Secretion of N-Terminal Fragment of γ-Human Atrial Natriuretic Polypeptide

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SUMMARY To elucidate the posttranslational processing of γ-human atrial natriuretic polypeptide (human atrial natriuretic factor-[1-126]), which is a prohormone of α-human atrial natriuretic polypeptide (human atrial natriuretic factor-[99-126]), and the secretion of γ-human atrial natriuretic polypeptide-derived peptides from the heart, we established a radioimmunoassay specific for the N-terminal sequence of γ-human atrial natriuretic polypeptide, γ-human atrial natriuretic polypeptide-(1-25), as well as a radioimmunoassay for α-human atrial natriuretic polypeptide. With the aid of the radioimmunoassays for γ-human atrial natriuretic polypeptide-(1-25) and for α-human atrial natriuretic polypeptide, we detected 290 ± 35.6 pg/ml of γ-human atrial natriuretic polypeptide-(1-25)-like immunoreactivity in plasma from healthy humans, while the simultaneously determined plasma α-human atrial natriuretic polypeptide, we detected 290 ± 35.6 pg/ml of γ-human atrial natriuretic polypeptide-(1-25)-like immunoreactivity in plasma from healthy humans, while the simultaneously determined plasma α-human atrial natriuretic polypeptide-like immunoreactivity level was 20.9 ± 2.8 pg/ml. Correlation between the two values was significant. High performance gel permeation chromatographic analysis revealed that the plasma γ-human atrial natriuretic polypeptide-(1-25)-like immunoreactivity was composed of a component (molecular weight, 10,000) without α-human atrial natriuretic polypeptide-like immunoreactivity, while the plasma α-human atrial natriuretic polypeptide-like immunoreactivity was composed of α-human atrial natriuretic polypeptide with a molecular weight of 3000. In patients with heart diseases, the plasma γ-human atrial natriuretic polypeptide-(1-25)-like immunoreactivity level showed a concomitant and graded increase, with the plasma α-human atrial natriuretic polypeptide-like immunoreactivity level in agreement with the severity of the disease. There were significant positive correlations between the two immunoreactivity levels and right or left atrial pressure. These results suggest cosecretion of 10 K N-terminal fragment of γ-human atrial natriuretic polypeptide and α-human atrial natriuretic polypeptide into the circulation in response to increased atrial stretch. (Hypertension 11 [Suppl I]: I-52-I-56, 1988)

KEY WORDS • posttranslational processing • prohormone • congestive heart failure • atrial pressure • radioimmunoassay • atrial natriuretic factor

THE epochal discovery by de Bold et al. that rat atrial extracts possess potent diuretic and natriuretic properties led to the identification of multiple forms of atrial natriuretic polypeptide (ANP) with high and low molecular weights. Secretion of ANP from the heart has been reported to be increased by volume loading or increases in intra-atrial pressure both in experimental animals and in humans. These findings suggest that the heart is a novel endocrine organ involved in the regulation of body fluid and blood pressure control.

Three distinct molecular forms of ANP, α-, β-, and γ-human ANP (hANP), have been isolated from the human atrium. α-hANP (human atrial natriuretic factor or ANF-[99-126]) is a biologically active form and comprises 28 amino acids with an intramolecular disulfide linkage; γ-hANP, or human ANF-(1-126), is a 126 amino acid prohormone, carrying the 28 amino acid sequence of α-hANP at its C-terminus; and β-hANP is an antiparallel dimer of α-hANP with intermolecular disulfide bridges. We demonstrated that the major circulating form of ANP secreted through the
coronary sinus as a hormone is $\alpha$-hANP.\textsuperscript{3,4} The sites and the mechanisms involved in processing the high-molecular-weight form of ANP, $\gamma$-hANP, to the low-molecular-weight form, $\alpha$-hANP, during the secretion from cardiocytes are still in debate, and knowledge about the cleaved N-terminal product of $\gamma$-hANP is currently lacking in humans.

