EVIDENCE IN VOLUME-DEPENDENT HYPERTENSION FOR AN AUGMENTING FACTOR FOR NOREPINEPHRINE OVERFLOW FROM SYMPATHETIC NERVE ENDINGS

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It has been reported that salt loading induces a blood pressure rise in rats whose renal mass has been reduced. We examined the involvement of the peripheral sympathetic nervous system in the pathogenesis of hypertension in subtotally nephrectomized and salt-loaded rats. Male Wistar rats (200–240g) underwent subtotal nephrectomy (removal of 70–80% of the renal mass). After surgery, 1% saline was given ad libitum as drinking water in the experimental group (E), while tap water was given to the controls (C). On the 10th day after nephrectomy, blood pressure was determined, and plasma samples were collected. Deproteinized plasma samples were diluted with Ringer-Locke solution (1/40 v/v), and were perfused into isolated mesenteric artery-intestinal loop preparations of normotensive male Wistar rats. Pressor responses and norepinephrine overflow induced by electrical nerve stimulation, and the pressor responses to exogenous norepinephrine were assessed before and after the addition of deproteinized plasma to the perfusate. Systolic blood pressure was significantly elevated in the experimental group (E: 150 ± 5 mmHg, C: 109 ± 4 mmHg, p < 0.01). When deproteinized plasma from the experimental group was perfused, pressor responses to electrical nerve stimulation were significantly increased (E: 167.2 ± 13.7%, C: 91.1 ± 6.4%, p < 0.01), as was norepinephrine overflow (E: 117.3 ± 6.4%, C: 90.7 ± 5.9%, p < 0.05), while responses to exogenous norepinephrine were only slightly augmented compared with the control group (E: 120.0 ± 2.7%, C: 105.5 ± 9.0%; n.s.). Deproteinized plasma from the experimental rats highly inhibited Na-K ATPase (E: 24.7 ± 3.8%, C: 2.4 ± 1.1%, p < 0.01) and strongly cross-reacted with antidigoxin antibodies being much more active than deproteinized plasma from control rats. Moreover, there was a significant positive correlation between Na-K ATPase inhibition and blood pressure (p < 0.01).

These data indicate that plasma from reduced renal mass-salt hypertensive rats contains an augmenting factor for norepinephrine overflow from sympathetic nerve endings. This factor might be Na-K ATPase inhibitor.

Key words:
Augmenting factor for norepinephrine overflow
Sympathetic nerve endings
Reduced renal mass-salt hypertensive rat
Na-K ATPase inhibitor
Mesenteric artery

EXCESSIVE dietary intake of sodium appears to play a significant role in the pathogenesis of human essential hypertension. To study the contribution of sodium to hypertension, several experimental models have been

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developed that require high sodium intake to induce blood pressure elevation. The reduced renal mass-saline model is one of these high sodium models for hypertension. Animals can be made hypertensive by removal of approximately 70–80% of the renal mass and the substitution of 1% saline for drinking water.6 Furthermore, rats with reduced renal mass hypertension have been reported to be volume-expanded and to have low plasma renin activity.3,4 Huot et al.3 observed that the release of a sodium-potassium adenosine triphosphatase (Na-K ATPase) inhibitor was implicated in this form of hypertension. It has been reported7 that deproteinized plasma from patients with essential hypertension contains elevated levels of a digitalis-like inhibitor of dog kidney Na-K ATPase. Moreover, Blaustein suggests that this inhibitor of the sodium pump may increase norepinephrine release from sympathetic nerve endings and reduce its re-uptake.8 We previously reported that blood pressure elevation induced by salt loading and partial nephrectomy in rats might be associated with increased plasma norepinephrine.2 However, the mechanism of increased sympathetic nerve activity in this form of hypertension is unclear.

In this study, we have examined the effects of deproteinized plasma from reduced renal mass-salt hypertensive rats on the pressor responses of rat mesenteric vasculatures to electrical nerve stimulation and exogenous norepinephrine. In addition, the effects of deproteinized plasma on norepinephrine release from sympathetic nerve endings were also studied.

