Atrial Natriuretic Factor-Specific Antibody As a Tool for Physiological Studies

Evidence for Role of Atrial Natriuretic Factor in Aldosterone and Renal Electrolyte Regulation

M. Audrey Rudd, Standford Plavin, Alan T. Hirsch, Julie R. Ingelfinger, and Victor J. Dzau

Numerous studies have shown that administration of atrial natriuretic factor (ANF) increases urinary sodium excretion and urine flow, decreases blood pressure, and inhibits renin and aldosterone release. However, the role of endogenous ANF in the regulation of renal sodium excretion, blood pressure, plasma renin activity, and aldosterone level remains to be elucidated. To examine this issue, endogenous ANF was blocked by administering rat ANF-(99-126) specific antiserum (Ab) to anesthetized rats (n=7). Control animals received either no injection (time controls, n=10) or preimmune serum (n=8). Blockade of endogenous ANF caused a 28±0.09%, 47±0.08%, and 51±0.08% fall in sodium excretion at 15, 30, and 45 minutes after Ab injection (p<0.05, p<0.01, p<0.01, respectively). Urine flow fell 35±7% at 45 minutes after ANF inhibition (p<0.05). Plasma ANF levels were suppressed to undetectable levels. However, there were no changes in blood pressure throughout the experiment nor plasma renin concentration when measured at 45 minutes after Ab injection. Interestingly, plasma aldosterone concentration increased significantly (by approximately 50%, p<0.025), in response to Ab. Completeness of blockade was demonstrated by the absence of sodium excretion response to exogenous ANF (500 ng). In either the time control or the preimmune serum group, urinary excretion, blood pressure, plasma ANF, plasma renin concentration, and plasma aldosterone concentration were unchanged throughout the experiment. In contrast to the Ab group, a challenge with exogenous ANF (500 ng) increased sodium excretion by 2.17 μeq/min in the preimmune serum group. These data suggest that endogenous ANF contributes to the regulation of urinary sodium excretion and plasma aldosterone concentration in the anesthetized rat. However, endogenous ANF does not appear to contribute to the regulation of blood pressure or plasma renin concentration. (Circulation Research 1989;65:1324-1329)

Since its discovery, the effect of atrial natriuretic factor (ANF) on volume regulation has been extensively studied. Administration of ANF can produce a dramatic increase in urinary sodium excretion (U_{Na,V}) and urine flow. Administration of ANF can produce a dramatic increase in urinary sodium excretion (U_{Na,V}) and urine flow.1-3 Increasing endogenous plasma levels of ANF by volume expansion or sodium loading has been shown to be associated with natriuresis and diuresis.4,5 Acute volume expansion increases plasma ANF levels and induces natriuresis, which can be attenuated or abolished by atrial appendectomy.6 Similarly, chronic sodium loading increases plasma ANF level, a phenomenon that precedes the natriuresis associated with this condition.3 The administration of ANF can also result in a decrease in systemic blood pressure and increases in renal blood flow and glomerular filtration rate.1,2,7 Continuous infusion with high doses of ANF reduces plasma renin concentration (PRC).8 However, infusion of lower doses that result in plasma ANF levels within the physiological range does not appear to influence PRC.10,11 In vitro studies using kidney slices have generally demonstrated either no effect or inhibition of renin release.12,13 Yet, Franco-Saenz et al4 recently reported stimulation of renin release from kidney slices by ANF. Finally, in vivo and in vitro experiments have shown that ANF inhibits aldosterone.
sterone release from the adrenal cortex. It is unclear whether this is a physiological or pharmacological effect.

Because most studies examined the responses to exogenous ANF or studied the association of renal sodium excretion to increased plasma ANF levels, there is a need to document the contribution of endogenous ANF to the physiological regulation of urinary excretion, blood pressure, and plasma renin by blocking ANF action. Since a pharmacological antagonist to ANF is not yet available, we used ANF-specific antibody as an inhibitor to examine this issue in the anesthetized rat.

