Symposium

The Change and Significance of the Na⁺-K⁺-ATPase α -Subunit in Ouabain-Hypertensive Rats

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Ouabain has recently been identified as an endogenous Na⁺-K⁺ pump inhibitor having a close association with hypertension. However, some patients with hypertension do not show high levels of endogenous ouabain(EO), and patients with high EO levels do not necessarily suffer from hypertension. It is believed that the Na⁺-K⁺-ATPase activity in essential hypertension does not undergo homogenous change. The present study was designed, therefore, to investigate the expression and the significance of the Na⁺-K⁺-ATPase α -subunit isoforms in kidney tissue in ouabain-hypertensive rats. Ouabain was administered chronically to establish a model of ouabain-hypertensive rats. Biochemical analysis, cytobiology and sABC immunohistochemistry were they used to assay for expression of Na⁺-K⁺-ATPase α -subunit isoforms in kidney tissue. After the first week of receiving ouabain, 65% ($n=13$) of rats had hypertension. After the second week, the blood pressure of these 13 hypertensive rats was increased significantly compared to the baseline and control levels ($p<0.05$). The plasma renin activity was normal, and angiotensin II and aldosterone levels were increased significantly in these rats ($p<0.05$). But in the other 35% ($n=7$) of rats of the experimental group, there was no apparent increase in blood pressure after receiving ouabain. The plasma ouabain level in the non-hypertensive subgroup was significantly higher than that in the hypertensive subgroup, but the ⁸⁶Rb intake and the number of ³H-ouabain binding sites did not decrease. The Na⁺-K⁺-ATPase activity showed non-homogeneous changes. In hypertensive rats, the expression levels of ouabain paralleled the degree of hypertension ($r=0.88$, $p<0.05$). The positive granules were mainly scattered in the cytoplasm of the reticular zone of adrenal cortex. There were thus different levels of expression of Na⁺-K⁺-ATPase α -subunit isoforms in this model. In the hypertension subgroup the α_1 was most strongly expressed, followed by the α_2 and α_3 isoforms. But in the non-hypertensive subgroup the order was $\alpha_3>\alpha_2>\alpha_1$. The positive granular was mainly scattered in the convoluted tubules of the kidney. These results suggest that the high level of ouabain and the change of the Na⁺-K⁺-ATPase α -subunit isoforms may play a critical role in hypertension. (*Hypertens Res* 2001; 24: 729-734)

Key Words: Na⁺-K⁺-ATPase, α -subunit, ouabain, ouabain-hypertensive rat

Introduction

The pathogenesis of hypertension is due to a variety of causes, including inherited predisposition and environmental factors (1). Although scientists are interested in the complex interplay of physiological and molecular factors, the actual causes of hypertension remain uninvestigated. Substantial

evidence implicates impaired renal excretion of sodium as the major culprit in the pathogenesis of hypertension (2-4). The key question is: how does the impairment of Na⁺ excretion lead to increased peripheral vascular resistance and elevation of blood pressure? The evidence suggests that elevated levels of a recently-discovered adrenal cortical hormone, endogenous ouabain (EO) and circulation of the Na⁺-K⁺-ATPase inhibitor play central roles in this process (5, 6).

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Remarkable increases in EO content have been observed in plasma, tissue, and urinary samples of many experimental models of essential and secondary hypertension. The increase parallels the average arterial pressure (7, 8).

However, some patients with hypertension do not show high levels of endogenous ouabain (EO), and patients with high EO levels do not necessarily suffer from hypertension. Hilton *et al.* (9) believe that the Na⁺-K⁺-ATPase activity in essential hypertension does not undergo homogenous change, and that it can be divided into two types: inhibition and non-inhibition Na⁺-K⁺-ATPase. The first type is related to the increase of plasma EO, and the manifestation of the latter type is the ionic transport defect of the cytomembrane, which is related to inheritance. Patients with hypertension sometimes show either or both of the two kinds of defects, which may manifest heterogeneity of the physiological and biochemical changes.

