

Participation of Ouabainlike Compound in Reduced Renal Mass–Saline Hypertension

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Abstract We examined the role of ouabainlike compound in reduced renal mass–saline hypertension using a population of rats immunized with ouabain. To develop ouabain-immunized rats, ouabain–bovine serum albumin conjugates were injected subcutaneously three times at 4-week intervals. Titer determinations were made 2 weeks after the third immunization, and rats with high titers were used in the study. Immunoglobulin G fractions from ouabain-immunized rats effectively inhibited the contractile response of guinea pig aorta to exogenous ouabain (150 nmol). Fourteen ouabain-immunized and seven nonimmunized control rats underwent subtotal nephrectomy. An additional eight ouabain-immunized and six nonimmunized rats served as sham-operated rats. Four groups of rats drank 1% NaCl solution for 3 weeks, and systolic blood pressure was measured weekly by the tail-cuff method. Two groups of sham-

operated rats remained normotensive. In contrast, two groups of subtotally nephrectomized rats developed hypertension. However, among these rats, systolic blood pressure was significantly lower in ouabain-immunized rats than in nonimmunized rats (161 ± 5 versus 180 ± 3 [\pm SEM] mm Hg, $P < .01$). The decrease in blood pressure was accompanied by a significant inhibition of aortic hypertrophy ($P < .05$). These results indicate that chronic blockade of circulating ouabainlike compound partly ameliorates reduced renal mass–saline hypertension and suggest that circulating ouabainlike compound may be involved in the pathophysiology in this model of hypertension. (*Hypertension*. 1994;23[suppl I]:I-110-I-113.)

Key Words • ouabain • hypertrophy • hypertension, experimental

There is much evidence to suggest that endogenous digitalis-like factors (EDLFs) can modulate sodium pump activity and could be involved in the regulation of sodium homeostasis.¹⁻³ Release of EDLF, the purpose of which is restoration of extracellular fluid volume via natriuresis, may lead secondarily to increased cytosolic calcium, arteriolar vasoconstriction, and hypertension.^{4,5} Hamlyn and colleagues⁶ identified from human plasma a ouabainlike compound (OLC), which is indistinguishable from the cardenolide ouabain, as a plausible EDLF.

It has been documented that animals develop a low-renin, volume-expanded type of hypertension after renal mass reduction and excessive sodium intake.⁷ As expected, on the basis of several assay methods,^{8,9} rats with this type of hypertension have been shown to have increased levels of EDLF in their plasma. However, the exact behavior of OLC with respect to blood pressure has yet to be examined in rats with reduced renal mass–saline (RRM-S) hypertension. Our previous studies have indicated that there is an increase in plasma OLC levels in rats with RRM-S hypertension.¹⁰ In this study, we developed a population of autoimmune rats sensitized against ouabain and investigated the effect of blocking the actions of circulating OLC on RRM-S hypertension in rats.

Methods

Immunization Protocol and Titer Determination

Male Wistar rats weighing 200 to 250 g ($n=25$) were immunized against ouabain. All procedures were in accordance with

the guidelines of the Animal Experiment Committee of the University of Tokyo. To render ouabain immunogenic, ouabain was covalently bound to bovine serum albumin (BSA) according to the method of Butler and Lindenbaum.¹¹ Initially, animals were injected subcutaneously with ouabain-BSA conjugate in an emulsion of Freund's complete adjuvant (0.4 mL). After 1 and 2 months, animals were given booster shots with ouabain-BSA conjugate in an emulsion of Freund's complete adjuvant (0.2 mL), and titer determination was made 2 weeks after the third immunization. For titer determination, rat serum was diluted 10^3 -fold by 10 mmol/L phosphate buffer. Then, 0.1 mL of the diluted serum was incubated with 0.3 mL of [³H]ouabain (17.1 Ci/mmol, 0.2 pmol) for 24 hours at 4°C. Free [³H]ouabain was precipitated with dextran-coated charcoal, and bound [³H]ouabain was determined by liquid scintillation.

