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Renal actions of synthetic Dendroaspis natriuretic peptide

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Renal actions of synthetic Dendroaspis natriuretic peptide.

Background. Dendroaspis natriuretic peptide (DNP), recently isolated from the venom of the green Mamba snake *Dendroaspis angusticeps*, is a 38 amino acid peptide containing a 17 amino acid disulfide ring structure similar to that of atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and C-type natriuretic peptide (CNP). DNP-like immunoreactivity (DNP-LI) was reported to be present in human plasma and atrial myocardium and to be elevated in human congestive heart failure. Although previously named DNP, it remains unknown if DNP is natriuretic or if is it present in canine plasma, urine, and atrial myocardium.

Method. Studies were performed *in vivo* in anesthetized dogs ($N = 6$) using intravenous infusion of synthetic DNP at 10 and 50 ng/kg/min. Employing a sensitive and specific radioimmunoassay for DNP, the presence of DNP-like peptide was assessed in the canine plasma and urine before, during, and following the administration of exogenous synthetic DNP. Additionally, we performed immunohistochemical studies using the indirect immunoperoxidase method with polyclonal DNP antiserum in normal atrial myocardium ($N = 10$). Atrial concentrations of DNP-LI were also assessed.

Results. We report that DNP is markedly natriuretic and diuretic, which, like ANP and BNP, is associated with the increase in urinary and plasma cGMP. DNP-like peptide is also detected in canine plasma, urine, and atrial myocardium.

Conclusion. These studies establish that DNP is a potent natriuretic and diuretic peptide with tubular actions linked to cGMP and that DNP may play a physiological role in the regulation of sodium excretion.

Investigations have established the existence of a family of structurally similar but genetically distinct natriuretic peptides that consist of atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) of myocardial cell origin, and C-type natriuretic peptide (CNP) of endothelial cell origin [1, 2]. ANP and BNP bind to the natriuretic peptide A-receptor (NPR-A), which, via 3′5′-cyclic guanosine monophosphate (cGMP), mediate the biological actions of natriuresis, vasodilation, renin inhibition, and lusitropism [3–5]. CNP lacks renal actions, but is vasodilating and growth inhibiting [2, 6]. CNP mediates its actions through the natriuretic peptide β-receptor (NPR-B), which is also linked to particulate guanylyl cyclase and cGMP generation [7]. Although this family of peptides now consists of three known peptides, the discovery of additional genes encoding putative guanylyl cyclases has raised the question about the possible existence of numerous ligands for these receptors yet to be discovered in mammal species [8]. Recently, additional natriuretic peptides have been isolated and characterized, particularly from the venom of the snakes *Dendroaspis angusticeps*, *Micrurus corallinus*, and *Bothrops jararaca* [9–11]. The physiological role of these peptides, as well as their presence in other species, remains unclear.

One of these newly characterized peptides possessing a structural similarity to the natriuretic peptide family, which was isolated from the venom of *Dendroaspis angusticeps*, or green Mamba snake [9], is a 38-amino acid peptide containing a 17-amino acid disulfide ring structure with a 15-residue C terminal extension (Fig. 1). This peptide potently vasorelaxes isolated rodent aorta [9] and canine coronary arteries with comparable potency to ANP (abstract; Wennberg and Burnett, *J Am Coll Cardiol* 29:305A, 1997). Additionally, this peptide augments the formation of cGMP in aortic endothelial and smooth muscle cells and displaces ANP binding from the natriuretic peptide receptors [9]. Most recently, we have reported that Dendroaspis natriuretic peptide (DNP)-like immunoreactivity (DNP-LI) is present in human plasma and atrial myocardium and is elevated in the plasma of humans with congestive heart failure [12]. We also determined that DNP levels in normal human plasma averaged 6 pg/ml with a range from 2 to 11 pg/ml. In human congestive heart failure (New York Heart Association guidelines; NYHA III or IV), we reported that DNP plasma levels averaged 37 pg/ml with a range from 3 to 200 pg/ml.

