Novel Chemiluminescent Enzyme Immunoassays for Individual Quantification of 3 Endogenous Molecular Forms of Atrial Natriuretic Peptide in Human Plasma

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Background: Atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP) are cardiac peptide hormones with pivotal roles in maintaining cardiovascular homeostasis. BNP and its precursor fragment are accepted as gold standard markers for heart failure (HF). Human ANP is present in the atria of the heart and plasma as 3 endogenous molecular forms designated α-ANP, β-ANP, and proANP. A previous study indicated that the ratios of these 3 ANP forms are altered in the plasma of HF patients. The purpose of our study was to establish immunoassays for quantifying the individual ANP forms to collect clinical information.

Methods: We developed 3 plate-based chemiluminescent enzyme immunoassays (CLEIAs) for measuring total ANP (i.e., sum of α-ANP, β-ANP, and proANP), β-ANP, and proANP levels. To minimize background signals, we added single-step PEGylation targeting the immobilized antibody in the conventional plate-based sandwich CLEIA procedure.

Results: CLEIAs with PEGylation showed sensitivity, specificity, reproducibility, and accuracy satisfying clinical requirements. Two of the CLEIAs enabled direct measurement in plasma samples. During treatments, acute decompensated HF patients exhibited marked decreases in plasma β-ANP levels but moderate decreases in plasma proANP level. The plasma ratios of α-ANP/total ANP and proANP/total ANP in acute decompensated HF patients were maintained, whereas the β-ANP/total ANP ratio was significantly decreased at discharge.

Conclusions: The combination of the 3 CLEIAs enabled accurate quantification of α-ANP, β-ANP, and proANP, even in plasma samples, and indicated the potential of β-ANP and proANP as circulating biomarkers for HF, with different characteristics from that of BNP.
Patients with acute decompensated heart failure will benefit from the information presented here. Evidence presented on the methods for individually measuring 3 endogenous forms of the human atrial natriuretic peptide (ANP) will allow better characterization of the concentration and ratio of each molecular form of ANP in plasma and the net bioactivity of circulating ANP. Knowledge in the field of chronological changes in the concentration of each ANP form in patients with acute decompensated heart failure during treatment will be advanced by the information presented.

Atrial natriuretic peptide (ANP) \(^7\) (1, 2) is a cardiac hormone belonging to the natriuretic peptide family that is highly conserved in vertebrates; members of this family share a homologous ring structure formed by an intramolecular disulfide bond (3), and this family also includes B-type natriuretic peptide (BNP) and C-type natriuretic peptide (CNP). All natriuretic peptides are synthesized as precursor polypeptides that are processed to bioactive forms (i.e., \(\alpha\)-ANP, BNP-32, and CNP-22/CNP-53), which are secreted into the circulation. ANP is synthesized mainly in atrial tissues (4) and plays an important role in the maintenance and regulation of cardiovascular homeostasis through natriuretic, diuretic, and vasodilation activities and an antagonistic effect on the renin-angiotensin-aldosterone system (3, 5); these functions are predominantly mediated via cGMP produced by guanylyl cyclase-A or natriuretic peptide receptor-A (5–7).

Because ANP and BNP are highly expressed in and secreted from cardiomyocytes upon stimulation by stretch tension and volume and pressure overload (8, 9), these peptides are used as biomarkers in clinical practice. For diagnosis for heart failure (HF), plasma levels of BNP and the N-terminal fragment of the BNP precursor (NT-proBNP), which show drastically altered profiles upon HF, are accepted as gold standard markers (10–12). In contrast, the plasma ANP level is not frequently measured because circulating ANP shows smaller changes than BNP during progression and remission of HF. Because the pathophysiology of HF is multifactorial and complicated, combined measurement of multiple biomarkers reflecting independent biological processes is required for accurate evaluation and management of HF. The strong demand for novel biomarkers has driven a number of studies for mining biomarker candidates that might complement or replace BNP to advance the diagnosis and prognosis of HF.

