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Review

Title: Atrial and brain natriuretic peptides: hormones secreted from the heart

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Highlights

- ANP and BNP are secreted by the heart and act as cardiac hormones.
- Human ANP has three molecular forms: $\alpha$-ANP, $\beta$-ANP, and proANP (or $\gamma$-ANP).
- ProANP and $\beta$-ANP are minor forms but are increased in patients with heart failure.
- ProBNP is secreted by the heart and is increased in patients with heart failure.
- miR30-GALNTs-dependent $O$-glycosylation contributes to the secretion of proBNP.

Abstract

The natriuretic peptide family consists of three biologically active peptides: atrial natriuretic peptide (ANP), brain (B-type) natriuretic peptide (BNP), and C-type natriuretic peptide (CNP). Among these, ANP and BNP are secreted by the heart and act as cardiac hormones. Both ANP and BNP preferentially bind to natriuretic peptide receptor-A (NPR-A or guanylyl cyclase-A) and exert similar effects through increases in intracellular cyclic guanosine monophosphate (cGMP) within target tissues. Expression and secretion of ANP and BNP are stimulated by various factors and are regulated via multiple signaling pathways. Human ANP has three molecular forms, $\alpha$-ANP, $\beta$-ANP, and proANP (or $\gamma$-ANP), with proANP predominating in healthy atrial tissue. During secretion proANP is proteolytically processed by corin, resulting in secretion of bioactive $\alpha$-ANP into the peripheral circulation. ProANP and $\beta$-ANP are minor forms in the circulation but are increased in patients with heart failure. The human BNP precursor proBNP is proteolytically processed to BNP$_{1-32}$ and N-terminal proBNP (NT-proBNP) within ventricular myocytes. Uncleaved proBNP as well as mature BNP$_{1-32}$ and NT-
proBNP is secreted from the heart, and its secretion is increased in patients with heart failure. Mature BNP, its metabolites including BNP3-32, BNP4-32, and BNP5-32, and proBNP are all detected as immunoreactive-BNP by the current BNP assay system. We recently developed an assay system that specifically detects human proBNP. Using this assay system, we observed that miR30-GALNTs-dependent O-glycosylation in the N-terminal region of proBNP contributes to regulation of the processing and secretion of proBNP from the heart.

**Abbreviations:** AIV, anterior inter-ventricular vein; ANP, atrial natriuretic peptide; AP-1, activator protein-1; AT1A, angiotensine II receptor type 1A; BNP, brain (B-type) natriuretic peptide; CAMK II, calcium/calmodulin kinase II; cGMP, cyclic guanosine monophosphate; CNP, C-type natriuretic peptide; CRE, cAMP-responsive element; CT-1, cardiotrophin 1; DPP4, dipeptidyl peptidase IV; ERK, extracellular signal-regulated kinase; ER-stress, endoplasmic reticulum stress; ET-1, endothelin-1; FR, flanking region; GALNT, N-acetylgalactosaminyltransferase; GP130, glycoprotein-130; GRE, glucocorticoid responsive element; GSK3β, glycogen synthase kinase 3β; hBNPluc, human BNP gene fused to a luciferase reporter gene; HPLC, HRE, hypoxia-respose element; JAK-STAT, IL-1β, interleukin 1β; IL-18, interleukin 18; IR-BNP, immunoreactive BNP; IR-proBNP, immunoreactive proBNP; JNK, Jun-N-tremial kinase; LIF, leukemia inhibitory factor; MAPK, mitogen-activated protein kinase; M-CAT, muscle-CAT binding site; MEF2, myocyte enhancer factor; NFAT, nuclear factor of
activated T-cells; NPR-A, natriuretic peptide receptor-A; NPR-B, natriuretic peptide receptor-B; NPR-C, natriuretic peptide receptor-C; NT-proBNP, N-terminal proBNP; NRSE, neuron restrictive silencer element; NRVMs, neonatal rat ventricular myocytes; SSRE, shear stress-responsive element; TGFβ, transforming growth factor β; TNFa, tumor necrosis factor alpha; TRE, thyroid hormone responsive element; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; proBNP, prohormone of BNP; ROCK, Rho-associate protein kinase; SRF, serum response factor; Tbx5, T-box transcription factor 5; URP, ubiquitin-related protein; UTR, untranslated region; WT, wild type; XBP1, X-box binding protein 1; Zfp260, zinc finger protein 260

