B-Type Natriuretic Peptide: From Posttranslational Processing to Clinical Measurement

Jens P. Goetze

BACKGROUND: Plasma cardiac natriuretic peptides and peptide fragments from their molecular precursors are markers of heart disease. Clinical studies have defined the current diagnostic utility of these markers, whereas biochemical elucidation of peptide structure and post-translational processing has revealed new plasma peptide forms of potential clinical use.

CONTENT: Natriuretic propeptide structures undergo variable degrees of endo- and exoproteolytic cleavages as well as amino acid modifications, which leave the plasma phase of the peptides highly heterogeneous and dependent on cardiac pathophysiology and capacity. An ongoing characterization of the molecular heterogeneity may not only help us to appreciate the biosynthetic capacity of the endocrine heart but may also lead to the discovery of new and more disease-specific targets for future molecular diagnosis.

SUMMARY: Peptides derived from pro–atrial natriuretic peptide and pro–B-type natriuretic peptide are useful plasma markers in heart failure. New data have defined cardiac myocytes as competent endocrine cells in post-translational processing and cellular secretion.
equimolar basis. A more comprehensive understanding of the biochemical structure may, therefore, provide new possibilities in molecular detection of cardiac diagnosis and prognosis. This review summarizes the present understanding of the posttranslational phase of cardiac BNP gene expression.

Structure of the BNP Precursor

Human proBNP comprises 108 amino acid residues. Mammalian precursor sequences have been deduced from cDNA sequences that encode the preprostructure (26–29). Amino acid homology between species is largely confined to the amino- and carboxy-terminal regions, whereas the remaining structure varies considerably between animals. Notably, the principal motifs for amino acid modifications and enzymatic processing are not well conserved between species. In addition to proBNP, human preproBNP contains an N-terminal hydrophobic signal peptide of 26 residues. This sequence is (theoretically) removed during translation before synthesis of the C-terminal part of the precursor is completed. PreproBNP does not, therefore, exist as an entity but is a theoretical structure. On the other hand, proBNP is an existing polypeptide as verified by gel filtration and sequence-specific immunoassays (19, 30–34). Nevertheless, the precursor molecule still remains to be purified together with the processing intermediates—apart from the C-terminal cleavage product, i.e., BNP-32, and the N-terminal region of the intact precursor. Whenever the primary proBNP structure is mentioned, it thus refers to the cDNA-deduced sequence and antibody-based data from chromatographic elution, Western blotting, and immunoassays.

PreproBNP and the Signal Peptide as an Entity in Plasma

As mentioned previously, signal peptides are removed during peptide translation in the cells and are usually not considered as peptide structures of interest in plasma measurement (Fig. 2). Recently, however, a peptide structure identical to preproBNP 17–26 has been identified in cardiac tissue and in blood plasma from patients (35). The clinical perspectives of this finding certainly deserve to be pursued, and the focus must include development of analytically sensitive and specific immunoassays. The result will, however, not be straightforward owing to the hydrophobic nature of signal peptides (36). Moreover, the new finding raises the hypothetical question of whether intact preproBNP is an existing peptide. Because the signal peptide is by definition highly hydrophobic, it is possible that preproBNP can be anchored in cell membranes or be associated with lipid compounds in circulation. For now, the preproBNP 17–26 peptide in plasma remains an interesting observation in urgent need of follow-up by biochemical and physiological experiments before actual clinical studies further evaluate this peptide for diagnostic use.

ProBNP-Derived Peptides

The posttranslational phase of BNP gene expression has become a new subject of interest. A hallmark of the study of cardiomyocyte BNP gene expression is the lack of useful in vitro cellular models. Although neonatal atrial myocytes can be cultured for short periods of time, they do not fully resemble differentiated atrial or ventricular myocytes. Furthermore, only a few immunoassays have been available for characterizing the molecular heterogeneity of the processing intermediates. Recent advances through mass spectrometry combined with the development of sequence-specific antibodies have nevertheless revealed a complex cardiac synthesis of natriuretic peptides.

