Abstract—Recent studies support the speculation that different molecular forms of the cardiac hormone BNP with differential biological activity may circulate in heart failure and be detected by conventional assays. In the current study we determined the ability of 3 widely used conventional assays to detect these different forms thought to circulate in heart failure. We also evaluated the ability of pro-BNP (1-108), N-terminal peptide (NT)–pro-BNP (1-76), and BNP 3-32, the latter a cleavage product of BNP 1-32 by dipeptidyl peptidase IV, on an equimolar basis to activate cGMP in cultured cardiac fibroblasts and cardiomyocytes compared with the biologically active mature BNP 1-32. Specifically, we observed that the Roche NT–pro-BNP assay detected both NT–pro-BNP 1-76 and pro-BNP 1-108 and that Biosite Triage and Shionogi detected both mature BNP 1-32 and the shortened BNP 3-32. Moreover, in cultured cardiac fibroblasts and cardiomyocytes, BNP 1-32 (10^-6 mol/L) activated cGMP. BNP 3-32 demonstrated a similar cGMP activating property in both cardiac cell types. In contrast, the cGMP response to pro-BNP 1-108 and NT–pro-BNP 1-76 was not significantly greater than no treatment alone. We conclude that widely used commercial assays for NT–pro-BNP 1-76 and BNP 1-32 cannot differentiate among pro-, processed, or degraded forms and, thus, may not thoroughly identify circulating BNP forms in heart failure patients. These findings also demonstrate differential cGMP activating properties of BNP forms and, importantly, that pro-BNP 1-108 and NT–pro-BNP 1-76 have reduced cGMP activity in vitro that may have biological relevance to human heart failure. (Hypertension. 2007;49:1114-1119.)

Key Words: peptides ■ natriuretic peptides ■ cGMP ■ heart failure ■ biomarkers

The BNP gene produces a 134 amino acid prepro-BNP precursor peptide, which, after the removal of a 26 amino acid signal peptide, results in a 108 amino acid prohormone1 (Figure 1). Further cleavage, presumably by the enzyme corin, produces ≥2 peptides, including the biologically active 32 amino acid BNP peptide (BNP 1-32), which contains a 17 amino acid disulfide ring and the linear 76 amino acid N-terminal peptide (NT)–pro-BNP 1-76.6 BNP 1-32 mediates it is biological actions by binding to the natriuretic peptide receptor A, which is a membrane-bound receptor widely localized in cells and tissues.3 Studies have suggested that BNP 1-32 may undergo further degradation by dipeptidyl peptidase IV that removes 2 N-terminal amino acids (Ser Pro) resulting in BNP 3-32.4 More recently it has been reported that pro-BNP is present in normal human plasma and increases with the severity of heart failure (HF) and that a defect in corin may also exist in HF, which would inhibit pro-BNP processing resulting in the release of uncleaved pro-BNP 1-108.5,7 However, the biological activity of such various circulating forms remains unclear.

Most recently, we have reported that, in a small group of patients with severe HF and markedly elevated plasma BNP as measured by commercially available assays, mature BNP 1-32 was not present when assessed by highly sensitive and specific state-of-the-art nano-liquid chromatography electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry.8 We speculated that other forms of BNP may circulate and be detected by conventional BNP assays. Such speculation would be consistent with previous work using liquid chromatography that supported the presence of both high and low molecular weight forms of BNP in plasma of humans with HF.9-11 Other evidence strongly supports the existence of additional molecular and posttranslational forms of BNP, such as glycosylated BNP 1-32, BNP 3-32, which, like BNP 1-32, also possesses the 17 amino acid disulfide ring structure, the linear NT–pro-BNP 1-76, and even the unprocessed, uncleaved complete pro-BNP 1-108.4-6,12 We also speculated that such forms could have reduced cGMP-activating properties, and, thus, immunoreactive BNP in HF patients would be less biologically effective. This may
explain in part how exogenous BNP is highly effective as a vasodilator in humans with acute decompensated HF, which is a stage of cardiac disease characterized by high levels of circulating BNP, of which the molecular forms have not been thoroughly investigated to date.13,14

