Clin Chem Lab Med 2011;49(12):1949–1954 © 2011 by Walter de Gruyter • Berlin • Boston. DOI 10.1515/CCLM.2011.686

Minireview

Measurement of the pro-hormone of brain type natriuretic peptide (proBNP): methodological considerations and pathophysiological relevance

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Abstract

Recent studies demonstrated that large amounts of the pro-hormone peptide of brain natriuretic peptide (proBNP) can be detected in plasma of healthy subjects and in particular of patients with heart failure. As a result, a great part of B-type natriuretic peptides measured in patients with cardiovascular disease may be devoid of biological activity. These findings stimulated the set up of specific immunoassay methods for the measurement of the intact proBNP peptide. The aim of this review article is to discuss the methodological characteristics and the possible clinical relevance of specific immunoassay methods for the measurement of the proBNP peptide. From an analytical point of view, a fully automated immunoassay of proBNP has some theoretical advantages (e.g., a more stable molecule with higher molecular weight than the derived peptides) compared to the active hormone BNP. Recent studies supported the concept that the precursor proBNP might be actually considered a circulating prohormone, which can be cleaved by specific plasma proteases in BNP, the active hormone, and NT-proBNP, an inactive peptide. The peripheral processing of circulating proBNP could likely be submitted to regulatory rules, which might be impaired in patients with heart failure, opening new perspectives in the treatment of heart failure (e.g., by studying drugs inducing the cleavage of the prohormone into active BNP). Furthermore, as a future perspective, the specific assay in the same plasma sample of the intact precursor proBNP and of the biologically active peptide BNP, could allow a more accurate estimation of the production/secretion of B-type related peptides from cardiomyocytes and of the global cardiac endocrine function.

Keywords: brain type natriuretic peptide (BNP); cardiac endocrine function; corin; heart failure; immunoassay.

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Received May 9, 2011; accepted July 26, 2011;
previously published online August 26, 2011

Introduction

Exactly 30 years ago, De Bold et al. (1) reported that intravenous injection of atrial extracts promotes a rapid and massive diuresis and natriuresis in rats, thus indicating that unknown endogenous substances, produced by cardiomyocytes, may have both diuretic and natriuretic properties. Only a few years after, a family of natriuretic and vasodilator peptides, named cardiac natriuretic hormones (CNHs), was isolated, purified and then chemically identified in human tissues (2, 3). CNHs now include atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and their related peptides. Other natriuretic peptides, such as C-type natriuretic peptide (CNP) and urodilatin are predominantly produced and secreted by other tissues. In particular, CNP is produced predominantly by endothelium, while urodilatin by renal tubular cells by the same gene of ANP, and so this peptide is present only in the urine (2, 3). All natriuretic peptides share a direct diuretic, natriuretic and vasodilator effect and an inhibitory and protective action on inflammatory processes of myocardium, endothelium and smooth muscle cells, in this way modulating coagulation and fibrinolysis pathways, and inhibiting platelet activation (2–7).

From a clinical point of view, CNHs gained interest especially when it was demonstrated that the measurement of B-type-related natriuretic peptides (including BNP and NT-proBNP) represents a significant improvement in the diagnostic and prognostic accuracy as well as a potential guide to treatment in the clinical setting of heart failure (8–13). For this reason, BNP/NT-proBNP assay is useful for diagnosis, risk stratification and follow-up of patients with heart failure (HF) (8–14).

Recent studies reported that many B-type-related peptides circulate in human blood (15–32). In particular, large amounts of pro-hormone peptide (i.e., proBNP) can be detected in plasma of patients with HF (15–22, 25, 32). These data may indicate that the post-translational maturation processing of BNP precursor is not efficient in heart failure (3, 20, 23). As a result, a great part of B-type natriuretic peptides assayed in healthy subjects and patients with cardiovascular disease may be devoid of biological activity. These findings stimulated the set up of specific immunoassay methods for the measurement of the intact proBNP peptide (18, 19, 22, 24, 25). The aim of this review article is to discuss the methodological characteristics and the possible clinical relevance of specific immunoassay methods for the measurement of the proBNP peptide.

Biosynthesis of B-type natriuretic peptides

CNHs are synthesized by cardiomyocytes as pro-hormones (i.e., proANP and proBNP), which are then split into two fragments at the time of secretion from cardiomyocytes: the longer fragment includes the NH_2-terminus (NT-proANP and NT-proBNP), while the shorter one (i.e., COOH-terminus fragment) represents the active hormone (ANP and BNP).

