B-Type Natriuretic Peptide Forms within the Heart, Coronary Sinus, and Peripheral Circulation in Humans: Evidence for Degradation before Secretion

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BACKGROUND: The B-type natriuretic peptides (BNP and N-terminal pro-BNP) are secreted by the heart and, in the case of BNP, serve to maintain circulatory homeostasis through renal and vascular actions and oppose many effects of the renin-angiotensin system. Recent evidence suggests that in patients with severe heart failure, circulating immunoreactive BNP is made up mainly of metabolites that may have reduced bioactivity. We hypothesized that BNP may be degraded before it even leaves the heart.

METHODS: Peripheral venous plasma plus atrial and ventricular tissue, obtained from explanted hearts at the time of transplantation, were collected from 3 patients with end-stage heart failure. In a separate study, plasma was collected from the coronary sinus and femoral artery of 3 separate patients undergoing cardiac catheterization. Plasma C18 reverse-phase extracts were separated on reverse-phase HPLC, and the collected fractions were subjected to RIAs with highly specific antisera directed to the amino- and carboxy-terminal ends of BNP(1–32).

RESULTS: ProBNP, BNP(1–32), and 2 major BNP metabolites were present in atrial and ventricular tissue, where BNP(1–32) represented 45% and 70% of total processed BNP, respectively. Neither BNP(1–32) nor the 2 metabolites were detected in peripheral venous plasma. Nor was BNP(1–32) detected in matching coronary sinus and femoral artery plasma from the 3 patients undergoing cardiac catheterization.

CONCLUSIONS: BNP(1–32) is partly degraded within the hearts of patients with end-stage heart failure, and even in patients with relatively well-preserved left ventricular systolic function, only BNP metabolites enter the systemic circulation.

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Human B-type natriuretic peptide (BNP),³ originally identified as a 32–amino acid peptide [BNP(1–32)] in atrial and ventricular tissue (1), is secreted by the heart. BNP increases sodium excretion, induces vasodilatation, and directly opposes many potentially deleterious effects of the renin-angiotensin system and aldosterone. Further, its antitrophic effects oppose vascular/cardiac hypertrophy and fibrosis. However, these actions are critically dependent on the intact or biologically active forms of BNP reaching their receptors in distant tissues. Recent studies have suggested that when cardiac function is severely impaired, only a small proportion of circulating immunoreactive BNP (irBNP) is represented by the known biologically active BNP(1–32) form, the majority of the material being metabolites that have lost amino-terminal residues 2–4, most likely owing to the enzyme dipeptidyl peptidase 4 (DPP-4). This enzyme, located on the endothelium of capillaries in many organs including the myocardium (5) and in plasma, rapidly removes a dipeptide from the amino-terminal end of BNP(1–32) to produce BNP(3–32) (6, 7), which circulates at low concentrations in humans (8). This peptide displays reduced bioactivity when its human form is infused into dogs (9), but whether this effect occurs in humans remains to be determined. In addition to DPP-4, neprilysin and insulin-degrading enzyme (IDE) can also remove amino-terminal residues and produce ring cleaved forms and, for IDE, carboxy-terminal deleted forms (10, 11). Although the effect of ring cleavage on BNP bioactivity is not clear, a single ring cleavage in

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³ Nonstandard abbreviations: BNP, B-type natriuretic peptide; ir, immunoreactive; DPP-4, dipeptidyl peptidase 4; IDE, insulin-degrading enzyme; ANP, atrial natriuretic peptide; AEBSF, 4-(2-aminoethyl)benzenesulfonyl-fluoride; LVEF, left ventricular ejection fraction; TFA, trifluoroacetic acid; DPP-4, dipeptidyl peptidase 4; GLP-1, glucagon-like peptide 1; NTproBNP, N-terminal pro-BNP.
atrial natriuretic peptide (ANP) either abolishes (12) or reduces (13) bioactivity and is likely to have a similar effect for BNP. Nevertheless, some BNP products of IDE action may have increased bioactivity (11).

