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Natriuretic Peptide Receptors and Neutral Endopeptidase in Mediating the Renal Actions of a New Therapeutic Synthetic Natriuretic Peptide Dendroaspis Natriuretic Peptide

Horng H. Chen, MB, BCH, John G. Lainchbury, MD, John C. Burnett, JR, MD
Rochester, Minnesota

OBJECTIVES The objectives of the current study were to define for the first time the roles of the natriuretic peptide (NP) receptors and neutral endopeptidase (NEP) in mediating and modulating the renal actions of Dendroaspis natriuretic peptide (DNP), a new therapeutic synthetic NP.

BACKGROUND Recent reports have advanced the therapeutic potential of a newly described synthetic NP called DNP. Dendroaspis natriuretic peptide is a 38-amino acid peptide recently isolated from the venom of *Dendroaspis augusticeps* (the green mamba snake).

METHODS Synthetic DNP was administered intra-renally at 5 ng/kg/min to 11 normal anesthetized dogs, 5 of which received the NP receptor antagonist HS-142-1 (3 mg/kg intravenous bolus) while the remaining 6 dogs received an infusion of the NEP inhibitor, candoxatrilat (8 and 80 μg/kg/min) (Pfizer, Sandwich United Kingdom).

RESULTS Intra-renal DNP resulted in marked natriuresis associated with increased urinary cyclic guanosine monophosphate excretion (UcGMPV), glomerular filtration rate (GFR), and renal blood flow (RBF) and decreased distal fractional sodium reabsorption (FNaR) compared with baseline. HS-142-1 attenuated the natriuretic response to DNP, resulting in decreased UcGMPV, GFR, and RBF and increased distal FNaR. In contrast, low and high doses of NEP inhibitor did not potentiate the renal actions of DNP.

CONCLUSIONS We report that the NP receptor blockade attenuated the renal actions of synthetic DNP and that the NEP inhibitor did not alter the renal response to DNP. This latter finding is a unique property of synthetic DNP, as distinguished from other known NPs, supporting its potential as a therapeutic agent. (J Am Coll Cardiol 2002;40:1186–91) © 2002 by the American College of Cardiology Foundation

Recent reports have advanced the therapeutic potential of a newly described synthetic natriuretic peptide called Dendroaspis natriuretic peptide (DNP) in the treatment of congestive heart failure (CHF) (1). Specifically, we have reported that intravenous infusion of DNP in experimental CHF increased the glomerular filtration rate (GFR) and sodium excretion despite reductions in arterial pressure. Synthetic DNP also increased plasma and urinary cyclic guanosine monophosphate (cGMP) and suppressed plasma renin activity. These renal and humoral actions of synthetic DNP also were associated with decreases in markedly elevated cardiac filling pressures. These properties of synthetic DNP suggest unique characteristics that support its development as a new intravenous agent for acutely decompensated CHF (1). Recent studies have also reported that synthetic DNP is a potent antiproliferative peptide and is also diuretic when infused directly into the brain (2,3).

Synthetic DNP was designed based on the sequence of the DNP originally isolated from venom of *Dendroaspis augusticeps* (the green mamba snake) (4). Synthetic DNP is a 38-amino-acid peptide that contains, similar to atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and C-type natriuretic peptide (CNP), a 17-amino-acid disulfide ring structure with a 15-amino-acid residue C-terminal extension (Fig. 1). This peptide potently vasorelaxes isolated precontracted rodent aorta and canine coronary arteries and augments the formation of 3′5′ cGMP in aortic endothelial and vascular smooth muscle cells (4,5). Dendroaspis natriuretic peptide has an extended C-terminus, which may result in greater resistance to peptide degradation in the kidney by neutral endopeptidase (NEP). In addition, although it is synthetic and without significant homology to the other known natriuretic peptides, the increases in plasma and urinary cGMP in response to synthetic DNP suggests an interaction with the known natriuretic peptide particulate-guanylyl-cyclase receptors.