Key words: myocardium, natriuretic hormones, cGMP, sodium excretion, *Dendroaspis angusticeps.*

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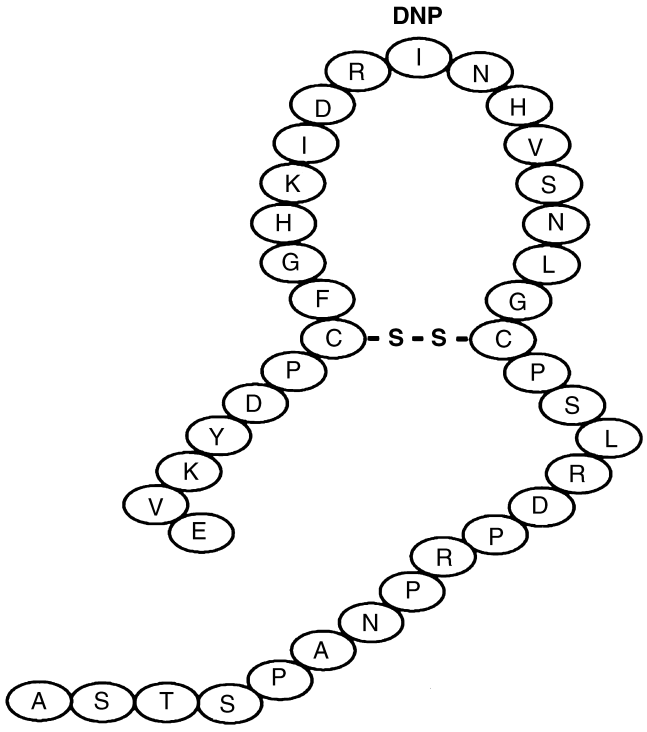

Fig. 1. Amino acid sequence and structure of Dendroaspis natriuretic peptide. The amino acid sequence was originally reported by Schweitz et al [9].

Although this peptide, based on its structural similarity to ANP, BNP, and CNP, was named DNP, it remains unknown if DNP is natriuretic. Indeed, the renal actions of DNP are undefined.

Based on the structural similarity to the other natriuretic peptides and the report that DNP activates cGMP *in vitro*, we hypothesized that DNP is a natriuretic and diuretic peptide. We therefore investigated the renal actions of synthetic DNP *in vivo* in normal anesthetized dogs. We also determined whether or not DNP-LI is present in canine plasma, urine, and atrial myocardium.

METHODS

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

On the evening before the experiment, 300 mg of lithium carbonate were administered orally for the assessment of renal tubular function, and dogs were fasted overnight. On the day of the acute experiment, all dogs were anesthetized with pentobarbital sodium given intravenously (30 mg/kg). Supplemental nonhypotensive doses of pentobarbital sodium were given as needed during the experiment. After tracheal intubation, dogs were mechanically ventilated (Harvard respirator; Harvard Apparatus, Millis, MA, USA) with 4 liter/min of supplemental oxygen.

Left lateral flank incisions were made, and the left kidney was exposed via a retroperitoneal approach. The ureter was cannulated with polyethylene catheters (PE-200) for a timed urine collection, and a calibrated noncannulating electromagnetic flow probe was placed carefully around the left renal artery and connected to a flowmeter (model FM 5010, Carolina Medical Electronics, King, NC, USA) for continuous monitoring of renal blood flow (RBF). Finally, the right femoral vein was cannulated with two polyethylene catheters (PE-240), one for infusion of inulin and the other for the infusion of DNP (DNP 1-38; Phoenix Pharmaceuticals, Inc., Mountain View, CA, USA). The right femoral artery was cannulated with a polyethylene catheter (PE-240) for direct arterial blood pressure measurement and arterial blood sampling.

After completion of the surgical preparation, a priming dose of inulin (ICN Biomedicals, Cleveland, OH, USA) dissolved in isotonic saline solution was injected, followed by a constant infusion of 1 ml/min to achieve a steady-state plasma inulin concentration between 40 and 60 mg/dl. The dogs were placed in dorsal suspension and allowed to equilibrate for 60 minutes without intervention. Body temperature was maintained by external warming (infrared heating lamp).

After an equilibration period of 60 minutes, a 30-minute baseline clearance (baseline) was performed. This was followed by a 15-minute lead-in period, during which DNP infusion at 10 ng/kg/min was begun intravenously, after which the second 30-minute clearance (DNP-10) period was performed. After the second clearance period, the intravenous infusion of DNP was changed to 50 ng/kg/min. After a 15-minute lead-in period with this dose of DNP, a 30-minute clearance (DNP-50) was performed. At the end of the third clearance, the DNP infusion was stopped and a 150-minute washout period followed with a 30-minute recovery clearance (recovery).

