Role of Endogenous Atrial Natriuretic Peptide in DOCA-Salt Hypertensive Rats

Effects of a Novel Nonpeptide Antagonist for Atrial Natriuretic Peptide Receptor

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Background. To explore roles of endogenous atrial natriuretic peptide (ANP) in blood pressure and volume regulation, we examined the effects of a newly developed ANP antagonist, HS-142-1 (HS) in deoxycorticosterone acetate (DOCA)-salt hypertensive rats.

Methods and Results. We examined 1) the effects of HS on ANP- or brain natriuretic peptide (BNP)-induced reductions in renal vascular resistance (RVR) of rat isolated perfused kidneys, 2) the effects of HS on cyclic GMP (cGMP) production in rat cultured vascular smooth muscle cells pretreated with ANP or BNP, and 3) the renal and systemic effects of HS in DOCA-salt-treated rats and control rats. We found that 1) HS dose-dependently reversed ANP- or BNP-induced decreases in RVR; 2) ANP or BNP at 100 nM caused an eightfold increase in cGMP production. These increases in cGMP were inhibited by HS in a dose-dependent fashion, and 300 µg/ml HS decreased cGMP to the control level. HS alone did not influence RVR or cGMP production; and 3) DOCA-salt rats showed higher plasma concentrations of ANP (198 versus 75 pg/ml) and BNP (23.7 versus 2.7 pg/ml, each p<0.01) than the control rats. Bolus administration of 8 mg/kg HS elevated blood pressure by 8% (p<0.01). This rise in blood pressure was attributed to an increase in systemic vascular resistance (+14%, p<0.05). Conversely, urinary excretion of sodium (=41%), glomerular filtration rate (-27%), and plasma (-77%) and urinary cGMP (-69%, each p<0.01) were decreased by administration of 8 mg/kg HS. These effects were dose dependent in DOCA-salt rats but slight or negligible in the control rats.

Conclusions. These results suggest that endogenous ANP and BNP may be involved in the regulation of blood pressure and body fluid volume in DOCA-salt rats in which ANP and BNP secretion is augmented. (Circulation 1993;87:554–561)

Key Words • sodium • cyclic GMP • atrial natriuretic peptide • brain natriuretic peptide

It is well established that atrial natriuretic peptide (ANP) has definite biological activities such as cardiovascular, renal, and hormonal actions. Whether ANP plays a pathophysiological role remains unclear, however, because these actions have been exhibited primarily with pharmacological doses. Since specific antagonists for ANP have not been available, the role of endogenous ANP has been studied by means of atrial appendectomy or ANP antiserum treatment. As a result, most studies suggest that endogenous ANP plays a given role in natriuresis caused by volume expansion. Whether ANP is actually involved in the regulation of blood pressure and body fluid volume in a physiological or disease condition, such as heart failure or hypertension, remains controversial, because different effects of ANP antiserum on blood pressure have been reported. The reasons for such controversy may be attributed at least in part to nonspecific effects rather than ANP inhibition per se brought about by these interventions. It cannot be denied that the effects of atrial appendectomy may be due to neural or mechanical derangements. Furthermore, intravenous injection of antiserum sometimes induces nonspecific or complement-related changes in blood pressure.

By screening of microbial culture broth for ANP receptor ligands, Morishita et al have recently discovered a microbial polysaccharide, HS-142-1 (HS), that selectively inhibits the binding of [125I]-ANP to guanylate cyclase–containing receptors in the kidney cortex. This antagonist for ANP receptors may provide clues with respect to the pathophysiological role of endogenous ANP. Thus, we examined the effects of HS on ANP- and brain natriuretic peptide (BNP)-induced
vasodilation and guanosine 3′:5′-cyclic monophosphate (cGMP) production in vitro systems and its renal effects in deoxycorticosterone acetate (DOCA)-salt hypertensive rats.

