Effects of Endogenous Ouabain on the Development of Hypertension in 1k1c Hypertensive Rats

Wei-Qing YUAN, Zhuo-Ren LU, Hao WANG, Yu-Kang YUAN*, and Hui-Xun REN*

This study was designed to evaluate the role of endogenous ouabain (EO) in the development of hypertension in 1k1c (one kidney, one clip) hypertensive rats. First, the EO content of the serum of 1k1c hypertensive rats and normal Sprague-Dawley (SD) rats was detected by the enzyme linked immunosorbent assay method (ELISA). Second, blood pressure changes in the 1k1c rats were recorded directly after the 1k1c rats were injected randomly with anti-ouabain antibody, normal rabbit IgG, and normal saline, respectively. The results showed that EO levels in the serum of 1k1c hypertensive rats were significantly higher than those of normal SD rats (2.25 ± 0.92 pg/I vs. 1.12 ± 0.17 pg/I, p< 0.01), and correlated significantly with systolic blood pressure (r=0.59, p<0.05). Anti-ouabain antibody was able to significantly decrease the blood pressure of 1k1c hypertensive rats in a dose-dependent manner, while normal rabbit IgG or normal saline was not. These results indicate that endogenous ouabain might play an important role in the development of hypertension in 1k1c hypertensive rats. (Hypertens Res 2000; 23 Suppl: S61-S65)

Key Words: ouabain, antibody, hypertension, rat, blood pressure

Introduction

Endogenous ouabain (EO), also referred to as endogenous sodium pump inhibitor, endogenous digitalislike substance, or endogenous digoxinlike factor (1-4), is a recently identified hormone that may be secreted from the adrenal cortex or the hypothalamus (5-8). Sodium load, blood volume extension, angiotensin II, and so on can stimulate EO secretion, and EO has been shown to play many physiological and pathological roles. A great deal of evidence has shown that elevated EO levels might be involved in the development of hypertension (7-11). However, the changes in EO content and the significance of EO in the mechanism of hypertension development in 1k1c (one kidney, one clip) rats, one of the most important hypertensive models, have not been studied previous-ly. In this study, our intent was to detect the EO content in the serum of 1k1c hypertensive rats by highly accurate and specific ELISA (enzyme linked immunosorbent assay), and to analyze the relationship between serum EO levels and blood pressure in 1k1c rats. In addition, the study was designed to explore the blood pressure effects of inhibiting EO activity through the administration of anti-ouabain antibody to 1k1c rats in order to further reveal the role of EO in the development of hypertension in this animal model.

Materials and Methods

Establishment of 1k1c Hypertensive Models

Sixty-five healthy adult male Sprague-Dawley (SD) rats (5-8 weeks old) weighing 180-210 g were purchased from

250 μl per well of rinse solution. Authentic ouabain (Sigma; Lot... 37°C. Before use, plates were washed with three rinses of 2% bovine serum albumin) to each well for 1 h at 200 μl of blocking buffer (10 mmol/l, pH 7.4 PBS containing 0.05% Tween-20). Unbound ovalbumin-ouabain was removed by washing each well three times with 250 μl of rinse solution (10 mmol/l, pH 7.4 PBS containing 0.05% Tween-20). Unoccupied protein-binding sites were blocked by adding 200 μl of blocking buffer (10 mmol/l, pH 7.4 PBS containing 2% bovine serum albumin) to each well for 1 h at 37°C. Before use, plates were washed with three rinses of 250 μl per well of rinse solution. Authentic ouabain (Sigma Chemicals Co., St. Louis, MO, USA) solution was diluted to 0.39, 0.78, 1.56, 3.125, 6.25, 12.5, 25, and 50 μg/l, respectively, with deionized water. The 50 μl samples or standards were added to successive wells. In addition, 50 μl of anti-ouabain antisera (produced by immunizing rabbits with BSA-ouabain conjugate (13), dilution of 1:12,000) was added to each well. Plates were incubated at 37°C for 2 h with continuous shaking followed by four rinses using 250 μl of rinse solution per well. A 100 μl aliquot of a 1:1,000 dilution of goat anti-rabbit IgG-peroxidase conjugate (Sino-American Biotechnology Co., China) was added to each well to react with any anti-ouabain antibodies remaining bound to the ovalbumin-ouabain, with the plates being incubated for an additional 30 min at 37°C with continuous shaking. Unbound anti-rabbit IgG-peroxidase conjugate was washed away by rinsing as described above. The presence of peroxidase remaining in each well was determined by the addition of 100 μl per well of OPD substrate solution. After 15 min at room temperature, the substrate reaction was terminated by the addition of 50 μl of 2 M H2SO4. The absorbance of each well was measured at an optical density 490 nm using the ELISA Reader (Model DG3022A, Huadong Electron Tube Factory, Shanghai, China). The concentrations of EO in each sample were calculated from the absorbance according to the ouabain standard curve.

