Decreased Density of Vascular Receptors for Atrial Natriuretic Peptide in DOCA-Salt Hypertensive Rats

ERNESTO L. SCHIFFRIN AND JEAN ST-LOUIS

SUMMARY We have previously found that vascular receptors for atrial natriuretic peptide (ANP) in the rat are down-regulated by volume expansion. For this reason vascular ANP receptor density and affinity were examined in a model of volume-expanded hypertension, the deoxycorticosterone acetate (DOCA)-salt hypertensive rat. The density of mesenteric vascular ANP binding sites was decreased in DOCA-salt hypertensive rats from a control value in uninephrectomized rats of 203 ± 25 fmol/mg protein to 60 ± 13 fmol/mg protein (p<0.01). The sensitivity of norepinephrine-precontracted aorta to ANP was significantly reduced in DOCA-salt hypertensive rats (p<0.001). DOCA-salt hypertensive rats infused intravenously for 4 days with ANP, 100 to 300 ng/hr, did not experience a lowering of blood pressure, in contrast to the significant reduction in blood pressure seen in two-kidney, one clip Goldblatt hypertensive rats similarly infused. In the latter there was no natriuretic response to ANP, while in the DOCA-salt hypertensive rats natriuresis occurred without lowering of blood pressure. In the DOCA-salt hypertensive rats plasma ANP concentration was increased to 68 ± 8 fmol/ml from 10 ± 1 fmol/ml in uninephrectomized rats. In conclusion, raised ANP concentration in plasma of volume-expanded hypertensive rats (DOCA-salt hypertension) may result in decreased density of ANP vascular receptors. These results suggest that a decrement in the number of ANP receptors may be a cause of decreased sensitivity of vascular responses to ANP in vitro and resistance to the blood pressure-lowering action of ANP in vivo. (Hypertension 9: 504–512, 1987)

KEY WORDS • atrial natriuretic factor • plasma atrial natriuretic peptide • vascular receptor regulation • vascular responsiveness • mesenteric vascular bed

THE atria of mammals have been shown to contain a family of peptides endowed with potent diuretic, natriuretic,1 vasorelaxant,2,3 and aldosterone-inhibiting properties.4,5 Atrial natriuretic factor or peptide (ANP) is the term applied to both the native peptides purified from atria and synthetic atrial natriuretic peptides. A 28-amino acid peptide (ANP 99–126) has been shown to be the main circulating form of ANP.6,7 Receptors mediating the vasorelaxant effect of ANP have been characterized in the mesenteric vascular bed of the rat.8 We have recently shown that these ANP binding sites are down-regulated after sodium-loading uninephrectomized rats and in one-kidney, one clip (1K1C) Goldblatt hypertensive rats,9 a model of non-renin-dependent, volume-expanded hypertension. After mineralocorticoid administration we have also found a decrement in the density of vascular ANP receptors.10 In all these experimental models sensitivity to ANP of norepinephrine (NE)-precontracted aortic strips was decreased. Infusion of ANP lowers the blood pressure of spontaneously hypertensive rats,11 a genetic model of hypertension, and of two-kidney, one clip (2K1C) Goldblatt hypertensive rats,12 a renin-dependent, non-volume-expanded form of experimental hypertension. In contrast, 1K1C Goldblatt hypertensive rats are relatively resistant to the hypotensive action of ANP.13 In 1K1C hypertensive rats, vascular ANP sites are decreased in density,9 while in the 2K1C model no down-regulation of vascular binding sites is present.9 We therefore hypothesized that in another experimental model of volume-expanded hypertension, the deoxycorticosterone acetate (DOCA)–salt
Iodination of ANP

Blood was withdrawn from the abdominal aorta. 50 μg of ANP was exposed to 1 mCi of 123I-labeled sodium iodide (New England Nuclear, Boston, MA, USA) in the presence of 5 μg of lactoperoxidase (Sigma) in a total volume of 80 μl of 0.05 M sodium phosphate buffer, pH 7.4. The reaction proceeded after the addition of 10 μl of 0.6% hydrogen peroxide (vol/vol) at three 5-minute intervals. Radiolabeled ANP was separated by high pressure liquid chromatography on a C18 Bondapak column (Waters, Milford, MA, USA) eluted with a linear gradient of 20 to 45% acetonitrile and 0.1% trifluoroacetic acid, with a slope of 0.5%/min and a flow rate of 1 ml/min. Specific activity, measured by radioreceptor assay and self-displacement, was approximately 1000 Ci/mmol.

Materials and Methods

Drugs

Synthetic rat ANP (Arg 101–Tyr 126) was obtained from Bachem (Torrance, CA, USA) through the Institut Armand Frappier (Laval, Quebec, Canada). Deoxycorticosterone was purchased from Sigma Chemical (St. Louis, MO, USA).