The goal of the current study was to extend previous studies and provide insight into the interactions of synthetic DNP with natriuretic peptide receptors and NEP. First, we sought to define an interaction between synthetic DNP and the natriuretic peptide particulate-guanylyl-cyclase receptors in mediating the renal actions of this new synthetic natriuretic peptide. This was accomplished by intra-renal administration of synthetic DNP in the presence of intravenous administration of the particulate-guanylyl-cyclase

From the Cardiorenal Research Laboratory, Division of Cardiovascular Diseases and Department of Physiology, Mayo Clinic and Foundation, Rochester, Minnesota. Supported by grants HL 36634 and HL 07111 from the National Institutes of Health, Miami Heart Research Institute, Mayo Foundation, the Joseph P. and Jeanne Sullivan Foundation, Bruce and Ruth Rappaport Program in Vascular Biology, National Kidney Foundation of Minnesota, Inc., and the General Mills Clinician Investigator Fellowship awarded to Dr. Chen.

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Abbreviations and Acronyms

ANP	= atrial natriuretic peptide
BNP	= brain natriuretic peptide
cGMP	= cyclic guanosine monophosphate
CHF	= congestive heart failure
CL_{Li}	= lithium clearance
CL_{Na}	= sodium clearance
CNP	= C-type natriuretic peptide
DFNaR	= distal fractional sodium reabsorption
DNP	= Dendroaspis natriuretic peptide
GFR	= glomerular filtration rate
MAP	= mean arterial blood pressure
NEP	= neutral endopeptidase 24-11
RBF	= renal blood flow
UcGMPV	= urinary cGMP excretion
UNaV	= urinary sodium excretion

receptor antagonist HS-142-1 (HS), which is known to block the renal actions of ANP and BNP (6). HS-142-1 is a novel polysaccharide isolated from culture broth of *Aureobasidium sp.*, which competitively and selectively inhibited ANP binding to its guanylyl-cyclase-containing blocked cGMP production elicited by ANP (7). We hypothesized that HS-142-1 would attenuate the renal actions of synthetic DNP. Our second goal was to demonstrate that synthetic DNP in vivo is resistant to degradation by NEP. To address this objective, DNP was administered in the presence and absence of low- and high-dose administration of the potent NEP inhibitor candoxatrilat. We hypothesized that the renal actions of DNP would not be potentiated by NEP inhibition. We found that natriuretic peptide receptor blockade attenuated the renal actions of synthetic DNP and that NEP inhibition did not alter the renal response to DNP. This latter finding is a unique property of synthetic DNP, as distinguished from other known natriuretic peptides, supporting its potential as a therapeutic agent.

METHODS

Studies were performed in 11 male mongrel dogs weighing between 20 and 25 kg. The dogs were maintained on a normal sodium diet with standard dog chow (Lab Canine Diet 5006; Purina Mills, St. Louis, Missouri) with free access to tap water. All studies conformed to the guidelines of the American Physiological Society and were approved by the Mayo Clinic Institutional Animal Care and Use Committee.

Experimental protocol. On the evening before the experiment, 300 mg of lithium carbonate was administered orally for the assessment of renal tubular function. On the day of the basic experiment, the dogs were anesthetized with sodium pentobarbital (15 mg/kg intravenous), intubated, and mechanically ventilated with supplemental oxygen (Harvard respirator, Amersham, Massachusetts) at 16 cycles/min. The femoral artery was cannulated for blood pressure monitoring and blood sampling. The femoral vein was also cannulated for normal saline infusion. The left kidney was exposed via a flank incision, and the ureter was cannulated for urine collection. A calibrated noncannulating electromagnetic flow probe was place around the renal artery for continuous monitoring of renal blood flow (RBF). In addition, a curved 23-gauge needle, attached to polyethylene tubing, was inserted into the renal artery proximal to the flow probe for intra-renal infusion of synthetic DNP (DNP 1-38, Phoenix Pharmaceuticals Inc., Mountain View, California). Furthermore, a curved 23-gauge needle attached to polyethylene tubing was inserted into the renal vein for collection of venous blood. Supplemental nonhypotensive