In humans, ANP occurs as 3 distinct endogenous molecular forms: bioactive \(\alpha\)-ANP (28 amino acid residues), an antiparallel dimer of \(\alpha\)-ANP (\(\beta\)-ANP; 56 residues), and an \(\alpha\)-ANP precursor (proANP or \(\gamma\)-ANP; 126 residues) (Fig. 1) (13, 14). \(\beta\)-ANP is not found in other species, and its synthesis and secretion processes remain unknown. The bioactivity of \(\beta\)-ANP and proANP is different from that of \(\alpha\)-ANP with regard to both duration and magnitude. All 3 forms are found in atrial tissues, with proANP being predominant (14–17), whereas \(\alpha\)-ANP dominates in normal plasma (18–21). Altered ratios of the 3 forms have been observed in atrial tissues (15, 16, 21) and plasma (22) of patients with HF. However, the utility of \(\beta\)-ANP and proANP as HF biomarkers remains to be evaluated, mainly because there has been no method to specifically measure \(\beta\)-ANP or proANP; previous studies used chromatography, which is not suited for high-throughput analysis.
In the present study, we aimed to reveal the clinical significance of individually measuring the 3 ANP forms in plasma. We developed quantitative plate-based sandwich chemiluminescent enzyme immunoassays (CLEIAs) for the 3 endogenous ANP forms, because currently available assay kits only enable measurement of total ANP (i.e., the sum of the 3 ANP forms). We tested the immunoassays using plasma from acute decompenated heart failure (ADHF) patients to monitor the changes in plasma levels of the 3 ANP forms and to measure their performance as biomarkers in comparison with the existing HF markers, BNP and NT-proBNP.

**MATERIALS AND METHODS**

**Products**

Chemically synthesized human α-ANP, human β-ANP, rat α-ANP, human BNP-32, and mammalian CNP-22 were purchased from the Peptide Institute. proANP [1–31]+Tyr was custom synthesized by Sigma-Aldrich Japan. Recombinant human proANP with an N-terminal FLAG tag (FLAG-proANP) was expressed in silkworms and purified using an anti-FLAG affinity column and reverse-phase (RP)-HPLC as described below. α-ANP fragment peptides were prepared by reduction, carboxymethylation, and subsequent digestion of α-ANP with endoproteinase Asp-N or trypsin (Promega), as described previously (23).

**Preparation and characterization of antibodies**

Anti-α-ANP mouse monoclonal antibodies (Abs), AC201 and KY-ANP-I, were donated by Shionogi & Co. Anti-α-ANP rabbit polyclonal antiserum (#131-7) was prepared as reported previously (24). Mouse monoclonal Abs specific to β-ANP (#32-3) and proANP (#95-5) were raised against immune complexes conjugated with reduced noncyclic α-ANP and proANP [1–31]+Tyr, respectively. All Abs were purified on an Affi-Gel protein A Sepharose column (Bio-Rad) and dia-lyzed against PBS. F(ab’)_2 fragments of AC201 and #95-5 prepared using an F(ab’)_2 preparation kit (Thermo Fisher Scientific) were labeled with alkaline phosphatase (ALP) using the ALP-labeling kit-SH (Dojindo); #32-3 was labeled with ALP using the ALP-labeling kit-NH₂ (Dojindo).
Competitive RIAs for epitope analysis of #32-3 and #95-5 were performed as described previously (23). Briefly, 100 μL each of the sample, Ab, and 125I-labeled tracer solution was mixed and incubated at 4 °C for 40 h. The radioactivity of the pellet was quantified using a γ counter (ARC-1000M; Aloka).

