Detection of Endogenous B-Type Natriuretic Peptide at Very Low Concentrations in Patients With Heart Failure

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Background—The myocardium secretes B-type natriuretic peptide (BNP) in response to stimuli associated with heart failure (HF). However, high immunoreactive-BNP levels in patients with HF are associated with a paradoxical lack of natriuretic response. We hypothesized that commercially available assays for immunoreactive BNP do not reflect the bioactivity of the natriuretic peptide system, because they measure both unprocessed inactive pro-BNP and mature BNP 1-32. We describe an assay for the detection of bioactive BNP 1-32 and confirm very low concentrations in plasma from HF patients.

Methods and Results—We developed a quantitative mass spectrometry immunoassay to capture endogenous BNP peptides using high affinity antibodies. Bound BNP and its truncated fragments were detected by matrix assisted laser desorption ionization–time of flight mass spectrometry based on their predicted masses. Mass spectrometry immunoassay revealed rapid in vitro degradation of BNP 1-32 in plasma, which requires plasma collection in the presence of high protease inhibitor concentrations. In 11 of 12 HF patients BNP 1-32 was detectable, ranging from 25 to 43 pg/mL. Several degraded forms of BNP were also detected at similarly low levels. In contrast, parallel measurements of immunoreactive BNP using the Biosite assay ranged from 900 to 5000 pg/mL.

Conclusions—Detection of endogenous BNP 1-32 requires special preservation of plasma samples. Mass spectrometry immunoassay technology demonstrates that HF patients have low levels of BNP 1-32. Commercially available immunoreactive-BNP assays overrepresent biological activity of the natriuretic peptide system because they cannot distinguish between active and inactive forms. This observation may, in part, explain the “natriuretic paradox.”

Key Words: natriuretic peptides ■ diagnosis ■ circulation ■ heart failure ■ mass spectrometry

B-type natriuretic peptide (BNP) is a cardiac hormone with mostly ventricular expression under conditions of volume overload and increased filling pressure.1–3 BNP is synthesized as a 108-aa propeptide and proteolytically processed to release an inactive 76-aa N-terminal fragment and bioactive mature BNP 1-32 into the bloodstream. Commercially available assays have proven utility for the diagnosis of heart failure (HF) with high specificity.4,5 At the same time, recombinant BNP 1-32 (nesiritide) has been approved for treatment of acute decompensated congestive HF; infusions of nesiritide lead to prompt symptomatic improvement in acutely ill HF patients.6,7 The seemingly paradoxical lack of natriuretic response to the high endogenous immunoreactive BNP (iBNP) levels as determined by commercially available assays has led investigators to hypothesize that altered and inactive circulating forms of BNP exist in HF patients.8–10 Indeed, experimental evidence suggests multiple molecular forms of BNP in HF patient plasma.11–14 Recent publications have shown that the intact precursor pro-BNP 1-108 is a major circulating component in HF patients, suggesting impaired processing mechanisms in the HF state.9,15 Moreover, pro-BNP has no16 or significantly reduced bioactivity compared with BNP 1-32,9 yet cross-reacts in commercial immunoassays because of common epitopes.9 Direct measurements of BNP 1-32 has been attempted by mass spectrometry17 and a combination of solid phase extraction, high-performance liquid chromatography, and immunodetection.11,12,18 Neither approach was able to detect the presence of the active BNP form, supporting the hypothesis of a somehow altered iBNP in HF patient samples. We describe a sensitive and quantitative mass spectrometry immunoassay (MSIA)19,20 to successfully measure BNP 1-32 in HF patients. We also present direct structural evidence for several proteolytically degraded forms of BNP in these patients. Further, we show that iBNP levels as detected by the Biosite Triage (Biosite Inc, San Diego, Calif) assay significantly overestimate the levels of bioactive, low molecular weight BNP, including BNP 1-32.
Materials
Protease inhibitors were from Calbiochem (4-(2-aminoethyl)benzenesulfonyl fluoride [AEBSF], benzamidine, pepstatin, H-Phe-Phe-Arg-chloromethylketone (PPACK II), protease inhibitor set III and VII) and Sigma (Leupeptin, aprotinin). The buffers HBS-N (0.01 mol/L HEPES, pH 7.4, containing 0.15 mol/L NaCl) and HBS-EP (0.01 mol/L HEPES, pH 7.4, containing 3 mmol/L EDTA, 0.15 mol/L NaCl, and 0.005% Tween20) were both from Biacore AB. Tween 20 and α-cyano-4-hydroxycinnamic acid were provided by Sigma-Aldrich. Sodium chloride, ammonium acetate, acetonitrile, trifluoroacetic acid, and ultrapure water were all purchased from American Sigma-Aldrich. Sodium chloride, ammonium acetate, acetonitrile, trifluoroacetic acid, and ultrapure water were all purchased from American Sigma-Aldrich. A mixture of equal concentrations (45 μg/mL) of Mabs 8.1 and 106.3 was described elsewhere and requires modification into storage tubes containing benzamidine and AEBSF (final inhibitor concentrations 10 mmol/L and 5 mmol/L, respectively) and stored at −70°C. Patients with Biosite Triage BNP assay results of >750 pg/mL were enrolled (Biosite Inc). Patient information is summarized in Table 1. Plasma from 10 consenting healthy volunteers was collected from the Lifescan Inc blood center (Milpitas, Calif) under identical conditions. Biosite Triage assay iBNP levels from these control subjects varied from <5 to 29 pg/mL.