Autoimmunity Validation

The specificity of OLC blockade by active immunization should be tested *in vivo* by studying the effect of injecting ouabain-immunized rats with exogenous ouabain. However, because rat tissues respond slowly to ouabain, guinea pig aorta was used to test the effects. The effects of immunoglobulin G (IgG) fractions on the contractile response to ouabain were evaluated *in vitro* using thoracic aorta from male guinea pigs (weight, 350 g) according to methods described previously.¹² In brief, aortic ring preparations were suspended in organ baths containing 15 mL of Krebs-Henseleit solution. The bathing solution was maintained at 37°C and bubbled with a mixture of 5% CO₂–95% O₂. After a 1-hour stabilization period, reproducible contractions were produced with 50 mmol/L KCl. Then, IgG fractions from either control or ouabain-immunized rats were added to the bath, and the contractile response to 150 nmol ouabain was examined 30 minutes later. IgG fractions were purified from 6 mL of rat serum according to the manufacturer's protocol using immobilized Protein G (Pierce, Rockford, Ill).

Preparation of Reduced Renal Mass–Saline Hypertension in Rats

Twenty-one autoimmune and 14 nonimmune control rats were divided into two groups. Fourteen autoimmune rats and

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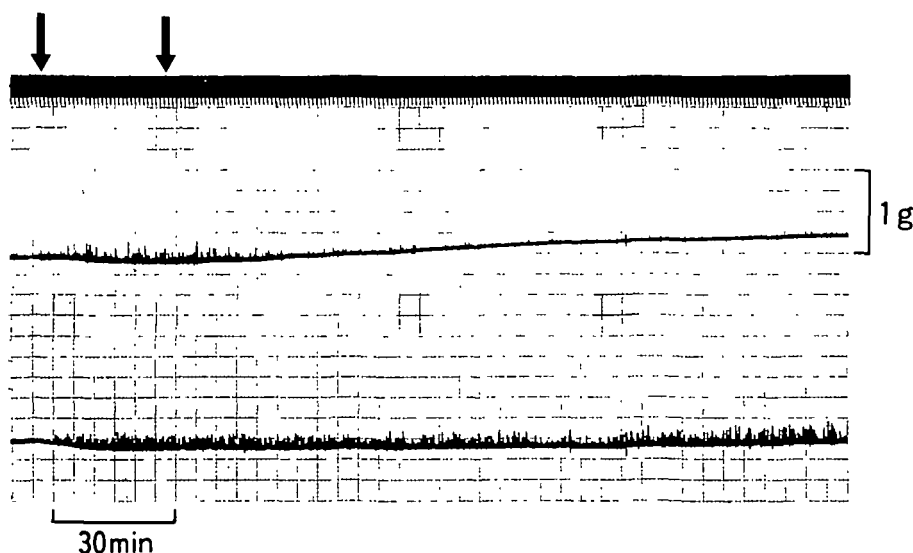


Fig 1. Representative tracing shows effects of Immunoglobulin G (IgG) fractions obtained from control (top) and ouabain-immunized (bottom) rats during the contractile response to ouabain in guinea pig aorta. Responses to ouabain (second arrow; 150 nmol) were compared after 30 minutes in the presence of IgG fractions (first arrow) obtained from 6 mL of serum. Final concentration of ouabain was approximately 10 μ mol/L in the organ bath. Fluctuation of tension was caused by the presence of foam due to the high protein content of IgG samples.

