

A Human Atrial Natriuretic Peptide Gene Mutation Reveals a Novel Peptide With Enhanced Blood Pressure-Lowering, Renal-Enhancing, and Aldosterone-Suppressing Actions

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Objectives	We sought to determine the physiologic actions and potential therapeutic applications of mutant atrial natriuretic peptide (mANP).
Background	The cardiac hormone atrial natriuretic peptide (ANP) is a 28-amino acid (AA) peptide that consists of a 17-AA ring structure together with a 6-AA N-terminus and a 5-AA C-terminus. In a targeted scan for sequence variants within the human ANP gene, a mutation was identified that results in a 40-AA peptide consisting of native ANP ₍₁₋₂₈₎ and a C-terminal extension of 12 AA. We have termed this peptide mutant ANP.
Methods	In vitro 3',5'-cyclic guanosine monophosphate (cGMP) activation in response to mANP was studied in cultured human cardiac fibroblasts known to express natriuretic peptide receptor A. The cardiorenal and neurohumoral properties of mANP compared with ANP were assessed in vivo in normal dogs.
Results	We observed an incremental in vitro cGMP dose response with increasing concentrations of mANP. In vivo with high-dose mANP (33 pmol/kg/min), we observed significantly greater plasma cGMP activation, diuretic, natriuretic, glomerular filtration rate enhancing, renin-angiotensin-aldosterone system inhibiting, cardiac unloading, and blood pressure lowering properties when compared with native ANP. Low-dose mANP (2 pmol/kg/min) has natriuretic and diuretic properties without altering systemic hemodynamics compared with no natriuretic or diuretic response with low-dose native ANP.
Conclusions	These studies establish that mANP activates cGMP in vitro and exerts greater and more sustained natriuretic, diuretic, glomerular filtration rate, and renal blood flow enhancing actions than native ANP in vivo. (J Am Coll Cardiol 2009;54:1024-32) © 2009 by the American College of Cardiology Foundation

Atrial natriuretic peptide (ANP) is a 28-amino acid (AA) peptide that consists of a 17-AA ring formed by a disulfide bond together with a 6-AA N-terminus and a 5-AA C-terminus. Studies in animal models of altered ANP production or receptor function as well as studies in humans with ANP infusion have demonstrated that ANP plays an important role in integrated cardiorenal function; ANP possesses natriuretic, vasodilatory, lusitropic, renal enhanc-

ing, and renin-angiotensin-aldosterone system (RAAS) inhibiting properties through activation of the natriuretic peptide receptor A (NPR-A) and generation of the second messenger 3',5'-cyclic guanosine monophosphate (cGMP) (1-6). By activating NPR-A, ANP also is antihypertrophic and antifibrotic, and genetic deletion of either the ANP

See page 1033

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gene (*Nppa*) or NPR-A results in hypertension, cardiac hypertrophy, and fibrosis (7-12). Importantly, ANP is cleared from the circulation through degradation by neutral endopeptidase (NEP) and by a receptor mechanism after binding to the natriuretic peptide receptor C (NPR-C) (13,14).

Most recently, mutations for the gene encoding pro-ANP have been reported. Rubattu et al. (15) reported that hypertensive subjects carrying an allelic variant in the ANP

gene promoter demonstrated increased left ventricular mass index as compared with the wild-type genotype in association with lower plasma pro-ANP levels. In contrast, a recent report involving the Woman's Health Study reported that the presence of an ANP promoter polymorphism is associated with a decrease in the development and progression of hypertension (16).

We recently identified an ANP gene mutation in a Caucasian family with familial atrial fibrillation (17). Translation of the mutant gene results in a fusion protein consisting of the normal 28-AA mature native ANP plus an anomalous C-terminus possessing 12 additional residues (Fig. 1). We have termed this 40-AA peptide mutant ANP (mANP).

Previous studies have demonstrated that the C-terminus of native ANP enhances the biological actions of ANP based upon augmenting interaction with NPR-A (18). Further, *Dendroaspis* natriuretic peptide (DNP), which also binds NPR-A, possesses an extended C-terminus (15 AA). The DNP possesses greater resistance to degradation by NEP compared with other natriuretic peptides, demonstrating that the extended C-terminus contributes to the potent biological properties of DNP (19).

In the current studies, we hypothesized that mANP would in vitro activate cGMP using cardiac fibroblasts, which are known to highly express NPR-A (20). Most importantly, using in vivo models, we hypothesized that mANP, compared with native ANP, would possess more sustained biological actions in the control of cardiorenal function and in suppression of the RAAS.

To test these hypotheses, we synthesized mANP and performed studies in vitro and in vivo to determine the ability of mANP and native ANP to activate cGMP, the second messenger of ANP, and to characterize the cardiorenal and RAAS-suppressing properties of mANP and native ANP. In vivo studies were performed in normal anesthetized dogs. The identification of this familial mutation provided the opportunity to better understand the

biology of ANP and the important role of the C-terminus in mediating biological activities.

Methods

Peptides. Mutant ANP was synthesized by the Mayo Protein Core Facility using solid phase methods, as previously described (17,21). Native ANP was purchased from Phoenix (Mountain View, California). The structure of each peptide was confirmed by mass spectrometry, and HPLC analysis confirmed the purity of each peptide to be >90%.

Cell culture. Human cardiac fibroblasts (ScienCell, San Diego, California) were cultured in fibroblast media (ScienCell) and supplemented with fibroblast growth serum, fetal bovine serum, and penicillin/streptomycin, as previously described (22). Cultured fibroblasts with 1 to 4 passages were treated with mANP or native ANP, and cGMP generation was determined (23).