AMINO ACID MODIFICATION

The overall proBNP structure appears simple (Fig. 2). In humans, it is divided into 2 principal regions by a cleavage site in position 73–76 (Arg-Ala-Pro-Arg),

![Fig. 1. Schematic presentation of human atrial, B-type, and C-type natriuretic peptides. Homolog amino acid residues between the natriuretic peptides are highlighted with red circles.](image-url)
leading to cleavage C-terminal to the site. The first region is the N-terminal fragment proBNP 1–76, and the second region is the C-terminal BNP-32 (proBNP 77–108). In contrast to other prohormones, proBNP does not contain a C-terminal–flanking region. The C-terminal region contains a ring structure formed by a disulfide bond between the cysteine residues in position 86 and 102 (Fig. 1). The protein disulfide isomerase family and thiol-disulfide oxidoreductases are likely enzymes involved in cardiac myocyte disulfide bond formation. Interestingly, cardiac expression of the protein disulfide isomerase transcript has been reported to be upregulated in cardiac disease [37]. Cellular experiments further suggest a direct cardioprotective effect of this regulation. It is speculated that not all cardiac natriuretic peptides are activated through this enzymatic process, which introduces the earliest possible regulatory step in natriuretic peptide biosynthesis and hormone activation. Regulation of protein disulfide isomerase has been classified as “endoplasmic reticulum stress,” which is a hallmark of several pathological disorders including diabetes mellitus, neurodegenerative disorders, and ischemic heart disease [38]. Other regions of the precursors may also be involved in disulfide bond formation. It has been shown for insulin biosynthesis that alterations in the proinsulin sequence can result in incorrect disulfide bonding and synthesis of insulin with altered chemical and biological properties [39].

For the cardiac natriuretic peptides, the recently reported frame-shift mutation in the human ANP gene [natriuretic peptide A (NPPA)], generating an elongated ANP peptide (C-terminally extended with the amino acid sequence RITAREDQWA-COOH) may be a natural peptide candidate for alterations in disulfide-bond formation [40].

The existence of larger forms of BNP than the purified BNP-32 was first indicated by gel filtration studies of cardiac tissue extracts and plasma from patients with cardiac disease [19, 30, 31, 41]. Some data also suggest the existence of molecular forms larger than the predicted precursor. Independently, several groups observed immunoreactive forms with molecular masses of 25–45 kDa in cardiac tissue and plasma. On the basis of the primary structure, however, intact proBNP has an expected mass of approximately 11 kDa. One report suggested that proANP and proBNP may oligomerize through a leucine zipper-like motif in the midregion [42]. The question of whether the peculiar elution profiles were chromatographic artifacts or represented peptide binding to other molecules was put aside when it was shown that human proBNP exists as an O-linked glycoprotein and that the modification is located precisely in the leucine zipper-like motif [43]. In the precursor structure, the midregion (proBNP 36–71) contains 7 serine and threonine residues, where O-linked glycosylation occurs either fully or partially (Fig. 2). This dramatic modification of a polypeptide apparently does not affect the overall 3-dimensional structure of the precursor in solution [44, 45]. On the other hand, the presence of carbohydrate groups will affect immunological measurement if the epitope recognition resides within this region—and also regulates en-
doproteolytic cleavage (46, 47). No specific immunoassay has been developed against only the glycosylated forms, and the ratio between glycosylated vs nonglycosylated proBNP products can be deduced only from assays that specifically measure the nonglycosylated forms or cross-react with both forms. Whether O-linked glycosylation is an “unlimited” posttranslational modification or is affected by increased BNP gene expression, as in heart disease, is an important question for future studies. It should also be reiterated that the ANP precursor may be subject to glycosylation (48). In addition, the proBNP sequence varies considerably between species in the midregion (25), which possibly renders glycosylation a species-dependent modification. Finally, it is unresolved whether atrial and ventricular myocytes possess the same biosynthetic capacity to glycosylate natriuretic precursor peptides.

