Pro–Atrial Natriuretic Peptide
A Novel Guanylyl Cyclase-A Receptor Activator That Goes Beyond Atrial and B-Type Natriuretic Peptides

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ABSTRACT

OBJECTIVES The aim of this study was to determine if the atrial natriuretic peptide (ANP) precursor proANP is biologically active compared with ANP and B-type natriuretic peptide (BNP).

BACKGROUND ProANP is produced in the atria and processed to ANP and activates the guanylyl cyclase receptor-A (GC-A) and its second messenger, cyclic guanosine monophosphate (cGMP). ProANP is found in the human circulation, but its bioavailability is undefined.

METHODS The in vivo actions of proANP compared with ANP, BNP, and placebo were investigated in normal canines (667 pmol/kg, n = 5/group). cGMP activation in human embryonic kidney 293 cells expressing GC-A or guanylyl cyclase receptor-B was also determined. ProANP processing and degradation were observed in serum from normal subjects (n = 13) and patients with heart failure (n = 14) ex vivo.

RESULTS ProANP had greater diuretic and natriuretic properties, with more sustained renal tubular actions, compared with ANP and BNP in vivo in normal canines, including marked renal vasodilation not observed with ANP or BNP. ProANP also resulted in greater and more prolonged cardiac unloading than ANP but much less hypotensive effects than BNP. ProANP stimulated cGMP generation by GC-A as much as ANP. ProANP was processed to ANP in serum from normal control subjects and patients with heart failure ex vivo.

CONCLUSIONS ProANP represents a novel activator of GC-A with enhanced diuretic, natriuretic, and renal vasodilating properties, and it may represent a key circulating natriuretic peptide in cardiorenal and blood pressure homeostasis. These results support the concepts that proANP may be a potential innovative therapeutic beyond ANP or BNP for cardiorenal diseases, including heart failure. (J Am Coll Cardiol HF 2015;3:715–23) © 2015 by the American College of Cardiology Foundation.

The prevalence of acute decompensated heart failure (HF) continues to grow, and the prognosis remains poor despite the availability of many drugs to treat HF (1). Conventional diuretic agents and vasodilators remain mainstays of therapy (1), but currently no new drugs have improved the prognosis of patients with acute decompensated HF (2–5). In addition, myocardial remodeling is driven by decreased cyclic guanosine monophosphate (cGMP) activity in cardiac myocytes in both HF with preserved ejection fraction (EF) and advanced HF with reduced EF (6), suggesting that cGMP replacement therapy may be useful in patients with HF. The cardiac guanylyl cyclase receptor-A (GC-A) activators atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP) play roles in circulating volume and blood pressure homeostasis (7,8). Carperitide (recombinant human ANP) and nesiritide (recombinant human BNP) have been used as therapeutic agents for patients with HF. The use of carperitide continues in Japan, with favorable results from clinical trials (9,10), but the use of nesiritide in patients with HF has decreased in the United States since the ASCEND-HF (Acute Study of Clinical Effectiveness of Nesiritide and Decompensated Heart Failure) trial because there was no evidence of...

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improved prognosis beyond conventional therapies (4).

The molecular precursor of ANP, proANP, is formed after removal of the signal peptide from pre-proANP, which is produced from the NPPA gene in the heart (11,12). ProANP is then processed into amino-terminal proANP and the biologically active ANP by corin (13,14) (Online Figure 1). ProANP is stored in secretory granules in atrial cardiomyocytes and cleaved to ANP upon secretion, in response to stretch (15). ANP is then degraded into smaller molecular fragments by nephrilysin and insulin-degrading enzyme (16-18). Hunter et al. (19) reported that proANP circulates in canines and humans. Although initially circulating pro-natriuretic peptides were not thought to be biologically active, we and others have recently established proBNP is active, has a longer half-life than ANP in rats (20), and can be processed to BNP in human serum (21), but it is unclear if this is true for proANP.