Plasma Sample Collection
The study was approved by the Institutional Review Board at Christ Hospital, Cincinnati. Blood samples from 12 New York Heart Association functional class III and IV HF patients were collected into EDTA vacutainers and transferred immediately after centrifugation into storage tubes containing benzamidine and AEBSF (final inhibitor concentrations 10 mmol/L and 5 mmol/L, respectively) and stored at −70°C. The study was approved by the Institutional Review Board at Christ Hospital, Cincinnati. Blood samples from 12 New York Heart Association functional class III and IV HF patients were collected into EDTA vacutainers and transferred immediately after centrifugation into storage tubes containing benzamidine and AEBSF (final inhibitor concentrations 10 mmol/L and 5 mmol/L, respectively) and stored at −70°C. Patients with Biosite Triage BNP assay results of >750 pg/mL were enrolled (Biosite Inc). Patient information is summarized in Table 1. Plasma from 10 consenting healthy volunteers was collected from the Lifescan Inc blood center (Milpitas, Calif) under identical conditions. Biosite Triage assay iBNP levels from these control subjects varied from <5 to 29 pg/mL.
The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.

**Results**

**Rapid Degradation of BNP 1-32 in Human Plasma**

Previous evidence suggests that proteases in the circulation degrade BNP after secretion.\(^{21,22}\) We reasoned, therefore, that plasma collection conditions are crucial to preserve the structural integrity of any BNP forms that might be present in HF patients at the point of sampling. To study the kinetics of proteolytic degradation of BNP 1-32 in human plasma and the resulting degradation products, we developed a highly sensitive and quantitative MSIA.\(^{19,20}\) MSIA uses covalent solid phase coupling of 2 high-affinity monoclonal antibodies against different epitopes of BNP 1-32 to allow for the capture of C- and N-terminal truncated forms of BNP 1-32. The antibody loaded resin is contained within a small pipette tip, which is crucial for efficient binding, washing, and elution without significant sample loss. Eluted peptides are spotted directly onto a MALDI sample plate and analyzed. The principle of MSIA is depicted in Figure 1. Figure 2 shows the time course of BNP 1-32 degradation spiked at 10 ng/mL into heparin plasma from a healthy volunteer. Incubation was carried out at 37°C to simulate degradation in vivo. A rapid disappearance of the intact BNP 1-32 peptide can be observed. After 10 minutes the mass spectrum shows new peaks that can be assigned to BNP 3-32, BNP 1-30, BNP 3-30, BNP 3-29, and the isobaric pair BNP 1-29/4-31. As the starting material continues to be degraded, the ratio of BNP 3-32 and BNP 1-32 increases and new degradation products appear at 15 and 30 minutes, eg, BNP 4-29, BNP 5-29. After 1 hour almost no BNP 1-32 is detectable.