Glycosylation could be a biochemical target for diagnostic applications if the modification is affected by cardiac disease and/or reflects changes in BNP gene expression. Also, the potential impact of early biosynthetic glycosylation on cellular sorting and the subsequent precursor processing may prove to be of diagnostic use. Because O-linked glycosylation can occur close to the principal prohormone maturation site in position 74–76 (on the threonyl residue in position 71), the presence of carbohydrate groups affects processing and hormonal maturation (46, 47). In turn, this modification regulates prohormone cleavage by blocking endoproteolytical enzymes, which leaves the propeptide with reduced or no biological activity. Conceptually, immunoreactive BNP with little or no biological activity has been nicknamed “junk-BNP.” This “junk,” however, may still prove to be the best peptide for clinical measurement.

**PROCESSING**

Human proBNP was first suggested to be cleaved by the ubiquitous endoprotease furin because the furin [furin (paired basic amino acid cleaving enzyme) (FURIN)] and BNP (NPPB) genes are coexpressed in cardiac myocytes (49). The Arg-Ala-Pro-Arg motif in position 73–76 in human proBNP has been shown to be a target for furin-mediated cleavage. Endoproteolytical processing can be blocked in vitro by inhibition of furin, and furin has been shown to be essential for maturation of the structurally related CNP (50). A different pro tease named corin has been identified in human heart cDNA and is a serine protease that can cleave both proANP and proBNP in vitro (51, 52). Corin contains a transmembrane domain anchored in the cell membrane and is thought to cleave the precursors upon secretion. A role of corin in the biosynthesis of cardiac natriuretic peptides has been substantiated in vivo by genetic coupling of corin mutations to clinical phenotypes that can be explained by reduced ANP and BNP bioactivity in circulation, e.g., hypertension (53, 54). Corin thus seems to be involved in the biosynthesis of natriuretic peptides, and one report even suggests that corin is active in the circulation (55). Of note, atrial posttranslational processing of proANP and proBNP is likely to differ from ventricular processing because isolated atrial granules have been reported to contain both unprocessed proANP and mature BNP-32 (56). Corin activity alone can therefore not fully explain the endoproteolytical maturation of cardiac natriuretic propeptides. The putative corin site in the BNP precursor is not conserved between mammals, and it may be worthwhile to examine whether human corin cleaves precursor peptides from other mammalian species.

The proprotein/prohormone convertases are a well-established family of intracellular processing enzymes, also known as the PCs. In addition to the already mentioned furin, the subtilisin-like endoproteases PC1/3 and PC2 are also expressed in the mammalian heart (57, 58), and PC1/3 expression has been demonstrated both in normal and pathological human cardiac tissue (59). Atrial myocytes transfected with an adenoviral vector expressing PC1/3 processes proANP to both mature ANP and to a truncated form (60). Although the precise cleavage site was not established, this finding underscores the possibility that other propeptides than furin and corin may be involved in the posttranslational endoproteolysis of proANP and proBNP. PC1/3 is active in secretory granules and could therefore be an important regulator of atrial proBNP processing, and cardiac PC1/3 expression has been reported to be upregulated in heart disease (61). At present, there are no data on other proBNP-derived fragments derived from endoproteolytical processing. This lack of information may reflect the lack of specific tools for the identification of new peptide fragments, which requires antibodies directed at other epitopes than the ones used so far for biochemical identification. However, the precursor sequence contains several basic amino acid residues that may represent cleavage sites for the PCs (11).