7 control rats underwent subtotal nephrectomy (NX group). The other 8 autoimmune rats and 6 control rats underwent sham operation (sham-operated group). Subtotal nephrectomy was achieved by removing the right kidney and 50% of the left kidney (both poles). The two poles of the left kidney were excised with scissors under the clamped left renal artery, and the cut surfaces were covered with glue (Alon-alpha, Towa Chemical, Tokyo, Japan). All rats were fed normal rat chow. Three days after the operation, all rats were given 1% NaCl solution to drink. Systolic blood pressure (SBP) was measured weekly by the tail-cuff method. Three weeks later, blood was collected from the abdominal aorta and processed for measurement of the following parameters. Some of the blood was used to measure hematocrit. Plasma renin activity and aldosterone concentration were measured by radioimmunoassay. Serum sodium and potassium concentrations were measured by flame photometry. Serum creatinine concentrations were determined by the colorimetric method with the Jaffe reaction.¹³ The heart, thoracic aorta, kidneys, and adrenals were excised and weighed. The aorta was cut longitudinally, and its surface area was measured.

Statistical Analysis

Values are given as mean \pm SEM. One-way and two-way analyses of variance were used for comparison of blood pressure, body weight, and other parameters of control and ouabain-immunized animals.

Results

Titer of Ouabain-Immunized Rats

Of 25 immunized rats, the percentages of bound compared with total [³H]ouabain were more than 50% in 10, between 25% and 50% in 12, and less than 25% in 3 rats. The last 3 rats were excluded from further study. Nonimmune rats showed no specific binding.

Autoimmunity Validation

In the presence of IgG fractions from nonimmunized rat serum, ouabain (150 nmol) caused a gradual contraction of guinea pig aorta (Fig 1). The contractile response to ouabain was totally abolished in vessel preparations pretreated with IgG fractions from ouabain-immunized rat serum.

Body Weight and Blood Pressure

Body weight at the third week of the experiment is shown in the Table. Body weight was significantly lower

in subtotally nephrectomized rats compared with sham-operated rats ($P < .01$). Fig 2 depicts the time course of SBP in the four groups: nonimmune and sham-operated rats (C-S), nonimmune rats with subtotal nephrectomy (C-NX), autoimmune and sham-operated rats (AI-S), and autoimmune rats with subtotal nephrectomy (AI-NX). SBP did not differ among the four groups before the operation and at the first week of saline consumption. SBP in the two groups of sham-operated rats remained unaltered during the 3 weeks. In contrast, SBP began to increase in the two groups of NX rats after 2 weeks. The SBP of C-NX rats progressively increased, but SBP remained unchanged between the second and third weeks in AI-NX rats. Therefore, a significant difference was observed in SBP between AI-NX and C-NX rats at the third week (161 ± 5 versus 180 ± 3 mm Hg, $P < .01$).

Other General and Hormonal Parameters

Significant increases in heart weight and aortic weight were observed in NX compared with sham-operated rats ($P < .01$) (Table). These changes apparently agree with hypertension found in NX rats. In the two groups of NX rats, heart and aortic weights were reduced in AI rats, and the latter change was significant ($P < .05$). Remnant kidney in the NX rats was markedly hypertrophied, and weights were 60% to 70% of that of the two intact kidneys in sham-operated rats. Furthermore, the weight of the adrenals increased significantly in NX rats ($P < .01$). However, these alterations were not modified by active immunization with ouabain.

As expected, a decrease in hematocrit and an increase in serum creatinine were found in NX rats. Active immunization with ouabain did not affect these changes. An increase in serum sodium concentration and decrease in serum potassium concentration were observed in NX rats compared with sham-operated rats. Significant changes were limited to rats immunized against ouabain (sodium, $P < .05$; potassium, $P < .01$). Active immunization with ouabain decreased serum potassium concentration in NX rats ($P < .05$).

Subtotal nephrectomy and excessive sodium intake inhibited the renin-aldosterone system in NX rats compared with sham-operated rats. Ouabain immunization did not affect renin inhibition but augmented the suppression of aldosterone in NX rats ($P < .05$).

