Developmental Change of Kidney Receptor for Atrial Natriuretic Factor in Spontaneously Hypertensive Rat

Toshio Ogura, Ichiro Yamamoto, and Norio Ogawa

Properties of human atrial natriuretic factor (ANF) binding to the crude membrane fraction of rat kidney were studied using the ANF-radiolabeled receptor assay; the developmental change of renal ANF receptors in three age groups of spontaneously hypertensive rats (SHR) was also investigated with the methods of radiolabeled receptor assay and the quantitative approach of in vitro macro-autoradiography. Temperature and incubation time greatly influenced ANF binding capacities because of the degradation of radiolabeled ligand. Addition of 5 mM MgCl₂ to assay buffer was useful for the stabilization of ANF specific binding. Scatchard analysis suggested that the crude membrane fraction of rat's kidney had a single binding site with the apparent dissociation constant of 0.55 nM. In the study of the developmental change of renal ANF receptor in SHR, systolic blood pressure of the SHR at the age of 5 weeks and 12 weeks was significantly higher than that of age-matched Wistar-Kyoto (WKY) rats, but there was no significant difference in blood pressure between SHR and WKY rats at the age of 3 weeks. Concerning the radiolabeled receptor assay of ANF, the apparent dissociation constant and maximum binding capacity in SHR were low in all age groups when compared with those of WKY rats. In the in vitro macro-autoradiographic observation, the specific binding of ANF was localized mainly in the renal cortex, and these binding patterns of SHR and WKY rats were the same in all age groups. The specific binding in the kidney of SHR was proved to be quantitatively smaller than that in WKY rats using the research analysis system (RAS 1,000) as investigated in radiolabeled receptor assay. These findings suggest that the kidneys of SHR have a higher affinity and lower capacity binding sites (possibly genetic in origin) for ANF from a young age, and these changes in the renal ANF receptor may affect the progression of the hypertensive state in SHR. (Hypertension 1989;13:449-455)

Atrial natriuretic factor (ANF), which was identified within a secretory-like granule from mammalian atria,¹ was purified from rat and human atria,² and the amino acid sequence determined.³ In previous studies, ANF was proved to have potent natriuretic and vasorelaxant actions.⁴-⁷ The actions of ANF were found by radiolabeled receptor assay⁸-¹⁰ and autoradiography¹¹,¹² to be mediated by receptors on the target organs (e.g., smooth muscle, kidney, adrenal gland, brain). Since continuous infusion of atrial peptides produces a marked natriuresis and diuresis,⁵-¹³ the kidney, which is involved in the control of fluid volume homeostasis, is one of the most important target organs of ANF. We previously reported that high affinity binding sites of ANF existed in the rat kidney, especially the renal cortex, by means of radiolabeled receptor assay¹⁴ and autoradiography.¹⁵,¹⁶ Recently, many investigators have reported on the relation between ANF and blood pressure, suggesting that ANF plays an important role in the hypertensive state.¹⁷,¹⁸ Although numerous studies on spontaneously hypertensive rats (SHR) have been reported as models for human essential hypertension, little is known about the pathogenesis of the hypertensive state in these animals.¹⁹,²⁰ We recently demonstrated that the ANF binding capacity in the kidneys of 12-week-old SHR was lower than that in the kidneys of Wistar-Kyoto (WKY) rats.²¹ A few studies were reported²² about SHR that concerned the change of renal ANF receptor. In the present study, we investigated the properties of renal ANF receptor bindings using the ANF radiolabeled receptor assay.
and also investigated the developmental changes in renal ANF receptors in SHR by means of radio-labeled receptor assay and the quantitative approach of in vitro macro-autoradiography.

Materials and Methods

Materials
Male Wistar rats (body weight, 250–300 g), male SHR, and normotensive WKY rats obtained from Charles River Japan (Kanagawa, Japan) were used. All rats were housed in climate-controlled metabolic cages with a 12-hour light/dark cycle and food (MF, Oriental Yeast Co., Tokyo, Japan) and water provided ad libitum. Wistar rats were used for the basic study on ANF binding assay. SHR were divided into three age groups: 3–4 weeks old (pre-hypertensive state), 5–8 weeks old (developmental hypertensive state), and 12–13 weeks old (established hypertensive state). Each group of SHR was compared with age-matched WKY rats. Blood pressure was measured routinely in conscious, unrestrained, and warmed rats with tail-cuff plethysmography (Ueda Seisakusho, Tokyo, Japan).

