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Urinary Excretion of Endogenous Digitalis-Like Natriuretic Substances in Healthy Subjects. Effect of Sodium Load

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In the current study digoxin-like immunoreactivity (DLIA), Na-K-ATPase inhibition and natriuretic activity of urinary extracts from 10 healthy volunteers following a low and a high-sodium intake, respectively, were measured. Detectable urinary DLIA (46.1 ± 5.6 ng eq digoxin/day), Na-K-ATPase inhibition (182.9 ± 22.7 nmol eq oub/day) and natriuretic activity ($U_{Na}V$: 0.38 ± 0.11 μEq/min) were observed during the low-sodium diet period in all subjects. High-sodium diet was associated with a significant increase in DLIA (87.9 ± 9.2 ng eq digoxin/day, $p < 0.001$) which parallelled changes in Na-K-ATPase inhibition (359.8 ± 51.9 nmol eq oub/day, $p < 0.005$) and natriuretic activity ($U_{Na}V$: 1.33 ± 0.3 μEq/min, $p < 0.025$). These results support the contention that DLIA is related to NH.

Key words: Natriuretic substances, Natriuretic hormone, Immunoreactivity, Na-K-ATPase.

Following the first report by DE WARDENER *et al.* (4), considerable evidence has emerged indicating the existence of an endogenous digitalis-like natriuretic substance(s) or natriuretic hormone which may contribute to the overall regulation of fluid balance by increasing sodium excretion in response to volume expansion (1, 5, 10). The natriuretic activity of natriuretic hormone has been related to its ability to inhibit Na-K-ATPase activity by binding to its digitalis receptor (8). Na-K-ATPase inhibition might also be responsible for other biological effects of natriuretic hormone, such as the increase in arterial pressure and vascular reactivity (5). Since the chemical nature of natriuretic hormone is unknown, studies on this hormone necessarily deal with biological activity. Methods to estimate na-

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triuretic hormone include the measurement of the Na-K-ATPase and ouabain binding inhibiting and natriuretic activities of plasma or urinary extracts (5, 10). Natriuretic hormone is contained in high quantities in the urine of humans and experimental animals submitted to acute iv plasma volume expansion. In normal conditions, smaller amounts of natriuretic hormone are also detectable in the urine and increase in response to high sodium intake (5).

More recently, a factor(s) with digoxin-like immunoreactive activity (DLIA) which behaves in a similar manner as natriuretic hormone has been detected in plasma and urine (11, 14). Plasma and urine DLIA increase following acute iv volume expansion (11). Furthermore, DLIA is mainly detected in the same chromatographic fraction as the natriuretic and Na-K-ATPase inhibiting activities of natriuretic hormone (11). Finally, the immunoprecipitate obtained after incubating the urine fraction containing natriuretic hormone with antidigoxin antibodies has an almost 10-fold greater natriuretic activity than that of the original fraction (14). These findings suggest that DLIA could be another expression of natriuretic hormone. However in the only published study in which DLIA and Na-K-ATPase inhibiting activity and natriuretic activity were simultaneously measured in the urine of healthy volunteers (15, 16), DLIA was found to be unrelated to the other measured parameters.

The current study was designed to further evaluate the relationship between urinary DLIA and the Na-K-ATPase inhibiting and natriuretic activities. The study was carried out in normal subjects during low and high sodium intake. The effect of changing sodium intake on plasma renin activity (PRA) and plasma levels of aldosterone (ALDO), norepinephrine (NE) and atrial natriuretic peptide (ANP) was also assessed in these subjects.

Materials and Methods

Ten healthy volunteers, 6 males and 4 females (mean age 29 ± 3 years, range 23-31 years), were given a diet containing about 50 mEq of sodium per day. On the fourth day a 24-hr urine collection was carefully obtained to measure sodium, DLIA and the Na-K-ATPase inhibiting activity and natriuretic activity. On the morning of the fifth day, after overnight fasting and following two hours of bed rest, an antecubital vein was catheterized. Forty-five minutes later a blood sample was taken to measure PRA, ALDO, NE, ANP and sodium. Subsequently, and for five days, the diet was supplemented with NaCl tablets, 300 mEq/day. Urinary and plasma measurements were repeated on the ninth and tenth day, respectively. Informed consent was obtained from each subject included in the study, which was approved by the Ethical Committee of the Hospital.