The study of ouabain is currently focused on its structure, elements, manner of secretion, relation with the renin-angiotensin-aldosterone system (RAAS) and relation to the adjustment of blood pressure (10–12), etc. Up to now there have been few reports on the expression of the Na⁺-K⁺-ATPase α -subunit isoforms in ouabain-hypertensive rats. Ouabain-hypertensive rats are the research subject of present study which also investigates the expression of the Na⁺-K⁺-ATPase α -subunit isoforms in kidney tissue and attempts to clarify the possible effects of ouabain on hypertension and relationship with the Na⁺-K⁺-ATPase.

Materials and Methods

General Protocol

Six-to-ten-week-old male Sprague-Dawley (SD) rats weighing 150–200g were used for the experiments. The rats were kept in a temperature-controlled room with a 12-h light/dark cycle and given free access to tap water and standard rat chow. The rats were randomly divided into experimental and control groups: the former ($n=20$) received a maintenance dose of ouabain (Sigma Chemical Co., St Louis, USA) intraperitoneally. On day 1, a loading dose of 34 $\mu\text{g/kg}$ IP was administered, followed by a dose of 27.8 $\mu\text{g/kg}$ per day IP for an additional 6 weeks. The latter group ($n=10$) received vehicle (0.9% saline) only. Ouabain was dissolved in sterile saline at 20 $\mu\text{g/ml}$ and was stored for up to 1 week at 4°C in the dark. Doses were determined based on previously published pharmacokinetic data for ouabain in rats (13). Doses administered were estimated to increase average plasma levels 500 to 1,000 pmol/l above the physiological level. After 6 weeks of treatment, 24 h after the last ouabain or vehicle injection, animals were anesthetized with 120 mg/kg IP thiobutabarbital, then 2 to 4 ml of blood was removed from the abdominal aorta, and the plastic syringe was washed with heparin or EDTA. This blood was centrifuged at 1,500 g and the plasma was removed and stored at –20°C for biochemical

assay. The heart, kidneys, and adrenals were removed, weighed, and stored at –70°C until assay for ouabain.

Blood Pressure Measurement

Systolic blood pressure was measured in all conscious rats weekly during the experiment using the indirect tail-cuff method. The rats were preconditioned to the experimental procedure for 1 week before actual measurements were conducted. The equipment included a blood pressure sensor/cuff, a blood pressure amplifier, and a digital recorder (Analytic Instrument Co., Tianjin, China). The average of four such recordings was taken as the individual systolic blood pressure. This method correlates highly with direct cannulation measurements (14).

Biochemical Measurements

Plasma and tissue ouabain levels were measured by the enzyme-linked immunosorbent assay (ELISA) method (15). Ouabain dialdehyde was coupled to C6-ovalbumin and prepared ouabain-ovalbumin conjugate. The resulting solution was freeze-dried. Ouabain-ovalbumin (10 mg) was suspended in 5 ml 0.9% saline and emulsified with 5 ml Freund's complete adjuvant. Each of two New Zealand White rabbits received a subcutaneous injection of the ouabain-ovalbumin solution with boosters at monthly intervals for 2 months. Blood samples were collected into EDTA tubes through a small incision on a marginal ear vein 2 weeks after the booster injections. Immunoreactivity to ouabain was detected in EDTA plasma from both rabbits. Plasma from the second bleeding of one rabbit was diluted 1:100 with assay buffer and stored in 100 μl aliquots at –20°C. A stock standard of 1 $\mu\text{g/ml}$ ouabain was prepared in ethanol and diluted in phosphate-buffered saline (PBS pH 7.4) to provide a series of standards with concentrations of 0, 0.23, 0.69, 2.1, 6.2, 18.7, and 56 nmol/l. Standards were stable at 4°C for at least 3 months. Venous blood samples were drawn from the research subjects into chilled tubes containing EDTA (1.5 mg/ml) and were centrifuged at 1,500 $\times g$ and 4°C for 5 min within 10 min of blood collection; the plasma was stored at –20°C. Bond Elute C18 cartridges were washed under vacuum with 3 ml methanol, followed by 3 ml distilled water. The plasma sample (1 ml) was added to the cartridge and allowed to pass through without vacuum, followed by three 3 ml washes with distilled water. Then 3 ml 25% acetonitrile/0.1% trifluoroacetic acid solution was used to elute ouabain. The concentrations of ouabain were detected on an automatic ELISA processing machine. Absorbance was read at 490 nm.