**MATERIALS AND METHODS**

1. Blood sample preparation

Thirty-six normotensive male Wistar rats, weighing 200–240g, were divided into control and experimental groups. Under pentobarbital anesthesia (intraperitoneal injection, 50 mg/kg), all rats underwent subtotal nephrectomy (70–80% renal mass removed). This was achieved by removing the right kidney and 50% of the left kidney (25% of upper and lower poles) by encircling them with loops of 4–0 silk suture and then tightening the loops. After nephrectomy, the control animals (n = 18) were allowed to drink water ad libitum while the experimental animals (n = 18) received 1% saline ad libitum. On the 9th day, the control rats (n = 8) and experimental rats (n = 9) were kept in metabolic cages for measurements of water intake, salt intake, urinary volume and urinary sodium excretion to be taken. Urinary sodium was analyzed with a flame photometer (Instrumentation Laboratory 643). On the 10th day, systolic blood pressure, heart rate (tail plethysmography) and body weight were measured. All rats were then anesthetized with pentobarbital and blood samples collected into chilled, heparinized tubes from the inferior vena cavae. Plasma from both groups was immediately separated from the cells by centrifugation (3,000 × g, 10 min, 4°C), and boiled for 2 min. After cooling, the resulting clot was disrupted and centrifuged for 60 min at 4°C and 10,000 × g. The supernatants (deproteinized plasma) were collected and stored at −20°C.

2. Effects of deproteinized plasma on the peripheral sympathetic nervous system

Male Wistar rats, weighing 300–340g, were anesthetized with pentobarbital (intraperitoneal injection, 50 mg/kg) and isolated mesenteric vasculatures were prepared according to the method described by Castellucci et al.10,11 The preparations were perfused with modified Ringer-Locke solution (mmol/L: NaCl 120.7, KCl 5.9, CaCl₂ 2.5, MgCl₂ 1.3, NaHCO₃ 15.5, NaH₂PO₄ 1.2, glucose 11.5; pH 7.4, 37°C). The solution was bubbled with a 95% O₂-5% CO₂ mixture and maintained at a flow rate of 0.8 ml/min with a peristaltic pump (Harvard Apparatus, Model 1200). The perfusion pressure was recorded via a side-arm with a pressure transducer connected to a polygraph (Nihon Kohden, Model CP-620G). Platinum electrodes were placed around the perirenal plexus of the mesenteric arteries. A 30-min equilibration period was allowed before starting the experiments. The intramuscular sympathetic nerves were stimulated at 40 volts for 1 min with biphasic rectangular pulses of 5 msec-duration at 15 Hz, with an electric stimulator (Nihon Kohden, Model SEN-3201). Exogenous 1-norepinephrine (3.3 μg) was given as a single injection in 0.1 ml of buffer into the arterial cannula. The fluid perfused through the mesenteric preparation was collected into tubes containing a mixture of EGTA (90 mg/ml) and glutathione (60 mg/ml) in a ratio of 20 μl/1 ml of perfusate, for a 3-min duration before and after electrical nerve stimulation. The latter collecting period consisted of a 1-min stimulatory period and a 2-min follow-up period.

Norepinephrine in the perfusate was adsorbed on to alumina, extracted in 0.1N perchloric acid.
### TABLE I  WATER INTAKE, URINARY VOLUME, SALT INTAKE AND URINARY SODIUM EXCRETION IN CONTROL AND EXPERIMENTAL RATS

<table>
<thead>
<tr>
<th></th>
<th>Control Rats (n = 8)</th>
<th>Na-Loaded Rats (n = 9)</th>
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<tbody>
<tr>
<td>Water intake (ml/day)</td>
<td>14.6 ± 4.2</td>
<td>26.0 ± 3.7</td>
</tr>
<tr>
<td>Urinary volume (ml/day)</td>
<td>19.2 ± 2.0</td>
<td>28.7 ± 2.0</td>
</tr>
<tr>
<td>Sodium intake (µEq/day)</td>
<td>393 ± 92</td>
<td>4719 ± 713</td>
</tr>
<tr>
<td>Urinary sodium excretion (µEq/day)</td>
<td>513 ± 73</td>
<td>4742 ± 663</td>
</tr>
</tbody>
</table>

*means ± SEM*

and then assayed using high pressure liquid chromatography (column: Biophase ODS 5 µm column; the solvent for the separation of norepinephrine was 0.1 M monochloric acid, pH 3.0, 2.0 mM EDTA-Na and 25 mg/L of sodium octyl sulfate) with an electrochemical detector (Bioanalytical Systems, Model LC-4A; carbon electrode; 700 mV). The norepinephrine release evoked by nerve stimulation was defined as the difference in norepinephrine content between the pre- and post-stimulatory periods.

