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.”

(Circ Heart Fail. 2008;1:258-264.)

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. 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. 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. 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. Indeed, experimental evidence suggests multiple molecular forms of BNP in HF patient plasma. 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. Moreover, pro-BNP has no or significantly reduced bioactivity compared with BNP 1-32, yet cross-reacts in commercial immunoassays because of common epitopes. Direct measurements of BNP 1-32 has been attempted by mass spectrometry and a combination of solid phase extraction, high-performance liquid chromatography, and immunodetection. 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) 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.
Methods

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-hydroxy cinnamic acid were provided by Sigma-Aldrich. Sodium chloride, ammonium acetate, acetonitrile, trifluor acetic acid, and ultrapure water were all purchased from American Bioanalytical, and n-octyl glucoside was from Roche Diagnostics.

Recombinant human BNP 1-32 was from Scios, Inc. All other BNP peptides were synthesized by the American Peptide Company with a BNP specific MSIA tip. For normalization biotinylated BNP (IRS) was added to each sample before extraction.

Mass Spectrometry Immunoassay

MSIA protein extraction was achieved using antibody derivatized affinity pipettes (Intrinsic Bioprobes, Inc). These affinity pipettes or MSIA-Tips were produced as described previously. A mixture of equal concentrations (45 pg/μL) of Mabs 8.1 and 106.3 were used as the affinity ligand. For nonspecific MSIA affinity tips an unrelated antibody against antihuman β2-microglobulin (β2M, Dako-Cytomation) was coupled.

Plasma was thawed at room temperature and incubated at 37°C for 10 minutes. Warmed plasma was centrifuged at 5000 g, 500 μL aliquots were transferred into a 96-well sample tray. Biotinylated BNP (bBNP) 1-32 was added to each well at a final concentration of 500 pg/mL to serve as an internal reference for MSIA.22 Samples were diluted 2-fold with HBS-EP, containing 1% Tween20 and 1 mol/L NaCl before MSIA.

Analyses of spiked standard curve samples, HF samples, and healthy control samples were performed in parallel. The workflow is depicted in Figure 1. Samples (1 mL) were first drawn 50 times through β2M affinity tips by automated aspiration/dispensing of 150 μL, followed by 300 times through BNP affinity tips. Next, each affinity tip was rinsed sequentially with HBS-EP, water, 25% acetonitrile in 2 mol/L ammonium acetate, 50 mmol/L n-octyl glucoside, and water (15 aspirations/dispenses at 150 μL for each step). Bound proteins were eluted from the affinity tip with 3.5 mL of matrix assisted laser desorption ionization (MALDI) matrix solution (6 mg/mL α-cyano-4-hydroxycinnamic acid in acetonitrile/water (1:2 vol/vol) with 0.8% trifluor oacetic acid) and deposited directly onto the MALDI-target.22 Mass spectra were acquired for each eluent by summing six 250-laser shot acquisitions using a linear Bruker Autoflex MALDI-time of flight (TOF) mass spectrometer. Resulting mass spectra were batch processed in Flex Analysis 2.4 (Bruker Daltonics) to label all spectral peaks with a signal-to-noise >20 and to internally calibrate the m/z axis using bBNP. In addition, spectral intensities were normalized to the peak intensities of bBNP for intersample comparison and quantification. Peak lists, containing peak characteristics (m/z, signal-to-noise, normalized intensities) were imported into spreadsheets for data analysis.

Data Analysis

MSIA peak lists were screened for m/z values matching the m/z values calculated for theoretical BNP fragments. We assumed an intact disulfide bond between Cys15 and Cys26. We also considered Met oxidation in one or both positions as a possible posttranslational modification. Last, we based the hypothetical peak list on the specificity of Mabs 8.1 and 106.3 and their known epitopes. MSIA mass peaks with a signal-to-noise of ≥20 and a mass error of ±212 ppm, compared with the theoretical mass were identified. Mass error tolerance was calculated by dividing the difference between the theoretical mass and the experimental mass by the theoretical mass. For BNP peptides for which a standard curve was generated, concentrations were calculated based on their peak intensities normalized to the internal reference standard (IRS) bBNP.

