We evaluated the urinary excretion of immunoreactive endogenous ouabain-like factor (OLF) and digoxin-like factor (DLF) to investigate their pathophysiological roles in sodium metabolism and blood pressure in 5/6-reduced renal mass rats, a model of volume-expanded hypertension. About five-sixths of the kidney mass (5/6 RRM, n = 9) was removed from male Sprague-Dawley rats, or the rats were sham operated (control, n = 10). Both groups were fed regular diets with tap water for 1 wk as a control period, followed by 1% saline solution for 4 wk. Systolic blood pressure (SBP), urine volume (UV), urinary sodium excretion (UNaV), DLF, and OLF were measured on the last 2 d of every week throughout the experimental period. SBP and UNaV were significantly higher in 5/6 RRM rats than in control rats. Urinary DLF significantly increased, reaching peak value in the first week, while OLF increased continuously, reaching peak value in the fourth week. In the first week, there were a significant positive correlations between the change in DLF and the changes in UNaV and SBP. However, the change in OLF was not correlated with changes in either UNaV or SBP. Both SBP and UNaV showed a significant positive correlation with OLF (p < 0.001, r = 0.547, p < 0.001, r = 0.658, respectively), whereas DLF significantly correlated with UNaV (p < 0.001, r = 0.584) but not with SBP in 5/6 RRM. These findings suggest that endogenous OLF and DLF coexist in rat urine and that an increased level of OLF, but not DLF, may contribute to the development and maintenance of hypertension. DLF may contribute to renal sodium excretion in this volume-expanded hypertensive rat model. (Hypertens Res 1998; 21: 193-199)

Key Words: hypertension, reduced renal mass, ouabain, digoxin

Cardiotonic steroids, which inhibit Na-K ATPase activity, increase cardiac contractility, peripheral resistance, blood vessel responsiveness to vasoactive agents, and arterial blood pressure. It is generally agreed that volume expansion stimulates circulating Na-K ATPase inhibitors. Increased levels of Na-K ATPase inhibitors are especially implicated in the pathogenesis of salt-dependent and low-renin types of hypertension (7). The search for an endogenous Na-K ATPase inhibitor has been supported by the speculation that naturally-occurring cardiotonic steroids exist in humans and other animals, and that there is a highly conserved binding site for such steroids. Many investigators have attempted to purify and identify such endogenous specific inhibitors of Na-K ATPase in plasma, urine, and tissues of humans and animals (2-6). To detect such endogenous Na-K ATPase inhibitors, several kinds of assays, based on measurement of ATPase inhibitory activity or assessment of competition to receptor binding capacity with the use of radio-labeled cardiac glycosides, have been employed. Gruber et al. (7) used digoxin-like immunoreactivity with readily available digoxin antibodies to measure endogenous Na-K ATPase inhibitors. We previously reported that increased digoxin-like immunoreactivity may play an important role in maintaining sodium metabolism and elevated blood pressure in 5/6-reduced renal mass hypertensive rats (5/6 RRM) (8) and in low-renin essential hypertension (9), both of which are known to involve volume-expanded hypertension.

Recently, Hamlyn et al. (10-13) showed that one such immunoreactive factor was indistinguishable from the cardiac glycoside ouabain, using several techniques based on mass spectrometry, immunoreactivity, and biochemistry. Harris et al. (14) have developed a reliable enzyme-linked immunosorbent assay (ELISA) for this ouabain-like factor (OLF).

In the present study, we continuously measured excretion of both OLF and digoxin-like factor (DLF) to investigate how OLF, DLF, or both are involved in blood pressure elevation and renal
Nineteen male Sprague-Dawley rats weighing 120 to 130 g were used in this study. Under intraperitoneal pentobarbital anesthesia, both upper and lower poles of the left kidney were removed. One week after the first operation, the right kidney was removed (5/6 RRM rats). Control rats underwent two sham operations. After the second operation, the rats were placed in metabolic cages and given tap-water for 1 wk (control period) followed by 1% saline for 4 wk (experimental period). The rats were given a regular rat chow and housed in temperature- and humidity-controlled metabolic cages with a 12-h light/dark cycle. Systolic blood pressure (SBP) was measured by a tail-cuff method, and 24-h urine was collected twice at the end of every week. Body weight was measured on the day of urine collection. Urine was immediately centrifuged and stored at −20°C for assay of DLF and OLF. Urinary sodium concentration was estimated by the ion electrode method.

