Effects of Volume Change on Circulating Immunoreactive Atrial Natriuretic Factor in Rats

MASAKAZU KOHNO, KERRY B. CLEGG, AND MOHINDER P. SAMBHI

SUMMARY The mammalian atrial hormone atrial natriuretic factor (ANF) has been shown to have potent natriuretic and diuretic actions as well as vasodilator effects when released into the circulation. To investigate how the levels of the circulating form of this peptide are affected by changes in intravascular fluid volume, we measured immunoreactive ANF in the plasma following acute saline load, acute furosemide treatment, and chronic water restriction. Circulating ANF increased significantly (p < 0.001) 1 minute after acute saline load and remained elevated within 5 minutes. Volume contraction induced by furosemide treatment significantly reduced the circulating immunoreactive ANF. These changes were consistent with the hypothesis that expansion causes an immediate release of immunoreactive ANF into the circulation, whereas volume contraction results in a decline of circulating levels of immunoreactive ANF. These results suggest that ANF is a sensitive indicator of intravascular fluid volume and may participate in the regulation of body fluids and blood pressure.

KEY WORDS • atrial natriuretic factor • chronic water restriction • acute furosemide treatment • acute saline load

INTRAVENOUS injection of rats with extracts of the atria, but not the ventricles, results in pronounced natriuretic and diuretic responses. Mammalian cardiac atria have been shown to contain a mixture of biologically active peptides, atrial natriuretic factors (ANFs), of heterogeneous molecular weights. Recently, the major circulating form of ANF has been characterized as the 28 amino acid α-ANF. All of the mechanisms controlling the secretion of ANF have yet to be determined. Mechanical distention of the left atrium, by obstructing the mitral orifice, has been shown to result in an increase in urine flow and sodium excretion. Similarly, an increase in perfusion pressure on isolated hearts resulted in an increased release of immunoreactive ANF (irANF) into the perfusate, and a positive correlation of ANF release with right atrial and pulmonary wedge pressure in humans has been reported. These data imply that ANF may be secreted from cardiac atria by stimulation of atrial stretch or baroreceptors and may play a role in the regulation of body fluids and blood pressure. Therefore, we investigated whether the circulating levels of ANF change with expansion as well as contraction of intravascular fluid volume. The circulating concentration of irANF in Wistar rats following acute saline load, acute furosemide administration, and chronic water restriction was quantitated by radioimmunoassay.

Materials and Methods

Determination of Immunoreactive ANF and Plasma Renin Activity

Immunoreactive ANF was extracted from 2 ml of plasma by binding to Sep-Pak C-18 cartridges (Millipore-Waters, Milford, MA, USA) using the procedure of Lang et al. The extracted peptides were dried by vacuum centrifugation (Savant, Hicksville, NY, USA) and dissolved in the assay buffer (0.01 M sodium phosphate, pH 7.4, 0.05 M NaCl, 0.1% bovine serum albumin).
albumin, 0.1% Nonidet P40, 0.01% NaCl). The recovery of ANF by extraction was calculated by adding known quantities of α-rat ANF (1-28), 100 to 200 pg/ml, to plasma previously treated with dextran-coated charcoal to remove endogenous ANF. Recovery was 63.9 ± 3.6%.

Radioimmunoassays were performed using antibody and [125I]ANF from Peninsula Laboratories (Belmont, CA, USA) directed against α-human ANF added to 100 µl of extract or plasma as previously described. Standard curves were prepared with 100 µl of α-rat ANF, 50 to 5000 pg/ml, in assay buffer. At the end of the incubation, 100 µl of each of normal rabbit serum diluted 1:50 in assay buffer and goat anti-rabbit IgG serum diluted 1:10 in assay buffer were added and allowed to precipitate for 24 hours. The samples were diluted with an additional 0.5 ml of assay buffer, and the precipitate was collected by centrifugation at 1700 g for 30 minutes. The unbound counts were aspirated, and the precipitates counted in a Micromedic gamma counter (Horsham, PA, USA). The effective range of the standard curve was between 5 and 200 pg of ANF, and 50% displacement was obtained with 22 pg of ANF. The interassay variation was 13.5% (n = 10), and the intra-assay variation was 5.0% (n = 20). Plasma renin activity (PRA) was measured by procedures previously reported.