Intracellular cGMP. Cells were treated at 80% to 90% confluency, as described previously (22). Briefly, cells were incubated in Hank's balanced salt solution (Invitrogen, Carlsbad, California) containing 20 mmol/l N-[2-hydroxyethyl]piperazine-N'[2-ethanesulfonic acid], 0.1% bovine serum albumin, and 0.5 mmol/l 3-isobutyl-1-methylxanthine (Sigma, St. Louis, Missouri). Treated cells received no treatment (control), ANP (10^{-6} M), or mANP (10^{-6} M, 10^{-8} M, or 10^{-11} M) for 10 min (Fig. 2). Of note, we performed preliminary studies evaluating cGMP generation to various incubation times with the 2 peptides, including 10, 30, and 60 min. We found that 10 min provided a maximal response in cGMP. Studies were performed in triplicate for each concentration of ANP, mANP, or control. Cells were then lysed in 6% TCA and sonicated for 10 min. The samples were ether extracted 4 times in ether, dried, and reconstituted in 500 μ l cGMP assay buffer. The samples were assayed using competitive radioimmunoassay cGMP (Perkin-Elmer, Boston, Massachusetts), as previously described (22).

In vivo experimental protocol. Studies were performed in normal male mongrel dogs (21 to 26 kg) on a fixed sodium diet (58 mEq/day, Hill's ID, Topeka, Kansas) for at least 5 days before experiments, with free access to drinking water. The study was performed in accordance with the Animal Welfare Act and with approval of the Mayo Clinic Institutional Animal Care and Use Committee.

Abbreviations and Acronyms

- AA** = amino acid
- ANP** = atrial natriuretic peptide
- cGMP** = 3',5'-cyclic guanosine monophosphate
- CL_L** = lithium clearance
- DFR_{Na}** = distal fractional reabsorption of sodium
- DNP** = *Dendroaspis* natriuretic peptide
- GFR** = glomerular filtration rate
- mANP** = mutant atrial natriuretic peptide
- MAP** = mean arterial blood pressure
- NEP** = neutral endopeptidase
- NPR** = natriuretic peptide receptor
- PCWP** = pulmonary capillary wedge pressure
- PFR_{Na}** = proximal fractional reabsorption of sodium
- RAAS** = renin-angiotensin-aldosterone system
- RF** = renal blood flow

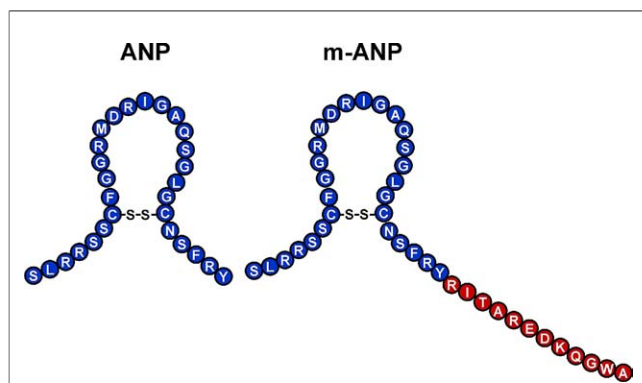
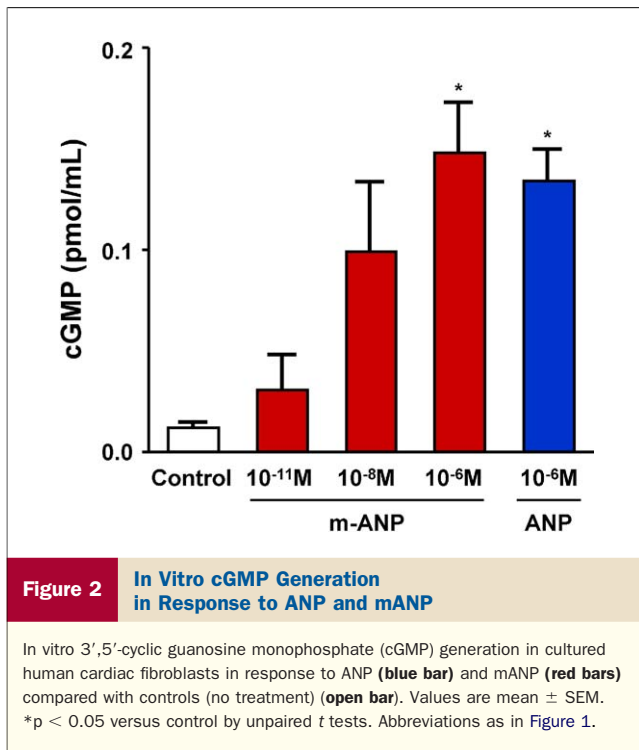


Figure 1. Amino Acid Sequence of ANP and mANP

Amino acid sequence and structure of atrial natriuretic peptide (ANP) and mutant atrial natriuretic peptide (mANP).



