 ± 0.7 ng/ml respectively). Rises in serum calcium concentration in SH and WKy rats at either age did not differ (Table 2).

Fig. 1 depicts the effect of PTH on the renal production of $1,25(OH)_2D$ in SH and WKy rats. The basal $1,25(OH)_2D$ productions by the renal mitochondria from SH rats were significantly higher than those from WKy rats both at 4 weeks of age (143.4 ± 17.6 vs. 48.0 ± 8.3 fmol/mg protein) and at 12 weeks of age (90.6 ± 16.4 vs. 45.8 ± 7.2 fmol/mg protein). Upon PTH injection, renal $1,25(OH)_2D$ production of SH rats at 4 weeks of age increased to 713.7 ± 23.4 fmol/mg of protein, a value that is not significantly different from that for WKy rats, namely 976.8 ± 120.2 fmol/mg of protein) (Fig. 1). On the basis of these data and the fact

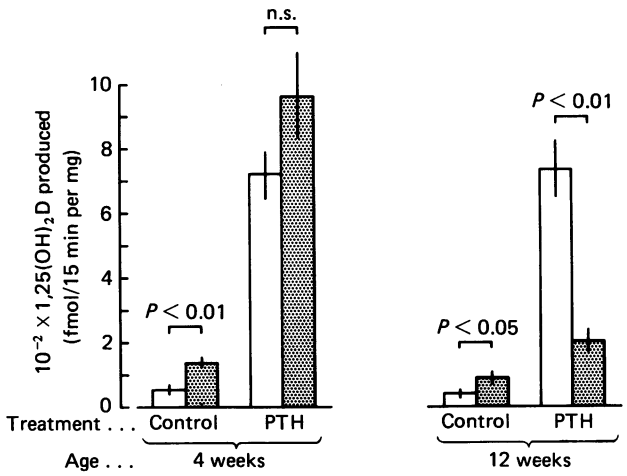

Fig. 1. Effect of PTH on the renal mitochondrial 1-hydroxylase activity in SH rats

Rats received subcutaneous injection of either PTH or vehicle at 0, 2, 4 and 6 h. Kidneys were excised at 8 h and mitochondria were prepared. $1,25(OH)_2D$ produced was determined as described in the text. The enzyme activity was expressed as fmol/mg of mitochondrial protein. Each column and bar represents the mean for four rats ± S.E.M. □, WKy; ▨, SH.

Table 3. Creatinine clearance in SH and WKy rats at 12 weeks of age

Rats were treated as described in Table 2 and anaesthetized with sodium pentobarbital. Rats were infused at a rate of 1 ml/h with a solution composed of 5 mM-$CaCl_2$, 5 mM-$MgCl_2$, 20 mM-NaCl, 2.5 mM-KCl and 4% (w/v) glucose, starting at 5 h. Urine was collected from 6 to 8 h via a catheter inserted into the bladder. Serum samples were obtained via a catheter inserted through the femoral vein. Abbreviation used: n.s., not significant.

Groups	No. of animals	Creatinine clearance (ml/h per 100 g body wt.) (mean ± S.E.M.) Control	PTH
WKy	4	0.68 ± 0.8	0.65 ± 0.7
SH	4	0.71 ± 0.8	0.63 ± 0.5
Significance (WKy versus SH) . . .		n.s.	n.s.

that the dosage level of PTH used in the present study is known to stimulate maximally the enzyme activity, thereby leading to a saturation of the enzyme activity, it is reasonable to assume that the responsiveness of the enzyme to exogenous PTH in SH rats at this age remains intact. By contrast, renal $1,25(OH)_2D$ production after PTH injection in SH rats at 12 weeks of age increased to only 202.3 ± 23.9 fmol/mg of protein, which is much less than in WKy rats, namely 738.8 ± 97.3 fmol/mg of protein) (Fig. 1).

As Table 3 shows, no difference in the creatinine clearance was observed between SH and WKy rats at 12 weeks of age.

