Original Article

Differential Regulation of the Sodium Pump α -Subunit Isoform Gene by Ouabain and Digoxin in Tissues of Rats

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The effects of ouabain and digoxin on both the systolic blood pressure (SBP) and sodium pump α -subunit expression in some tissues of rats were compared. Normal rats were injected with ouabain, digoxin, and normal saline (NS), respectively, everyday, and indirect SBP was recorded once a week. Six weeks later, all the rats were killed, and sodium pump α_1 -, α_2 -, and α_3 -subunit mRNA levels were detected in the myocardium, kidney, adrenal gland, aortic smooth muscle, and hypothalamus by the RT-PCR method. The results showed that the SBP of rats infused with ouabain increased significantly at the end of week 6, while no difference in SBP was found between the digoxin and NS groups. The effects of ouabain and digoxin on sodium pump α -subunit isoform expression were also different. Myocardium: both ouabain and digoxin stimulated expression of the α_3 -isoform whereas α_2 was unchanged. Levels of the α_1 isoform decreased significantly in the ouabain group and decreased slightly in the digoxin group, respectively. Kidney: digoxin had the same effects as ouabain. α_1 levels increased, but those of α_2 and α_3 remained unchanged. Adrenal gland: α_2 and α_3 levels increased, but those of α_1 decreased in the ouabain group. α_1 and α_3 levels increased and those of α_2 remained unchanged in the digoxin group. Aortic smooth muscle: both ouabain and digoxin increased α_1 and α_3 expression. α_2 levels decreased in the digoxin group but remained unchanged in the ouabain group. Hypothalamus: both ouabain and digoxin stimulated α_1 expression, while α_2 and α_3 levels remained unchanged. The results of this study have shown that ouabain and digoxin have the different effects on both the systolic blood pressure and expression of sodium pump α -subunit isoforms in some tissues in rats. Further studies on the expression of sodium pump α -subunit isoforms might be helpful for the understanding of the physiological role of endogenous ouabain and the molecular mechanisms involved in the pathogenesis of hypertension.

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Key Words: ouabain, digoxin, sodium pump, hypertension, rat

Introduction

Accumulating evidence indicates that endogenous ligands of the digitalis receptor, which have recently been reported to be indistinguishable from ouabain, may exist in the mammalian body (1, 2). Endogenous ouabain (EO) might induce many cytobiological changes and play an im-

portant role in regulating water and sodium metabolism and vascular tone in the body (3, 4). Many studies have shown that the EO content in both hypertensive patients and hypertensive animals is much greater than that of normal subjects, suggesting that higher EO levels might be involved in the development of hypertension (5-7). Hamlyn and colleagues (8, 9) have reported that the administration of ouabain to either Wistar rats or

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Table 1. Primer Sequences for Amplifying Sodium Pump α_1 -, α_2 -, α_3 -Subunit Isoforms and G3PDH

Primer specificity		Position of bp in cDNA	Sequence (5'→3')	Length of product (bp)
α_1 -isoform	P1	1,617-1,638	AAGGACGCCTTTCAGAATGCCT	247
	P2	1,863-1,843	TGACCATGATGACCTTAATCC	
α_2 -isoform	P1	2,690-2,710	CACCTACTTTGTAATACTGGC	264
	P2	2,953-2,931	ATCAGGATCTTGTCTTCATGCC	
α_3 -isoform	P1	1,602-1,621	GACCCCAATGACAACCGATA	285
	P2	1,886-1,866	CATGGACATGAGACCCACGAA	
G3PDH	P1	550-569	ACCACAGTCCATGCCATCAC	452
	P2	1,001-982	TCCACCACCCTGTTGCTGTT	

Sprague-Dawley (SD) rats can induce hypertension. However, the mechanism by which ouabain induces hypertension is not completely clear. It has been suggested that EO induces a series of changes with cytobiological effects and leads to hypertension by changing the sodium pump (Na^+, K^+ -ATPase) configuration and inhibiting its activity, and that this might be the only effect of EO on the sodium pump according to traditional views (3, 4).

The sodium pump, acting as a "receptor" for cardiac glycosides such as digoxin and ouabain, consists of genes encoding for at least three α -subunit isoforms and two β -subunit isoforms. The α -subunit contains the catalytic and ouabain binding sites and possesses specialized functions, and the β -subunit is a glycoprotein that is essential for the normal function and assembly of the Na^+, K^+ -ATPase (10). These isoforms are expressed in a tissue- and cell-specific fashion, and are controlled by developmental and hormonal regulatory influences (10-12). In addition, this gene expression can be affected in some diseased conditions such as hypertension and a hypertrophied myocardium (13, 14). But the effects of ouabain on sodium pump α -subunit gene expression is not well understood. This study was designed to investigate the effects of ouabain on sodium pump α -subunit gene expression *in vivo* and to compare these effects with those of digoxin.