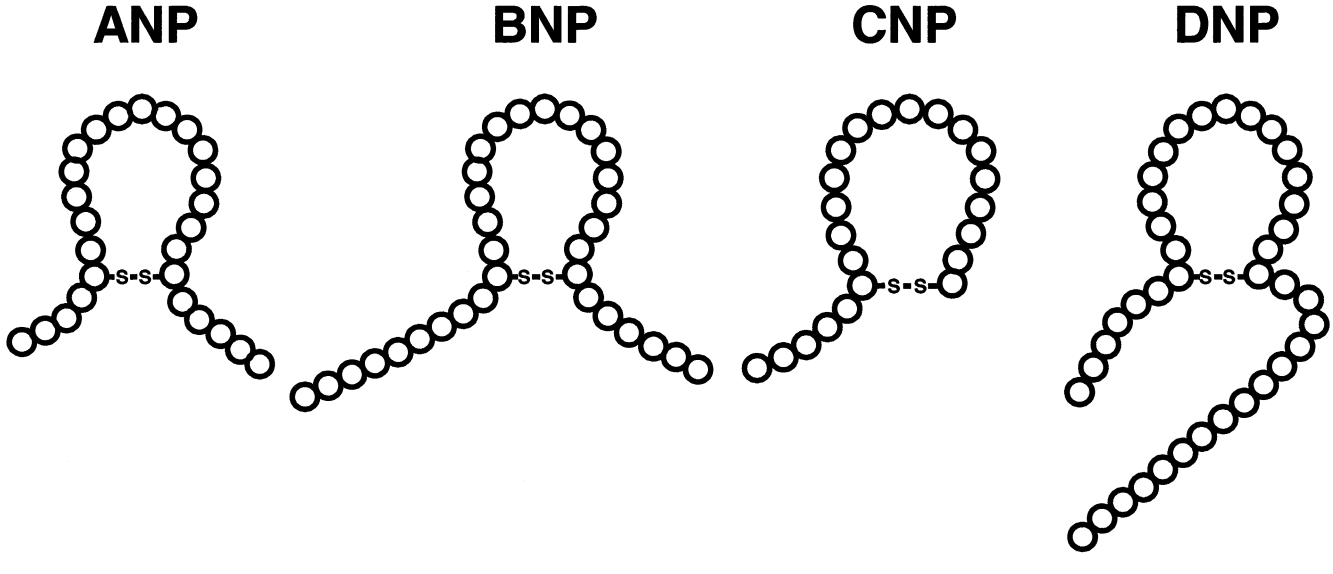

Figure 1. Amino acid sequence and structure of natriuretic peptides. ANP = atrial natriuretic peptide; BNP = brain natriuretic peptide; CNP = C-type natriuretic peptide; DNP = Dendroaspsis natriuretic peptide.

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doses of pentobarbital sodium were given as needed during the experiment.

After completion of the surgical preparation, a priming dose of insulin (ICN Biomedicals, Cleveland, Ohio) dissolved in isotonic saline was administered intravenously followed by a constant infusion of 1 ml/min to achieve a steady-state plasma inulin concentration between 40 to 60 mg/dl. After an equilibration period of 60 min, a 30-min baseline clearance was performed; hemodynamic recordings, plasma, and urine were collected for hormonal determination. This was followed by a 15-min lead-in period during which DNP was infused intra-renally at 5 ng/kg/min in all 11 dogs, after which the second 30-min clearance was performed.

After this second 30-min clearance, five dogs received intravenous bolus injection of the natriuretic peptide receptor antagonist HS-142-1 3 mg/kg (a gift from Yuzuru Matsuda, Tokyo Research Laboratories, Kyowa Hakko Kogyo Co., Japan) in addition to the continuous intra-renal infusion of DNP. This was followed by four 30-min clearances.

The remaining six dogs received continuous intravenous infusion of the NEP inhibitor candoxatrilat (Pfizer) at a dose of 8 μg/kg/min (low dose) in addition to the continuous intra-renal infusion of DNP. After a 15-min lead-in period, a 30-min clearance was performed, after which the dose of candoxatrilat was increased to 80 μg/kg/min (high dose), and, after a 15-min lead-in period, another 30-min clearance was performed. The dose of 80 μg/kg/min represents the peak dose response curve for candoxatrilat (unpublished data, Pfizer).

Mean arterial blood pressure (MAP) was assessed via direct measurement from the femoral arterial catheter. Urine was collected on ice for assessment of urine volume, electrolytes, and inulin. Urine collected for cGMP analysis was heated to more than 90°C before storage. Blood was collected in heparin and ethylene diamine tetra acetate tubes and immediately placed on ice. After centrifugation at 2,500 rpm at 4°C, plasma was decanted and stored at −20°C until analysis. The GFR was determined by the clearance of inulin.