The effects of deproteinized plasma from the control rats (n = 9) and experimental rats (n = 8) were assessed by the change in both pressor response and norepinephrine overflow induced by electrical nerve stimulation at 15 Hz, and the pressor response induced by 3.3 µg of exogenous norepinephrine, which caused almost maximal vasodepression. After control stimulation (S1), deproteinized plasma was added to the perfusion medium at a final concentration of 1/40 vol/vol. The next electrical nerve stimulation or exogenous norepinephrine application were performed 15 min (S2) and 27 min (S3) after the start of plasma infusion. The effects of the deproteinized plasma were evaluated by comparing the value of S1 and that of (S2 + S3)/2 in each preparation. In preliminary experiments we have already shown that an electrically-induced increase in norepinephrine overflow was completely blocked when guanethidine was added to the perfusion medium. This evidence indicated that the periartrial electrical stimulation was neuronal in nature. It was also demonstrated that the pressor responses and norepinephrine overflow did not significantly change during at least seven repeated stimuli to the same preparation, thus indicating reproducibility for this experimental technique.

3. Examination of Na-K ATPase inhibitor

Deproteinized plasma samples from the control rats (n = 9) and experimental rats (n = 10) were examined for their ability to inhibit dog kidney Na-K ATPase activity using an affinity chromatography technique for Na-K ATPase inhibitor described by Henning et al.

Dog kidney Na-K ATPase (EC3.6.1.3, Sigma) was immobilized by aggregation of ovalbumin (final concentration 4%) with 1 mg Na-K ATPase protein, in the presence of 0.36% glutaraldehyde, 2.5 mM EGTA, and 63 mM phosphate buffer, pH 7.6, in a final volume of 3.5 ml. The solution was frozen at −20°C for 4 h and then thawed in a cold room at 4°C, after which a sponge-like protein polymer was easily removed from the tube. After rinsing with distilled water the insoluble aggregate was lyophilized. A light hydrophilic porous powder was obtained and stored at −20°C.

Batch-wise affinity chromatography was performed by incubation of the immobilized enzyme with deproteinized plasma. One ml of deproteinized plasma was mixed with 5 mg of immobilized Na-K ATPase powder and this mixture was incubated for 30 min at 37°C. The reaction was stopped by centrifugation at 4°C, the pellets were washed at 4°C three times with 1 ml of distilled water, once with 1 ml of 0.5 M ammonium acetate and once with 1 ml of 1.0 M ammonium acetate. The last supernatant was collected, lyophilized, reconstituted in 100 µl of
water and tested for its inhibitory effect on non-immobilized dog kidney Na-K ATPase activity.

Dog kidney Na-K ATPase (1.6 μg protein) was incubated at 37°C for 45 min in 120 μl of the following (a), (b) and (c) media: (a) 80 mM Tris-HCl, 100 mM NaCl, 5 mM KCl, 3 mM MgCl₂, 1 mM EGTA, 2 mM ATP (vanadate-free, Sigma), 20 nCi/ml of [γ-³²P]-ATP (3,000 Ci/mmol, Amersham), pH 7.4; (b) medium (a) plus 0.1 mM ouabain; (c), medium (a) plus 10 μl of fractionated plasma. The reaction was stopped by sudden cooling at 4°C and by addition of cold perchloric acid (10% final concentration). Then, 0.5 ml of cold charcoal suspension (20%, w/v) was added, and after 5 min the mixture was centrifuged for 3 min at 15,000 x g. The resulting supernatant was analyzed for its ³²P content in a liquid scintillation counter. The effects of the plasma fractions were expressed as percentages inhibition of ouabain-sensitive Na-K ATPase activity, obtained by the equation, [(a)–(c)]/[(a)–(b)].