**Key words:** natriuretic peptides, ANP, BNP, O-glycosylation
1. Introduction

Atrial natriuretic peptide (ANP), the first identified member of the natriuretic peptide family, was discovered in 1983-1984\(^1,2\) as a peptide hormone responsible for the natriuretic and diuretic activity elicited by atrial tissue extract.\(^3\) Four years later, brain (or B-type) natriuretic peptide (BNP) was isolated from porcine brain tissues, and C-type natriuretic peptide (CNP) was subsequently also isolated from porcine brain.\(^4,5\) Later studies, however, showed that, both ANP and BNP are mainly produced in cardiac tissue,\(^6\) and CNP is expressed in variety of central nervous system and peripheral tissues.\(^7-10\) In addition, no appreciable amounts of BNP mRNA have been detected in the rat or human brain, suggesting BNP expression in the brain differs among species.

The natriuretic peptide system also includes three membrane-bound natriuretic peptide receptors: natriuretic peptide receptor-A (NPR-A or guanylyl cyclase-A), NPR-B (or guanylyl cyclase-B), and NPR-C (or clearance receptor). Evidence suggests this system plays important roles in the regulation of blood pressure and body fluid volume through its pleiotropic effects. For example, ANP and BNP are secreted from cardiomyocytes into the circulation in response to stretching of the myocardium. The circulating peptides then induce a decrease in vascular tone, an immediate increase in electrolyte and water excretion via the kidney, and antifibrotic and antihypertrophic effects in the heart, all of which functionally antagonize the renin-angiotensin-aldosterone system.\(^6\) Based on these effects, ANP and BNP are clinically administered as therapeutic agents to patients with acute heart failure, while an agent that inhibits neprilysin, a proteolytic enzyme that degrades and inactivates ANP and BNP, is used for patients with
chronic heart failure. In addition, BNP is widely used as an established biomarker for heart diseases. It was recently shown that human proBNP, a BNP precursor, circulates in peripheral blood and that O-glycosylation in its N-terminal region contributes to the increase in plasma proBNP levels seen in patients with heart failure.\textsuperscript{11} In this review, we describe what is currently known about the biochemistry and molecular biology of ANP and BNP, with a focus on proBNP.\textsuperscript{12}

2. Structure of ANP and BNP peptides and genes

ANP was isolated from human atrial tissue, and its amino acid sequence was completely determined in 1984.\textsuperscript{2} It was then revealed that ANP is synthesized and secreted from the heart as a cardiac hormone in response to atrial stretch. The major molecular form of circulating human ANP is a 28 amino-acid peptide (\(\alpha\)-ANP) that contains a ring structure with a disulfide linkage (Figure 1).\textsuperscript{13} The gene encoding ANP contains three exons, and its transcript is translated to a 151-amino acid precursor, preproANP. The 25-amino acid signal peptide is then removed, yielding 126-amino acid proANP (\(\gamma\)-ANP), which is the tissue form of the hormone. ProANP is thought to be proteolytically converted to ANP by the transmembrane enzyme corin during its secretion (Figure 1).\textsuperscript{14} \(\beta\)-ANP, which is an anti-parallel dimer of \(\alpha\)-ANP, is also isolated from the human failing heart, and plasma levels of both \(\beta\)-ANP and proANP are increased in cases of severe heart failure\textsuperscript{12, 15}, though the precise mechanism regulating the formation of \(\beta\)-ANP has not been clarified.

BNP was first identified in porcine brain tissue in 1988 by Sudoh et al. as a peptide with natriuretic-diuretic, hypotensive, and smooth muscle relaxant properties similar to
those of ANP. Soon thereafter, Saito et al. reported that human BNP was produced and secreted from the heart as a cardiac hormone. The predominant circulating form of BNP is a 26-, 45- or 32-amino acid peptide in pig, rat and human, respectively. Thus, whereas the amino acid sequence of ANP is well conserved among species, the amino acid sequences of BNP are diverse.