Hormone and electrolyte analysis. After plasma extraction plasma and urine ANP, BNP, DNP, renin, and cGMP were measured by radioimmunoassay (5). Urinary and plasma inulin were measured by the anthrone method. Urinary and plasma lithium were determined by flame emission spectrophotometry (model 357, Instrumentation Laboratory, Wilmington, Massachusetts). Employing the lithium clearance (CL_{Li}) technique, distal fractional reabsorption of sodium (DFNaR) was calculated using the following equation: $DFNaR = [(CL_{Li} - CL_{Na})/CL_{Li} \times 100]$, where CL_{Li} = [(urine Li × urine flow)/plasma Li] and CL_{Na} = [(urine Na × urine flow)/plasma Na].

Statistical analysis. Results of the quantitative studies were expressed as mean ± SEM. Data were assessed by repeated measures analysis of variance for comparisons within groups, using GraphPad Prism software (GraphPad Software Inc., San Diego, California) and post-hoc Dunnett's test for multiple comparisons. In the group receiving NEP inhibition, there were four repeated measures; in the group receiving HS-142-1, there were six repeated measures. Statistical significance was accepted as $p < 0.05$.

Table 1. Renal and Humoral Actions of Intra-Renal DNP

	Baseline	Intra-Renal DNP
MAP, mm Hg	130 ± 3	126 ± 3
UNaV, μEq/min	67 ± 30	355 ± 41*
DFNaR, %	97 ± 0.8	86 ± 2.8*
GFR, ml/min	33 ± 4	52 ± 5*
RBF, ml/min	285 ± 23	319 ± 17*
UcGMPV, pmol/min	1,012 ± 115	2,033 ± 235*
Plasma cGMP, pmol/ml	11 ± 1	20 ± 1*
Renal vein renin, ng/ml/h	8 ± 1	5 ± 1*

*$p < 0.05$ versus baseline. Mean ± SEM.

DFNaR = distal fractional sodium reabsorption; DNP = Dendroaspis natriuretic peptide; GFR = glomerular filtration rate; MAP = mean arterial blood pressure; RBF = renal blood flow; UcGMPV = urinary cGMP excretion; UNaV = urinary sodium excretion.

RESULTS

Renal and humoral actions of intra-renal DNP. Table 1 reports the renal and humoral actions of intra-renal infusion of DNP (n = 11). Mean arterial blood pressure was not significantly altered with the intra-renal infusion of DNP. Urinary sodium excretion (UNaV) was significantly increased, associated with a decrease in DFNaR. Both GFR and renal blood flow increased with the intra-renal infusion of DNP. Urinary cGMP excretion (UcGMPV) and plasma cGMP increased, while renal vein renin activity was suppressed with intra-renal infusion of DNP. Both plasma and urinary ANP and BNP remained unchanged (data not shown).

Natriuretic peptide receptor antagonist HS-142-1 on DNP. The effects of HS-142-1, a natriuretic peptide receptor antagonist on UNaV, DFNaR, GFR, and RBF are illustrated in Figure 2 (n = 5). HS-142-1 attenuated the natriuretic response (UNaV) to DNP, which was secondary to a decrease in GFR and RBF and increased DFNaR. The attenuated renal response to DNP in the presence of HS is associated with decreases in plasma cGMP and UcGMP, the second messenger of DNP (Fig. 3), in the absence of any change in plasma and urinary DNP excretion (data not shown). Renal vein renin activity increased with HS-142-1, from 3.8 ± 1 ng/ml/h with DNP alone to 5.3 ± 2 ng/ml/h ($p < 0.05$) after HS-142-1.

NEP inhibition on DNP. Both low- and high-dose NEP inhibition did not potentiate the renal actions of DNP. Urinary sodium excretion, DFNaR, RBF, and GFR remained unchanged with either low-dose or high-dose NEP inhibitor (Table 2) (n = 6). Plasma ANP, BNP, DNP, and urinary BNP and DNP excretion did not increase with NEP inhibitor; however, urinary ANP excretion did increase (Table 2). There was a trend for UcGMPV to