All assays were performed in duplicate, and statistical significance was determined by paired and unpaired Student's t tests.

Acute Saline Load

Twenty male Wistar rats (weight, 300–400 g; Simonsen Labs, Gilroy, CA, USA) that had been maintained on ad libitum food and water were divided into four groups. The rats were anesthetized intraperitoneally with sodium pentobarbital (6 mg/100 g), and the left jugular vein and left carotid artery cannulated with polyethylene tubing (PE-50, Clay Adams, Parsippany, NJ, USA). The trachea was also cannulated with a catheter (PE-240). Thirty minutes after operation, a 5-ml sample of blood was withdrawn from the carotid artery of Control Group 1 (n = 5). The remaining animals were infused with isotonic saline, 2 ml/100 g, over a period of 15 seconds into the jugular vein. After infusion, 5-ml blood samples were withdrawn at 1 minute in Group 2 (n = 5); 3 minutes in Group 3 (n = 5), and 5 minutes in Group 4 (n = 5). An additional 20 animals underwent sham operation and samples were taken as described.

All samples were immediately placed into ice-cold tubes containing 10 µl of aprotinin (250 KIU; Sigma Chemical, St. Louis, MO, USA) and heparin, 10 IU/ml of blood. Plasma was separated by a 10-minute centrifugation at 1700 g, 4°C and immediately frozen at −20°C for subsequent radioimmunoassay of ANF. Assays were performed within 24 hours of collecting the samples, and prolonged storage was avoided.

Acute Furosemide Administration

Furosemide (1 mg/100 g) was administered intraperitoneally to seven male Wistar rats (weight, 300–400 g), and the rats were placed in metabolic cages. After 5 hours, the rats were removed from the cages and weighed and the urine volume was measured. The animals were immediately killed by decapitation, and the blood collected as described. Circulating irANF, PRA, and hematocrit were measured. Blood samples from seven control rats (weight, 300–400 g) without furosemide administration were collected and assayed in parallel.

Chronic Water Restriction

Female Wistar rats (weight, 210–300 g) were maintained on a normal sodium diet. The rats were randomly assigned to a water-restricted group (n = 9) or a normal control group (n = 7) given free access to water. The water-restricted group was allowed a total water intake of 1 ml/100 g body weight for 4 days and then no water for 24 hours before death. The rats were weighed at the beginning and end of the experiment. The rats were placed in a metabolic cage during the last 24 hours. Urine was collected, and the volume was measured. The rats were killed by decapitation, and the blood was collected. Plasma was prepared as already described for radioimmunoassay of ANF and PRA.

Results

Acute Saline Load

Table 1 shows the concentration of circulating irANF in groups of rats before and 1, 3, and 5 minutes after saline load. The irANF was measured after extraction of the plasma with C-18 silica. The concentration of irANF at 1 minute was significantly higher compared with control level (p<0.001). Although circulating irANF declined at 3 minutes compared with that at 1 minute (p<0.02), it was still significantly higher than the control level (p<0.05). Five minutes after infusion, the concentration of irANF (115 ± 18 pg/ml) approached the basal level (92 ± 13 pg/ml). In sham-operated rats the circulating level of irANF did not vary significantly over the course of the experiment and remained near the control level, thus demonstrating that the withdrawal-replacement sampling technique did not severely alter the ANF level.

Volume Depletion by Furosemide

Table 2 shows the effect of furosemide administration on body weight, urine volume, PRA, and hematocrit, and plasma irANF. The irANF concentration was decreased significantly (p<0.005) from the control level, while the PRA and hematocrit were significantly increased (p<0.05). Initially, the average body weight of the control and experimental animals was identical. However, 5 hours after furosemide treatment, body weight significantly declined (p<0.01) and was accompanied by increased urine volume.

Chronic Water Restriction

Table 3 shows the body weight, 24-hour urine volume/100 g body weight, PRA, and concentration of irANF in control animals (n = 7) and animals with
chronic water restriction (n = 9). The circulating irANF concentration was significantly greater in the control animals than in the water-restricted group (p < 0.001) for both extracted and unextracted plasma. Initially, the average body weight of the control and experimental rats was identical. However, severe water restriction significantly reduced the body weights of the experimental group (p < 0.001). As expected, the 24-hour urine volume was reduced in the volume-depleted group (p < 0.001) and the PRA was significantly elevated (p < 0.001) as compared with the control group.