The night before experimentation, dogs were fasted and given 300 mg lithium carbonate for assessment of renal tubular function. All studies were initiated between 8:00 AM and 10:00 AM. On the day of the experiment, dogs were anesthetized with pentobarbital sodium (15 mg/kg intravenous), intubated, and mechanically ventilated with supplemental oxygen (Harvard respirator, Amersham, Massachusetts) at 12 cycles/min. A flow-directed balloon-tipped thermodilution catheter was advanced to the pulmonary artery through the external jugular vein for measurement of cardiac filling pressures and cardiac output. The femoral artery was cannulated for mean arterial blood pressure (MAP) monitoring and blood sampling. The femoral vein was cannulated for inulin and normal saline infusion. Through a left lateral flank incision, the left kidney was exposed and the ureter was cannulated for urine sampling. A calibrated electromagnetic flow probe (Carolina Medical Electronics, East Bend, North Carolina) was placed around the renal artery to measure renal blood flow (RBF). Supplemental nonhypotensive doses of pentobarbital were administered as needed during the experiment.

The study protocol started with the administration of a weight-adjusted inulin bolus. Continuous inulin and saline infusions at a rate of 1 ml/min each were started. After 60 min of equilibrium, a baseline clearance was performed. All clearances lasted 30 min and consisted of urine collection over 30 min. Arterial blood sampling and hemodynamic measurements were measured midway through each clearance. After the baseline clearance, the saline infusion was replaced by either high-dose native ANP (n = 7), high-dose mANP (n = 7), low-dose native ANP (n = 7), or low-dose

mANP (n = 7). High dose was defined as 33 pmol/kg/min and low dose as 2 pmol/kg/min. Peptides were infused for a total of 45 min, which included a 15-min lead-in period followed by a 30-min clearance. Peptide infusion was then discontinued, and 4 30-min clearances were performed (washout, recovery 1, recovery 2, and recovery 3).

Cardiovascular parameters measured included MAP, cardiac output, and pulmonary capillary wedge pressure (PCWP). Cardiac output was measured by thermodilution (cardiac output computer model 9510-A, American Edwards Laboratories, Irvine, California). Systemic vascular resistance (SVR) was calculated as (MAP minus right atrial pressure) divided by cardiac output. Glomerular filtration rate (GFR) was measured by inulin clearance.

Neurohormonal and electrolyte analysis. Plasma and urine ANP were measured by radioimmunoassay as previously described (24). There is high cross-reactivity for mANP with the above ANP assay. Plasma and urinary samples for cGMP were measured by radioimmunoassay using the method of Steiner et al. (23). Plasma renin activity (25), angiotensin II (26), and aldosterone (27) were determined by commercially available radioimmunoassays as described previously. Inulin concentrations were measured using the anthrone method for GFR analysis, as previously described (28). Electrolytes, including lithium, were measured by flame photometry (IL943, Instrumentation Laboratory, London, United Kingdom), and GFR was measured by the clearance of inulin. Employing the lithium clearance (CL_{Li}) technique, we calculated the proximal fractional reabsorption of sodium (PFR_{Na}) according to the equation: $PFR_{Na} = [1 - (CL_{Li} / GFR)] \times 100$, and we calculated the distal fractional reabsorption of sodium (DFR_{Na}) according to the equation: $DFR_{Na} = [(CL_{Li} - CL_{Na}) / CL_{Li}] \times 100$, in which $CL_{Li} = [(urine Li \times urine flow) / plasma Li]$ and $CL_{Na} = [(urine Na \times urine flow) / plasma Na]$.

Statistical analysis. Results are expressed as mean ± SE. Student unpaired *t* tests were employed for single comparisons between groups. Comparisons within a group were made by 1-way analysis of variance (ANOVA) for repeated measures followed by Dunnett's post-test analysis. The baseline measurement was used as the "control" in Dunnett's analysis. Two-way ANOVA was used to compare the main group effects of mutant ANP versus native ANP, and group differences at specific time points were evaluated by Bonferroni post-test analysis. GraphPad Prism software (GraphPad Software, La Jolla, California) was used for the above calculations. Statistical significance was accepted as $p < 0.05$.

Results

Cyclic GMP generation in cardiac fibroblasts. In vitro studies measuring cGMP generation after exposure to ANP and mANP were performed in cultured human cardiac fibroblasts, and results are shown in Figure 2. We observed an incremental cGMP generation dose response with increasing concentrations of mANP consistent with

activation of NPR-A. There was no significant difference in cGMP generation with ANP (10^{-6} M) and mANP (10^{-6} M).

Cardiorenal and neurohormonal function, high dose. Systemic hemodynamics, renal hemodynamics, and cGMP responses are reported in Table 1. There was an overall greater and more sustained decrease in MAP with mANP compared with native ANP. Despite the greater reduction in MAP, there was a greater and more sustained increase in RBF and GFR with mANP compared with native ANP. There was a trend ($p = 0.058$) toward greater suppression of PCWP with mANP compared with ANP. There also was greater activation of plasma cGMP with mANP compared with native ANP, with a trend ($p = 0.064$) for greater urinary cGMP activation.

Figures 3A and 3B illustrate urine flow and urinary sodium excretion with high-dose (33 pmol/kg/min) infusion of mANP and native ANP. There was a greater peak and an overall greater increase in urine flow and sodium excretion with mANP infusion when compared with native ANP. The increased natriuresis with mANP infusion was localized to the distal nephron, where there was a greater decrease in DFR_{Na} with mANP infusion compared with native ANP (Fig. 3C). There was no difference in PFR_{Na} between the 2 peptides.

Assessment of the RAAS is reported in Figure 4. There was no significant difference in baseline values of plasma renin, angiotensin II, or aldosterone between mANP and native ANP groups as measured by Student unpaired *t* test. Overall, there was a greater decrease in plasma renin activity with mANP infusion compared

with native ANP (Fig. 4A). Further, there was a significantly greater and sustained decrease in both angiotensin II (Fig. 4B) and aldosterone (Fig. 4C) from baseline with mANP infusion compared with native ANP. Figure 5A illustrates the significantly greater increase in urinary excretion of ANP immunoreactivity with mANP compared with native ANP.