Blood samples

Human blood samples were collected with informed consent, and all studies using human samples were conducted in accordance with the protocols approved by the ethics committee for human experiments of our center (M23-90). Peripheral venous blood was collected with test tubes containing aprotinin (500 kallikrein inhibitor units/mL) and EDTA-2Na (1.5 g/L) from ADHF patients [43 males and 17 females; age 73.0 (66.8–80.0) years (median and interquartile ranges, first to third quartiles), height 160.5 (154.0–168.3) cm] who received no ANP treatment during the study period, at the following time points: first, within 12 h after admission; second, 1–2 days after admission; third, 5–8 days after admission; and fourth, before discharge [21.0 (14.5–33.3) days after admission]. The collected blood samples were used to prepare plasma samples. Plasma samples separated from blood obtained from healthy volunteers [5 males and 5 females; age 42.0 (31.3–47.0) years] were obtained by Kohjin Bio. The plasma samples were aliquoted and stored frozen until measurements. Plasma extract was prepared using a Sep-Pak C18 Plus cartridge (Waters) by washing the cartridge with 10% acetonitrile/0.1% trifluoroacetic acid, followed by elution with 40% acetonitrile/0.1% trifluoroacetic acid. The eluate was lyophilized and reconstituted in the desired buffer.

Sandwich CLEIA

PEGylated anti-α-ANP antibody-coated immunoassay plates were prepared as described previously (23). Briefly, a 96-well plate was coated with the purified Ab of interest and then treated with 5 μmol/L methyl-PEG12-NES ester (Thermo Fisher Scientific) for PEGylation of the primary amine groups on the immobilized Abs. After blocking with Tris-HCl buffer containing 2% BlockAce (DS Pharma Biomedical), 5% horse serum, and 20% sucrose, the plates were dried and stored at −20 °C until use.

Sandwich CLEIA was performed as described previously (23). Briefly, after washing the plate, 50 μL of assay buffer containing 0.5 mmol/L EDTA-2Na, 5% BSA, 0.05% Triton X-100, and 500 U/mL aprotinin was added to each well, and 50 μL of a standard or a test sample was added in duplicate. After incubation at 4 °C for 24 h, the plate was washed and incubated with buffer containing the ALP-labeled Ab of interest at room temperature for 1 h. After washing, the plate was incubated with CDP-Star (Applied Biosystems) solution at room temperature for 20 min, and the chemiluminescence intensity was measured using a SpectraMax L luminometer (Molecular Devices).

The limit of detection (LOD) and limit of quantification (LOQ) were calculated as 3.3 σ/slope and 10 σ/slope, respectively (σ, SD of the intensity of the zero standards; slope, the slope of the calibration curve around the LOD). Spiking of peptides was conducted using 50 μL of the assay buffer containing an appropriate concentration of the peptide in place of the assay buffer as the pre-added buffer.

RP-HPLC analysis of plasma samples

The plasma extract was prepared as described above and reconstituted in 0.1% trifluoroacetic acid containing 0.05% Triton X-100 and then subjected to RP-HPLC on a Symmetry300 C18 column (4.6 × 250 mm, 5 μm, Waters) using linear gradient elution with 10–60% acetonitrile in 0.1% trifluoroacetic acid for 50 min at a flow rate of 1 mL/min. The fractions were collected and lyophilized, and the immunoreactivity of each fraction was measured using CLEIA.
Blood biochemical markers

Plasma levels of BNP, renin activity, and aldosterone were measured by SRL Inc. using the Lumipulse Presto II BNP kit, PRA-FR kit, and SPAC-S Aldosterone kit (Fujirebio Diagnostics). Serum NT-proBNP and plasma cGMP levels were quantified using the Elecsys proBNP II (Roche Diagnostics) and cyclic GMP assay kit (Yamasa Shoyu). Blood urea nitrogen and serum creatinine levels were measured using an automated analyzer (Labspect 008; Hitachi). The analytical performance of these measurements is summarized in Supplemental Table 1 in the Data Supplement that accompanies the online version of this article at http://www.jalm.org/content/vol1/issue1. Estimated glomerular filtration rate was calculated according to the published equation for Japanese individuals: 194 × serum creatinine −1.094 × age −0.287 (× 0.739 for females) [unit, mL · min −1 · (1.73 m2) −1], where serum creatinine is expressed as mg/dL (25).