Plasma renin activity and angiotensin II and aldosterone contents were determined by commercial radioimmunoassay (Northern Biological Technique Co., Beijing, China). Erythrocyte sodium pump quantity and activity was measured by the method reported by Lu (16).

Streptavidin-Biotin-Peroxidase Complex Immunohistochemical Assay

The tissue samples were fixed with 10% formalin and embedded in paraffin, deparaffinized in xylene, and immersed in a solution of 0.3% hydrogen peroxide, then incubated with 5% normal goat serum for 30 min. The primary antibodies used were polyclonal rabbit anti-human ouabain antibody (the generous gift of Dr. Hamlyn, University of Maryland, Maryland, USA) at a 1:100 dilution, and monoclonal rat anti-human $\text{Na}^+-\text{K}^+-\text{ATPase } \alpha_1, \alpha_2, \alpha_3$ -subunit isoforms antibodies (Santa Cruz Chemical Co., Santa Cruz, USA) at a 1:50 dilution for 2 h at room temperature. The samples were then incubated with biotin-conjugated anti-mouse immunoglobulin-G, after that they were incubated with an avidin-biotinylated peroxidase conjugate. The sections finally were counterstained with hematoxylin. Negative controls were established in which normal rabbit serum was used as the primary antibody. The positive expression rates were determined by an image analyzer SAMBA 200 (SAMBA Technologies, Grenoble, France) (17).

Statistical Analyses

All data were processed by computer with an SPSS 10.0

Fig. 1. Line graph shows tail systolic blood pressure (BP) in SD rats treated with ouabain ($n = 20$) and saline ($n = 10$).

Table 1. Plasma Renin Activity and Angiotensin II and Aldosterone Contents

Group	N	Renin activity (ng Ang I · ml ⁻¹ /h)	Angiotensin II (ng/ml)	Aldosterone (pg/ml)
Ouabain	20			
Hypertensive	13	2.25±0.26	9.26±0.88*	438.43±30.58*
Non-hypertensive	7	2.23±0.17	7.75±0.32	336.91±28.16
Saline	10	2.29±0.44	7.93±0.25	323.36±29.11

* $p < 0.05$ vs. saline-treated controls.

Table 2. Plasma and Tissue Ouabain Levels

Group	N	Plasma (ng/ml)	Kidney (ng/g tissue)	Adrenal (ng/g tissue)	Heart (ng/g tissue)
Ouabain	20				
Hypertensive	13	1.06±0.17	5.19±1.16*	26.35±3.27	2.66±1.35
Non-hypertensive	7	2.79±0.13* ^Δ	8.06±0.12* ^Δ	28.24±3.21	3.97±1.63
Saline	10	0.91±0.04	2.41±1.37	29.52±4.16	3.94±1.18

* $p < 0.05$ vs. saline-treated controls; ^Δ $p < 0.05$ vs. ouabain-hypertensive rats.

software package, and were expressed as the mean ± SEM. Comparisons between two independent groups were made with the Student's *t*-test. Comparisons between more than two independent groups were made with one-way ANOVA. Regression analyses were used to compare the relationship between systolic blood pressure and ouabain levels. A value of $p < 0.05$ was considered to indicate statistical significance.

Results

Sixty five percent ($n = 13$) of the ouabain-treated rats achieved great tail systolic blood pressures after the first week of receiving ouabain, compared with their respective saline-controls. From the second week, tail systolic blood pressure in SD rats receiving 27.8 μg/kg per day ouabain was significantly greater than that of baseline or in the respective saline-controls. A stable administration of ouabain was able to maintain blood pressure at a high level. But in the other 37% ($n = 7$) of ouabain-treated rats, the blood pressure showed no apparent increase after ouabain administration (Fig. 1). Plasma renin activity was unchanged in rats receiving intraperitoneal ouabain, but angiotensin II and aldosterone increased remarkably in hypertensive rats compared with non-hypertensive rats and controls (Table 1).