Animal Experiments

DOCA-salt hypertension was induced by the method of Ormsbee and Ryan.14 Male Sprague-Dawley rats (Charles River Laboratories, St-Constant, Quebec, Canada), weighing 200 g, were uninephrectomized under ether anesthesia. Silicone rubber impregnated or not with deoxycorticosterone acetate (DOCA), 100 mg per rat, was implanted in all experimental groups, and rats were offered 1% saline to drink. Rats were studied within 2 weeks of becoming hypertensive. The 2K1C hypertensive rats were prepared by placing a silver clip on the left renal artery under ether anesthesia. The 2K1C rats that did not become hypertensive (blood pressure > 150 mm Hg was considered hypertension) served as controls. Experiments were performed on the fourth week after development of hypertension. The 1K1C rats were prepared similarly, but right nephrectomy was also performed when the silver clip was placed. Hypertension developed in most of these rats, and uninephrectomized rats served as controls. Blood pressure was taken weekly by the tail cuff method with the rats under light ether anesthesia and recorded on a Grass Model 7 polygraph (Quincy, MA, USA) fitted with a 7-P8 preamplifier and a Model 1010 Grass crystal microphone as a pulse detector. The average of three pressure readings was recorded.

Intravenous infusion of ANP was performed with ALZET osmotic minipumps (Model 2001; Alza Corp., Palo Alto, CA, USA) containing ANP in acidified saline, implanted subcutaneously. A PE-60 catheter connected to the pump was inserted into the jugular vein. Controls were sham-infused. Rats were housed in metabolic cages for this experiment, and arterial pressure was monitored as a pulse detector. The average of three pressure readings was recorded.

For measurement of plasma ANP all groups of rats were prepared separately. Rats were anesthetized intraperitoneally with pentobarbital, 60 mg/kg, and blood was withdrawn from the abdominal aorta.

Iodination of ANP

Synthetic rat ANP (101–126) was iodinated with iodine-125 by the lactoperoxidase method. In brief, 5 μg of ANP was exposed to 1 mCi of 125I-labeled sodium iodide (New England Nuclear, Boston, MA, USA) in the presence of 5 μg of lactoperoxidase in a total volume of 80 μl of 0.05 M sodium phosphate buffer, pH 7.4. The reaction proceeded after the addition of 10 μl of 0.6% hydrogen peroxide (vol/vol) at three 5-minute intervals. Radiolabeled ANP was separated by high pressure liquid chromatography on a C18 Bondapak column (Waters, Milford, MA, USA) eluted with a linear gradient of 20 to 45% acetonitrile and 0.1% trifluoroacetic acid, with a slope of 0.5%/min and a flow rate of 1 ml/min. Specific activity, measured by radioreceptor assay and self-displacement, was approximately 1000 Ci/mmol.

Membrane Preparation

Rats were killed by decapitation. The mesenteric vascular bed was isolated as previously described.16 Rat thoracic aorta was dissected, and the first 0.5 cm of the proximal end was discarded. The tissues were then immersed in 0.25 M sucrose solution, finely minced with scissors, and homogenized in a polytron (Kinetica, Lucerne, Switzerland; Setting 8; two 10-second homogenizations). The homogenate was centrifuged at 1500 g for 10 minutes at 4°C, the supernatant was decanted and recentrifuged. The final supernatant was filtered through cheesecloth, then centrifuged at 104,000 g for 30 minutes. The pellet was resuspended in a 0.05 M Tris HCl buffer (pH 7.4), containing 5 mM MgCl2, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 0.1% bacitracin, and 1 μM aprotinin. Proteins were measured by the Coomassie blue method. Bovine serum albumin was then added at a concentration of 0.2%, and the membranes were diluted to a protein concentration of 0.5 mg/ml for mesenteric arteries and 2 mg/ml for aorta in the Tris HCl buffer containing 0.2% albumin (assay buffer).

ANP Binding Assay

The binding assay was performed as previously described.8 Briefly, 10 to 30 pM of labeled ANP and at least 14 different concentrations of unlabeled ANP (10−12–10−6 M) were employed in competition experiments with 50 μg of protein per tube for mesenteric arteries and 200 μg of protein per tube for aorta at 4°C for 60 minutes. All assays were performed in duplicate. Separation of bound and free radioactivities was achieved by rapid filtration through polyethyleneimine-treated Whatman GF/C filters (Clifton, NJ, USA) soaked with 0.5 ml of assay buffer. The filters were washed twice with 3 ml of 0.9% NaCl, then allowed to dry and counted in a Rackgamma LKB counter (Turku, Finland) with 65% efficiency.

Response of Vascular Strips to ANP

Rat thoracic aorta from the different experimental groups was dissected and cut in helical strips. The first 0.5 cm of the proximal end of the strips was discarded, and the next 2.5 cm was prepared for incubation in a glass-jacketed, 15-ml tissue bath at 37°C subjected to a tension of 1.0 g. The bathing medium was Kreb’s bicarbonate with the following composition (mM) NaCl,
118; MgSO₄, 1.18; KH₂PO₄, 1.18; dextrose, 5.5; NaHCO₃, 25.0; CaCl₂, 2.5; and KCl, 4.7. The solution was bubbled with a mixture of 95% O₂ and 5% CO₂. Tissues were equilibrated for 90 minutes, until challenged with 1 μM NE. One hour later, a cumulative dose-response curve to NE was obtained. After full relaxation, 70 to 90 minutes later, tissues were contracted with 0.1 μM NE. When the plateau of contraction was well established (after 7–10 minutes), a cumulative dose-response curve to ANP (0.1–100 nM) was obtained. After maximum relaxation to ANP was reached, the tissues were washed at frequent intervals and were rechallenged 50 minutes later with 0.1 μM NE.