To investigate the posttranslational processing of $\gamma$-hANP and the secretion of $\gamma$-hANP-derived peptides from the heart, we examined $\gamma$-hANP-derived peptides in human plasma with the aid of two different radioimmunoassays (RIAs), one detecting $\alpha$-hANP and the other detecting the N-terminal sequence of $\gamma$-hANP.

**Subjects and Methods**

Eight normal male volunteers, aged 23 to 38 years (mean, 30 years), were studied. Blood was withdrawn at 0900 from the right antecubital vein in recumbent position after overnight fast.

Sixteen patients, 11 men and 5 women, aged 32 to 77 years (mean, 53 years), who were undergoing consecutive catheterization of the right or left sides of the heart, were investigated. Six had ischemic heart disease, five had valvular disease, four had dilated cardiomyopathy, and one had hypertension. Nine patients (mean age, 52 years) were classified according to New York Heart Association (NYHA) Functional Classification as having class I or II disease and seven (mean, 53 years) as having class III or IV disease. Fourteen patients were in normal sinus rhythm and two had atrial fibrillation. Right atrial pressure (RAP), pulmonary arterial pressure (PAP), and pulmonary capillary wedge pressure (PCWP) were continuously recorded with a Swan-Ganz catheter (heparin-coated thermodilution catheter, Model SP5107, Gould Inc., Oxnard, CA, USA). The patients with NYHA class III or IV disease showed significantly elevated RAP ($6.7 \pm 1.6$ vs $2.6 \pm 0.4$ mm Hg; $p<0.01$), PAP ($38.9 \pm 3.2$ vs $11.8 \pm 1.3$ mm Hg; $p<0.001$), and PCWP ($23.8 \pm 3.2$ vs $10.2 \pm 2.5$ mm Hg; $p<0.001$) compared with the patients with NYHA class I or II disease. Blood was taken through an indwelling cannula inserted into the antecubital or femoral vein before catheterization studies.

**Blood Sampling**

Blood was drawn into chilled plastic syringes and transferred to chilled polypropylene tubes containing aprotinin (Ohkura Pharmaceutical, Kyoto, Japan), 1000 kallikrein inhibiting units/ml, and EDTA (Sigma Chemical, St. Louis, MO, USA) 1 mg/ml.\textsuperscript{6,8} After centrifugation at 3000 rpm for 15 minutes, plasma was immediately frozen at $-20^\circ$C until processed and thawed only once at the assay.

**Peptides**

The $\alpha$-hANP was purchased from Protein Research Foundation, Osaka, Japan. The $\gamma$-hANP-(1-16) (human Cardiodilatin 1-16, Lot 008412) and $\gamma$-hANP-(1-67) (Cardiodilatin 1-67 or prepro-ANF-[26-92], Lot 008101) were purchased from Peninsula Laboratories Inc. (Belmont, CA, USA). The $\gamma$-hANP-(1-10) (human ANF-[1-10]), $\gamma$-hANP-(1-25) (human ANF-[1-25]), and $\gamma$-hANP-(17-25) (human ANF-[17-25]) were donated from Shionogi Research Laboratories (Shionogi & Co., Osaka, Japan). The homogeneity of the peptides was confirmed by reverse phase high performance liquid chromatography and amino acid analysis.

**Radioimmunoassay for $\alpha$-hANP (Human ANF-[99-126])**

The plasma $\alpha$-hANP level was determined by a RIA that recognizes a common C-terminal sequence of $\alpha$-hANP and $\alpha$-rat ANP ($\alpha$-rANP), as described previously in detail.\textsuperscript{6-9}