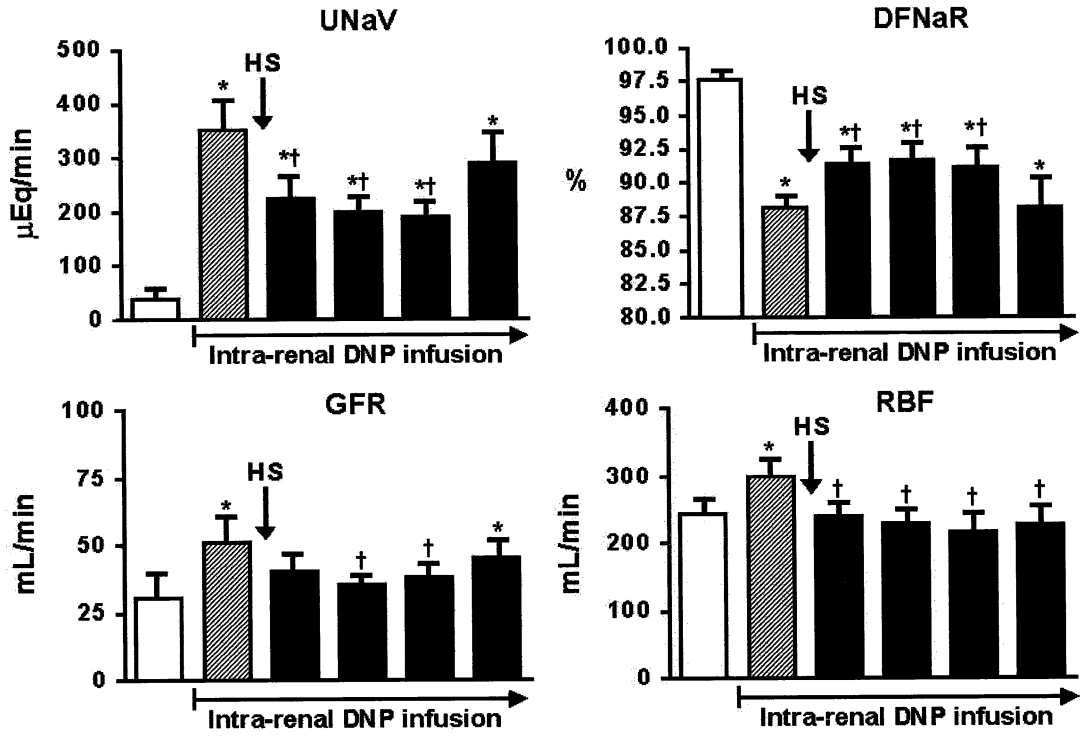

Figure 2. Urinary sodium excretion (UNaV), distal tubular fractional reabsorption of sodium (DFNaR), glomerular filtration rate (GFR), and renal blood flow (RBF) at baseline **(open bars)**, with intra-renal Dendroaspis natriuretic peptide (DNP) infusion alone **(hatched bars)** and 30, 60, 90, and 120 min after intravenous bolus administration of HS-142-1 (HS) **(solid bars)** in addition to continuous intra-renal infusion of DNP. *$p < 0.05$ versus baseline; †$p < 0.05$ versus intra-renal DNP alone.

increase with NEP inhibition; however, this did not reach statistical significance (Table 2).

DISCUSSION

Our current findings demonstrate that the natriuretic and renal hemodynamic actions of synthetic DNP, a potentially important new therapeutic agent, are attenuated by natriuretic peptide receptor antagonism, while NEP inhibition had no effect. These findings suggest that the biological actions of DNP are mediated, in part, via the natriuretic peptide particulate-guanylyl-cyclase receptors and that DNP is either resistant to degradation by NEP or is not a substrate at all. The current findings confirm previous experimental studies establishing that synthetic DNP has natriuretic, diuretic, renin-inhibiting properties and enhances RBF and GFR. More importantly, the current study extends previous investigations and defines the role of the natriuretic peptide particulate-guanylyl-cyclase receptors and NEP in mediating the renal actions of synthetic DNP.

Dendroaspis natriuretic peptide was administered intra-renally in order to define its renal actions without altering the MAP. Intra-renal DNP resulted in marked natriuresis, diuresis which was associated with increases in RBF, GFR, plasma and urinary cGMP excretion, and a decrease in distal fractional sodium reabsorption without a change in renal perfusion pressure. Despite the marked natriuresis and diuresis, renal vein renin activity was suppressed consistent with the renin-inhibiting actions of DNP. Furthermore, plasma and urinary cGMP increased with intra-renal DNP despite the fact the both plasma and urinary ANP and BNP were unchanged, therefore confirming that cGMP is indeed the second messenger of DNP.

