Evidence for Functional Heterogeneity of Circulating B-Type Natriuretic Peptide

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Objectives
These studies describe molecular forms of circulating B-type natriuretic peptide (BNP) as well as their biological activity.

Background
Increased circulating levels of immunoreactive BNP correlate with the severity of heart failure and are considered a sensitive biomarker. However, little is known about the molecular forms of circulating BNP and their biological activity.

Methods
Western blot analysis was used to characterize immunoreactive BNP species in heart failure plasma. Recombinant proBNP was assessed for reactivity in commercially available BNP assays and cell activity by cyclic guanosine monophosphate production in vascular cells.

Results
Heart failure plasma contained both low- (LMW-BNP) and high-molecular-weight (HMW-BNP) forms. The LMW-BNP migrated similarly to a 32-amino acid BNP standard, whereas HMW-BNP, when deglycosylated, was similar to deglycosylated recombinant proBNP. Recombinant proBNP and BNP were equally recognized by the Triage BNP assay (Biosite, San Diego, California). Furthermore, recombinant proBNP and BNP were both recognized by the Advia Centaur BNP test (Bayer Diagnostics, Tarrytown, New York), but only recombinant proBNP was recognized by the Elecsys NTproBNP assay (Roche Diagnostics, Indianapolis, Indiana). Recombinant proBNP exerted significantly less biological activity than BNP on human endothelial and vascular smooth muscle cells. Comparison of effective concentration (50%) values indicates that proBNP is 6- to 8-fold less potent than BNP in these human cells.

Conclusions
This study demonstrates that proBNP, constituting a substantial portion of immunoreactive BNP in heart failure plasma, possesses significantly lower biological activity than the processed 32-amino acid hormone. These results implicate a discordance in heart failure between the high circulating levels of immunoreactive BNP and hormone activity, suggesting that some patients may be in a state of natriuretic peptide deficiency. (J Am Coll Cardiol 2007;49:1071–8) © 2007 by the American College of Cardiology Foundation

The cardiac-derived hormones atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP) play important roles in the regulation of cardiovascular and renal homeostasis (1). B-type natriuretic peptide is synthesized as a 108-amino acid prohormone termed proBNP that is proteolytically cleaved to release the 32-amino acid C-terminal BNP peptide (Fig. 1) (2,3). Both of these forms of BNP (proBNP and BNP) were purified and characterized by amino acid sequence analysis from BNP-containing peptides isolated from human atrial extracts (4).

In clinical heart failure (HF) syndromes, cardiac BNP expression and secretion increases in response to hemodynamic forces such as elevated filling pressure due to fluid overload and increased afterload from the effects of neurohormones angiotensin II and endothelin (5–8). Numerous studies have reported that increased circulating levels of BNP correlate with the severity of HF and that elevated BNP levels provide a sensitive biomarker for the diagnosis and prognosis of HF (9–12). Although HF patients frequently have increased plasma concentrations of immunoreactive BNP (iBNP), they show a paradoxic lack of BNP activity with sodium and fluid retention associ-
and vascular smooth muscle cells results in the cellular synthesis of cyclic guanosine monophosphate (cGMP), which mediates the vascular effects of this peptide (20–22).

We hypothesized that profiling the molecular species of iBNP in plasma from HF patients and determining the relative biological activity of unprocessed proBNP and processed BNP would advance our understanding of the true hormonal state of HF patients. Therefore, we studied the physical forms of circulating immunoreactive BNP in HF using Western blot techniques and the biological activity of recombinant forms of proBNP as determined by stimulation of cGMP production in cell culture assays. We also searched for evidence of peripheral activation of proBNP and examined the specificity of various commercial assays for 32-amino acid BNP, N-terminal (NT) proBNP, and intact proBNP.