With this in mind, we hypothesized that commercially available assay systems to determine BNP plasma concentrations in humans are not specific for mature BNP 1-32 and may detect a wide spectrum of different molecular forms of BNP. We further hypothesized that these different forms would possess a diverse ability to generate cGMP in vitro, recognizing cGMP to be the second messenger of the natriuretic peptides. Therefore, the first goal of the current study was to assess the ability of 2 commercial BNP 1-32 assays (Biosite and Shionogi), as well as the commercial Roche NT-pro-BNP 1-76 assay to detect equimolar concentrations of BNP 1-32, BNP 3-32, and NT-pro-BNP 1-76 when added to normal human plasma. In a separate experiment using the Biosite Triage and Roche assays we also evaluated the addition of 2500, 5000, and 10 000 pg/mL of these 3 forms. For pro-BNP 1-108, 4 concentrations were added to normal human plasma at 500, 2500, 5000, and 10 000 pg/100 µL. Interassay and intra-assay coefficients of variations were 7.2% and 8.0%, respectively, for Shionogi BNP, 8.8% and 9.9%, respectively, for Biosite, and 3.1% and 2.5%, respectively, for Roche NT-pro-BNP.

Methods

Immunoreactivity of BNP Molecular Forms by Conventional Assays

These studies were approved by the Mayo Institutional Review Board. Three distinct assays were used in the current study. Immunoreactivity of various molecular forms of BNP was determined by the immunoradiometric assay using antibody to human BNP (Shionogi Co Ltd) and by the fluorescence immunoassay (Biosite Diagnostics), as described previously.15–18 We also used the Elecsys pro-BNP electrochemiluminescence immunoassay (Roche Diagnostics), also as described previously.19–20 A stock solution of BNP 1-32, BNP 3-32, and NT-pro-BNP 1-76 was made with a final concentration of 500 pg/100 µL that was added to 1-mL plasma aliquots of normal human plasma. In a separate experiment using the Biosite Triage and Roche assays we also evaluated the addition of 2500, 5000, and 10 000 pg/mL of these 3 forms. For pro-BNP 1-108, 4 concentrations were added to normal human plasma at 500, 2500, 5000, and 10 000 pg/100 µL. Interassay and intra-assay coefficients of variations were 7.2% and 8.0%, respectively, for Shionogi BNP, 8.8% and 9.9%, respectively, for Biosite, and 3.1% and 2.5%, respectively, for Roche NT-pro-BNP.

Cell Culture and Measurement of cGMP

For the in vitro studies, human cardiac fibroblasts (ScienCell) were cultured in manufacturer fibroblast medium (ScienCell) supplemented with fibroblast growth serum, FBS, and penicillin/streptomycin. Cells were treated at 80% to 90% confluence. Only cell passages 1 through 4 were used for experiments. For cardiomyocytes, we used human cardiomyocytes (ScienCell), which were cultured in manufacturer cardiomyocyte medium (ScienCell) supplemented with cardiomycocyte growth supplement, FBS, and penicillin/streptomycin supplied as supplements on poly-L-lysine coated (Becton Dickinson) plates. Cells were treated at 80% to 90% confluence. Only cell passages 1 through 3 were used for experiments.

Cells were first exposed to BNP 1-32, BNP 3-32, NT-pro-BNP 1-76, and pro-BNP 1-108 for 10 minutes at 10⁻⁶ mol/L. After exposure, cells were lysed in 6% trichloroacetic acid and sonicated for 10 minutes. The samples were extracted four times in 4 volumes of ether/water, dried, and reconstituted in 300 µL of cGMP assay buffer. The samples were assayed using a competitive radioimmunoassay cGMP kit (Perkin-Elmer). Briefly, samples and standards are incubated with anti-human cGMP polyclonal antibody and ¹²⁵I-antigen for 18 hours. cGMP assay buffer was added to the samples, and they were centrifuged for 20 minutes at 2500 rpm. The free fraction was aspirated off, and the bound fraction was counted and concentrations determined. Samples are corrected for dilution factors and protein concentration. Values are expressed as picomoles.
per milliliter. There was no cross-reactivity with ANP, BNP 1-32, or CNP.

**Reagents**

BNP 1-32 and NT–pro-BNP 1-76 were purchased from Phoenix Pharmaceuticals; BNP 3-32 was synthesized in the Mayo Protein Core Laboratory, and nonglycosylated recombinant pro-BNP 1-108 produced in *Escherichia coli* was purchased from HyTest.

**Statistical Analysis**

cGMP generation for the different peptides was compared with Kruskal–Wallis test and posthoc Dunn’s test with the unspiked normal plasma as control. Likewise, immunoreactivity for the different peptides was compared separately for each assay with Kruskal–Wallis test and posthoc Dunn’s test with the unspiked normal plasma as control. Slopes for the concentration–immunoreactivity curve for the detection of pro-BNP 1-108 were determined and compared with linear regression analysis. Data are reported as mean ± SEM. A *P* value <0.05 was considered significant.