Cardiorenal and neurohormonal function, low dose. Figures 6A and 6B illustrate urine flow and urinary sodium excretion with low-dose (2 pmol/kg/min) infusion of mANP and ANP. A significant increase in urine flow was observed only after mANP administration. There was a significantly greater overall increase in sodium excretion with mANP infusion when compared with native ANP, and this increase was sustained for 120 min after infusion. Again, the greater natriuresis was localized to the distal nephron, with a greater decrease in DFR_{Na} with mANP infusion (Fig. 6C).

The systemic and renal hemodynamics with low-dose infusion are reported in Table 2. In contrast to high-dose infusion, there was no decrease in MAP after low-dose mANP infusion. Further, there was no difference in MAP between mANP and native ANP.

Plasma levels of renin, angiotensin II, and aldosterone levels were not significantly different between the 2 peptides (data not shown). Plasma cGMP was greater with ANP infusion but was increased with both peptides. Urinary cGMP excretion was increased with both peptides (Table 2). Figure 5B illustrates that there was no difference in urinary excretion of ANP immunoreactivity with mANP and native ANP at low dose.

Table 1 Cardiovascular, Renal Hemodynamics, and cGMP-Activating Properties With High-Dose (33 pmol·kg⁻¹·min⁻¹) mANP and Native ANP

	Peptide	Baseline	High-Dose Infusion	30-Min Post-Infusion	120-Min Post-Infusion
MAP, mm Hg*	mANP	133 ± 6	120 ± 5†	120 ± 5†	122 ± 6†
	Native ANP	136 ± 4	127 ± 4†	132 ± 3	134 ± 4
CO, l/min*	mANP	3.9 ± 0.3	3.6 ± 0.3	2.9 ± 0.3†	2.8 ± 0.2†
	Native ANP	3.8 ± 0.3	3.6 ± 0.3	3.5 ± 0.3	3.3 ± 0.2
PCWP, mm Hg	mANP	4.8 ± 0.6	2.3 ± 0.7†	2.3 ± 0.7†	3.7 ± 1.0†
	Native ANP	4.7 ± 0.5	2.9 ± 0.4†	3.3 ± 0.4	5.0 ± 0.9
SVR, mm Hg·l ⁻¹ ·min ⁻¹	mANP	33.8 ± 2.1	33.8 ± 2.8	42.4 ± 3.7†	43.0 ± 3.1†
	Native ANP	35.7 ± 3.7	35.0 ± 3.6	37.7 ± 4.0	40.6 ± 4.1
RBF, ml/min*	mANP	251 ± 30	333 ± 21†	317 ± 16†	305 ± 15†
	Native ANP	245 ± 25	288 ± 22†	282 ± 21†	280 ± 20†
GFR, ml/min*	mANP	41.5 ± 5.2	65.4 ± 6.6†	53.2 ± 5.7‡	49.1 ± 2.8
	Native ANP	36.1 ± 4.3	54.1 ± 4.3†	33.9 ± 3.9	43.1 ± 6.4
Plasma ANP, pg/ml	mANP	38.9 ± 2.6	313.8 ± 25.7†	53.7 ± 1.3	35.4 ± 2.4
	Native ANP	33.4 ± 2.8	478.1 ± 103.8†	31.8 ± 4.7	38.2 ± 4.1
Plasma cGMP, nmol/ml*	mANP	12.8 ± 2.7	52.4 ± 3.0†	33.0 ± 4.1†	10.6 ± 1.4
	Native ANP	11.6 ± 1.5	47.1 ± 3.6†	22.7 ± 2.3†	11.6 ± 2.4
Urine cGMP, pmol/min	mANP	946 ± 100	8,684 ± 1,433†	6,281 ± 1,374†	1,451 ± 266
	Native ANP	1,000 ± 155	6,521 ± 1,105†	4,487 ± 773†	1,358 ± 223

Values are mean ± SE. * $p < 0.05$ for main group effect of mANP versus native ANP (2-way analysis of variance [ANOVA]); † $p < 0.05$ versus baseline (1-way ANOVA); and ‡ $p < 0.05$ for mANP versus ANP at a specific time point (2-way ANOVA and Bonferroni post-tests).

ANP = atrial natriuretic peptide; cGMP = 3',5'-cyclic guanosine monophosphate; CO = cardiac output; mANP = mutant atrial natriuretic peptide; GFR = glomerular filtration rate; MAP = mean arterial pressure; PCWP = pulmonary capillary wedge pressure; RBF = renal blood flow; SVR = systemic vascular resistance.

