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 (~6%), glomerular filtration rate (~27%), and plasma (~77%) and urinary cGMP (~77%), 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.

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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 antisera 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 antisera on blood pressure have been reported. The reasons for such controversy may be attributed to 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 antisera 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 (=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 equilibration, 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 cannulas 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% paraaminobenzoic 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 indoxyline 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 determined.
Assays

Plasma concentrations of ANP and BNP were determined by radioimmunoassay as described previously. The plasma was extracted with Sep-Pak C\textsubscript{18} cartridges (Millipore, Milford, Mass.) and eluted with 60% CH\textsubscript{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% Na\textsubscript{2}SO\textsubscript{4}. Peptide standards or samples were preincubated with antiserum against each peptide for 24 hours, after which \textsuperscript{125}I-ANP or \textsuperscript{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.

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). 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. \textsuperscript{1}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\textsuperscript{-1}·g\textsuperscript{-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 exhib-
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.

Study 4

Figure 4 presents a typical course of MBP after HS administration to a DOCA-salt rat. Bolus injection of HS (8 mg/kg i.v.) gradually elevated MBP. The peak effect of HS was observed around 30 minutes after the HS treatment, and MBP returned to baseline levels 60 minutes after the injection. Figures 5–8 summarize the changes in variables measured during HS administration. Average baseline values for each measurement are listed in Table 1 (study 4). MBP, urinary excretions of sodium (U_Na), and protein (U_protein), and plasma and urinary cGMP were significantly higher in DOCA-salt rats than in control rats with or without correction for body weight. Other variables measured were not significantly different between the two groups of rats.

As shown in Figure 5, HS at 8 mg/kg significantly increased MBP by 8.0 ± 2.2% in DOCA-salt rats, whereas no effects were observed in the normotensive control rats (−0.1 ± 3.2%, NS). U_Na also decreased in DOCA-salt rats in a dose-dependent fashion (8 mg/kg, -41 ± 15%, p < 0.01). Furthermore, HS decreased the hematocrit significantly in DOCA-salt rats (8 mg/kg, -4.1 ± 0.7%, p < 0.01). These effects were not seen in the controls.

Figure 6 presents a comparison of the renal effects of HS. In DOCA-salt rats, GFR was decreased (8 mg/kg, -27 ± 6%, p < 0.05), whereas renal blood flow was unchanged (-18 ± 17%, NS). These variables were not changed in the control rats. In parallel with decreases in U_Na and GFR, U_protein was decreased by HS only in DOCA-salt rats (Figure 7). These changes were associated with a remarkable reduction in plasma (8 mg/kg, -77 ± 9%, p < 0.01) and urinary cGMP (-68 ± 11%, p < 0.01) in DOCA-salt rats. In contrast with the effects on MBP or renal function, HS substantially decreased plasma cGMP concentration (8 mg/kg, -49 ± 17%, p < 0.05), even in the control rats.

Study 5

The baseline hemodynamic data are listed in Table 1 (study 5). Although MBP was significantly higher in DOCA-salt rats than in control rats, other variables measured were similar in both groups. However, when the CO and systemic vascular resistance were not corrected for body weight, systemic vascular resistance but not CO was significantly greater in DOCA-salt rats than in control rats (systemic vascular resistance, 2.39 ± 0.11 versus 1.84 ± 0.18 mm Hg · min · ml⁻¹, p < 0.05; CO, 73.1 ± 6.2 versus 73.1 ± 6.4 ml/min, NS). Figure 8 demonstrates the effects of HS on systemic hemodynamics. MBP was again increased by 8 mg/kg HS (+9.7 ± 1.2%, p < 0.01), whereas neither heart rate nor CO was changed, resulting in a significant increase in systemic vascular resistance (+14.9 ± 4.5%, p < 0.05). These effects were not observed in the control rats.

Discussion

HS inhibited ANP- and BNP-induced vasorelaxation in the isolated kidney. The effects of HS were not

Figure 2. Histological evaluation of renal tissue sections from control rats and HS-treated rats. (A) Representative photomicrographs of renal cortex from control rats (left) and HS-treated rats (right) showing glomerular hyalinization and tubular atrophy. (B) Quantitative analysis of glomerular area from control rats (open bars) and HS-treated rats (closed bars). (C) Quantitative analysis of tubular cast area from control rats (open bars) and HS-treated rats (closed bars).
those 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. showed that HS antagonized the binding of 125I-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. 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 guanyl clyase domain in bovine adrenocortical membrane but not 60-kd guanylate cyclase-free receptors in bovine lung membranes. Furthermore, HS inhibits natriuretic peptide–induced cGMP production in PC12, NG108-15, and LLC-PK1 cells. 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. 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. showed a significant increase in blood pressure by repetitive passive immunization, whereas Greenwald et al. did not find any changes in blood pressure of autoimmunized rats. Similar controversial findings have been obtained in DOCA-salt rats. Itoh et al. again demonstrated blood pressure elevation, whereas Naruse et al. did not. One of the reasons for such conflicting results is probably the polyglobal (rather than monoclonal) antibody.
FIGURE 7. Graphs showing changes in plasma cyclic GMP (cGMP) concentration, urinary cGMP excretion, and urinary protein excretion by a bolus injection of vehicle or HS-142-1 (HS; 0.5, 2.0, and 8.0 mg/kg) in deoxycorticosterone acetate (DOCA)-salt rats and the control rats of study 4. Each circle indicates the average changes in parameters of five or six rats (0 and 8.0 mg/kg in DOCA-salt rats) before and after HS or vehicle administration. The effects of HS were compared with those with vehicle using Dunnett's test after two-way ANOVA.

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. Accordinly, full suppression of ANP action may lead to elevated blood pressure in some types of hypertension.

Our result is consistent with the report by Panmami et al. 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. Sasaki et al 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. Furthermore, Drexler et al 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 antibody and with clinical studies that have shown that elevated ANP exerts vasodilator effects.

HS acutely brought about as much as 40% decreases in UNaV in DOCA-salt rats, suggesting ANP's important role in volume regulation under conditions of augmented ANP secretion. This decrease in UNaV was
associated with a decrease in GFR. It has been reported that antisera raised against ANP significantly decreased UNaV without changing GFR in rats with congestive heart failure and those with reduced renal mass. However, the latter authors found that the antisera 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 UNaV. This phenomenon does not seem to result simply from decreases in GFR or UNaV, because ANP has a specific proteinemic effect. We have reported that dopamine did not increase UNaV despite a substantial increase in GFR and UNaV, whereas ANP increased it in the same patients with glomerulonephritis. This implies that ANP may contribute to an increase in UNaV in addition to its natriuretic action in a condition with augmented ANP secretion and glomerular damage.

HS decreased not only UNaV 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 UNaV, 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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