Analytical methods

Plasma for electrolyte and inulin measurements was obtained from blood collected in heparinized tubes. Plasma and urine electrolytes including lithium were measured by flame-emission spectrophotometer (IL943, Flame Photometer; Instrumentation Laboratory, Lexington, MA, USA). Plasma and urine inulin concentrations were measured by the anthrone method, and the glomerular filtration rate (GFR) was measured by the clearance of inulin. The lithium clearance technique was

employed to estimate the proximal and distal fractional reabsorption of sodium. Proximal fractional reabsorption was calculated by the following formula: [1 − (lithium clearance/GFR)] × 100. Distal fractional reabsorption of sodium was calculated by this formula: [(lithium clearance − sodium clearance)/lithium clearance] × 100.

Plasma and urinary cGMP were measured by radioimmunoassay using the method of Steiner, Parker, and Kipnis [13]. Urine for cGMP measurement was heated to 90°C before storage at −20°C to inhibit degradative enzymatic activity.

Plasma and urinary DNP-LI was determined using a specific and sensitive radioimmunoassay for DNP before, during, and following the DNP administration. Blood was collected in chilled tubes containing ethylenediaminetetraacetic acid (EDTA) and immediately placed on ice. After centrifugation at 2500 r.p.m. at 4°C for 10 minutes, the plasma was decanted and stored at −20°C until analyzed. Plasma (1 ml) was extracted on C-8 Bond Elut cartridges, which were washed with methanol and distilled water. DNP was eluted with 95% methanol containing 1% trifluoroacetic acid (TFA). Concentrated elutes were then assayed with a specific and sensitive radioimmunoassay for DNP (Phoenix Pharmaceuticals). Urinary DNP-LI was measured directly by this radioimmunoassay without extraction. Samples and standards were incubated with rabbit anti-DNP at 4°C for 18 hours. ^{125}I-labeled DNP (100 μl) was added and incubated for another 18 hours at 4°C. Free and bound fractions were then separated by the addition of a second antibody/normal rabbit serum solution and were centrifuged. Radioactivity of the bound fraction was measured with a γ counter. The minimal detectable level for this assay was 0.5 pg per tube, and the IC_{50} of the standard curve was 29.0 pg. Recovery was 83.0 ± 1.8%, and the intra-assay coefficient of variation (CV) was 10.0 ± 1.8% and interassay CV was 12.0 ± 1.5%. There was no cross-reactivity between the DNP assay and the ANP, BNP, or CNP assays. We challenged the DNP radioimmunoassay with increasing concentrations of ANP, BNP, and CNP over a range from 0.5 to 500 pg while also challenging the ANP, BNP, and CNP assay with increasing concentrations of DNP over a range from 0.5 to 500 pg. We detected no cross-reactivity between the ANP, BNP, CNP, and DNP assays. The second methodology, which was used to test the specificity of the radioimmunoassay for DNP, was immunoblotting. Immunoblotting was performed employing antibodies to DNP using synthetic ANP, BNP, CNP, and DNP at the concentrations of 100, 10, 1, and 0.1 ng, with no cross-reactive staining for ANP, BNP, or CNP. Positive and dose-dependent immunostaining was noted with the DNP antibody at concentrations of DNP at 100, 10, and 1 ng.

Atrial tissue concentration of DNP-LI was determined in the 10 normal dogs not exposed to DNP infusion employing the same sensitive and specific radioimmunoassay.

Immunohistochemical methods

Sections for immunohistochemical staining were taken from atrial free walls from 10 normal dogs not exposed to exogenous DNP. Immunohistochemical studies were performed by the indirect immunoperoxidase method as described previously [14]. Tissues were immediately fixed with 10% buffered formalin and were embedded in paraffin, and sections 6 μm thick were cut and mounted on salinized glass slides. The sections were deparaffinized with graded concentrations of xylene and were hydrated with ethanol. To block the activity of endogenous peroxidase, the slides were incubated with 0.6% hydrogen peroxide in methanol for 20 minutes at room temperature. After being washed, sections were incubated in 5% normal goat serum (Dako, Carpinteria, CA, USA) for 10 minutes at room temperature to reduce nonspecific background staining and were then incubated with polyclonal rabbit anti-DNP (Phoenix Pharmaceuticals) at a dilution of 1:500 (in normal goat serum) in humidified chambers for 18 hours at room temperature. All slides were incubated for 30 minutes with the second antibody-horseradish peroxidase conjugate (BioSource, Camarillo, CA, USA). The reaction was visualized by incubating the sections with a solution of 3′-amino-9′-ethylcarbazole (Sigma, St. Louis, MO, USA) in dimethylformamide and sodium acetate. The sections were counterstained with hematoxylin and were coverslipped and reviewed with an Olympus microscope. Control sections were stained with 1% nonimmune goat serum. Specificity of immunostaining was confirmed by adsorption testing.