HS-142-1 is an antagonist to natriuretic peptide particulate-guanylyl-cyclase receptors (6). This antagonist, which has been well-characterized, attenuated the natriuretic and diuretic actions of intra-renal DNP and abolished the increase in RBF and GFR. More importantly, both plasma and urinary cGMP returned to baseline with HS-142-1 despite continued intra-renal infusion of DNP, therefore supporting the conclusion that the renal actions of DNP are, in part, mediated via the natriuretic peptide particulate-guanylyl-cyclase receptors producing cGMP as a second messenger. Of note, HS-142-1 attenuated the natriuretic response and the decrease in DFNaR but did not abolish these actions completely. This may be due to the fact that these effects may persist for a longer duration as compared with the second messenger or DNP could also act on other receptors producing a different second messenger, which contributes to these actions. Further studies will be needed to address this issue.

Neutral endopeptidase 24-11 is an ectoenzyme that is found in abundance in the proximal tubules in the kidney (8). It metabolizes ANP, BNP, CNP, adrenomedullin, bradykinin, and other vasoactive peptides (9). Candoxatrilat

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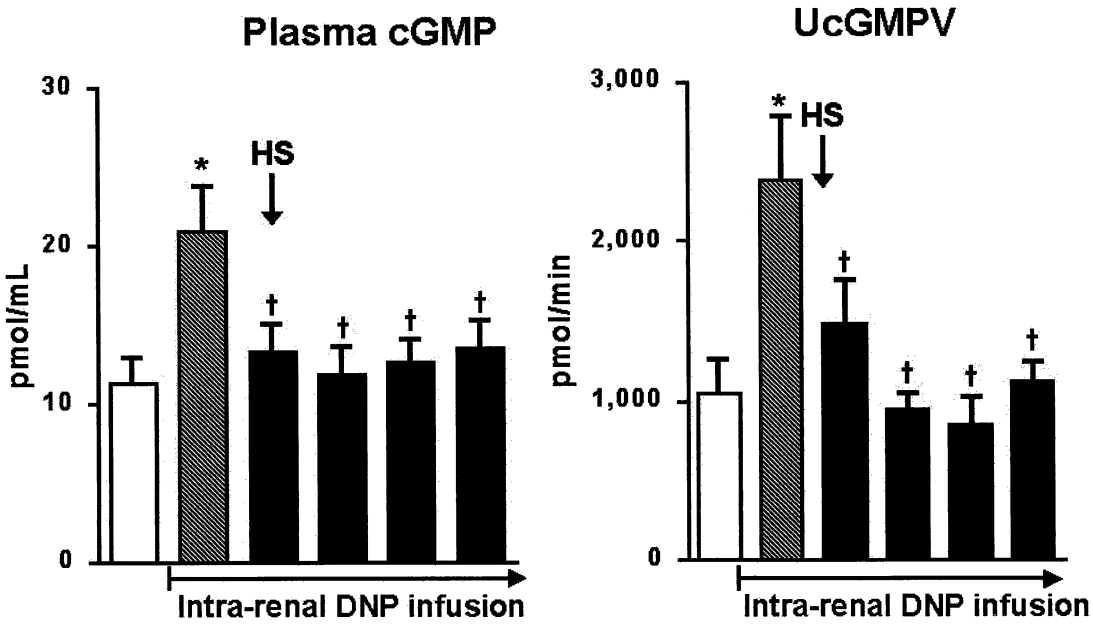

Figure 3. Plasma cGMP and urinary cGMP excretion (UcGMPV) at baseline **(open bars),** with intra-renal Dendroaspis natriuretic peptide (DNP) infusion alone **(hatched bars)** and 30, 60, 90, and 120 min after intravenous bolus administration of HS-142-1 (HS) **(solid bars)** in addition to continuous intra-renal infusion of DNP. *$p < 0.05$ versus baseline; $p < 0.05$ versus intra-renal DNP alone.

is an NEP inhibitor that potentiates the effects of both ANP and BNP by inhibiting their degradation by NEP. To ensure that there was adequate inhibition of NEP, we administered a higher dose of candoxatrilat, 80 μg/kg/min, which represents the peak dose response curve for candoxatrilat (unpublished data, Pfizer) to ensure that maximum inhibition was achieved (10).