Materials and Methods

Ouabain and Digoxin Infusion and Blood Pressure Measurement

Twenty-four healthy adult male SD rats (6-10 weeks old) weighing 200-250 g were purchased from the Experimental Animal Center of Xi'an Medical University. They were given free access to tap water and standard rat chow. After 1 week, the body weight and systolic blood pressures of the rats were determined to document normotension. The rats were divided randomly into three groups: the ouabain group (O group, $n=8$), digoxin group (D group, $n=8$) and normal saline group (N group, $n=8$). Animals in these three groups were given ouabain (Sigma Chemical Co., $20 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$) (8, 9), digoxin (Sigma Chemical Co., $32 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$), and

normal saline ($1 \text{ ml} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$) intraperitoneally daily, respectively. Feeding and living conditions were the same among these groups throughout the study. Indirect systolic blood pressure (SBP) was recorded by the tail cuff method once a week using the Heart Rate & Blood Pressure Recorder for Rats (Model MRB-III A, Shanghai Hypertension Institute, Shanghai, China). At the end of week 6, all of the rats were fasted overnight and killed by decapitation on the following morning. The left ventricular myocardium, cortex of the kidney, adrenal gland, thoracic aortic smooth muscle, and hypothalamus were collected for detecting mRNA levels of the sodium pump α -subunit isoform using reverse transcription polymerase chain reaction (RT-PCR) techniques. Those procedures were in accordance with the institutional and the National Research Council's guidelines for animal experiments.

RT-PCR

Total RNA was extracted from the above tissues as previously described (12), followed by digestion with 5 U RNase-free DNase (Boehringer Mannheim, Germany). Total RNA, 1 μg , was used to synthesize the first strand of cDNA. RNA samples were heated to 95°C for 4 min, chilled on ice, and reverse-transcribed at 48°C for 45 min in 23 μl containing 10 pmol reverse primer, 200 U reverse transcriptase, 400 $\mu\text{mol/l}$ of each deoxynucleotide triphosphate (dNTP: dATP, dTTP, dCTP, and dGTP), 50 mmol/l Tris-HCl (pH 8.3), 75 mmol/l KCl, and 3 mmol/l MgCl_2 . The reaction was terminated by heating to 65°C for 10 min. Equal volumes of resultant product were amplified in a final volume of 50 μl containing the following: 200 $\mu\text{mol/l}$ dNTP, 5 pmol each of forward and reverse primers, 10 mmol/l Tris-HCl (pH 8.8), 50 mmol/l KCl, 1.5 mmol/l MgCl_2 , 0.1% TritonX 100, and 2.5 U *taq* polymerase (Promega, Madison, WI). Samples were denatured at 94°C for 2 min and cycled 25 times through the following steps: 1 min at 94°C , 1 min at 52°C , and 2 min at 68°C . The final cycle was extended for 7 min at 68°C . PCR products were electrophoresed on 2% agarose gels and quantitated by scanning densitometry (Gelworks ID Intermediate 3.01). RT-PCR of the glyceraldehyde-3-phosphate-dehydrogenase (G3PDH) gene was performed

Fig. 1. Amplification curve of PCR

simultaneously as a control (15). The mRNA levels of the α_1 -, α_2 -, α_3 -subunit isoforms were expressed as relative units compared with the control (G3PDH), respectively.

Specific primers for PCR were chosen with the help of a suite of genetics data base programs (Oligo 5.0) and were synthesized by the Shanghai Institute of Cell Biology, Chinese Academy of Science. Primer sequences are listed in Table 1.

The PCR products quantitatively reflect the amount of initial template DNA only before the reaction reaches the plateau of the amplification curves. Therefore, we must determine the PCR cycles in this reaction system to compare the amount of initial template DNA using RT-PCR methods (16). Figure 1 shows the results found when equivalent RNA aliquots of the α_3 -subunit were amplified with varying numbers of PCR cycles. The amplification curves of the α_1 -, α_2 -, α_3 -subunits of the sodium pumps and G3PDH were similar. As shown, the plateau in PCR amplification occurred at about cycle 30, and the amount of PCR product increased exponentially at cycle 25 of this reaction for the sodium pump α_1 -, α_2 -, α_3 -subunit isoforms and for G3PDH.

Statistical Analysis

Values are expressed as mean $\pm$ SD. The Student's *t*-test was performed with Microsoft Excel 5.0 statistical software. *P* values of less than 0.05 were considered to indicate statistical significance.

Results

Effects of Continued Infusion of Ouabain or Digoxin on SBP

The SBP of rats began to increase after 2 weeks of oua-

Fig. 2. Changes in the SBP of each group during the experiment. Points and bars represent means $\pm$ SD ($n=8$ in each group). Statistically significant difference: * $p<0.05$, ** $p<0.01$ (compared with N group).