Furthermore, plasma fractions fractionated by affinity chromatography were tested for their cross-reactivity with antidiogxin antibodies using a kit from Dainabot.

Values are represented as means ± SEM. Statistical significance was determined by the Student's t-test and analysis of variance. A level of p < 0.05 was taken as significant.

RESULTS

Systolic blood pressure (BP), heart rate (HR) and body weight (BW) of the control and experimental rats were not significantly different before subtotal nephrectomy. On the 10th day after surgery, systolic blood pressure and heart rate were significantly elevated in the saline-drinking rats compared with those drinking water (BP: 150 ± 5 mmHg, v.s. 109 ± 4 mmHg, p < 0.01, HR: 475 ± 8 beats/min, v.s. 440 ± 13 beats/min, p < 0.05), while body weight was significantly lower in the experimental group (BW: 237 ± 6g, v.s. 253 ± 7g, p < 0.05).

Table I shows water and sodium balance. Water intake, urinary volume, sodium intake and urinary sodium excretion were significantly higher in the saline-drinking than in the water-drinking rats.

Figure 1 shows an example of vascular reactivity and norepinephrine overflow induced by electrical nerve stimulation of isolated mesenteric artery-intestinal loop preparations perfused with control or experimental rat deproteinized plasma samples. The baseline perfusion pressure did not change following addition of deproteinized plasma from either control or experimental rats. There was no significant difference in the basal norepinephrine overflow (3-min non-stimulated period) in the absence or presence of plasma (without plasma: 0.22 ± 0.03 ng/g wet tissue weight, n = 9; with plasma: 0.23 ± 0.02 ng/g wet tissue weight, n = 9). Both vascular reactivity and norepinephrine overflow following electrical stimulation were increased by addition of deproteinized plasma samples from the experimental group, but these changes were not ob-
Augmenting Factor for Norepinephrine Overflow

Fig. 2. The effects of deproteinized plasma from control and experimental rats on (A) pressor responses, (B) norepinephrine overflow during electrical nerve stimulation (ES; 15 Hz), and (C) pressor responses to exogenous norepinephrine (NE; 3.3 μg) in isolated mesenteric artery-loop preparations of normotensive Wistar rats. Values are represented as percentages of the corresponding control values in the absence of deproteinized plasma.

TABLE II
THE EFFECTS OF DEPROTEINIZED PLASMA FROM CONTROL AND EXPERIMENTAL RATS ON (A) PRESSOR RESPONSES, (B) NOREPINEPHRINE OVERFLOW DURING ELECTRICAL NERVE STIMULATION (ES; 15 Hz), AND (C) PRESSOR RESPONSES TO EXOGENOUS NOREPINEPHRINE (NE; 3.3 μg) IN ISOLATED MESENTERIC ARTERY-LOOP PREPARATIONS OF NORMOTENSIVE WISTAR RATS

<table>
<thead>
<tr>
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<th>Control Rats (n)</th>
<th>Na-Loaded Rats (n)</th>
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<tr>
<td>(A) Pressor Response to ES (mmHg)</td>
<td></td>
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<tr>
<td>Plasma (-)</td>
<td>53.7 ± 1.6 (5)</td>
<td>56.3 ± 2.8 (4)</td>
</tr>
<tr>
<td>Plasma (+)</td>
<td>49.0 ± 1.4 (5)</td>
<td>94.2 ± 4.8 (4)</td>
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<tr>
<td></td>
<td></td>
<td>p &lt; 0.01</td>
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<tr>
<td>(B) NE Overflow by ES (ng/g wet tissue weight)</td>
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<td></td>
</tr>
<tr>
<td>Plasma (-)</td>
<td>0.91 ± 0.04 (5)</td>
<td>1.03 ± 0.02 (4)</td>
</tr>
<tr>
<td>Plasma (+)</td>
<td>0.82 ± 0.03 (5)</td>
<td>1.21 ± 0.02 (4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p &lt; 0.05</td>
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<tr>
<td>(C) Pressor Response to Exogenous NE (mmHg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma (-)</td>
<td>49.9 ± 7.5 (4)</td>
<td>50.9 ± 5.9 (5)</td>
</tr>
<tr>
<td>Plasma (+)</td>
<td>54.0 ± 7.3 (4)</td>
<td>61.2 ± 6.9 (5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>n.s.</td>
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Values are represented as means ± SEM in the absence of deproteinized plasma (plasma (-)) and in its presence (plasma (+)).

served in the case of the controls.