Detection of Endogenous Ouabain Content in the Serum of 1k1c and Normal SD Rats by the ELISA Method

Sample collection and assay (6): At the end of the study the rats were fasted overnight and killed by decapitation on the following morning. Trunk blood was collected and centrifuged immediately. Ouabain was extracted from serum samples by mixing the serum with an equal volume of 0.1% distilled trifluoroacetic acid for 3 h at room temperature followed by centrifugation (3,000 × g for 30 min) to pellet any insoluble material. The supernatant was passed through C-18 disposable Bond Elute columns (Analytichem International, Harbor City, CA, USA). The column was washed several times with water to remove unbound materials. The ouabain was then eluted with 25% acetonitrile. The eluates were dried with a vacuum centrifuge, and the extracts were redissolved with phosphate-buffered saline (PBS, 0.01 mol/l, pH 7.4). The serum ouabain content was detected by ELISA, as described previously (13). In brief, enzyme immunoassay plates were coated with ovalbumin-ouabain by adding to each well 100 μl of 1 μg/ml ovalbumin-ouabain in coating buffer (10 mmol/l, pH 9.6 carbonate-buffered saline). Plates were treated for storage for 18–24 h at 4°C before use. Unbound ovalbumin-ouabain was removed by washing each well three times with 250 μl of rinse solution (10 mmol/l, pH 7.4 PBS containing 0.05% Tween-20). Unoccupied protein-binding sites were blocked by adding 200 μl of blocking buffer (10 mmol/l, pH 7.4 PBS containing 2% bovine serum albumin) to each well for 1 h at 37°C. Before use, plates were washed with three rinses of 250 μl per well of rinse solution. Authentic ouabain (Sigma Chemicals Co., St. Louis, MO, USA) solution was diluted to 0.39, 0.78, 1.56, 3.125, 6.25, 12.5, 25, and 50 μg/l, respectively, with deionized water. The 50 μl samples or standards were added to successive wells. In addition, 50 μl of anti-ouabain antisera (produced by immunizing rabbits with BSA-ouabain conjugate (13), dilution of 1:12,000) was added to each well. Plates were incubated at 37°C for 2 h with continuous shaking followed by four rinses using 250 μl of rinse solution per well. A 100 μl aliquot of a 1:1,000 dilution of goat anti-rabbit IgG-peroxidase conjugate (Sino-American Biotechnology Co., China) was added to each well to react with any anti-ouabain antibodies remaining bound to the ovalbumin-ouabain, with the plates being incubated for an additional 30 min at 37°C with continuous shaking. Unbound anti-rabbit IgG-peroxidase conjugate was washed away by rinsing as described above. The presence of peroxidase remaining in each well was determined by the addition of 100 μl per well of OPD substrate solution. After 15 min at room temperature, the substrate reaction was terminated by the addition of 50 μl of 2 M H2SO4. The absorbance of each well was measured at an optical density 490 nm using the ELISA Reader (Model DG3022A, Huadong Electron Tube Factory, Shanghai, China). The concentrations of EO in each sample were calculated from the absorbance according to the ouabain standard curve.