Urinary OLF was measured with a ouabain EIA Reagent Pack (DuPont, Daiichi Kagaku, Tokyo, Japan), based on the study by Hamlyn et al. (14). Each urine sample (0.5 ml) was mixed with two volumes of 0.1% distilled trifluoroacetic acid and incubated at room temperature for 1 h and then at −4°C overnight. After centrifugation (3,000 g, 30 min, 4°C), the supernatant was passed through a preactivated Sep-Pak C-18 cartridge (Waters, Millipore, Milford, MA) and eluted with 25% acetonitrile after washing with 10 ml of distilled water. The evaporates were dissolved in the assay buffer (phosphate-buffered saline 10 mM, pH 7.0, with 0.85% NaCl), containing 2% bovine serum albumin and 0.05% Tween-20 (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The sensitivity of the enzymatic immunoassay was 0.4 pg/well and 7 pg/well with 50% displacement. The dilution curves of the urine extracts were parallel to the standard curve (Fig. 1). The cross-reactivity of the anti-ouabain antibody with digoxin was 3.0%. With these extraction procedures, the recovery of ouabain was 108.0 ± 3.4%, and that of digoxin was 0.67 ± 0.04%.

Urinary DLF was measured by using digoxin radioimmunoassay as reported previously (9). In brief, urine was extracted with a Sep-Pak C-18 cartridge and eluted with 60% acetonitrile. The evaporated elute was dissolved in the assay buffer (phosphate-buffered saline 10 mM, pH 7.0, with 0.85% NaCl), containing 1% bovine serum albumin. The assay buffer was added to both standard digoxin (Sigma, St Louis, MO) and to the samples to yield 200 µl. Then, 100 µl of antibody (Mallinckrodt, St Louis, MO) and 100 µl of 125I-digoxigenin (Amalex, Tokyo, Japan) were added. After 3 h of incubation, 400 µl of 0.1% bovine γ-globulin and 800 µl of polyethylene glycol (25%) were added. The tubes were then centrifuged at 3,000 rpm, and the supernatant was aspirated. The radioactivity in the precipitate was counted. The sensitivity of the assay was 0.78 pg per tube, and 50% displacement was at 10 pg/tube. The cross-reactivity of anti-digoxin antibody with ouabain was 0.05% (9). With these extraction procedures, the recovery of digoxin was 68.7 ± 3.5%, and that of ouabain was 2.9 ± 0.9%.

Statistical Analysis
Values are expressed as means ± SEM. One-way analysis of variance (ANOVA) was used to assess the significance of differences between groups, and unpaired Student's t-test was used to compare the two groups. Two-way repeated-measures ANOVA was used for comparisons within groups. Regression coefficients were obtained by the least-squares method. p < 0.05 was considered to indicate statistical significance.