In particular, human BNP is synthesized as a 134-amino acid (aa) precursor protein (pre-proBNP) and is subsequently processed to form a 108-aa propeptide, named proBNP. The propetide hormones of the cardiac natriuretic peptides can be enzymatically cleaved by proprotein convertases produced in the cardiomyocytes, such as corin and furin (3). ProBNP is then processed to form the 76-aa N-terminal peptide (i.e., NT-proBNP) and the biologically active 32-aa C-terminal peptide (i.e., BNP_{1-32}) (Figure 1). Some of the biosynthesized pro-hormone ($proBNP_{1-108}$) is *O*-glycosylated within the Golgi apparatus (3, 28–31). If *O*-glycosylation does not occur, $proBNP_{1-108}$ can be cleaved to BNP_{77-108} and NT-$proBNP_{1-76}$ by the processing enzymes within the trans-Golgi network. If *O*-glycosylation occurs, glycosylated-$proBNP_{1-108}$ cannot be cleaved, and uncleaved glycosylated $proBNP_{1-108}$ is secreted into the circulation. Finally, a smaller part of intact pro-hormone is not glycosylated and cleaved, and so this peptide can be present into circulation in intact form as $proBNP_{1-108}$. As indicated in the Figure 1, the glycosylation on the threonyl residue in position 71 (Thr 71) may regulate pro-hormone cleavage by either blocking or guiding endoproteolytical enzymes (3, 31).

Circulating B-type-related peptides: biochemical characteristics

According to the biosynthetic pathways described above, in addition to the bioactive hormone BNP, a huge number of circulating proBNP-derived fragments can be identified by chromatographic procedures in plasma of experimental animals and patients with HF (3, 19, 20, 26, 27, 32). Moreover, the $proBNP_{1-108}$ and NT-$proBNP_{1-76}$ (and probably also other shorter peptides derived from these precursors) are present in plasma in both glycosylated and non-glycosylated form, especially in plasma samples of patients with heart failure (3, 20, 29).

Some studies suggested that proBNP may be the major BNP-immunoreactive form in human blood, especially in patients with congestive heart failure (18–20, 32). In particular, Seferian et al. (29) recently reported that the plasma pool of the endogenous NT-proBNP contains a small portion (about 5%) of non-glycosylated or incompletely glycosylated protein, and that this portion can be detected by antibodies specific to the central part of the molecule.

A still open question is whether the circulating proBNP is also present in polymer form, such as trimer (26, 27), or not (20). Some more recent studies seem to indicate that the high-molecular forms of proBNP, found in older studies, were more probably due to the highly glycosylated forms of the precursor peptide rather than to the polymerization forms of the peptide (20).

It is usually assumed that the active peptide BNP_{1-32} should have a shorter plasma half-life and consequently lower plasma concentration, compared to $proBNP_{1-108}$ and NT-$proBNP_{1-76}$ (Table 1) (2, 3, 16–18). However, there is no study set up with the specific aim to accurately evaluate and compare the respective plasma half-life of BNP, NT-proBNP and proBNP in vivo in humans, but there are only a few studies available in the literature, which use experimental animal models. Using an experimental model in sheep, Pemberton et al. (33) reported that the plasma half-life of BNP is about 4.8 ± 1.0 min in vivo, while that of NT-proBNP was 69.6 ± 10.8 min. Using an experimental rat model, Semenov et al. (34) recently reported that the terminal half-life in vivo for human glycosylated proBNP was 9.0 ± 0.5 min compared with 6.4 ± 0.5 min for BNP. Moreover, the terminal half-life was 15.7 ± 1.4 min and 15.5 ± 1.3 min for glycosylated and non-glycosylated forms of NT-proBNP, respectively (34).

The proBNP assay: methodological considerations

As hypothesized several years ago (35), but only recently demonstrated (36), all immunoassays, which were considered specific for BNP or NT-proBNP peptides, actually show some cross-reactivity with proBNP. From an analytical point of view, this finding points out an important methodological problem: the need to set up more specific immunoassay methods, not only for the biologically active peptide BNP_{1-32}, but also for all the other B-type related peptides, including the precursor $proBNP_{1-108}$.

