Effects of Intracerebroventricular Infusion of Fab Fragments of Digoxin Antibody (Digibind) on Development of Reduced Renal Mass-Saline Hypertension in Rats

Kaoru Yamada, Atsuo Goto*, Chen Hui*, Hiroshi Nagoshi*, and Masao Omata*

To clarify the role of brain ouabain-like compound in reduced renal mass-saline hypertension, we examined the effects of intracerebroventricular infusion of the Fab fragments of antidigoxin antibody (Digibind) on the change in blood pressure of saline-drinking subtotally nephrectomized rats. Twenty male Wistar rats weighing 250 g each underwent subtotal nephrectomy. Two groups of 10 rats received intracerebroventricular infusion of Digibind (20 mg/ml) or normal sheep IgG (20 mg/ml) at a rate of 0.5 μl/h for 11 days. All rats began to drink 1% NaCl solution after two days of infusion. Systolic blood pressure was measured by the tail-cuff method on days 2, 6 and 9 of infusion. Two groups of saline-drinking rats with reduced renal mass developed hypertension. However, systolic blood pressure was significantly higher in Digibind-infused rats than in IgG-infused rats (day 2, 144 ± 3(SEM) vs. 133 ± 1 mmHg, p<0.05; day 6, 161 ± 4 vs. 151 ± 2 mmHg, 0.05<p<0.1, day 9, 181 ± 8 vs. 155 ± 2 mmHg, p < 0.05). In spite of similar renal dysfunction, plasma aldosterone concentrations, and plasma OLC levels, the accelerated increase in blood pressure was accompanied by a significantly impaired pressure-natriuresis relationship (0.089 ± 0.013 vs. 0.131 ± 0.013 mmol/day/mmHg, p < 0.05). These results indicate that chronic intracerebroventricular infusion of Digibind augmented reduced renal mass-saline hypertension in rats and suggest that brain ouabain-like compound may play a protective role against the elevation of blood pressure, at least in this model of hypertension. (Hypertens Res 1995;18:145-150)

Key Words: ouabain-like compound, reduced renal mass-saline hypertension, Digibind

Much evidence suggests that endogenous ouabain-like compound (OLC) or Na+,K+-ATPase inhibitor could modulate sodium pump activity and could be involved in the regulation of sodium homeostasis (1-3). Release of OLC, the purpose of which is restoration of extracellular fluid volume via natriuresis, may secondarily lead to increased cytosolic calcium, arteriolar vasoconstriction, and hypertension. As such, circulating OLC has been the focus of much attention. By contrast, recent evidence suggests that OLC or Na+,K+-ATPase inhibitor in the central nervous system (CNS) may participate in the regulation of central sympathetic outflow and/or renal sodium excretion in response to central high sodium (4-7). Theoretically, the regulation of these two systems, i.e., the sympathetic nervous system and urinary sodium excretion, could be importantly involved in the development of high blood pressure in volume-expanded hypertension.

It has been documented that animals develop low renin, volume-expanded hypertension following renal mass reduction and excess sodium intake (8-10). As expected, rats with this type of hypertension have been shown to have increased levels of circulating OLC or Na+,K+-ATPase inhibitor in their plasma (11, 12). However, the role of brain OLC in reduced renal mass-saline (RRM-S) hypertension has not been examined yet. The present study was undertaken to determine whether brain OLC is involved in the development of RRM-S hypertension. For this, we evaluated the effects of intracerebroventricular (ICV) infusion of Digibind on the changes in blood pressure of saline-loaded subtotally nephrectomized rats.

Methods

Experiments were done on 20 male Wistar rats (250 g) purchased from Douken, Ibaragi, Japan. All procedures were in accordance with the guidelines of the Animal Experiment Committee of the University of Tokyo. Rats were maintained on normal rat chow and tap water. All rats underwent subtotal nephrectomy, and two days later an osmotic minipump was implanted into each rat for chronic ICV infusion of Digibind or normal sheep IgG. Then all rats were given 1% saline solution to drink and the changes in blood pressure were observed. Due to
various technical difficulties (e.g., incorrect cannula or catheter failure), three rats were discarded so that results were finally compiled from 17 rats.

**Chronic Measurements in Awake Rats**

Systolic blood pressure (BP) was measured by tail-cuff method before, and on days 2, 6 and 9 after the start of ICV infusion. Body weight was measured at the same time. Each BP measurement was obtained by averaging five individual readings.