Effects of Anti-Ouabain Antibody on the Blood Pressure of 1k1c Rats

Anti-ouabain immunoglobulin G (IgG) and normal rabbit IgG were extracted from anti-ouabain antisera and normal rabbit serum, respectively, with a 2.5 × 20 cm DEAE-Sephadex (Pharmacia Co.) A-50 column. The 1k1c hypertensive rats (n = 43) were divided randomly into five groups: one administered a small dose of anti-ouabain antibody (As group, n = 8), one an intermediate dose of anti-ouabain antibody (Ai group, n = 9), one a large dose of anti-ouabain antibody (Al group, n = 10), one normal rabbit IgG (IG group, n = 8), and one normal saline (NS group, n = 8). All of the rats were anesthetized with 25% ethyl carbamate, and the direct systolic blood pressure was measured through a cannulated carotid artery. After blood pressure stabilization, the rats of the As, Ai, and Al groups were injected with anti-ouabain antibody (three different doses of 2.3, 4.6, and 23 mg/kg, respectively) and the rats of the IG and NS groups were injected with normal rabbit IgG (4.6 mg/kg) and normal saline (equal volume to the antibody), respectively. The direct systolic blood pressure of all the rats was recorded for 3 h after administration.
Statistical Analysis

Values were expressed as mean ± SD. The Student’s t-test was performed, and the correlation coefficients were calculated by Microsoft Excel 5.0 statistical software. P values of less than 0.05 were considered to indicate statistical significance.

Results

Changes in the Systolic Blood Pressure of 1k1c Rats after Surgery

Systolic blood pressure began to increase 1 week after nephrectomy compared with the pressures before surgery (131 ± 13.5 mmHg vs. 104 ± 12.3 mmHg, p < 0.01). Systolic blood pressure increased significantly 4 weeks after nephrectomy (165 ± 16.9 mmHg vs. 104 ± 12.3 mmHg, p < 0.001). However, the systolic blood pressure of normal SD rats did not change significantly during the period of this study (109 ± 10.3 mmHg vs. 103 ± 9.6 mmHg, p > 0.05).

Serum EO Content of 1k1c Hypertensive Rats Compared with That of Normal SD Rats

Figure 1 shows that the serum EO content of 1k1c hypertensive rats was significantly higher than that of normal SD rats (2.25 ± 0.92 µg/l vs. 1.12 ± 0.17 µg/l, p < 0.01).

Correlation between Serum EO Levels and Blood Pressure in 1k1c Rats

Figure 2 shows that the serum EO content of 1k1c hypertensive rats was significantly correlated with systolic blood pressure (r = 0.59, p < 0.05).

Hemodynamic Effects of Anti-Ouabain Antibody on the Blood Pressure of 1k1c Hypertensive Rats

The administration of anti-ouabain antibody was able to significantly decrease the blood pressure of 1k1c hypertensive rats as compared with the results obtained with normal saline. The administration of normal rabbit IgG, however, did not decrease blood pressure (Fig. 3). Two different kinds of changes in blood pressure appeared to result from the administration of anti-ouabain antibody. First, the blood pressure of 1k1c hypertensive rats began to decrease 1 min after and reached the lowest point 2-5
min after the antibody administration. It took approximately 30 min for the blood pressure to be restored to pre-administration levels. Second, the anti-ouabain antibody decreased blood pressure in a dose-dependent manner, with the decreases in blood pressure correlating positively with the doses of antibody used.

**Discussion**

Our results show that the serum EO content of 1k1c hypertensive rats is significantly higher than that of normal SD rats, possibly due to the obvious sodium and water retention, activated renin-angiotensin systems, and increased sympathetic nervous system activity in 1k1c hypertensive rats (12). In addition, all of the following changes have been reported to play a role in the induction of EO secretion:

**Blood Volume Expansion**

Much evidence has shown that blood volume expansion can significantly increase EO secretion. Elevated EO levels in the body could play an important role in the mechanism of blood pressure increases in hypertensive models such as “DOCA-salt” and “partially nephrectomized” hypertensive rats in which there is sodium retention and an expansion of blood volume (8, 10, 11).