Fig. 3. Two percent agarose gel of PCR products of sodium pump α_1 -, α_2 -, α_3 -subunit isoforms and G3PDH. Lane 1: 100 bp DNA ladder; Lanes 2, 6: 247 bp products of RT-PCR from α_1 -isoform; Lanes 3, 7: 264 bp products of RT-PCR from the α_2 -isoform; Lanes 4, 8: 285 bp products of RT-PCR from α_3 -isoform; Lanes 5, 9: 452 bp products of RT-PCR from G3PDH. Products of lanes 2-5 for one tissue and lanes 6-9 for another tissue.

bain administration and increased significantly after 6 weeks compared with the control group, which received normal saline (132.6 ± 9.0 vs. 115.7 ± 8.2 mmHg, $p<0.05$). No difference in systolic blood pressure was found between the rats administered digoxin and those administered normal saline (Fig. 2).

Sodium Pump α -Subunit Isoform Abundance in Normal SD Rats

The sodium pump α_1 -, α_2 -, and α_3 -subunit isoforms are

Fig. 4. The α_1 -, α_2 -, and α_3 -isoform mRNA levels in various tissues were quantified by densitometric scanning and expressed as relative units compared with the control (G3PDH). Columns and bars show means $\pm$ SD (n=8 in each group). Statistically significant difference: *p<0.05, **p<0.01 (compared with N group).

distributed in a tissue-specific fashion in normal rats. The α_1 -isoform was found to be ubiquitous in most tissues, and its expression was greater than that of the other two isoforms in the ventricular myocardium, kidney, and adrenal gland. The predominance of the α_1 -isoform was especially found in the kidney. In the hypothalamus, however, expression of α_1 -isoform was less than that of the α_2 - and α_3 -isoforms. Second, the α_2 -isoform was expressed abundantly in the ventricular myocardium, aortic smooth muscle, and hypothalamus. The α_2 -isoform was more abundant than α_1 or α_3 in aortic smooth muscle. Third, the α_3 -isoform was expressed abundantly in aortic smooth muscle and the hypothalamus. In addition, we can describe the sodium pump α -subunit isoform abundance in each tissue: α_1 and α_2 were the predominant isoforms, while much less of the α_3 was expressed in the ventricular myocardium; α_1 was abundant, while less of α_2 and α_3 were expressed in the kidney; slightly more α_1 than the α_2 - and α_3 -isoforms were present in the adrenal gland, while slightly more α_2 was present in the aortic

smooth muscle; more α_2 and α_3 than α_1 were expressed in the hypothalamus.

Effects of Ouabain and Digoxin on the Expression of the Sodium Pump α -Subunit Isoform (Figs. 3, 4)

Expression of the sodium pump α -subunit isoform is regulated by either ouabain or digoxin in a tissue-specific fashion. 1) Ventricular myocardium (Fig. 4a): Both ouabain and digoxin stimulated expression of the α_3 -isoform, whereas the expression of α_2 remained unchanged. The α_1 expression decreased significantly in the ouabain group and decreased slightly in the digoxin group, respectively. 2) Kidney (Fig. 4b): Digoxin had the same effect on sodium pump α -subunit isoform expression as ouabain. The α_1 levels increased, while those of α_2 and α_3 remained unchanged. 3) Adrenal gland (Fig. 4c): α_2 and α_3 levels increased, while those of α_1 decreased in the ouabain group. In the digoxin group, α_1 and α_3 levels increased, and those of α_2 remained unchanged. 4) Aortic

smooth muscle (Fig. 4d): Both ouabain and digoxin increased α_1 and α_3 expression. The α_2 levels decreased in the digoxin group but remained unchanged in the ouabain group. 5) Hypothalamus (Fig. 4e): Both ouabain and digoxin, respectively, stimulated α_1 expression, which is generally relatively low in normal SD rats, while α_2 and α_3 levels remained unchanged.

Discussion

It has been demonstrated that ouabain administered intraperitoneally is readily absorbed, and that plasma ouabain levels are not significantly different between intraperitoneal and intravenous groups at 10 min after administration (8, 9). The doses of ouabain infused in our study were determined based on both pharmacokinetic data for ouabain and data obtained from the literature (8, 9). According to ouabain pharmacokinetics in animals, the doses of ouabain infused to the experimental rats in the present study are estimated to increase plasma levels of ouabain to 3-8 times higher (2-5 nmol/l) than physiological levels (the plasma ouabain concentrations in normal animals and human beings are approximately 0.07-0.11 nmol/l), which is similar to the levels present in many pathologic states, including hypertension and congestive heart failure (5). Moreover, based on weight gain, behavior changes, and cardiac rhythms, the pressor effects of ouabain did not appear to be associated with substantial toxicity.

