Natriuretic Peptide System in Human Heart Failure

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Background. Atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and C-type natriuretic peptide (CNP) are a family of structurally related peptides that participate in the integrated control of renal and cardiovascular function. Previous studies suggest a functional role for these hormonal peptides in cardio renal regulation in congestive heart failure (CHF).

Methods and Results. The present studies were performed in normal subjects (n=6) and in patients with mild (New York Heart Association [NYHA] class I to II, n=20) and severe (NYHA class III to IV, n=20) CHF by use of radioimmunoassay and immunohistochemical staining (IHCS). Plasma ANP was significantly increased in both mild and severe CHF compared with normal subjects. In contrast, plasma BNP was only moderately increased in the severe CHF group, and plasma CNP concentration was unchanged in CHF compared with normal subjects. Atrial tissue concentrations of the natriuretic peptides did not parallel circulating concentrations. ANP predominated in normal atrial tissue, but BNP predominated in CHF. In ventricular tissue, IHCS staining was present for all three peptides in normal ventricular myocardium and was markedly enhanced in CHF.

Conclusions. These studies support a differential regulation of ANP, BNP, and CNP circulating concentrations and tissue activity in human CHF. (Circulation. 1993;88:1004-1009.)

Key Words • natriuretic peptides • heart failure • left ventricle

Investigations have demonstrated the existence of a family of structurally related peptides, atrial (ANP), brain (BNP), and C-type (CNP) natriuretic peptides, that function in the integrated control of renal and cardiovascular function. ANP is a 28-amino acid peptide that is secreted from the atria and possesses natriuretic, vasoactive, and renin-inhibiting actions.1,2 BNP is a recently isolated 32-amino acid peptide, also found in the heart, that shares structural and biological similarity to ANP.3 CNP is a newly identified 22-amino acid peptide that also demonstrates structural similarity to the cardiac hormones ANP and BNP and, like ANP and BNP, has a 17-amino acid ring formed by a disulfide bond. Unlike these two previously identified cardiac peptides, CNP lacks the COOH-terminal amino acid extension from the ring structure. ANP, BNP, and CNP decrease cardiac preload, but unlike ANP and BNP, CNP is not natriuretic.5 Whereas ANP and BNP have been localized to the heart, recent investigations have failed to detect CNP messenger RNA (mRNA) in the human myocardium,6 although small concentrations of CNP are detectable in the porcine myocardium.7 CNP was originally localized to the brain, but recent investigations have localized it to endothelial cells, consistent with a paracrine role for CNP in the control of vascular tone.8-10 Both ANP and BNP function biologically via a guanylate cyclase receptor (ANPR-A) that is highly expressed in endothelial cells.11 CNP functions via a separate guanylate cyclase receptor (ANPR-B) that is highly expressed in vascular smooth muscle. All three peptides are cleared and degraded intracellularly by a common clearance receptor (ANPR-C).11 Most recently, Nunez and coworkers12 have reported that mRNA transcripts for these three receptors are all detectable in rat and human heart, underscoring a potential role for all three natriuretic peptides as possible paracrine factors in modulation of cardiac contractility or cardiac reflexes.

In chronic congestive heart failure (CHF), plasma concentrations of ANP are significantly increased in the circulation secondary to enhanced atrial and ventricular synthesis13-16 and decreased ANP clearance in liver and kidneys.17 Circulating BNP is also increased as a result of enhanced cardiac production.6,18 Recognizing the biological actions of these circulating peptides, numerous studies support a functional role for these hormones in cardiorenal regulation in CHF.19-21 Although studies have recently documented the presence of CNP in normal human and canine plasma, the circulating and cardiac tissue concentrations of this potent vasoactive peptide of endothelial cell origin in CHF remain undefined.5,22

The present study was designed with two objectives. The first was to determine concentrations of ANP, BNP,
and CNP in plasma and in atrial tissue during the evolution of CHF by investigating these cardiovascular peptides in patients with asymptomatic, mild, and severe CHF. The second was to determine the presence and extent of ventricular ANP, BNP, and CNP, recognizing the importance of the ventricular myocardium as a source and potential target of these cardiovascular peptides.