Statistical analysis

Results of quantitative studies are expressed as mean ± SE. Statistical comparisons were performed using repeated-measures analysis of variance, followed by the post hoc Bonferroni test. Statistical significance was accepted for a P value of less than 0.05.

RESULTS

Plasma DNP and urinary DNP excretion are shown in Figure 2. Employing the specific and sensitive radioimmunoassay, plasma and urinary DNP-LI were detectable prior to infusion of exogenous DNP. Both plasma and urinary DNP excretion increased during DNP infusion and decreased during the recovery period.

Urinary sodium excretion ($U_{Na}V$), urine flow (UV), plasma cGMP, and urinary cGMP excretion ($U_{cGMP}V$) in response to exogenous DNP are illustrated in Figure 3. Low-dose DNP (10 ng/kg/min) increased $U_{Na}V$ in association with increases in plasma cGMP and $U_{cGMP}V$. High-dose DNP (50 ng/kg/min) further increased $U_{Na}V$ with a

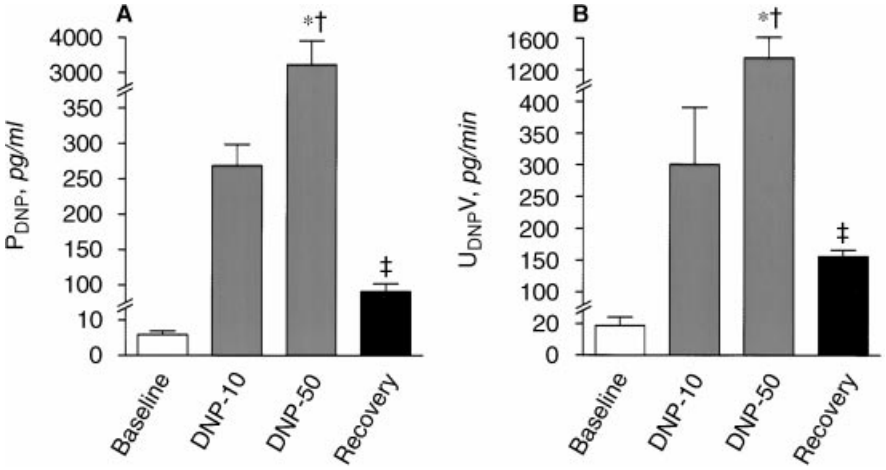

Fig. 2. Plasma Dendroaspis natriuretic peptide (P_{DNP}; *A*) and urinary DNP excretion ($U_{DNP}V$; *B*). Abbreviations are: Baseline, baseline 30-minute clearance; DNP-10, 30-minute clearance with 10 ng/kg intravenous infusion of DNP; DNP-50, 30-minute clearance with 50 ng/kg intravenous infusion of DNP; Recovery, 30-minute clearance after a 150-minute washout period. Repeated-measures analysis of variance followed by post hoc Bonferroni test was used for statistical comparisons between clearances. $*P < 0.05$ vs. Baseline; $\dagger P < 0.05$ vs. DNP-10; $\ddagger P < 0.05$ vs. DNP-50.

significant increase in UV. These natriuretic and diuretic responses to high-dose DNP (DNP-50 clearance) were also associated with increases in plasma cGMP and $U_{cGMP}V$ compared with baseline and with the previous clearance (DNP-10 clearance). Following DNP infusion, $U_{Na}V$ and UV decreased to the baseline values. These decreases were accompanied by decreases in plasma cGMP and $U_{cGMP}V$.