**Subtotal Nephrectomy**

Subtotal nephrectomy (removal of 70-80% renal mass) was performed under pentobarbital anesthesia (40 mg/kg) by removing the right kidney and 50% of the left kidney (both poles). The two poles of the left kidney were excised by scissors while clamping of the left renal artery, and the cut surfaces were covered with glue (Alon-alpha; Towa Chemical, Tokyo, Japan). Each rat received procaine penicillin G, 60,000 U i.m., postoperatively.

**Chronic Intracerebroventricular Infusion of Digibind**

Each rat was anesthetized with sodium pentobarbital (40 mg/kg). A skin incision was made to expose the upper part of the skull and a 1-mm hole was drilled through the left parietal bone, 0.5 mm posterior to the bregma and 1.5 mm lateral to the mid-sagittal suture. A cannula consisting of 28-gauge stainless steel tubing was inserted stereotaxically into the left lateral ventricle (4.5 mm below the skull surface) and was fixed to the skull with acrylic cement. An osmotic minipump (model 2002, Alza Corp., Palo Alto, CA, USA) was then implanted subcutaneously in the mid scapular area of the back and connected through Teflon tubing to the brain cannula. Each rat received procaine penicillin G, 60,000 U i.m., postoperatively.

Osmotic minipumps were filled with 0.2 ml of either Digibind (20 mg/ml) or normal sheep IgG (20 mg/ml, Sigma, St. Louis, MO, USA) dissolved in physiological saline solution. At a mean pumping rate of 0.5 μl/h, the pumps were estimated to have a pumping duration of 14 days. Two groups of 10 rats received ICV infusion of Digibind or normal sheep IgG, respectively.

**Urine Collection**

Urine was collected from several rats using metabolic cages for 24 h on days 6 and 10 of infusion. Urine volume, urinary sodium excretion, and urinary potassium excretion were determined.

**Measurements of Serum Electrolytes, Creatinine and Hormones**

After 11 days of chronic ICV infusion, blood was collected from the abdominal aorta into plastic syringes under pentobarbital anesthesia. Each blood sample was divided into two parts and was centrifuged at 3,000 rpm for 10 min at 4°C to obtain serum and plasma. A part of the blood sample was used to measure hematocrit. Plasma aldosterone concentration was determined by radioimmunoassay. Plasma OLC levels were measured by radioimmunoassay of ouabain as described below. Serum sodium and potassium concentrations were measured by the colorimetric method of the Jaffe reaction (13). Heart, thoracic aorta, kidneys and adrenals were excised and weighed. The length of the aorta was measured.

**Measurement of Plasma OLC Levels**

Each plasma sample (1 ml) was diluted with an equal volume of 0.1% trifluoro acetic acid (TFA) in water and was applied to a Sep-Pak C18 cartridge (Waters, Milford, USA) that had been activated with methanol and equilibrated with distilled water. After complete washing with 30 ml of distilled water, OLC was eluted with 3 ml of 25% acetonitrile in water. The eluent was evaporated, lyophilized, and assayed for OLC based on radioimmunoassay for ouabain.

For the immunoassay, anti-ouabain antibody (IgG) was diluted 1 × 10⁵-fold by 10 mmol/l phosphate buffer containing bovine serum albumin (5 g/l), 154 mmol/l NaCl, and 15 mmol/l sodium azide. The freeze-dried sample was redissolved in 0.25 ml of phosphate buffer, and 0.1 ml of samples or standard solutions of ouabain were incubated with 0.1 ml of diluted anti-ouabain IgG, 0.3 ml of phosphate buffer, and 0.1 ml of [3H] ouabain (15.6 Ci/mmol, 450 pmol/l, New England Nuclear, Boston, USA) for 24 h at 4°C. Then, 0.2 ml of goat anti-rabbit IgG antibody (Immunobead Second Antibody Reagent, Bio-Rad, Richmond, CA, USA) was added and incubation was continued for 24 h at 4°C. Antibody-bound ouabain was precipitated by centrifugation at 4°C for 20 min (3,000 rpm) and was counted by liquid scintillation.

**Statistics**

Values are given as mean ± SEM. Paired or non-paired t-test was used for the statistical evaluation as appropriate. P values less than 0.05 were considered to indicate significance.

**Results**

**Body Weight and Blood Pressure**

Although body weights in Digibind-infused rats were slightly lower than those of IgG-infused rats on days 2, 6, 9 and 11 of infusion, no significant differences were found during the observation period (on day 11, Digibind 238 ± 36 g, IgG 253 ± 36 g, NS).