Urine fractionation. — Urine aliquots corresponding to 4-h urine collections were frozen, lyophilized to dryness and stored at −80 °C until further processed. Lyophilized samples were redissolved in 10 ml distilled water, acidified to pH 3.5 with concentrated acetic acid, and centrifuged 10 min at 4 °C and 1,500 *g*. The supernatant was then applied to a 2.5 × 55 cm column packed with Sephadex G-25 Fine (Pharmacia, Uppsala, Sweden) and eluted with 0.1 M acetate buffer (pH 3.5) at a flow rate of 2 ml/min. Fractions of 3 ml each were collected with an automated fraction collector (LKB 2070 Ultrorac, Uppsala, Sweden). The concentration of sodium and potassium was measured in each fraction. The 25 fractions (75 ml) immediately after the salt peak were pooled and divided into three aliquots corresponding to 15, 30 and 180 min urine collection to be assayed for Na-K-ATPase inhibition, DLIA and natriuretic activity, respectively. These ali-

quots were then lyophilized to dryness and stored at −80 °C until assayed. In separate experiments, labelled human ANP (^{125}I-ANP, Ser 99-Tyr 126), prostaglandin E_2 (^{3}H-PGE), 6-keto prostaglandin $F_{1\alpha}$ (^{3}H-6-keto-$PGF_{1\alpha}$) and prostaglandin D_2 (^{3}H-PGD_2) were added to previously lyophilized aliquots from the same 24 hours urine collection in order to assess their elution pattern in the Sephadex G-25 column.

Digoxin-like immunoreactivity.— Each 30 min aliquot was dissolved in 500 µl of 0.01 M phosphate buffer, pH 7.4, containing bovine serum albumin 0.1 %, NaCl 0.9 % and sodium azide 0.1 %. DLIA was assessed with the RIANEN™ digoxin ^{125}I radioimmunoassay kit (New England Nuclear, Boston MA). Results are given as ng equivalents of digoxin/day (ng eq digoxin/day).

Na-K-ATPase inhibiting activity. — This was assessed by evaluating the capacity of urinary extracts to inhibit the activity of a preparation of pig cerebral cortex Na-K-ATPase (Sigma). Na-K-ATPase activity was estimated by measuring the rate of inorganic phosphate released from ATP.

Each 15 min aliquot was dissolved in 1 ml imidazole buffer (10 mM, pH 7.2), and 300 µl of this solution were incubated for 5 min at 37 °C with 500 µl of imidazole buffer containing ATP-Na_2 (1 mM), $MgCl\text{-}6H_2O$ (1 mM), NaCl (100 mM), KCl (20 mM) and EGTA (0.1 mM). Five min later, 100 µl of Na-K-ATPase (250 U/ml) was added. Each sample was assayed in duplicate and a blank tube was also prepared by adding 100 µl distilled H_2O instead of the enzyme. After 10 min, the reaction was stopped with 1 ml of ice-cold TCA (10 %). Samples were then centrifuged at 2000 rpm for 5 min and the inorganic phosphate contained in the supernatant (100 µl) was measured according to the method of LANZETTA *et al.* (17). A dose-response curve for ouabain (range 10 nM to 1 mM) was generated for each set of unknowns and values for each unknown were calculated from the ouabain curve and expressed as nmol equivalents of ouabain/day (nmol eq oub/day).

Natriuretic activity. — Twenty male Wistar rats (270-290 g) fed with a normal sodium chow (70 mEq of Na per kg of food) were used as bioassay animals. Under ketamine HCl anesthesia (100 mg/kg b.w., i.m.), catheters were placed in the carotid and right jugular and femoral veins. The bladder was exposed through a suprapubic incision and catheterized. Once the surgical procedure was completed a priming dose (1.5 ml) of saline containing ^{125}I-iothalamate (0.25 µCi/ml) and PAH (1.2 mg/ml) was given through the femoral vein catheter followed by a constant infusion (0.05 ml/min) throughout the experiment. Mean arterial pressure (MAP) was continuously recorded through the carotid catheter using a highly sensitive transducer and a multichannel recorder (MX4P and MT4, Lectromed Lt, Jersey Channels Islands). After allowing 1 hour for equilibration, three 20 min urine collections were completed before and after the jugular vein infusion (0.08 ml/min for 20 min) of the 180 min aliquot dissolved in 2 ml saline. At the midpoint of each clearance period a blood sample was taken to measure sodium, PAH and ^{125}I-iothalamate. Urine samples were collected in preweighed tubes, and volume was determined gravimetrically. Urinary sodium, PAH and ^{125}I-Iothalamate were also measured. PAH and ^{125}I-iothalamate clearances were used to estimate renal plasma flow (RPF) and glomerular filtration rate (GFR), respectively. Results obtained from each rat are expressed as the mean of the 3 clearance periods performed before and after the administration of the urine extract, respectively.