Considering the proBNP assay, some different methodological approaches may be considered for the measurement of the peptide (35). In plasma or tissue extracts, proBNP was usually isolated by means of chromatographic procedures, in particular by HPLC, and then identified and measured by immunoassay or more accurately by mass spectrometry (16–32, 35). These methods are highly specific for the peptide, but also complex, time consuming and not available for the clinical laboratory routine (35). To solve these problems, at least three different methodological approaches were developed with the aim to set up a highly specific immunoassay method for the measurement of proBNP (18, 24, 37).

Goetze et al. (24) set up a radioimmunoassay (RIA) for proBNP assay based on the measurement of plasma treated with trypsin, which cleaves all proBNP-related peptides to the small 1–21 fragment. The aim of these authors was to develop a processing-independent analysis (PIA) for accurate quantification of proBNP and its fragments in plasma. This method uses an antibody specific for a processing-independent epitope of human proBNP. This antibody was directed against the first 10 amino acids of proBNP peptide and the same peptide, radio-iodinated with chloramines-T, was used as a tracer in the RIA. Using this method, these authors determined the total concentration of proBNP and its

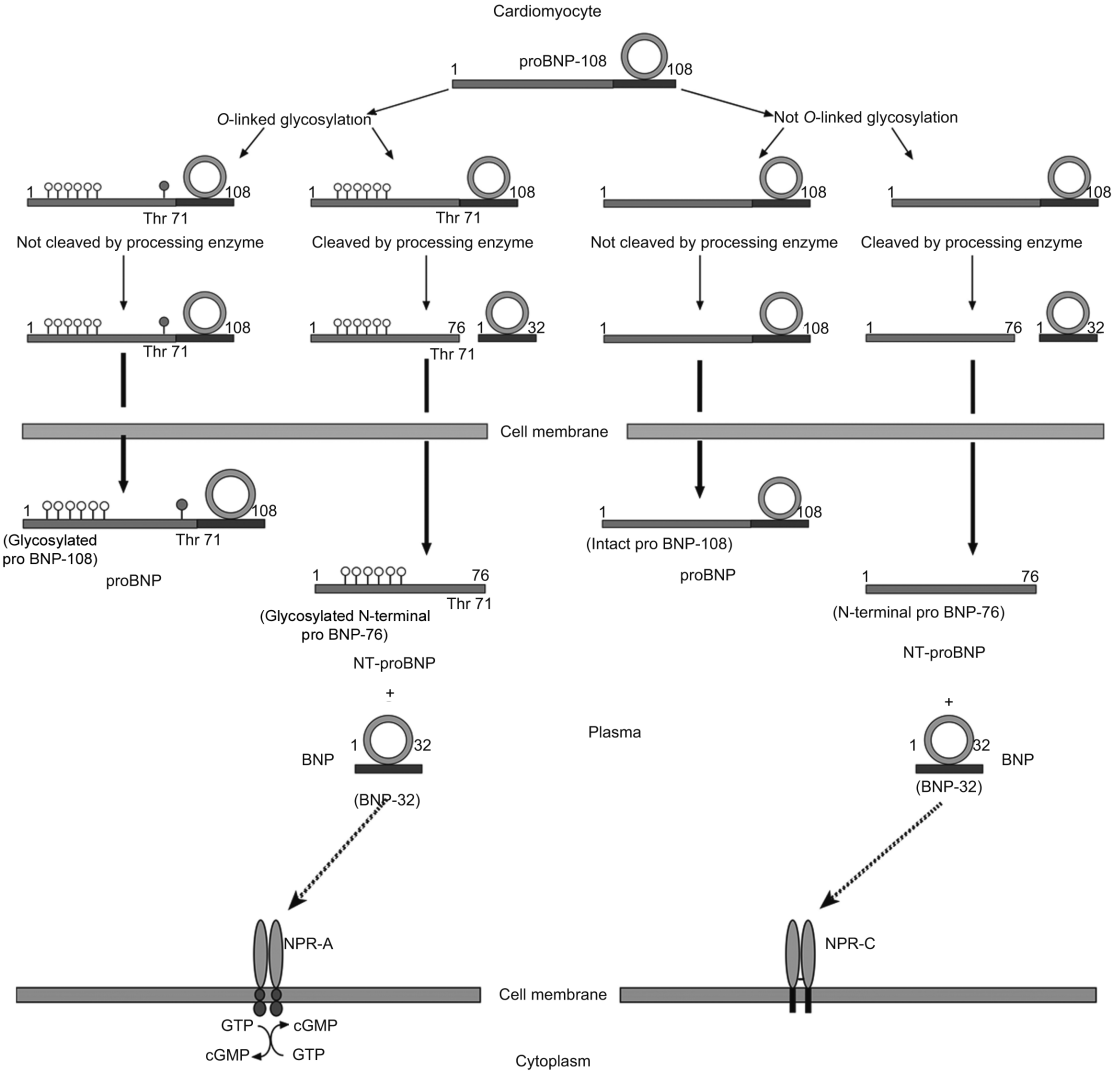

Figure 1 Schematic representation of biosynthesis, secretion and distribution of B-type related natriuretic peptides.
Some of the biosynthesized pro-hormone (proBNP-108) is *O*-glycosylated within the Golgi apparatus. If *O*-glycosylation does not occur, proBNP-108 can be cleaved to BNP-32 and NT-proBNP-76 by the processing enzymes within the trans-Golgi network. If *O*-glycosylation occurs, glycosylated-proBNP-108 cannot be cleaved, and uncleaved glycosylated proBNP-108 is secreted into the circulation. Finally, a smaller part of intact pro-hormone is not glycosylated and cleaved, and so this peptide can be present into circulation in intact form as proBNP-108. As indicated in the Figure, the glycosylation on the threonyl residue in position 71 (Thr 71) could regulate pro-hormone cleavage by either blocking or guiding endoproteolytical enzymes [see the reference (3) for more details]. Only BNP_{1-32}, which is the active hormone, is able to bind the specific receptors, NPR-A and NPR-C. NPR-A is a guanylate cyclase-coupled receptor, which mediates the biological effects of cardiac natriuretic peptides. NPR-C, not coupled to a guanylate cyclase, has essentially a clearance function for all natriuretic peptides [see the reference (2) for more details].