As with proBNP (20), we hypothesized that proANP would be biologically active in vivo, with a longer half-life and longer lasting cardiorenal actions than ANP or BNP. Furthermore, we hypothesized that proANP can stimulate cGMP production via GC-A receptors and be processed to ANP in the circulation. These studies advance our understanding of proANP, ANP, GC-A, and cGMP signaling in the circulation, with potential physiological and therapeutic implications.

METHODS

All human and animal experimental protocols used in the present study were approved by the Institutional Review Board and the Animal Care and Use Committee at the Mayo Clinic. Detailed methods are provided in the Online Appendix.

**ProANP, ANP, BNP, AND C-TYPE NATRIURETIC PEPTIDE SYNTHESIS AND REAGENTS.** Recombinant human and canine proANP were synthesized by ProMab Biotechnologies (Richmond, California). ANP, canine BNP, and C-type natriuretic peptide (CNP) were from Phoenix Laboratories (Burlingame, California). Human proANP was tagged with Trx on the N-terminus and with 6-histidine (His) on the C-terminus for peptide isolation, but canine proANP was untagged.

**IN VIVO STUDIES IN NORMAL CANINES.** We performed procedures (22-24) to assess the actions by intravenous bolus injection of equimolar doses (667 pmol/kg) (25) of canine proANP, ANP, canine BNP, and placebo (0.9% saline) in normal canines (21 to 30 kg, n = 5 of each group). Short-term procedures permitted the characterization of pharmacokinetics and cardiorenal function up to 3 h after peptide or placebo injection. In brief, lithium carbonate tablets were given the night before the study to assess renal tubular function. Hemodynamic parameters were assessed with an arterial line and a Swan-Ganz catheter. Blood and urine were collected from an arterial line and a ureter catheter, respectively. Renal blood flow was monitored with an electromagnetic flow probe placed on the renal artery. Inulin was continuously infused for measurement of glomerular filtration rate, which was calculated by inulin clearance. Plasma and urinary sodium, potassium, and lithium levels were determined. Urinary excretion of each parameter (X), such as cGMP, sodium, or potassium, was determined as X/× urine volume (UV) (milliliters per minute). Using the lithium clearance technique, we calculated renal tubular function, proximal fractional reabsorption of sodium (PFRNa), and distal fractional reabsorption of sodium (DFRNa) (23,24). Levels of cGMP were assayed using the RIA cGMP kit (Perkin-Elmer, Waltham, Massachusetts) (22-24). Pharmacokinetic profiles were calculated by noncompartmental analysis using Phoenix WinNon-line 6.3 software (Certara, Princeton, New Jersey) (26).

**IN VITRO STUDY IN HUMAN EMBRYONIC KIDNEY 293 CELLS**

Human embryonic kidney 293 (HEK293) cells were stably transfected with either human GC-A or guanyl cyclase receptor-B (GC-B) using Lipofectamine (Invitrogen, Grand Island, New York) (27). Cells were treated with or without 10−10 to 10−8 mol/l proANP, ANP, BNP, or CNP for 10 min, and intracellular cGMP levels were measured.

**EX VIVO HUMAN STUDIES.** Fresh serum was obtained from healthy volunteers (normal control subjects) and patients with HF (New York Heart Association functional class II to IV), with informed consent. Fresh serum (100 μl) with or without 500 ng Trx- and His-tagged proANP (1.9 × 10−7 mol/l) added was incubated for varying times from 5 to 180 min at 37 C, then immunoprecipitated and Western-blotted using His-tag antibody (21,28).

**STATISTICAL ANALYSIS.** Data are reported as mean ± SEM. Unpaired Student t tests were performed for comparison between groups. Comparisons within a group in time-course ex vivo and in vivo studies were made by 1-way analysis of variance followed by Bonferroni multiple-comparison post-test analyses.
FIGURE 1  In Vivo Urinary Changes

(A,B) Time course of urine volume (UV) (A) and urinary sodium excretion (UNaV) (B) after peptide injections. Data are calculated from the difference from baseline. *p < 0.05 versus baseline (0 min); †p < 0.05 versus placebo; ‡p < 0.05 versus atrial natriuretic peptide (ANP), and §p < 0.05 versus B-type natriuretic peptide (BNP).