**Inhibition of Protease Activity**

BNP 1-32 was shown to be an efficient substrate for dipeptidyl peptidase IV, which leads to rapid formation of BNP 3-32 as assessed by mass spectrometry.\(^{23}\) In addition, Kallikrein-like activities have previously been implicated in the C-terminal degradation of BNP 1-32 as assessed by immunoassay.\(^{24}\) To minimize the effect of protease degradation after blood sampling we tested a broad range of inhibitors individually and in combination using MSIA. A mixture of AEBSF and leupeptin at 2.5 mmol/L each was determined to be most effective. An alternative inhibitor for leupeptin was benzamidine, 10 mmol/L of which prevented degradation of BNP 1-32 in combination with 5 mmol/L AEBSF. Incubation was carried out at room temperature. Figure 2 shows BNP 1-32 stability in EDTA plasma in the presence of effective protease inhibitors concentrations for up to 2 hours.

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**Figure 2.** Time course for BNP 1-32 hydrolysis in heparin plasma. The peptide was added at 10 ng/mL and allowed to incubate at 37°C for 1 hour. Aliquots were removed and analyzed by BNP-MSIA as described in Materials and Methods section.

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**Figure 3.** Prevention of BNP 1-32 hydrolysis. A, EDTA plasma from a healthy donor was spiked with 10 ng/mL of BNP 1-32 in the presence of 10 mmol/L benzamidine and 5 mmol/L AEBSF. Incubation was carried out at room temperature. B, Both protease inhibitors were added 10 minutes after the BNP spike into plasma. At that point a plasma aliquot was frozen and stored at −70°C. Six-month stability of this sample was assessed by MSIA.
3B shows long-term stability of such treated samples for up to 6 months, when stored at −70°C.

Quantitative MSIA Assay Development

Based on initial qualitative MSIA screening of HF patient plasma samples we synthesized BNP peptides with N- and C-terminal truncations. These peptides (BNP 1-32, BNP 3-32, BNP 2-31, BNP 4-32, BNP 5-32, BNP 5-31, BNP 4-30, and BNP 4-27) were added at equimolar concentrations to normal plasma to generate external standard curves for each peptide. MSIA achieved a sensitivity of 3.1 pmol/L for each peptide (lower level of quantification) with a signal-to-noise ratio of 35 even at the lowest concentration. This corresponds to roughly 10 pg/mL of BNP 1-32 and slightly lower pg/mL concentrations for the shorter peptides, depending on their molecular weights. Further, we included an N-terminally biotinylated BNP 1-32 (bBNP) at 500 pg/mL as an IRS to account for potential variations associated with both the affinity purification and MALDI processes. By choosing an IRS with a higher molecular weight compared with BNP 1-32, we avoided any overlap with naturally occurring BNP fragments in the mass spectrum. Figure 4A shows a typical standard curve for 3 of the 8 standard BNP peptides. We observed a nonlinear relationship, reflecting binding behavior of the BNP antibodies to the various BNP peptides during the MSIA process at very low peptide concentrations. Panel B depicts 4 mass spectra of the standard peptide mixture at 6.25, 25, 100, and 400 pmol/L, respectively. In all samples, the IRS was present at 500 pg/mL. Different ion intensities observed for different peptides relative to the IRS reflect different ionization efficiencies of each peptide and can thus be normalized.