Other analytical methods. — Plasma renin activity was determined by the ra-

dioimmunoassay (Clinical Assay, Baxter, Cambridge MA) of generated angiotensin I after 3 h incubation at pH 7.4 and 37 °C in conditions inhibiting further conversion of angiotensin I. ALDO and NE were measured using commercially available kits (Coat-A-Count Aldosterone, Diagnostic and Products Corporation, Los Angeles, CA and Cathecolamines ^{3}H-Radioenzymatic Assay, Amersham International, UK, respectively). ANP was extracted on C18 Sep-Pack cartridges (Waters Associates, Milford, MA) and assayed by a previously described radioimmunoassay technique (7). ^{125}I-iothalamate was measured in a gamma counter. Sodium, potassium and PAH were measured by standard techniques.

Statistical analysis of results was performed by the paired and unpaired Student's t test and the two variable regression analyses, as appropriate. Results are given as the mean ± SEM.

Results

At the end of the low sodium diet period urinary sodium excretion was 35 ± 4 mEq/day (range 7-45 mEq/day) in all subjects, and increased up to 340 ± 16 mEq/day (range 304-415 mEq/day) following sodium supplementation.

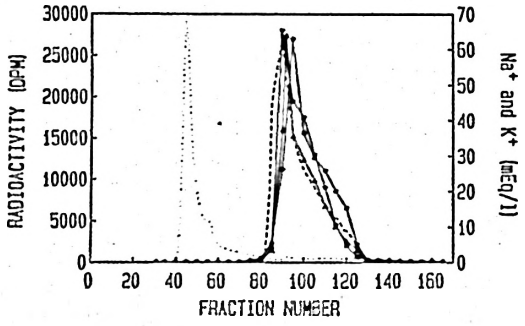

Fig. 1. *Chromatographic pattern of sodium and potassium (– – –), ^{125}I-ANP (····), ^{3}H-PGE$_2$ (▲), ^{3}H-6-keto-PGF$_{1\alpha}$ (●) and ^{3}H-PGD$_2$ (*) in the Sephadex G-25 column.*
No radioactivity was detected in the 25 post-salt fractions used for natriuretic hormone measurement.

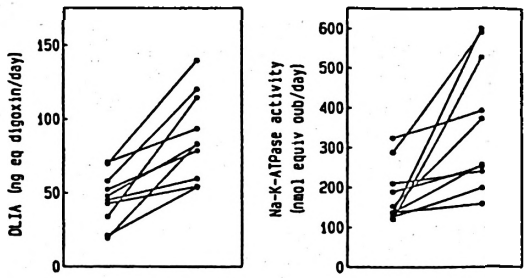

Fig. 2. *Digoxin-like immunoreactive activity (DLIA) and Na-K-ATPase inhibiting activity (Na-K-ATPase inhibition) in urinary extracts from 10 healthy subjects studied during low and high sodium intake.*

The Sephadex G-25 pattern of sodium and potassium, ^{125}I-ANP, ^{3}H-PGE$_2$, ^{3}H-6-teko-PGF$_{1\alpha}$ and ^{3}H-PGD$_2$ is shown in figure 1. All labelled prostaglandins eluted between fractions 80 and 130, together with the salts. ^{125}I-ANP eluted in about the 40-60 fractions, as corresponds to high molecular weight material. No radioactivity was detected in the 25 post-salt fractions used to measure DLIA and the natriuretic and Na-K-ATPase inhibiting activities.