Methods

Normal and CHF Subjects for Circulating Peptide Characterization

Forty-six patients (34 men and 12 women, 62±2 [mean±SEM] years old) were studied. Patients were classified by New York Heart Association (NYHA) functional class according to their cardiac symptoms into four groups (NYHA class I, n=11; NYHA class II, n=9; NYHA class III, n=7; NYHA class IV, n=13). Six subjects without cardiac disease served as a normal group. The causes of the ventricular dysfunction included idiopathic dilated cardiomyopathy, ischemic cardiomyopathy, valvular heart disease, amyloid heart disease, and hemochromatosis. All patients had a complete physical and laboratory evaluation. Patients with renal failure (serum creatinine >176 μmol/L) were excluded from the study. All patients with symptomatic CHF were on treatment, which included digitalis, diuretics, and/or vasodilators. Venipuncture for measurement of ANP, BNP, and CNP was performed in the supine position before radionuclide angiography.

Normal and CHF Subjects for Cardiac Tissue Characterization

Six patients undergoing coronary artery bypass graft surgery (four men and two women, aged 55±5 [mean±SEM] years) with NYHA class I or II served as a source for atrial tissue for determination of peptide concentrations. Six patients (five men and one woman, aged 49±2 years) with end-stage heart failure undergoing cardiac transplantation served as sources of atrial tissue for peptide concentrations and ventricular myocardium for immunohistochemistry (NYHA class IV). Six normal donor hearts being used for cardiac transplantation at the Mayo Clinic also served as a source for normal atrial tissue peptide concentrations. Normal ventricular myocardium was also obtained by biopsy immediately before cardiac transplantation for immunohistochemistry. Venous plasma samples were obtained in the operating room immediately before surgery. After tissue was removed, it was then immediately placed in liquid nitrogen and stored at −70°C until further processing for tissue concentration studies.15,23

Quantification of Plasma and Tissue ANP

Arterial blood for hormone assay was collected in EDTA tubes and immediately placed on ice. After centrifugation at 2500 rpm at 4°C, the plasma was decanted and stored at −20°C until analysis. Plasma and right atrial tissue ANP were measured by a specific radioimmunoassay (RIA) as previously described.13 After the cardiac tissue was removed, the tissue was immediately frozen in liquid nitrogen and stored at −70°C until further processing for ANP tissue content. The tissue from each subject was pulverized, boiled for 5 minutes in 10 volumes of 1 mol/L acetic acid/20 mmol/L hydrochloric acid solution to abolish intrinsic proteolytic activity, and then homogenized.15,23 The homogenate was centrifuged for 30 minutes at 15 000 rpm and 4°C. The supernatant was then stored at −20°C and analyzed by a specific RIA for ANP.

Quantification of Plasma and Tissue BNP

Plasma or right atrial tissue BNP was determined by a specific RIA. One milliliter of plasma or tissue supernatant (as used for ANP) was preacidified and extracted on C-8 Bond Elut cartridges and eluted with 1% trifluoroacetic acid/95% methanol. Concentrated eluates were then assayed using a BNP RIA kit (Peninsula Laboratories, Inc, Belmont, Calif). Samples or standards were incubated for 24 hours at 4°C with 100 μL of antibody raised against human BNP. 125I-labeled BNP (100 μL) was added and incubated another 24 hours at 4°C. Free and bound fractions were then separated by addition of a second antibody and centrifuged, and the pellet was counted. Minimum detectable concentration for the assay is 0.5 pg per tube. Recovery was 75±11%, and interassay and intra-assay variabilities are 12% and 10%, respectively. The cross-reactivity of the BNP assay to other structurally related peptides was determined by the addition of synthetic ANP-28 (Peninsula Laboratories) in concentrations ranging from 0.5 to 500 pg per tube. There was no detectable immunoreactivity to ANP, demonstrating a cross-reactivity of <1%. In addition, synthetic BNP-32 (Peninsula Laboratories) was added to the ANP assay in concentrations ranging from 0.5 to 500 pg per tube with no detectable immunoreactivity, demonstrating a cross-reactivity of <1%.