The human gene encoding BNP contains three exons (Figure 1), and its transcript is translated to a 134-amino acid peptide, preproBNP. The 26-amino acid signal peptide is then removed, yielding the 108-amino acid proBNP, which is in turn cleaved to the 32-amino acid, biologically active BNP and the 76-amino acid NT-proBNP, which is inactive. Both are secreted from the ventricles into the peripheral circulation via a constitutive pathway (Figure 1). One of the enzymes involved in the processing of proBNP to BNP is thought to be furin, which is a protein convertase localized mainly in the trans-Golgi network. Furin typically cleaves at sites marked by a consensus Arg-Xaa-(Lys/Arg)-Arg sequence, which is present within the amino acid sequence of proBNP. In addition to this canonical secretion of mature BNP, recent studies have shown that uncleaved proBNP is also secreted from the heart and is present in human plasma. O-glycosylation at a site near the cleavage site reportedly inhibits the processing of proBNP. As described later in detail, human proBNP has seven O-glycosylation sites, one of which is located near the cleavage site. By contrast, rat proBNP has two O-glycosylation sites, both of which are located relatively far from the cleavage site. This difference between humans and rats likely explains why BNP-45 is the major circulating form in rats, irrespective of the presence or absence of heart failure, while proBNP is the major circulating form in both
healthy subjects and heart failure patients. Moreover, the ratio of secreted proBNP to mature BNP appears to be increased in patients with severe heart failure. We recently reported a new regulatory mechanism underlying the processing and secretion of proBNP in the heart, which will be discussed in a later section.

3. Physiological actions of ANP and BNP

Both ANP and BNP preferentially bind to NPR-A, which is linked to guanylyl cyclase, leading to production of the intracellular messenger cyclic guanosine monophosphate (cGMP) in target cells. By contrast, CNP preferentially binds to NPR-B (guanylyl cyclase-B), and all three natriuretic peptides can bind to NPR-C, or clearance receptor, which is not linked to guanylyl cyclase and appears to act to clear the peptides from the circulation. Thus, the physiological actions of ANP and BNP at target cells and organs are similar and commonly mediated via NPR-A. The effects of ANP/BNP include diuresis, natriuresis, vasodilation, and inhibition of aldosterone synthesis and renin secretion, which enable these hormones to play key roles in regulating blood pressure and fluid volume (reviewed in ). After secretion from the heart, ANP/BNP immediately affect electrolyte and water excretion in the kidney and functionally antagonize the renal actions of the renin-angiotensin-aldosterone system. ANP/BNP affect blood pressure acutely by acting on vascular smooth muscle cells and chronically by affecting vascular endothelial permeability. They also reportedly prevent vascular smooth muscle cell growth and proliferation and vascular fibrosis. In addition, ANP/BNP act on the heart itself to suppress cardiac hypertrophy and fibrosis.
The analysis of mice genetically lacking or overexpressing NPR-A has enabled us to better understand the physiological actions of ANP and BNP within the cardiovascular system. In NPR-A straight knockout mice, blood pressures are significantly higher than in wild-type mice, as are heart-to-body weight ratios.\textsuperscript{23, 24} Echocardiographic and histological analyses revealed marked cardiac hypertrophy with interstitial myocardial fibrosis in NPR-A knockout mice.\textsuperscript{24} These effects were attenuated in NPR-A and angiotensin (Ang) II type 1A (AT\textsubscript{1A}) receptor double-knockout mice and in NPR-A knockout mice administered the selective AT\textsubscript{1A} receptor antagonist CS-866; that is, by deletion or pharmacological blockade of AT\textsubscript{1A} receptors.\textsuperscript{25} To clarify the impact of the blood pressure reduction, per se, on cardiac hypertrophy and fibrosis in NPR-A knockout mice, 6-hydroxydopamine (6-OHDA) was administered to lower blood pressure. Interestingly, although the decline of blood pressure elicited by 6-OHDA in NPR-A knockout mice was comparable to that induced by AT\textsubscript{1A} knockout or CS-866, it had no effect on cardiac hypertrophy or fibrosis.\textsuperscript{25} On the other hand, while infusion of a subpressor dose of Ang II had no effect on systolic blood pressure, cardiac hypertrophy, or fibrosis in wild-type mice, the same Ang II dose greatly increased cardiac hypertrophy and fibrosis in NPR-A knockout mice without affecting blood pressure.