Figure 2 depicts the individual results of urinary DLIA and Na-K-ATPase inhibiting activity obtained at the end of the two study periods. DLIA (87.9 ± 9.2 vs 46.1 ± 5.6 ng eq digoxin/day) and Na-K-ATPase inhibiting activity 359.8 ± 51.9 vs 182.9 ± 22.7 nmol eq oub/day) were significantly higher ($p < 0.001$ and $p < 0.005$, respectively) following the high sodium intake period than at the end of the low sodium diet period. The increase in DLIA and Na-K-ATPase inhibition activity after sodium supplementation was observed in all individuals. There was a significant direct correlation between Na-K-ATPase inhibiting activity and DLIA

Table I. *Renal plasma flow (RPF), glomerular filtration rate (GFR), urine volume (UV), urinary sodium excretion ($U_{Na}V$), fractional sodium excretion (FENa) and mean arterial pressure (MAP) observed in the bioassay rats before (basal) and after the administration of the urinary extracts corresponding to the low (low Na) and high sodium (high Na) intake periods.*

	Basal	Low Na	p value (vs basal)	Basal	High Na	p value (vs basal)
RPF (ml/min)	4.67 ± 0.63	4.88 ± 0.37	NS	5.02 ± 0.37	5.35 ± 0.45	NS
GFR (ml/min)	1.35 ± 0.12	1.29 ± 0.12	NS	1.39 ± 0.13	1.46 ± 0.36	NS
UV (µl/min)	5.47 ± 0.80	11.36 ± 1.94	< 0.01	5.35 ± 0.74	14.59 ± 2.46	< 0.01
$U_{Na}V$ (µEq/min)	0.22 ± 0.06	0.60 ± 0.15	< 0.01	0.24 ± 0.03	1.57 ± 0.32*	< 0.001
FENa (%)	0.10 ± 0.02	0.32 ± 0.07	< 0.01	0.10 ± 0.02	0.63 ± 0.10*	< 0.001
MAP (mmHg)	124 ± 3	122 ± 4	NS	120 ± 3	133 ± 4**	< 0.005

* = $p < 0.025$ and ** = $p < 0.05$ vs low Na

values obtained in the whole group of samples analyzed ($r = 0.50$, $p < 0.05$).

Urinary extracts from both the high and low-sodium diet periods caused a significant increase in urine volume, sodium excretion and fractional sodium excretion with respect to baseline values, without affecting RPF and GFR. The natriuretic potency of urinary extracts obtained following high sodium intake, however, was remarkably higher than that of urinary extracts obtained during low sodium diet, the absolute increase in sodium excretion induced by extracts obtained during high and low sodium intake being 1.33 ± 0.31 µEq/min and 0.38 ± 0.11 µEq/min, respectively ($p < 0.025$). Extracts obtained at the end of the low-sodium diet did not affect MAP of the bioassay animals. On the contrary, MAP significantly increased in rats to which the extracts of the high-sodium diet period were administered (table I).

The mean plasma levels of PRA, ALDO, NE and ANP observed at the end of the two study periods in all subjects is shown in table II. The increase in sodium intake suppressed the renin-aldosterone system and increased ANP levels. A reduction in plasma NE was also observed following high sodium intake, although the difference was not significant.

Discussion

An endogenous factor(s) with DLIA was first described by GRUBER *et al.* (11) in plasma of hydropenic dogs. Saline infusion to these animals was followed by a marked increase in plasma DLIA levels. Na-K-ATPase inhibiting activity in the same plasma fraction also increased after saline infusion in these animals. The authors suggested that both DLIA and Na-K-ATPase inhibition were due to the same

Table II. *Plasma renin activity and plasma concentrations of aldosterone, atrial natriuretic peptide and norepinephrine in the studied subjects during low (low Na) and high (high Na) sodium intake.*

	Low Na	High Na	p
Plasma renin activity (ng/ml · h)	1.32 ± 0.26	0.51 ± 0.11	< 0.025
Aldosterone (ng/100 ml)	24.4 ± 2.7	9.0 ± 1.1	< 0.01
Atrial natriuretic peptide (fmol/ml)	6.01 ± 1.15	9.89 ± 1.71	< 0.05
Norepinephrine (pg/ml)	351.2 ± 46.6	268.9 ± 24.1	NS

factor and that DLIA estimated natriuretic hormone. Subsequent studies in animals and humans have given support to this hypothesis. CASTAÑEDA-HERNÁNDEZ and GODFRAIND (2) found DLIA in rat plasma, which increased during high sodium intake. Similar findings were obtained by GAULT *et al.* in hypertensive patients (6). High plasma and urinary DLIA and Na-K-ATPase inhibiting activity have also been reported in patients and experimental animals with arterial hypertension and chronic renal failure (1, 5, 9, 10, 13). Finally, KLINGMÜLLER *et al.* were able to concentrate the natriuretic material contained in the urine of healthy subjects on high sodium intake using an anti-digoxin antiserum (14).