Whereas research has focused on the degradation of BNP(1–32) within the circulation, no attention has been paid to the possibility that degradation occurs within the heart before secretion. We previously observed that BNP(1–32) may be substantially degraded before release from the heart into the general circulation (14), and in this study we have assessed this issue in more detail. Our goal was to characterize the BNP forms present in explanted human heart tissue and matching plasma in patients with end-stage heart failure and also characterize the newly secreted forms of BNP in coronary sinus plasma in patients with preserved left ventricular systolic function undergoing cardiac catheterization.

Materials and Methods

Human BNP(1–32) and proBNP were purchased from American Peptide Company and Hytest, respectively. Other peptides were synthesized by Mimotopes.

STUDY PATIENTS, TISSUE, AND PLASMA COLLECTION

The studies were approved by the New Zealand Multi-Region Ethics Committee and the Upper South B Regional Ethics Committee, Australian and New Zealand Clinical Trials Registry number ACTRN1260600136505. All study patients provided written informed consent.

STUDY 1

We obtained atrial and ventricular tissue from explanted hearts of 3 patients with end-stage heart failure at the time of cardiac transplantation. Patient 1 had a severe congenital etiology, patient 2 had ischemic cardiomyopathy, and patient 3 had terminal dilated cardiomyopathy. At the time of cardiac transplantation, the atrial appendage and left ventricular free wall (midline) were excised and rapidly frozen at −80 °C. Peripheral venous blood from the same patients was collected just before removal of their heart and before transplantation into chilled tubes containing EDTA and immediately centrifuged at 4 °C, and the plasma was frozen at −80 °C. Characteristics for these patients are provided in Supplemental Table 1, which accompanies the online version of this article at http://www.clinchem.org/content/vol60/issue3.

STUDY 2

In this separate study to characterize secreted and circulating forms of BNP, coronary sinus and matching femoral artery samples were collected into chilled 9-mL glass tubes containing 140 µg EDTA, 380 µg leupeptin, and 5.4 mg 4-(2-aminoethyl)benzenesulfonyl-fluoride (AEBSF) (4) from 3 patients undergoing routine cardiac catheterization for assessment of coronary artery disease (15). All 3 individuals had left ventricular ejection fraction (LVEF) >40% and healthy kidney function and were clinically free of heart failure. Patients 4 and 5 had suffered a recent acute myocardial infarction, and patient 6 had severe valvular disease. All 3 individuals were selected for the high concentrations of irBNP in their coronary sinus plasma, to enable multiple immunoassay measurements. Coronary sinus plasma from patients 4, 5, and 6 contained 1198, 281, and 232 ng/L (345, 81, and 67 pmol/L) irBNP, respectively, and their matching arterial samples contained 378, 111, and 111 ng/L (109, 32, and 32 pmol/L) irBNP. Blood samples were centrifuged at 1500 g for 10 min at 4 °C, and the plasma was immediately frozen and stored at −80 °C.

EXTRACTION OF BNP FROM TISSUE AND PLASMA

Peptides in cardiac tissues were extracted by use of a previously published method for the purification of BNP from heart tissues (1). Briefly, 2 g ventricle or 1 g atrial tissue were diced at 4 °C, boiled for 5 min in 10 mL of 0.1% Triton X-100 to inactivate enzymes, acidified with acetic acid, and homogenized for 2 min at 24 000 rpm (Ultra-Turrax T25, IKA). After centrifugation for 20 min at 6600 g at 4 °C, the supernatant was made up to 10 mL with 1 mol/L acetic acid containing 0.01% Triton-X 100 and stored at −80 °C. Plasma samples were extracted on Sep-Pak C18 cartridges (Waters Corp.) as described previously (16). Recovery of BNP(1–32) was 70%. BNP(3–32) should exhibit a similar recovery.

HPLC

Reverse-phase HPLC used a 250 × 4.6-mm, 5-μm Jupiter C18 column (Phenomenex) at 40 °C with a gradient from 16.8% to 24% acetonitrile in 0.1% trifluoroacetic acid (TFA) over 30 min and then to 60% acetonitrile in 0.1% TFA over 5 min at 1 mL/min. The eluate was collected in 0.5-mL fractions, dried, and reconstituted in assay buffer for RIA. The column was calibrated with synthetic BNP(1–32) and, in most instances, BNP(3–32) each day that samples were processed.