Quantification of Plasma and Tissue CNP

Plasma or right atrial tissue CNP was determined by a specific RIA. For the RIA of plasma or tissue content, samples were extracted by the Vycor glass technique modified from the method of Gutkowska et al.24 Briefly, 1 mL plasma or tissue supernatant was gently mixed with 0.5 mL Vycor glass suspension for 1 hour at 4°C. The Vycor was washed with water, and the CNP was eluted from the Vycor with 60% acetone in 0.05 mol/L HCl. Eluates were concentrated on a Savant, and pellets were resuspended in assay buffer for RIA. CNP immunoreactivity was then determined by a double antibody RIA. A specific antibody to human CNP-22 was used in the assay (Peninsula Laboratories). Recovery of CNP was 72±6%, as determined by addition of synthetic CNP to plasma. The lower limit of detection was 2 pg per tube. Intra-assay variability was determined to be 5.2%. Cross-reactivity of the CNP-22 antibody with CNP-53 was established by addition of synthetic CNP-53 to the CNP-22 assay at concentrations ranging from 2 to 500 pg per tube. The cross-reactivity was determined to be 97±6%. There is no reported cross-reactivity between the specific CNP-22 antibody and endothelin, ANP, or BNP. This was verified by addition of synthetic endothelin, ANP, and BNP (Peninsula Laboratories) to the CNP at concentrations ranging from 0.5 to 500 pg per tube with no detectable immunoreactivity.

Immunohistochemistry

Full-thickness ventricular sections were obtained from the middle one third of the left ventricular lateral
free wall. Tissues were immediately fixed in 10% buffered formalin. After fixation, the tissue was dehydrated and embedded in paraffin. Serial sections were cut at a thickness of 5 μm. The presence of cardiac ventricular ANP, BNP, and CNP in normal and CHF cardiac tissues was documented by a specific immunohistochemical staining technique for each peptide. Briefly, this was a modification of the immunohistochemical technique of Chapeau and coworkers25 using a two-stage process, as we have previously described8,9,1523,26 using antibodies to ANP, BNP, and CNP. Lack of cross-reactivity to the specific ANP antibody was determined by addition of BNP and CNP (Peninsula Laboratories) to the ANP RIA with no detectable immunoreactivity. Lack of cross-reactivity to the specific BNP antibody was determined by addition of ANP and CNP to the BNP RIA with no detectable immunoreactivity. Lack of cross-reactivity to the specific CNP antibody was determined by addition of ANP and BNP to the CNP RIA with no detectable immunoreactivity. Two trained observers reviewed the sections without knowledge as to the respective groups from which the tissue was harvested. The presence of ANP, BNP, or CNP immunohistochemical staining was assessed by microscopic examination of the final slides and evaluated as to (1) quantification of the degree of staining as 0.5 (minimal intensity), 1.0 (mild intensity), 1.5 (moderate intensity), and 2.0 (maximal intensity) (a score of zero represented an absence of ANP, BNP, or CNP immunohistochemical stain), and (2) percentage of positive staining in the entire section examined.

Statistics

Results of the quantitative studies are expressed as mean±SEM. Statistical comparisons within each group were performed by ANOVA for repeated measures followed by Fisher’s least significant difference test of repeated measures when appropriate, whereas comparisons between groups were performed by factorial ANOVA followed by Fisher’s least significant difference test of repeated measures. Statistical significance was accepted for P<.05.

Results

Table 1 and Fig 1 report the clinical characteristics and plasma and cardiac tissue concentrations of ANP, BNP, and CNP. Cardiac index and left ventricular ejection fraction progressively decreased and left ventricular end-diastolic volume index increased with the severity of functional class. In patients in NYHA classes I and II, only plasma ANP was increased. Atrial ANP was decreased compared with normal subjects. In contrast, atrial BNP was greater than atrial ANP and increased compared with normal atrial tissue. Atrial CNP was greater than normal concentrations but remained less than ANP. Finally, in severe CHF (NYHA III and IV), plasma ANP increased further and plasma BNP increased from normal and from NYHA I and II but was less than ANP. Plasma CNP remained unchanged from normal concentrations. Atrial ANP increased to concentrations observed in normal subjects. Atrial BNP increased further and remained greater than ANP. Atrial CNP remained modestly but significantly increased compared with normal concentrations.