**Methods**

**Study 1: Effects of HS on Renal Vascular Resistance in Isolated Kidney**

Kidneys from male Wistar rats (n = 300 g) were isolated and perfused as described previously. Briefly, the animals were anesthetized with pentobarbital (30 mg/kg i.p.). A double-lumen needle was inserted into the right renal artery via the superior mesenteric artery, and the artery was perfused without ischemia. The perfusate was composed of a Krebs-Henseleit buffer that contained 6.7 g/dl of fraction V bovine serum albumin, 20 kinds of essential amino acids, and 5 mM glucose. After isolation of the kidney, 120 ml of the perfusate was recirculated at 37°C with continuous oxygenation with 95% O2/5% CO2. Renal perfusion pressure was monitored at the renal artery through the double-lumen needle. After a period of equilibrium, the perfusion pressure was initially set at 100 mm Hg, and the perfusion flow was maintained constant throughout the study. The effects of 0.33 mM α-rat ANP or 0.33 mM BNP-45 (Protein Research Foundation, Osaka, Japan) on renal perfusion pressure were first examined. HS (1.25, 2.5, and 5 μg/ml; Kyowa Hakko Kogyo Co. Ltd., Tokyo) or vehicle was then added to the perfusate in a cumulative manner. Changes in renal perfusion pressure were consecutively examined at a constant perfusion flow (n = 5 each).

**Study 2: Effects of HS on cGMP Production by Cultured Vascular Smooth Muscle Cells**

The effects of HS, ANP, and BNP on cGMP production by cultured vascular smooth muscle cells (VSMCs) were studied. Rat aortic smooth muscle cells were cultured by the explant method. Primary cultures of rat aortic smooth muscle cells were passaged into 24 well plates. After confluent growth was observed, the cultured cells were incubated with serum-free medium for 24 hours and used for assaying cGMP. Cultured cells were incubated with 0.95 ml RPMI 1640 medium containing 0.5 mM 3-isobutyl-1-methylxanthine at 37°C for 5 minutes, after which 0.05 ml of each test agent was added before incubation at 37°C for another 5 minutes. The reaction was stopped by addition of 0.01 ml HClO4 (60%) to each well. After neutralization with KOH, the cells were homogenized, and the cGMP concentration in the supernatant was measured by radioimmunoassay.

**Study 3: Plasma Concentrations of ANP and BNP in DOCA-Salt Hypertension**

The plasma concentrations of ANP and BNP were examined in 12 DOCA-salt rats and 10 control rats. DOCA-salt hypertensive rats were prepared according to a method previously reported. Briefly, male Wistar rats about 6 weeks old were anesthetized with light ether anesthesia and uninephrectomized, and a Silastic pellet (Dow-Corning, Midland, Mich.) containing 200 mg/kg DOCA was implanted subcutaneously. The rats were given 0.9% NaCl drinking water for 6 weeks. The control rats were also uninephrectomized but were given normal tap water. Blood pressure in these rats was measured by a tail-cuff method. Blood was collected by puncturing the abdominal aorta under inactin anesthesia. The assay methods for plasma ANP and BNP are described below.

**Study 4: Renal Effects of HS in DOCA-Salt Hypertensive Rats**

DOCA-salt rats and control rats were anesthetized with inactin (100 mg/kg i.p.), and canulas were inserted into the trachea, right carotid artery, right jugular vein, and bladder. Blood pressure was monitored through the carotid artery, and urine was collected into preweighed tubes through a PE50 bladder cannula. A bolus injection of lactated Ringer’s solution containing 5% inulin and 5% paraaminohippuric acid (PAH; 1.67 ml/kg body wt) was followed by a continuous infusion of both agents (1%) through one of the two jugular vein cannulas at a rate of 4 ml·hr⁻¹·kg⁻¹ body wt. After a 60-minute equilibration period, two baseline urine collections were repeated every 20 minutes. Thereafter, HS was administered through the other cannula. The dose of HS injected was 0 (vehicle), 0.5, 2.0, or 8.0 mg/kg body wt. Each dose group consisted of five or six rats. Urine collection was resumed 15 minutes after HS injection and continued for 30 minutes. At the midpoint of the urine collection, 0.5 ml of blood was drawn for hematocrit, PAH, inulin, and cGMP measurements through the arterial line. This blood loss was immediately replaced by the same volume of heparinized blood derived from other donor rats (Wistar). Mean blood pressure (MBP) was measured with a pressure transducer (Statham Instruments Division, Gould, Inc., Oxnard, Calif.). Urine volume was determined by weight, Na concentration in the urine by flame photometry, and urinary protein concentration by the modified Lowry method. PAH and inulin concentrations in the plasma and urine were measured by Marshall’s and anthrone methods, respectively. Effective renal blood flow was obtained by PAH clearance and hematocrit and glomerular filtration rate (GFR) by inulin clearance. Filtration fraction was calculated as GFR/PAH clearance.