Plasma ouabain levels were not significantly greater in ouabain-hypertensive rats compared with controls, but were significantly greater in ouabain non-hypertensive rats compared with ouabain-hypertensive subgroup and controls. Total kidney ouabain content was increased in rats receiving ouabain vs. controls. The level in the ouabain non-hypertensive subgroup was the greatest. Total adrenal and heart (ventricles and atria) ouabain levels were not significantly different between the experimental groups and controls (Table 2).

⁸⁶Rb intake and the number of ³H-ouabain binding sites apparently decreased in hypertensive rats compared with controls, but were unchanged in the non-hypertensive subgroups

Table 3. Erythrocyte Sodium Pump Quantity and Activity

Group	N	³ H-Ouabain binding site (number/cell)	⁸⁶ Rb uptake (10 ⁻⁸ mmol·cell ⁻¹ /min)
Ouabain	20		
Hypertensive	13	307±31*	0.02±0.01*
Non-hypertensive	7	443±55* ^Δ	0.17±0.01* ^Δ
Saline	10	462±78	0.19±0.03

* $p < 0.05$ vs. saline-treated controls; ^Δ $p < 0.05$ vs. ouabain-hypertensive rats.

Table 4. Erythrocyte Na⁺,K⁺ Content

Group	N	Na ⁺ Content (mmol/kgHb)	K ⁺ Content (mmol/kgHb)
Ouabain	20		
Hypertensive	13	19.11±10.01*	300.47±24.15**
Non-hypertensive	7	53.61±20.34**	261.13±33.82**
Saline	10	27.21±13.70	257.08±25.16

* $p < 0.05$ vs. saline-treated controls; ** $p < 0.01$ vs. saline-treated controls.

Table 5. Expression of Na⁺-K⁺-ATPase α Isoforms in Ouabain-Hypertensive Rats

Group	N	α_1 (%)	α_2 (%)	α_3 (%)
Ouabain	20			
Hypertensive	13	49.98±18.72	35.85±11.48	23.10±9.02
Non-hypertensive	7	29.58±6.37*	49.49±10.54*	62.73±11.49*

* $p < 0.05$ vs. ouabain-hypertensive rats.

Fig. 2. The relationship of ouabain expression and SBP.

compared with the hypertensive groups and controls. The Na⁺-K⁺-ATPase activity showed non-homogeneous changes in animals receiving ouabain. This also can be described as "sodium-pump-inhibition-type" and "non-sodium-pump-inhibition-type" (Table 3).

Erythrocyte Na⁺ content was significantly decreased in hypertensive and increased in non-hypertensive rats; K⁺ content was significantly increased in hypertensive and decreased in non-hypertensive compared with controls, respectively (Table 4).

In the hypertensive sub group, the ouabain expression in the adrenal glands paralleled the degree of hypertension ($r =$

0.88, $p < 0.05$, Fig. 2). There were significant differences in the expression levels of Na⁺-K⁺-ATPase α -subunit isoforms in adrenal tissue between hypertensive and non-hypertensive subgroups (Table 5). The α_3 was most strongly expressed, followed by the α_2 and α_1 isoforms. But in hypertensive rats the order was $\alpha_1 > \alpha_2 > \alpha_3$. The positive granules for each isoform appeared yellow-brown and were mainly scattered in the cytoplasm of the reticular zone of the adrenal cortex (Figs. 3–5) and the convoluted tubule of kidney (Fig. 6).

Discussion

A circulating sodium pump inhibitor, endogenous ouabain, has been implicated in the pathogenesis of human disorders such as essential hypertension, pregnancy-induced hypertension, primary aldosteronism and cardiac failure (7, 8). These findings suggest that ouabain probably has a role in hypertension. Although both essential and secondary hypertension have EO increase, it is still not clear whether the increase is the primary or secondary change. The present model of ouabain-hypertension revealed normal plasma renin activity, but angiotensin II and aldosterone were increased remarkably in hypertensive rats. These results showed that long-term administration of ouabain produces a sustained elevation of blood pressure in most subjects, but others had no

Fig. 3. Positive expression of the α_1 subunit in adrenal tissues. The positive granules were mainly scattered in the cytoplasm of the reticular zone of the adrenal cortex (10×20).

