Glomerular Atrial Natriuretic Factor Receptors in Spontaneously Hypertensive Rats

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There are differences in the renal handling of sodium between spontaneously hypertensive rats (SHR) and their normotensive controls. We investigated whether this difference may be associated with changes in plasma and tissue atrial natriuretic factor (ANF) levels and with alterations in glomerular ANF receptors at 4, 8, 12, and 16 weeks of age. Age-matched Wistar-Kyoto (WKY) and Wistar rats were used as normotensive controls. Systolic blood pressure was higher in SHR at 8, 12, and 16 weeks, and cardiac hypertrophy was also present in these animals at 4 weeks. Plasma ANF C- and N-terminal concentrations were greater than in both normotensive groups at 8 and 16 weeks. ANF in the right atrium was higher in SHR than in WKY rats and identical to that in the Wistar group at 4 and 8 weeks. ANF in the left atrium was lower in SHR than in both control groups at week 12. No differences were found in ventricular ANF content. The density of glomerular ANF binding sites increased with age in WKY and Wistar rats but not in SHR. At weeks 8, 12, and 16, both normotensive groups had a higher density of binding sites than SHR, but binding site affinity was greater in SHR at weeks 8 and 12. After incubation with increasing concentrations of ANF, the production of cyclic guanosine monophosphate (cGMP) by isolated glomeruli from 16-week-old rats was lower in SHR than in both normotensive groups. We conclude that the development of hypertension in SHR is associated with higher plasma ANF levels and decreased glomerular ANF receptor density and glomerular cGMP production. Through modulation of ANF glomerular receptors, ANF in SHR, when compared with normotensive counterparts, may contribute to the difference in renal sodium handling. (Hypertension 1989;13:567–574)

The natriuretic effect of atrial natriuretic factor (ANF) may be secondary to an increased glomerular filtration rate by enhancement of absolute and fractional sodium excretion. However, experimental evidence indicates that other factors, such as inner medullary washout, could also be important. Experiments on the isolated dog glomerulus have suggested that the augmented glomerular filtration rate could be due to a dual mechanism, an increase in both glomerular capillary hydrostatic pressure and the glomerular capillary ultrafiltration coefficient.

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plasma ANF levels at different stages in the development of hypertension in SHR. Since cyclic guanosine monophosphate (cGMP) has been proposed as a biologic marker of ANF, the renal glomerulus has been identified as a specific target of ANF, and the production of cGMP by isolated glomeruli was also investigated.

### Materials and Methods

Three-, 7-, 11-, and 15-week-old SHR and their age-matched normotensive Wistar and WKY rat controls (Taconic Farms, Inc., Germantown, New York) were kept on regular rat chow and tap water ad libitum. Six days later, with prior warming (37°C for 10 minutes), the systolic blood pressure was measured indirectly by the tail-cuff method (Narco Bio-Systems, Houston, Texas). The rats were decapitated 24 hours later. Twenty animals were used for each age and strain group.

#### Biochemical Methods

Trunk blood was collected immediately after decapitation. Blood for ANF measurement was collected in tubes that contained the following protease inhibitors at a final concentration of: 1.0 × 10⁻² mol/l EDTA, 5 × 10⁻⁶ mol/l pepstatin, and 3 × 10⁻³ mol/l phenylmethylsulfonyl fluoride (PMSF). The samples were immediately centrifuged at 2,000g for 10 minutes at 4°C. ANF-(99-126) was extracted from plasma with Vycor glass beads (Corning Glass Works, Corning, New York) and measured by radioimmunossay (RIA), as described elsewhere. The detection limit of the assay was 0.75 pg/tube. The interassay and intra-assay coefficient of variance was below 14%, and the recovery of added [¹²⁵I]ANF was 75%.

Plasma samples were directly assayed for the measurement of ANF-(1-98), as described previously. The ED₉₀ of the displacement curve was 0.97±2.5 (n=12), and the ED₉₀ was 44±10.5 femtomole/tube. Intragroup and intergroup variations in the assay were lower than 15%. The antibody showed 100% cross-reactivity to rat ANF-(1-126), rat ANF-(11-137), and human ANF-(1-30). No cross-reactivity was detected with 300 pmol of either ANF-(99-126) or ANF-(103-126). The assay was validated by the addition of 194, 1,540, and 19,120 fmol/ml plasma of ANF-(1-98) (n=10 for each) and the recovery was 115, 105, and 85%, respectively.

