PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 150, 142–147 (1975)

Analysis of the Renin–Angiotensin System during Fasting in Adult Male Rabbits (38991)

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The male rabbit, when deprived of food, develops a syndrome characterized by a triad of natriuresis, polyuria and polydipsia (1–3). Though a well-known phenomenon, the mechanism of the urinary Na^+ loss accompanying fasting is not known. In agreement with human studies (4), the natriuresis is due to the caloric deprivation and not to the concomitant Na^+ depletion (unpublished data). This Na^+ loss has been detected in the rabbit as early as 6 hr after food withdrawal and may well represent the primary, initial event that leads to an increased urine flow, reduced plasma volume and finally, the polydipsia. In view of the negative Na^+ balance, it is not surprising that an increased adrenal mineralocorticoid secretion occurs which limits this loss during caloric deprivation in the rabbit (5, 6), and that increases in aldosterone secretion as measured by direct or indirect indices have been observed during some phase of fasting (7–10), though not all reports are in agreement (11, 12). However, the increased mineralocorticoid secretion limits but does not prevent the natriuresis, and in fact, an unresponsiveness to the sodium retaining action of mineralocorticoid hormone during early starvation has been reported (10). A rise in plasma renin activity during fasting has been reported (8, 13), but in more recent and detailed clinical studies of the natriuresis of a total fast, the plasma renin activity fell (10), while the plasma renin concentration decreased at first and then rose markedly at a time when the Na^+ loss was diminished (9). Since dissociation between plasma aldosterone and renin in relation to Na^+ excretion occurred in early fasting, i.e., the Na^+ loss occurred when plasma aldosterone was elevated, while the renin activity and concentration were reduced, some other factor or condition must have counterbalanced the effectiveness of aldosterone. Studies in the human (14), rat (15–17), and dog (18) indicate that measurements of multiple, if not all, components of the renin-angiotensin system are required for proper analysis of changes. Accordingly, these studies were initiated to analyze more completely the changes which occur in the various components of the renin–angiotensin system during caloric deprivation. The rabbit was chosen since it has been used extensively as an experimental model of physiologic adjustments to caloric and sodium deprivation (1–3, 5, 6, 19–22).

Methods. Nine adult male New Zealand rabbits weighing 2.5–3.0 kg were used to obtain blood samples after a 4 day control period (fed) and after 3 days of total food deprivation; water was available, *ad libitum*, throughout. Each rabbit was cycled through this regimen several times following appropriate rest periods. An additional 12 male rabbits were similarly treated and used for the analysis of the pulmonary converting enzyme. Methods for the daily measurement of body weight, water intake and urinary volume and electrolytes have been reported (3, 6, 22). Results were statistically evaluated by means of Students' t test of significance.

Plasma Renin Activity (PRA). A 2.5-ml sample of blood was obtained by nicking the marginal ear vein with a scalpel and allowing the blood to drip into prechilled 6-ml hematocrit tubes (Sanford–Magath) containing 1.0 mg EDTA and setting in an ice-filled beaker. Plasma was separated by centrifugation at 4° and stored frozen at −20°. A 1.0 ml aliquot of the thawed (at 4°) plasma was transferred to incubation medium containing 2.0 ml of 0.2 M maleate buffer (pH 6.0), 10 μl of dimercaprol (BAL) and 10 μl of hydroxyquinoline. A 1.0 ml aliquot of this sample-incubation mixture was incubated for 1 hr at 37° and another 1.0 ml at 4°. The amount of angiotensin I formed in each incubation tube was measured by radioimmunoassay (RIA) using the New

England Nuclear Kit for angiotensin I. The PRA is expressed as ng of angiotensin I formed/ml plasma/hr.

Plasma Renin Concentration (PRC). The PRC was determined by measuring the angiotensin I formed (NEN RIA Kit) in plasma incubates containing an excess of renin substrate. The renin substrate was supplied by adding to the incubation medium plasma from a male rabbit nephrectomized 5 hr earlier. After 5 hr, the plasma of the nephrectomized rabbits contained negligible quantities of renin (0.75 ng angiotensin I formed/ml/hr), while the plasma angiotensinogen concentration (PAC) increased markedly to 3519 ng/ml; PAC in our intact rabbits is 483 ± 129 ng/ml. The incubation mixture contained 50 μl BAL, 50 μl hydroxyquinoline, 0.2 ml of test plasma, 0.9 ml maleate buffer and 0.8 ml of plasma from a nephrectomized rabbit and was incubated for 30 min at 37° in a shaker bath.