Methods

Materials. Human recombinant BNP was provided by Scios (Fremont, California). Recombinant NT-proBNP 1–76 was purchased from Phoenix Pharmaceuticals (Belmont, California). Complete Protease Inhibitor Cocktail was from Roche Applied Science (Indianapolis, Indiana). Endo-α-N-acetylgalactosamidase, α-3,6,8,9-neuraminidase, and β-1,2,3,4,6-N-acetyl-glucosaminidase were obtained from EMD Biosciences (San Diego, California). β-(1→4)-Galactosidase was obtained from Sigma (St. Louis, Missouri).

Expression and purification of proBNP in CHO cells and E. coli. Expression and purification of recombinant proBNP in Chinese hamster ovary (CHO) cells is described in detail elsewhere (23). Briefly, a plasmid carrying the genes for preproBNP and glutamine synthase was constructed to transfect CHO cells. Stably transfected cell lines were generated by selection for resistance to methionine sulfoximine. The level of secreted proBNP was determined by sandwich ELISA using 2 monoclonal antibodies directed against full-length BNP32 and the C-terminus (amino acids 26 to 32) of BNP32, respectively. The most promising cell line was expanded, and proBNP in the medium was purified by antibody-affinity chromatography and further by strong cation-exchange chromatography (23).

For expression of proBNP in Escherichia coli, the gene encoding a full length of proBNP (amino acids 1 to 108) was cloned into expression vector pET24 (EMD Biosciences). An N-terminal His6-tag and linker sequence, followed by an rTEV protease cleavage site was added to aid purification. The translated protein therefore has the N-terminal sequence MHHHHHHHNPIPTTENLYFQG preceding proBNP (molecular weight 14,515.4 Da). Escherichia coli strain Rosetta DE3, pLysS (EMD Biosciences) was used for expression induced with IPTG. Soluble proBNP was purified by immobilized metal affinity chromatography followed by strong cation-exchange chromatography to achieve the desired purity. For the present studies, the purification tag was not removed.
Protein identity and concentration for both recombinant proBNP proteins (derived from mammalian cells and bacteria) were determined by N-terminal sequencing and amino acid analysis, respectively. In both cases, purity of the final product was >95% by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

Immunoprecipitation and Western blot analysis. Plasma samples from 5 consenting patients with New York Heart Association functional class IV chronic HF were analyzed. The protocol for collection of these samples was approved by the Veterans Administration Medical Center Institutional Review Board, San Diego, California. For immunoprecipitation, a monoclonal antibody directed against BNP32 was cross-linked to POROS XL ID resin according to the manufacturer's instructions (Applied Biosystems, Foster City, California). The plasma (2.5 ml) was incubated to the manufacturer's instructions (Applied Biosystems, Foster City, California). The plasma (2.5 ml) was incubated with 3 μl BNP antibody-conjugated beads for 16 hours at 4°C. The immunoprecipitates were washed with phosphate-buffered solution, then electrophoresed and transferred onto PVDF membrane for Western blotting. A monoclonal antibody directed against the C-terminus of BNP32 was used as the primary detection antibody, and bands were visualized on film using ECL Advanced detection (GE Healthcare, Piscataway, New Jersey).

Deglycosylation of proBNP. ProBNP derived from CHO and the immunoprecipitates from HF patients were incubated with a deglycosylation enzyme cocktail consisting of endo-α-N-acetylgalactosaminidase, α-3,6,8,9-neuraminidase, β-1, 2,3,4,6-α-N-acetyl-glucosaminidase, and β-(1→4)-galactosidase in 50 mmol/l sodium phosphate buffer, pH 6.0 at 37°C for 8 hours. Reactions were stopped by addition of SDS-PAGE sample buffer and heating, then subjected to Western blot analysis.

Cell culture. Primary human aortic endothelial cells, primary human coronary artery endothelial cells, primary human umbilical endothelial cells, and primary human aortic smooth muscle cells were purchased from Cambrex (Walkersville, Maryland). Cells at passages 3 through 5 were grown in EGM-2 MV complete medium containing 5% fetal bovine serum (FBS). After confluence, cells were split and cultured in 6-well plates for the experiments.