**Effect of Decapitation and Anesthesia on Immunoreactive ANF**

Figure 1 shows the effect of the different methods of collecting blood samples on the basal level of extracted irANF measured in control rats. The control level of irANF in plasma prepared from blood collected following decapitation was significantly higher (p < 0.001) than that in plasma obtained from carotid artery cannulation with the rats under pentobarbital anesthesia. The plasma level of irANF in another group of control animals, anesthetized with pentobarbital 30 minutes before decapitation, was intermediate between the values found with decapitation or anesthesia alone.

**Discussion**

Mammalian cardiac atria contain biologically active peptides, ANFs, that are capable of producing diuresis, natriuresis, and vasodilation. The amino acid sequence of ANF peptides has been determined, peptides that mimic the actions of endogenous factors have been synthesized, and the structure and sequence of the ANF gene have been determined. Further, ANF has been shown to be synthesized by cardiocytes in vitro and secreted as the high molecular weight 126 amino acid proANF, which is subsequently converted to a 28 amino acid circulating form of these peptides in the presence of serum. ANF release by the atria may play a role in the regulation of body fluids and blood pressure. Lang et al. have demonstrated that acute volume load serves as a trigger for the release of ANF into the circulation. Available evidence is currently compatible with the hypothesis that volume expansion causes an increase in ANF; however, evidence that volume contraction, particularly chronic contraction, causes a decrease in circulating ANF has been limited. We have presented data here that demonstrates the effect of both acute volume expansion and acute and chronic volume contraction on the increase and decrease, respectively, of circulating ANF.

The effect of rapid volume expansion induced by saline infusion on the irANF level in extracted plasma from groups of rats at various time points was determined. The basal level of irANF (92 pg/ml) in extracted samples increased within 1 minute after the infusion by 140 to 150 pg/ml. This result indicates that acute volume expansion, induced through bolus injection of normal saline, causes the immediate release of ANF into the general circulation. Circulating levels of irANF were decreased 3 minutes after infusion but had returned to basal levels 5 minutes after infusion.

The rapid increase and subsequent decrease of irANF during volume load are consistent with the findings of Lang et al., in which increased atrial perfusion pressure in vitro increased ANF release. The rapid clearance of irANF and subsequent return to control levels is indicative of a circulating half-life of approximately 2 minutes for the released ANF. This rate of

**Table 1. Effect of Acute Saline Load on Plasma Immunoreactive ANF**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control (n = 7)</th>
<th>Furosemide (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before administration</td>
<td>354 ± 10</td>
<td>354 ± 12</td>
</tr>
<tr>
<td>After administration</td>
<td>346 ± 11</td>
<td>328 ± 7*</td>
</tr>
<tr>
<td>Urine volume (ml/5 hr)</td>
<td>4.3 ± 0.6</td>
<td>16.2 ± 2.1†</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>36.3 ± 0.7</td>
<td>44.6 ± 2.4‡</td>
</tr>
<tr>
<td>PRA (ng ANG I/ml/hr)</td>
<td>5.6 ± 1.7</td>
<td>16.9 ± 8.9†</td>
</tr>
<tr>
<td>irANF (pg/ml)</td>
<td>196 ± 33</td>
<td>130 ± 26§</td>
</tr>
</tbody>
</table>

Values are means ± SD. irANF = immunoreactive ANF, ANG I = angiotensin I. *p < 0.01, †p < 0.001, ‡p < 0.02, §p < 0.005, compared with control values.

**Table 2. Effect of Furosemide Administration on Immunoreactive ANF**

**Table 3. Effect of Chronic Water Restriction on Plasma Immunoreactive ANF**

<table>
<thead>
<tr>
<th>Group</th>
<th>Weight (g)</th>
<th>24-hour urine volume (ml/100 g)</th>
<th>PRA (ng ANG I/ml/hr)</th>
<th>Plasma irANF (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 7)</td>
<td>249 ± 29</td>
<td>5.9 ± 0.8</td>
<td>6.0 ± 1.4</td>
<td>190 ± 30</td>
</tr>
<tr>
<td>Water restricted (n = 9)</td>
<td>255 ± 33</td>
<td>216 ± 33*</td>
<td>1.5 ± 0.2*</td>
<td>12.2 ± 3.4*</td>
</tr>
</tbody>
</table>

Values are means ± SD. ANG I = angiotensin I; irANF = immunoreactive ANF.