Plasma Angiotensinogen Concentration (PAC). PAC was measured by incubating 1.0 μl of plasma, obtained as described above, for 30 min at 37° in medium (pH 5.5) containing 0.96 ml of 0.2 *M* maleate buffer, 20 μl of BAL, 20 μl of 8-hydroxyquinoline and an excess of hog renin at 5 Goldblatt units/ml (Nutritional Biochemicals). The amount of angiotensin I formed was assayed by RIA, as above. The PAC is expressed as ng angiotensin I generated/ml plasma under these conditions. In preliminary experiments using 0, 1.0, 2.5, and 5.0 units of renin for 0, 15, 30, and 60 min of incubation, it was found that 5.0 U/ml clearly utilized all of the renin substrate in 15 min of incubation, while 2.5 U/ml nearly did so; 5.0 units/ml was selected as the excess level for these experiments.

The influence of PAC on the rate of generation of angiotensin I was determined by adding 0–0.8 ml of plasma from a nephrectomized rabbit (PAC = 3519 ng/ml; PRA = 0.75 ng/ml/hr) to incubation tubes containing 0.2 ml normal rabbit plasma (PAC = 483 ng/ml; PRA = 4.5 ng/ml/hr) and determining the amount of angiotensin I formed in 30 min by RIA.

Pulmonary Angiotensin I Converting Enzyme (PCE). The angiotensin I CE from pulmonary tissue was estimated by measuring the quantity of labeled His–Leu and Leu released from angiotensin I labeled with tritium in the COOH-terminal leucine by combining the method of Soffer, Reza and Caldwell (23) with modifications from the method of Boucher *et al.* (24). Following sacrifice of the rabbits by stunning and cervical fracture, the lungs were quickly removed and transferred to the cold room. All subsequent procedures were performed at 4°.

The lung tissue was washed free of blood with cold running tap water, dissected free of fat, large bronchi and conducting airways, and rewashed with cold homogenizing buffer. The single whole lung was then blotted, weighed, chopped and homogenized (DiVitris, setting 5) for three 30-sec periods in an ice bath; a single dissected lung provided an average of 10.0g of parenchymal tissue. The homogenizing medium (2 ml medium/g tissue) was composed of 20 m*M* Tris HCl buffer (pH 7.8) containing 5 m*M* Mg acetate, 30 m*M* KCl and 0.25 *M* sucrose. The homogenate was centrifuged at 700*g* for 10 min. An 0.2 ml aliquot of the supernatant was transferred to the reaction mixture containing 0.3 ml of 50 m*M* Tris HCl (pH 7.4) with 30 m*M* NaCl and 50 μl ^{3}H-angiotensin I (sp act = 29.4 mCi/mg, Schwarz–Mann) labeled at terminal leucine (1 μCi) with 0.4 m*M* unlabeled angiotensin I. The reaction mixture was incubated at 37° for 20 min in a shaker bath. The reaction was stopped with 0.5 ml of 0.2 *M* pyridinium acetate (pH 3.1).

A 0.5 ml aliquot of the reaction mixture was applied to a 6 cm resin column (Dowex 50W-X2) contained in a 1-ml Tuberculin glass syringe that had been equilibrated with the starting buffer. Three fractions were collected into scintillation vials: (a) Leucine was eluted with 0.8 ml of 0.2 *M* pyridinium acetate (pH 3.1), (b) The dipeptide, His–Leu was eluted with 0.8 ml of 0.9 *M* pyridinium acetate (pH 4.2), and (c) angiotensin I was eluted with 0.8 ml of 0.2 *N* NH_4OH followed by 0.8 ml of 0.1 *N* diethylamine. The radioactivity of the fractions was counted with a Packard Tri-Carb Spectrometer using 10 ml of PCS (Amersham/Searle) scintillation fluid. Recovery of radioactivity from the columns averaged

95% and ranged from 91 to 99%. The sum of the ^{3}H-Leu and His–^{3}H-Leu cpm divided by the total counts of the three fractions × 100 equals the percent conversion of ^{3}H-angiotensin I to His-Leu and Leu. The validity of including the Leu counts can be questioned, but with 20 min incubation, only 1–2% of the total counts were found in the Leu fraction, and this was true for tissues from control and deprived rabbits. There was no significant difference in values for PCE when calculated as conversion to His–Leu only versus His–Leu + Leu. The lung CE is expressed as the μg angiotensin I converted/hr/ml supernatant of the 700*g* spun homogenate.