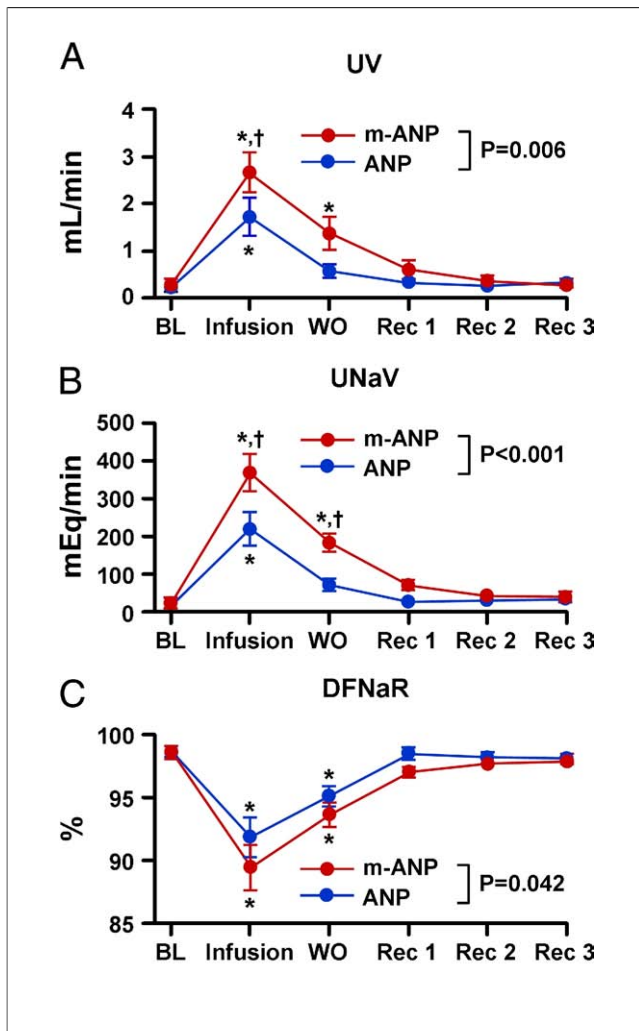


Figure 3 Renal Excretory Response to High-Dose ANP and mANP

(A) Urine flow (UV), (B) urine sodium excretion (UNaV), and (C) distal tubular fractional sodium reabsorption (DFNaR) after infusion of high-dose (33 pmol/kg/min) ANP (blue lines) and mANP (red lines) in normal dogs. Values are mean \pm SEM. * $p < 0.05$ versus baseline by 1-way analysis of variance (ANOVA). $\dagger p < 0.05$ for mANP versus ANP at a specific time point as measured by 2-way ANOVA and Bonferroni post-tests. The p value shown represents the main group effect between ANP and mANP as measured by 2-way ANOVA. BL = baseline; infusion = infusion of high-dose (33 pmol/kg/min) mANP or ANP; Rec 1 = 30 to 60 min post-infusion; Rec 2 = 60 to 90 min post-infusion; Rec 3 = 90 to 120 min post-infusion; WO = washout (0 to 30 min post-infusion); other abbreviations as in Figure 1.

Discussion

In this study, we have demonstrated that mANP, comprising native ANP and a 12-AA addition to the C-terminus, activates cGMP in vitro, thus indicating the mANP is capable, despite its extended C-terminus, to interact with its particulate guanylyl cyclase receptor, NPR-A. High-dose mANP in normal dogs demonstrated greater blood pressure lowering properties together with greater diuretic, natriuretic, GFR enhancing, and RAAS inhibiting properties when compared with native ANP. These enhanced cardio-

renal and neurohumoral properties observed with mANP were associated with a greater increase in plasma cGMP than with native peptide. We also found that low-dose mANP, in normal dogs at nonhypotensive concentrations, has natriuretic and diuretic properties that were not observed with native ANP.

We and others have pursued in the past the molecular design of chimeric natriuretic peptides that combine selected AA sequences from the native natriuretic peptides so as to produce novel designer hormones whose biological actions go beyond those of the native natriuretic peptides. In the current study, we have utilized information from an ANP gene mutation found in a Caucasian family with familial atrial fibrillation (17). Specifically, this new ANP is a result of the translation of the mutant gene resulting in a fusion protein consisting of the normal 28-AA mature