The results summarized above suggest that cardiac responsiveness to Ang II is enhanced in NPR-A knockout mice and that NPR-A signaling suppresses cardiac hypertrophy and pathological cardiac remodeling in part by antagonizing AT\textsubscript{1A} signaling.\textsuperscript{25} Consistent with that idea, cardiac-specific overexpression of NPR-A using the myosin heavy chain promoter led to reductions in cardiomyocyte size in the transgenic
mice as compared to wild-type mice. Moreover, crossing transgenic mice cardiac-specifically overexpressing NPR-A with NPR-A knockout mice resulted in cardiac-specific NPR-A rescue. Although blood pressures and heart rates did not differ from those in NPR-A knockout mice, cardiomyocytes in the NPR-A rescued mice were smaller than in either NPR-A knockout or wild-type hearts. Consistent with the reduction in myocyte size, levels of both ANP mRNA and peptide were significantly reduced by cardiac overexpression of NPR-A, irrespective of background genotype. By contrast, mice with cardiomyocyte-restricted NPR-A deletion exhibited mild cardiac hypertrophy and markedly increased levels of ANP mRNA expression and systemic circulating ANP, despite blood pressures that were 7-10 mmHg lower than in wild type mice. This reduction in blood pressure likely reflects the elevated systemic ANP levels and its endocrine actions. Furthermore, in cardiac-specific NPR-A knockout mice, cardiac hypertrophic responses to pressure overload on the heart were enhanced and accompanied by marked deterioration of cardiac systolic function. All these observation demonstrate that ANP and ANP/BNP-NPR-A-cGMP signaling play an important role in suppressing cardiac hypertrophy and pathological cardiac remodeling at least in part by antagonizing signaling from the renin-angiotensin system.

Lin et al. demonstrated that, like cardiomyocytes, cardiac fibroblasts also express NPR-A, NPR-B and NPR-C, and that cardiac fibroblasts show larger increases in intracellular cGMP in response to NPR-A or NPR-B stimulation than do cardiomyocytes. Both ANP and BNP increase cGMP production in cardiac fibroblasts and inhibit Ang II-induced increases in preproendothelin-1 mRNA expression. ANP and BNP also inhibit
agonist- and stretch-stimulated cardiac fibroblast proliferation. ANP/BNP-NPR-A signaling is thus an important regulator of the growth and proliferation in cardiac fibroblasts.\textsuperscript{30}

As mentioned above, marked cardiac hypertrophy with interstitial fibrosis was observed in NPR-A knockout mice,\textsuperscript{24} and pressure overload in these mice leads to reduced left ventricular systolic function.\textsuperscript{31} On the other hand, although ANP knockout mice show salt-sensitive hypertension, there is no obvious cardiac hypertrophy under basal conditions.\textsuperscript{32} However, pressure overload leads to robust interstitial and perivascular fibrosis with cardiac hypertrophy and reduced fractioning shortening.\textsuperscript{33} BNP knockout mice exhibit multifocal fibrotic lesions within cardiac ventricles with no signs of systemic hypertension or ventricular hypertrophy.\textsuperscript{34} In response to ventricular pressure overload, the focal fibrotic lesions are worsened in BNP knockout mice but not in wild-type mice, despite similar increases in ventricular weights in the two groups.\textsuperscript{34} These results indicate ANP and BNP exert anti-fibrotic effects in vivo and serve as important local regulators of ventricular remodeling. The difference in phenotypes of ANP and BNP knockout mice may indicate the predominant paracrine effects of BNP in the ventricles.

In addition, using an in vitro model, it was observed that recombinant proBNP has significantly less biological activity than mature BNP in human endothelial and vascular smooth muscle cells.\textsuperscript{35} Comparison of the concentrations required to elicit a 50\% of maximum response (EC\textsubscript{50}) indicated proBNP has less than 12\%-17\% of the bioactivity of BNP in human cells. Consequently, the combined overall bioactivity of all endogenous BNP molecular forms would depend on the proportion of biologically less active proBNP
relative to the total BNP forms, and this proportion may affect pathophysiological conditions in heart diseases.

4. Distributions of ANP and BNP within the heart

Within the heart, ANP is expressed in both atrial and ventricular tissues, though levels in the atria are 250-1000 times higher than in the ventricles. Likewise, ANP mRNA levels are higher in the atria than ventricles, indicating the atria are the major source of cardiac ANP production. Interestingly, although high levels of both ANP and its mRNA are present in neonatal ventricular myocytes, they rapidly decline with changes in cardiac contractile protein isoforms after birth. Under pathological conditions in adult hearts, however, ventricular expression of ANP is re-induced.