Recombinant expression of the biological receptor human NPRA in CHO cells. A 3.2-kb cDNA encoding the full length of human NPRA was subcloned into the Xba I/Sal I sites of plasmid pAXneoRX. The NPRA constructs were transfected into CHO-K1 cells. The transfected cells were selected for growth in the medium containing the neomycin analog G418, and positive clones were screened by determination of 125I-ANP binding and ANP-stimulated cyclic GMP accumulation. The CHO cells stably overexpressing human NPRA were named CHO-GCA cells. Cells between passages 7 through 12 were grown in medium containing 45% DMEM 21, 45% Coon’s F12, 10% FBS, 10 mmol/l HEPES, and 2 mmol/l glutamine.

Determination of intracellular cGMP accumulation. Cells were changed to serum-free medium and preincubated with 0.1 mmol/l 3-isobutyl-1-methylxanthine for 1 h, then treated with varying concentrations of human BNP or CHO-derived human proBNP ranging from 0.1 nmol/l to 1,000 nmol/l for 10 min. Cells were lysed with 0.1 mol/l HCl at room temperature for 20 min. The lysates were centrifuged at 600 g, and the levels of cGMP in the supernatant were measured using a cGMP enzyme immunoassay kit from Assay Designs (Ann Arbor, Michigan).

Measurement of BNP and proBNP by the Triage BNP assay, Advia Centaur BNP assay, and the Elecsys 2010 proBNP assay. Known amounts of BNP or proBNP were added to normal human plasma (obtained from VWR, West Chester, Pennsylvania), and samples were measured by the Triage BNP test (Biosite, San Diego, California), Advia Centaur BNP test (Bayer Diagnostics, Tarrytown, New York), and the Elecsys 2010 NT-proBNP assay that determines NT-proBNP (Roche Diagnostics, Indianapolis, Indiana) according to manufacturers’ instructions.

Data analysis. The data were analyzed and plotted using GraphPad Prism 4 software (GraphPad, San Diego, California). Effective concentration (50%) (EC50) values were calculated with Sigmoidal dose-response equation. Statistical analysis was performed using a t test with the Satterthwaite approximation for the degrees of freedom. A confirmatory Wilcoxon nonparametric test was also prepared. All calculations were prepared in SAS version 8 (SAS Institute, Cary, North Carolina).

Results

Characterization of circulating iBNP in patients with advanced HF demonstrated that proBNP is a major form of immunoreactive BNP in HF plasma. Immunoreactive BNP species in HF plasma were evaluated by Western blot analysis using antibodies directed against BNP. The analysis identified high-molecular-weight (HMW) and low-molecular-weight (LMW) BNP species (Fig. 2A). The HMW-BNP species migrated at an approximate molecular weight range of 20 kDa to 22 kDa, and the recombinant proBNP used as a standard had an estimated molecular weight range of 22 kDa to 24 kDa. The LMW-BNP species migrated in a manner similar to that of the 32-amino acid species. In 4 of the 5 samples tested, the HMW-BNP represented a significant amount of the total immunoreactive BNP material compared with the LMW-BNP.

The mobility of the HMW-BNP species was lower than predicted for a 108-amino acid protein, suggesting the protein may be glycosylated, a typical post-translational modification of plasma proteins. Structural characterization of the mammalian cell-derived recombinant proBNP has determined that there are 7 sites of O-linked oligosaccharide attachment within the propeptide region (23). Therefore, deglycosylation of both the recombinant protein and the immunoprecipitates from patients were performed to...
enzymatically remove O-linked oligosaccharides. Western blot analysis (Fig. 2B) revealed a reduction in the molecular weight of the HMW-BNP species present in patient plasma to approximately 12 kDa, the predicted size of the 108-amino acid form. The LMW-BNP species appeared unaltered. The deglycosylated HMW-BNP species comigrated with the deglycosylated CHO-derived proBNP, suggesting that the 2 species were similar. For this reason, differences in migration of recombinant proBNP and HMW-BNP before deglycosylation probably represent different patterns of glycosylation between the mammalian cells used for recombinant expression (CHO) and humans.

These results suggest that HMW forms of BNP are prevalent in HF plasma. Furthermore, this immunoreactive HMW-BNP material is similar to proBNP, the 108-amino acid unprocessed form of the hormone.