**Study 5: Cardiovascular Effects of HS in DOCA-Salt Hypertensive Rats**

The effects of HS on systemic hemodynamics in another group of DOCA-salt rats and control rats (n = 5 for each tested dose) under the same conditions as in study 4 were examined. Vehicle or HS (2 or 8 mg/kg body wt) was injected as a bolus through one of the two jugular vein cannulas. Before and 30 minutes after HS administration, blood pressure, heart rate, and cardiac output (CO) were determined. The CO was measured by a dye-dilution method using a microcuvette as previously described. Briefly, the rat’s right carotid artery and jugular vein were cannulated. The arterial line was connected with a pressure transducer and cuvette (0.5×3×5 mm inside dimensions) with a three-way stopcock. After the recovery period from surgery, 0.1 mg of indocyanine green (0.02 ml) was injected into the other jugular vein cannula and flushed with 0.05 ml of physiological saline. Arterial blood was drawn with a peristaltic pump (Minipuls 2, Gilson, France), and the dye concentration of the arterial blood was continuously
recorded with a densitometer (EN-900, Erma, Tokyo) attached to the cuvette. About 0.5 ml of blood was propelled out during measurement of the dye concentration, after which it was reversed with the pump. This procedure did not influence blood pressure. The CO was calculated according to the Stewart-Hamilton formula. The CO measurements were duplicated. Systemic vascular resistance was calculated as MBP/CO.

**Assays**

Plasma concentrations of ANP and BNP were determined by radioimmunoassay as described previously.\(^{22,24}\) The plasma was extracted with Sep-Pak C\(_18\) cartridges (Millipore, Milford, Mass.) and eluted with 60% CH\(_3\)CN containing 0.1% trifluoroacetic acid. After evaporation, the extracts were reconstituted with the radioimmunoassay buffer, which consisted of 50 mM sodium phosphate (pH 7.4), 0.25% bovine serum albumin, 0.1% Triton X-100, 80 mM NaCl, 25 mM EDTA, and 0.05% NaN\(_3\). Peptide standards or samples were preincubated with antiserum against each peptide for 24 hours, after which \(^{125}\)I-ANP or \(^{125}\)I-BNP was added and incubated for another 36 hours. Free and bound tracers were separated by the double-antibody method. This radioimmunoassay system for ANP and BNP showed <0.01% cross-reactivity between the two. The plasma, urinary, and medium concentrations of cGMP were determined by radioimmunoassay after succinylation.\(^{23}\)

**Statistical Analysis**

Values are expressed as mean±SEM. The in vivo effects of HS on variables measured in DOCA-salt hypertensive and control rats were compared with the effects of vehicle by Dunnett's method after two-way ANOVA (studies 4 and 5).\(^{25}\) The effects of HS on renal perfusion pressure and cGMP production by the VSMCs were assessed on the basis of one-way ANOVA followed by Tukey's test (studies 1 and 2). Differences in baseline values of hormonal and cardiorenal function between the DOCA-salt and control rats were compared by Student's \(t\) test (studies 3, 4, and 5). Differences were considered significant at \(p<0.05\).