The effects of deproteinized plasma on the pressor responses and norepinephrine overflow during electrical nerve stimulation (15 Hz), and on the pressor responses to exogenous norepinephrine (3.3 μg) in the isolated mesenteric vasculature are shown in Fig. 2 and Table II. Pressor responses to electrical nerve stimulation
were greater following the addition of experimental rat deproteinized plasma than following addition of control rat deproteinized plasma (167.2 ± 13.7%, v.s. 91.1 ± 6.4%; p < 0.01). Norepinephrine overflow from sympathetic nerve endings following electrical stimulation was also significantly greater in tissues perfused with experimental group deproteinized plasma than in those perfused with control deproteinized plasma (117.3 ± 6.4%, v.s. 90.7 ± 5.9%, p < 0.05).

However, vasoconstrictor responses to exogenous norepinephrine were only slightly higher in tissues perfused with experimental rat deproteinized plasma than in the controls (120.0 ± 2.7%, v.s. 105.5 ± 9.0%; n.s.).

Figure 3 shows the percentage inhibition of Na-K ATPase and the equivalent digoxin concentration. The inhibitory effect was significantly higher with deproteinized plasma from salt-loaded rats than with that from controls (24.7 ± 3.8%, v.s. 2.4 ± 1.1%; p < 0.01). Deproteinized plasma from the saline-drinking rats cross-reacted with antidigoxin antibody (0.61 ± 0.08 ng/ml), then value obtained being significantly higher than that obtained with control rat plasma (0.03 ± 0.03 ng/ml). Moreover, there was a significant positive correlation between Na-K ATPase inhibition and blood pressure (Fig. 4).

**DISCUSSION**

It has been reported that rats with reduced renal mass-salt hypertension (RRM-salt hypertensive rats) are volume-expanded and have low plasma renin activity⁴,⁵. If the model used here is volume-expanded, vasopressin might induce water retention and volume expansion. There is in fact evidence for a marked increase in vasopressin release from the neurohypophysis in RRM-salt hypertensive rats⁶.⁷ Gavras⁸ observed that when subtotally nephrectomized rats (85% removal of renal mass) were maintained on a high salt diet for one week, they had mild

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hypertension (systolic BP; 154 mmHg), and that injection of arginine-vasopressin antagonist resulted in only a small decrease in blood pressure, indicating that vasopressin was only minimally responsible for the maintenance of high blood pressure at that stage. Moreover, he reported that plasma levels of norepinephrine were higher in the high salt group.

Some studies have demonstrated that sympathetic nerve activity is increased under conditions of impaired sodium excretion from the kidney\textsuperscript{9,15–18}. Watanabe\textsuperscript{16} showed that plasma norepinephrine was increased in hypertensive nephritic rats with salt loading. Moreover, Ishii et al\textsuperscript{18} reported that there was a highly significant positive correlation between mean blood pressure and plasma norepinephrine concentration in nephritic patients (serum creatinine concentrations less than 1.6 mg/dl). However, the mechanisms involved in the enhancement of sympathetic nerve activity in sodium-induced hypertension remain unclear.

The present experiments were designed to investigate the humoral factor affecting the peripheral sympathetic nervous system in the pathogenesis of hypertension in subtotaly nephrectomized salt-loaded rats. It was demonstrated that endogenous norepinephrine overflow following electrical nerve stimulation of mesenteric vascular beds was enhanced by addition of deproteinized plasma from RRM-salt hypertensive rats above levels produced in the presence of deproteinized plasma from RRM-normotensive rats. Vasconstrictor responses to electrical nerve stimulation were also significantly increased in the presence of experimental group deproteinized plasma compared with controls. However, there was no significant difference in the vasconstrictor response to exogenous noradrenaline between tissues perfused with deproteinized plasma from the experimental group or from the controls. These results suggest that plasma from RRM-salt hypertensive rats contains an augmenting factor for norepinephrine overflow from sympathetic nerve endings.