Hearts and kidneys were rapidly removed after decapitation. The right and left atria were dissected separately, and atrial ANF concentrations were measured by RIA. Briefly, the atria were homogenized for 1 minute with a Polytron (setting 8) (Kinematica GMBH, Luzerne, Switzerland) in 2 ml 0.1 M acetic acid containing the same protease inhibitors as described above. The ventricles were carefully dissected, minced, and boiled in 1 M acetic acid for 5 minutes (10 ml acetic acid/g tissue), then cooled to room temperature and homogenized in a Polytron (30 seconds, set at 8). The homogenates were centrifuged at 15,000 rpm for 10 minutes, and the supernatant was applied to a C₄ Sep-Pak cartridge (Waters Associates, Milford, Massachusetts) that had previously been activated with 5 ml 100% acetonitrile. The cartridges were washed twice with 5 ml 0.1% trifluoroacetic acid in H₂O, and the ANF adsorbed on the columns was eluted with 80% acetonitrile in 0.1% trifluoroacetic acid. The material was lyophilized in a Speed-Vac (Savant Instrs. Inc., Farmingdale, New York) and kept at -70°C until ANF was measured by RIA. The detection limit of the assay was 3 pg/tube. The interassay and intra-assay coefficients of variance were below 16%, and the recovery of added [¹²⁵I]ANF was 85%. Atrial and ventricular protein content was measured by a modification of the method of Bradford. Plasma ANF levels and tissue ANF content were not corrected for recovery.

#### Preparation of Glomerular Membranes

Kidneys were excised from the renal capsules, placed in ice-cold 0.9% NaCl solution, dissected longitudinally, and the medulla and papilla eliminated. They were then homogenized by passing the cortical tissue through a 0.5-mm mesh. The mesh was diluted with 0.9% NaCl solution and filtered through a 200-µm, 50-µm, 100-µm, and 150-µm mesh nylon sieve. Glomeruli retained in the sieve were collected in a 50-ml centrifuge tube and washed by centrifugation (4°C, 2,000g). After the last centrifugation, they were suspended in 0.05 mol/l Tris-HCl (pH 7.2) and kept at -70°C. The purity of the glomerular suspension was assessed by light microscopy and was estimated to be about 95% at the end of the preparation process. This glomerular suspension was homogenized for 1 minute with a Polytron (set at 6), centrifuged at 30,000g for 30 minutes and resuspended in 1 ml 0.05 M Tris-HCl (pH 7.2). An aliquot was taken for protein determination by a modification of the method of Bradford.

#### Glomerular Membrane Binding Assay

The binding assay of ANF to glomeruli was performed as follows: Aliquots (35 µg) of glomerular membrane protein were incubated in duplicate for 60 minutes at 22°C, as described by Carrier et al, in the presence of increasing concentrations of unlabeled ANF (10⁻¹²-10⁻⁷ mol/l) and 20 pM [¹²⁵I]ANF in a final volume of 1 ml. The reaction was stopped by dilution with 3.5 ml assay buffer and rapid filtration through polyethyleneimine-treated Whatman GF/C filters, which were then rinsed three times with 3 ml Tris-HCl (pH 7.2), allowed to dry, and counted in a LKB Gamma Counter (Turku, Finland) with 65% efficiency. [¹²⁵I]ANF was prepared by the lactoperoxidase method. It had a specific activity of approximately 1,000 Ci/mmol. Rat ANF-(99-126) was purchased from BioMega Inc. (Laval, Quebec, Canada).
The glomerular ANF receptor binding assay has been previously validated. Binding equilibrium was achieved after 60 minutes of incubation. No degradation of ANF was detected by high-pressure liquid chromatography (HPLC) after incubation.26