Statistical analysis

Data are expressed as medians and interquartile range (first to third quartiles). The Wilcoxon signed-rank test and the Friedman test were used to compare the values of 2 and 4 different time points, respectively. The statistical analyses were performed using R version 2.8.1 (http://cran.r-project.org). The differences were considered significant when \( P < 0.05 \).

RESULTS

Preparation of \( \beta \)-ANP– and proANP-specific Abs

To develop CLEIAs for selective measurement of \( \beta \)-ANP and proANP, we raised monoclonal Abs specific for \( \beta \)-ANP and proANP, respectively. The proANP-specific Ab #95-5 showed affinity to its antigen proANP [1–31]+Tyr with a dissociation constant \((K_d)\) of 7.66 \( \times 10^{-11} \) mol/L, but not for \( \alpha \)-ANP or \( \beta \)-ANP (see online Supplemental Fig. 1A and Supplemental Table 2). The \( \beta \)-ANP–specific Ab #32-3 exhibited higher affinity for \( \beta \)-ANP \((K_d = 1.69 \times 10^{-11} \) mol/L) than for \( \alpha \)-ANP \((K_d = 1.34 \times 10^{-8} \) mol/L) (see online Supplemental Fig. 1B).

Development of CLEIAs for the 3 ANP forms

Fig. 1 shows each pair of sandwich Abs used in the CLEIAs. KY-ANP-I and #131-7, which recognize the middle portion of \( \alpha \)-ANP, were used as capture Abs. AC201, which recognizes the C-terminal tail of ANP, #32-3, a \( \beta \)-ANP–specific Ab, and #95-5, targeting the N-terminal portion of proANP, were used as detection Abs to measure total ANP, \( \beta \)-ANP, and proANP levels, respectively. As a calibration standard for the total ANP and proANP CLEIAs, we prepared recombinant FLAG-proANP, which had a purity higher than 95% as determined by densitometric analysis (see online Supplemental Fig. 2). Total ANP and proANP were measured directly in plasma samples; however, \( \beta \)-ANP required sample extraction because of its lower plasma concentration. To minimize nonspecific binding, we used single-step PEGylation mainly targeting the Fc region of the immobilized Abs. PEGylation markedly improved the sensitivity and linearity of the 3 CLEIAs and reduced the LODs and LOQs by 6.2- to 14.6-fold compared to the assays without PEGylation (Fig. 2 and Table 1).

Validation of the CLEIAs

The intra- and interassay CVs for 20 replicates of standard peptides and plasma samples were <10% (Table 1; also see online Supplemental Fig. 3). We evaluated the cross-reactivity of the CLEIAs with each of the 3 ANP forms and with other natriuretic peptides. \( \beta \)-ANP and recombinant FLAG-proANP were specifically detected by the \( \beta \)-ANP and proANP CLEIA, respectively. In the CLEIA for total ANP, \( \alpha \)-ANP, \( \beta \)-ANP, and FLAG-ANP were detected at a substantial ratio of 1:2:1, reflecting that \( \beta \)-ANP is an \( \alpha \)-ANP homodimer. We observed no or negligible cross-reactivity with the other analogs.
Next, we evaluated whether the 3 CLEIAs enabled for direct measurement of the levels of the respective ANP forms in plasma samples. To examine the specificity of detection in plasma samples, extracts from 2 samples were fractionated by RP-HPLC, and the immunoreactivity of each fraction was measured using the 3 CLEIAs. Each CLEIA specifically detected its target ANP forms, and the immunoreactivity of β-ANP and proANP measured with the total ANP CLEIA showed good agreement with that measured with the β-ANP and proANP CLEIAs separately (Fig. 3). We also investigated the effect of the presence of an excess of α-ANP on the measurement of β-ANP with the β-ANP CLEIA because the plasma concentration of β-ANP has been shown to be lower than that of α-ANP (18–21). Up to 1 nmol/L α-ANP did not affect the measurements (see online Supplemental Fig. 4). We further examined the accuracy of the CLEIAs using spike recovery tests. The recovery rates were 100% (15%) [mean (SD)], indicating sufficient accuracy for clinical tests (Table 1). We also conducted dilution tests; a linear correlation was observed when the concentrations measured in the serially diluted plasma samples were plotted against the dilutions, indicating that plasma components did not perturb the CLEIAs (see online Supplemental Fig. 5).