**Activated Renin-Angiotensin System**

Recently, Laredo et al. (6) have found that angiotensin II can stimulate the secretion of endogenous ouabain from cultured bovine adrenocortical cells. This result provides direct evidence that angiotensin II is a powerful factor in the stimulation of EO secretion.

**Increased Sympathetic Nervous System Activity**

Yamada and his colleagues (14) have reported that the central noradrenergic denervation induced by 6-hydroxydopamine can decrease the EO content of the hypothalamus and serum by 90% and 70%, respectively, indicating that higher sympathetic nervous system activity might be one of the stimulatory causes of EO secretion.

In order to explore the role of circulating digitalislike factor in the development of hypertension, several studies on the effects of anti-digoxin antibody on blood pressure have previously been carried out in DOCA-salt rats, spontaneously hypertensive rats (SHR), corticotrophin (ACTH)-induced hypertensive rats, and aortic coarctate hypertensive rats (1-4). The results and the corresponding conclusions from these studies, however, have not been consistent. Much recent evidence has shown that endogenous digitalislike factor, digoxinlike factor, and sodium pump inhibitor are indistinguishable from ouabain (5-8). This study is the first to have used anti-ouabain antibody both in determining endogenous ouabain levels and in exploring the changes in blood pressure after the inhibition of EO activity in the 1k1c hypertensive model. In addition, we used normal rabbit IgG in this study to rule out the possibility that the effects of antibody on blood pressure might be due to the nonspecific hypotensive side effects of immunoglobulin.

The results of this study indicate that the EO levels of serum correlate significantly with systolic blood pressure, and that the administration of anti-ouabain antibody can decrease blood pressure in 1k1c hypertensive rats in a dose-dependent manner. These findings suggest that higher EO levels in the body might play an important role in the pathogenesis of hypertension and participate in the development of hypertension, as other groups have reported (7-11). The mechanism by which ouabain increases blood pressure has been thought to be the result of the inhibition of sodium pump activity in heart muscle, the smooth muscle of vessels, sympathetic nerves, and so on (7-11). The inhibition of sodium pumping could increase intracellular Na⁺ levels and, consequently, result in the development of hypertension in a variety of ways (7-11): 1) more intracellular sodium could result from suppressed Na⁺-Ca²⁺ exchanges, and Ca²⁺ concentrations in cells could thereby increase, which would also increase the vascular tone; 2) increasing the activity of the sympathetic nervous system; 3) sensitizing the resistant vasculature to other endogenous vasopressors, and decreasing the reactive response of vasculature to some vasorelaxing factors; 4) affecting the “Renin-Angiotensin-Aldosterone System”; and 5) enhancing collagen production and promoting hypertrophy and hyperplasia of arterial smooth muscle cells, which increases the peripheral vascular resistance.

In summary, the results of this study indicate that EO might play an important role in the development of hypertension in 1k1c hypertensive rats. Mechanisms related to the rise of blood pressure in 1k1c rats are similar, at least in part, to those of human hypertension. Therefore, this study might be helpful for further exploring the roles of EO in the development of human hypertension.

**Abbreviations**

Abbreviations used in this paper: ACTH, adrenocorticotropic hormone; BSA, bovine serum albumin; C-18, octadecylsilane; DOCA, deoxycorticosterone acetate; ELISA, enzyme linked immunosorbent assay; EO, endogenous ouabain; IgG, immunoglobulin G; NS, normal saline; 1k1c, one kidney, one clip; OPD, 0-phenylenediamine; PBS, phosphate-buffered saline; SBP, systolic blood pressure; SD, Sprague-Dawley; SHR, spontaneously hypertensive rat.

**Acknowledgements**

The authors thank Dr. Yong-Xiao Cao of the Department of Pharmacology, Xi'an Medical University for his valuable support and advice in conducting this work.
References