Cyclic Nucleotide Measurement

Glomerular protein (35 μg) was preincubated at 37° C for 15 minutes in 400 μl HBSS. Isobutylmethylxantine was then added to achieve a final concentration of 0.5 mmol/l. Two minutes later, ANF diluted in 50 μl buffer was added to final concentrations of 10^{-13}, 10^{-12}, and 10^{-11} mol/l; the same volume of buffer alone was added for baseline cGMP concentration. Sixty seconds later, the incubation reaction was stopped by the addition of 500 μl trichloroacetic acid (120 g/l) and sonication. The preparation was centrifuged at 2,000g for 15 minutes, and the supernatant was extracted five times with water-saturated ethyl ether and evaporated. Each sample was suspended in 500 μl 0.05 mol/l sodium acetate buffer, pH 7.4. An aliquot of 100 μl was acetylated, and the concentration of cGMP was measured by RIA with a commercial kit (New England Nuclear, Boston, Massachusetts). For each ANF concentration, two experiments were performed in duplicate.

Analysis of Data

The results are expressed as mean±SEM. The data were evaluated by two-way analysis of variance, and an a posteriori contrast test, according to Bonferroni's method, was applied whenever a level of significance was found ($p<0.05$). The binding data were analyzed with the computer-based LIGAND program,27 after preliminary treatment with the EBDA program,28 to determine the density and affinity of binding sites in the competition experiments. These results were evaluated by one-way analysis of variance and an a posteriori test according to Bonferroni's method and were considered significant at $p<0.05$.

Results

Blood pressure, body and heart weight, and hematocrit values of SHR, WKY rats, and Wistar rats are depicted in Figure 1. At 4 weeks, the blood pressure readings of SHR were already slightly but significantly higher than those of Wistar but not of WKY rats. Blood pressure in the SHR rose significantly with age and was higher than in both normotensive groups at 8, 12, and 16 weeks. Body weight was lower in SHR than in Wistar rats at 4 weeks and less than in both WKY and Wistar rats at 8, 12, and 16 weeks. The body weight of WKY rats was also lower than that of Wistar rats during the same period of time. Relative heart weight was already higher in SHR than in their normotensive counterparts at 4 weeks and continued to be so at all study periods. It was also higher in WKY than in Wistar rats at 8 and 12 weeks. The SHR had higher hematocrit values than either normotensive group at weeks 8 and 16, higher than Wistar rats at week 4, and higher than WKY rats at week 12.

ANF concentration in the right and left atria is shown in Figure 2. At week 4, ANF in the right atrium of SHR was higher than in both normotensive groups; at week 8, it was higher than in WKY rats, and at week 12 it was lower than in either normotensive control group. No differences were found among all three groups at 16 weeks. ANF concentration in the left atrium was higher in Wistar rats than in either WKY rats or SHR at weeks 4 and 8. At week 12, the SHR showed lower left atrial ANF values than either normotensive group. No differences were noted at week 16. Ventricular ANF tended to be higher in SHR at weeks 4, 8, and 16, but no statistical difference was evident. Ventricular ANF concentration at 4 weeks was: 3.27±1.03, 2.48±0.59, and 7.43±1.94 ng/mg protein; at 8 weeks: 43.13±8.49, 40.35±14.41, and 79.45±9.67 ng/mg protein; at 12 weeks: 46.00±11.63, 28.81±6.77, and 21.59±10.57 ng/mg protein.

![Figure 1. Systolic blood pressure (BP), body and relative heart weights, and hematocrit in spontaneously hypertensive rats (SHR), Wistar-Kyoto (WKY) rats, and Wistar rats.](image-url)
protein; and at 16 weeks: 11.95±4.65, 8.86±4.00, and 25.21±7.23 ng/mg protein in Wistar rats, WKY rats, and SHR, respectively.

Figure 3 illustrates the plasma levels of ANF C-terminal [ANF-(99-126)] and ANF N-terminal [ANF-(1-98)] as well as the density of glomerular ANF receptors. At 4 weeks, plasma ANF levels were higher in SHR than in Wistar but not WKY rats. At weeks 8 and 16, the SHR had higher plasma ANF-(99-126) values than both normotensive controls. No differences were found at 12 weeks. Because of the animal size, no samples were obtained to measure ANF-(1-98) at 4 weeks. As with ANF-(99-126), plasma ANF-(1-98) levels were higher in SHR than in normotensive rats at 8 and 16 weeks.