Fig 2 and Table 2 report immunohistochemical studies of ventricular myocardium for ANP, BNP, and CNP in normal hearts (donor hearts) and severe CHF hearts (end-stage CHF recipient hearts). Nonimmune staining demonstrated no staining for these peptides (not illustrated). In normal ventricular tissue, specific staining intensity for ANP was scored 1.0, with 10±2% of the section staining positive. Similarly, the BNP and CNP

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<th>ANP, using antibodies</th>
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**TABLE 1. Clinical Characteristics and Plasma and Cardiac Atrial Tissue Concentrations of ANP, BNP, and CNP of Normal Subjects and Congestive Heart Failure Patients**

- ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; CNP, C-type natriuretic peptide; CI, cardiac index; LVEF, left ventricular ejection fraction; LVEDVI, left ventricular end-diastolic volume index; NYHA, New York Heart Association. Values are mean±SEM.
- *P<.05 vs normal subjects group; †P<.05 vs mild congestive heart failure group; §P<.05 vs ANP.
scores for staining intensity were 0.5, with 8±3% and 6±2% of the section staining positive for both peptides. In contrast, the ventricular tissue from the NYHA class IV severe CHF patients stained strongly for all three peptides. For the severe CHF group, ANP, BNP, and CNP all scored 2.0 for intensity, and correspondingly, there was 70±5% positive stain area for ANP, 60±9% area for BNP, and 50±8% area for CNP.

Discussion

The present study confirms that circulating ANP progressively increases with the severity of symptomatic CHF and extends previous reports by demonstrating that plasma BNP increases moderately and only in severe CHF. In the present study, we report for the first time that plasma CNP in chronic CHF in humans is unchanged. Atrial tissue concentrations for these structurally related peptides did not parallel changes in circulating concentrations. In normal subjects, tissue ANP exceeds concentrations for BNP and CNP. With increasing severity of CHF, BNP is predominant in atrial myocardium. Despite the lack of increase in circulating CNP, a modest but significant increase in atrial CNP was noted. In ventricular myocardium, immunohistochemical staining was present for all three peptides in normal subjects and was markedly enhanced in CHF.

The present investigation confirms numerous previous studies in human CHF and demonstrates the early and progressive elevation of circulating ANP with worsening left ventricular dysfunction. Thus, ANP emerges as a marker not only for severe CHF but also for asymptomatic ventricular dysfunction, as recently suggested by Francis et al and by Lerman et al. In mild CHF, the atrial tissue concentration of ANP was decreased, which may be explained by ANP depletion in the early stage of CHF, consistent with studies in experimental CHF. However, in the severe CHF group, atrial tissue concentration of ANP was significantly increased. The mechanism of this elevation in atrial ANP in the severe CHF group probably represents enhanced production, as has been demonstrated in previous studies in the absence of decreases in metabolic clearance. Nonetheless, increases in circulating ANP may also involve reduced hepatic and renal clearance secondary to impaired hepatic and renal function in human heart failure. The increase in ventricular ANP presence as demonstrated with immunohistochemistry and the increase in atrial and plasma concentrations support such a conclusion.

**TABLE 2.** Percentage of Positive Immunohistochemical Staining for ANP, BNP, and CNP in Ventricular Tissues of Normal (Donor) and Failing (Recipient) Hearts