Figure 1 depicts the time course of BP in two groups of rats, Digibind-infused rats (n = 8) and IgG-infused rats (n = 9). BP did not differ between the groups before ICV infusion of Digibind or normal sheep IgG (Digibind 126 ± 3 vs. IgG 125 ± 2 mmHg). Even before the start of saline loading, BP began to rise in the two groups of rats after 2 days of infusion. BP was significantly higher in Digibind-infused rats than in IgG-infused rats (Digibind 144 ± 9 vs. 133 ± 2 mmHg, p < 0.05). After the start of saline drinking, BP in both groups progressively increased. Elevation of BP was more remarkable in Digibind-infused rats than in IgG-infused rats on
days 6 and 9 of infusion, and the difference was statistically significant on day 9 (day 6, Digibind 161 ± 13 vs. IgG 151 ± 6 mmHg, 0.05 < p < 0.1; day 9, Digibind 181 ± 23 vs. IgG 155 ± 7 mmHg, p < 0.05).

Other General and Hormonal Parameters (Table 1) Between the two groups of RRM-S rats, there was no difference in heart weight in spite of a 26-mmHg difference in BP. Thus, higher BP levels were not accompanied by greater cardiac enlargement in Digibind-infused rats. No difference was observed in aortic, renal, or adrenal weights, either. The two groups of RRM-S rats also showed similar serum creatinine, sodium, potassium, and hematocrit levels.

To clarify the mechanisms by which ICV infusion of Digibind caused the acceleration of RRM-S hypertension, we measured several variables associated with sodium excretion. Absolute urine volume and urinary sodium excretion were lower in Digibind-infused rats than in IgG-infused rats, but the difference was not significant, probably due to large variation among individual rats. Since urinary sodium excretion in rats with RRM-S correlates with the rise in BP during the first 10 days following saline loading (II), we examined the relationships between urinary sodium excretion and BP in two

Table 1. Blood Pressure, Body Weight and Other Measured Variables

<table>
<thead>
<tr>
<th>Group</th>
<th>Normal IgG</th>
<th>Digibind</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td>Blood pressure (mmHg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9th day</td>
<td>155 ± 2</td>
<td>181 ± 8*</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>253 ± 12</td>
<td>238 ± 13</td>
</tr>
<tr>
<td>Heart weight (%)</td>
<td>0.321 ± 0.014</td>
<td>0.347 ± 0.023</td>
</tr>
<tr>
<td>Aortic weight (mg/mm)</td>
<td>1.185 ± 0.023</td>
<td>1.126 ± 0.044</td>
</tr>
<tr>
<td>Kidney weight (%)</td>
<td>0.512 ± 0.038</td>
<td>0.562 ± 0.038</td>
</tr>
<tr>
<td>Adrenal weight (%)</td>
<td>0.0273 ± 0.0015</td>
<td>0.0272 ± 0.0014</td>
</tr>
<tr>
<td>Hematocrit</td>
<td>0.357 ± 0.022</td>
<td>0.345 ± 0.017</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>0.76 ± 0.04</td>
<td>0.84 ± 0.10</td>
</tr>
<tr>
<td>Serum sodium (mmol/l)</td>
<td>145.7 ± 2.0</td>
<td>146.0 ± 1.4</td>
</tr>
<tr>
<td>Serum potassium (mmol/l)</td>
<td>5.50 ± 0.23</td>
<td>5.04 ± 0.18</td>
</tr>
<tr>
<td>PAC (pg/ml)</td>
<td>33.0 ± 12.2</td>
<td>24.4 ± 7.9</td>
</tr>
<tr>
<td>OLC (pmol/l)</td>
<td>68.3 ± 15.5</td>
<td>83.9 ± 9.3</td>
</tr>
<tr>
<td>Urine volume (ml/d)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6th day</td>
<td>138 ± 59</td>
<td>112 ± 78</td>
</tr>
<tr>
<td>10th day</td>
<td>158 ± 82</td>
<td>109 ± 60</td>
</tr>
<tr>
<td>Urine sodium excretion (mmol/d)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6th day</td>
<td>19.7 ± 8.3</td>
<td>15.5 ± 9.4</td>
</tr>
<tr>
<td>10th day</td>
<td>21.4 ± 10.1</td>
<td>13.8 ± 7.1</td>
</tr>
<tr>
<td>Urine potassium excretion (mmol/d)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6th day</td>
<td>1.9 ± 0.4</td>
<td>1.8 ± 0.5</td>
</tr>
<tr>
<td>10th day</td>
<td>2.2 ± 0.4</td>
<td>1.9 ± 0.8</td>
</tr>
</tbody>
</table>