(C,D) Total UV (C) and sodium excretion (D) in the kidneys for 180 min after drug injection.

FIGURE 2  In Vivo RBF and Hct

(A) Renal blood flow (RBF) and (B) hematocrit (Hct). Data are calculated from the difference from baseline. *p < 0.05 versus baseline (0 min); †p < 0.05 versus placebo; ‡p < 0.05 versus atrial natriuretic peptide (ANP); and §p < 0.05 vs B-type natriuretic peptide (BNP).
when the global test was significant. Two-way analysis of variance was used to compare between groups in time-course in vivo studies, followed by Bonferroni post-tests. GraphPad Prism 5 (GraphPad Software, La Jolla, California) and JMP 10 (SAS Institute, Cary, North Carolina) were used for these calculations. Statistical significance was accepted at \( p < 0.05 \).

RESULTS

IN VIVO RENAL ACTIONS. Online Table 1 illustrates baseline characteristics in each group. There was no statistical difference among the groups. We calculated the value of the difference from baseline (0 min) before drug injection for each parameter to compare among the groups.

ProANP resulted in biphasic increases in UV and urinary sodium excretion with the first peak at 10 min and the second peak between 60 and 90 min, whereas ANP and BNP showed a monophasic increase peak at 10 min (Figures 1A and 1B). ANP also had a lower peak than proANP and BNP. Urinary potassium excretion followed the same pattern with proANP, whereas ANP and BNP decreased sharply in the first 45 min, with BNP remaining low while ANP slowly increased over the next 150 min (Online Table 2). ProANP had significantly greater total UV for 3 h, but neither ANP nor BNP significantly changed from placebo (Figure 1C). ProANP and BNP had significantly greater total sodium excretion than placebo, but ANP did not (Figure 1D). All 3 peptides had no significant differences compared with placebo in total potassium excretion (data not shown).

ProANP significantly increased renal blood flow from 45 to 150 min compared with ANP and BNP (Figure 2A), and proANP had a greater increase in glomerular filtration rate at 10 min compared with ANP or BNP (Online Table 2). ProANP showed the greatest increase in hematocrit from 30 to 180 min, possibly because of
Volume loss caused by diuresis (Figure 2B). To determine which nephron segment was involved in the greater natriuretic response to proANP, we analyzed PFRNa and DFRNa, as shown in Online Table 2. ProANP significantly decreased PFRNa at 10 min, similar to ANP and BNP but showed a biphasic effect like UV (Figure 1A) and urinary sodium excretion (Figure 1B), with a significantly prolonged decrease of PFRNa over 120 min compared with the other 3 groups. ProANP also significantly decreased DFRNa, with a peak at 10 min, and showed a significantly prolonged decrease of DFRNa for 60 min compared with its baseline as well as placebo. ANP and BNP lacked the prolonged effects.

**IN VIVO HEMODYNAMIC ACTIONS.** Figure 3 and Online Table 2 report hemodynamic responses before and after natriuretic peptide injection compared with placebo. All 3 natriuretic peptides showed significant decreases in mean arterial pressure, but only proANP and BNP decreased pulmonary capillary wedge pressure (PCWP) and systemic vascular resistance compared with placebo (Figures 3A to 3C). BNP had the most potent sustained decreases of mean arterial pressure, PCWP, and systemic vascular resistance. ProANP decreased mean arterial pressure similar to ANP but had a prolonged decrease in PCWP for 2 h, whereas ANP did not. BNP increased heart rate (HR) for the first 20 min (Figure 3D), but after 30 min, proANP resulted in the greatest HR increase, together with increased hematocrit (Figure 2B).

**IN VIVO PLASMA AND URINARY cGMP IMMUNOREACTIVITY.** Online Figures 2A and 2B illustrate plasma and urinary cGMP levels. BNP had the greatest and most persistent increases of plasma and urinary cGMP compared with proANP or ANP, whereas proANP had longer effects on both plasma and urinary cGMP levels than ANP. In pharmacokinetic analyses (Table 1), BNP showed the highest plasma and urinary cGMP maximal concentration and area under the curve. ProANP had significant increases on both parameters with a longer half-life compared with either ANP or BNP.