Table 1 reports the mean arterial pressure (MAP) and renal function effects of exogenous DNP. Low-dose DNP increased fractional sodium excretion (FE_{Na}) and decreased distal fractional reabsorption of sodium (DFR_{Na}). MAP, GFR, and RBF were unchanged. High-dose DNP decreased MAP in association with a further increase in FE_{Na} and decrease in DFR_{Na} without changes in GFR or RBF. During the recovery period, MAP returned to near baseline levels with a decrease in RBF. GFR remained unchanged during the experiment, whereas DFR_{Na} increased to baseline levels during recovery period. Proximal fractional reabsorption of sodium tended to decrease during DNP administration (PFR_{Na} at baseline, 63 ± 6; low-dose DNP, 58 ± 7; high-dose DNP, 58 ± 4; recovery, 72 ± 6%) but did not reach a significant level. Plasma ANP, BNP, or CNP did not increase during or after DNP administration (Table 2).

Atrial myocardial presence of DNP immunoreactivity was determined by measurement of tissue DNP concentration in normal dogs not exposed to exogenous DNP administration. Atrial tissue concentrations of DNP were detectable at a concentration of 1950 ± 197 pg/g of wet tissue. DNP-LI was also present in atrial myocardium of these dogs using immunohistochemical studies (Fig. 4). DNP immunoreactivity was located in the perinuclear region of canine atrial myocytes.

DISCUSSION

This study demonstrates for the first time, to our knowledge, that DNP has potent natriuretic and diuretic properties in normal dogs and that DNP-LI is present in canine plasma, urine, and atrial myocardium. DNP-mediated natriuresis and diuresis were associated with parallel increases in plasma cGMP and urinary cGMP excretion independent of increases in plasma ANP, BNP, or CNP. Finally, the excretory responses to exogenous DNP administration were associated with decreases in distal tubular reabsorption of sodium and arterial pressure in the absence of alterations in GFR or RBF.

Similar to the renal responses to ANP and BNP [15, 16], DNP increased urinary cGMP excretion and decreased distal tubular reabsorption of sodium. These observations support the conclusion that DNP may function through the NPR-A receptor, which is linked to particulate guanylyl cyclase and cGMP generation and is also highly expressed in inner medullary collecting duct cells [15]. Such a conclusion is also supported by the report that DNP displaces ANP binding in vascular smooth muscle cells [9]. We cannot, however, exclude the existence of a new, yet to be identified natriuretic peptide receptor. In addition, the lack of increase in plasma ANP, BNP, and CNP in this study during DNP administration also suggests that the renal actions of DNP are direct and not mediated by the other natriuretic peptides. In response to high-dose DNP, MAP decreased and returned to near baseline following discontinuation of DNP administration. Such hypotensive action is shared by the other natriuretic peptides, which is secondary to decreases in both cardiac preload and afterload [17–19].

In this study, we cannot exclude that DNP may also enhance sodium excretion by increasing the medullary blood flow. The decrease in RBF observed during the recovery period, which followed a 150-minute washout, may reflect responses to intravascular volume contraction following natriuresis and diuresis. An additional explanation could be a similarity to the intrarenal actions of ANP, which were initially reported to be associated