Materials and Methods

Antibody Preparation

Antibodies were produced by use of the procedure previously described. Rat ANF-(99-126) was conjugated with thyroglobulin by the carbodiimide method. After dialysis, the ANF-protein conjugate was emulsified with 3 vol complete Freund’s adjuvant, and 2 ml was injected subcutaneously into New Zealand White rabbits. Booster injections (2 ml) consisting of ANF conjugate and incomplete Freund’s adjuvant were given 4-6 weeks apart. Approximately 4-5 weeks after each booster injection, blood was drawn via the central ear artery and spun, and serum was obtained for antibody screening and titering.

The antiserum (#413851) has a titer of 1:30,000 against rat ANF-(99-126) and cross-reacts with rat ANF-(103-123) (74%), rat ANF-(103-125) (75%), rat ANF-(103-126) (63%), and rat ANF-(92-126) (100%) as published previously. It does not cross-react with rat ANF-(99-109), rat ANF-(116-126), or human ANF-(99-126). It is highly specific for ANF and does not recognize bradykinin, angiotensin II, arginine vasopressin, renin, hemoglobin, or albumin.

Animal Studies

Surgical preparation. Male Sprague-Dawley rats (12-16 weeks old) were purchased from Charles River Breeding Laboratory (Wilmington, Massachusetts) and were fed regular Purina Lab Chow. They were allowed free access to food and water the night before the experiment. Animals were anesthetized with sodium pentobarbital (50 mg/kg i.p.). Once anesthesia was induced, a tracheostomy was performed to maintain a patent airway. The femoral artery was cannulated with polyethylene tubing (PE-50) for blood pressure measurement. Cannulae were placed in the femoral vein for infusion of fluids (PE-50) and injection (PE-10). The bladder was catheterized for urine collection. A blood sample was taken at this point for hematocrit measurement before receiving the infusion. To maintain anesthesia, saline containing pentobarbital (4 ug/min) was infused at a rate of 20 μl/min. Approximately 1 hour was allowed for stabilization before urine collections were begun.

Radiolmmunoassay. Plasma ANF levels were measured by direct radioimmunoassay (RIA) by the method as described. The interassay and intrassay coefficients of variation of this assay are 10% for both. We attempted initially to measure ANF in plasma after Sep-Pak extraction as described. Unfortunately, we found that this method could not be used successfully for the determination of circulating free ANF in the samples from rats that received anti-ANF antibody. Because in these samples plasma ANF is completely bound by the injected antibody, the plasma level of free ANF is undetectable by direct RIA. However, after Sep-Pak extraction of these plasma, ANF dissociated from the antibody in vitro, and the antibody was retained by the column while dissociated ANF fell through. Therefore, RIA of the eluate provided a measurement for total ANF and yielded a misleading value for the in vivo level.

Protocol. Animals were divided into three groups: antisera (Ab, n=7), preimmune serum (PRE, n=8), and time control (TC, n=10) (see Figure 1 for diagram of protocol). Once the animals were stable, three 15-minute control urine collections (Ci, Cz, and C3) were taken. At this point, a blood sample (0.7 ml) (P1) was taken and replaced with an equal volume of saline. Care was taken during the withdrawal and infusion not to cause any changes in blood pressure or heart rate. Injections of either ANF Ab (0.2 ml) or control PRE (0.2 ml) were given at this time. After the serum injection, three additional 15-minute urine collections (E1, E2, and E3) were taken. After this, another 0.7 ml blood sample (P2) was drawn and was followed by an equivolume saline replacement. To test the effectiveness of Ab, a challenge of exogenous ANF (500 ng) was given to the PRE and Ab groups. This was followed by three additional 15-minute urine collections (E4, E5, and E6) urine collections. A final blood sample (P3) was taken 5 minutes after ANF challenge. Animals in the TC group did not receive injections at any time and were used to demonstrate stability of the animal preparation.

Figure 1. Diagram of treatment protocol. After surgery (S), animals were allowed to recover (R) before starting control collections (Ci, Cz, C3). After injection of preimmune serum (PRE) or atrial natriuretic factor (ANF) antiserum (Ab; AB in figure), three additional urine collections (E1, E2, E3) were taken. E4, E5, and E6 represent urine samples taken after an exogenous ANF challenge in both PRE and Ab groups. Blood samples were taken after the control collections (P1), 45 minutes after Ab or PRE (P2), and 5 minutes after ANF challenge (P3). Although blood sampling was done in the time control group, they did not receive any injections.
of free ANF in plasma. For this reason, we resort to direct RIA in this study.