**IN VITRO cGMP ACTIVATION IN HEK293 CELLS.** We investigated the ability of proANP to activate cGMP generation compared with ANP and BNP in HEK293 cells expressing GC-A and GC-B (Figures 4A and 4B). CNP was used as a positive GC-B control. BNP, ANP, and proANP all significantly induced GC-A-stimulated cGMP production in a dose-dependent manner (Figure 4A). ProANP had significantly more effects than BNP and a similar effect to ANP at the highest dose ($10^{-8}$ mol/l). BNP, ANP, and proANP showed little cGMP stimulation in GC-B cells (Figure 4B).

**EX VIVO ProANP PROCESSING IN HUMAN SERUM.** We assessed whether proANP is processed in the normal or HF human circulation ex vivo. Characteristics of normal control subjects and patients with HF and individual characteristics of patients with HF are shown in Online Tables 3 and 4. All patients were in the hospital and had moderate to severe HF.

As shown in Figure 5A, proANP was produced with an N-terminal Trx-tag to aid in peptide purification and a C-terminal His-tag to aid in the isolation of ANP forms after processing. His-tag antibodies will detect unprocessed proANP, proANP without the
(A) Exogenous pro-atrial natriuretic peptide (proANP) was incubated in fresh human serum for indicated times at 37°C. Unprocessed or processed proANP was isolated by immunoprecipitation and detected by Western blot. (B,C) Representative Western blot for normal serum (B) and heart failure (HF) serum (C). (D) Densitometric analysis of ANP. *p < 0.05 versus 0 min.

### TABLE 1 cGMP Pharmacokinetics

<table>
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<th></th>
<th>PcGMP Cmax (pmol/ml)</th>
<th>PcGMP Tmax (min)</th>
<th>PcGMP AUClast (pmol min/ml)</th>
<th>PcGMP T1/2 (min)</th>
<th>UcGMPV Cmax (pmol/ml)</th>
<th>UcGMPV Tmax (min)</th>
<th>UcGMPV AUClast (pmol min/ml)</th>
<th>UcGMPV T1/2 (min)</th>
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<td>ANP</td>
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<td>1,205.6 ± 176.9</td>
<td>13.3 ± 1.8</td>
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<td>BNP</td>
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<td>9.0 ± 1.8</td>
<td>3,498.6 ± 488.1</td>
<td>17.7 ± 1.6</td>
<td>10,415.2 ± 1,190.6*</td>
<td>18.0 ± 3.7</td>
<td>498,780.3 ± 51,977.7*</td>
<td>23.6 ± 2.2</td>
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<td>ProANP</td>
<td>51.5 ± 4.7†</td>
<td>7.6 ± 0.7</td>
<td>2,720.0 ± 342.9*</td>
<td>40.3 ± 6.7†</td>
<td>5,984.9 ± 417.9</td>
<td>20.0 ± 3.2</td>
<td>383,668.1 ± 28,010.2</td>
<td>44.8 ± 3.2†</td>
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* p < 0.05 versus ANP, † p < 0.05 versus BNP.

ANP = atrial natriuretic peptide; AUClast = area under the concentration-time curve from time 0 to the last measurable concentration (180 min); BNP = B-type natriuretic peptide; cGMP = cyclic guanosine monophosphate; Cmax = maximum concentration; PcGMP = plasma cyclic guanosine monophosphate level; Tmax = time to maximum concentration; T1/2 = half-life; UcGMPV = urinary cyclic guanosine monophosphate excretion.
Trx-tag, and ANP but not processed aminoterminal proANP (Figure 5A). ProANP (~23 kDa) was processed into a smaller molecular weight form (~4 kDa) (Figures 5B and 5C) in serum from normal control subjects and patients with HF, which was confirmed to be ANP by sequencing. In some subjects, a faint band around 13 kDa was determined to be proANP without the Trx-tag (Figure 5B).