Recently, it has been reported that deproteinized plasma from patients with essential hypertension and from experimental animals with volume-dependent hypertension contains elevated levels of a digitalis-like inhibitor of dog kidney Na-K ATPase, called natriuretic hormone. Huot et al\textsuperscript{19} reported that the level of a circulating Na\textsuperscript{+} transport inhibitor increased and vascular Na-K pump activity and cardiac microsomal Na-K ATPase activity decreased in RRM-salt hypertensive rats. Moreover, they reported that both anteroventral third ventricle (AV3V) lesions and central sympathectomy prevented the development of RRM-salt hypertension. These results suggest that the Na-K ATPase inhibitor is released from the AV3V area of the brain. According to Blaustein's hypothesis, inhibition of the sodium pump may increase norepinephrine release from, and reduce re-uptake by the presynaptic nerve ending as a result of increased intracellular sodium.

There are a number of substances, such as angiotensin II and β-stimulant, which increase norepinephrine release from sympathetic nerve endings, though they are not heat-stable. Furthermore, in RRM-salt hypertensive rats plasma angiotensin II levels may be low since these animals have low plasma renin activity.\textsuperscript{9} Hence, in this type of hypertension these substances might not be responsible for the increased norepinephrine overflow from the nerve endings. In the present study, it was demonstrated that deproteinized plasma obtained by boiling from RRM-salt hypertensive rats was highly inhibitory to Na-K ATPase activity compared with that from RRM-water normotensive rats. Moreover, deproteinized plasma from the salt-loaded hypertensive rats cross-reacted with antidiuretic hormones although much more strongly than did the controls. Therefore, it appears that the plasma of RRM-salt hypertensive rats contains a digitalis-like substance. Consequently, it is suggested that an augmenting factor for norepinephrine overflow in RRM-salt hypertensive rats might be natriuretic hormone.

We previously observed\textsuperscript{9} that plasma and urinary norepinephrine were increased on the 10th day after subtotal nephrectomy in the saline-loaded hypertensive rats and that blood pressure was significantly correlated with plasma norepinephrine in this type of hypertension. Increased plasma norepinephrine in RRM-salt hypertensive rats may be attributable to the humoral factor seen in this study to be increasing the norepinephrine overflow from sympathetic nerve endings.

In addition, it was reported that there might be a vascular sensitizing factor to pressor agents in the plasma of hypertensive animals. Mizuhashi et al\textsuperscript{20} showed that arterial pressure and vascular sensitivity to angiotensin or norepinephrine rose following injection of plasma from hypertensive subjects into nephrectomized,
pentolinium-treated rats. Furthermore, Plunkett et al. reported that a vascular sensitizing factor in saline-loaded dogs might be the same substance as the putative natriuretic hormone. In the present study, when deproteinized plasma obtained from RRM-salt hypertensive rats was added to the mesenteric vascular bed perfusate, it slightly increased vascular sensitivity to exogenous norepinephrine, compared to the reaction seen using deproteinized plasma from RRM-normotensive rats, showing that plasma from RRM-salt hypertensive rats might contain a vascular sensitizing factor to norepinephrine. Hence, addition of deproteinized plasma from salt-loaded rats to the perfusate might cause electrical nerve stimulation to induce greater changes in pressor responses than in norepinephrine overflow. If the augmenting factor for norepinephrine overflow is the same substance as natriuretic hormone, our findings support the hypothesis that natriuretic hormone increases norepinephrine overflow from sympathetic nerve endings as a result of increased intracellular sodium. Although the nature and chemical properties of both this augmenting factor and the natriuretic hormone have not been determined, preliminary findings suggest that both substances are heat-stable.

It is concluded that plasma from RRM-salt hypertensive rats might contain an augmenting factor for norepinephrine overflow from sympathetic nerve endings, and that this factor might be a Na-K ATPase inhibitor. The results of the present study provide some new insight into volume-dependent hypertension.

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