All data except blood pressure and urinary electrolytes excretion are for the 11th day of the intracerebroventricular infusion. Values are given as means ± SEM. Weights of heart, kidney, and adrenals indicate the ratio to body weight. Aortic weight is corrected for the length. PAC = plasma aldosterone concentration, OLC = plasma concentration of ouabain-like compound. * p < 0.05.
groups of rats. There was no significant correlation between urinary sodium excretion and BP in either group of rat. When we divided the absolute levels of urinary sodium excretion by BP levels, a significant difference was observed in urinary sodium excretion corrected for BP levels (0.089 ± 0.013 vs. 0.131 ± 0.013 mmol/day/mmHg, p < 0.05) in spite of a similar degree of renal dysfunction (Fig. 2). No difference was found in either urinary potassium excretion or urinary sodium/potassium ratio. Two humoral factors which could influence renal sodium excretion were measured. However, ICV infusion of Digibind did not affect either PAC or plasma OLC levels.

Discussion

The mechanism by which hypertension occurs in a volume-dependent salt-sensitive model remains unclear. One major line of investigation has involved the premise that a circulating OLC or Na⁺,K⁺-ATPase inhibitor contributes to hypertension in volume-dependent models through an action on vascular smooth muscle sodium/calcium exchange. Actually, the roles of the circulating OLC or Na⁺, K⁺-ATPase inhibitor based on several different assay methods have been well documented in RRM-S hypertension (11, 12). Further, administration of canrenone, which may act as an antagonist at ouabain binding site of Na⁺,K⁺-ATPase, caused a decline in the blood pressure of RRM-S rats (15). Our previous observation that the rise in blood pressure was significantly inhibited in rats immunized with ouabain provides evidence for the participation of circulating OLC in the hypertensive mechanisms in rats with RRM-S (16). Taken together, these findings suggest that circulating OLC may play an important role in RRM-S hypertension. Furthermore, it appears that the CNS and peripheral sympathetic nervous system also contribute to the elevation of blood pressure in animals with RRM-S (11, 12). Anteroventral third ventricle lesions and central sympathectomy with ICV injection of 6-hydroxydopamine prevented the development of RRM-S hypertension in rats.

Excess sodium intake intermittently or chronically could increase sodium concentrations in CSF, resulting in an activation of central sodium receptors (17, 18). Several investigators have recently focused on the roles of brain OLC, as opposed to circulating OLC, in the sympathoexcitatory and pressor actions of ICV infusion of hypertonic NaCl solution (4-6, 19). On the other hand, we found that ICV preinjection of Digibind caused an impaired natriuresis following ICV infusion of hypertonic NaCl solution and pointed to the role of brain OLC in CNS-mediated natriuresis (7). The possible actions of brain OLC have been examined only in acute experiments in these previous studies and, therefore, longer-term investigations are clearly needed to elucidate more fully the actual role of brain OLC. Since two factors, i.e., sympathetic outflow and urinary sodium excretion, are the key regulators of blood pressure per se, long-term actions of brain OLC, if any, would influence the levels of blood pressure. Our assumption was that chronic ICV infusion of Digibind would affect the development of hypertension if brain OLC is actually involved in the long-term regulation of the sympathetic nervous system and/or urinary sodium excretion in rats with RRM-S.

We used Digibind as an antagonist of OLC also in this study. Digibind is a commercial preparation of Fab fragments raised in sheep immunized with digoxin-human serum albumin and reverses the signs and symptoms of digitalis intoxication (20). Recent findings suggest that Digibind may also recognize the structure of ouabain and inhibit its biological actions (5, 7, 21). Although Digibind has been found to be remarkably specific, it appears that sufficient amounts of Digibind more or less recognize cardiotonic steroids with cis-trans-cis configuration. Digibind could recognize OLC since OLC may actually be ouabain or its isomer (22-24).