Given the above, the combination of the CLEIAs enabled accurate quantification of β-ANP and proANP, even in plasma samples, which enabled calculation of the α-ANP level by subtracting the values for β-ANP and proANP from the value for total ANP.

**Plasma concentrations of the 3 ANP forms**

By using the 3 CLEIAs, the concentrations of α-ANP, β-ANP, and proANP in 10 normal plasma samples were determined to be 47.9 (30.7–77.8) pmol/L [median and interquartile range (first to third quartiles)], 0.87 (0.66–1.24) pmol/L, and 1.52 (0.82–2.44) pmol/L, respectively. The ratios of α-ANP, β-ANP, and proANP to total ANP were 96.0% (93.1–97.0%), 1.75% (1.05–2.23%), and 2.57% (1.56–4.12%), respectively.

We analyzed the plasma samples of ADHF patients at 4 time points between admission and discharge. We observed marked therapeutic effects during hospitalization by increased fractional shortening, and
decreases in body weight and blood pressure, in addition to the maintained kidney function (i.e., blood urea nitrogen and creatinine levels and estimated glomerular filtration rate) (Table 2). During the course of the treatments, the plasma concentrations of total ANP and 3 ANP forms were significantly decreased (Table 2 and Fig. 4). Significant decreases in levels of β-ANP, BNP, NT-proBNP, and cGMP were observed between the first and second time points, whereas levels of total ANP, α-ANP, and proANP decreased more moderately over the entire time course (Fig. 4). The α-ANP/total ANP and proANP/total ANP ratios were maintained over the test period; in contrast, the β-ANP/total ANP ratio was significantly decreased at the fourth time point. These results indicate that the chronological alterations in plasma levels of α-ANP and proANP, along with ratios of α-ANP, β-ANP, and proANP to total ANP were different from those in plasma levels of BNP and NT-proBNP.

**DISCUSSION**

The importance of monitoring β-ANP and proANP levels in atrial tissues and plasma from patients with cardiac diseases has been indicated

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**Table 1. Characteristics of the 3 established CLEIAs.**

<table>
<thead>
<tr>
<th>CLEIA</th>
<th>Total ANP</th>
<th>β-ANP</th>
<th>proANP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard</td>
<td>FLAG-proANP</td>
<td>β-ANP</td>
<td>FLAG-proANP</td>
</tr>
<tr>
<td>Plasma sample</td>
<td>Intact plasma</td>
<td>Plasma extract</td>
<td>Intact plasma</td>
</tr>
<tr>
<td>Linearity range, pmol/L</td>
<td>0.2–250</td>
<td>0.1–250</td>
<td>0.1–250</td>
</tr>
<tr>
<td>LOD, pmol/L</td>
<td>PEGylation (+)</td>
<td>0.076</td>
<td>0.077</td>
</tr>
<tr>
<td></td>
<td>PEGylation (−)</td>
<td>0.998</td>
<td>1.125</td>
</tr>
<tr>
<td>LOD, pmol/L</td>
<td>PEGylation (+)</td>
<td>0.26</td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td>PEGylation (−)</td>
<td>3.03</td>
<td>3.41</td>
</tr>
<tr>
<td>Intraassay CV, %&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Standard</td>
<td>0.9–4.6</td>
<td>0.3–2.8</td>
</tr>
<tr>
<td></td>
<td>Plasma</td>
<td>3.1–5.2</td>
<td>1.5–2.1</td>
</tr>
<tr>
<td>Interassay CV, %&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Standard</td>
<td>2.5–9.0</td>
<td>4.0–6.2</td>
</tr>
<tr>
<td></td>
<td>Plasma</td>
<td>4.7–7.9</td>
<td>4.8–5.5</td>
</tr>
<tr>
<td>Cross-reactivity, %&lt;sup&gt;b&lt;/sup&gt;</td>
<td>α-ANP</td>
<td>100.0</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>β-ANP</td>
<td>202.4</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>FLAG-proANP</td>
<td>99.0</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>BNP-32</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>CNP-22</td>
<td>0.0</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>proANP[1–31]+Tyr</td>
<td>0.1</td>
<td>0.0</td>
</tr>
<tr>
<td>Recovery, %&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Low concentration</td>
<td>109.2–111.6</td>
<td>103.1–110.8</td>
</tr>
<tr>
<td></td>
<td>Medium concentration</td>
<td>103.4–114.5</td>
<td>92.4–100.7</td>
</tr>
<tr>
<td></td>
<td>High concentration</td>
<td>94.6–106.1</td>
<td>99.1–104.8</td>
</tr>
</tbody>
</table>