Biochemical Methods

Blood was collected from the trunk in all experimental groups within the first 5 seconds after decapitation into chilled tubes containing EDTA and centrifuged at 4°C (1500 g for 30 minutes). Plasma renin activity (PRA) was measured by radioimmunoassay of angiotensin I generated in a 2-hour incubation period at 37°C and pH 6.5 in the presence of EDTA and 8-hydroxyquinoline.¹⁷

The concentration of ANP was measured in plasma from rats prepared separately. Blood was withdrawn from the aorta of rats anesthetized intraperitoneally with pentobarbital, 60 mg/kg, into tubes containing EDTA to which 10 μM PMSF and 5 μM pepstatin had been added. Radioimmunoassay was performed after extraction of acidified plasma (pH 4.0) through C18 Sep-Pak cartridges with acidified ethanol (pH 4.0). The antibody against ANP was kindly provided by Dr. J. Gutkowska of our institute. The antibody cross-reacted 100% with ANP (99–126), ANP (101–126), and atropine III and 30% with atropine II. The minimal detectable concentration was 1 pg/tube, the binding of ¹²⁵I-labeled ANP to the antibody was inhibited by 50% by 28 pg/tube. Recovery of added ANP during extraction was 78%. Within-assay variation was 5%, and interassay variation was 8%.

5'-Nucleotidase was measured by a modification of the technique of Song and Bodansky¹⁸ to verify that selective enrichment in membrane components did not occur in individual experimental groups.

Analysis of Data

Binding data were analyzed by computer-assisted nonlinear regression analysis using the LIGAND program (Biomedical Computing Technology Information Center, Nashville, TN, USA) for determination of density and affinity of binding sites. The ALLFIT program (Biomedical Computing Technology Information Center), based on the four-parameter logistic equation, was used to fit the data from dose-response curves. Curves were compared by performing a partial F test on the sum of squares of residuals after fitting the data with or without constraining a parameter such as the concentration producing 50% relaxation (IC₅₀) to be shared or equal. A significant improvement in the goodness of fit (statistically significant reduction in the sum of squares of residuals) when the parameter was not shared indicated that this parameter was different for each curve. The deviations of mean values on the fitted curves (residuals) were weighted according to the reciprocal of the variance of the values obtained in individual experiments. The number of degrees of freedom for each fit was obtained by subtracting the number of parameters fitted from the number of data points. Since the effective concentration producing 50% of the maximal response (EC₅₀) to NE on aortic strips was not identical in the different groups examined and since the concentration of ANP producing 50% relaxation (IC₅₀) of NE-contracted aorta is dependent on the sensitivity to NE, the apparent pKᵢ of ANP (−log of the inhibitory constant or Kᵢ in M) was calculated from the IC₅₀ of ANP, the EC₅₀ of NE, and the concentration of NE used on each individual strip, according to the model of functional antagonism described by van den Brink¹⁹ using the equation of Cheng and Prusoff.²⁰ Results are reported as means ± SEM. Statistical differences between means were analyzed by one-way analysis of variance or analysis of variance for repeated measures, as appropriate. Differences were considered significant at a p level below 0.05.

Results

ANP Binding Sites in the Mesenteric Vasculature and Aorta of Hypertensive Rats

When unilaterally nephrectomized rats were offered 1% saline to drink, renin was suppressed and mesenteric vascular ANP binding sites were significantly (p<0.001) decreased in density, as expected from previous studies (Table 1 and Figure 1). DOCA-salt hypertensive rats exhibited a similar decrement in density of vascular ANP binding sites (see Table 1 and Figure 1). Similar results were observed in the aorta in a limited number of experiments that could be performed (due to the larger amount of protein necessary to detect an adequate signal in the binding assay of the aorta because of the smaller number of binding sites; Table 2). In seven different groups of rats from this study and previous studies (one- and two-kidney controls, 1K1C and 2K1C hypertensive, DOCA-infused or one-kidney salt-loaded rats, and DOCA-salt hypertensive rats), the decrease in mean density of binding sites for ANP in mesenteric vessels and in the aorta were closely correlated (r = 0.95, n = 7 groups, p<0.01).

Relaxation Responses in Aorta of DOCA-Salt Hypertensive Rats

Aortic strips from DOCA-salt hypertensive rats and sodium-loaded unilaterally nephrectomized rats, precontracted with an EC₅₀ of NE, exhibited a significantly decreased sensitivity to ANP (F = 83.5, p<0.001 for DOCA-salt hypertensive rats vs uninephrectomized rats; F = 37.3, p<0.001 for salt-loaded uninephrectomized rats vs uninephrectomized rats; Figure 2; see Table 1). This decrease in sensitivity of aortic strips to ANP agreed with the reduction in density of 50% relaxation, 70 to 90 minutes later, tissues were contracted with 0.1 μM NE. When the plateau of contraction was well established (after 7–10 minutes), a cumulative dose-response curve to ANP (0.1–100 nM) was obtained. After maximum relaxation to ANP was reached, the tissues were washed at frequent intervals and were rechallenged 50 minutes later with 0.1 μM NE.