Physiologic assay of cGMP production demonstrated marked limitation of cellular bioactivity of recombinant proBNP. Because HMW material similar to proBNP appears to constitute a substantial portion of the immunoreactive BNP in HF plasma, we evaluated the stimulation of cGMP production as an index of the biological activity of proBNP and BNP in primary human endothelial cells prepared from aorta, coronary artery, and umbilical vein as well as primary human aortic smooth muscle cells. Cells were treated with increasing concentrations of BNP or proBNP for 10 min, and intracellular cGMP levels were determined. As shown in Figure 3, both BNP and proBNP dose-dependently stimulated intracellular cGMP production in all 3 of these endothelial cells. However, proBNP was significantly less potent than BNP in stimulating cGMP production in these cells (p < 0.05). The calculated EC50 values of proBNP were 6- to 8-fold higher than the EC50 values of BNP in all 3 types of endothelial cells studied here (Table 1), confirming that proBNP possesses significantly lower activity.

In primary human aortic smooth muscle cells, proBNP and BNP both induced a dose-dependent increase in cGMP. However, proBNP was significantly reduced in potency compared with BNP (Fig. 3) (p < 0.05). Derived EC50 values suggest that proBNP is about 8-fold less potent than BNP on activation of the NPRA in these smooth muscle cells (Table 1).

To further characterize proBNP activity, we evaluated its cGMP-stimulating activity in a cell line (CHO-GCA) that overexpresses the human NPRA receptor. Both BNP and proBNP stimulated intracellular cGMP production in a concentration-dependent manner (data not shown). Consistent with the findings in human vascular cells, proBNP is significantly less potent than BNP on stimulating intracellular cGMP synthesis in CHO-GCA cells, with derived EC50 values of 111 ± 12 nmol/l and 7 ± 0.4 nmol/l, respectively (p < 0.05).

To determine whether the glycosylation interferes with proBNP activity, we expressed and purified nonglycosylated proBNP from E. coli. As shown in Figure 4, there was no significant difference between recombinant mammalian cell-derived proBNP and E. coli-expressed proBNP on stimulation of cGMP production in HAECS and CHO-GCA cells, indicating that the 2 types of recombinant proBNP exert the same biological activity and that glycosylation does not appear to affect proBNP activity.
Because proBNP showed low cellular bioactivity, we evaluated whether proBNP modulates the cellular activity of BNP. The EC50 concentrations of each peptide, 30 nM and 300 nM for BNP and proBNP respectively, were selected to treat human coronary artery endothelial cells (HCAECs) alone or in combination. Both BNP and proBNP each gave an equivalent cGMP response, whereas combined they stimulated approximately 2 times greater cGMP production than each peptide alone, suggesting BNP and proBNP function in an additive fashion (data not shown). We also tested whether NT-proBNP interferes with BNP or proBNP activity. The results showed that NT-proBNP had no significant effect on BNP or proBNP-stimulated cGMP production in HCAECs (data not shown). In other words, neither proBNP nor NT-proBNP functions as an effective receptor-blocking agent. ProBNP is apparently not processed to a more active form in the periphery (lack of detectable cell processing of recombinant proBNP).

It is possible that proBNP is proteolytically processed to an active form in these vascular cell assays, thereby overestimating its biological activity. The inclusion of a protease inhibitor cocktail in the cell assay did not alter the activity profile of proBNP (Fig. 5). In addition, Western blot analysis of the conditioned medium from proBNP-treated cells could not identify any LMW-BNP bands (data not shown). These data suggest that the proteolysis of recombinant proBNP to an active form is not contributing to the activity profile of proBNP in these assays. However, the possibility that some processing of proBNP to an active form does occur in these cell assays cannot be ruled out.