In contrast, in the only study specifically investigating the relationship between DLIA and the natriuretic and Na-K-ATPase inhibiting activities in the urine of healthy subjects on low and high-sodium diet, KRAMER *et al.* (15, 16) found a clear dissociation between these parameters. In that study, urine was processed with the same method as the present one and the assays were performed in the salt and post salt fractions. Natriuretic activity and Na-K-ATPase inhibition were found only in the post salt fractions. DLIA was also mainly present in the post salt fraction although a small activity was detected in the salt fraction. High sodium intake induced a significant increase in Na-K-ATPase inhibition and natriuretic activity. On the contrary, DLIA significantly decreased in the post salt fraction following high sodium intake. Based on these data, KRAMER *et al.* (16) suggested that DLIA does not reflect NH.

The current study re-evaluates the effect of sodium intake on DLIA and the Na-K-ATPase inhibiting and natriuretic activities in urine samples of healthy subjects. All assays were performed in the post-salt fraction after Sephadex G-25 chromatography, since it is the only fraction containing natriuretic activity (3, 14). This fraction does not contain PGE_2, bradykinin and substance P (14), nor does it contain ANP (present study).

All urinary extracts obtained in the present study had a natriuretic activity when infused into euvolemic bioassay rats, the natriuretic potency being greater in samples obtained at the end of the high sodium intake period. This natriuretic effect was associated with an increased fractional sodium excretion, indicating a tubular action of the extracts. This biological effect is recognized as being related to natriuretic hormone (1, 5, 10). Urinary extracts from sodium-loaded subjects also induced a significant increase in MAP, which migth have contributed to the greater natriuretic effect observed with these extracts. All urinary extracts also showed Na-K-ATPase inhibiting activity, which was greater following high sodium intake. These findings suggest the presence of natriuretic hormone in the urine fraction studied.

In the current study, DLIA was clearly related to the *in vivo* and *in vitro* activities associated with natriuretic hormone. DLIA was detected in the same urine fraction containing Na-K-ATPase inhibiting and natriuretic activities. High sodium diet, which increased the Na-K-ATPase inhibiting activity and natriuretic activity of urinary extracts, also induced a parallel change in the urinary excretion of DLIA. Reasons for the discrepancy between these results and those of KRAMER *et al.* (16) are difficult to ascertain. The experimental design of both studies, as well as the methodology used were almost identical. Possible interferences by dietary factors, which affected DLIA measurements in experimental animals (12), were avoided by giving the subjects an identical diet throughout the study and NaCl supplementation during the high sodium intake period. Further studies are clearly needed to better understand the complex relationship between the different biological activities attributed to natriuretic hormone.

In conclusion, the post-salt fraction obtained by Sephadex G-25 chromatography from urine samples of healthy subjects, which shows biological activities associated to natriuretic hormone (natriuretic activity *in vivo* and Na-K-ATPase inhibition *in vitro*) also contains DLIA. The administration of a sodium load to these subjects induced an increase in DLIA which paralleled changes in the Na-K-ATPase inhibiting activity and natriuretic activity. These findings are consistent with the contention that DLIA is related to natriuretic hormone.

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Resumen

Se determinan las actividades inmunoreactivas similares a la digoxina (DLIA), inhibidora de Na-K-ATPasa y natriurética, de los extractos urinarios obtenidos de 10 voluntarios sanos después de 5 días de dieta hipo- e hipersódica, sucesivamente. En todos los individuos se observan, durante la dieta hiposódica, niveles urinarios detectables de actividad DLIA (46,1 ± 5,6 ng eq digoxina/día), inhibidora NA-K-ATPasa (182,9 ± 22,7 nmol eq oub/día) y natriurética ($U_{Na}V$: 0,38 ± 0,11 µEq/min). La ingesta rica en sodio se asocia a un incremento en la actividad DLIA (87,9 ± 9,2 ng eq digoxina/día, $p < 0,001$) y a cambios paralelos en las actividades inhibidora Na-K-ATPasa (359,8 ± 51,9 nmol eq oub/día, $p < 0,005$) y natriurética ($U_{Na}V$: 1,33 ± 0,3 µEq/min, $p < 0,025$). Estos resultados indican que la actividad DLIA está relacionada con la hormona natriurética.

Palabras clave: Sustancias natriuréticas, Hormona natriurética, Inmunorreactividad, Na-K-ATPasa.

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