**Results**

**Immunoreactivity of BNP Molecular Forms by Conventional BNP Assays**

BNP immunoreactivity for BNP 1-32, BNP 3-32, and NT–pro-BNP 1-76 as determined by the Biosite, Shionogi, and Roche assays is illustrated in Figure 2. The Biosite assay, which recognizes the BNP 17 amino acid disulfide ring and an N-terminal epitope, detected BNP 1-32 (60%) and BNP 3-32 (76%). In contrast, the Biosite assay minimally recognized NT–pro-BNP 1-76 (<3%).

The Shionogi assay, which recognizes the ring of BNP and an epitope on the C-terminus, also detected BNP 1-32 (60%) and similarly BNP 3-32 (76%). As with the Biosite assay, there was minimal detection of NT–pro-BNP 1-76 (<1%).

In contrast to the conventional assays directed at the BNP disulfide ring, the Roche NT–pro-BNP assay (Figure 2) is specifically directed at 2 sites in the 1-76 sequence of pro-BNP 1-108. The NT–pro-BNP 1-76 assay was highly specific for NT–pro-BNP 1-76 (92%) with very little detection of BNP 1-32 and BNP 3-32 (<2%).

The Table reports the findings of a broader range (2500 to 10 000 pg/mL) of these 3 BNP forms when assayed with the Biosite Triage and Roche assays. The findings here were similar to the findings with the 500 pg/mL concentration, as illustrated in Figure 2.

Figure 3 illustrates the BNP immunoreactivity of pro-BNP 1-108 using all 3 of the assays. Increasing concentrations of pro-BNP 1-108 were used to mimic the high concentrations of NT–pro-BNP reported in HF. The data reveal that the Roche NT–pro-BNP assay detected ∼30% of immunoreactive pro-BNP 1-108, whereas both the Shionogi and Biosite assays detected <2%. In a separate experiment using lower concentrations of pro-BNP 1-108 (500 pg/mL), there was minimal detection by either the Biosite or Roche assays (data not shown).

**cGMP Activating Actions of BNP Molecular Forms in Cultured Human Cardiac Fibroblasts and Human Cardiomyocytes**

Figure 4 illustrates the cGMP activating actions of the various molecular BNP forms in cultured human cardiac fibroblasts and cardiomyocytes. At equimolar concentrations in cardiac fibroblasts (10^-6 mol/L), BNP 3-32 and mature BNP 1-32 resulted in similar generation of cGMP (0.126 ± 0.013 and 0.137 ± 0.004 pmol/mL, respectively) that was significantly greater compared with no treatment (*P*<0.0001 for both molecular forms). Neither NT–pro-BNP 1-76 nor pro-BNP

<table>
<thead>
<tr>
<th>Picograms Added</th>
<th>10 000</th>
<th>5000</th>
<th>2500</th>
</tr>
</thead>
<tbody>
<tr>
<td>BNP 1-32</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triage, pg/mL</td>
<td>&gt;5000</td>
<td>&gt;5000</td>
<td>2450 ± 108</td>
</tr>
<tr>
<td>Roche, pg/mL</td>
<td>24 ± 1</td>
<td>22 ± 3</td>
<td>21 ± 2</td>
</tr>
<tr>
<td>BNP 3-32</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triage, pg/mL</td>
<td>&gt;5000</td>
<td>4810 ± 501</td>
<td>3220 ± 209</td>
</tr>
<tr>
<td>Roche, pg/mL</td>
<td>24 ± 2</td>
<td>25 ± 2</td>
<td>27 ± 2</td>
</tr>
<tr>
<td>BNP 1-76</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triage, pg/mL</td>
<td>17 ± 3</td>
<td>8 ± 0.2</td>
<td>5 ± 0.3</td>
</tr>
<tr>
<td>Roche, pg/mL</td>
<td>8331 ± 483</td>
<td>4210 ± 454</td>
<td>2125 ± 245</td>
</tr>
</tbody>
</table>
1-108 was able to significantly increase cGMP in these cultured cells compared with no treatment alone. At equimolar concentrations in cardiomyocytes (10^{-6} \text{ mol/L}), BNP 3-32 and mature BNP 1-32 resulted in similar generation of cGMP (0.682 \pm 0.027 and 1.034 \pm 0.123 pmol/mL, respectively) that was significantly greater compared with no treatment (P < 0.0001 for both molecular forms). Neither NT–pro-BNP 1-76 nor pro-BNP 1-108 was able to significantly increase cGMP in these cultured cells compared with no treatment alone.