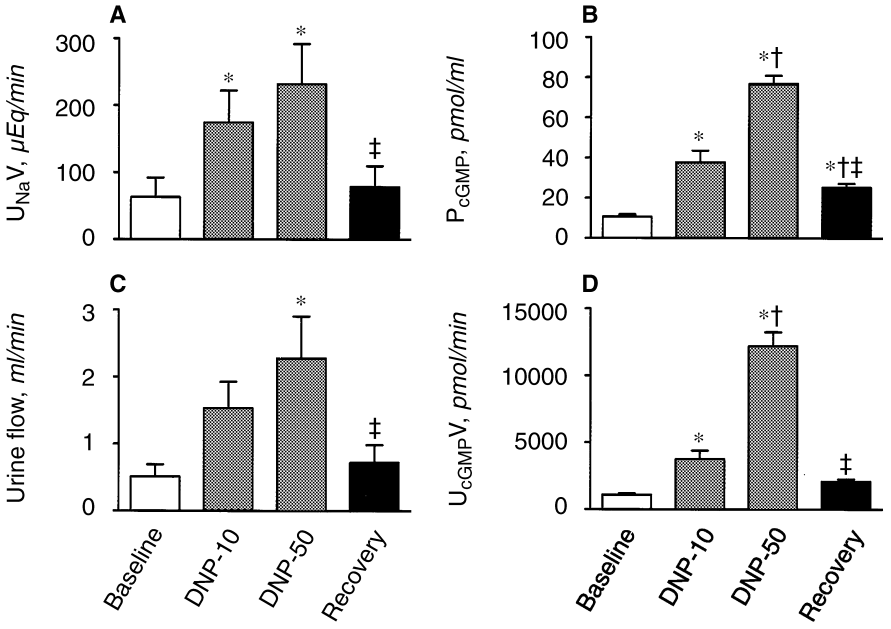

Fig. 3. Humoral and renal responses to Dendroaspis natriuretic peptide (DNP) administration. Abbreviations are: Baseline, baseline 30-minute clearance; DNP-10, 30-minute clearance with 10 ng/kg intravenous infusion of DNP; DNP-50, 30-minute clearance with 50 ng/kg intravenous infusion of DNP; Recovery, 30-minute clearance after a 150-minute washout period. A repeated measures analysis of variance followed by post hoc Bonferroni test was used for statistical comparisons between clearances. *$P < 0.05$ vs. Baseline; †$P < 0.05$ vs. DNP-10; ‡$P < 0.05$ vs. DNP-50.

Table 1. Dendroaspis natriuretic peptide (DNP)-mediated changes in renal function

	Baseline	DNP-10	DNP-50	Recovery
MAP *mm Hg*	141 ± 6	128 ± 8	109 ± 7[ab]	132 ± 8
GFR *ml/min*	37 ± 2	41 ± 4	39 ± 2	26 ± 7
RBF *ml/min*	303 ± 17	279 ± 21	276 ± 43	220 ± 32
FE_{Na} %	1.2 ± 0.5	2.9 ± 1.0[a]	3.9 ± 0.9[a]	1.2 ± 0.4
DFR_{Na} %	97 ± 1	94 ± 2[a]	91 ± 2[a]	95 ± 1

Abbreviations are: Baseline, baseline 30-min clearance; DNP-10, 30-min clearance with 10 ng/kg i.v. infusion of DNP; DNP-50, 30-min clearance with 50 ng/kg i.v. infusion of DNP; Recovery, 30-min clearance after 150-min washout period; MAP, mean arterial pressure; GFR, glomerular filtration rate; RBF, renal blood flor; FE_{Na}, fractional sodium excretion; DFR_{Na}, distal fractional sodium reabsorption. Values are expressed as means ± standard errors.

[a] $P < 0.05$ vs. Baseline

[b] $P < 0.05$ vs. DNP-10

Table 2. Plasma natriuretic peptides during DNP administration

	Baseline	DNP-10	DNP-50	Recovery
ANP *pg/ml*	17.4 ± 0.7	17.6 ± 0.4	18.1 ± 1.2	16.1 ± 0.6
BNP *pg/ml*	10.1 ± 1.4	12.9 ± 2.6	12.6 ± 2.0	9.6 ± 2.3
CNP *pg/ml*	9.7 ± 0.5	9.1 ± 0.5	9.5 ± 0.9	9.8 ± 0.8

Abbreviations are: Baseline, baseline 30-min clearance; DNP-10, 30-min clearance with 10 ng/kg i.v. infusion of DNP; DNP-50, 30-min clearance with 50 ng/kg i.v. infusion of DNP; Recovery, 30-min clearance after 150-min washout period; ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; CNP, C-type natriuretic peptide. Values are expressed as means ± standard errors.

with a transient renal vasodilation followed by renal vasoconstriction [4, 20].

We chose two different doses for DNP to establish a broad range of plasma concentrations. Importantly, the lower dose achieved circulating concentrations of approximately 250 pg/ml, which we now know are at the upper range of those observed in human heart failure and thus may be considered pathophysiological. The higher dose clearly establishes the pharmacological actions of synthetic DNP by achieving concentrations of approximately 3000 pg/ml. Although the high dose represents pharmacological actions, the observed renal responses provide insight into the potential therapeutic utility of DNP.

In a recent study, our group reported the presence of DNP-LI in human plasma and atrial myocardium, with elevated concentrations in plasma in humans with congestive heart failure [12]. This study importantly confirms this previous report and extends the report in humans to a nonhuman mammalian species. Specifically, we report DNP-LI presence in canine plasma, urine, and atrial myocardium employing radioimmunoassay and immunohistochemical methods, which possess no cross-reactivity to the other known natriuretic peptides.