Plasma Concentrations of BNP 1-32 and Related Fragments

After informed consent, we prospectively collected plasma with the inclusion of appropriate protease inhibitors benzamidine and AEBSF from 12 patients who had advanced HF. Each sample was analyzed by a 2-step MSIA as illustrated in Figure 1, with the inclusion of the IRS and external standard curves. We also analyzed 10 confirmed healthy donor samples. The resulting peak lists from all MSIA runs were scrutinized for the presence of predicted BNP-related peptides as described under Materials and Methods section. Mass peaks were assigned based on predicted masses for BNP peptide fragments. Of note, mass peaks observed in either the normal controls or the MSIA extractions using the control antibody could not be assigned to any BNP-related peptide, validating the high specificity of the MSIA approach with BNP monoclonal antibodies 8.1 and 106.3. Figure 5 shows representative mass spectra from 2 HF patients and 2 normal controls after BNP specific extraction. The quantitative re-
Results for all 12 HF samples are summarized in Table 2. In 11 of 12 patients we measured low levels of intact BNP 1-32 ranging from 25 to 43 pg/mL. BNP 3-32, BNP 4-32, and BNP 5-32 were present in all patients. In some patients, we detected small amounts of methionine-oxidized BNP 3-32, BNP 4-32, and BNP 5-32. BNP peptides with C-terminal truncations could also be assigned in 3 patients (Figure 5) but were not quantifiable because they were below the levels of quantification.

Comparison With Triage Biosite BNP Assay

Samples from all HF patients were analyzed using the Triage Biosite BNP assay at study enrollment and blood sample collection. Figure 6 highlights the differences between the iBNP concentrations obtained with the Biosite assay and the sum of all BNP fragments determined by MSIA. We did not include patients 2, 4, and 6 in this comparison, because we were unable to quantify the C-terminally truncated BNP peptides in these samples.

Discussion

Our data lend further support to the hypothesis that high levels of iBNP as measured in a commercial point of care (POC) assay do not reflect the bioactivity of the natriuretic peptide system. Yandle et al. first showed the presence of circulating unprocessed pro-BNP 1-108 in HF patients. We and others found that recombinantly expressed pro-BNP has significantly reduced or no activity at all, and we provided Western blot evidence that pro-BNP constitutes a major plasma component in HF patients. Similarly, Seferian et al. measured pro-BNP; BNP ratios of up to 10:8:1 in patients with HF. Further, because the epitopes are preserved in pro-BNP, we have also shown that at least 2 of the commercial POC assays for BNP cross-react with the inactive forms of pro-BNP.
speculate that even shorter peptides may have reduced natriuretic hormone response in HF patients, especially in the acutely decompensated state, and why infusion with bioactive BNP 1-32 (nesiritide) generally provides symptomatic relief.

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Disclosures

Dr Menon is a consultant and shareholder of CHF Solutions Inc.

References

CLINICAL PERSPECTIVE

The important physiologic actions of the natriuretic peptide system become attenuated in the setting of advanced heart failure. This process is complex and involves multiple mechanisms. We developed a uniquely sensitive mass spectrometry-based method for the specific detection of endogenous forms of B-type natriuretic peptide (BNP). We also established rigorous patient plasma sampling methods allowing precise analysis and showed that structural preservation of endogenous BNP forms requires unusually high concentrations of protease inhibitors. This enabled us to detect and quantify BNP 1-32, as well as multiple other forms of BNP, in plasma from 12 patients with advanced heart failure. All patients in our study had markedly elevated endogenous immunoreactive BNP levels, as assessed with the Biosite POC assay. In contrast, the actual BNP 1-32 levels in the patients were very low. Our data confirm that currently available diagnostic BNP assays do not assess the levels of biologically active BNP. We also demonstrated the presence of shorter proteolytically processed forms of BNP 1-32. The clinical significance of these forms remains to be investigated. The well-documented clinical utility of rapid BNP immunoassays remains unchallenged; however, our data help to explain the blunting of the expected physiologic responses to apparently high levels of BNP. We hypothesize that some patients with advanced heart failure may actually be in a state of natriuretic peptide deficiency and may benefit from exogenous administration of recombinant BNP 1-32. Our data provide novel insights that provide strong support for the hypothesis of inefficient prohormone processing in advanced heart failure.
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