DISCUSSION

The present study supports the previous observations by others (Shedl *et al.*, 1984; Stern *et al.*, 1984) that serum $1,25(OH)_2D$ in SH rats is in the normal range. Furthermore, our data demonstrate that rises in serum $1,25(OH)_2D$ in response to PTH injection are attenuated at 12 weeks of age but not at 4 weeks of age.

The reduced response of serum $1,25(OH)_2D$ concentration to PTH injection may be due to either reduced stimulation of 1-hydroxylase in the kidney or enhanced metabolism of $1,25(OH)_2D$. To test these possibilities, we measured 1-hydroxylase activity in the kidney. The enzyme activity in renal mitochondria from SH rats at 4 weeks of age responded properly to PTH (Fig. 1). By contrast, that from SH rats at 12 weeks of age showed a markedly reduced response, suggesting that the decreased serum $1,25(OH)_2D$ in response to PTH injection may be mainly due to the impaired response to PTH of 1-hydroxylase (Fig. 1).

Although normal renal function has been reported for SH rats as compared with age-matched WKy rats, it is possible that the observed reduction in response to PTH of 1-hydroxylase in SH rats at 12 weeks in the present study may have been a result of reduced renal function. It turned out not to be the case, however, since creatinine clearance in SH rats at this age did not differ from that in WKy rats (Table 3). Thus the attenuated response to PTH of 1-hydroxylase seems to be attributable to the decreased responsiveness of the enzyme activity itself. Two possible mechanisms may account for the attenuated responsiveness of the enzyme activity to PTH. One is that, with existing high levels of PTH in the SH rats, the renal 1-hydroxylase is incapable of further substantial increase in activity. This may be partly true, since basal $1,25(OH)_2D$ production by the kidney was higher in the SH rats as compared with that in WKy rats (Fig. 1), a finding that is consistent with the reported hyperparathyroidism. However, since PTH has been known to inhibit phosphate reabsorption in the renal proximal convoluted tubules where 60–70% of filtered load of phosphate is reabsorbed, and since the phosphaturic effect of PTH was greater in SH rats than in WKy rats (670 ± 85 mg/100 ml of urine as against 432 ± 69 mg/100 ml of urine) in the present study, it is unlikely that the inhibitory effect of PTH on the reabsorption of phosphate in this nephron segment is impaired in SH rats. It is well established that PTH activates the adenylate cyclase system through binding to its receptor in the proximal convoluted tubules, which, in turn, leads to the inhibition of phosphate reabsorption as well as to the stimulation of 1-hydroxylase. Taken together, the attenuated response of 1-hydroxylase to PTH in SH rats observed in the present study could not be explained by a simple down-regulation of receptor due to the existence of higher PTH levels. Another possibility is that the SH rat may have a general defect in the transport of cell calcium that itself could result in secondary impairment of the control of the 1-hydroxylase. Decreases in urinary calcium excretion after PTH injection were, however, in the same range in SH and WKy rats (5.1 ± 0.6 and 3.8 ± 0.5 mg/ml respectively), thus suggesting that cellular calcium transport in the kidney of SH rat remains intact, although the possibility that the intracellular calcium concentration remains higher in SH rats is not ruled out.

No data are available on serum PTH in SH rats at 4 weeks of age. However, as shown in Fig. 1, basal $1,25(OH)_2D$ production by the kidney from SH rats was higher than that in WKy rats. These data suggest hyperparathyroidism or the presence of agents that stimulate the renal 1-hydroxylase activity in SH rats even at 4 weeks of age, when blood pressure is normal. Similar serum $1,25(OH)_2D$ levels in SH and WKy rats, notwithstanding the higher basal $1,25(OH)_2D_3$ production by the kidney in SH rats (Fig. 1), suggest possible enhancement of metabolism and/or excretion of $1,25(OH)_2D$.

These data suggest that abnormality of calcium metabolism and, in particular, vitamin D metabolism, may contribute to the development of hypertension. Elucidation of (1) the mechanism underlying the effect of age on the impaired response to PTH of renal 1-hydroxylase and (2) the cause of higher basal enzyme activity even before the onset of hypertension, as well as the cause of enhanced metabolism of $1,25(OH)_2D$, may provide further insight into pathophysiology of hypertension.

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