Membrane Preparations
The kidney was quickly removed and decapsulated in ice-cold Buffer A (50 mM Tris-HCl buffer, pH 7.6), containing 5 μg/ml chymostatin, 5 μg/ml leupeptin, and 750 kallikrein inhibition equivalents (KIE)/ml of aprotinin. The tissue was minced with a Polytron PT-10 (Kinematica, Luzen, Switzerland) at full speed for 10 seconds and homogenized by five strokes in a glass homogenizer in ice. The homogenate was centrifuged at 800g for 10 minutes at 4°C, and the pellet (nuclear fraction) was discarded. The supernatant was centrifuged at 18,000g for 20 minutes at 4°C. The final pellets, whole kidney membrane preparations, were resuspended in Buffer A and again centrifuged in Buffer A and used for the radiolabeled receptor assay immediately. Protein determinations were carried out according to the procedure of the Bio-Rad protein assay kit (Bio-Rad Labs., Richmond, California).

Iodination of Atrial Natriuretic Factor
Iodine-125-labeled ANF was preparing according to the lactoperoxidase method with slight modification as previously reported.14 Synthetic ANF (5 μg, α-human, 1–28 ANP, Peptide Institute, Osaka, Japan) in 5 μl 0.1 M acetic acid, was combined with 20 μl 0.5 M NaH2PO4 solution (pH 7.0), 1 mCi iodine-125–labeled sodium (Amersham Co., Tokyo, Japan), and 5 μg lactoperoxidase (Sigma Chemical Co., St. Louis, Missouri) in 5 μl 0.1 M sodium acetate buffer (pH 5.6). Iodination was initiated by addition of 5 μl 0.002% H2O2. After 1 minute, an additional 5 μl H2O2 was added. After 1 minute, the reaction was stopped by dilution with 1 ml ice-cold 0.3 M acetic acid (pH 2.4). The reaction mixture was immediately applied to a column (1 cm×70 cm) of Sephadex G 50 equilibrated and eluted with 0.3 M acetic acid at 4°C, and 1-ml fractions were collected in tubes that contained 200 μl 2.5% bovine serum albumin (BSA) (crystallized and lyophilized, Sigma Chemical Co.). Radioactivity in 10-μl portions of each fraction was counted by an auto-well gamma system (Aloka ARC 501, Aloka Co., Tokyo, Japan). Since the highest binding to the rat kidney membrane preparation was observed during the descending part of the first peak in two peaks, these fractions were used for the binding assay as [125I]ANF.

Binding (Radiolabeled Receptor Assay)
The ANF binding assay was carried out essentially as described previously.14 Briefly, the membrane preparation of rat kidney (0.10–0.12 mg protein per assay tube) was mixed with increasing concentrations of [125I]ANF (0.1–1.2 nM) in the presence or absence of unlabeled 2 μM ANF at a final volume of 0.5 ml Buffer B (Buffer A with 5 mM MgCl2, 0.1% BSA, 750 KIE/ml aprotonin) in a micro test tube (Ratio-lab. Co., Dreteich, Germany). Samples were incubated in an ice bath for 15 minutes. After the reaction, the assay tubes were centrifuged at 10,000g for 3 minutes. The radioactivity in these pellets was counted by the auto-well gamma system. The specific binding of ANF to the membrane preparations was obtained by the difference between the radioactivity bound to the receptor in the presence of an excess of 2 μM ANF and that bound in its absence. Scatchard plots of the data were analyzed with a computer (NEC-9801) according to the method of Marquardt.24

In Vitro Macro-Autoradiography
In vitro macro-autoradiography was performed as previously reported.15 Briefly, the kidney was quickly frozen by dry ice acetone. Sections (10-μm thick) were cut on a cryostat microtome at −30°C and thaw mounted onto slides. The freshly thaw-mounted sections on the slides were dried under an air stream at room temperature. These sections were preincubated for 2 minutes in ice-cold Buffer B into which 0.05% polyethyleneimine was added. Then each section was incubated in ice-cold Buffer B and [125I]ANF, with or without unlabeled excess ANF (2 μM), for 5 minutes. After the incubation, the sections were rinsed in ice-cold Buffer C (50 mM Tris-HCl buffer, pH 7.6) for 5 minutes and desiccated. These slides of kidney sections were fixed in 2.5% glutaraldehyde and then dehydrated through a graded series of ethanol concentrations before being embedded in paraffin. Thin sections were cut on a Leitz microtome and placed on glass slides. In some cases, sections were stained with uranyl acetate and lead citrate. The sections were observed under a transmission electron microscope (HITACHI H-7000).