In normal rats, chronic ICV infusion of hypertonic NaCl solution elevates blood pressure by depressing the sympatho-inhibition in the anterior hypothalamus (25, 26). If brain OLC is involved in this process, as suggested in acute experiments, then chronic inhibition of brain OLC would prevent
that chronic ICV infusion of Digibind prevented salt-induced hypertension in Dahl salt-sensitive (S) rats is compatible with this view. If, on the other hand, brain OLC may play a more important role in promoting natriuresis than in facilitating sympathetic outflow, chronic inhibition of brain OLC could worsen sodium retention and would require other natriuretic systems, including pressure-natriuresis mechanism, to maintain sodium balance. In the present study, BP levels of rats receiving ICV infusion of Digibind were higher than those of rats receiving ICV infusion of normal sheep IgG on days 2, 6 (although not significant) and 9 of infusion. This was an unexpected finding and implies that brain OLC may play a protective role against RRM-S hypertension. Our observations probably suggest that brain OLC may be more important as a regulator of natriuresis than that of the sympathetic nervous system in the long-term regulation of blood pressure, at least in rats with RRM-S. The reason why the roles of brain OLC may be different between Dahl S rats and rats with RRM-S is unclear. Apparently, renal function is impaired in rats with RRM-S, judging from the elevated levels of serum creatinine, and the contribution of CNS-mediated natriuresis to sodium balance may be much greater in these rats than in Dahl S rats with intact kidneys. Since the relationship between sympathetic outflow and increased brain OLC is reportedly different between SHR and WKY rats (6), differences in the model of hypertension, i.e., genetic versus artificially-induced, may account for the discrepancy between the results of Leenen and coworkers and ours.

Although our present data suggest a protective role of brain OLC in RRM-S hypertension, the mechanisms by which brain OLC mediates its effects are mostly speculative. Absolute urinary sodium excretion was lower in Digibind-infused rats than in IgG-infused rats, but the difference was not significant, probably due to large variation among individual rats. The only difference was observed in urinary sodium excretion corrected for BP levels. Therefore, we would favor the view that chronic blockade of brain OLC impaired sodium excretion and, at least partly, participated in the acceleration of hypertension in Digibind-infused rats. In the present study, two humoral factors that could influence renal sodium excretion were measured. However, ICV infusion of Digibind did not affect either PAC or plasma OLC levels. Gutman et al. found a tremendous increase in sodium excretion, which may be mediated by the suppression of mineralocorticoid secretion, following implantation of ouabain in the hypothalamus (28). Manunta et al. found lower OLC levels in selected central structures in rats that received intraarterial injection of ouabain for 5 weeks and suggested that CNS levels of OLC may be regulated via a negative feedback mechanism (29). No data supporting these previous communications were obtained in this study. Higher BP levels were not accompanied by greater cardiac enlargement. This may be due to the facts that both groups developed hypertension and that heart weight is not a sensitive enough index to detect the small difference in BP levels. Recent studies suggest that many factors other than BP levels per se affect cardiac hypertrophy and clear differences in BP levels have not been reflected in significant differences in heart weight in several experiments (30, 31).

Since saline loading started after BP measurement on day 2, it appears that impaired natriuresis alone cannot account for the significant increase in BP on day 2 of infusion in Digibind-infused rats. Therefore, we cannot exclude the contribution of the sympathetic nervous system to the higher BP levels in the Digibind group. It is well known that ouabain exerts important effects through the regulation of cell membrane Na+,K+-ATPase on many CNS neurotransmitters, including norepinephrine, dopamine, serotonin, acetylcholine, and GABA (32). ICV administration of ouabain affects central autonomic activity through the modulation of each neurotransmitter, but the ensuing effects may be dependent on the neurotransmitters involved and/or the exact CNS areas involved. For example, activation of forebrain and spinal cord or hindbrain noradrenergic mechanisms can result in an increase or a decrease in sympathetic outflow, respectively. Further, the effects of ouabain appears to be biphasic. High doses of ouabain produce CNS stimulation characterized by convulsions and death, while lower doses induce CNS depression characterized by loss of locomotor activity and lack of response to external stimuli (33). Although ICV administration of exogenous ouabain surely induces sympathoexcitiation and a rise in blood pressure, the physiological roles of endogenous brain OLC could be quite different from those estimated from acute experiments, and could depend upon the concentrations and the CNS areas.

In conclusion, BP levels were significantly higher in saline-drinking subtotally-nephrectomized rats which received chronic ICV infusion of Digibind than in rats which received ICV infusion of normal sheep IgG. These observations suggest that brain OLC may play a protective role against the development of reduced renal mass-saline hypertension in rats. The mechanism of protection and the exact CNS areas of OLC action remain to be determined.

Acknowledgments
We are indebted to Burroughs Wellcome Co., Research Triangle Park, NC, U.S.A., for the generous gift of Digibind.

References