RIA

Tissue and plasma BNP forms were characterized with 2 RIAs that we developed and named the “N-terminal assay” and “C-terminal assay.” These assays were specific for the amino (N) and carboxy (C)-terminal ends of BNP(1–32) as described below. The assays used antisera raised in rabbits against human BNP(1–13) and BNP(19–32) conjugated, respectively, to ovalbumin and bovine serum albumin through their cysteine res-
A third locally developed RIA that we named the “commercial assay” recognized the inner portion of the N-terminal arm of BNP and used a diluted commercial antiserum to BNP(1–32) (T-4021, Bachem). All 3 assays were performed as previously described (14) but used the antisera above. For each assay, 50 μL BNP(1–32) calibrator or sample was incubated with 50 μL antiserum at 1:3000, 1:10 000, or the manufacturer’s dilution in assay buffer for 22 h at 4 °C when radiolabeled BNP(1–32) peptide (2500 cpm in 50 μL) was added. After another 22-h incubation at 4 °C, 0.5 mL of 5% Sac-Cell (Immunodiagnostics) in 2% polyethylene glycol in assay buffer was added, incubated for 30 min, and centrifuged at 1500g for 15 min. The supernatants were aspirated, and the sediment was counted in a γ counter. A fourth RIA, using an antiserum against proBNP(1–13) (17), was used to locate proBNP and N-terminal pro-BNP (NTproBNP) on chromatograms. Assay characteristics are provided in online Supplemental Table 2.

Results

CROSS-REACTIVITY OF ANTISERA

The 3 antisera directed to components of the BNP(1–32) molecule recognized different epitopes (Fig. 1). Cross-reactivities relative to BNP(1–32) 100% were, for the N-terminal antiserum, BNP (2–32) 0.42%, BNP(3–32) <0.003%; for the commercial antiserum, BNP(1–32) 100%, BNP(2–32) 27.3%, BNP(3–32) 2.6%; for the C-terminal antiserum, BNP(19–29) <0.004%, BNP(19–30) <0.004%, BNP(19–31) <0.004%, BNP(19–32) 46.3%, and BNP(19–32)Ala33 [BNP(19–32) with an additional nonsequence alanine added at its C terminus] 2%.

The commercial antiserum to BNP(1–32) recognized the inner portion of the amino-terminal arm of BNP and the precursor proBNP, which contains this epitope. In contrast, the BNP N-terminal antiserum, raised against BNP(1–13), had an absolute requirement for residue 1 (serine). It did not cross-react with proBNP (cross-reactivity 0.8%) indicating that the free primary amine was essential for recognition by this antibody. Consequently, this antiserum recognized only BNP(1–32) or its metabolites containing a complete amino-terminal end. Similarly, the C-terminal antiserum, raised against BNP(19–32), had an absolute requirement for the C-terminal histidine at position 32 and had very low cross-reactivity with the synthetic C-terminal extended peptide BNP(19–32)Ala33. This antiserum also recognized proBNP. Hence it reacted
only with BNP(1–32) and proBNP or their fragments with complete C-terminal ends.

STUDY 1

Reverse-phase HPLC revealed 4 main peaks of BNP immunoreactivity in atrial and ventricular extracts (peaks 1–3 and 4-proBNP in Figs. 2 and 3 and online Supplemental Figs. 1 and 2). Peak 1 eluted at the position of BNP(1–32) and cross-reacted in all 3 BNP assays, but not NT-proBNP (not shown) assays, identifying this peak as BNP(1–32). Peaks 2 and 3 eluted later than the BNP(1–32) standard and had similar cross-reactivities to peak 1, indicating the presence of complete N- and C-terminal ends in both these peptides. These peptides were not N- or C-terminally extended BNP forms, as they reacted in the N- and C-terminal assays that did not recognize extended BNP forms. They most likely represented ring-cleaved BNP metabolites or posttranslationally modified forms. The fourth peak, comprising proBNP, eluted late in all 4 chromatograms at the position of proBNP standard and accordingly reacted with the NT-proBNP (not shown), commercial, and C-terminal BNP assays. A number of minor components present in the atrial and ventricular profiles reacted predominantly in the C-terminal assay, suggesting that the C-terminal end of BNP was more resistant to degradation than the N-terminal end. Included in this group was a small shoulder on the leading edge of peak 1 that eluted at the position of BNP(3–32) and had an immunoreactive profile consistent with this metabolite. BNP(1–32) (peak 1) represented only 45% of total processed BNP immunoreactivity in the atrium and 70% in the ventricle (mean of 3 study patients) when calculated from the areas under peaks 1–3, but excluding proBNP (peak 4).