**Results**

**Study 1**

Figure 1 shows a representative tracing of perfusion pressure in isolated kidneys. \(\alpha\)-Rat ANP dose-dependently lowered renal perfusion pressure, whereas the addition of HS elevated it toward the baseline level. However, acetylcholine still brought about a quick decrease in pressure even in the presence of HS. HS alone did not change the perfusion pressure. Furthermore, HS did not influence the effects of sodium nitroprusside, endothelin-1, angiotensin II, or norepinephrine on perfusion pressure (data not shown). Figure 2 summarizes the effects of ANP and HS on renal vascular resistance. Average renal perfusion flow was 34.0±1.2 ml·min\(^{-1}\)·g\(^{-1}\) kidney wt. HS reversed ANP-induced reductions in renal vascular resistance in a dose-dependent manner (renal vascular resistance, 0.33 nM ANP, −26±7% versus 0.33 nM ANP + 5 \(\mu\)g/ml HS, −3±3%, \(p<0.01\)). HS also antagonized BNP-induced vasorelaxation dose-dependently (renal vascular resistance, 0.33 nM BNP, −34±6% versus 0.33 nM BNP + 5 \(\mu\)g/ml HS, −10±7%, \(p<0.05\)). Figure 2 includes data for the vehicle control. Vehicle did not change the ANP-induced decrease in renal vascular resistance.

**Study 2**

Figure 3 demonstrates the effects of HS, ANP, and BNP on cGMP production in rat cultured VSMCs. ANP at 100 nM caused an eightfold increase in cGMP production (4.0±1.5 versus 31.1±1.9 pmol per well, \(p<0.01\)), whereas HS alone did not influence the basal production of cGMP (HS 300 \(\mu\)g/ml, 4.2±2.3 pmol per well, NS). However, HS dose-dependently suppressed ANP-stimulated cGMP production. HS at concentrations higher than 30 \(\mu\)g/ml completely abolished the effects of ANP on cGMP production (ANP 100 nM + HS 300 \(\mu\)g/ml, 3.6±1.7 pmol per well, NS). BNP-stimulated cGMP production was also decreased by HS.

**Study 3**

DOCA-salt treatment for 6 weeks significantly elevated systolic blood pressure (218±14 versus 139±8 mm Hg, \(p<0.01\)). Plasma ANP was significantly higher in DOCA-salt rats than in control rats (198±41 versus 75±10 pg/ml, \(p<0.01\)). Similarly, DOCA-salt rats exhibit...
Figure 3. Bar graph showing cyclic GMP (cGMP) production from rat cultured vascular smooth muscle cells during incubation with HS-142-1 (HS), atrial natriuretic peptide (ANP), and brain natriuretic peptide (BNP) in study 2. Each bar consists of four wells. To analyze the effects of HS in the presence of 100 nM ANP or BNP, each bar treated with HS in the ANP or BNP group was compared with the untreated bar with HS (ANP/BNP 100, HS 0) using one-way ANOVA and Tukey’s test. Similarly, to examine whether or not the antagonistic effects of HS were complete, the control (ANP/BNP 0, HS 0) was compared with each treated group.

Figure 4. Representative tracing of time course changes in mean blood pressure (MBP) of a deoxycorticosterone acetate (DOCA)-salt treated rat after intravenous administration of 8 mg/kg HS-142-1 (HS) in study 4.
nonspecific or toxic, because this compound did not antagonize other major vasoactive peptides and because the isolated kidney could still respond to acetylcholine with respect to vasodilation, even in the presence of high doses of HS. Morishita et al.\textsuperscript{15} showed that HS antagonized the binding of \textsuperscript{125}I-ANP to the renal cortex tissue in a competitive manner. This suggests that HS exerts the antagonistic action at the receptor level. Since high doses of HS alone did not show any effects in our in vitro systems, this compound seems to be devoid of agonistic activities, which are often accompanied by peptide antagonists. Furthermore, ANP- and BNP-induced cGMP production was also inhibited by HS. Morishita et al.\textsuperscript{15} showed that HS inhibited the binding of ANP to the renal cortex membrane but not that of atriopeptin I. Atriopeptin I is considered to be a specific agonist of clearance receptors. It has also been demonstrated by use of affinity cross-linking techniques that HS recognizes only 135-kd ANP receptors with a guanylate cyclase domain in bovine adrenocortical membrane but not 60-kd guanylate cyclase-free receptors in bovine lung membranes.\textsuperscript{20} Furthermore, HS inhibits natriuretic peptide–induced cGMP production in PC12, NG108-15,\textsuperscript{27} and LLC-PK\textsubscript{1} cells.\textsuperscript{28} Accordingly, HS is an antagonist of guanylate cyclase–coupled ANP receptors, and its in vivo application seems to be suitable for estimating the roles of endogenous ANP and BNP.