Ouabain has been used clinically primarily as a short-term, intravenous drug for patients with congestive heart failure or atrial arrhythmias who may be unable to demonstrate a pressor response. However, clinical experiences have suggested that the long-term administration of digoxin does not produce hypertension. The present results also show that digoxin does not induce hypertension, indicating that there is a big difference between the effects of ouabain and those of digoxin on blood pressure, although both drugs belong to the digitalis family. Moreover, it has been found that both digoxin and digitoxin can prevent some forms of hypertension (17, 18). The reasons for the difference in effects of ouabain and digoxin on blood pressure are unknown.

According to the traditional view, EO may induce a series of changes in cytobiological effects, thus leading to hypertension by changing the sodium pump configuration and inhibiting its activity; it is possible that this inhibition is the only effect of ouabain on sodium pumps (3, 4). There are two central questions in relation to this view, however. First, digoxin is another member of the digitalis family, and it, like ouabain, causes cardiac and vascular smooth muscle to contract by inhibiting the activity of sodium pumps; clinical experience, however, and the results of the present study, suggest that digoxin does not induce hypertension with long-term administration and

might even prevent the development of hypertension (17, 18). Therefore, why is there a great difference between the effects of ouabain and those of digoxin on blood pressure? Second, EO levels in physiological and even in some diseased conditions are not as high as those used in most *in vitro* studies intending to suppress sodium pumps, indicating that unless there are ouabain-sensitive forms of the sodium pump α -subunit (α_2 and α_3), the lower concentrations of circulating ouabain might not be adequate to inhibit the enzyme and to play an important role in the control of vascular tone under physiological and pathophysiological conditions (5, 10). The results of this study have revealed abnormal expressions of sodium pump α -subunit isoform gene in ouabain-hypertensive rats, which might not be the consequences of hypertension because: 1) It is well known that ouabain could increase intracellular Na^+ and Ca^{2+} content and plasma angiotensin II and aldosterone levels (3, 4), all of which could regulate the gene expressions of sodium pump α -subunit isoforms (10, 19); 2) There were great differences of sodium pump α -subunit isoform gene expressions among different hypertensive animals (13), including the ouabain-hypertensive rat in the present study. Based on our previous studies and related research papers that the α_1 -, α_2 -, and α_3 -isoforms have different affinities for Na^+ and cardiac glycosides (3, 4, 10), we could put forward the hypothesis that ouabain induces some changes in the biological effects on related tissues and increases blood pressure not only by changing the sodium pump configuration and inhibiting its activity, but also, at least in part, by causing an abnormal expression of the sodium pump α -subunit isoforms gene. The relationships among ouabain, the sodium pump, and blood pressure could be presented in the following schema. But this hypothesis needs more evidence to be verified.

The sodium pump, acting as a "receptor" for cardiac glycosides such as digoxin and ouabain, plays a central role in a variety of physiological processes, including transepithelial ion transport, the regulation of cell

volume, Na⁺-coupled uptake of metabolic substrates (glucose, amino acids), and propagation of the action potential of muscle and nerve (3, 4, 10). It is well known that the α_1 -, α_2 -, and α_3 -isoforms have different affinities for Na⁺ and cardiac glycosides (3, 4, 10). According to our new hypothesis, ouabain could affect the activity of sodium pumps by changing either the configuration or the gene expression of sodium pump α -isoforms. Changes in sodium pump activity might: 1) in turn regulate the gene expression of sodium pump α -isoforms by changing intracellular Na⁺, Ca²⁺, and other ion concentrations (10, 19); and 2) interact with the complex neurohumoral regulation, which might then lead to hypertension in many ways, and interact with both sodium pump α -subunit isoform expression (10) and endogenous ouabain secretion (2).

In summary, there was a great difference between the effects of ouabain and those of digoxin on the blood pressure of rats. Prolonged administration of small doses of ouabain induced hypertension in SD rats, while administration of digoxin did not. Ouabain had a significant effect on the expression of sodium pump α_1 -, α_2 -, and α_3 -subunit isoforms in a tissue-specific fashion, and there were great differences between the effects of ouabain and those of digoxin on α -mRNA levels. Further determination of the significance of the regulations of sodium pump α -subunit isoform expression might be great helpful for the insight into the physiological role of endogenous ouabain and the molecular mechanism involved in the pathogenesis of hypertension.

Abbreviations

Abbreviations used in this paper: bp, base pair; cDNA, complementary DNA; DNA, deoxyribonucleic acid; dNTP, deoxynucleotide triphosphate; EO, endogenous ouabain; G3PDH, glyceraldehyde-3-phosphate-dehydrogenase; mRNA, message RNA; NS, normal saline; PCR, polymerase chain reaction; RNA, ribonucleic acid; RT, reverse transcription; SBP, systolic blood pressure; SD, Sprague-Dawley.

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