**Plasma renin concentration.** Plasma collected in chilled EDTA tubes was mixed with sheep anephric plasma as a source of substrate. This plasma had been shown previously to be devoid of renin activity. This mixture was then assayed for renin activity by incubation at 37°C for 1 hour, and subsequent RIA was performed for angiotensin I generation as described.21

**Plasma aldosterone concentration.** Aldosterone in unextracted plasma was measured with a solid phase RIA kit (Coat-A-Count, Diagnostics Products, Los Angeles, California). The sensitivity of the assay was from 2.5 to 120 ng/dl.

**Urine analysis.** Urine samples were analyzed for Na⁺ and K⁺ by use of a flame photometer (model IL 450, Instrumentation Laboratory, Lexington, Massachusetts).

**Statistical Analysis**

All the time-course data were first subjected to ANOVA and subsequently to the Newman-Keuls multiple-comparison test. Analysis among groups was done by ANOVA and Duncan's multiple-comparisons test. All values are mean±SEM.

**Results**

There were no differences in basal urinary excretion, blood pressure, and heart rate (Table 1), or plasma ANF, PRC, and plasma aldosterone concentration (PAC) among the three groups (Table 2).

**Urinary Excretion**

As shown in Figure 2, U₄NaV did not change throughout the experiment in the TC group. Injection of PRE did not influence U₄NaV, potassium excretion, or urine flow. In contrast, after ANF Ab administration, U₄NaV decreased from a baseline of 0.61 μeq/min to 0.42, 0.32, and 0.28 μeq/min at 15, 30, and 45 (E₁, E₂, and E₃) minutes after Ab injection (p<0.05, p<0.01, and p<0.01, respectively). Urinary potassium excretion did not change. Urine flow (Table 1) fell during E₃ from 14.5 μl/min to 9.43 μl/min, (p<0.05, p<0.01, and p<0.01, respectively).

**Blood Pressure, Heart Rate, and Hematocrit**

ANF blockade did not produce any measurable change in blood pressure or heart rate during the entire study period (Table 1). Similarly, there were no changes in blood pressure in either the PRE or the TC groups. In selective animals, we measured hematocrit from the blood samples taken at the control period (P₀) and 45 minutes after Ab or PRE injection (P₀). Hematocrit did not change in any of the groups between P₀ and P₃ [PRE, 44.4% vs. 44.8% (n=3); Ab, 45.5% vs. 44.8% (n=2), respectively].

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**Table 1. Urinary Excretion and Blood Pressure Response After Preimmune and Atrial Natriuretic Factor Antiserum**

<table>
<thead>
<tr>
<th></th>
<th>MC</th>
<th>E₁</th>
<th>E₂</th>
<th>E₃</th>
<th>E₄</th>
<th>E₅</th>
<th>E₆</th>
</tr>
</thead>
<tbody>
<tr>
<td>U₄NaV (μeq/min)</td>
<td>0.78±0.19</td>
<td>0.78±0.15</td>
<td>0.77±0.13</td>
<td>0.81±0.14</td>
<td>0.64±0.14</td>
<td>0.68±0.15</td>
<td>0.62±0.12</td>
</tr>
<tr>
<td>V (μl/min)</td>
<td>16.9±2.4</td>
<td>15.8±2.1</td>
<td>15.7±2.7</td>
<td>15.2±1.7</td>
<td>40.7±11.1*</td>
<td>14.8±4.2*</td>
<td>17.4±4.0</td>
</tr>
<tr>
<td>MAP (mm Hg)</td>
<td>128±4</td>
<td>130±7</td>
<td>131±6</td>
<td>130±6</td>
<td>129±6</td>
<td>128±7</td>
<td>130±8</td>
</tr>
<tr>
<td>HR (beats/min)</td>
<td>405±13</td>
<td>416±22</td>
<td>413±17</td>
<td>424±17</td>
<td>424±15</td>
<td>416±17</td>
<td>416±17</td>
</tr>
</tbody>
</table>