The density of glomerular ANF receptors was similar in all experimental groups at 4 weeks. In both normotensive control groups, a significant progressive increase in receptor density was observed with age. No such increase was seen in SHR. Both Wistar and WKY rats presented a higher receptor number than SHR at 8, 12, and 16 weeks. This lower glomerular ANF receptor density in SHR was accompanied by a higher affinity (Kd) at weeks 8 and 12 (Table 1). Figure 4 shows representative ANF binding curves for glomerular membranes in SHR, Wistar rats, and WKY rats at 4, 8, 12, and 16 weeks of age.

The in vitro production of cGMP by glomeruli isolated from 16-week-old SHR, Wistar rats, and WKY rats is depicted in Figure 5. No differences were observed in cGMP production at a low ANF concentration (10^{-9}-3.3×10^{-8} M). At higher ANF concentrations (10^{-7}-10^{-6} M), cGMP production by the glomeruli of Wistar rats was significantly higher than in the other two groups. At ANF concentrations of 3.3×10^{-8} M and 10^{-7} M, cGMP production by the glomeruli of both normotensive groups was higher than that of SHR.
Discussion

It has previously been reported that SHR present a sodium preference when compared with either normotensive rats or with other models of genetic hypertension; this characteristic is not dependent on the level of blood pressure. Furthermore, exchangeable body sodium is higher in SHR than in WKY rats, even on a low sodium diet, which is not related to blood pressure either. Moreover, basal urinary sodium excretion and urinary volume may be lower in adult SHR than in normotensive WKY rats. A lower whole kidney glomerular filtration rate has also been reported in SHR during the development of hypertension. All this accumulated evidence suggests that abnormalities in renal function may be involved in the pathogenesis of hypertension in SHR, more so when kidneys transplanted from SHR into F1 hybrids significantly raise blood pressure in the latter. SHR are known to respond with exaggerated natriuresis when challenged with an acute sodium load, however, this response is abolished when renal perfusion pressure is acutely reduced to normotensive levels, mainly through a decrease in the glomerular filtration rate. This characteristic suggests that the kidneys of adult SHR require a higher blood pressure than those of their normotensive controls to excrete an acute sodium load.

Widely discrepant results have been reported with regard to plasma ANF levels and atrial ANF content when comparing SHR with WKY rats. This discrepancy has also been found in studies on ANF binding sites in renal tissue, and could be partially due to the biologic variability of WKY rats. Another factor that may explain this controversy is the daily fluctuation of plasma ANF levels in SHR. To reduce the possibility of differential results due to this biologic variability, we chose to study two rat strains as the normotensive controls of SHR.

As early as 4 weeks of age, blood pressure was higher in SHR than in Wistar but not in WKY rats. It rose steadily in the older SHR, reaching higher levels than in both normotensive groups. Body weight was lower and relative heart weight was greater in SHR than in WKY rats in all age periods. The body weight of SHR can be lower, equal to, or greater than WKY rats, but cardiac hypertrophy in the prehypertensive period is a well-known characteristic of SHR. The finding of higher hematocrit values in SHR, when compared with WKY rats at age 8, 12, and 16 weeks, suggests a lower plasma volume in the hypertensive group. This finding has...
been previously reported, but whether there is a plasma volume difference between SHR and WKY rats is highly controversial.