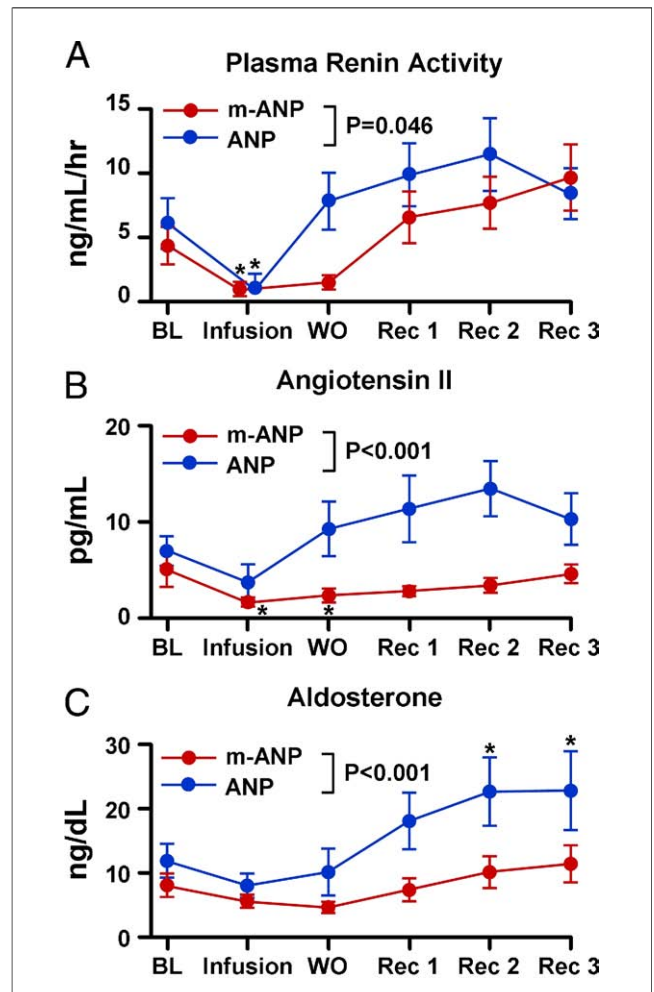
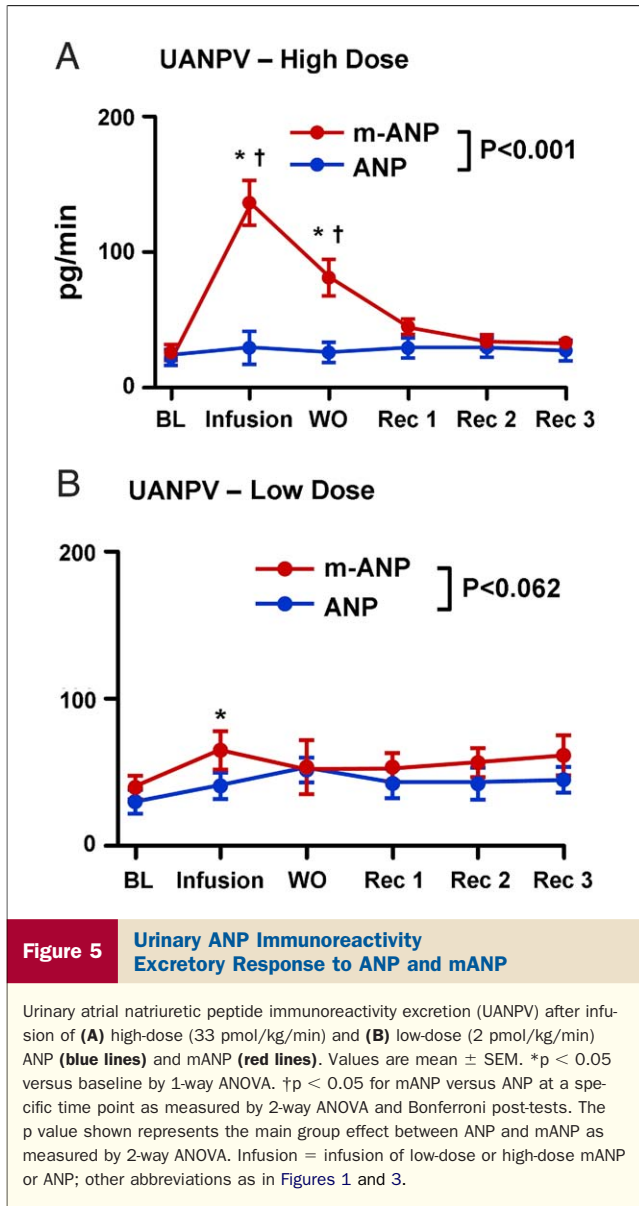


Figure 4 RAA Response to High-Dose ANP and mANP

Measurement of the renin-angiotensin-aldosterone (RAA) system after infusion of high-dose (33 pmol/kg/min) ANP (blue lines) and mANP (red lines) in normal dogs: (A) plasma renin activity, (B) angiotensin II, and (C) aldosterone. Values are mean \pm SEM. * $p < 0.05$ versus baseline by 1-way ANOVA. The p value shown represents the main group effect between ANP and mANP as measured by 2-way ANOVA. Abbreviations as in Figures 1 and 3.

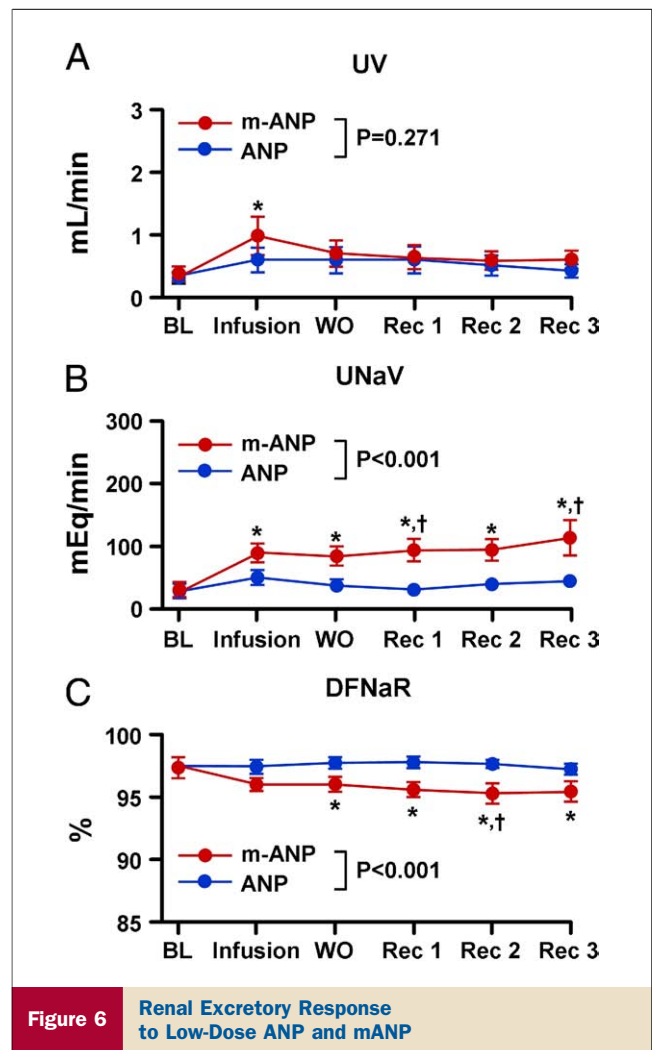


native ANP plus an anomalous C-terminus possessing 12 additional residues (Fig. 1).