Neither BNP(1–32) nor the metabolite peaks 2 and 3 seen in the atrial and ventricular tissue extracts (Figs. 2 and 3 and online Supplemental Figs. 1 and 2) were present in peripheral venous plasma (Fig. 4 and online Supplemental Fig. 3) drawn immediately before transplant from the same 3 study patients. No irBNP eluted at the position of BNP(1–32) in 2 patients [pa-
tient 1 (Fig. 4) and patient 3 (see online Supplemental Fig. 3), whereas in patient 2 (Fig. 4), a small peak eluting at the BNP(1–32) position did not cross-react in the N-terminal assay, indicating loss of 1 or more amino acids from the N-terminal end. Two further immunoreactive components (peaks A and B), along with other minor early-eluting components, had immunoreactivity profiles consistent with N- but not C-terminal truncation. Peaks C (patient 2) and D (patients 1 and 2) contained complete carboxy- and amino-termini but eluted later than BNP(1–32), indicating loss of amino acids from within the ring structure. As expected in these patients with end-sage heart failure, the plasma extracts contained proBNP (peak 4).

**STUDY 2**

The proteolytic enzyme inhibitors used for the prevention of BNP degradation (4) in this study interfered in our N- and C-terminal assays, producing markedly increased results. Separate HPLC experiments with inhibitors alone showed that this interference, mainly due to AEBSF, resulted in large peaks (shaded areas in Figs. 5 and 6 and online Supplemental Fig. 4) that were well resolved from the main irBNP peaks that eluted between them.

No irBNP eluted at the position of BNP(1–32) in coronary sinus or arterial plasma from the 3 study patients tested (Figs. 5 and 6 and online Supplemental Fig. 4) apart from small amounts of C-terminal immunoreactivity in 2 patients. Coronary sinus plasma from all 3 patients contained at least 2 major peaks of irBNP that immediately preceded the elution position of the BNP(1–32) standard. Peak F (fractions 46–50) contained at least 2 components in which the later eluting portion (fraction 49) reacted with all 3 BNP antisera, indicating it is a BNP form with intact C- and N-terminal ends. Because this peak eluted earlier than BNP(1–32), we concluded it was a ring cleaved BNP metabolite. However the complete absence of BNP(1–32) rested on the elution of peak F ahead of the BNP(1–32) standard. Although standards were run before and after each set of HPLC runs, we cannot exclude minor
variations in HPLC retention times contributing to this difference. The shoulder on the leading edge of peak F (Figs. 5 and 6 and online Supplemental Fig. 4) is likely to be BNP(3–32) because it eluted at the position of this standard and had an immunoreactive profile consistent with BNP(3–32). The earlier eluting peak (peak E) had a similar immunoreactive profile. Both of these peaks were also present in the matching arterial plasma in 1 patient (Fig. 5) but were barely detectable in arterial plasma from the other 2 patients.

Discussion

Rapid degradation of BNP(1–32) in plasma by enzymes such as DPP-4 has been observed (7), supporting the notion that BNP degradation may occur in the circulation, either in plasma or on cell surfaces. In contrast, the possibility that BNP is degraded within the heart before secretion has not been assessed. We therefore undertook 2 studies to investigate whether BNP is degraded in the heart before entering the coronary sinus or systemic circulation. In the first study, we characterized the irBNP forms in atrial and ventricular tissue, and in peripheral venous plasma obtained just before removal of the diseased heart. In the second study, we characterized the forms of BNP in the venous effluent of the heart (coronary sinus plasma) and in matched arterial plasma samples in patients undergoing cardiac catheterization. Importantly, the combination of our N-terminal assay, which did not recognize products of DPP-4 action such as BNP(3–32), with our C-terminal or commercial assays allowed us to characterize peptides that may have been subjected to DPP-4 action, although mass spectrometry would have provided a more definitive identification.