In the present study, plasma concentrations of ANP and BNP were higher in DOCA-salt rats than in the normotensive control rats. This is compatible with previous reports.\textsuperscript{21,29} An ANP antagonist, HS, brought about a small but significant increase in blood pressure of DOCA-salt hypertensive rats but not in control rats. This suggests a susceptibility to ANP antagonists in animals with increased basal concentrations of plasma ANP and BNP. However, conflicting results on the effects of the ANP antiserum on blood pressure in hypertensive rats have been reported by several groups. In spontaneously hypertensive rats, Itoh et al.\textsuperscript{11} showed a significant increase in blood pressure by repetitive passive immunization, whereas Greenwald et al.\textsuperscript{10} did not find any changes in blood pressure of autoimmunized rats. Similar controversial findings have been obtained in DOCA-salt rats. Itoh et al.\textsuperscript{11} again demonstrated blood pressure elevation, whereas Naruse et al.\textsuperscript{11} did not. One of the reasons for such conflicting results is probably the polyglobal (rather than monoclonal) antibody
used with unspecific effects. Moreover, the efficacies of these interventions on suppression of ANP actions could be evaluated by measuring cGMP levels. Suppression of the plasma cGMP to a degree similar to that in the present study was confirmed only in the report by Itoh et al.11 Accordingly, full suppression of ANP action may lead to elevated blood pressure in some types of hypertension.

Our result is consistent with the report by Pandiani et al.13 They found that ANP antibody elevated blood pressure from the normotensive level and increased body weight in rats with 60% reduced renal mass who drank 1% saline. Their blood pressure remained normal when normal rabbit serum was administered. This suggests that endogenous ANP may play a compensatory role in blood pressure elevation in normotensive as well as hypertensive states.

HS-induced blood pressure elevation was attributed to a rise in systemic vascular resistance. We cannot determine from this study how much the antinatriuretic effect of HS directly contributes to this blood pressure elevation. It is frequently reported that ANP decreases blood pressure as well as CO in rats, including DOCA-salt hypertensive rats.30 Sasaki et al10 showed that ANP antiserum elevated both blood pressure and CO in normotensive rats and spontaneously hypertensive rats, suggesting effects opposite to those of ANP administration. In humans, however, ANP exhibits vasodilatory effects and simultaneous increases in CO like other vasodilators.31 Furthermore, Drexler et al12 reported that the antiserum for ANP decreased CO and elevated systemic vascular resistance without changing blood pressure in rats with congestive heart failure. The different results may be caused by the different experimental methods and conditions. However, the present observation is consistent with experimental studies using monoclonal antibody12 and with clinical studies31 that have shown that elevated ANP exerts vasodilator effects.

HS acutely brought about as much as 40% decreases in \( U_{NaV} \) in DOCA-salt rats, suggesting ANP's important role in volume regulation under conditions of augmented ANP secretion. This decrease in \( U_{NaV} \) was
TABLE I.  Average Basal Values of the Renal (Study 4) and Systemic Hemodynamics (Study 5) in DOCA-Salt Hypertensive Rats and Control Rats