**Discussion**

The current study confirms and extends previous investigations with 3 widely used commercial assays for quantifying BNP and reports that molecular forms other than the mature biologically active BNP 1-32 can be potentially detected by these 3 assays. We further demonstrate that these various molecular forms of BNP that are reported to circulate in human plasma possess differential cGMP, generating potency in cultured human cardiac fibroblasts and cardiomyocytes. These studies, therefore, provide new insights into BNP as a biomarker and also into the biology of this endocrine system that is markedly activated in human HF.

The cardiac hormone BNP is widely used as a biomarker in the diagnosis and prognosis of human HF and cardiovascular disease.\textsuperscript{15–23} Indeed, in advanced overt HF plasma, BNP values have been reported to be markedly elevated into concentrations of thousands of picograms. In a recent study by Hawkridge et al\textsuperscript{8} in humans with severe HF in which plasma BNP was significantly increased based on a commercial BNP assay, these investigators using highly sensitive Fourier transform ion cyclotron resonance mass spectrometry reported the absence of mature biologically active BNP 1-32 despite its detection by a conventional assay system. This observation suggested that commercial assays for BNP 1-32 might also detect other molecular forms of BNP. Of interest, these studies detected an 8-kDa species that coeluted with BNP 1-32 but did not appear to be related to BNP. Thus, it remains unclear exactly which forms of BNP are being detected by conventional BNP assays. Indeed, evidence strongly supports the existence of additional molecular and posttranslational forms of BNP, such as glycosylated BNP 1-32, BNP 3-32, the linear NT–pro-BNP 1-76, and even the unprocessed, uncleaved complete pro-BNP 1-108.\textsuperscript{4–6,12} If there were an increase in these various molecular forms in the circulation in HF, and if their biological activity is altered, it would impact our understanding of the compensatory role of the BNP system in HF and also influence our therapeutic approach to this disease process. In contrast to HF, it should be noted that, in early hypertension, the hypertensive heart is associated with an actual suppression of NT–pro-BNP 1-76, which suggests the lack of a compensatory response of the BNP system in a disease mostly associated with HF with preserved systolic function.\textsuperscript{24}

Our studies confirm and extend previous reports. Specifically, Rawlins et al\textsuperscript{25} determined immunoreactivity in normal

**Figure 3.** BNP immunoreactivity of pro-BNP 1-108 as determined by the Roche NT–pro-BNP 1-76, Biosite Triage 1-32, and Shionogi 1-32 assays. Pro-BNP 1-108 was added to normal plasma at concentrations of 2500, 5000, and 10 000 pg/100 \( \mu \text{L} \).

**Figure 4.** cGMP generation in human cardiac fibroblasts (A) and cardiomyocytes (B) to BNP molecular forms compared with no treatment. Cells were treated with respective BNP molecular forms at 10 to 6 mol/L. Values are the mean \pm SEM of 3 samples per treatment from 4 experiments. Passages 1 to 4 were used. *P < 0.0001 vs no treatment.
human plasma to which BNP 1-32 and 3-32 were added using automated assays based on the Biosite and Shionogi assays. As did we, these investigators reported that both widely used assays detected both BNP 1-32 and 3-32 with greater detection by the Shionogi compared with the Biosite assay. We extended this previous report by also determining the immunoreactivity of NT–pro-BNP 1-76 and pro-BNP 1-108 using these 2 assays. In our studies, these 2 BNP assays to BNP 1-32 demonstrated minimal detection of NT–pro-BNP 1-76 and pro-BNP 1-108. In contrast, the Roche NT–pro-BNP 1-76 assay was much more sensitive in the detection of NT–pro-BNP 1-76 and also pro-BNP 1-108 hormone, but in the detection of pro-BNP 1-108, it was only approximately one third of immunoreactivity. There was minimal detection by the NT–pro-BNP 1-76 assay of BNP 1-32 or pro-BNP 3-32.