Our *in vitro* assay clearly demonstrated that mANP can activate natriuretic peptide receptors linked to particulate guanylate cyclase as cGMP generation increased with mANP in cultured human fibroblasts that are known to express NPR-A (20). Increasing mANP concentrations resulted in incremental increases in cGMP generation. These *in vitro* results establish that mANP, characterized by the 12-AA extension to native ANP, maintains biological activity. When we compared cGMP generation of mANP with ANP in cultured fibroblasts, there was no significant difference between the 2 peptides. This finding suggests that the *in vivo* differences seen between mANP and ANP are likely secondary to altered degradation of mANP and not to enhanced

activation of NPR-A. Further studies will be needed to clarify this speculation.

It is well documented that native ANP promotes diuresis and natriuresis through its direct actions on the kidneys and inhibition of the RAAS together with renal vasodilatory properties (29–32). In this study using normal dogs, we observed a greater natriuretic response with both high- and low-dose mANP when compared with native ANP. The greater natriuretic properties of both high- and low-dose mANP appear to be at least partially localized to the distal nephron, where NPR-A is highly expressed (31), with a significantly greater reduction in DFR_{Na} when compared with native ANP. We also demonstrated a greater increase in GFR and RBF with mANP compared with native ANP.



(A) Urine flow (UV), (B) urine sodium excretion (UNaV), and (C) distal tubular fractional sodium reabsorption (DFNaR) after infusion of low-dose (2 pmol/kg/min) ANP (blue lines) and mANP (red lines) in normal dogs. Values are mean ± SEM. *p < 0.05 versus baseline by 1-way ANOVA. †p < 0.05 for mANP versus ANP at a specific time point as measured by 2-way ANOVA and Bonferroni post-tests. The p value shown represents the main group effect between ANP and mANP as measured by 2-way ANOVA. Infusion = infusion of low-dose (2 pmol/kg/min) mANP or ANP; other abbreviations as in Figures 1 and 3.

Table 2 Cardiovascular, Renal Hemodynamics, and cGMP-Activating Properties With Low-Dose ($2 \text{ pmol}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$) mANP and Native ANP

	Peptide	Baseline	Low-Dose Infusion	30-Min Post-Infusion	120-Min Post-Infusion
MAP, mm Hg	mANP	133 ± 3	137 ± 3	137 ± 3	136 ± 3
	Native ANP	131 ± 6	131 ± 6	132 ± 6	135 ± 7
CO, l/min	mANP	3.1 ± 0.1	2.6 ± 0.2	2.6 ± 0.3	2.7 ± 0.2
	Native ANP	3.3 ± 0.4	2.6 ± 0.3*	2.3 ± 0.2*	2.5 ± 0.3*
PCWP, mm Hg	mANP	4.1 ± 1.0	4.1 ± 0.9	4.5 ± 1.1	5.0 ± 1.1
	Native ANP	3.6 ± 0.4	3.8 ± 0.5	4.5 ± 0.5	6.5 ± 1.5*
SVR, mm Hg·l ⁻¹ ·min ⁻¹	mANP	48.5 ± 3.1	54.2 ± 5.2	56.0 ± 6.7	54.0 ± 5.8
	Native ANP	52.4 ± 5.7	55.9 ± 4.8	59.9 ± 5.3	58.9 ± 6.1
RBF, ml/min	mANP	255 ± 13	256 ± 6	258 ± 13	265 ± 14
	Native ANP	251 ± 32	219 ± 14	223 ± 17	232 ± 20
GFR, ml/min	mANP	41.9 ± 4.2	50.1 ± 5.1	49.2 ± 5.5	45.9 ± 6.5
	Native ANP	37.1 ± 5.2	51.0 ± 4.0	42.0 ± 4.8	46.2 ± 6.5
Plasma ANP, pg/ml	mANP	55.1 ± 3.9	81.8 ± 7.8*	63.1 ± 7.4	69.8 ± 11.3
	Native ANP	51.9 ± 2.4	121.6 ± 7.1*	69.6 ± 3.4	70.9 ± 5.7*
Plasma cGMP, nmol/ml†	mANP	10.4 ± 0.7	14.1 ± 1.0*	10.9 ± 1.0	10.4 ± 1.0
	Native ANP	12.1 ± 0.7	19.9 ± 1.9*	14.2 ± 1.2	12.3 ± 1.2
Urine cGMP, pmol/min	mANP	1,271 ± 192	2,013 ± 241*	1,665 ± 265	1,436 ± 228
	Native ANP	1,221 ± 164	2,505 ± 345*	2,007 ± 320	1,402 ± 143

Values are mean ± SE. *p < 0.05 versus baseline (1-way ANOVA); †p < 0.05 for main group effect of mANP versus native ANP (2-way ANOVA); and ‡p < 0.05 for mANP versus ANP at a specific time point (2-way ANOVA and Bonferroni post-hoc tests).

Abbreviations as in Table 1.

This too is consistent with high expression of the NPR-A in the glomerulus and renal vasculature (33).