In agreement with earlier results, we now report that the ANF content of the right atrium of prehypertensive SHR (4 weeks) is higher than in both normotensive groups. This difference is still present at 8 weeks but only when compared with WKY rats. At week 12, once hypertension is well established, a lower concentration of ANF is observed in the right atrium. This observation is similar to that previously reported in two-volume-expanded models of nonspontaneous experimental hypertension, one-kidney, one clip and non–renin-dependent two-kidney, one clip rats. However, we do not have any evidence of a similar mechanism in SHR, more so when higher hematocrit values are found at this stage of development (Figure 1). At a later stage (16 weeks), right atrial ANF content is depressed and equal in all groups. Whether this is an age-related phenomenon remains to be established. A lower ANF content is also observed in the left atrium of SHR at 12 weeks of age. This finding has been interpreted as secondary to atrial stretch produced by the higher atrial pressure known to occur in SHR, as in the right atrium. This difference disappears at week 16. No difference in the ventricular content of ANF is found at any stage. Our results contrast with those of other investigators who have reported an increased ANF content in the ventricles of SHR. Whether this difference is due to the variability of the control groups or to the methodology is not clear at the present time.

Plasma ANF C-terminal [ANF-(99–126)] concentrations are elevated in SHR at weeks 8 and 16, but not at week 12. ANF N-terminal [ANF-(1–98)] levels follow exactly the same pattern. The fact that both C- and N-terminal peptides were measured in the same samples by a completely different RIA adds credibility to the belief that these fluctuations are due to a true biologic variability in plasma ANF during the development of hypertension in SHR, and may explain the controversy found in the literature over the values of atrial and plasma ANF, especially when the latter may present wide daily variations.

The density of glomerular ANF binding sites increases with age in both normotensive strains. No such rise is observed in SHR and, indeed, the number of binding sites is significantly lower at 8, 12, and 16 weeks than in both Wistar and WKY rats (Figure 3). This lower density of glomerular ANF receptors in SHR is accompanied by a higher affinity (lower Kd) at weeks 8 and 12 (Table 1). Such a reciprocal change has also been noted in the clipped kidney of two-kidney, one clip hypertensive rats. This decreased density of glomerular ANF receptors in SHR is in agreement with results from other investigators, who used different methodologies or whole kidney membranes. The fact that we can find a greatly decreased number of glomerular binding sites in SHR with similar plasma levels of ANF (12 weeks) suggests that plasma ANF concentrations may not be the only factor involved in glomerular ANF receptor regulation. A similar situation has been observed in renin-dependent two-kidney, one clip hypertensive animals in which the clipped and nonclipped kidneys have a significantly different number of ANF binding sites in the presence of the same plasma ANF concentration.

It has been reported that there are two types of ANF binding sites but only one, present in low density, stimulates cGMP production. Copurification of ANF receptors and particulate guanylate cyclase has been reported in extrarenal tissues as well as in renal tissue. Our finding of lower cGMP production by the in vitro incubation of glomeruli from 16-week-old SHR, together with a decreased density of glomerular ANF binding sites, suggests that the down-regulated receptor population is that associated with guanylate cyclase. Our results are compatible with those of Marsh et al, who noted lower basal and ANF-stimulated urinary cGMP secretion rates in conscious SHR.

Acute administration of ANF at low doses may produce a similar decrease in blood pressure, diuresis, and natriuresis in SHR and WKY rats. In higher doses, a marked drop in blood pressure is observed in SHR accompanied by lower diuresis and natriuresis than in normotensive controls. This decrease of natriuresis with higher doses of ANF could be explained by a reduction in renal perfusion pressure, a well-known factor mediating the effect of the peptide. However, the drop in renal perfusion pressure may very well not be the only factor involved. The decrease in renal perfusion pressure in SHR to normal levels abolishes the exaggerated natriuretic response to a stimulus of ANF release, an acute sodium load. The decrease in the natriuretic response to ANF or to a sodium load in SHR with normal renal perfusion pressure could be mediated in part by the lower density of glomerular ANF receptors.

In summary, depending on their developmental stage, SHR may have higher, lower, or equal ANF content in the right atrium, and equal or lower ANF content in the left atrium when compared with normotensive rats. Plasma ANF C- and N-terminal concentrations were more elevated in SHR than in WKY rats at weeks 8 and 16. The number of glomerular ANF binding sites increased with age in both normotensive strains, but not in SHR. The lower density of ANF binding sites was accompanied by a higher affinity and by a decrease in the in vitro production of cGMP by SHR glomeruli. The down-regulation of glomerular ANF receptors in SHR may explain in part the differential sodium handling of this hypertensive model when compared with WKY rats.

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