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. 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 INP levels from these control subjects varied from <5 to 29 pg/mL.

### Table 1. Data for Heart Failure Patients Used in this Study

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age/Sex</th>
<th>NYHA Classification</th>
<th>Biosite Triage BNP Value (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>75/F</td>
<td>IIIA</td>
<td>5239</td>
</tr>
<tr>
<td>2</td>
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<td>IV</td>
<td>2782</td>
</tr>
<tr>
<td>3</td>
<td>79/M</td>
<td>IV</td>
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</tr>
<tr>
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<td>78/F</td>
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<tr>
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<td>56/F</td>
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</tr>
<tr>
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<td>2120</td>
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<td>9</td>
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<td>12</td>
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<td>IIIA</td>
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</tbody>
</table>

NYHA indicates New York Heart Association; BNP, B-type natriuretic peptide.
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. Similar degradation was observed when BNP was spiked into whole blood, before plasma preparation and MSIA analysis (data not shown). We also tested BNP degradation in EDTA plasma, which had no effect on the rapid transformation of BNP 1-32 to 3-32, but slowed down further conversion significantly (data not shown). The same was true for incubation at 4°C, which slowed degradation only somewhat. We concluded that commonly used collection conditions are inefficient to prevent BNP 1-32 degradation during and after sample collection.

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. In a typical experiment 10 ng/mL of BNP 1-32 were added to normal EDTA plasma in the presence of 1 or several protease inhibitors. We found no or little effect of 0.5 mmol/L bestatin, 0.1 mmol/L pepstatin A, and up to 1 mmol/L E64. PPACK I and II at 1 mmol/L effectively prevented degradation at the C terminus but did not inhibit dipeptidyl peptidase activity at the N terminus. AEBSF, leupeptin, and benzamidine were tested up to 5 mmol/L individually and in various combinations. 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. Figure 3A shows BNP 1-32 stability in EDTA plasma in the presence of 1 or several protease inhibitors. We found no or little effect of 0.5 mmol/L bestatin, 0.1 mmol/L pepstatin A, and up to 1 mmol/L E64. PPACK I and II at 1 mmol/L effectively prevented degradation at the C terminus but did not inhibit dipeptidyl peptidase activity at the N terminus. AEBSF, leupeptin, and benzamidine were tested up to 5 mmol/L individually and in various combinations. 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. Figure 3A shows BNP 1-32 stability in EDTA plasma stored at room temperature in the presence of effective protease inhibitor concentrations for up to 2 hours. We also tested whether these protease inhibitors were effective when added 10 minutes after the addition of exogenous BNP 1-32 to allow for some degradation to BNP 3-32. Figure 3B shows that when these protease inhibitors were added 10 minutes after the BNP spike into plasma, no or little degradation could be observed. 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\textsuperscript{14} 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\textsuperscript{9} or no activity at all,\textsuperscript{16} and we provided Western blot evidence that pro-BNP constitutes a major plasma component in HF patients.\textsuperscript{9} Similarly, Seferian et al\textsuperscript{15} 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

<table>
<thead>
<tr>
<th>BNP Peptide (pg/mL)</th>
<th>HF Patient No.</th>
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<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>1-32</td>
<td>28</td>
</tr>
<tr>
<td>3-32</td>
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</tr>
<tr>
<td>4-32</td>
<td>86</td>
</tr>
<tr>
<td>4-32(_{ox})</td>
<td>17</td>
</tr>
<tr>
<td>5-32</td>
<td>228</td>
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<tr>
<td>5-32(_{ox})</td>
<td>&lt;LOQ</td>
</tr>
<tr>
<td>5-31</td>
<td>&lt;LOQ</td>
</tr>
<tr>
<td>5-27</td>
<td>NQ</td>
</tr>
<tr>
<td>5-26</td>
<td>NQ</td>
</tr>
</tbody>
</table>