Values are mean±SEM for time control (TC) group (n=10), preimmune serum (PRE) group (n=8), and atrial natriuretic factor (ANF) antiserum (Ab) group (n=7). MC, baseline control urine collections; E₁, E₂, and E₃, urine collections after injection of PRE or ANF Ab; E₄, E₅, and E₆, urine collections after an exogenous ANF challenge; U₄NaV, urine sodium excretion rate; V, urine flow rate; U₄V, urinary potassium excretion; MAP, mean arterial pressure; HR, heart rate.

tp<0.05 vs. MC.

tp<0.01 vs. MC.
### TABLE 2. Plasma Hormones Before and After Preimmune and Atrial Natriuretic Factor Antiserum

<table>
<thead>
<tr>
<th></th>
<th>TC (n=9)</th>
<th>P1</th>
<th>P2</th>
<th>P3</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC (n=9)</td>
<td>376±30</td>
<td>362±34</td>
<td>381±30</td>
<td></td>
</tr>
<tr>
<td>ANF (pg/ml)</td>
<td>353±25</td>
<td>353±86</td>
<td>794±134*</td>
<td></td>
</tr>
<tr>
<td>PRC (ng/ml/hr)</td>
<td>Ab (n=7)</td>
<td>264±30</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>PRC (ng/ml/hr)</td>
<td>Ab (n=7)</td>
<td>17.3±5.4</td>
<td>14.4±5.3</td>
<td>9.4±2.4*</td>
</tr>
<tr>
<td>PAC (ng/dl)</td>
<td>PRE (n=7)</td>
<td>12.9±4.2</td>
<td>11.2±3.7</td>
<td>15.2±5.9*</td>
</tr>
<tr>
<td>PAC (ng/dl)</td>
<td>Ab (n=7)</td>
<td>14.8±2.9</td>
<td>13.6±2.3</td>
<td>15.0±2.1</td>
</tr>
<tr>
<td>PAC (ng/dl)</td>
<td>PRE (n=7)</td>
<td>18.0±5.8</td>
<td>9.9±5.6</td>
<td>15.6±4.0</td>
</tr>
<tr>
<td>PAC (ng/dl)</td>
<td>Ab (n=4)</td>
<td>15.3±7.8</td>
<td>22.3±8.4*</td>
<td>22.8±5.6*</td>
</tr>
</tbody>
</table>

Values are mean±SEM for time control (TC), preimmune serum (PRE), and antiserum (Ab) groups. P1, blood samples after control collections; P2, blood samples 45 minutes after Ab or PRE; P3, blood samples after atrial natriuretic factor (ANF) challenge; PRC, plasma renin concentration; PAC, plasma aldosterone concentration; ND, not detectable. *p<0.05 vs. P1.

### Plasma Hormonal Levels

Baseline ANF values for the Ab group was 264 pg/ml and fell to below detectable levels (<80 pg/ml, unextracted assay) 45 minutes after Ab administration (see Table 2 for these and following values). Basal values in the PRE and TC groups were 353 and 376 pg/ml, respectively, and remained unchanged in both groups during this same time period.

PRC was measured at 45 minutes after Ab or PRE injection. This time point was estimated to correspond to the peak of Ab action as we have described previously.22,23 At this point, plasma ANF level was undetectable. PRC was unchanged in both groups of animals; it remained at 14±3 and 13±4 ng angiotensin I/ml/hr in the Ab and PRE groups, respectively (p=NS). Similarly, PRC of the TC animals was unaltered during this period.

PAC was 15.3±7.8 ng/dl at baseline but increased to 22.3±8.4 ng/dl (p<0.025) at 45 minutes after Ab injection as plasma ANF fell to an undetectable level. Basal PAC in the PRE group was 18.0±5.8 ng/dl and did not change significantly after PRE administration (9.9±5.6 ng/dl, p=NS). PAC did not change significantly (15.6±4.0 ng/dl) during P3 after ANF challenge. The failure of PAC to decrease as plasma ANF increased was probably due to the very short time period (5 minutes) after ANF injection.

### ANF Challenge

To test the effectiveness of the antisem, an exogenous ANF challenge of 500 ng was given 45 minutes after administering the Ab or PRE. Sodium excretion increased 2.19 μeq/min (p<0.05) in the PRE-treated animals (Figure 2). In contrast, the injection of ANF did not increase U_{NaV} in the Ab-treated group. In fact, U_{NaV} continued to fall in the Ab group over the next 45 minutes. Urine flow also increased in the PRE group after ANF (from 75.8 to 40.7 μl/min, p<0.05). During the ANF challenge, plasma ANF concentration remained below detectable levels in animals receiving ANF Ab whereas it rose in the PRE group from 330 to 527 pg/ml.