N-terminal trimming has been noted for proBNP-derived peptides, in which both the N-terminus of the biosynthetic precursor and the C-terminal bioactive BNP-32 product contain amino acid motifs for aminopeptidase recognition and cleavage. More precisely, the N-terminus of proBNP and BNP-32 (proBNP 77–108) contains a prolyl residue in position 2 (His-Pro and Ser-Pro, respectively). Prolyl residues are important for peptide structure and folding, and they are also involved in exoproteolytic trimming. N-terminal trimming has been demonstrated for BNP in vitro, in which synthetic BNP-32 (proBNP 77–108) incubated in the
presence of dipeptidyl peptidase (DPP)-IV are cleaved after the N-terminal Ser-Pro residues (62). DPP-IV, an enzyme located mainly on endothelial cells and in circulation, preferentially cleaves N-termini with either prolyl or alanyl residues in the second position (63). Thus, this DPP-IV cleavage may not be part of the biosynthetic maturation but rather may be related to the elimination phase. An N-terminally trimmed form of proBNP lacking the His-Pro residues in position 1–2 has also been reported in heart failure patients (64). This report disclosed that a truncated proBNP 3–108 form circulates in increased concentrations in heart failure patients. Experiments in our laboratory have shown that the human proBNP N-terminus can be trimmed in vitro by DPP-IV and fully blocked by inhibition of DPP-IV. In this context, it is noteworthy that the first report on glycosylated proBNP in a recombinant expression system (Chinese hamster ovary cells) also identified a truncated proBNP 3–108 form in cell extracts (43). Although this finding may be explained by experimental handling of extracts and medium, it could also indicate that N-terminal exoproteolysis is in fact a part of the intracellular maturation (65, 66). Whether the trimming of BNP and its molecular precursor has an actual regulatory function in cardiac natriuretic peptide physiology remains a question for future experimental research. One could speculate that amino-terminal trimming affects the metabolic fate of the peptides and thus their turnover in circulation. There are, however, no data available on the actual biological relevance of these trimmings. Comparison between mammalian species reveals homology at the N-terminus of proBNP but not for the N-terminus of BNP, indicating that the N-terminus of the precursor has been subjected to phylogenetic conservation through a selection process, perhaps related to the removal of the signal peptide.

Cellular Storage and Secretion

BNP gene expression takes place in both atrial and ventricular myocytes. In the normal heart, the main regional site of BNP expression is in the atria (67, 68). Ventricular BNP gene expression increases drastically in cardiac disease that affects the ventricles, i.e., congestive heart failure (69). The observation of ventricular BNP gene expression in ventricular disease may have given rise to the common notion that BNP is mainly a ventricular hormone. Atrial and ventricular myocytes, however, differ considerably with respect to their endocrine phenotypes, and it is reasonable to expect marked differences in peptide storage and secretion patterns (70). Atrial granules contain a mix of intact precursors and biosynthetic end-products, i.e., bioactive ANP-28 and BNP-32. In contrast, normal ventricular myocytes do not seem to form such granules, and normal ventricular myocytes do not contain proBNP-derived peptides (68). A few reports describe observed granules and proBNP-derived peptides in ventricular myocytes from pathological hearts (71, 72). Thus, ventricular myocytes not only regulate the BNP gene at the transcriptional and posttranslational level but also seem to be able to differentiate with respect to the basic biosynthetic apparatus. One report even suggests the presence of different classes of granules, in which one class contains only ANP-related products, and another class contains both ANP and BNP peptides (73). In this context, the proANP structure has been implicated in granule formation through calcium-mediated aggregation in the trans-Golgi network, where substitution of the acidic residues in the N-terminal region changes the size and the shape of intracellular vesicles and their ability to dock with the plasma membrane (74, 75). In addition to these findings, it should be mentioned that observation by electron microscopy of atrial myocytes from ANP-gene–deficient mice did not reveal secretory granules (76). Cardiac BNP expression in ANP-deficient mice is also affected by decreased BNP mRNA contents in the atria and increased expression in the ventricles (77). BNP peptide contents in these tissues paralleled the mRNA findings, with no peptide in atrial regions and borderline detectable contents in ventricular samples. The formation of granules in atrial and ventricular myocytes consequently differs and may be dependent on the 2 cardiac natriuretic peptide systems. To fully understand these mechanisms, further experiments addressing the role of the proBNP-derived peptides in granule formation and docking should be pursued. Characterization of ANP expression in BNP-deficient animals could also prove to be informative because atrial granules have in fact been observed in these animals (78). The general perception of cardiac secretion nevertheless refers to atrial release as a regulated process, whereas ventricular release resembles constitutive or constitutive-like secretion.