BNP(1–32) has previously been characterized in human atrial and ventricular tissue (1, 18). Our study confirms the presence of BNP(1–32) in atrial and ventricular tissue in these patients. However, we also identified 2 additional forms of BNP in both the atria and ventricles. These forms contain intact N- and C-termini, and their cross-reaction in the N- and C-terminal assays precludes them being N-terminal extended forms similar to proBNP or C-terminal extended forms as reported by Pan et al. (19). They may be BNP metabolites that have lost residues from the peptide ring, carry posttransla-

Fig. 4. Study 1: Reverse-phase HPLC of peripheral venous plasma extracts from patients 1 and 2. These results match their tissue results in Figs. 2 and 3. Each fraction was assayed with the 3 immunoassays used in Fig. 2. Line and arrow codes are given in the Fig. 2 legend.
tional modifications, or are BNP dimers similar to \( \beta \)ANP. Such ring cleaved forms could be produced in atrial granules by peptidases located in them (20, 21) or after secretion. Interestingly, the 2 metabolite peaks in ventricular samples were proportionately smaller than their corresponding BNP(1–32) peak compared to those in the atrial extracts. This may be related to the different secretion pathways in these tissues. Together, the 2 metabolites represented 55% of the total processed BNP in the atrial tissue and 30% in the ventricle; however, much of the unprocessed proBNP in these samples could be processed to BNP during secretion or in the circulation.

Collection of venous plasma samples immediately before removal of the diseased heart allowed us to compare the forms of BNP circulating in plasma with those in the tissues that secreted them. In contrast to the rather “clean” HPLC profiles of the heart tissue extracts, the profiles in the matching peripheral venous plasma samples were much more complex, with multiple metabolites present. Importantly, we did not detect BNP(1–32) or either of the 2 metabolites detected in atrial and ventricular tissue in any of these matching plasma samples. All of the irBNP components observed in these plasma extracts, apart from peaks C and D (Fig. 4), had lost N-terminal immunoreactivity, whereas most contained a complete C terminus and usually reacted with the commercial antiserum. Because the latter antiserum tolerates the loss of only a few amino acids from the N-terminal end before losing immunoreactivity, it is likely that these components have lost 1 or 2 amino acids from the N-terminal end. The presence of BNP(1–32) plus 2 metabolites in atrial and ventricular tissue, but their complete absence in peripheral venous plasma, clearly indicates substantial metabolism of BNP either during secretion or within the circulation.

We also examined whether BNP could be degraded even before it reaches the systemic circulation by collecting matching coronary sinus and arterial

Fig. 5. Study 2: Reverse-phase HPLC of extracts from coronary sinus and femoral artery plasma from patient 4. Each fraction was assayed with the 3 immunoassays used in Fig. 2. Line and arrow codes are given in the Fig. 2 legend. Major peaks have been labeled to correspond with those in the text. The hatched areas cover large peaks due to interference in our assay from proteolytic enzyme inhibitors used for blood collection.
blood from 3 patients undergoing coronary artery catheterization, and subjecting the plasma extracts to HPLC. We did not identify BNP(1–32) in coronary sinus or arterial plasma from any of the patients, although we took great care to avoid postsampling degradation. In all 3 coronary sinus samples and 1 arterial sample, we found a single peak (peak F in Figs. 5 and 6 and online Supplemental Fig. 4) that contained complete N- and C-terminal arms, but which consistently eluted earlier than BNP(1–32), indicating that it was a ring cleaved metabolite of BNP. In agreement with an earlier study on venous plasma (4), we found that BNP(3–32) and other N-terminally truncated metabolites were present, consistent with the actions of the enzyme DPP-4, neprilysin, or IDE.