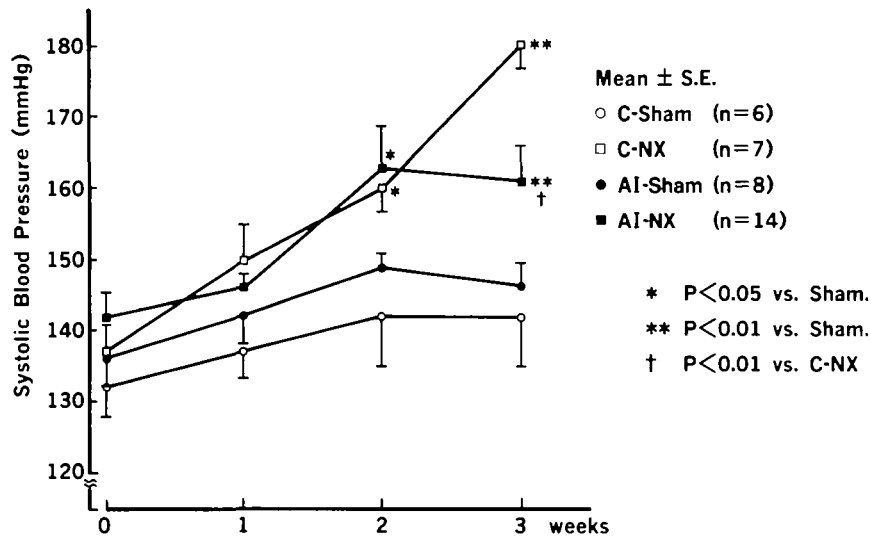


FIG 2. Line graph shows systolic blood pressure changes in control-sham-operated (C-Sham), control-subtotally nephrectomized (C-NX), autoimmune-sham-operated (AI-Sham), and autoimmune-subtotally nephrectomized (AI-NX) rats. Systolic blood pressure was measured by the indirect tail-cuff method before operation (0 weeks) and then weekly until 3 weeks after the start of saline consumption.

Discussion

The mechanism by which hypertension occurs in a volume-dependent, salt-sensitive model remains unclear. One major line of investigation has involved the premise that a circulating EDLF contributes to hypertension in volume-dependent models through an action on vascular smooth muscle Na^+Ca^{2+} exchange. Previous investigations support the role of the circulating EDLF in RRM-S hypertension. Incubation of normal rat tail arteries in

supernates of boiled plasma from RRM-S hypertensive rats inhibits ouabain-sensitive ^{86}Rb uptake.⁸ Decreased Na^+,K^+ -ATPase activity in cardiac microsomes and decreased sodium pump activity in tail artery have also been observed in RRM-S hypertensive rats.⁹

Numerous candidates for EDLF have been detected in mammalian preparations, although many are unlikely to be physiological regulators of the sodium pump.² Recently, Hamlyn et al⁶ were able to purify OLC as an

Blood Pressure, Body Weight, and Other Parameters

	Rat Group			
	Sham Operation		Subtotal Nephrectomy	
	C	AI	C	AI
No. of rats	6	8	7	14
SBP, mm Hg	142±7	146±3	180±3*	161±5*†
BW, g	521±27	529±18	431±20*	453±16*
Heart weight, g (% of BW)	1.299±0.040 (0.252±0.012)	1.346±0.037 (0.255±0.004)	1.326±0.065 (0.308±0.012)*	1.326±0.044 (0.300±0.017)*
Aortic weight, mg (mg/mm ²)	38.6±1.1 (0.224±0.004)	42.2±1.0 (0.237±0.007)	48.5±1.5* (0.299±0.009)*	47.4±1.8* (0.271±0.008)*‡
Kidney weight, g (% of BW)	2.895±0.122 (0.558±0.014)	3.146±0.102 (0.596±0.017)	1.728±0.093* (0.402±0.015)*	1.677±0.068* (0.373±0.013)*
Weight of adrenals, mg (% of BW)	53.3±3.5 (0.0107±0.0004)	59.9±1.9 (0.0114±0.0004)	71.9±2.8* (0.0169±0.0010)*	77.7±3.4* (0.0163±0.0014)*
Hematocrit	0.423±0.004	0.417±0.005	0.344±0.014*	0.355±0.009*
Creatinine, mg/dL	0.52±0.03	0.55±0.03	1.05±0.05*	1.08±0.03*
Sodium, mEq/L	148.9±0.3	148.0±0.5	152.7±1.8	152.0±0.9§
Potassium, mEq/L	5.18±0.19	5.30±0.17	4.84±0.11	4.53±0.09*‡
PRA, ng · mL ⁻¹ · h ⁻¹	21.3±1.8	22.4±1.7	2.9±0.7*	3.0±0.5*
PAC, pg/mL	142±10	162±29	118±13	64±9*‡