<table>
<thead>
<tr>
<th></th>
<th>DOCA-salt rats</th>
<th>Control rats</th>
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<tbody>
<tr>
<td>Study 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>22</td>
<td>20</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>369±13</td>
<td>403±33</td>
</tr>
<tr>
<td>Mean blood pressure (mm Hg)</td>
<td>173±7*</td>
<td>124±8</td>
</tr>
<tr>
<td>Urinary sodium excretion (neighbors kg⁻¹ m⁻¹)</td>
<td>8.14±1.32†</td>
<td>1.33±0.59</td>
</tr>
<tr>
<td>Urinary protein excretion (µg kg⁻¹ m⁻¹)</td>
<td>729±138†</td>
<td>315±53</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>47±2</td>
<td>49±1</td>
</tr>
<tr>
<td>Glomerular filtration rate (ml kg⁻¹ m⁻¹)</td>
<td>3.01±0.37</td>
<td>2.67±0.34</td>
</tr>
<tr>
<td>Renal blood flow (ml kg⁻¹ m⁻¹)</td>
<td>24.3±3.3</td>
<td>26.8±5.3</td>
</tr>
<tr>
<td>Filtration fraction</td>
<td>0.24±0.03</td>
<td>0.21±0.02</td>
</tr>
<tr>
<td>Plasma cGMP (pmol/ml)</td>
<td>16.7±5.2t</td>
<td>5.8±1.8</td>
</tr>
<tr>
<td>Urinary cGMP (pmol kg⁻¹ m⁻¹)</td>
<td>1.71±0.32‡</td>
<td>0.71±0.40</td>
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<tr>
<td>Study 5</td>
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<tr>
<td>n</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>350±16</td>
<td>408±46</td>
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<tr>
<td>Mean blood pressure (mm Hg)</td>
<td>174±6*</td>
<td>130±9</td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>393±6</td>
<td>371±9</td>
</tr>
<tr>
<td>Cardiac output (ml kg⁻¹ m⁻¹)</td>
<td>209±24</td>
<td>180±18</td>
</tr>
<tr>
<td>Systemic vascular resistance (mm Hg kg⁻¹ m⁻¹)</td>
<td>0.83±0.05</td>
<td>0.70±0.06</td>
</tr>
</tbody>
</table>

DOCA, deoxycorticosterone acetate; cGMP, cyclic GMP; bpm, beats per minute. Values are mean±SEM.
*p<0.01, †p<0.02, ‡p<0.05 vs. control.

associated with a decrease in GFR. It has been reported that antiserum raised against ANP significantly decreased U₉ₙV without changing GFR in rats with congestive heart failure and those with reduced renal mass. However, the latter authors found that the antiserum treatment caused substantial decreases in GFR in diabetic rats. This suggests that the contribution of ANP to regulation of GFR may be different in each disease condition. HS also decreases U₉ₙV. This phenomenon does not seem to result simply from decreases in GFR or U₉ₙV, because ANP has a specific proteinuric effect. We have reported that dopamine did not increase U₉ₙV despite a substantial increase in GFR and U₉ₙV, whereas ANP increased it in the same patients with glomerulonephritis. This implies that ANP may contribute to an increase in U₉ₙV in addition to its natriuretic action in a condition with augmented ANP secretion and glomerular damage.

HS decreased not only U₉ₙV but also hematocrit. Antinatriuresis caused by HS in the present study may result in the decrease in hematocrit. However, it has already been pointed out that ANP increases hematocrit independent of its natriuretic effects. Because ANP has been shown to increase hematocrit even in bilaterally nephrectomized rats, ANP has been shown to shift plasma components to the extravascular space by some mechanism. Thus, HS-induced reduction in hematocrit also seems to be attributable to the blockade of ANP effects.

Significant cardiovascular and renal effects of HS were manifested only in DOCA-salt rats. This may depend mainly on a difference in the basal level of ANP. Because BNP causes hypotensive and natriuretic effects with potency comparable to or greater than that of ANP, increased BNP levels probably contributed to the effect observed in DOCA-salt rats. On the other hand, unlike the effects on blood pressure or U₉ₙV, HS significantly reduced the plasma concentration of cGMP even in the control rats. This suggests that ANP at physiological levels may contribute to the actual level of the plasma cGMP concentration. However, a small rise in cGMP as observed in control animals may not be enough to exert detectable changes in systemic hemodynamics.

In conclusion, an application of a newly developed ANP antagonist, HS-142-1, enabled us to evaluate the pathophysiological roles of endogenous ANP and BNP. This compound exerted pressor and antinatriuretic effects in DOCA-salt hypertensive rats with elevated plasma levels of ANP and BNP, suggesting compensatory roles of ANP and BNP in the regulation of blood pressure and body fluid volume.

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Role of endogenous atrial natriuretic peptide in DOCA-salt hypertensive rats. Effects of a novel nonpeptide antagonist for atrial natriuretic peptide receptor.

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