In preliminary studies, ^{3}H-Leu, unlabeled His–Leu and ^{3}H-angiotensin I were applied individually and in combination to the column to identify and quantify their presence in the three fractions in order to characterize the column separation. Unlabeled His–Leu was identified and quantified with the ninhydrin reaction. 99% of ^{3}H-Leu was recovered in Fraction a, 96% of His–Leu in Fraction b and 98% of ^{3}H-angiotensin I in Fraction c. The percent conversion of blanks (no tissue) at 37° and tissue reaction mixtures incubated at 4° averaged <1.0%.

Plasma angiotensin I and II. Plasma angiotensin I was measured by RIA (NEN Kit). The total plasma angiotensin I and II were measured by the bioassay method of Boucher *et al.* (24) with modifications from that of Lazar *et al.* (25), utilizing the rise in blood pressure of pentolinium treated, anaesthetized rats following injection of angiotensin II standards (Hypertensin, Ciba) and plasma extracts from normal or food-deprived rabbits; the plasma angiotensin I and II were isolated and purified by column (Dowex 50W-X2) and paper chromatography (24). Blood pressure was measured with a Statham P23Db transducer using femoral artery cannulation and recorded with a Physiograph (E & M Instruments Co.). Injections were made via femoral vein cannulation. All samples were assayed in triplicate with standard bracketing. Only those assay animals displaying a 10 mm Hg or greater response to 1 ng of angiotensin II standard were used.

Results. In all experiments, the complete withdrawal of calories for 3 days resulted in the development of a polydipsia, polyuria and natriuresis; all three increments were highly significant ($P < .001$, Table I; $P < .005$, Table II). These enhanced daily urinary Na$^+$ excretions and fluid exchanges were returned to their respective normal control values by refeeding, *ad libitum*, for 1 day (Table I).

The average normal control PRA was 4.5 ± 0.2, expressed as ng angiotensin I

TABLE I. ANALYSIS OF THE RENIN–ANGIOTENSIN SYSTEM FOLLOWING 3 DAYS OF CALORIC DEPRIVATION IN ADULT MALE RABBITS[a]

	Control (fed)	Caloric deprivation (for 3 days)	Recovery (refed 1 day)
Body weight (kg)	2.8±0.1 (21)	2.6±0.1 (20)	2.9±0.2 (7)
Water intake (ml/kg/day)	137±7 (17)	300±21* (20)	128±11 (7)
Urine volume (ml/kg/day)	50±4 (17)	206±20* (20)	46±4 (7)
Urinary Na$^+$ (mEq/kg/day)	0.005±0.008 (17)	0.2±0.7* (16)	.01±.001 (4)
PRA (ng angiotensin I/ml/hr)	4.5±0.2 (14)	4.9±0.3 (14)	4.6±0.3 (8)
PRC (ng angiotensin I/ml/hr)	42.5±5.4 (8)	11.5±1.5* (8)	—
PAC (ng angiotensin I/ml)	483±129 (6)	1841±90* (3)	—
Plasma angiotensin I (ng/ml)	3.2±0.3 (11)	.02±0* (14)	2.6±0.1 (7)
Plasma angiotensin I and II (ng/ml)	3.9±0.7 (6)	7.9±1.1** (6)	—

[a] PRA = plasma renin activity in ng angiotensin I generated/ml/hr. PRC = plasma renin concentration in ng angiotensin I generated/ml/hr. PAC = plasma angiotensinogen conc. in ng angiotensin I generated/ml plasma. (#) = number of measurements.

* $P < 0.001$, ** $P < 0.005$ compared with its control. Each value is the mean ± SE for the last day of control, deprivation or refeeding.

TABLE II. INFLUENCE OF CALORIC DEPRIVATION ON PULMONARY CONVERTING ENZYME (PCE) IN THE ADULT MALE RABBIT.

	Control (fed)	Caloric deprivation (for 3 days)
Number of rabbits	6	6
Body weight (kg)	3.2±0.1	3.1±0.2
Water intake (ml/kg/day)	116±7	211±13[a]
Urine volume (ml/kg/day)	33±6	191±48[a]
Urinary Na^+ (mEq/kg/day)	0.003±0	0.383±0.11[a]
Lung converting enzyme (PCE):		
% conversion Angio[b] I → II	59±3	78±1[a]
μg Angio I converted/hr/ml[c]	244.4±10.9	324.2±4.4[a]

[a] $P < .005$ when compared with control value.