In the current study, NEP inhibition with low- and high-dose candoxatrilat did not potentiate the renal actions of DNP. In contrast, this level of NEP inhibition has been shown in previous studies to potentiate the renal actions of exogenous ANP and BNP (8,11). Neither plasma nor urinary DNP concentration increased with NEP inhibition. However, urinary ANP excretion was significantly increased, therefore suggesting effective inhibition of NEP. There was a trend for urinary cGMP excretion to increase with NEP inhibition; however, this did not reach statistical significance. The lack of significant increase in UcGMPV with the NEP inhibitor candoxatrilat in normal dogs, despite increases in urinary ANP excretion, is consistent with our previous study (10) where we found similar results. This is most likely due to the normal baseline ANP level, which is not activated in normal dogs. Kinetic studies have shown that the rank order of hydrolysis by NEP is CNP > ANP > BNP, and it has been suggested that the longer length of the C-terminus of the peptide results in greater resistance to hydrolysis by NEP (12). Indeed, BNP has a longer C-terminus as compared with ANP, which, in turn, has a longer C-terminus than CNP. Dendroaspis natriuretic peptide, in contrast, has a 15-residue C-terminus that is longer when compared with BNP, ANP, and CNP. This structural difference may explain DNP's resistance to NEP degradation. Importantly, this resistance to degradation by NEP makes synthetic DNP unique among the known natriuretic peptides and underscores a therapeutic potential for synthetic DNP, especially in CHF in which NEP has been reported to be unregulated (13).

Conclusions. The current study is the first to report the role of the natriuretic peptide particulate-guanylyl-cyclase receptors and NEP in mediating the renal actions of synthetic DNP. The natriuretic and renal hemodynamic actions of DNP are attenuated by HS-142-1, while NEP inhibition did not potentiate the renal actions of DNP. These findings support our conclusion that the biological actions of DNP are mediated, in part, via the natriuretic

Table 2. Low- and High-Dose NEP Inhibition on DNP

	DNP	DNP + Low-Dose NEPI	DNP + High-Dose NEPI
UNaV, μEq/min	353 ± 61	370 ± 70	374 ± 54
DFNaR, %	85 ± 5	84 ± 5	83 ± 6
GFR, ml/min	53 ± 7	49 ± 3	60 ± 14
RBF, ml/min	335 ± 27	318 ± 23	321 ± 39
Plasma ANP, pg/ml	79 ± 23	74 ± 14	172 ± 78
UANPV, pg/min	24 ± 8	131 ± 53*	365 ± 125*
Plasma DNP, pg/ml	86 ± 43	105 ± 54	108 ± 60
UDNPV, pg/min	160 ± 82	170 ± 60	265 ± 40
Plasma BNP, pg/ml	19 ± 4	22 ± 7	20 ± 4
UBNPV, pg/min	13 ± 8	12 ± 6	13 ± 8
UcGMPV, pmol/min	1,737 ± 244	2,601 ± 384	3,204 ± 628

*$p < 0.05$ versus DNP. Mean ± SEM.

DFNaR = distal fractional sodium reabsorption; DNP = Dendroaspis natriuretic peptide; GFR = glomerular filtration rate; NEP = neutral endopeptidase 24-11; NEPI = neutral endopeptidase 24-11 inhibitor; RBF = renal blood flow; UANPV = urinary atrial natriuretic peptide excretion; UBNPV = urinary brain natriuretic peptide; UcGMP = urinary cGMP excretion; UDNPV = urinary excretion; UNaV = urinary sodium excretion.

peptide particulate-guanylyl-cyclase receptors and that DNP is either resistant to degradation by NEP or not a substrate at all. These studies also establish the unique property of DNP, which is its resistance to NEP degradation, supporting its potential as a therapy for cardiovascular diseases such as CHF.

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Reprint requests and correspondence: Dr. Horng H. Chen, Cardiorenal Research Laboratory, Guggenheim 915, Mayo Clinic and Foundation, 200 First Street Southwest, Rochester, Minnesota 55905. E-mail: chen.horng@mayo.edu.

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