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<th>ANP (%)</th>
<th>BNP (%)</th>
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<td>Normal ventricles (n=6)</td>
<td>10±2</td>
<td>8±3</td>
<td>6±2</td>
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<tr>
<td>CHF ventricles (n=6)</td>
<td>70±5*</td>
<td>60±9*</td>
<td>50±8*</td>
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ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; CNP, C-type natriuretic peptide; CHF, congestive heart failure. Values are mean±SEM. *P<.05 vs normal ventricles.
Mukoyama and coworkers\(^\text{18}\) reported the elevation of circulating BNP in humans with CHF. Our findings confirm these observations. In contrast to Mukoyama et al, however, our studies reveal that circulating BNP is not elevated in the early stages of ventricular dysfunction but rather is increased only in subjects with moderate and severe CHF. The difference between these two studies may be related to a greater separation of patients in our study, which used both evaluation of symptoms and assessment of ventricular function and/or the lack of detection of large-molecular-weight BNP with our assay. In contrast to circulating concentrations of BNP, atrial BNP concentrations were markedly increased in CHF. The higher atrial BNP in mild and severe CHF (Table 1) compared with ANP tissue immunoreactivity together with much greater increases in circulating ANP than BNP may suggest that the processing and/or release of ANP and BNP are different. Indeed, recent studies in isolated cardiac atrial myocytes support this interpretation.\(^\text{30}\) Alternatively, synthesis and release of ANP may be regulated, whereas BNP is constitutive. Preliminary studies suggest that the pathophysiological concentrations of BNP observed in the present study have biological activity.\(^\text{3}\)

The present investigation documents for the first time the concentration of circulating and atrial BNP in humans with and without CHF. CNP was present in normal human plasma, as has recently been reported by Stingo and coworkers.\(^\text{9}\) Unlike ANP and BNP, plasma CNP is not elevated in mild or severe CHF. The lack of increase in circulating CNP may in part be explained by its localization to endothelial cells rather than to myocytes. Indeed, since the endothelium-derived vasodilator nitric oxide may be decreased in CHF, perhaps in response to a decrease in arterial pressure and endothelial cell shear stress,\(^\text{31}\) CNP may not be increased because of a decrease in the mechanical stimulus for its release.

Despite the lack of increase in circulating CNP in CHF, atrial concentrations by radioimmunoassay and ventricular immunoreactivity by immunohistochemistry were increased. The mechanism of this increase is unclear. Since previous reports by Takahashi et al\(^\text{2}\) have failed to show CNP mRNA in human myocardium but recent studies have demonstrated CNP presence and synthesis in cultured human and bovine endothelial cells, a possible explanation of the present findings could be binding of CNP to CNP receptors. This explanation is also supported by the report of CNP receptors in human and rodent myocardium.\(^\text{12}\) This explanation would be consistent with a paracrine role for CNP in myocardial regulation. Further studies investigating the local synthesis of CNP in harvested coronary endothelial cells will be required to resolve if increased coronary endothelial synthesis of CNP is the mechanism for the increase in cardiac tissue CNP, as observed in the present study.

Investigations during the past decade support the existence of a family of structurally related peptides ANP, BNP, and CNP. Evidence supports their function in the integrated control of renal and cardiovascular homeostasis. From the present investigation and knowledge of the reported biological actions of these peptides, a unifying concept emerges of their potential role in the diagnosis and pathophysiology of human CHF. The plasma elevation of ANP in the absence of elevation of BNP may serve as a noninvasive marker for early asymptomatic ventricular dysfunction. This isolated elevation of ANP may contribute to the pathophysiological compensation of early ventricular dysfunction, as supported by studies in experimental CHF in which activation of ANP in acute CHF served to inhibit activation of the renin-angiotensin-aldosterone system and maintain sodium balance despite the stimulus of arterial hypotension.\(^\text{21}\) With chronic severe CHF, the renin-angiotensin system may escape from the inhibitory action of ANP, and both ANP and renin are recruited together.\(^\text{32}\) The elevation of circulating BNP may be a marker for symptomatic CHF. The contribution of BNP to the regulation of cardio renal function remains unclear, with recent studies demonstrating either an enhanced natriuretic action in CHF in humans\(^\text{33}\) or a markedly blunted renal and cardiovascular action to pharmacological concentrations of BNP in experimental CHF.\(^\text{34,35}\) CNP elevation, unlike ANP and BNP, is not characteristic of human CHF, although a possible paracrine role in the heart in CHF will need to be explored. The functional significance of a lack of increase in circulating CNP, a potent vasoactive peptide,\(^\text{36}\) remains to be defined.

In summary, the present investigations report for the first time circulating concentrations and cardiac tissue activity of the three structurally related cardio renal-acting peptides ANP, BNP, and CNP in human CHF. These studies support a differential regulation of their circulating and cardiac concentrations.

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