Regulation of Aldosterone Receptor in Rat Kidney Cytosol by Atrial Natriuretic Factor

Masatsugu Horiuchi, Noriyuki Kohashi, Hisashi Nishiyama, Junkichi Hama, Toshihiko Takenaka, Hirokazu Kondo, and Ryo Katori

We investigated the effect of atrial natriuretic factor (ANF) on aldosterone receptors in the kidney cytosol, because the binding of aldosterone to aldosterone receptors in the cytosol is considered a critical step of its action. Rat atriopeptin III was injected into male Sprague-Dawley rats (200–250 g) via the femoral vein while under pentobarbital anesthesia, and aldosterone receptors in the kidney cytosol were determined. The maximum binding capacity and dissociation constant were calculated by the Scatchard analysis. Maximum binding capacity of both types of aldosterone receptor (Type I, high affinity and low binding capacity and Type II, low affinity and high binding capacity) gradually decreased after ANF injection, reached the lowest level after 2 hours, and then slightly recovered. When more than 2.5 µg/kg of rat atriopeptin III was injected, the density of aldosterone receptors significantly decreased.

Injection of 12.5 µg/kg of rat atriopeptin III decreased maximum binding capacity of Type I receptor from 42.3±2.4 (mean±SD, n=6) to 22.8±3.2 femtomole/mg protein (n=6) (p<0.01), and that of Type II receptor decreased from 388±46 to 285±30 fmol/mg protein (p<0.01). Dissociation constant of both types of receptors did not change significantly after ANF injection. Plasma aldosterone concentration showed no significant change after ANF injection, and a significant change was noted after ANF administration on aldosterone receptors in the experiments on adrenalectomized rats 7 days after operation. Furosemide had no significant effect on aldosterone receptors in both normal and adrenalectomized rats. These results suggest that ANF may inhibit the action of aldosterone by decreasing aldosterone receptor density in the kidney cytosol. (Hypertension 1989:13:334–340)

The interaction of many hormones is very important in the regulation of blood pressure. Recently, atrial natriuretic factor (ANF) has been discovered to play some important roles in the regulation of water-electrolyte balance, and aldosterone is widely known to play an important role in the regulation of body fluid. In the kidney, ANF and aldosterone have opposite and apparently counterbalancing effects on sodium excretion, with ANF reducing sodium reabsorption and aldosterone increasing it. Recently, it has been reported that ANF inhibits the biosynthesis or secretion of aldosterone under some experimental conditions. Thus, it is increasingly important to understand the interaction of ANF and aldosterone in the regulation of plasma volume and control of systemic blood pressure.

It is generally accepted that aldosterone, similar to the other steroid hormones, binds to a specific receptor protein in the cytoplasm, and then hormone-bound receptor transforms to "activated" receptor-aldosterone complex; this activated complex can translocate into the nuclei, resulting in the expression of specific messenger ribonucleic acid (mRNA) for aldosterone-induced enzymes and proteins. The action of aldosterone may be regulated at each of these steps, but the binding capacity of the receptors in the cytoplasm and their affinity for aldosterone are the most important factors for regulating the action of aldosterone. Therefore, we examined the effect of ANF on the aldosterone receptor density and affinity in the cytoplasm to reveal the interaction of ANF and aldosterone.