Our studies also address in vitro biological activity of these various molecular forms of BNP. Specifically, we compared the cGMP activating properties of 3 additional molecular forms of BNP that included the unprocessed uncleaved pro-BNP 1-108, the NT–pro-BNP 1-76, and then the further processed form of BNP 1-32, which is BNP 3-32 that is processed by dipeptidyl peptidase IV. We used cultured human cardiac fibroblasts and cardiomyocytes based on the increasing importance of both these cardiac cell types as a source and target for BNP. In our investigations, we found that BNP 3-32 retained similar cGMP activating properties as compared with BNP 1-32 in both fibroblasts and cardiomyocytes. In contrast, the linear NT–pro-BNP 1-76 that lacks the 17 amino acid disulfide bridge had no cGMP activity. Furthermore, pro-BNP 1-108, despite the disulfide ring, also lacked cGMP-activating properties. This later finding takes on clinical significance, as Dries et al. have reported that corin, which cleaves pro-BNP 1-108 to active BNP 1-32 and nonbiologically active NT–pro-BNP 1-76, may be downregulated in severe HF resulting in a higher percentage of pro-BNP compared with BNP 1-32 in the circulation. Such a downregulation of corin has also been reported in a model of experimental HF supporting the concept of impaired pro-BNP processing in this disease state. Importantly, Giuliani et al. have reported that pro-BNP 1-108 clearly increases with the severity of human HF. Thus, one could speculate that activation of the BNP system in advanced HF may represent the release of less biologically active BNP that results in a biologically relative BNP deficiency state.

The current findings have diagnostic and biological significance in considering the use of these 3 widely used commercial assays for BNP. First, it should be noted that NT–pro-BNP has emerged as potentially slightly superior to BNP in predicting cardiovascular outcomes in population studies and clinical trials. One could speculate that this could be because of a potential alteration in processing of pro-BNP 1-108 in which pro-BNP 1-108 concentrations would increase, whereas mature BNP 1-32, as well as NT–pro-BNP 1-76, concentrations would decrease. Based on what we observe in the current study, the Roche NT–pro-BNP 1-76 could detect such shift, whereas the assays directed at mature BNP 1-32 may not. Therefore, it may be valuable to evaluate the ratio of NT–pro-BNP 1-76/BNP 1-32 together with plasma cGMP to possibly enhance diagnostic and prognostic outcomes. An additional important conclusion from the current studies, however, is that the widely used commercial assays for BNP are not completely specific for either BNP 1-32 or NT–pro-BNP 1-76, and this has implications for their interpretation.

In summary, our studies further demonstrate that 3 widely used commercial assays for the BNP system are not entirely specific for BNP 1-32 or NT–pro-BNP 1-76 and may detect other molecular forms of BNP reported to circulate. Furthermore, we report the first study to characterize various molecular forms of BNP in activating in vitro the cGMP system in cultured human cardiac human fibroblasts and cardiomyocytes. Specifically, we find a diversity of cGMP actions in which only the mature biologically active BNP 1-32 peptide and the dipeptidyl peptidase IV processed from BNP 3-32 possess potent cGMP activating properties. In contrast, other forms reported to circulate in humans, specifically NT–pro-BNP 1-76 and pro-BNP 1-108, lack such cGMP activity. These findings are significant, and if these forms with reduced activity are increased in human HF, one may conclude that HF may represent a state of relative BNP deficiency. Altogether, such information has important implications for the interpretation of BNP as a biomarker in human cardiovascular disease.

Perspectives

The biology and biochemistry of BNP and NT–pro-BNP are far from being fully understood. As demonstrated in the current study, the presence of the ring structure is not sufficient to elicit the biological action of BNP. Indeed, it appears that only small or short molecular forms of BNP are capable of activating the natriuretic peptide receptor A receptor leading to the generation of cGMP from particulate guanylyte cyclase. From these findings, one can speculate that, whereas BNP 1-32 or shorter forms, such as BNP 3-32, have preserved biological activities, larger forms of BNP, like NT–pro-BNP 1-76 and pro-BNP 1-108, regardless of the presence of the ring structure, are unable to bind to the receptor and elicit biological actions, likely because of, at least in part, steric resistance. Importantly, the existence of larger molecular forms, rather than smaller, has been reported in humans with overt HF, explaining in part reduced cardiovascular and renal effects of high levels of BNP circulating in severe stages of cardiovascular disease. Finally, one may conclude that currently available assays are not specific in distinguishing among all of the possible circulating molecular forms of BNP, nor can help to differentiate their biological abilities, although clearly the use of these assays has enhanced the diagnosis of HF. Thus, these studies lay the groundwork for future investigations to further define the structure and the biological actions of different molecular forms of BNP. Indeed, a greater knowledge of this biological system may markedly increase the clinical usefulness of these biomarkers.

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Disclosures

None.

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Immunoreactivity and Guanosine 3',5'-Cyclic Monophosphate Activating Actions of Various Molecular Forms of Human B-Type Natriuretic Peptide
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