Substantial cross-reactivity occurs between BNP, NT-proBNP, and proBNP in the Triage BNP, Advia Centaur BNP, and Elecsys NT-proBNP assays. Because plasma BNP is a biomarker used to diagnose HF, we tested whether with BNP or proBNP activity. The results showed that NT-proBNP had no significant effect on BNP or proBNP-stimulated cGMP production in HCAECs (data not shown). In other words, neither proBNP nor NT-proBNP functions as an effective receptor-blocking agent. ProBNP is apparently not processed to a more active form in the periphery (lack of detectable cell processing of recombinant proBNP). It is possible that proBNP is proteolytically processed to an active form in these vascular cell assays, thereby overestimating its biological activity. The inclusion of a protease inhibitor cocktail in the cell assay did not alter the activity profile of proBNP (Fig. 5). In addition, Western blot analysis of the conditioned medium from proBNP-treated cells could not identify any LMW-BNP bands (data not shown). These data suggest that the proteolysis of recombinant proBNP to an active form is not contributing to the activity profile of proBNP in these assays. However, the possibility that some processing of proBNP to an active form does occur in these cell assays cannot be ruled out.

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<th>Table 1</th>
<th>Potency for Stimulation of cGMP Production in Primary Human Vascular Cells Treated With BNP and proBNP</th>
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<tr>
<td>Cell Type</td>
<td>BNP (nmol/l)</td>
</tr>
<tr>
<td>HAEC</td>
<td>36 ± 3</td>
</tr>
<tr>
<td>HCAEC</td>
<td>58 ± 23</td>
</tr>
<tr>
<td>HUVEC</td>
<td>66 ± 12</td>
</tr>
<tr>
<td>HASMC</td>
<td>28 ± 6</td>
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Human aortic endothelial cells (HAEC), coronary artery endothelial cells (HCAEC), umbilical vein endothelial cells (HUVEC) and arterial smooth muscle cells (HASMC), were incubated with B-type natriuretic peptide (BNP) and recombinant proBNP, and the derived effective concentration (50%) (EC50) values are indicated. Data are mean ± standard deviation from 3 separate experiments.
the Triage BNP assay, a widely used diagnostic test, could distinguish between proBNP and BNP. Equimolar amounts of BNP or recombinant proBNP were added to normal human plasma, and the samples were then subjected to measurement. As shown in Figure 6A, the Triage assay recognizes BNP and proBNP equally well, suggesting this clinical BNP assay cannot distinguish between the fully active 32-amino acid hormone and the unprocessed prohormone with significantly reduced activity. Similar results were obtained with the Advia Centaur BNP assay, which recognized recombinant proBNP and BNP, with a slightly higher reactivity toward BNP (Fig. 6B).

The Elecsys NT-proBNP assay, recognized recombinant proBNP but not BNP (Fig. 7). Although there is a linear relationship between the concentration of recombinant proBNP in the sample and the NT-proBNP assay value (R² = 0.9996), the assay significantly underestimates the molar amount of recombinant proBNP.

These results are summarized in Table 2.

**Discussion**

The present data offer several new insights into the hormonal state of HF. First, in patients with advanced HF, much of circulating iBNP appears to be proBNP. Second, proBNP is 6- to 8-fold less active than BNP in our endothelial cell assay. Third, we found no evidence of peripheral processing of proBNP to more active forms. Finally, we found that the currently available commercial assays for BNP and NT-proBNP do not clearly distinguish between the various circulating forms BNP, NT-proBNP, and proBNP.