Materials and Methods

Animals and Treatment

Male Sprague-Dawley rats, 6 weeks of age, weighing 170–200 g, were purchased from Shizuoka Laboratory Animal Center (Shizuoka, Japan). They had free access to food that contained 0.17 meq/g NaCl (CE-2, Clea Japan Inc., Tokyo, Japan), and drink-
ing water was provided ad libitum. Rats weighing 200–250 g were used in all experiments, and in some experiments, rats were adrenalectomized bilaterally, given 0.9% NaCl solution to drink, and then were used for experiments 7 days after the operation. Under pentobarbital anesthesia (6 mg/100 g i.p.) (Pitman Moore/NC, New Jersey), polyethylene tubes (Becton Dickinson & Co., New Jersey) were inserted in the trachea, the urinary bladder for collecting urine, and the femoral vein. Rat atriopeptin III (rAP III) (0.5–25 μg/kg) or furosemide (500 μg/kg), dissolved in 1 ml of phosphate-buffered saline, was injected via a femoral vein for 1 minute, and urine was collected for 10 minutes after injection. At times indicated in the text, rats were killed by decapitation after procuring blood samples from the abdominal aorta, and kidney cytosol was prepared as follows for the determination of aldosterone receptors.

Materials

The sources of materials used in this work are as follows: [1,2,6,7-3H]aldosterone (75 Ci/mmol) was obtained from Radiochemical Center, Amersham (Buckinghamshire, United Kingdom); unlabeled aldosterone, bovine serum albumin, and Tris from Sigma Chemical, St. Louis, Missouri; dextran T-500 from Pharmacia Fine Chemicals, Uppsala, Sweden; aqueous counting scintillant (ACS II) from Amersham/Searle Corp., Arlington Heights, Illinois; radioimmunoassay kit for plasma aldosterone (PAC) from Dai-ichi, Chiba, Japan. rAP III was synthesized and kindly provided by Dr. Makoto Tamura of Kaken Pharmaceutical Co., Otsu, Japan. All other chemicals (reagent grade) were obtained from Wako Pure Chemical Industries, Ltd., Osaka, Japan.

Receptor binding assay. All animals were killed by decapitation after blood sampling, and the kidneys were rapidly perfused in situ with 10 ml ice-cold 50 mM Tris HCl buffer (pH 7.5) that contained 250 mM sucrose, 50 mM KCl, 3 mM MgCl2, 20 mM sodium molybdate, and 1 mM mercaptoethanol. The kidneys were removed, decapsulated, rinsed with ice-cold buffer, minced with scissors, and then homogenized in two volumes of the same ice-cold buffer by five strokes of a Potter-Elvehjem homogenizer with a loose-fitting Teflon pestle. The homogenates were centrifuged at 105,000g by Hitachi Ultracentrifuge 65P (Hitachi Ltd., Tokyo, Japan) for 1 hour at 2° C to obtain cytoplasmic supernatant fraction (cytosol). Cytosol fraction of 125 μl was incubated with 25 μl of tritiated aldosterone diluted with ethyleneglycol so that final concentrations were 2×10^-6 to 2×10^-10 M. Incubation was carried out at 0° C for 24 hours; this incubation time is thought to be enough for exchanging endogenous aldosterone contained in the prepared cytosol with tritiated aldosterone added in the incubation mixture. After incubation, 50 μl of dextran-coated charcoal (450 mg dextran T-500, 4.5 g charcoal suspended in 100 ml of 10 mM Tris HCl buffer, pH 7.5) was added, and 10 minutes after incubation at 4° C, the assay mixture was centrifuged at 8,500g for 2 minutes. The resulting supernatant fraction of 50 μl was used for the determination of aldosterone receptors with addition of 5 ml ACS II. For determination of nonspecific binding, 1,000-fold excess of unlabeled aldosterone was added, and specific binding was determined by subtracting nonspecific binding from total binding. Radioactivity was counted by Packard Tri-Carb 460C (Downers Grove, Illinois) with efficiency of 35–40%. Maximum binding capacity (Bmax) and dissociation constant (Kd) were analyzed by Scatchard plot. In experiments using adrenalectomized rats, higher concentrations of tritiated aldosterone were used (6×10^-8 to 4×10^-10 M).

Effect of rat atriopeptin III on binding of tritiated aldosterone to aldosterone receptor and on stability of unbound aldosterone receptor. Cytosol prepared from adrenalectomized rats with the buffer not containing sodium molybdate was used for the study of receptor stability. Cytosol was preincubated with various concentrations (10^-6 to 10^-11 M) of rAP III in the buffer were added to the assay mixtures, and 1×10^-9 M of tritiated aldosterone was used for labeling aldosterone receptor. After 24 hours of incubation at 0° C, aldosterone receptor density was determined.