In the normal human heart, the tissue concentrations of both BNP and its mRNA are higher in the atria than the ventricles, but as the ventricles are larger than the atria, total ventricular content of BNP and its mRNA respectively correspond to ~30% and ~70% of that in the whole heart. The difference between the BNP mRNA and protein contents may be attributable to the fact that, unlike ANP, BNP is secreted from the ventricles into the peripheral circulation via a constitutive pathway, without storage within ventricular tissue. In control subjects, human plasma BNP levels are much lower than those of ANP. In patients with congestive heart failure, however, myocardial levels of BNP mRNA and circulating levels of BNP and NT-proBNP are markedly increased as compared with those of ANP, which suggests BNP functions in the emergency defense against ventricular overload under disease conditions. In both healthy subjects and patients with dilated
cardiomyopathy, there is a significant step-up in plasma BNP levels between the aortic root and the anterior inter-ventricular vein (AIV), but there is no significant change from the AIV to the coronary sinus.\textsuperscript{38} This indicates BNP is secreted mainly from the left ventricle and not from the atria.

5. Transcriptional regulation of ANP and BNP gene expression

Expression and secretion of ANP and BNP are increased under pathological conditions in the heart. Mechanical stress stimulates the synthesis and secretion of ANP and BNP in both atrial and ventricular cells.\textsuperscript{39} Neurohumoral factors, such as endothelin-1,\textsuperscript{40, 41}\textalpha-textit{-}adrenergic agonists,\textsuperscript{42, 43} and Ang II,\textsuperscript{44} as well as various growth factors and cytokines, including interleukin (IL) -1\textbeta,\textsuperscript{45-47} IL-18,\textsuperscript{48} Leukemia inhibitory factor (LIF), Cardiotrophin (CT) -1,\textsuperscript{49, 50} tumor necrosis factor (TNF) \textalpha,\textsuperscript{46, 47, 51} and transforming growth factor (TGF) -\textbeta,\textsuperscript{52} all stimulate ANP and/or BNP synthesis in cultured cardiomyocytes. ER stress also induces BNP gene expression in cardiomyocytes.\textsuperscript{53} Multiple signaling pathways involving MAPKs including ERK1/2 (p44/p42 MAPK), p38MAPK, INK, and ERK5, GP130/JAK-STAT, CaMKII, PKCs, PI3K-Akt-GSK3\textbeta, Rho-ROCK, and calcineurin-NFATs reportedly contribute to the up-regulation of ANP and/or BNP gene expression induced by mechanical or neurohumoral stimulation.\textsuperscript{39, 54-59}

Several cis-acting regulatory elements have been shown to be involved in the basal and inducible expression of ANP and BNP genes. Luciferase reporter assays showed that the proximal (less than 3-kbp) 5’-flanking region (FR) of the ANP gene in human, rat and mouse is sufficient to confer cardiac-restricted gene expression and to reproduce the
spatial and temporal expression of the endogenous ANP gene.\textsuperscript{60-62} This suggests the proximal 5′-FR of the ANP gene contains sequences essential for regulation of the gene expression, and it is now known that this region contains binding elements for the SRF, NKX2.5, Tbx5, GATA4/6, MEF2C and Zfp260 transcriptional factors.\textsuperscript{55, 63} These elements participate individually or cooperatively to regulate basal and inducible activation of ANP promoter activity within cardiac cells.\textsuperscript{53-69} NRSE, hypoxia-response element (HRE), and glucocorticoid responsive element (GRE) are located outside the proximal promoter, but are also reportedly involved in inducing ANP gene transcription.\textsuperscript{70-73}

Several studies have investigated the significance of the 5′-FR in the regulation of cardiac-specific and inducible BNP gene expression. A study using transgenic mice harboring a 1.8-kbp or 400-bp segment of the human BNP 5′-FR fused to a luciferase reporter gene (-1818hBNPluc and -400hBNPluc, respectively) showed that the proximal region of the human BNP promoter is sufficient to confer ventricle-specific expression.\textsuperscript{74} In addition, BNP mRNA has an AT-rich region in its 3′-untranslated region, which is related to the instability of the transcript, implying post-transcriptional control of BNP expression.\textsuperscript{75, 76} This is consistent with the half-life of BNP mRNA being shorter than that of ANP mRNA. Moreover, deletion analysis showed that the region of the human BNP 5′-FR located between -127 to -40 confers cardiac-specific expression.\textsuperscript{77} This proximal region of the human BNP promoter contains potential GATA, M-CAT and AP-1/CRE-like binding elements, which are conserved among humans, rats and mice and are known to regulate cardiac-specific gene expression and to mediate both basal and inducible BNP
gene expression. In cardiomyocytes, for example, X-box binding protein 1 (XBP1), a major URP-linked transcriptional factor, regulates the ER stress-inducible BNP gene expression via the proximal AP1/CRE-like binding element. CArG-like sequences, which are conserved among various species and mediate hypertrophic stimulus-inducible BNP gene transcription in a SRF-dependent manner, are located a short distance upstream of the aforementioned elements (-193 to -184 in human BNP gene). Other elements located in more distal regions of the human BNP 5'-FR include NRSE (-552), shear stress responsive elements (SSREs) (-652, -641 and 161), thyroid hormone responsive element (TRE) (-1000) and a nuclear factor of activated T-cells (NF-AT) binding site (-927), which also mediate inducible activation of the human BNP promoter. It thus appears that a broad array of mediators and signaling pathways contribute to the increases in ANP and BNP synthesis and secretion observed under pathological conditions.