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TABLE 1. Effect of Salt and DOCA-Salt Hypertension on ANP Binding and ANP-Induced Relaxation of Contracted Aorta

<table>
<thead>
<tr>
<th>Group</th>
<th>$B_{max}$ (fmol/mg protein)</th>
<th>$K_d$ (nM)</th>
<th>$5'$-nucleotidase (μmol P/hr/mg protein)</th>
<th>Aortic relaxation</th>
<th>PRA (ng ANG I/ml/hr)</th>
<th>Blood pressure (mm Hg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2K control</td>
<td>255 ± 60</td>
<td>0.33 ± 0.10</td>
<td>20.5 ± 2.4</td>
<td>1.94 ± 0.10</td>
<td>10.58 ± 0.12</td>
<td>5.80 ± 0.63</td>
</tr>
<tr>
<td>Uninephrectomized</td>
<td>203 ± 25</td>
<td>0.38 ± 0.03</td>
<td>21.6 ± 2.9</td>
<td>1.43 ± 0.10</td>
<td>10.73 ± 0.13</td>
<td>4.48 ± 0.32</td>
</tr>
<tr>
<td>Uninephrectomized + NaCl</td>
<td>103 ± 28*</td>
<td>0.29 ± 0.08</td>
<td>21.9 ± 2.8</td>
<td>2.69 ± 0.14</td>
<td>10.44 ± 0.11</td>
<td>0.84 ± 0.76*</td>
</tr>
<tr>
<td>Uninephrectomized + NaCl + DOCA</td>
<td>60 ± 13*</td>
<td>0.53 ± 0.11</td>
<td>24.1 ± 2.6</td>
<td>3.42 ± 0.22</td>
<td>10.04 ± 0.09</td>
<td>0.00 ± 0.00§</td>
</tr>
</tbody>
</table>

Values are results of five binding experiments and eight aortic relaxation studies (means ± SEM). $B_{max}$ = density of binding sites; $P_i$ = inorganic phosphate; ANG I = angiotensin I; 2K = two-kidney. The IC$_{50}$ values are means ± SEM estimated by the computer program ALLFIT by simultaneous weighted fitting of all dose-response curves of each group and were not significantly different from the means of the IC$_{50}$ of individually fitted curves for each group. The pK$_{i}$ values are means ± SEM of $-\log K_i$. $K_i$ was calculated from the IC$_{50}$ for ANP, the EC$_{50}$ of NE, both fitted individually for each experiment, and the corresponding EC$_{50}$ of NE, using the equation of Cheng and Prusoff.

Figure 1. Representative experiment of binding of $^{125}$I-labeled ANP (20 PM) to membranes (0.05 mg protein per tube in 0.25 ml) prepared from the mesenteric vascular bed of control, uninephrectomized (Uni-Nx), uninephrectomized and salt-loaded (Uni-Nx + NaCl), and DOCA-salt hypertensive (Uni-Nx + NaCl + DOCA) rats. Results are represented as bound/total (B/T) versus total ANP (labeled + unlabeled). Density and affinity of binding sites were calculated using the computer program LIGAND. Control density of binding sites ($B_{max}$ = 225 fmol/mg protein, $K_d$ = 0.3 nM; Uni-Nx: $B_{max}$ = 230 fmol/mg protein, $K_d$ = 0.3 nM; Uni-Nx + NaCl: $B_{max}$ = 84 fmol/mg protein, $K_d$ = 0.2 nM; DOCA-salt hypertensive (Uni-Nx + NaCl + DOCA) $B_{max}$ = 100 fmol/mg protein, $K_d$ = 0.4 nM.

ANP binding sites in the mesenteric vascular bed (see Table 1) and aorta (see Table 2). If, instead of an analysis of the whole dose-response curve to ANP by a partial $F$ test on the sum of squares of residuals after curve fitting with or without shared $K_v$ control or uninephrectomized group (one-way analysis of variance).  

Effect of Salt and DOCA-Salt Hypertension on ANP Binding and ANP-Induced Relaxation of Contracted Aorta

Plasma ANP Concentration in Hypertensive Rats

In a parallel group of rats it was found that salt-loaded one-kidney or DOCA-salt hypertensive rats had an increased concentration of ANP in plasma (Table 2).