<sup>a</sup> Calculated from 20 replicates of 7 concentrations of 0.1–1250 pmol/L standards or plasma samples from 5 healthy volunteers.
<sup>b</sup> Calculated from 7 concentrations of 0.2–1250 pmol/L standards.
<sup>c</sup> The standard was spiked at 2, 10, and 100 pmol/L for total ANP CLEIA and at 1, 5, and 25 pmol/L for β-ANP and proANP CLEIAs, respectively.
in previous studies, which were mainly based on chromatographic analysis (15–17, 21, 22). Because of the lack of a quantification method that is highly specific to each ANP form and enables high-throughput measurement, the clinical significance of individual ANP forms as biomarkers has not been fully examined. In the present study, we developed 3 plate-based CLEIAs to quantify individual ANP forms, 2 of which allowed direct analysis of plasma samples without extraction. The combined usage of the 3 CLEIAs enabled accurate quantification of the 3 ANP forms based on the number of α-ANP units.

We examined the effect of PEGylation of the immobilized Abs because PEGylation was expected to minimize the background signal by preventing nonspecific binding and interaction of plasma components recognizing the Fc region (23). As expected, PEGylation of the monoclonal KY-ANP-I and the polyclonal #131-7 significantly improved the sensitivity of the CLEIA, with a LOD of <0.1 pmol/L, which was consistent with our previous results (23). These data indicate the general usefulness of PEGylation for plate-based immunoassays with IgG class Abs.

The development of an immunoassay specific for β-ANP was challenging because β-ANP is an α-ANP homodimer and therefore has 2 amino acid sequences identical to α-ANP. Only one trial has been reported to date for β-ANP-specific detection, which could not entirely exclude detection of α-ANP present in the samples (26). To overcome this problem, we focused on the differences in 3-dimensional structure of α-ANP and β-ANP; the peptide backbone of α-ANP has a ring structure with the presence of an intramolecular disulfide bond, whereas β-ANP has 2 intermolecular disulfide bonds with 2 peptide backbones that provides a more flexible and extended structure. By using noncyclic ANP as an antigen in several trials, we successfully produced the β-ANP–specific Ab #32-3. The monoclonal #32-3 exhibited 1000-fold higher affinity for β-ANP than α-ANP, resulting in high specificity of the developed CLEIA for β-ANP. Thus, this CLEIA might recognize parallel β-ANP together with the naturally occurring antiparallel β-ANP (14).