Quantitative Approach of Autoradiography
The autoradiogram was analyzed with the quantitative approach using the research analysis system (RAS 1,000, Amersham, Tokyo, Japan). The specific binding of ANF in the autoradiogram was obtained.
by the subtraction data between total binding and nonspecific binding. On the basis of optical density in the microscale (Amersham), the value of radioactivity that was calculated with optical density in the subtraction film was analyzed with RAS 1,000. Moreover, 20 points of 1-mm square radioactivity in the specific binding of the autoradiogram of SHR were randomly measured using the spot analysis system of RAS 1,000 and were compared with those of WKY rats.

Statistical Analysis
The results were expressed as mean±SEM, and statistically analyzed by means of unpaired Student’s t test.

Results
Optimum Conditions for Radiolabeled Receptor Assay
Specific binding of [125I]ANF to the renal membrane fraction was time and temperature dependent (Figure 1). At 4°C without MgCl2 and aprotinin, the peak of specific binding was attained within 5 minutes and then rapidly decreased. In the presence of 5 mM MgCl2 and 750 KIE/ml aprotinin at 4°C, the highest and longest specific binding of ANF was observed at 15 minutes compared with other conditions. However, at 24°C the specific binding of [125I]ANF to the renal membrane fraction was proportional to the membrane protein concentration from 0.12 to 0.25 mg/tube (Figure 2).

Stability of [125I]Atrial Natriuretic Factor
Figure 3 shows the gel filtration pattern of [125I]ANF in the supernatant of incubation medium after 0 (fresh [125I]ANF), 15, and 30 minutes incubation. The incubation medium at 15 minutes formed the single major peak in the gel filtration pattern, which was recognized as being in the same position as that of the fresh [125I]ANF, whereas several low peaks were observed in the gel filtration pattern of incubation medium at 30 minutes. This suggested that [125I]ANF in the incubation medium was stable for 15 minutes, but it was degraded for 30 minutes at 4°C.

Saturation Curve and Scatchard Plot
Figure 4 shows a representative saturation curve and Scatchard analysis of the [125I]ANF binding to the whole kidney membrane fraction of Wistar rats. Scatchard analysis suggested that rat whole kidney has a single class of binding sites with apparent dissociation constant (Kd) of 0.55±0.04 nM (mean±SEM, n=6) and maximum binding capacity (Bmax) of 23.0±0.78 fmol/0.1 mg (mean±SEM, n=6).
Radiolabeled Receptor Assay of Atrial Natriuretic Factor in Spontaneously Hypertensive Rats

Body weight, systolic blood pressure, and data of Scatchard analysis of ANF binding assay of SHR and WKY rats are shown in Table 1. The systolic blood pressure in SHR at the age of 3 weeks was the same as that in control rats, but the systolic blood pressure at the ages of 5 and 12 weeks was significantly higher than that in the age-matched control rats. Concerning the data of Scatchard analysis in the kidney of these rats, the apparent $K_d$ and $B^\text{max}$ of SHR were lower than those in WKY rats in all three age groups.

Computer Analyzed Autoradiogram of Atrial Natriuretic Factor in Spontaneously Hypertensive Rats

In the autoradiogram, specific binding of $[125\text{I}]$ANF was mainly localized in the renal cortex (Figure 5). Figure 6 shows the specific binding in SHR and WKY rats at age 4 weeks using the RAS 1,000. The specific binding of ANF in SHR clearly decreased compared with that in WKY rats. To quantify these visual changes, we investigated the radioactivity in the film by using the spot analysis system of RAS 1,000. Figure 7 summarizes the radioactivity of ANF specific binding in the kidney of SHR and WKY rats at the ages of 4, 8, and 13 weeks. The specific binding of ANF in SHR was significantly lower in all age groups, although the localization of ANF binding in the kidney of SHR was similar to the WKY rats in all three age groups.