FIGURE 1. Representative experiment of binding of $^{125}$I-labeled ANP (20 PM) to membranes (0.05 mg protein per tube in 0.25 ml) prepared from the mesenteric vascular bed of control, uninephrectomized (Uni-Nx), uninephrectomized and salt-loaded (Uni-Nx + NaCl), and DOCA-salt hypertensive (Uni-Nx + NaCl + DOCA) rats. Results are represented as bound/total (B/T) versus total ANP (labeled + unlabeled). Density and affinity of binding sites were calculated using the computer program LIGAND. Control density of binding sites ($B_{max}$ = 225 fmol/mg protein, $K_d$ = 0.3 nM; Uni-Nx: $B_{max}$ = 230 fmol/mg protein, $K_d$ = 0.3 nM; Uni-Nx + NaCl: $B_{max}$ = 84 fmol/mg protein, $K_d$ = 0.2 nM; DOCA-salt hypertensive (Uni-Nx + NaCl + DOCA) $B_{max}$ = 100 fmol/mg protein, $K_d$ = 0.4 nM.

TABLE 2. Aortic Binding Sites

<table>
<thead>
<tr>
<th>Group</th>
<th>$B_{max}$ (fmol/mg protein)</th>
<th>$K_d$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2K control</td>
<td>40.0 ± 11.1</td>
<td>0.47 ± 0.16</td>
</tr>
<tr>
<td>Uninephrectomized</td>
<td>37.8 ± 6.6</td>
<td>0.28 ± 0.01</td>
</tr>
<tr>
<td>Uninephrectomized + NaCl</td>
<td>16.7 ± 2.3*</td>
<td>0.12 ± 0.01</td>
</tr>
<tr>
<td>2K + DOCA (n = 3)</td>
<td>14.2 ± 5.8*</td>
<td>0.20 ± 0.09</td>
</tr>
<tr>
<td>DOCA-salt hypertensive (n = 3)</td>
<td>13.2 ± 4.2*</td>
<td>0.25 ± 0.06</td>
</tr>
<tr>
<td>1KIC hypertensive (n = 2)</td>
<td>19.8 ± 3.0*</td>
<td>0.26 ± 0.03</td>
</tr>
<tr>
<td>2K1C hypertensive (n = 5)</td>
<td>30.0 ± 5.0</td>
<td>0.40 ± 0.13</td>
</tr>
</tbody>
</table>

Values are means ± SEM. Number of binding experiments is shown in parentheses. $B_{max}$ = density of binding sites; 2K = two kidney.

*p<0.05, compared with 2K control or uninephrectomized rats.
**FIGURE 2.** Relaxation of norepinephrine (NE)-precontracted rat aorta from control, uninephrectomized (Uni-Nx), uninephrectomized and salt-loaded (Uni-Nx + NaCl), and DOCA-salt hypertensive rats (Uni-Nx + NaCl + DOCA). The dose of NE was submaximal (EC_{50}) and a cumulative response to ANP was obtained. The curves were fitted by constraining the IC_{50} of ANP to be equal and shared by the four curves, by pairs of curves to be compared, or by none. The latter fit provided the lowest sum of squares of residuals, indicating that the IC_{50} of the Uni-Nx + NaCl and the DOCA-salt hypertensive rats was significantly different from both one-kidney and two-kidney controls (F = 83.5 for DOCA-salt hypertensive rats; F = 37.3 for Uni-Nx + NaCl; p < 0.001 compared with one-kidney controls). The calculated IC_{50} was also treated according to the model of functional antagonism of van den Brink,\(^9\) using the EC_{50} of NE and the concentration of NE used (EC_{90}) in the equation of Cheng and Prusoff,\(^{20}\) to obtain the apparent K_{a} of ANP. The apparent pK_{a} (\(-\log \text{apparent } K_{a} \text{ in M}\)) for the DOCA-salt hypertensive rats was significantly different from that of one-kidney controls (p < 0.01).

3). Interestingly, a similar increase in plasma ANP levels was present in 1K1C hypertensive rats (see Table 3), in which we previously found a decreased density of vascular ANP receptors.\(^9\) The 2K1C hypertensive rats also had a higher concentration of ANP than controls (see Table 3) but significantly lower than the two other hypertensive models. The decrease in the mean density of binding sites for ANP in the vasculature of the different groups of rats in this study and previous studies\(^9,^{10}\) was inversely proportional to the mean concentration of plasma ANP in each group (r = -0.67, n = 7 groups, p < 0.05).

**In Vivo Response to ANP Infusion in DOCA-Salt and 2K1C Goldblatt Hypertensive Rats**

The decreased density of vascular ANP binding sites and the decrement in the relaxation responses of aorta to ANP in DOCA-salt hypertensive rats suggested that DOCA-salt hypertensive rats infused with ANP should be resistant to the hypotensive effect of the peptide. ANP was therefore infused intravenously with osmotic minipumps implanted subcutaneously, at a dose of 100 ng/hr, which has previously been shown to lower blood pressure of 2K1C hypertensive rats.\(^{12}\) This dose inexplicably but repeatedly did not result in a significant increment in the plasma concentration of ANP in either 2K1C or DOCA-salt hypertensive rats compared with sham-infused hypertensive control rats after 4 days (results not shown), although in normal rats plasma levels of ANP did rise from 16.1 ± 1.7 to 29.6 ± 4.5 fmol/ml (n = 6, p < 0.05). When ANP was infused intravenously at a dose of 100 ng/hr into 2K1C