The β-ANP CLEIA revealed that the circulating β-ANP level was approximately 1 pmol/L in healthy subjects, which is consistent with reported values and is below or near the LOD (18–21). The increased β-ANP level observed in HF patients at admission decreased during hospitalization to the levels observed in younger healthy subjects. This result indicates that the plasma β-ANP level is affected by severity of HF rather than by age (16), as has been previously suggested (22). The decrease in β-ANP level from

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Fig. 3. Specificity of the 3 CLEIAs for measuring plasma samples.
Two plasma extracts were fractionated by RP-HPLC, and the immunoreactivity of each fraction was measured using the CLEIAs. The elution time of the 3 ANP forms is indicated by the arrows.
admission to discharge was comparable to that of BNP and NT-proBNP, indicating that circulating β-ANP levels reflect features of HF pathophysiology. In addition, the circulating β-ANP level exhibited a larger decrease rate than the total ANP level, decreasing to the normal level at discharge, which suggests that this ANP form is more indicative of the severity of HF than total ANP. Although β-ANP can be converted into 2 molar equivalents of α-ANP in plasma, the conversion of α-ANP to β-ANP seems to rarely occur in plasma. Collectively, the circulating β-ANP level is concluded to be a biomarker candidate for HF, which can be used even in patients who are administered α-ANP as a treatment.

proANP is processed by a serine protease corin to produce mature α-ANP and an N-terminal fragment peptide that are secreted from the cardiomyocytes; thus, the plasma level of proANP is substantially lower than that of α-ANP. Although previous studies have failed to detect proANP in plasma of healthy subjects, our proANP CLEIA exhibited high sensitivity, enabling quantification of low concentrations of circulating proANP. In the plasma, proANP is more resistant to protease digestion than α-ANP and thus more stably present. In addition, the aggregation of proANP observed in atrial tissues is unlikely to occur in plasma, as indicated by chromatographic analyses. In this context, a possible influence of proANP aggregation on Ab detection is excluded when this CLEIA is used with plasma samples. Therefore, the immunoreactivity measured by the proANP CLEIA is considered to approximate the total amount of unprocessed precursors in plasma.

In contrast to the proANP/total ANP ratio of approximately 2.5% in healthy subjects, ADHF pa-

Table 2. Pathophysiological parameters of 60 patients with ADHF at the first (on admission) and fourth (before discharge) time points of measurement.

<table>
<thead>
<tr>
<th></th>
<th>First time point</th>
<th>Fourth time point</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>New York Heart Association functional class</td>
<td>3.0 (3.0–4.0)</td>
<td>2.0 (2.0–2.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Body weight, kg</td>
<td>58.1 (51.2–70.4)</td>
<td>52.4 (46.3–60.8)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Mean blood pressure, mmHg</td>
<td>94.8 (78.8–110.1)</td>
<td>72.5 (66.6–78.6)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Ejection fraction, %</td>
<td>31.3 (22.5–42.5)</td>
<td>38.0 (27.8–52.6)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Total ANP, pmol/L</td>
<td>226.5 (123.7–365.8)</td>
<td>126.1 (64.2–228.9)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>α-ANP, pmol/L</td>
<td>193.5 (95.0–301.8)</td>
<td>106.7 (52.1–211.2)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>β-ANP, pmol/L</td>
<td>5.70 (2.09–17.2)</td>
<td>1.46 (0.16–3.91)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>proANP, pmol/L</td>
<td>26.0 (11.2–42.1)</td>
<td>12.8 (5.84–28.1)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>α-ANP/total ANP, %</td>
<td>87.4 (79.7–91.4)</td>
<td>88.2 (77.2–92.7)</td>
<td>0.083</td>
</tr>
<tr>
<td>β-ANP/total ANP, %</td>
<td>2.27 (0.98–4.31)</td>
<td>0.94 (0.10–3.18)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>proANP/total ANP, %</td>
<td>10.1 (6.63–16.5)</td>
<td>9.80 (6.54–20.3)</td>
<td>0.515</td>
</tr>
<tr>
<td>BNP, ng/L</td>
<td>785.0 (405.0–1040.0)</td>
<td>248.0 (113.8–495.5)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>NT-proBNP, μg/L</td>
<td>5.89 (2.98–14.3)</td>
<td>1.56 (0.68–4.01)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Plasma renin activity, μg/L/h</td>
<td>1.35 (0.70–7.65)</td>
<td>3.65 (1.10–11.0)</td>
<td>0.058</td>
</tr>
<tr>
<td>Aldosterone, nmol/L</td>
<td>2.11 (1.31–4.29)</td>
<td>3.07 (1.95–7.04)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>cGMP, nmol/L</td>
<td>12.0 (7.03–16.5)</td>
<td>6.90 (5.15–10.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Blood urea nitrogen, mmol/L</td>
<td>9.46 (6.69–13.4)</td>
<td>9.64 (7.14–12.9)</td>
<td>0.204</td>
</tr>
<tr>
<td>Creatinine, μmol/L</td>
<td>103.0 (81.5–149.6)</td>
<td>113.6 (84.6–144.3)</td>
<td>0.459</td>
</tr>
<tr>
<td>Estimated glomerular filtration rate, ml · min⁻¹ · (1.73 m²)⁻¹</td>
<td>41.4 (28.7–56.2)</td>
<td>41.9 (29.2–53.9)</td>
<td>0.244</td>
</tr>
</tbody>
</table>