### Discussion

Blockade of endogenous ANF with Ab significantly reduced U_{NaV}, urine flow, and potassium excretion in the anesthesized Sprague-Dawley rat. This occurred without any measurable changes in blood pressure or PRC. Our data on U_{NaV} support a role of endogenous ANF on renal sodium regulation in the anesthesized rat on a regular sodium intake. Recently, Naruse et al24 and Sasaki et al25 also observed a reduction in U_{NaV} after injection of ANF Ab in anesthesized Wistar and Wistar-Kyoto strains, respectively. However, in both studies the...
fall in UNaV was short-lived, lasting less than 30 minutes. Since antibodies, when given in excess, should have a much longer half-life, one may conclude that the quantities of antibody given by these investigators were quite limited and were readily saturated by the endogenous ANF in the circulation. A second, but less likely possibility, is that the UNaV and blood pressure responses in the previous studies were nonspecific (i.e., mediated by complement activation). Plasma ANF was not measured in the study of Naruse et al. Indeed, neither of these investigators examined the effectiveness of blockade nor the sufficiency of in vivo with an exogenous ANF challenge. In our study, UNaV in the Ab-treated group remained below basal level even after an ANF challenge. Finally, it is possible that the sodium or volume state of the animals differed between the studies. When urinary excretion was used as an index, the animals in the study of Sasaki et al^28 excreted 50% less sodium than our animals whereas the animals in the study of Naruse et al had an urine flow that was greater than that of our animals (ranging from 20–200 μl/min).

Another effect of ANF administration is the inhibition of the renin angiotensin system. Continuous infusion of ANF in doses ranging from 70–100 ng/kg/min has been shown to inhibit renin release. However, lower doses or a single bolus injection have no effect on renin release. Franco-Saenz et al^24 have recently demonstrated an increase in plasma renin activity with continuous infusion of ANF. Studies using kidney slices have also shown mixed results: either no effect or an inhibition of renin release by ANF. In our study, PRC did not change significantly when plasma ANF was completely suppressed. However, Naruse et al^24 found plasma renin activity to rise after ANF blockade and to peak at 45 minutes after injection. The reason for this difference is unclear. The increase in plasma renin activity may be stimulated by the hypotension seen in Naruse's experiment. Blood pressure did not change in our study. The failure to see a rise in PRC in our study did not appear to be due to inadequate ANF blockade since plasma ANF was undetectable and exogenous challenge with a large dose of ANF did not result in an increased plasma ANF or UNaV.

Several groups of investigators have reported that ANF inhibits aldosterone secretion in vivo. This effect appears to be a direct influence of ANF on the adrenal cortex. Our observation that PAC increased in response to ANF blockade without any changes in PRC is consistent with the above reports. The increased PAC seen in our experiments was not due to alterations in potassium balance since urinary potassium concentration did not change. With regards to sodium balance, UNaV fell and should theoretically induce a positive sodium balance and reduce PAC. Thus, the increase in PAC was likely to be due to a blockade of the direct ANF effect on aldosterone release. It is interesting to consider that this increase in PAC may have contributed, in part, to the antinatriuresis seen with ANF blockade in these rats.

Finally, administration of exogenous ANF has been reported to reduce systemic blood pressure. Therefore, we monitored systemic blood pressure and heart rate before and during ANF inhibition. Our data failed to demonstrate an important role of endogenous ANF in blood pressure regulation in the anesthetized rat.

In summary, blockade of endogenous basal ANF results in a reduction of urine flow and UNaV associated with an increase in PAC without any detectable change in PRC or blood pressure. This suggests that ANF may not contribute significantly to basal blood pressure maintenance nor to the basal release of renin in the anesthetized rat. Future studies in the conscious rat and during various pathological states will be important in furthering our understanding of the role of ANF in volume and blood pressure regulation.

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References


Key Words • atrial natriuretic factor • antisera • sodium excretion • plasma renin concentration
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