Despite numerous studies associating plasma BNP levels with HF severity and clinical outcomes, little is known about its structure and bioactivity. It has previously been shown that HMW forms of BNP are present in significant amounts in HF patients and that this material is structurally similar if not identical to proBNP (14,15,24). Consistent with these reports, our Western blot analysis of plasma from 5 patients with advanced HF for immunoreactive BNP material demonstrates significant amounts of both HMW and LMW species. Endogenous proBNP as well as recombinant, mammalian cell-derived proBNP have been shown to be O-glycosylated (data reported here as well as reference 23). Analysis of the recombinant proBNP has shown that glycosylation is on 7 specific sites, all within the propeptide region and not within the amino-terminal 32-amino acid BNP portion (23). Plasma HMW-BNP behaves similarly to the recombinant proBNP standard when treated with deglycosylation enzymes, confirming that endogenous HMW-BNP is a form of glycosylated proBNP. By this criterion, recombinant mammalian cell-derived proBNP appears to be very reminiscent of endogenous HMW-BNP.
We demonstrated that recombinant proBNP has reduced (6- to 8-fold) biological activity compared with the 32-amino acid BNP peptide on both endothelial and vascular smooth muscle cells. Recombinant proBNP also has reduced potency for activating human NPRA expressed recombinantly in mammalian cells, suggesting that the results noted in primary human endothelial cells and vascular smooth muscle cells are NPRA mediated. The vasculature is a key target organ for BNP physiology, and both endothelial cells and smooth muscle cells express the hormone’s biological receptor, NPRA (19). B-Type natriuretic peptide acts directly on endothelial cells to reduce endothelin-1 production as well as modulate fluid permeability (1). Acting on vascular smooth muscle, BNP has been shown to be a vasodilator (25). The cellular effects of proBNP and BNP described here are thus a critical part of the physiology of this hormone. These data suggest that the unprocessed hormone has significantly reduced activity compared with the mature BNP peptide.

The mechanism(s) leading to significantly increased amounts of circulating proBNP in the setting of HF is unknown. We explored this by inclusion of protease inhibitors in our cell-line cGMP assay. Our findings support the hypothesis that physiologically important peripheral conversion of proBNP to active BNP does not occur. Activation of the gene’s promoter in HF leads to a marked increase in proBNP expression by cardiac myocytes (6,8,26). The increased production of proBNP may overwhelm the ability of cardiac myocytes to process it to the mature 32-amino acid peptide, thereby resulting in the release of significant amounts of proBNP rather than BNP. Alternatively, the processing and/or metabolism of proBNP in circulation may be decreased or the metabolism of BNP may be increased. B-type natriuretic peptide is metabolized predominantly by natriuretic peptide clearance receptor and neutral endopeptidase cleavage (27–29). Whether proBNP shares metabolic pathways similar to those of BNP remains to be determined. Further studies on the processing and metabolism of proBNP should advance our understanding of molecular forms of BNP circulating in HF.

Finally, we addressed the ability of commercially available assays to distinguish the different BNP forms circulating in patients with advanced HF. Recombinant mammalian cell-derived proBNP is as effectively detected by the Triage BNP assay kit as BNP. The available literature suggests that this

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<th>Assay</th>
<th>BNP Forms Detected</th>
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<tr>
<td>Triage</td>
<td>Yes</td>
</tr>
<tr>
<td>Advia Centaur</td>
<td>No</td>
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<td>Elecsys</td>
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BNP = B-type natriuretic peptide.
assay uses a capture antibody that recognizes the ring structure of the BNP peptide and a detection antibody that recognizes the amino terminus of BNP (30). Thus, one would predict that this assay should detect proBNP. These results were confirmed by the Advia Centaur BNP test, another widely used BNP diagnostic assay. The Elecsys NT-proBNP assay also detected recombinant proBNP; however, it significantly underestimated the amount of protein present. It is possible that the glycosylation of the proBNP protein in the NT region (amino acids 1 to 76) interferes with the assay. Because endogenous proBNP is also glycosylated, it is anticipated that values generated with this assay may not reflect actual blood levels (data here and reference 23).

In summary, the present data offer at least a partial explanation of why HF patients with high levels of immunoreactive plasma BNP may still suffer from severe symptoms, e.g., congestion and edema, in that BNP forms with little physiologic activity, such as proBNP, seem to constitute much of the circulating iBNP in advanced HF. Whether the levels of active 32-amino acid BNP rise proportionately, as suggested by Giuliani et al. (16), or are relatively deficient, remains a topic for further study. Additional mechanisms limiting the activity of BNP in the HF setting may include activation of cGMP phosphodiesterases or down-modulation of NPRA. Further work in this area will better define the structure and activity of endogenous natriuretic peptides in the setting of heart disease.

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REFERENCES