Cytosol prepared from adrenalectomized rats with the buffer not containing sodium molybdate was used for the study of receptor stability. Cytosol was preincubated with various concentrations (10^-6 to 10^-11 M) of rAP III dissolved in the buffer were added to the assay mixtures, and 1×10^-9 M of tritiated aldosterone, and remaining contents of aldosterone receptor were determined.

Other procedures. Urinary sodium concentrations were determined by the flame photometer, and changes in urinary sodium volume (ΔUNaV) were determined as follows: ΔUNaV (μeq/kg/10 min)= Urinary sodium content for 30 minutes before drug injection–(Urinary sodium content for 30 minutes before drug injection)/3. PAC was measured by the radioimmunoassay method. Protein was determined by the method of Lowry et al10 with bovine serum albumin as a standard. Results in this work are expressed as mean±SD. Student's t test was used to determine statistical significance, and time-dependent effect of ANF was analyzed by Kruskal-Wallis's test (nonparametric one-way analysis of variance) because the homogeneity of variance between groups was not recognized by Bartlett's method; then the difference from control group was evaluated by Bonferroni's criterion.

Results

Scatchard plot analysis of aldosterone binding in the cytosol of normal rat kidney is shown in Figure 1. Computer analysis, according to the method of Rosenthal,11 revealed that the aldosterone receptor consists of two different types of receptors, as previously reported by others. One is termed...
Type I receptor with a high affinity and low binding capacity for aldosterone, and the other is termed Type II receptor with a low affinity and high binding capacity for aldosterone. Glucocorticoid has a relatively high affinity for Type II sites but much less than the affinity of aldosterone for Type I sites. In Figure 1, the line "a" indicates Type I receptor and the line "b" indicates Type II receptor.

Bolus injection of 12.5 µg/kg of rAP III was done via polyethylene catheter inserted into the femoral vein under pentobarbital anesthesia, and renal aldosterone receptor in the cytoplasm was measured at various times after infusion (Figure 2). As shown in Figure 2, B_max of Type I aldosterone receptor gradually decreased after ANF injection; a statistically significant decrease in B_max was observed 90 minutes after injection, and a maximum decrease in B_max was obtained at 120 minutes. B_max of Type I receptor was significantly decreased from 44.3±3.1 to 22.8±3.2 femtomole/mg protein (p<0.01) at 120 minutes. This decreased aldosterone receptor density recovered slightly 3 hours after ANF injection (Figure 2). Type II aldosterone receptor B_max was decreased in a similar manner, but K_d of both types of receptor did not change significantly (data are not shown). Control rats injected with phosphate-buffered saline showed no detectable changes in aldosterone receptors during the time course of the study, and thus, the effect of the period of anesthesia on the receptor was excluded. Next, we examined the dose-dependent effect of rAP III on aldosterone receptors. Various concentrations of rAP III were injected into rats, and the density of aldosterone receptors was determined 2 hours after rAP III injection. Results of B_max of Type I receptor are shown in Figure 3. More than 2.5 µg/kg of rAP III was effective in decreasing receptor density, but a significant dose-dependent effect of rAP III more than 2.5 µg/kg was not observed to decrease the B_max of the receptors, whereas significant natriuretic
effect was observed for the initial 10 minutes with more than 6.0 μg/kg rAP III injection and reached a plateau with 15.0 μg/kg. These results indicate that natriuresis caused by rAP III injection seems unlikely to influence directly the aldosterone receptor.