To more precisely assess proANP processing in the circulation, we examined proANP processing in serum from normal control subjects versus patients with HF at 5 time points: 0, 5, 15, 60, and 180 min. As a control, we pre-treated samples for 0 min with ethylenediaminetetraacetic acid, which inhibits proANP processing. In both normal control subjects and patients with HF, ANP appeared after 5 min incubation and persisted for 15 min, then began to decrease at 60 min through 180 min (Figures 5B and 5C). The density of the ANP band from Western blots in each group was significantly higher at each time point compared with 0 min, and there was no significant difference between normal control subjects and patients with HF (Figure 5D).

**DISCUSSION**

This is the first study to investigate proANP as a GC-A activator, with more sustained natriuretic, diuretic, and renal vasodilating actions than ANP or BNP and with greater and more sustained cardiac unloading properties than ANP. Our results suggest that proANP is a biologically active natriuretic peptide with both physiological and therapeutic implications (Online Figure 3).

To date, the in vivo physiological actions of proANP have not been defined, although de Bold (29) previously reported that extracts from the atrial myocardium, which contained atrial natriuretic factor, but most likely proANP as well, had potent diuretic, natriuretic, and blood pressure-lowering properties. In our study, we observed that proANP had greater and more prolonged cGMP activation than ANP, while the area under the curve of BNP for both plasma cGMP and urinary cGMP excretion (Online Figure 2) was the highest of all 3 peptides.

Unexpectedly, proANP had significantly greater natriuretic and diuretic actions than ANP or BNP (Figures 1A and 1B). These greater renal actions were a result of greater tubular actions and greater increases in renal blood flow and glomerular filtration rate. The cardioenal differences between proANP and mature ANP may be due to: 1) the longer half-life of proANP; 2) “dual” activating properties of proANP via proANP and “processed to ANP” forms; and/or 3) conversion of proANP to active ANP forms; such as vessel dilator (ANP31-67), which works in the kidney through a non-cGMP pathway (30). Indeed, we speculate that the vessel dilator form may explain the second peak of proANP urinary actions, which seems to occur via non-cGMP activation, as urinary cGMP was not biphasic.

Beyond the kidney, we report that proANP reduced cardiac filling pressures, including reductions in pulmonary artery pressure, PCWP, right atrial pressure, and systemic vascular resistance, with increases in HR and hematocrit compared with baseline. In contrast, these hemodynamic effects were not observed with ANP. Notably, proANP markedly increased HR over 120 min (Figure 3D). We speculate that the increased HR might be in response to volume loss associated with decreased hematocrit (Figure 2B), but this increase in HR may lead to adverse clinical events due to increased myocardial oxygen demand. Further studies are required to observe HR in an HF model and to investigate the modulating actions of proANP on sympathetic activity.

Both proANP and ANP reduced blood pressure, most likely because of actions on both the kidney and the systemic circulation, but with one-half the hypotensive effects of BNP. Surprisingly, BNP showed stronger and more sustained systemic vasodilation together with greater cardiac unloading effects, but with much less natriuresis, diuresis, and renal vasodilation compared with proANP. We cannot explain why BNP plays a different role from ANP or proANP, as all are GC-A activators. Recognizing that plasma and urinary cGMP reflects guanylyl cyclase activation, we observed that proANP had greater and more prolonged cGMP activation than ANP, but the area under the curve of BNP for both plasma cGMP and urinary cGMP excretion was greatest for all 3 peptides (Online Figure 2, Table 1). Possible explanations include BNP signaling through an unknown receptor, differences in processing and degradation, or resistance to degrading enzymes (31).

We demonstrate that proANP activates cGMP generation in GC-A- but not GC-B-overexpressing HEK293 cells. Although less than ANP, proANP clearly increased cGMP production, consistent with the properties of a GC-A activator. This may be a direct action of proANP on GC-A, as it has been reported that HEK293 cells lack corin, which processes proANP into ANP (13). GC-A-activating actions by proANP support our concept that proANP may represent a biologically active peptide itself, complementing mature processed ANP actions.