C indicates nonimmune; AI, autoimmune; SBP, systolic blood pressure; BW, body weight; PRA, plasma renin activity; and PAC, plasma aldosterone concentration. Values are given as mean±SEM.

All data are from the third week of the experiments.

Weights of heart, kidney(s), and adrenals are expressed as absolute weights and percentage of body weight. Aortic weight is expressed as absolute weight and value corrected for surface area.

*P<.01, §P<.05 vs corresponding rats with sham operation.

†P<.01, ‡P<.05 vs C with subtotal nephrectomy.

EDLF from human plasma. Because OLC is indistinguishable from ouabain, the inhibition of sodium pump activity with OLC could cause increased cardiac contractility, increased peripheral resistance, increased blood vessel responsiveness to vasoactive agents, and increased arterial blood pressure. Recent findings clearly indicate that chronic inhibition of Na^+, K^+ -ATPase over 4 weeks gradually leads to the development of hypertension in rats.³

We have recently measured plasma OLC levels to examine the role of OLC in rats with RRM-S hypertension. At 3 weeks after subtotal nephrectomy and excessive sodium intake, plasma OLC level was significantly higher in NX rats than in control rats. Furthermore, OLC values correlated significantly with SBP levels. In the present study, we hypothesized that prolonged OLC deprivation could be attained by immunizing rats against ouabain, effectively producing autoimmune rats. Similar immunization methods have been successfully used to investigate the role of atriopeptin or renin in rats.^{14,15}

We first studied whether rats that developed antibodies to ouabain were resistant to exogenously infused ouabain. However, it was very difficult to observe a consistent cardiovascular response in rats, probably because of the delayed sensitivity of rats to cardiac glycosides. Instead, we confirmed that IgG fractions obtained from ouabain-immunized rats are effective in blocking the vasoconstrictive actions of exogenous ouabain on guinea pig aorta. The amount of IgG used in one experiment corresponded to that obtained from all of the serum of one rat. The final concentration of ouabain was $10 \mu\text{mol/L}$ and was much higher than reported plasma OLC levels.³ We consider autoimmune rats to be chronically devoid of the effects of circulating OLC.

In the present study, SBP of AI-NX rats was significantly lower than that of C-NX rats at the end of 3 weeks. In parallel with this finding, aortic hypertrophy also was inhibited in AI-NX rats compared with C-NX rats. Our findings suggest that chronic blockade of circulating OLC partly prevents RRM-S hypertension and that OLC may play a role in the hypertensive mechanism of this model.

The enlargement of the adrenals was found in NX rats, but the significance of adrenal hypertrophy is unclear. This finding is in agreement with a previous study.¹⁶ Although OLC is supposedly produced in the adrenals,³ immunization with ouabain had no effect on adrenal hypertrophy. In NX rats, ouabain immunization decreased aldosterone and potassium concentrations. OLC could mediate these two phenomena. It is suggested that ouabain administration may elevate plasma aldosterone levels in rats.³ Furthermore, OLC may

function as one of the determinants of serum potassium concentration through the regulation of transmembrane potassium gradient.

In summary, chronic blockade of the circulating OLC system partly inhibited the development of RRM-S hypertension in rats. It appears that RRM-S hypertension requires the presence of circulating OLC for its full expression. Our observation provides evidence for the participation of endogenous OLC in the hypertensive mechanisms in rats with reduced renal mass and excessive sodium intake.

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Hypertension. 1994;23:I110

doi: 10.1161/01.HYP.23.1_Suppl.I110

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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