**Radioimmunoassay for $\gamma$-hANP-(1-25) (Human ANF-[1-25])**

The $\gamma$-hANP-(1-25) was radioiodinated by the chloramine-T method as reported previously.\textsuperscript{9} The specific activity of $^{125}$I-$\gamma$-hANP-(1-25) ranged from 600 to 900 $\mu$Ci/µg. Labeled $\gamma$-hANP-(1-25) was diluted in assay buffer (0.1 M potassium phosphate buffer, pH 7.4, containing 0.1% gelatin [E. Merck, Darmstadt, West Germany], 1 mM Na$_2$EDTA, 0.2 mM cystine, 0.1% Triton X-100, and 0.01% merthiolate) to a concentration of 15,000 cpm as a total count per tube. Rabbit antiserum was raised against $\gamma$-hANP-(1-30) (human ANF-[1-30]) and used at a final dilution of 1:25,000. The standard curve was constructed by serial dilution of synthetic $\gamma$-hANP-(1-25). The antibody and standard or sample were incubated for 24 hours at 4°C, after which labeled $\gamma$-hANP-(1-25) was added and incubated for an additional 24 hours at 4°C. Bound and free ligands were separated by adding a suspension of dextran-coated charcoal consisting of 600 mg of Norit SX Plus (Norit, Holland) and 60 mg of Dextran T-70 (Pharmacia, Uppsala, Sweden) in 0.05 M potassium phosphate buffer, pH 7.4, containing 0.01% merthiolate. After centrifugation, the supernate was counted in a gamma counter. Nonspecific binding of $\gamma$-hANP-(1-25) was consistently 0.6 to 1.0%. Intra-assay and interassay coefficients of variation were 7.2% ($n = 7$) and 12.2% ($n = 13$), respectively.

**Determination of Plasma $\alpha$-hANP-Like and $\gamma$-hANP-(1-25)-Like Immunoreactivities**

Measurements of plasma $\alpha$-hANP-like immunoreactivity (LI) and $\gamma$-hANP-(1-25)-LI were performed with or without extraction. In the direct assay without extraction, 25 µl of plasma was added to the incubation mixture. Hormone-free plasma was prepared by passing through a Sep-Pak C$_8$ cartridge and used for constructing the standard curves and for diluting plasma samples. Extraction of ANP from plasma was carried out according to the method described previously.\textsuperscript{7,8} Plasma was acidified by the addition of 0.25 ml of 2 M HCl/ml plasma and was applied to a Sep-Pak C$_8$ cartridge. Adsorbed peptides were eluted with 2 ml of 50% acetonitrile in 5 mM trifluoroacetic acid.
acid. The eluate was evaporated to dryness under reduced pressure and reconstituted in the assay buffer and subjected to the RIA. Recoveries of 50 pg of α-hANP and 50 pg of γ-hANP-(1-25) were 70% and 67%, respectively.

High Performance Gel Permeation Chromatography

Extracts from 50 ml of pooled human plasma were subjected to high performance gel permeation chromatography (HP-GPC) on a TSK-GEL G2000 SW column (7.5 x 600 mm; Toyo Soda, Tokyo, Japan) and eluted with 10 mM trifluoroacetic acid containing 0.3 M sodium chloride and 30% acetonitrile as a solvent (described previously). The flow rate was 0.3 ml/min and the fraction volume was 0.36 ml. The column was calibrated with a polypeptide molecular weight calibration kit (Pharmacia) and synthetic α-hANP.

Statistical Analysis

All values are expressed as means ± SEM. Paired or unpaired Student’s t test was used to test the significance of single comparisons. Linear regression analysis was performed with the method of least squares.

Results

Radioimmunoassay for γ-hANP-(1-25)

A typical standard curve of γ-hANP-(1-25) in the RIA is shown in Figure 1A. The minimal detectable quantity in the RIA was as little as 1.7 fmol (5 pg) of synthetic γ-hANP-(1-25) and the 50% binding intercept was 20.5 fmol (60 pg). Dilution curves of various γ-hANP-related peptides and α-hANP are also shown in Figure 1A. The cross-reactivities of γ-hANP-(1-16) (human ANF-[1-16]) and γ-hANP-(1-67) (human ANF-[1-67]) were 10.9% and 74.9%, respectively, on a molar basis. Since γ-hANP-(17-25) did not show significant cross-reactivity, the RIA was presumed to recognize the N-terminal portion of γ-hANP. The α-hANP did not cross-react in this RIA.