products in healthy volunteers and HF patients, showing that proBNP values were greatly higher in patients (24). However, this RIA cannot be recommended for the clinical routine because this assay requires a preliminary treatment of plasma samples with trypsin, a very long incubation time (i.e., up to 5 days), and the use of radiolabeled material. Furthermore, it is theoretically conceivable that this RIA measures all the peptides containing the N-terminal part of the precursor $proBNP_{1-108}$, and so even the NT-proBNP. As a consequence, for an accurate measurement of circulating levels of the intact $proBNP_{1-108}$ is also necessary to accurately and independently assay the NT-proBNP concentration. From a pathophysiological point of view, it is important to note that this RIA can allow an estimation of the overall production by cardiomyocytes of the precursor proBNP, including both the intact circulating $proBNP_{1-108}$ and the part of the precursor split before secretion (or in plasma) in NT-proBNP (and BNP).

Tamm et al. (37) developed a sandwich immunofluorescence assay for the quantification of BNP and its precursor proBNP. Authors reported that this immunofluorescence assay recognizes with the same efficiency the BNP as well as the recombinant glycosylated and non-glycosylated forms of proBNP (37). This immunoassay method (named ‘‘single-

Table 1 Biochemical and physiological characteristics of BNP, NT-proBNP and proBNP peptides.

	BNP	NT-proBNP	proBNP
Molecular mass	3462 Da	8457 Da[a]	11900 Da[a]
Amino acids	32	76	108
Biological function	Active hormone	Inactive	Pro-hormone
Plasma half life	15–20 min	>60 min	>60 min
Glycosylation	Not glycosylated	Highly glycosylated	Highly glycosylated
In vitro stability	Rapidly degraded in serum	More stable	More stable
Clearance mechanisms	Clearance receptors and enzymatic degradation	Enzymatic degradation	Enzymatic degradation

[a]The molecular mass (MM) of NT-proBNP and proBNP depends to the degree of glycosylation of the peptide; in the Table are reported the MM of non-glycosylated peptides.

epitope sandwich assay'') is different from the conventional sandwich assay since it requires only one epitope for antigen immunodetection by two different monoclonal antibodies. In this novel immunoassay, the first antibody is used as a capture antibody and is specific for the region consisting of BNP amino acid residues 11–22, which is the most stable part of the peptide and includes the biologically active cysteine ring. The second antibody is used as a detection antibody and recognizes the immune complex, including the antigen (i.e., BNP_{11-22}) bound to the first antibody. In other words, the second antibody does not recognize the free antigen, but the primary immune complex, consisting of the first antibody and BNP, operates as antigen for the second antibody. It is also important to note that this immunoassay method is not theoretically specific for the intact $proBNP_{1-108}$ peptide. Indeed, the single-epitope sandwich assay should also recognize all the peptides (shorter than proBNP), which include the region consisting of amino acid residues 11–22 of BNP and are also able to form a complex with the first antibody.