To evaluate whether the decrease of aldosterone receptor density is specific for ANF, we next compared the effect of furosemide with ANF on aldosterone receptors. Comparable effects on natriuresis were obtained with 500 μg/kg of furosemide and 12.5 μg/kg of rAP III (Figure 4A). We injected these concentrations of furosemide and rAP III into anesthetized rats, and renal cytoplasmic aldosterone receptors were determined 2 hours after drug injection. Results are shown in Table 1. No remarkable changes in Bmax were observed in furosemide-treated rats compared with the phosphate-buffered, saline-injected rats (control), whereas rAP III decreased the Bmax of both types of aldosterone receptor. On the other hand, Ka of aldosterone receptor showed no significant differences in either experiment.

In our experimental conditions, injection of both rAP III and furosemide gave no significant change in PAC (Figure 4B). This result suggests that the effect of ANF on aldosterone receptor may be independent of plasma aldosterone. To confirm this possibility, we used adrenalectomized rats 7 days after operation to examine the effect of ANF on aldosterone receptors without the influence of endogenous aldosterone. As shown in Table 2, Bmax of both Type I and Type II receptors was decreased significantly by ANF injection compared with those of phosphate-buffered, saline-injected rats, and Ka of both types of receptors did not change significantly. Furthermore, no marked changes of Bmax and Ka were observed in furosemide-injected rats. These results support the idea that the decrease of receptor density by ANF might have no relation to plasma aldosterone. The number of renal cytoplasmic aldosterone receptors is higher in adrenalectomized rats than in normal rats (Table 2). As reported before, this phenomenon is perhaps due to the “up-regulation” of receptor in response to the diminution of aldosterone15 or recycling of the nuclear receptor into the cytoplasm.16

To examine the direct effect of ANF on aldosterone receptor, we performed the following experiments in vitro. As shown in Figure 5A, rAP III did not affect the binding of tritiated aldosterone to aldosterone receptor. It is generally accepted that steroid hormone receptor, including aldosterone receptor, is labile if receptor protein is maintained in its unbound form. Because of this instability, we used the buffer containing 20 mM sodium molybdate17 in preparing cytosol and the receptor binding assay to protect receptor protein. As shown in Figure 5B, cytosol prepared without sodium molybdate gradually decreased active receptor content even at 4° C, and a relatively higher concentration of rAP III could not practically keep the receptor protein in active form.

Table 1. Effects of Rat Atriopeptin III and Furosemide on Aldosterone Binding Properties of Rat Kidney Cytosol

<table>
<thead>
<tr>
<th>Experimental condition</th>
<th>Type I</th>
<th>Type II</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bmax (fmol/mg protein)</td>
<td>Ka (nM)</td>
</tr>
<tr>
<td>Control (n=5) (PBS-injected)</td>
<td>42.3±2.4</td>
<td>7.21±0.38</td>
</tr>
<tr>
<td>rAP III-injected (n=6)</td>
<td>22.8±3.2*</td>
<td>6.84±0.94</td>
</tr>
<tr>
<td>Furosemide-injected (n=6)</td>
<td>41.4±4.9</td>
<td>7.84±0.31</td>
</tr>
</tbody>
</table>

Values are mean±SD. Rat atriopeptin (rAP) III (12.5 μg/kg), furosemide (50 μg/kg), or phosphate-buffered solution (PBS) were injected into rats with intact adrenal glands, and renal cytosol, prepared 2 hours after injection, was used for receptor binding assay. Ka, dissociation constant.

* p<0.01 versus control.

Discussion

Like the other steroid hormones, aldosterone initiates its action by binding to the specific receptor protein in the cytoplasm, which subsequently binds...
TABLE 2. Effects of Rat Atriopeptin III and Furosemide on Aldosterone Binding Properties of Adrenalectomized Rat Kidney Cytosol