Although our studies support proANP as a direct activator of GC-A, in fact a dual mechanism for GC-A activation may be at play, as we demonstrate that proANP can be processed to biologically active ANP in
human serum, similar to our previous studies on proBNP processing in the circulation (21). It has been reported that corin is present in the circulation and kidneys (32), supporting potential proANP cleavage to ANP in these locations (13,14). Similar to normal human serum, proANP was rapidly processed to ANP in patients with HF. Thus, our human proANP could activate GC-A either directly or indirectly through processing to ANP in either normal control subjects or patients with HF. These results are in contrast to those from our studies of proBNP processing and degradation, in which the cGMP activity of proBNP was much less than BNP, proBNP processing to BNP was rapid with degradation complete within 60 min in normal control subjects, and proBNP processing was delayed (28). These differences in metabolism and bioactivity between proANP and proBNP may be key factors in cardiovascular homeostasis.

The concept of a cGMP-deficient state in HF raises a therapeutic opportunity, especially in HF with preserved EF and advanced HF with reduced EF (6,33), despite results from the ASCEND-HF trial, which were negative, and supported by encouraging (6,33), despite results from the ASCEND-HF trial, which were negative, and supported by encouraging findings from the PARAMOUNT (Prospective Comparison of ARNI with ARB on Management of Heart Failure With Preserved Ejection Fraction) (35), and reduced risk for mortality and rehospitalization in patients with HF with reduced EF compared with valsartan (PARAMOUNT (Prospective Comparison of ARNI with ARB on Management of Heart Failure With Preserved Ejection Fraction)) (35), and reduced risk for mortality and rehospitalization in patients with HF with reduced EF compared with enalapril (PARADIGM-HF [Efficacy and Safety of LCZ696 Compared to Enalapril on Morbidity and Mortality of Patients With Chronic Heart Failure]) (36) and increased cGMP (37). These trials suggest that enhancement of the cGMP pathway may be useful treatment for patients with HF.

STUDY LIMITATIONS. A limitation of our study was the relatively small number of canines in each group. However, large normal canines compared with small animal studies lack hemodynamic variability and are well established in our laboratory, thus providing statistical significance using a smaller number of canines. A second limitation was that proANP effects in animals may not translate to humans, so clinical studies are needed to confirm these results.

CONCLUSIONS

ProANP represents a novel GC-A activator with a longer half-life and beneficial cardiorenal actions beyond ANP and BNP. The bioactivity of exogenous proANP may be through direct stimulation of GC-A, as well as through processing into ANP, as proANP is processed in the circulation of both normal control subjects and patients with HF. Our findings lay the rationale for further investigations of proANP with a focus on not only physiological mechanisms of action, but therapeutic implications in HF.

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PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: ProANP may be a unique “dual” activator of GC-A by either direct activation or through processing to ANP, contributing to sodium and volume regulation. ProANP’s in vivo cardiorenal actions continued for up to 3 h, and processing of proANP remained intact in patients with HF, so HF may be a target disease for proANP treatment. ProANP may serve as a long-lasting novel diuretic drug that can be administered by bolus injection and be more useful in the clinical setting compared with carperitide (ANP) or nesiritide (BNP). For widespread clinical application, proANP should be produced as a recombinant peptide on the basis of its extended amino acid length.

TRANSLATIONAL OUTLOOK: Our studies advance our understanding of the heart as an endocrine organ and the proANP, ANP, GC-A, and cGMP system with biological, diagnostic, and therapeutic implications. Because proANP circulates, we must now consider it as a biologically active hormone, and it may be useful to develop assays that explore its biomarker potential. Finally, its unique renal and cardiac unloading actions may also lay the foundation for its development as a new therapy for cardiorenal disease.
REFERENCES


KEY WORDS
atrial natriuretic peptides, cGMP, kidney

APPENDIX For an expanded Methods section and supplemental tables and figures, please see the online version of this article.