### Table 3. Plasma ANP in Hypertensive Rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Plasma ANP (fmol/ml)</th>
<th>Blood pressure (mm Hg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2K control (n = 6)</td>
<td>14 ± 3 (6-22)</td>
<td>107 ± 4</td>
</tr>
<tr>
<td>Uninephrectomized (n = 6)</td>
<td>10 ± 1 (7-15)</td>
<td>101 ± 4</td>
</tr>
<tr>
<td>2K1C hypertensive (n = 5)</td>
<td>41 ± 8* (21-66)</td>
<td>171 ± 8*</td>
</tr>
<tr>
<td>1K1C hypertensive (n = 6)</td>
<td>70 ± 7* (39-86)</td>
<td>213 ± 4†</td>
</tr>
<tr>
<td>DOCA-salt hypertensive (n = 5)</td>
<td>68 ± 8 (47-104)</td>
<td>162 ± 2</td>
</tr>
</tbody>
</table>

Values are means ± SEM. Range of values is shown in parentheses. 2K = two kidney.

\(^{*}p<0.01\), compared with 2K control values.

\(^{†}p<0.05\), compared with values for 2K1C hypertensive rats.
hypertensive rats, blood pressure decreased significantly \( (p<0.01, \text{analysis of variance for repeated measures; Figure 4}) \), as expected from previous studies. In DOCA-salt hypertensive rats infused similarly at the same time, blood pressure remained elevated. Interestingly, in DOCA-salt hypertensive rats in which blood pressure did not decrease, there was a tendency to a natriuretic response to the ANP infusion. In the 2K1C hypertensive rats in which blood pressure was reduced by ANP, there was no natriuretic response to ANP.

To further investigate the effects of ANP on blood pressure and natriuresis in DOCA-salt hypertension, an additional group of DOCA-salt hypertensive rats was sham-infused or infused with ANP at 100 or 300 ng/hr for 4 days (results not shown). Blood pressure failed to decrease in the ANP-infused rats at either dose, although natriuresis on Day 4 showed a tendency to increase from 19.6 ± 1.9 mEq/day in sham-infused DOCA-salt hypertensive rats to 25.7 ± 3.4 mEq/day after intravenous infusion of ANP, 100 ng/hr, and to 27.9 ± 4.3 mEq/day after ANP, 300 ng/hr. Plasma levels of ANP tended to rise at the higher dose of 300 ng/hr (from 53 ± 10 to 88 ± 30 fmol/ml) versus control DOCA-salt hypertensive rats, but the difference did not reach statistical significance because of the wide scatter of values in the ANP-infused group.

**Discussion**

Our study shows that the density of vascular ANP binding sites is reduced and aortic responsiveness to ANP is decreased in DOCA-salt hypertensive rats, a model of renin-suppressed, volume-expanded hypertension. These findings were associated with an elevated plasma concentration of ANP, which has also been reported in DOCA-salt hypertensive rats in a recent study published after ours was completed. In these DOCA-salt hypertensive rats, the intravenous infusion of ANP at two doses (100 and 300 ng/hr) did not result in a lowering of blood pressure. In contrast to DOCA-salt hypertensive rats, 2K1C hypertensive rats responded to the lower (100 ng/hr) dose of ANP with a lowering of blood pressure despite the absence of a natriuretic response, results that were similar to those obtained by Garcia et al. In these rats ANP binding sites in vessels tend to be lower but the decrease is not statistically significant. In the 2K1C hypertensive rats plasma levels of ANP were elevated but to a lesser degree than that in DOCA-salt hypertensive rats or 1K1C hypertensive rats (see Table 3), another model in which we have previously demonstrated significant decrement in the density of ANP vascular receptors. This finding suggests an inverse relationship between the density of vascular ANP binding sites and the plasma concentration of ANP in rats.

The differences in plasma concentration of ANP were not related to the level of high blood pressure. Hypertension was more severe in 1K1C hypertensive rats than in DOCA-salt or 2K1C hypertensive rats. The differences in plasma concentration of ANP in hypertensive rats may be related to the degree of volume expansion, which is greater in DOCA-salt and 1K1C hypertensive rats than in 2K1C hypertensive rats. Volume expansion is a potent stimulus for the secretion of ANP. Since ANP has been shown to down-regulate ANP binding sites and cyclic guanosine 3',5'-monophosphate responses to ANP in cultured vascular smooth muscle cells, the high levels of plasma ANP may be the cause of the decrease in density of ANP vascular receptors. As shown in this and previous studies, the decrement in the number of ANP receptors may result in a decreased vascular relaxation response, which consequently may produce a diminished hypertensive effect of ANP in DOCA-salt hypertensive rats and in 1K1C hypertensive rats, in contrast to 2K1C hypertensive rats. In contrast to our results showing no lowering of blood pressure with ANP in DOCA-salt hypertensive rats and its effectiveness in 2K1C hypertensive rats, Seymour et al. found that ANP lowers blood pressure more effectively in DOCA-salt than in 2K1C hypertensive rats. However, these were short-term (20-minute) experiments and the dose used was larger, which may explain the differences between that study and the present one.