Results
Body weight in 5/6 RRM rats was sustained at a significantly lower level than that in control rats throughout the study period (270 ± 20 vs. 370 ± 9 g, p < 0.05). Figure 2 shows changes in SBP and urinary sodium excretion (UNaV) in control and 5/6 RRM rats. There was no difference in SBP between the two groups during the control period. SBP increased significantly in 5/6 RRM rats after saline administration. The control group also showed a slight increase in SBP after the third week. However, SBP in 5/6 RRM rats was significantly higher than that in control rats throughout the 4-wk study period (223 ± 5 vs. 135 ± 4 mmHg at the fourth week, p < 0.05). UNaV in both groups increased rapidly in the first week and was maintained at a similar level over the 4-wk period of 1% saline ingestion. Moreover, UNaV was significantly higher in 5/6 RRM rats than in the control rats throughout the experimental period.
Urinary DLF in 5/6 RRM showed a moderate, significant increase in the first week, reaching peak value ($1.91 \pm 0.19$ to $5.84 \pm 0.64$ ng·digoxin/kg/d, $p < 0.05$), and remained at a similar level, which was significantly higher than that in the control group, for the duration of the experimental period (Fig. 3). In the control rats, a transient increase in urinary DLF was seen only in the first week. Urinary OLF
in the 5/6 RRM group, however, increased gradually and reached peak value in the fourth week, while in the control group urinary OLF returned to the baseline level in the second week after an initial transient increase in the first week, similar to urinary DLF (Fig. 3). Thus, urinary OLF in 5/6 RRM rats was maintained at a significantly higher level than that in the control group after the second week. Furthermore, the profile of SBP (Fig. 2) differed significantly from that of urinary DLF, although it did not differ from that of urinary OLF using two-way repeated-measures ANOVA. In the first week, the change in urinary DLF positively correlated not only with that in UNaV but also with that in SBP ($r = 0.757$, $p < 0.05$, $r = 0.503$, $p < 0.05$, respectively), while no correlation was observed between the change in urinary OLF and the change in either UNaV or SBP (Fig. 4, 5).
Overall, there were significant positive correlations between urinary excretion of sodium and both urinary DLF and urinary OLF in 5/6 RRM rats throughout the study (Fig. 6). On the other hand, in 5/6 RRM rats SBP showed a significant positive correlation with urinary OLF ($r = 0.547$, $p < 0.001$), but not with urinary DLF (Fig. 7).

**Discussion**

The results of the present study demonstrate new findings concerning the role of two different endogenous sodium pump inhibitors, OLF and DLF, in blood pressure elevation and sodium metabolism.
in 5/6 RRM rats. After de Wardener et al. (15) and Kramer et al. (16) suggested that a hypothetical humoral factor, a circulating Na-K-adenosine triphosphate (ATPase) inhibitor, might play an important role in the pathogenesis of hypertension, elevated circulating levels of this factor have been shown in several low-renin hypertensive models, including 1KIC (17), reduced renal mass rats (18), and DOCA-salt hypertensive rats (19). Numerous candidate substances for this factor have been reported in plasma, tissues, and urine in humans and other species over the past three decades. However, many such candidates were nongenomic or of low affinity to Na-K-ATPase and do not strictly fulfill the criteria for endogenous digitalis-like factor (20). Hamlyn et al. (13) were able to identify and purify this factor from human plasma and concluded that it was immunologically, spectrometrically, and biochemically identical to ouabain or an isomer of ouabain. Recently, Tymia et al. (21) have reported that a compound isolated from bovine hypothalamus (hypothalamic inhibitory factor (HIF)) is an isomer of ouabain and provided additional, interesting evidence that mammalian endogenous ouabain is also an isomer of ouabain, using nanogram scale circular dichroic structural analysis (22). Since ouabain was proposed as an endogenous Na-K ATPase inhibitor, similar reports of ouabain-like immunoactivity in various kinds of hypertension have been made by several groups of investigators. Other steroids, however, remain as possible candidates. It has been shown that two peaks inhibit $^3$H-ouabain binding to erythrocytes in human urine (20). Goto et al. (23) reported that a different, less polar, urinary substance, which is indistinguishable from digoxin, exists in three HPLC and thin-layer chromatography systems, except for one steroidal structure that is quite similar to that of ouabain. Shaikh et al. (24) also reported the existence of a digoxin-like immunoreactive factor in mammalian adrenal cortex, which was observed in a less polar fraction of plasma and was associated with the inhibition of red blood cell Na-K ATPase activity. These results suggest that more than one substance may function as endogenous Na-K ATPase inhibitors.