ProBNP-Derived Peptides in Plasma

ProBNP-derived peptides are secreted by cardiac myocytes and circulate in plasma. Their molecular heterogeneity has been characterized by chromatography in combination with sequence-specific immunoassays. Most of our conception of the cellular synthesis is in fact derived from the plasma phase, which represents the net sum of secretion and metabolism. The picomolar concentrations in plasma limit the possibilities for full biochemical identification and underscore the importance of epitope recognition by the immunoassays. With this in mind, it is established that bioactive BNP is secreted from the heart and circulates without binding
to plasma proteins (79). Synthetic BNP-32 (proBNP 77–108) is trimmed when incubated in whole blood, generating a BNP form lacking the 2 N-terminal amino acid residues (41, 80). As mentioned before, this molecular form can also be generated in vitro by enzymatic DPP-IV trimming and possibly also other aminopeptidases. Further processing of plasma BNP seems to involve degradation with a loss of bioactivity though disruption of the ring structure mediated by neutral endopeptidase (NEP 24.11) or by receptor-mediated cellular uptake. Although this has been known for some time, the therapeutic potential of inhibiting neutral endopeptidase with increased plasma concentrations of “beneficial” natriuretic activity is still a clinically unproven strategy (81). The metabolic half-life of BNP-32 has been reported to be 13–20 min (82, 83). Immunoactive BNP is also excreted in urine, but the precise contribution of renal excretion to overall metabolism is still not clarified. A minor degree of hepatic clearance has also been observed, but is not significantly altered in patients with liver failure (84).

In addition to bioactive BNP, other proBNP-derived fragments circulate in plasma. These fragments are commonly referred to as N-terminal proBNP, but the molecular heterogeneity also includes the intact precursor, in particular in heart failure patients (19, 85, 86, 87). Cardiac secretion of proBNP and its N-terminal fragments has been demonstrated by blood sampling from the coronary sinus. The molar ratio of secreted proBNP 1–76 to intact proBNP is not yet fully clarified but is likely to depend on cardiac status, i.e., more unprocessed precursor compared to biosynthetic cleavage products in severe heart failure. On the metabolic phase, there are still major discrepancies in the suggested half-life of N-terminal precursor fragments, which at least partially reflect epitope recognition in the assays. Theoretically, the half-life of proBNP 1–76 in circulation should be around 25 min (88) and thus not differ much from the established metabolism of BNP-32 (proBNP 77–108). One report, however, suggested a considerably longer half-life (approximately 90 min after cardiac pacing), which would explain the higher plasma concentrations of N-terminal proBNP fragments compared to bioactive BNP in healthy individuals and in patients (89).

Interestingly, processing of proBNP to bioactive BNP can also occur in circulation, which traditionally may be attributed to an “elimination mechanism” (90). Conversion of proBNP to BNP is, however, an activating event in terms of hormone bioactivity and thus challenges the concept of biosynthesis as the sole prohormone maturation mechanism. As our perception of the molecular heterogeneity in plasma has changed radically over the last few years, there is a renewed—and rather urgent—need for classic pharmacokinetic studies to better separate the biosynthetic phase from the peripheral elimination.

**Assay Calibration**

Cardiac natriuretic peptide biosynthesis is a complex process that produces a variety of peptides targeted for cellular secretion (25). The different phases of gene expression are not only region specific but also depend on changes within the secretory apparatus in cardiac myocytes. The main clinical applications of peptides today strongly relate to plasma measurement in cardiovascular diagnosis and prognosis. The clinical immunoassays must be designed with careful insight into the biosynthesis of the peptides. Another defining aspect of immunoassay measurement is the choice of calibrator. This aspect has not been scrutinized by researchers apart from the observation of disturbingly large molar discrepancies between the different assays (91). On the other hand, it has not been possible to raise meaningful assay calibration issues until now, with the establishment of the existence of a complex molecular heterogeneity. One way of bypassing this lack of information has been introduced as a “processing–independent assay,” which simply quantifies 1 in vitro cleavage product that represents all secreted precursor molecules on a molar basis (31, 48, 92); for reviews see (93, 94). This approach has several advantages. First, measurement of a standardized peptide fragment allows for quantification of the total amount of peptide products derived from mRNA translation, because each fragment will reflect 1 mRNA reading and translation. Second, the use of well-defined, small epitopes renders monoclonal antibodies less important in situations in which such antibodies still cross-react in various degrees with the different endogenous forms. Last, the use of an in vitro–generated peptide fragment allows for accurate calibration by use of the same peptide fragment. Many automated assays today still cross-react to different forms even with the use of monoclonal antibodies, and a standardized calibrator is still far from resolution.