<table>
<thead>
<tr>
<th>Experimental condition</th>
<th>Type I</th>
<th>Type II</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$B_{\text{max}}$ (fmol/mg protein)</td>
<td>$K_d$ (nM)</td>
</tr>
<tr>
<td>Control (n=6) (PBS-injected)</td>
<td>384±28</td>
<td>3.52±0.94</td>
</tr>
<tr>
<td>rAP III-injected (n=6)</td>
<td>207±34*</td>
<td>3.84±0.88</td>
</tr>
<tr>
<td>Furosemide-injected (n=4)</td>
<td>418±40</td>
<td>3.93±0.75</td>
</tr>
</tbody>
</table>

Values are mean±SD. Rat atriopeptin (rAP) III (12.5 μg/kg), furosemide (500 μg/kg), or phosphate-buffered solution (PBS) were injected into adrenalectomized rats, and renal cytosol, prepared 2 hours after injection, was used for receptor binding assay. $K_d$, dissociation constant.

* $p<0.01$ versus control.

FIGURE 5. Line graphs showing the effect of rat atriopeptin (rAP) III on the binding of tritiated ($[^{3}H]$)aldosterone to aldosterone receptor (panel A) and unbound receptor stability at 4°C without sodium molybdate (panel B). Cytosol prepared from adrenalectomized rats was incubated with $10^{-8}$ to $10^{-11}$ M rAP III with $1\times10^{-9}$ M $[^{3}H]$aldosterone (panel A), and receptor binding capacity was determined. Cytosol preincubated with $10^{-8}$ M rAP III for various times indicated in the figure was used for receptor binding assay (panel B). Data are mean of duplicate determinations in three separate experiments.

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terms, it will be interesting to evaluate aldosterone receptor density and aldosterone responsiveness in chronic ANF excess states such as hypertension and congestive heart failure. However, there are some difficulties in discussing aldosterone responsiveness simply in relation to the aldosterone receptor, for example, because some factors, including aldosterone itself, which is reported to be decreased by ANF, modulate the aldosterone receptor and its responsiveness, and sodium reabsorption is influenced by both ANF, PAC, and their receptors. Since we cannot separate these factors for evaluating aldosterone responsiveness in the whole animal, we think it is important to examine the exact site in which aldosterone receptor decreases in the kidney in relation to the ANF acting site.

In the present study, we indicated that the decrease of the aldosterone receptor by ANF injection is independent of plasma aldosterone, because this decrease occurred similarly in adrenalectomized rats (Table 2). These data suggest that the decreased density of aldosterone receptor by ANF infusion might not be related to so-called down regulation. In adrenalectomized rats, it is also considered that steroid hormone receptor scarcely translocates into the nuclei because of a trace amount of steroid hormones, and nuclear receptor may be recycled into the cytoplasm. Thus, it is reasonable to suggest that the decrease of Bmax of aldosterone receptor in the cytoplasm by ANF is practically due to the degradation or inactivation of receptor protein in the cytoplasm. ANF is generally considered to exert its action by initially binding to the receptor on the plasma membrane, consequently activating guanylate cyclase, and recently a new cyclic guanosine 5'-monophosphate (cGMP)-independent mechanism of ANF action has also been reported. In our experiments, ANF did not affect directly the binding of aldosterone to the receptor or the stability of receptor protein. For these reasons, it is thought that the ANF effect on the aldosterone receptors is a secondary effect. There is a possibility that ANF induces or activates some kinds of enzymes that modify the receptor protein in the cytoplasm. In fact, steroid hormone receptors are reported to be transformed to an inactive form, which cannot bind hormone by phosphatases and is degraded by proteases. In addition to the possibility that ANF induces or activates these enzymes, other unknown substances, which affect the aldosterone receptor, might be induced by ANF.

Our results suggest the possibility that the natriuretic activity of ANF will be enhanced in the kidney by an antialdosterone function of ANF due to decreasing the aldosterone receptor density in the cytosol and that the interaction of ANF with aldosterone may play an important role in the regulation of water-electrolyte balance and blood pressure in vivo. The present study will give also a new insight for understanding molecular mechanism of ANF action by evaluating ANF-induced enzymes or substances further.

Acknowledgments
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