*p < 0.001, compared with control values.
Figure 1. Anesthesia-induced variation in extracted concentration of immunoreactive ANF. Plasma immunoreactive ANF was measured in blood samples recovered by (A) immediate decapitation; (B) decapitation 30 minutes after pentobarbital anesthesia (6 mg/kg i.p.); (C) carotid artery cannulation 30 minutes after pentobarbital anesthesia. Single asterisk indicates significant difference compared with B or C value (p<0.001). Double asterisk indicates significant difference compared with C value (p<0.01).

Clearance is in agreement with previous volume expansion data. The mechanism of ANF clearance has yet to be determined; however, ANF has been shown to be very sensitive to inactivation by several proteolytic enzymes. Whether degradation occurs at the tissue level or in the general circulation is not certain, but the recent data of Crozier et al. indicate that circulating levels of ANF are reduced approximately 50% by passage through major organs. The data of Bloch et al. further indicate that the low molecular weight ANF peptide is reasonably stable in serum for the duration of pro-ANF cleavage (60 minutes at 21 °C; 30 minutes at 37 °C) and thus suggests that tissue degradation or binding is responsible for the loss of irANF from the circulation.

The basal levels of irANF in the acute saline load experiments were lower than control values observed for furosemide administration or chronic water restriction. In the latter experiments blood samples were collected by decapitation, whereas blood samples in the former experiments were withdrawn from the carotid artery with the rats under anesthesia. The observed differences in the controls may be explained by two mechanisms. First, pentobarbital anesthesia is associated strongly with a decline in circulating irANF. Second, decapitation and the associated tachycardia induced by the procedure and resulting hemodynamic changes may raise the level of irANF. This contention is supported by preliminary data (see Figure 1) comparing decapitation and anesthesia on the basal level of extracted irANF in rats. Decapitation in the presence of pentobarbital anesthesia resulted in irANF values intermediate (111 ± 12 pg/ml; n = 8) between those found with decapitation (193 ± 19 pg/ml; n = 10) or pentobarbital alone (93 ± 12 pg/ml; n = 10). Further studies will be necessary to elucidate the mechanism of ANF release under these experimental conditions.

The effects of acute and chronic volume depletion on the irANF levels were examined by rapid diuresis and chronic water deprivation. The administration of furosemide resulted in significant volume depletion, as indicated by weight loss, higher PRA, and increased hematocrit. These data suggest that the amount of ANF released by the atria in response to volume depletion is reduced. This lower concentration of ANF is consistent with the apparent physiological role of ANF and indicates that the atria respond to the decrease in fluid volume by reducing the rate of ANF release. In agreement with this acute response, chronic water deprivation, which also resulted in a severe volume contraction (a 14% weight loss and a 200% increase in PRA), was similarly associated with a decreased level of circulating irANF. Consistent with our observation, atrial intracellular granule concentrations have been shown to be higher in rats after water deprivation, which has led to the suggestion that these granules are the storage site for ANF. How the atria regulate the production and secretion of ANF remains to be determined; however, the observed increase in cellular granularity and lower circulating ANF concentrations can be interpreted as a retention of ANF by the atria. The retention of ANF by the atria during water deprivation is accompanied by a reduction in atrial ANF messenger RNA, suggesting that the tissue level of ANF is more dependent on the rate of release than on the rate of biosynthesis.

Consistent with the role of ANF in diuresis, urine volumes were also significantly lower in water-restricted rats compared with the values in control rats. Many factors, including a reduction in the putative
hypothalamic natriuretic hormone and increased activity of the renin-angiotensin-aldosterone system and vasopressin, may be associated with the reduction of urine volume after water restriction. However, a decline in the circulating level of ANF may also play a major role in the reduction of urine volume.

In the present study, we demonstrated that the circulating concentration of irANF increases after volume expansion and decreases after volume contraction. Since the atria are in an ideal location to sense changes in the intravascular fluid volume, the atria may respond to chronic as well as acute changes in intravascular fluid volume by altering the level of ANF release, thereby participating in the regulation of body fluids and, possibly, blood pressure.

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Effects of volume change on circulating immunoreactive atrial natriuretic factor in rats.

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