Despite a decrease in MAP, we observed a significant inhibition of the RAAS with high-dose mANP as compared with native ANP. The reduction in renin secretion was likely secondary to increased sodium delivery to the macula densa as previous studies have demonstrated an inverse relationship between sodium delivery to the macula densa and renin secretion in view of the enhanced GFR with mANP (34). A direct cGMP-dependent action of ANP has also been demonstrated in juxtaglomerular cells, which may be an alternative mechanism for greater renin suppression (35). The reduction in angiotensin II most likely results from suppression of renin but may also be secondary to increased renal perfusion. Regarding aldosterone, native ANP is known to directly inhibit both the basal and angiotensin II-induced secretion of aldosterone from the zona glomerulosa, where there is a high concentration of NPR-A receptors (33,36,37). The suppression of aldosterone activation observed with mANP is likely multifactorial, including reduced angiotensin II levels as well as greater or prolonged activation of the NPR-A receptors in the adrenal glands and may have contributed to the greater diuresis and natriuresis of mANP.

A hallmark of the natriuretic peptides especially ANP, B-type natriuretic peptide (BNP), and *Dendroaspis* natriuretic peptide (DNP) is their ability to unload the heart by arterial and venodilation together with a reduction in pre-load through diuresis and natriuresis. Of note, mANP demonstrated a more sustained reduction in PCWP compared with native ANP. Further, as stated in the preceding text, reduction in arterial pressure and increase in RBF were

greater with mANP compared with native ANP. Thus, despite a greater reduction in arterial pressure with mANP at the high dose employed with the current study, renal function was more enhanced. This feature is unique compared with other conventional vasodilators that tend to reduce renal perfusion and thus might be a highly favorable characteristic with clinical implications.

With low-dose mANP, we did not observe significant MAP reduction in this study, nor did we demonstrate an inhibition of the RAAS or changes in GFR or RBF. However, it should be noted that low-dose mANP resulted in a significant and sustained natriuresis when compared with native ANP. Indeed, this sustained effect on sodium excretion and DFR_{Na} was more prolonged than at high dose, underscoring the intrinsic natriuretic properties of mANP and the importance of renal perfusion in modulating the renal response to natriuretic peptides.

Regarding the mechanisms of the greater and sustained actions of mANP, it is possible that the elongated C-terminus of mANP renders the peptide more resistant to degradation by either NEP or clearance by NPR-C. Kinetic studies have shown the rank order for hydrolysis by NEP is C-type natriuretic peptide > ANP > BNP, suggesting that the longer the C-terminus, the greater resistance to NEP degradation by the peptide (19,38). Indeed, studies show that DNP with a 15-AA C-terminus is highly resistant to NEP degradation and potently natriuretic, which has been attributed to resistance to degradation by NEP (19,39,40). As mANP has a 17-AA C terminus, longer than the 15-AA C-terminus of DNP, resistance to hydrolysis by NEP, thereby potentiating mANP's actions, is plausible. Importantly, we noted that high-dose mANP resulted in signifi-

cantly greater urinary ANP excretion compared with native ANP, consistent with greater resistance to renal degradation by NEP. Alternatively, Shimake et al. (18) demonstrated that the C-terminus of ANP enhances ANP interactions with the NPR-A, resulting in greater cGMP activation, enhanced vasorelaxing actions, and augmented renal responses. It is possible that the extended C-terminus of mANP also enhances ligand-receptor interactions. Further studies are needed to address this issue, including defining the biological actions of the novel entire C-terminus itself. The concept that the C-terminus itself has biological actions is consistent with the report that the 15-AA C-terminus of DNP has intrinsic natriuretic and diuretic actions (21).

Native ANP is currently approved for the treatment of heart failure in Japan. The greater and sustained properties of mANP underscore its own therapeutic potential. Recent studies demonstrate the increasing prevalence of systolic hypertension in the setting of acute heart failure (41). Thus, mANP may provide a reduction in blood pressure while improving renal hemodynamics, natriuresis, and diuresis with suppression of the RAAS. Alternatively, in acute heart failure patients with low blood pressure and subsequent renal compromise, low-dose mANP could increase natriuresis without affecting blood pressure.

Study limitations. There are several limitations to the current study, including the lack of time controls for the in vivo studies. It is therefore possible that the experimental protocol may account for some of the changes seen with either ANP or mANP infusion. However, we believe that the hemodynamic and neurohumoral data from washout and recovery periods suggest that the changes are secondary to peptide infusion and not the experimental protocol. Furthermore, anesthesia may alter the response to ANP and mANP, and extrapolation of the data to conscious subjects (animals and humans) should be done cautiously. Finally, in this study, we sought to define the pharmacodynamics of mANP, and future studies will need to be performed to define the pharmacokinetics of mANP.

Conclusions

In summary, these studies highlight the important biology of the C-terminus of ANP, especially the novel properties of a C-terminus defined by a human ANP mutation. Here, we demonstrate the ability in vitro of mANP to activate the NPR-A linked to cGMP. This novel mANP, which possesses a longer C-terminus (17-AA) than native ANP—and indeed the longest C-terminus of known natriuretic peptides—exhibits greater and more sustained natriuretic, diuretic, GFR, and RBF enhancing actions together with cardiac unloading and RAAS suppressing properties as compared with native ANP. The greater cardiorenal and neurohumoral actions of mANP may be secondary to increased resistance to NEP degradation and/or clearance by NPR-C. Additionally, greater interactions with NPR-A are also

possible. These biological properties underscore the therapeutic potential of mANP in cardiorenal disease syndromes and warrant further studies.

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