Further studies are clearly required specifically to identify the DNP gene and to determine precise species amino acid sequences for DNP. Future studies employing acute and chronic increases in cardiac filling pressures as well as DNP antibodies will be needed to address release mechanisms for endogenous DNP, as well as the physiological role of DNP in sodium homeostasis. These studies are beyond the scope of this study, which defined, to our knowledge for the first time, the renal actions of synthetic DNP.

In summary, we report that intravenous administration of synthetic DNP results in natriuresis and diuresis in normal dogs and that DNP-LI is present in canine plasma, urine, and atrial myocardium. This natriuresis and diuresis is associated with activation of the cGMP

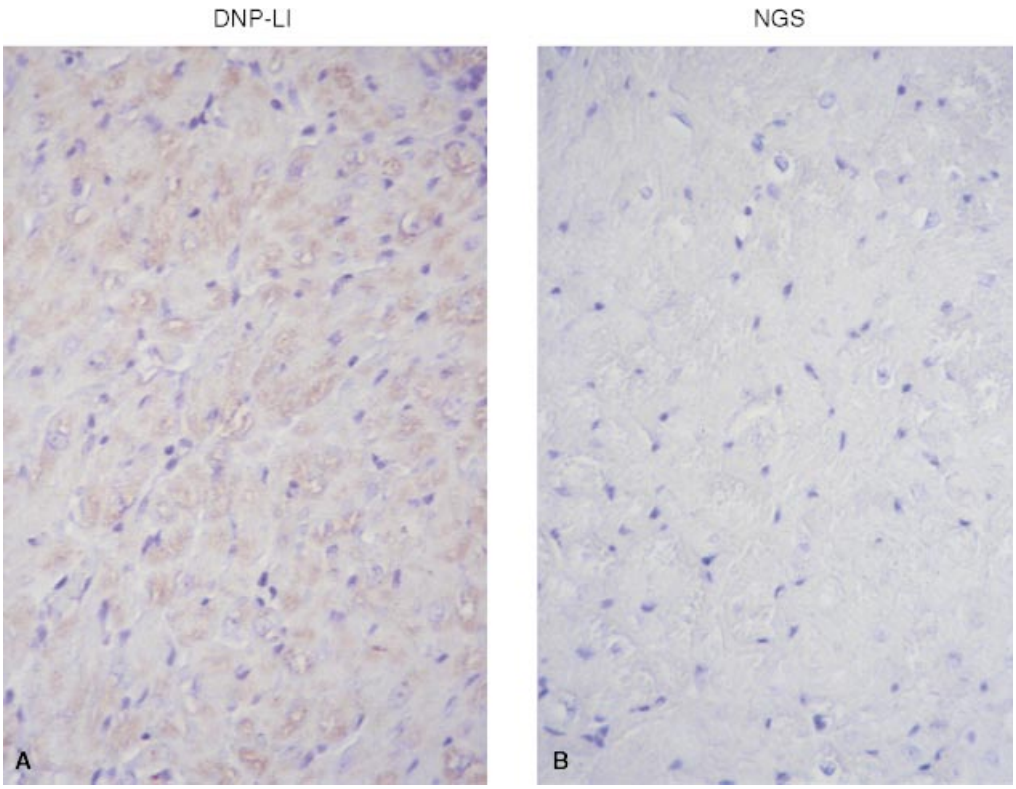

Fig. 4. Immunohistochemical staining for DNP-like peptide in the canine atrium. Representative sample of atrial myocardium from 10 normal dogs not exposed to exogenous DNP. NGS means normal goat serum (original magnification, ×400).

system and is localized to the terminal nephron independent of changes in GFR, RBF, or plasma concentrations of ANP, BNP, or CNP.

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APPENDIX

Abbreviations used in this article are: ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; cGMP, cyclic 3′5′ guanosine monophosphate; CNP, C-type natriuretic peptide; DFR_{Na}, distal fractional reabsorption of sodium; DNP, Dendroaspis natriuretic peptide; DNP-LI, DNP-like immunoreactivity; GFR, glomerular filtration rate; MAP, mean arterial pressure; PFR_{Na}, proximal fractional reabsorption of sodium; RBF, renal blood flow; RIA, radioimmunoassay; $U_{cGMP}V$, urinary cGMP excretion; $U_{Na}V$, urinary sodium excretion; UV, urine flow.

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