[b] Angio = angiotensin.

[c] PCE expressed as μg angiotensin I converted to II/hr/ml supernatant of the 700*g* spun homogenate. Each value is the mean ± SE for the last day of control or deprivation.

formed/ml plasma/hr; the PRA remained at normal levels (4.9 ± 0.3) following 3 days of caloric deprivation (Table I).

The average normal PRC was 42.5 ± 5.4, expressed as ng angiotensin I formed/ml plasma/hr; the PRC was significantly reduced ($P < .001$) to about a fourth of normal, 11.5 ± 1.5, after 3 days of deprivation (Table I).

Caloric deprivation induced a significant ($P < .001$) increase in the PAC; the PAC rose from 483 ± 129 to 1841 ± 90, expressed as ng angiotensin I generated/ml plasma (Table I).

Plasma angiotensin I (ng/ml) was reduced ($P < .001$) to an extremely low level, 0.02 ± 0, as compared with the average normal control value of 3.2 ± 0.3 ng/ml (Table I). In contrast, the combined plasma angiotensin I and II, which must therefore represent mostly angiotensin II, was elevated ($P < .005$) by about 100 percent, from 3.9 ± 0.7 to 7.9 ± 1.1 ng/ml (Table I).

Using a separate set of 12 male rabbits, 3 days of caloric deprivation significantly ($P < .005$; Table II) increased the amount of converting enzyme in lung parenchymal tissue, when expressed as the percent conversion of angiotensin I to angiotensin II, (from 59 ± 3 to 78 ± 1%), or as μg angiotensin I converted to II/hr/ml of 700*g* supernatant, (from 244.4 ± 10.9 to 324.2 ± 4.4 μg/hr/ml supernatant). When basing the % conversion on the His–Leu counts only, their values (%) are: 57 ± 2 (control) and 77 ± 2 (deprived).

Discussion. Consistent with prior reports (1–3), total food deprivation for 3 days resulted in significant increases in daily urinary Na^+ excretion, urinary volume and fluid intake in all of the male rabbits studied; refeeding for 1 day returned all parameters to normal. Of these changes, the enhanced Na^+ excretion has been detected as early as 6 hr following caloric deprivation, and its progressive, continuous nature accounts for a significant reduction in plasma volume and the consequent stimulation of thirst with a reduction in plasma osmolality (unpublished data). Reduced renal tubular water reabsorption occurs (22) as a consequence of reduced vasopression secretion (19–21) and maintained adrenal glucocortical hormone secretion; the latter is obligatory for the enhanced fluid exchange to occur (6). The mechanisms of the enhanced fluid exchanges and their inhibition by estrogens have been the subject of previous publications (1–3, 5, 6, 19–22).

Since food deprivation leads to a Na^+ deficit, a stimulus to the renin–angiotensin–aldosterone system, it is not surprising that plasma angiotensin II was significantly elevated. Such a finding is consonant with our demonstration that adrenal mineralocorticoid secretion is enhanced and limits the Na^+ loss during caloric deprivation in the rabbit. Treatment of food-deprived male rabbits with a blocker of mineralocortical hormone action or adrenalectomy of hydrocortisone-maintained, food-deprived male rabbits induces an increase in urinary Na^+ loss as compared with intact food-deprived males (5, 6). Others have reported increases in aldosterone secretion during fasting (7, 10), though some have failed to detect an increase (11, 12). The elevated plasma angiotensin II is consistent with and could account for the enhanced mineralocorticoid secretion during caloric deprivation in the male rabbit. The plasma angiotensin I, as measured by RIA was drastically reduced during deprivation, hence the increased total plasma angiotensin I and II, measured by bioassay, must have been solely due to an increase in the circulating angiotensin II. The normal plasma angiotensin II concentration, obtained by subtracting angiotensin I from the I plus II concentration, was 0.7 ng/ml; the latter was elevated to about 7.9 ng/ml during deprivation (Table I).