If an endogenously occurring proBNP-derived peptide is used for assay calibration, it becomes tricky. Because the ratio of bioactive BNP to intact precursor shifts toward less-processed biosynthetic products, one would perhaps choose the dominant “disease” form over the more prevalent forms in healthy individuals. However, large comparative studies have failed to reveal major differences between BNP and proBNP measurements in terms of overall clinical performance, although plasma measurement based on assays directed against the N-terminal proBNP fragment is greatly influenced by the degree of O-linked glycosylation. Clearly, this issue is far from settled, and our present
perception of “normal” concentrations of the biosynthetic products may have to be reconsidered.

From Quantification to Qualitative Assessment

Accurate measurement of plasma markers is a hallmark in clinical laboratories. In this context, the natriuretic peptide field has been dramatically expanded during the last few years, so that we now perceive cardiac biosynthesis as a complex process including disulfide bonding, endoproteolytic cleavage(s), possibly exoproteolytic trimming, and major amino acid modifications (25). The next-generation assays must therefore be designed with the intent for more specific markers in cardiac disease. And the task will not be simple. By analogy, clinical measurement of the peptide hormone gastrin, a hormone known for almost 100 years, still suffers from biochemical assay pitfalls that can be directly explained by antibody specificity and differences in calibration (95). Perhaps the next-generation assays for natriuretic peptide measurement should include not only a quantitative measure but also a qualitative evaluation. As glycosylation and endoproteolytic maturation varies between normal to pathological cardiomyocytes—and clinically between patients with the same degree of cardiac dysfunction—so does the plasma profile of the different peptide forms. The biosynthetic apparatus may not be able to process efficiently in disease characterized by increased BNP gene expression, which may then be followed by secretion of less processed and modified peptide forms to circulation. This concept is by no means new in a physiological context, but the clinical applications of this “molecular shift” have not yet been pursued. For instance, a measure of proBNP concentrations (antigen test) combined with an evaluation of bioactivity of the endogenous peptides, including a Western blot profile of proBNP-derived peptides, may help further our understanding of the individual patient’s ability to produce mature cardiac natriuretic peptides. In turn, this information could be used to identify patients with either “good” or “bad” natriuretic peptides—the good forms being the bioactive hormones that cause renal excretion of sodium and water. Lastly, this type of laboratory evaluation could help individualize treatment, which is still a 2-edged sword in the care of patients with heart failure (96).

Concluding Remarks

Since the discovery of the endocrine heart 30 years ago, natriuretic peptides have been implicated in normal human physiology and in cardiovascular disease (97). An overwhelming amount of research has also identified peptides derived from proANP and proBNP as useful plasma markers in heart failure. Our understanding of cardiac synthesis, secretion, and elimination is starting to take form, however, and many of the immunoassays used today need renewed evaluation. Cellular expression, including posttranslational maturation, has revealed a complex biosynthetic phase with major regional as well as cellular differences in storage and secretion between healthy and diseased states. An investigative focus on molecular heterogeneity could, therefore, reveal new diagnostic possibilities because different biosynthetic products are not equal markers of the same pathophysiological processes. In a time when natriuretic peptide measurement has been firmly introduced to the clinical setting, the analytical possibilities in human cardiovascular disease must be reconsidered with more specific markers. The next-generation assays may soon be here.

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