LOQ indicates lower level of quantification, 3.1 pmol/L for each peptide; NQ, mass peaks for these peptides were detectable, but were not quantified; MSIA, mass spectrometry immunoassay; HF, heart failure; BNP, B-type natriuretic peptide.
Figure 6. Comparison of total BNP levels as determined by the Triage Biosite assay (white bars) and MSIA (black bars). All BNP fragments detected and quantified by MSIA as shown in Table 2 were added together for this figure to represent the total amount of low molecular weight BNP.

precursor. Other investigators have attempted direct detection of active BNP 1-32 using different extraction and detection methods, including mass spectrometry. In our study, we were able to quantify active BNP 1-32, for the first time, in 11 of 12 patients diagnosed with HF. Our unique approach to capture circulating BNP peptides with high affinity monoclonal antibodies, coupled with high sensitivity MALDI-TOF mass spectrometry detection allowed us to study the molecular complexity of BNP degradation in human plasma. We found that effective protease inhibition is crucial to preserve the structural integrity of BNP 1-32 during and after blood collection. Although commonly used EDTA anticoagulation tubes are sufficient to preserve iBNP, additional inhibitors in the millimolar range are required for complete protease inactivation. This may explain why other investigators were unable to detect and quantify BNP 1-32 in vivo.

The presence of BNP 3-32 attributable to action of dipeptidyl peptidases has been suggested, and we were able to detect this BNP analog in every single patient we studied. We also detected further proteolysed BNP peptides, eg, BNP 4-32, BNP 5-32, BNP 5-31, BNP 1-25, and BNP 1-26. We tested the bioactivity of each of these peptides in a cell-based assay measuring cGMP production and found no differences compared with BNP 1-32 (Jessica O’Rear, unpublished data, 2007). As has been shown for BNP 3-32, one can only speculate that even shorter peptides may have reduced natriuretic activity in vivo, despite their apparent in vitro activity.

Even assuming activity, the sum of all BNP peptides measured in every patient by MSIA would still represent only a fraction of the total iBNP measured by the Biosite assay (Figure 6). The BNP antibodies used in our MSIA also bind pro-BNP. Although we verified that the presence of up to 10 ng/mL of recombinant pro-BNP does not interfere with the quantification of low molecular weight BNP forms, we were unable to quantify endogenous pro-BNP levels with this MSIA method (Eric Niederkofler, unpublished data, 2007). Mass spectrometry is uniquely suited to detect and quantify multiple forms of endogenous BNP, and our results highlight the need for more specific clinical immunoassays to address questions of abnormal pro-BNP processing and to provide an accurate measure of bioactive BNP concentrations.

The presence of BNP fragments with one oxidized methionine residue is also noteworthy. Although it is possible to introduce methionine oxidation during sample preparation and MALDI, we observed this modification only in patient samples and never in our standard peptides or spiked plasma samples. Posttranslational oxidation might be a reflection of oxidative stress, a hallmark of cardiac disease. The exact site and role of this modification needs to be further studied, but we have previously shown that modification of Met10 leads to inactivation of BNP 1-32 (Andy Protter, unpublished data, 2002).

In summary, the data from our study strongly suggest that concentrations of bioactive BNP 1-32 are very low in patients with symptomatic HF. Commercial POC assays measure very high iBNP levels presumably due to a large molar excess of cross-reacting unprocessed pro-BNP. Although the small patient number was insufficient to establish a relation between HF stage and specific hormone levels, our findings may help to explain the paradoxical lack of physiological 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.

Acknowledgments

The authors thank Dr Roger Mills for critical reading of this manuscript, his helpful advice, and general support of this project. Further, the authors acknowledge Dr Jan Scardina and Pat Hummel for the production of monoclonal BNP antibodies 8.1 and 106.3.

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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Circ Heart Fail. 2008;1:258-264; originally published online October 14, 2008;
doi: 10.1161/CIRCHEARTFAILURE.108.790774

Circulation: Heart Failure is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1941-3289. Online ISSN: 1941-3297

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