6. Diversity of molecular forms of BNP

There are three endogenous molecular forms of ANP in human: bioactive α-ANP, an antiparallel α-ANP homodimer designated β-ANP, and the α-ANP precursor proANP (γ-ANP). Healthy atrial tissues contain all three molecular forms of ANP, with proANP absolutely predominating. Because ProANP is proteolytically converted to α-ANP by corin during secretion (Figure 1), the major circulating form is α-ANP. Nonetheless, ProANP is detected in normal human plasma in some cases, though β-ANP was not. The relative levels of the different ANP molecular forms in tissue and plasma are often altered in patients with cardiac diseases. For example, all three ANP forms are detected
in the atrial extracts from hearts with congestive failure, but β-ANP levels are especially high\textsuperscript{12,15,82}. A similar molecular pattern is observed for circulating ANP in patients with heart failure, in whom increased levels of β-ANP is detected along with increased plasma proANP levels. This suggests the pattern of the molecular ratios among the three ANP forms varies and may reflect the presence and severity of cardiac disease.\textsuperscript{12,15} However the biosynthesis, localization and metabolism of β-ANP remain unclear.

The diversity of the molecular forms of circulating BNP has recently been revealed.\textsuperscript{85-87} During the process of BNP secretion in humans, BNP mRNA is initially translated into preproBNP and then converted to proBNP through removal of signal peptides within cardiomyocytes.\textsuperscript{6,88,89} ProBNP is then cleaved to BNP and N-terminal proBNP (NT-proBNP), presumably by the proteolytic enzyme furin, and the two are secreted from the heart in equimolar fashion.\textsuperscript{17-19} Using nano-liquid chromatography-electrospray ionization-FT-ICR-MS (Fourier transform ion cyclotron resonance mass spectrometry), which enables precise analysis of trace peptides, Hawkridge et al surprisingly observed that, despite exceedingly high levels of circulating BNP in New York Heart Association class IV patients, no endogenous BNP\textsubscript{1-32} was detected in patients with heart failure.\textsuperscript{85} Criticisms of this report suggested that BNP\textsubscript{1-32} may have decomposed after blood collection or that BNP\textsubscript{1-32} was lost due to the poor recovery rates during the various chromatography steps. However, Niederkofler et al. later reported that despite careful preservation of BNP\textsubscript{1-32}, mass spectrometry immunoassay technology revealed that levels of BNP\textsubscript{1-32} are low in heart failure patients and that various other forms of BNP, including BNP\textsubscript{3-32}, BNP\textsubscript{4-32}, and BNP\textsubscript{5-32}, are present.\textsuperscript{86} Similarly, analysis
using quantitative mass spectrometry immunoassays for intact BNP$_{1-32}$ and its fragments showed that actual BNP$_{1-32}$ levels are much lower than the serum BNP levels measured using the conventional BNP assay system, and that BNP$_{3-32}$, BNP$_{4-32}$, and BNP$_{5-32}$ are more abundant and associated with the observed plasma BNP levels. This is because the present BNP assay system detects BNP$_{1-32}$ and its metabolites, including BNP$_{3-32}$, BNP$_{4-32}$, and BNP$_{5-32}$. It appears that once BNP$_{1-32}$ is secreted into the circulation, dipeptidyl peptidase IV (DPP4) promptly removes the two N-terminal amino acids (Ser-Pro) to generate BNP$_{3-32}$, and that plasma BNP$_{3-32}$ levels are increased in patients with heart failure. Other aminopeptidases presumably further digest the N-terminal region of BNP$_{1-32}$ and/or BNP$_{3-32}$, yielding the BNP$_{4-32}$, and BNP$_{5-32}$ circulating in the peripheral blood. The inability of the conventional BNP assay system to discriminate BNP$_{1-32}$ and its metabolites from other BNP forms is thought to be the reason why their levels are low compared to overall BNP immunoreactivity.