Giuliani et al. (18) selected a specific monoclonal antibody (named ''mAb Hinge76'') that recognizes the cleavage site of $proBNP_{1-108}$, an epitope found only in the precursor form (see Figure 1). This monoclonal antibody recognizes the recombinant $proBNP_{1-108}$ in a dose-dependent manner, without any significant cross-reactivity with either recombinant NT-$proBNP_{1-76}$ or synthetic BNP_{1-32}. These authors set up a sandwich immunoassay for the measurement of proBNP, by combining the monoclonal antibody mAb Hinge76 with a polyclonal antibody directed against BNP_{1-32}. An automated version of this method on the BioPlex™ 2200 analyzer was then set up and their analytical characteristics were evaluated (38). More recently, some studies reported the clinical results obtained in general community (25) and in patients with heart failure (39) or chronic renal disease (40) by measuring proBNP concentrations with of this immunoassay method.

Should we need the proBNP assay?

From an analytical point of view, a fully automated immunoassay of proBNP has some theoretical advantages as biomarker (i.e., more stable molecule, higher molecular weight, lower biological variability) compared to the measurement of the active hormone BNP (Table 1). As a future perspective, the simultaneous measurement in the same plasma sample with two methods, one specific for the intact precursor $proBNP_{1-108,}$ and the other for active peptide BNP_{1-32}, could allow a more accurate estimation of both production/secretion of B-type related peptides from cardiomyocytes and overall activity of the cardiac endocrine function, compared to the single assay of either peptide (41). Information obtained by contemporaneous measurement of proBNP and BNP with specific assays should likely extend our present understanding of pathophysiological mechanisms linking together disease progression and cardiac endocrine dysfunction. Indeed, a recent study in ambulatory patients with chronic systolic HF showed that the combined assessment of conventional BNP and proBNP immunoassays provides additional information in determining the risk of adverse clinical outcomes, particularly in patients with low BNP values (22). However, designed studies will be necessary in order to estimate and compare the diagnostic and prognostic accuracy of specific assays for different B-type related peptides: BNP, NT-proBNP and intact proBNP, used either alone or in combination.

From a pathophysiological point of view, a blunted natriuretic response after pharmacological doses of ANP and BNP has been observed in experimental models and in patients with chronic heart failure, suggesting a resistance to the biological effects of CNH, principally to natriuresis (2, 42, 43). As discussed in detail previously (2), resistance to the biological action of CNH can be attributed at least to three kinds of different causes/mechanisms, acting at pre-receptor, receptor and post-receptor level, respectively.

Considering the possible causes of resistance at the pre-receptor level, recent findings (16–32, 34, 44, 45) suggest that in patients with heart failure there may be an insufficient post-translation maturation of the biosynthetic precursors of B-type natriuretic peptide system. According to these findings (16–32, 34, 44, 45), it is theoretically conceivable that the active hormone (i.e., BNP) may be produced even in vivo from the circulating precursor $proBNP_{1-108}$ by plasma enzyme degradation. Indeed, the soluble form of corin, a transmembrane serine protease able to cleave proBNP, is also capable of processing the circulating intact precursor of natriuretic hormones (44). Dong et al. (45) recently confirmed that soluble corin is measurable in human blood. Furthermore, these authors reported that plasma corin levels are significantly lower in heart failure patients than in healthy

controls and that the reduction in plasma enzyme is correlated to the severity of the disease (45). Finally, Semenov et al. demonstrate that synthetic or recombinant human proBNP can be processed to active BNP in the circulating blood, when injected in the femoral vein of rats (34).

The above-mentioned studies (16–32, 34, 44, 45) open a new and more complex scenario regarding the B-type natriuretic peptides, the precursor proBNP actually acting as a circulating pro-hormone. The peripheral processing of circulating proBNP could likely be submitted to regulatory rules, which might be impaired in patients with heart failure, opening new perspectives in the treatment of heart failure. A novel pharmacological target may be pharmacodynamic actions of drugs inducing and/or modulating the maturation of the prohormone into active hormone (i.e., BNP). However, further studies are necessary to demonstrate whether proBNP may be an equivalent alternative to BNP/NT-proBNP assay or may add incremental value to these existing biomarkers of cardiac dysfunction.

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