Detection of Plasma γ-hANP-(1-25)-Like Immunoreactivity and Its Characterization

The serial dilution curves of human plasma were parallel to the standard curve of γ-hANP-(1-25). HP-GPC coupled with the two RIAs for γ-hANP-(1-25) and for α-hANP revealed that γ-hANP-(1-25)-LI in extracts of pooled plasma from normal subjects was eluted at an approximate molecular weight of 10,000 and was not detected by the RIA for α-hANP (see Figure 1B). In contrast, the peak with α-hANP-LI was eluted at the position corresponding to authentic α-hANP. The plasma γ-hANP-(1-25)-LI level in healthy subjects was 290 ± 35.6 pg/ml, while the plasma α-hANP-LI level determined simultaneously was 20.9 ± 2.8 pg/ml. There was a significant correlation between the two values (r = 0.76, p < 0.01), as shown in Figure 2A. The apparent molar ratio of plasma γ-hANP-(1-25)-LI to α-hANP-LI levels was 15.9 ± 2.1.

Plasma Levels of γ-hANP-(1-25)-Like Immunoreactivity in Patients with Heart Disease

Plasma γ-hANP-(1-25)-LI levels in patients with heart disease were 1810 ± 380 pg/ml for NYHA class I or II and 11,600 ± 3370 pg/ml for NYHA class III or IV, while the plasma α-hANP-LI levels were 201 ± 36.9 pg/ml and 1600 ± 415 pg/ml, respectively.

![Figure 1](http://hyper.ahajournals.org/)

**Figure 1.** A. A representative standard curve of γ-hANP-(1-25) (closed circles) and dilution curves of various γ-hANP-related peptides and α-hANP. B. Gel filtration profiles on a TSK-GEL G2000 SW column of pooled plasma immunoreactivities from healthy subjects detected by the RIA for γ-hANP-(1-25) (closed circles) and by the RIA for α-hANP (open circles). Arrows denote the elution positions of a series of myoglobins of a polypeptide molecular weight calibration kit (Pharmacia), void volume (Vo), total volume (Vt), and synthetic α-hANP.
Correlation between the two values was significant ($r = 0.81$, $p < 0.001$). The results show graded and concomitant increases of plasma $\gamma$-hANP-(1-25)-LI and $\alpha$-hANP-LI levels according to severity of disease, as illustrated in Figure 2B. Both plasma $\gamma$-hANP-(1-25)-LI and $\alpha$-hANP-LI levels were significantly correlated with RAP ($r = 0.80$, $p < 0.01$ and $r = 0.93$, $p < 0.01$, respectively), PAP ($r = 0.90$, $p < 0.01$ and $r = 0.83$, $p < 0.01$, respectively), and PCWP ($r = 0.62$, $p < 0.05$ and $r = 0.61$, $p < 0.05$, respectively).

**Discussion**

In the present study we established a highly sensitive and specific RIA that can recognize the N-terminal sequence of $\gamma$-hANP, $\gamma$-hANP-(1-25). Using this RIA, we detected appreciable amounts of $\gamma$-hANP-(1-25)-LI in normal human plasma; on HP-GPC study, the substance was shown to have an approximate molecular weight of 10,000. The $\gamma$-hANP-(1-25) immunoreactive peak showed no cross-reactivity in the RIA for $\alpha$-hANP, indicating that $\gamma$-hANP-(1-25) does not contain the antigenetic sequence of $\alpha$-hANP. These results suggest that plasma $\gamma$-hANP-(1-25)-LI reflects the N-terminal fragment produced by the removal of $\alpha$-hANP from $\gamma$-hANP, which possibly corresponds to $\gamma$-hANP-(1-98) (human ANF-[1-98]), and that at least two circulating $\gamma$-hANP-derived peptides are present in normal human plasma, N-terminal fragment of $\gamma$-hANP and $\alpha$-hANP (Figure 3).