We previously reported that an increase in digoxin-like immunoreactivity may play important roles in both sodium excretion and blood pressure elevation in 5/6 RRM rats (25). Therefore, in this study, we measured not only digoxin-like immunoreactivity but also ouabain-like immunoreactivity, using reliable methods established and developed in Hamlyn’s laboratory. The present data indicate that at least two different substances, with digoxin-like and ouabain-like immunoreactivity, exist in rat urine. We measured immunoreactivity by means of suitable extraction procedures as described above. As mentioned previously, the eluate for the ouabain measurement contained little digoxin-like immunoreactivity, and anti-ouabain antibody showed only 3% cross-reactivity with digoxin. Similarly, the extract for digoxin radioimmunoassay did not include ouabain, and the anti-digoxin antibody showed less than 0.1% cross-reactivity with ouabain. Thus, each measurement of digoxin-like or ouabain-like immunoreactivity in this study is considered reliable and free of the effect of contamination. This observation is supported by evidence presented by Tamura et al. (26) showing that the two Na-K ATPase inhibitory activities derived from 5/6 RRM rat urine exhibit cross-reactivity to both anti-ouabain antibody and anti-digoxin antibody. They also reported, however, that the source of most inhibitors in rat urine, except for ouabain-like inhibitor, was a regular diet. We could not confirm this in our study, because the same standard rat chow was given throughout the experiment period to all rats. Although we did not measure the exact amount of chow each rat ate, the increment in urinary DLF was about 3-fold and that in ouabain was about 2.5-fold after saline ingestion. Regardless of whether or not the rats ate 2.5- or 3-fold more chow after drinking saline, neither the increase in urinary DLF nor that in ouabain was attributed only to diet. Further experiments using synthetic rat chow are needed to evaluate the exact amount of the endogenous inhibitor itself.

This study also presented an interesting difference between digoxin-like immunoreactivity and ouabain-like immunoreactivity with respect to time course and involvement in blood pressure regulation. In 5/6 RRM rats, DLF showed a moderate increase and reached its peak level soon after the start of saline ingestion. DLF then decreased and remained at a similar level throughout the experimental period. In contrast, OLF increased gradually after saline loading and reached its peak level at the end of the experimental period. OLF was considered responsible for the blood pressure elevation in 5/6 RRM rats. Furthermore, a significant positive correlation with blood pressure was observed only for OLF, while sodium excretion showed significant positive correlations with both OLF and DLF. These discrepancies suggest that regulatory mechanisms, governing the production or stimulation, or both, of OLF and DLF differ, and that each compound acts selectively in the establishment of hypertension. It is possible that OLF is more involved than DLF in blood pressure regulation. This hypothesis reflects our previous data and conclusion (25) that a transient rise in digoxin-like immunoreactivity may be related to the maintenance of sodium and water homeostasis but does not contribute to blood pressure elevation. Although we did not measure the plasma concentration of ouabain in this study, Yamada et al. (27) reported that plasma ouabain-like immunoreactivity in reduced renal mass saline-drinking rats was 7-fold higher than that in control rats. These results suggest that increased levels of OLF may contribute to the establishment of hypertension. Inhibited Na-K ATPase activity has been evaluated in the vascular smooth muscle cells of 5/6 RRM rats from the first week to the fifth week of 1% NaCl solution ingestion (18). It has been reported that chronic administration of digoxin in humans does not increase blood pressure (28), whereas intraperitoneal admin-
istration of ouabain chronically increases blood pressure in rats (29). These observations may suggest that there are variations related to species or to rapidity or completeness of binding, and that all pump inhibitors do not have the same potential to increase blood pressure. However, there is currently no evidence to support these assumptions.

In conclusion, our findings demonstrate that both immunoreactive OLF and DLF exist as endogenous digitalis-like factors in RRM rat urine, and that OLF may be more closely related than DLF to the sustained blood pressure elevation in this model of volume-expanded hypertension. However, the mechanisms of OLF and DLF and differences in their target organ specificity and affinity remain unknown. Further study is needed to identify modulators of their production and secretion, and to clarify their physiological roles in blood pressure regulation and sodium metabolism.

References