Fig. 4. Positive expression of the α_2 subunit in adrenal tissues. The positive results are the same in Fig. 3 (10×10).

Fig. 5. Positive expression of the α_3 subunit in adrenal tissues. The positive results are the same as in Fig. 3 (10×10).

Fig. 6. Positive expression of the α_3 subunit in the kidney. The positive granules appeared yellow-brown and were mainly scattered in the convoluted tubule of kidney. The vascular also had the positive expression (10×20).

change in blood pressure. The $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity showed non-homogeneity changes in hypertension and non-hypertension subgroups respectively. The possible reason is ouabain could increase intracellular Na^+ and Ca^{2+} content and plasma angiotensin II and aldosterone levels, effect the activation of the RAAS, then regulate the the $\text{Na}^+\text{-K}^+\text{-ATPase}$ α -subunits isoforms expression and its activity in experimental hypertension model, which may be important in the pathogenesis of hypertension.

$\text{Na}^+\text{-K}^+\text{-ATPase}$ is an integral membrane protein found in the cells of all higher eukaryotes and is responsible for translocating the $\text{Na}^+\text{-K}^+$ ions across the cell membrane. It is the receptor for cardiac glycosides, including ouabain and digoxin. The former study of our scientific group observed the different effects of ouabain and digoxin on both the systolic blood pressure and expression of sodium pump α subunit isoforms in tissues in rats (18).

Utilizing an immunohistochemistry assay, the present paper mainly focuses on studying the characteristics of the ex-

pression of each of the α -subunit in kidney tissues of hypertensive and non-hypertensive rats as represented by the comparison of the functional properties of $\text{Na}^+\text{-K}^+\text{-ATPase}$ molecules composed of each of the three α -subunit isoforms and the structure-function relationships of the $\text{Na}^+\text{-K}^+\text{-ATPase}$. The results of the present study indicate that the expression rates of α -subunit isoforms and erythrocyte $\text{Na}^+\text{-K}^+$ contents showed different changes in hypertensive and non-hypertensive rats. In non-hypertensive subgroup the sodium pump activity was not inhibited and ouabain resistance was present.

The ouabain resistance is that the biological effect of ouabain does not as good as expected on the aspects of inhibiting $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity. The combining power for ouabain and ouabain binding site of the $\text{Na}^+\text{-K}^+\text{-ATPase}$ are decreased. Under the condition of high level ouabain, $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity can't be inhibited consequently, so

leading to kidney sodium excretion disturbance (6). Multiple extracellular and transmembrane regions are involved in determining ouabain sensitivity. Price *et al.* (19) used the site-directed mutagenesis technique to identify substitutions at amino acid residues Gln-111 and Asn-122 of the sheep α_1 subunit that alter ouabain sensitivity of $\text{Na}^+\text{-K}^+\text{-ATPase}$. Three transmembrane amino acids, Tyr-108 and Cys-104 mainly in the H₁-H₂ transmembrane regions have been found to be important for ouabain sensitivity (20). However, it is possible that some of the amino acid substitution were in the binding site which others might only affect binding at a distance through conformation changes.

In summary, the $\text{Na}^+\text{-K}^+\text{-ATPase}$ may be regulated in the specific $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity types by the extent to which isoforms function in response to certain physiological demands. The identification and characterization of the change of the $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity non-homogeneity, the function and gene expression variation of the $\text{Na}^+\text{-K}^+\text{-ATPase}$ α -subunits isoforms may act as an important joint between cytomembrane ions transport and ouabain, ouabain could regulate the gene expression of sodium pump α -subunits isoforms, and sodium pump activity variation and genomic mutation lead to the ionic transport defect and sodium drain obstruct then effect blood pressure, so ouabain and $\text{Na}^+\text{-K}^+\text{-ATPase}$ might play a critical role in hypertension.

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