The current BNP assay system also detects proBNP, which recent studies show is also secreted into the circulation with BNP$_{1-32}$ and NT-proBNP and is increased in patients with heart failure (Figure 2). BNP measurement are currently made using a sandwich immunoassay employing two monoclonal antibodies, each of which recognizes regions common to both BNP and proBNP. Through gel filtration HPLC analysis combined with an immunofluorescent BNP assay, high and low molecular weight forms of BNP (proBNP and mature BNP, respectively) were detected in plasma from both healthy subjects and patients with heart failure. The peak for immunoreactive-BNP (IR-BNP), which includes mature BNP and its metabolites plus proBNP, is predominant as
compared to the IR-proBNP peak in patients with heart failure due to atrial overload, including those with mitral stenosis, mitral regurgitation or an atrial septal defect. By contrast, the IR-proBNP and IR-BNP peaks are nearly equivalent in heart failure patients with ventricular overload, including those with aortic regurgitation and aortic stenosis. Two IR-BNP peaks are also observed with tissue extracts from cardiac atria and ventricles. Interestingly, in atrial tissue, the low molecular weight form corresponding to mature BNP is the dominant form of IR-BNP, whereas the high molecular weight IR-BNP peak corresponding to proBNP is the dominant form in ventricular tissue. Collectively then, both BNP and proBNP are present in plasma from healthy subjects and heart failure patients, and the proportion of proBNP to total IR-BNP, which is the sum of proBNP and BNP, including BNP$_{1-32}$, BNP$_{3-32}$, BNP$_{4-32}$, and BNP$_{5-32}$, appears to be altered depending on the mechanism and strength of the cardiac overload.

7. Mechanisms underlying proBNP secretion: the role of $O$-glycosylation

The precise molecular mechanisms by which the processing of proBNP to BNP and NT-proBNP is regulated in cardiomyocytes has not been fully elucidated. We recently reported on the significance to proBNP processing of $O$-glycosylation in the proBNP N-terminal region via the miR-30-GALNTs axis. Several studies have shown that the N-terminal region of circulating proBNP is heavily $O$-glycosylated. Within the Golgi apparatus of ventricular myocytes, human proBNP is post-translationally glycosylated to various degrees at Ser36, Thr37, Thr44, Thr48, Thr53, Ser58 and Thr71 (Figure 2). The $O$-glycosylated proBNP is then transported to the trans-Golgi network, where it is cleaved
to BNP and NT-proBNP by furin (Figure 2).\textsuperscript{17-19} Semenov et al. reported that \textit{O-}
glycosylation at Thr71 inhibits processing of recombinant human proBNP in HEK293
cells,\textsuperscript{98} which suggests \textit{O}-glycosylation of proBNP at Thr71 is involved in the regulation
of proBNP processing. On the other hand, Peng et al. reported that both wild-type and a
proBNP mutant lacking \textit{O}-glycosylation at Thr71 due to alanine substitution (T71A) were
processed similarly in HL-1 cells, an atrial cardiomyocyte cell line,\textsuperscript{99} which suggests \textit{O-}
glycosylation at Th71 does not affect proBNP processing in atrial cardiomyocytes. From
these studies, therefore, the significance of \textit{O}-glycosylation in the N-terminal region of
human proBNP to its processing and secretion remained unclear.

To clarify the regulatory function of \textit{O}-glycosylation with regard to human proBNP
secretion from ventricular myocytes, we introduced into lentivirus vectors cDNA
encoding human wild-type pre-proBNP or pre-proBNP mutant in which combinations of
glycosylation sites were replaced with alanine, thereby blocking glycosylation. After
infecting cultured primary neonatal rat ventricular myocytes (NRVMs) with the lentivirus
vectors, we measured the levels of proBNP and BNP in conditioned medium using both
our recently developed proBNP-specific assay system and the conventional BNP assay
system, which yields the sum of proBNP, mature BNP and its metabolites. In medium
conditioned by NRVMs expressing wild-type proBNP, the proBNP/BNP ratio, which
indicates the efficacy of BNP processing, was about 0.4. This value is similar to that in
plasma samples collected from the coronary sinus of heart failure patients and in medium
conditioned by human iPS-derived cardiomyocytes.\textsuperscript{11} In medium conditioned by NRVMs
expressing the proBNP T71A mutant, the proBNP/BNP ratio was significantly lower
(about 0.1) than in medium conditioned by NRVMs expressing wild-type proBNP. This confirms that O-glycosylation at Thr71 plays a key regulatory role in the processing and secretion of proBNP in ventricular myocytes. In addition, in a proBNP mutant in which all glycosylation sites in the N-terminal region were substituted with Ala (non-glyco mutant), the proBNP/BNP ratio was even lower (about 0.03) than the ratio with the T71A mutant, which suggests that glycosylation sites other than Thr71 also participate in the regulation of proBNP secretion. Indeed, O-glycosylation at Thr48 and Thr71 additively inhibit proBNP processing, and glycolsylation at the other five sites (Ser36, Thr37, Thr44, Thr53, and Ser58) appear to support the inhibitory effect of glycans attached at Thr48 and Thr71, thereby increasing proBNP secretion from the ventricular myocytes.11