The sensitivity to ANP infusion in vivo may also be related to renin status. ANP may antagonize the vaso-
constrictor effect of increased concentrations of angiotensin II.21 Indeed, two models of non-renin-dependent hypertension (DOCA-salt and 1K1C) did not respond or responded less to ANP while the model of high renin hypertension (2K1C) did experience a significant lowering of blood pressure with ANP infusion. Thus, it is possible that an effect on peripheral resistance will be exerted mainly in the presence of a high concentration of angiotensin II, such as is found in the 2K1C hypertensive rat. This may represent an added factor to receptor status in the difference in responsiveness to ANP in renin-dependent and non-renin-dependent models of experimental hypertension.

The mechanism whereby ANP lowers blood pressure remains controversial but may be more complex than initially suspected. Lappe et al.22 have found that ANP may exert its hypotensive effect by decreasing cardiac output, presumably by decreasing venous return as a result of ANP-induced venodilation. Arteriolar constriction, probably secondary to a neurohumoral response rather than arterial dilatation, appears to characterize the short-term hemodynamic effects of ANP in both conscious and anesthetized rats. The precise anatomical location of vascular receptors in the mesenteric vascular bed has not been previously demonstrated. In unpublished observations (1986), we have found the density of ANP binding sites to be highest in the main branches of the mesenteric artery (approximately 300 fmol/mg protein), with smaller numbers in secondary branches of the mesenteric artery and even lower density in mesenteric veins (approximately 60 fmol/mg protein). In DOCA-salt hypertensive rats the density of venous ANP receptors is decreased (unpublished results, 1986), which could result in reduced venodilation. On the other hand, a role of larger arterial vessels in the decrease in blood pressure cannot be excluded altogether, since there are few data on the hemodynamic responses during long-term low-dose ANP infusion. Small resistance arterioles, however, have recently been shown to be unresponsive to ANP,29 and this may explain in part the lack of increased mesenteric blood flow found after infusion of ANP.28 Results from different laboratories thus suggest a complex mechanism for the hemodynamic effect of ANP, including dilatation of larger arterial vessels, venodilatation, decreased cardiac output, or decreased blood volume.28,30,31 Our results do not permit us to draw conclusions on any of these possibilities except that an increment in natriuresis, and therefore a decrease in blood volume, does not appear to be involved, since the rats whose blood pressure was more sensitive to ANP did not experience increased natriuresis in this or previous studies,18 while increased natriuresis after ANP appears to characterize responses to ANP in volume-expanded states.13,32

With respect to vascular responses, our receptor preparation may be considered a model of the behavior of vascular ANP receptors, arterial, venous, or perhaps in capillaries, which play a role in the hemodynamic responses to ANP in hypertensive rats.

The role of elevated blood pressure on the decreased density of vascular ANP receptors is unclear. In a previous study2 vascular ANP sites in 2K1C hypertensive rats tended to decrease but not significantly, similarly to the data in aorta in Table 2, in spite of the significant elevation of plasma ANP in this experimental model. In uninephrectomized salt-loaded rats and in DOCA-infused rats,19 all of which are normotensive volume-expanded models, the increment in plasma ANP is smaller than that in 2K1C hypertensive rats, but a significant reduction in density of vascular ANP sites occurs (to 35-50% of control values). If normotensive volume-expanded rats are eliminated, the inverse correlation between receptor density and plasma ANP becomes highly significant (r = -0.95, n = 5 groups, p < 0.005). The slope of the regression is significantly less acute than that for normotensive rats. This result suggests that there may be a certain resistance to the down-regulating effect of ANP on ANP vascular receptors in hypertensive rats.

We have studied ANP binding sites in the mesenteric vascular bed,5,29 as a model of vascular ANP receptors, and relaxation responses to ANP of NE-precontracted aorta, as a model for biological responses to ANP, in order to investigate both phenomena in the same rats. We have recently demonstrated that the order of potency of the synthetic 28- and 26-amino acid ANP, as well as that of atriopeptin I, II, and III, is similar in the radioligand binding assay on membranes of rat mesenteric vessels and aorta (E. L. Schiffrin and J. St-Louis, unpublished observations, 1986). Furthermore, the potency of these five ANPs is similar for relaxing phenylephrine-precontracted mesenteric artery rings and aortic strips (unpublished observations, 1986). Finally, preliminary results (see Table 2 and unpublished results, 1986) show that aortic ANP binding sites are also decreased by sodium loading and mineralocorticoids and in 1K1C or DOCA-salt hypertension, as we have previously shown for the mesenteric vasculature.5,10 As shown in this study and previous studies,5,10 the correlation between change in density of ANP sites in mesenteric vessels and aorta was very close (r = 0.95). This finding indicates that the molecular requirements for ANP binding and biological effects as well as the regulation of these sites are similar on mesenteric vessels and aorta in the rat. Thus, the investigation of changes in ANP binding sites in the mesenteric vasculature and relaxation responses to ANP in the aorta appears justified to obtain binding and biological data in the same rats.