The PRC decreased, while plasma aldosterone rose during the early (natriuretic) phase of total fasting in human subjects; PRC then began to increase after about 5 days when Na^+ conservation occurred (9). This dissociation between PRC and plasma aldosterone concentration occurred only in the early (natriuretic) phase of fasting but not at all during Na^+ deprivation in which both parameters were increased. A similar dissociation between aldosterone secretory rate (high) and PRA (low) during the natriuresis of starvation has been reported (10); the aldosterone secretory rate fell and the PRA rose during the antinatriuretic phase of refeeding. The stimulus causing the increased aldosterone secretion cannot be discerned because only a single component of the renin–angiotensin system was determined. Our findings are similar to those of Chinn (9) in that the PRC was reduced during caloric withdrawal, but in contrast, the PRA remained at normal levels. For this to be true, the renin substrate, PAC, must have increased. Indeed, both the PAC and the PCE were increased by about 280% and 33%, respectively. These data explain the maintenance of normal levels of PRA, the decrease in angiotensin I and the increase in angiotensin II plasma concentrations despite a 73% reduction in PRC. Hence, the enhanced mineralocorticoid secretion and limited Na^+ loss are explained.

It is implicit in the above argument that the renin–renin substrate reaction normally is limited by substrate concentration in the rabbit. When the PAC was varied over a wide range (100–2200 ng/ml), the velocity of angiotensin I formation increased. Maximal velocity (32 ng angiotensin I formed/ml/hr) occurred at a substrate concentration of 2200 ng angiotensin I formed/ml. One-half maximal velocity (16 ng angiotensin I formed/ml/hr) corresponds to a PAC of 820 ng angiotensin I formed/ml. Since the normal PAC for our rabbits is about 500 ng angiotensin I formed/ml, the velocity of angiotensin I formation at any given concentration of renin is well below the one-half maximum; thus, PAC is rate limiting in the adult male New Zealand rabbit as it is in dog, man, and rat (18, 26, 17).

The significant increase in PCE is probably not related to the attendant Na^+ deficit. Kurihara and his co-workers (27, 28) found that long-term changes in Na^+ intake affected PRA but not the plasma CE activity in rabbits; they did not determine the pulmonary CE activity which would have provided a more relevant comparison.

The changes in the renin–angiotensin system detected in this study could account for the increased mineralocorticoid secretion and minimized Na^+ loss in the calorically deprived male rabbit (5, 6). However, their magnitude or effectiveness is apparently not sufficient to prevent the characteristically continued Na^+ loss. An unresponsiveness to the Na^+ retaining action of exogenous mineralocorticoid during early starvation in the human has been reported (10). We found the natriuresis of caloric deprivation unaltered by exogenous mineralocorticoid (0.1 mg/kg/day × 7 days of deoxycorticosterone acetate, unpublished data). This is not unexpected since endogenous mineralocorticoid secretion is already elevated under these conditions (5, 6), and the physiological status may represent an unfair test condition to study sensitivity to Na^+-retaining hormones, particularly in view of the zero Na^+ intake. The marked changes in the angiotensin system noted in the plasma and lungs during caloric deprivation may also occur within renal tissue and influence renal Na^+ excretion independently of circulating mineralocortical hormone. The renal vascular resistance of the isolated rabbit kidney (30) and intrarenal blood distribution of the isolated dog kidney (31) have a dependency on the concentration of renin substrate in the perfusate. An intrarenal effect of the components of the renin-angiotensin system on Na$^+$ excretion has been proposed (29–32); such an action may be the mechanism of the natriuresis of fasting and warrants further study.

Summary. Caloric deprivation for 3 days in adult male rabbits induced significant increases in daily urinary Na$^+$ excretion, urinary volume and fluid intake as previously reported. These changes were accompanied by: (a) a significant reduction in plasma renin concentration; (b) an unchanged plasma renin activity; (c) a marked increase in the plasma angiotensinogen concentration; (d) a significant reduction in plasma angiotensin I; and (e) a significant increase in plasma angiotensin II. In a separate group of adult male rabbits, 3 days of caloric deprivation significantly increased the amount of converting enzyme in pulmonary parenchymal tissue. These findings correlate with the previously reported enhancement of mineralocortical hormone secretion and limiting effect of the latter on the natriuresis of caloric withdrawal. Since the increased mineralocortical hormone secretion does not prevent the natriuresis, the possibility that these striking changes in the components of the renin–angiotensin system during caloric deprivation may exert intrarenal effects is discussed.

The authors wish to thank Dr. R. O. Soffer, Albert Einstein College of Medicine, for his helpful suggestions and for providing us with a preprint of his method for determining the pulmonary converting enzyme. We also thank Mr. John Hegmann for his excellent technical assistance.

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Received March 10, 1975. P.S.E.B.M. 1975, Vol. 150.