Recently, Michener et al.\textsuperscript{10} reported the presence of N-terminal immunoreactivity in anesthetized rat plasma with an approximate molecular weight of 14,000 detected in Western blots, which was smaller than the atriopeptin prohormone or $\gamma$-rANP (molecular weight, 17,000) and was not detected by the antiserum against the C-terminal sequence of $\gamma$-rANP or $\alpha$-rANP. Our findings in humans are compatible with their results in rats. The elucidation of the exact nature of $\gamma$-hANP-(1-25)-LI in plasma, however, requires further investigations.

The positive correlation between plasma $\gamma$-hANP-(1-25)-LI and $\alpha$-hANP-LI levels demonstrated in the present study suggests cosecretion of N-terminal fragment of $\gamma$-hANP and $\alpha$-hANP from the heart in humans. Since synthetic $\gamma$-hANP-(1-98) is not available at present, we cannot estimate the cross-reactivity of N-terminal fragment of $\gamma$-hANP in our RIA for $\gamma$-hANP-(1-25). Since $\gamma$-hANP-(1-67) shows quite higher cross-reactivity (74.9\%) than the shorter N-terminal fragment, $\gamma$-hANP-(1-16) (10.9\%), it seems likely that 10K N-terminal fragment of $\gamma$-hANP cross-reacts nearly equally with $\gamma$-hANP-(1-25). Based on this assumption, the calculated apparent molar ratio of plasma $\gamma$-hANP-(1-25)-LI to $\alpha$-hANP-LI levels (15.9 ± 2.1) might reflect the exact molar ratio of circulating N-terminal fragment of $\gamma$-hANP to $\alpha$-hANP. The high apparent molar ratio possibly...
indicates relatively slower disappearance and smaller distribution volume of N-terminal fragment of γ-hANP in the circulation compared with α-hANP. Michener et al. reported that the basal plasma level of N-terminal fragment of γ-rANP in rats is also much higher than the basal α-rANP level. Our observations may be compared to the relation between C-peptide and insulin. The molar ratio of C-peptide to insulin is around 10, and this high ratio in the systemic circulation is ascribed primarily to marked differences in the hepatic clearance of the two peptides.11

In patients with congestive heart failure, elevated plasma ANP level has been reported,12,13 and this is interpreted to reflect the accelerated release in response to the enhanced preload on the heart. In the present study, we confirmed that the plasma α-hANP-LI level showed a graded increase according to the severity of heart disease, and there was a positive correlation between the plasma α-hANP-LI level and central hemodynamic parameters, such as RAP, PAP, and PCWP. In these patients, the plasma γ-hANP-(1-25)-LI level increased in parallel with the plasma α-hANP-LI level, suggesting that an increase in atrial pressure or atrial stretch can be the common stimulus for cosecretion of N-terminal fragment of γ-hANP and α-hANP. We also observed a parallel increase of plasma γ-hANP-(1-25)-LI and α-hANP-LI levels during the infusion of isotonic saline (2 L) into healthy men (unpublished observation). The finding further supports the possible cosecretion of N-terminal fragment of γ-hANP and α-hANP.

Elucidation of the functional significance of N-terminal fragment of γ-hANP must await further investigation. However, the striking sequence conservation of the N-terminal region of γ-ANP among species raises the possibility of unknown biological actions of N-terminal fragment of γ-hANP. The simultaneous measurement of plasma N-terminal fragment of γ-hANP and α-hANP levels may also provide insight into the evaluation of the secretory function of the heart during the therapeutic administration of α-hANP11,13 or of possible pathophysiological conditions producing release of structurally abnormal ANP into the circulation or change of receptors for ANP.

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