True down-regulation of receptors has to be distinguished from apparent down-regulation by prior receptor occupancy. The mean plasma concentration of ANP in DOCA-salt hypertensive rats was 70 pm and the ANP concentration of ANP a small percentage (<20%) of receptors is occupied by ANP. Thus, a 70% decrease in density of ANP binding sites would not be expected to occur at these plasma concentrations of ANP because of retention of circulating ANP on receptors (prior receptor occupancy). Since the true affinity may be greater in vivo, as shown in cultured vascular smooth muscle cells, the possibility of prior receptor occupancy cannot be eliminated. Half-life of dissociation of ANP is 69 minutes in this
preparation. The procedure leading to preparation of the membranes and the binding experiment takes several hours; thus, it is unlikely that significant amounts of retained peptide are still present when the binding experiment is performed. Furthermore, in cultured vascular smooth muscle cells, we have previously found that exposure to ANP results in decreased binding sites. In that study it was shown that prior receptor occupancy did not play a role in the decrease of density of ANP sites found. In another study, human platelet ANP binding sites were decreased by sodium loading, which resulted in higher ANP concentrations. That study also demonstrated that prior receptor occupancy was not responsible for the decrease in density of ANP binding sites. Both tissues have higher affinity than that found in vascular tissue in this report; as a result, retention of circulating peptide would be expected to play a more important role in the decrease in ANP receptors in platelets or cultured vascular smooth muscle cells than in the rat blood vessels in this study. Moreover, in 2K1C hypertensive rats, the ANP concentration in plasma is elevated (as shown in this study) but ANP vascular sites are not significantly down-regulated. Thus, in this instance prior receptor occupancy is not involved. There is no apparent reason for prior receptor occupancy to occur in DOCA-salt hypertensive rats but not in 2K1C Goldblatt hypertensive rats. For all these reasons prior occupation of receptors by increased circulating ANP appears to be an improbable explanation for the decrease in density of ANP sites in DOCA-salt hypertensive rats.

In this and previous studies, we showed that, when the density of vascular ANP binding sites is reduced, the sensitivity of vascular relaxation responses to ANP is decreased (increase in IC50) without a change in the maximal response to ANP. For relaxation studies we chose a NE concentration equal to that producing 90% of the maximal contraction (EC50). As a result the IC50 for ANP was in the nanomolar range. In unpublished studies we have observed, on both mesenteric arteries and aortic strips, that if an EC50 of NE or phenylephrine is used instead of an EC50, the IC50 of ANP falls to approximately 30 to 50 pM. Lower concentrations of vasoconstrictor may not be used since the response will be too small and variable to adequately assess the relaxation response to ANP. This finding indicates that the affinity (Kd) of ANP for relaxation responses is actually to the left of the affinity (Ki) of ANP binding sites. This phenomenon may be explained by hypothesizing that there are "spare" ANP receptors. The presence of putative "spare" receptors for ANP in vessels would explain why decreases in density of ANP binding sites will determine a decrement in the sensitivity to ANP (greater EC50) and not a reduction in the efficacy of the response (lower maximal response).

The doses of ANP used in this study and infused intravenously for 4 days did not result in higher concentrations of ANP in plasma in either 2K1C or DOCA-salt hypertensive rats, although a significant increment could be detected in normal rats. At the dose of 300 ng/hr the mean plasma levels of ANP were higher, but this difference did not achieve statistical significance due to the wide scatter of values in ANP-infused rats. That ANP was effectively infused is shown 1) by the lowering of blood pressure in 2K1C hypertensive rats, similar to previous reports, 2) by the tendency to an increased natriuresis in ANP-infused DOCA-salt hypertensive rats (although it did not achieve statistical significance), and 3) by the verified emptying of the osmotic minipumps. The inability to detect an effective increase in the plasma concentration of ANP may have been due to the variability of the already high levels of ANP in hypertensive rats, since a small increase was found in normal rats. The mechanism of blood pressure lowering in absence of a detectable increase in plasma ANP remains to be determined but is highly reproducible in 2K1C hypertensive rats, as shown in this and previous studies.

In conclusion, we have demonstrated that a decreased density of ANP receptors in mesenteric vessels and aorta is associated with high ANP plasma concentrations in DOCA-salt hypertensive rats. These ANP sites may be a model of the behavior of ANP receptors elsewhere in the vasculature. A decreased sensitivity of the vasculature in vitro to the relaxing effect of ANP may result from the reduced number of ANP receptors. A decrement in the in vivo responsiveness to the blood pressure-lowering effect of ANP in part may be a consequence of the reduced potency of ANP-induced vasorelaxation, although the exact mechanism for the lowering of blood pressure by ANP remains uncertain. Variable down-regulation of ANP receptors may determine the degree of responsiveness of different experimental hypertensive models to ANP. Finally, although the precise vascular site of action of ANP is undetermined (arterial or possibly venous), the vascular actions of ANP appear to be more important than its natriuretic effects, in the absence of hypotensive effect of ANP in volume-expanded experimental models of hypertension such as the DOCA-salt hypertensive rat.

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