Hypertrophic stimuli induce increases in the proBNP/BNP ratio through O-glycosylation-dependent mechanisms.11 The N-acetylgalactosaminyltransferases (GalNac-T; GALNT) are a family of enzymes that catalyze O-glycosylation of various molecules. Recent studies indicate that GALNT-mediated O-glycosylation significantly affects protein processing essential to pathophysiological processes.100 For example, secretion of fibroblast growth factor-23 requires GALNT3-mediated O-glycosylation, which inhibits the protein’s cleavage into inactivated fragments.21 We found that expression of GALNT1/2 is increased in NRVMs exposed to hypertrophic stimuli and in failing rat hearts, and that proBNP/BNP ratios were decreased in medium conditioned by NRVMs expressing wild-type proBNP along with siRNA targeting GALNT1/2. This suggests GALNT-dependent regulation of O-glycosylation of proBNP contributes to the increase in cardiac secretion of proBNP under pathological conditions. In addition, we
found that in rats expression of miR-30s, microRNAs abundantly expressed in cardiomyocytes, is decreased in hypertrophic cardiomyocytes and in failing hearts. This is consistent with earlier observations in pathologically remodeled hearts from both rodents and humans. We also showed that miR-30s negatively regulate expression of GALNT1/2 in NRVMs and in the mouse heart in vivo. Collectively, these observations indicate that under pathological conditions in the heart, stress-induced increases in GALNT1/2 expression resulting from decreases in miR-30 expression facilitate O-glycosylation-dependent processing failure and the resultant secretion of proBNP.

Consistent with this conclusion, Vodovar et al. reported an association between greater glycosylation of proBNP at Thr71 and diminished proBNP processing in patients with acute decompensated heart failure, though glycosylation at other sites, including Thr48, was not evaluated in that study (Figure 2). Further studies of the structures of the glycans attached to proBNP and the molecular mechanisms underlying proBNP glycosylation may shed light on the novel molecular pathways regulating intracardiac BNP metabolism and provide a better understanding of the pathological signaling contributing to the pathogenesis of heart failure. In addition, further evaluation of proBNP levels and proBNP/BNP ratios may give us more detailed information about pathological conditions of the heart.

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Disclosures

None.
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Figure legends

Figure 1

Structure of the gene and biosynthetic pathway of human ANP and BNP.

After transcription, proANP is proteolytically converted to ANP by corin during the hormone’s secretion. The precise mechanism regulating the formation of β-ANP remains unknown. On ProBNP is cleaved to BNP and NT-proBNP in the Golgi apparatus by furin. ProBNP is also secreted from cardiac myocytes.
Figure 1
Figure 2

Schematic presentation of glycosylation, processing and secretion of B-type natriuretic peptide (BNP) in cardiomyocytes and the plasma molecular forms of BNP.

After transcription, proBNP is post-translationally O-glycosylated within the Golgi apparatus and cleaved to BNP and NT-proBNP in equimolar fashion by furin within the trans-Golgi network. ProBNP is often heavily O-glycosylated in its N-terminal region. Furin cannot easily cleave O-glycosylated proBNP, especially when Thr48 and/or Thr71 are O-glycosylated. Because the precise structure of the O-glycan attached to proBNP and degree of O-glycosylation of proBNP have not been fully elucidated, the hypothetical structure of O-glycosylated proBNP and NT-proBNP are illustrated with a question mark. Unprocessed proBNP is also secreted into the peripheral circulation along with BNP and NT-proBNP. However, the current BNP assay system utilizes antibodies that recognize both proBNP and BNP and its metabolites. Thus, immunoreactive BNP (IR-BNP) comprises both BNP and proBNP.