Discussion

The ANF binding capacity was greatly influenced by temperature and incubation time (Figure 1) as Napier et al. reported. To elucidate the stability of $[125\text{I}]$ANF influenced by incubation time and temperature, we investigated the fractional pattern of $[125\text{I}]$ANF, with the result that the degraded radioactive ligand influenced the decreased specific binding of ANF. Since the $[125\text{I}]$ANF became stable within 15 minutes of incubation at $4^\circ\text{C}$, we conducted the ANF radiolabeled receptor assay at $4^\circ\text{C}$ for 15 minutes. In these assay conditions, Scatchard analysis suggests that rat kidney has single binding sites with an apparent $K_d$ of 0.55 nM. As we

<table>
<thead>
<tr>
<th>Variable</th>
<th>3-week-old</th>
<th>5-week-old</th>
<th>12-week-old</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WKY</td>
<td>SHR</td>
<td>WKY</td>
</tr>
<tr>
<td>Body wt (g)</td>
<td>45.6±0.5</td>
<td>42.1±0.7*</td>
<td>94.0±1.9</td>
</tr>
<tr>
<td>BP (mm Hg)</td>
<td>102.6±6.8</td>
<td>112.6±3.1</td>
<td>115.2±4.4</td>
</tr>
<tr>
<td>$K_d$ (nM)</td>
<td>0.3±0.0</td>
<td>0.16±0.02†</td>
<td>0.27±0.03</td>
</tr>
<tr>
<td>$B^\text{max}$ (fmol/0.1 mg protein)</td>
<td>6.70±0.17</td>
<td>3.95±0.47†</td>
<td>13.6±0.87</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SEM of five rats. WKY, Wistar-Kyoto rats; SHR, spontaneously hypertensive rats; BP, blood pressure; $K_d$, dissociation constant; $B^\text{max}$, maximum binding capacity.

* $p<0.05$, † $p<0.01$ compared with values for WKY rats (unpaired Student's $t$ test).
Macro-autoradiographic localization of iodine-125-labeled atrial natriuretic factor (ANP) binding to the Wistar rat kidney section. Kidney sections (10 μm) were incubated with 0.5 nM [125I]ANP at 4°C for 5 minutes in the absence (Panel A) or presence (Panel B) of 2 μM ANP. Dense grains were seen in the renal cortex in a punctuated pattern. Linear deposits from cortex to outer medulla were observed in the renal cortex. These dense grains disappeared with the addition of 2 μM ANP (Panel B).

Previously reported,\textsuperscript{15,16} the ANF binding sites mainly exist in the renal cortex. However, since the physiological action of ANF in the medullary collecting tubules was not confirmed and the result of autoradiography was intended to contrast with that of RRA, we performed the ANF binding assay using the rat whole kidney.

Autoradiography is considered a valuable method to determine the receptor localization. In the autoradiogram of ANF there were no different patterns of ANF binding sites between SHR and WKY rats in all three age groups. Nevertheless, when using the quantitative analysis of autoradiography, the number of ANF receptors in the kidney of SHR was proved to be lower than that in WKY rats. The results from autoradiography agreed with the data from the radiolabeled receptor assay of ANF.

Several investigators have shown that ANF affects blood pressure,\textsuperscript{6,25,26} which suggests that ANF takes a part in blood pressure regulation. Plasma ANF levels in hypertensive patients are controversial. Some groups have reported increased plasma ANF levels in patients with essential hypertension,\textsuperscript{18,27,28} and other groups have reported no difference compared with control subjects.\textsuperscript{29,30} In secondary hypertensive animal models such as deoxycorticosterone acetate-salt hypertensive rats and one-kidney, one clip rats, plasma ANF levels were reported to be elevated compared with control rats and the down-regulation of ANF was suggested.\textsuperscript{25,31,32} The present study demonstrated that the ANF receptor binding in the kidney of SHR was lower than that in the kidney of WKY rats by means of radiolabeled receptor assay and autoradiography. As these low binding levels were observed even in 3-week-old SHR, which were normotensive, these differences are possibly genetic in origin. Saito et al\textsuperscript{22} reported the decrease in ANF binding to basolateral membranes in 14- and 15-week-old SHR and SHR-stroke prone strain, but not in 5-week-old rats. As the reduction of ANF binding sites of SHR might be due to the increased plasma ANF concentration, they suggested that these phenomena were caused by the down-regulation of the receptor. As shown in Table 1, the changes in ANF receptors may partly correspond with blood pressure. However, despite the increased level of ANF in the blood,\textsuperscript{22,23} SHR...
had enhanced natriuretic and hypotensive responsiveness to exogenous ANF, 33-35 which suggests that these phenomena cannot be explained by the down-regulation of the ANF receptor. We also observed a gradual increase in the number of ANF receptors in the kidney of both SHR and WKY rats with age. Similar age-associated changes were found due to the high affinity of ANF receptors and the development of ANF receptors in SHR are related to the progression of hypertension or not, is now being investigated using renin-aldosterone system in dogs. Am J Med 1984; 77:1069-1075


KEY WORDS • atrial natriuretic factor • autoradiography • kidney • spontaneously hypertensive rat
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