*Values are presented as medians and interquartile range (first to third quartiles).
Patients exhibited a proANP ratio of approximately 12% at admission, which was consistent with the values observed in plasma of severe HF patients (22, 33). The increased proANP/total ANP ratio in HF reflects an aberrant ratio of unprocessed proANP and mature α-ANP. This result may be explained by impaired processing of proANP in the cardiomyocytes, since reduced activity of the rate-limiting corin in failing hearts has been suggested by several studies (30, 34). In this context, the increased proANP/total ANP ratio may reflect insufficient corin activity compared with the demand for bioactive ANP to improve the stress condition of the failing heart.

Interestingly, the high proANP/total ANP ratio at admission was sustained until discharge. Renal function, which affects the ANP clearance rate (35, 36), was not altered over the study period; thus, the sustained high proANP/total ANP ratio was likely to reflect sustained secretion of proANP from cardiomyocytes, probably as a result of insufficient processing, even at discharge, as hypothesized above. In contrast, the circulating proBNP/total BNP ratio was altered in response to HF deterioration and improvement (37), suggesting that the activity of convertase to process proBNP is also regulated by the severity of HF. The difference observed between proANP and proBNP
may have resulted from greater augmentation of the expression of BNP than that of ANP in the failing heart and the significant reduction in plasma BNP level during hospitalization. The time course of plasma levels of cGMP, a downstream mediator of ANP and BNP signaling, displayed a decreasing profile similar to that observed for plasma ANP rather than that for plasma BNP; this cGMP profile is likely to reflect the larger contribution of ANP than BNP to the guanylyl cyclase-A–mediated signaling responsible for compensatory function of the natriuretic peptides. Therefore, the increased proANP/total ANP ratio is considered to reflect aberrant processing of proANP in the failing heart, and thus endogenous proANP may be a circulating biomarker for HF with implications different from those of the more stable surrogate marker, midregional proANP (corresponding to proANP[53–98]) (38).

The present study has several limitations. First, quantification of circulating β-ANP requires sample extraction, leading to experimental error of the assay. In addition, further improvement of the current plate-based method is necessary to achieve a high-throughput test for clinical use. Finally, because evaluation of the clinical significance was preliminary in terms of a single-center study performed in a limited number of ADHF patients, further validation with a larger number of patients is required.

In conclusion, concomitant use of the 3 CLEIAs with high sensitivity, specificity, reproducibility, and accuracy is a tool to monitor endogenous α-ANP, β-ANP, and proANP, enabling evaluation of the net bioactivity of circulating ANP. Using these new tools, we provided a possibility that circulating β-ANP and proANP are candidate HF biomarkers that may reflect pathophysiological phases different from those indicated by BNP and NT-proBNP. Our present study also provides clues to elucidate the molecular mechanisms underlying the aberrant expression and secretion of β-ANP and proANP, furthering elucidating the pathophysiology of HF.

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