These results confirm that BNP(1–32) is present in the hearts of patients with end-stage heart failure, but that it comprises only 45%–70% of total processed BNP in cardiac tissues, with the remainder comprising BNP metabolites. However, neither BNP(1–32) nor these metabolites were detected in peripheral venous plasma containing irBNP secreted by these same cardiac tissues. Although circulating BNP(1–32) has been identified in heart failure, its concentrations are low and detectable in only 56% of patients (3). Thus, the absence of BNP(1–32) in plasma from our study patients may reflect the small number of patients in our study. In a separate group of patients without heart failure, we were unable to detect BNP(1–32) in venous (coronary sinus) blood draining atrial and ventricular tissue. Instead we found several metabolites in which amino acids had been deleted from the amino-terminal end of the molecule and others that most likely have cleaved or depleted ring structures. Whether these metabolites are bioactive or not is uncertain, although BNP(3–32), which was present, has been reported to have reduced bioactivity in dogs (9). Conversely, it is possible that 1 or more “metabolites” are in fact a more biologically active BNP product (11).

Fig. 6. Study 2: Reverse-phase HPLC of extracts from coronary sinus and femoral artery plasma from patient 5. Each fraction was assayed with the 3 immunoassays used in Fig. 2. Line and arrow codes are given in the Fig. 2 legend. Major peaks have been labeled to correspond with those in the text. The hatched areas cover large peaks due to interference in our assay from proteolytic enzyme inhibitors used for blood collection.
Conversion of BNP(1–32), which is present in the heart, to metabolites in the coronary sinus implies exposure of the newly secreted BNP to proteolytic enzymes and rapid degradation between secretion and exit from the heart. Rapid degradation like this has been shown for the incretin peptide glucagon-like peptide 1 (GLP-1), where only 33% to 54% of the peptide remains intact by the time it leaves the local capillary bed in the gut, most likely owing to the action of DPP-4 in the capillaries (22).

Like GLP-1, BNP(1–32) is susceptible to the actions of DPP-4. Indeed, the soluble form of DPP-4 in plasma rapidly removes the N-terminal dipeptide, giving rise to the metabolite BNP(3–32) (7). DPP-4 is well placed to cleave BNP during secretion, as it is located in capillaries of the myocardium (23) and capillaries of rat ventricles (5). Our finding of small amounts of BNP(3–32)-like immunoreactivity in coronary sinus plasma but proportionately much less in stored forms in atrial and ventricular tissue is consistent with the action of DPP-4 during secretion. However, although BNP(1–32) is a relatively poor substrate for neprilysin and IDE, it is also possible that that the N-terminal truncated forms could have arisen from their action.

Limitations in our studies should be noted. First, the number of participants in each of the 2 studies is small for the obvious reason that the work required for all analyses in each individual is considerable. Although we are confident that the data we present are accurate, we cannot be certain that exactly the same findings will pertain to all patients with cardiac disorders. Second, although our observations in study 2 were in patients with a LVEF >40%, it is not possible to extrapolate with confidence these findings to healthy people. Blood sampling from the coronary sinus and femoral (or other) artery in healthy volunteers is needed to determine whether a profile of BNP forms similar to those we have documented exists in healthy humans.

In conclusion, we have shown that BNP(1–32) and 2 BNP metabolites are present in the hearts of patients with end-stage heart failure, and that BNP(1–32) represents 45% to 70% of the total processed iBNP forms present. The identity and potential bioactivity of the 2 metabolite forms remains to be determined. In contrast, BNP(1–32) was not detected in peripheral venous blood from the end-stage heart failure patients or coronary sinus blood from a separate group of patients without heart failure undergoing cardiac catheterization for assessment of coronary artery disease, although a number of BNP metabolites were detected. Together, these data indicate that BNP(1–32) is substantially metabolized in severely failed hearts and that even in cardiac disease without heart failure, little if any BNP(1–32) enters the circulation via the coronary sinus. Whether BNP(1–32) is similarly degraded in truly healthy people remains to be determined.

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