Elevated Plasma B-Type Natriuretic Peptide Concentrations Directly Inhibit Circulating Neprilysin Activity in Heart Failure

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ABSTRACT

OBJECTIVES This study sought to hypothesize that elevated B-type natriuretic peptide (BNP) could act as an endogenous neprilysin inhibitor.

BACKGROUND A hallmark of acute decompensated heart failure (ADHF) is the overproduction of natriuretic peptides (NPs) by stretched cardiomyocytes. Various strategies have been developed to potentiate the beneficial effect of the NPs, including the recent use of neprilysin angiotensin receptor inhibitors. Contrary to rodents, human BNP is poorly sensitive to neprilysin degradation while retaining affinity to neprilysin.

METHODS We enrolled 638 patients presenting to the emergency department with acute dyspnea of which 468 had ADHF and 169 had dyspnea of noncardiac origin. We also included 46 patients with stable chronic heart failure (HF) and 10 age-matched healthy subjects. Plasma samples were collected within 4 hours after emergency department admission. BNP, neprilysin concentration and activity, and the neprilysin substrate substance P concentration were measured.

RESULTS We found that when plasma BNP rose above 916 pg/ml, neprilysin activity was markedly reduced \((p < 0.0001)\) and stratified 95% of the population into 2 groups: BNP < 916 pg/ml/neprilysin activity ≥ 0.21 nmol/ml/min and BNP ≥ 916 pg/ml/neprilysin activity < 0.21 nmol/ml/min with very different prognoses. In vitro, BNP was responsible for neprilysin inhibition. Neprilysin activity was inversely correlated with the concentration of substance P \((r = −0.80; p < 0.0001)\).

CONCLUSIONS Besides being an effector of the cardiac response to cardiomyocyte stretching in ADHF, elevated plasma BNP is also an endogenous neprilysin inhibitor. A biologically relevant BNP threshold discriminates 2 populations of HF patients with different vasoactive peptide profiles and outcome. If confirmed, this may identify an important threshold for managing HF patients. (J Am Coll Cardiol HF 2015;3:629–36) © 2015 by the American College of Cardiology Foundation.
One of the hallmarks of acute decompensated heart failure (ADHF) is the rapid large-scale production of natriuretic peptides (NPs), which occurs in response to mechanical and neurohormonal effects on cardiomyocytes. B-type NP (BNP) and its N-terminal pro-peptide equivalent (NT-proBNP) are established biomarkers for the diagnosis and prognosis of ADHF as articulated in international clinical practice guidelines (1,2). It is worth noting that immunoreactive BNP (irBNP), as quantified by conventional routine BNP assays, corresponds to both bioactive BNP and its precursor (proBNP) (3). Furthermore, it has been shown that proBNP was predominant in patients with severe heart failure (HF) and high irBNP values (4–6).

Production of BNP by stretched cardiomyocytes promotes natriuresis, diuresis, and vasorelaxation to mitigate cardiac overload (7). In addition, BNP also exhibits antifibrotic (8), antihypertrophic (9), anti-inflammatory (10), and angiogenic (11) actions, and acutely opposes the renin-angiotensin-aldosterone (12) and sympathetic nervous systems. Various strategies have been attempted to potentiate the beneficial effects of NPs in both ADHF and stable chronic HF (CHF) patients (13). These have included the infusion of either recombinant (e.g., nesiritide (14) or orlatriptide (15)) or synthetic NPs (e.g., vasonatrin (16) or CD-NP (17)) and inhibition of the NP-degrading neprilysin (also known as neutral endopeptidase) in combination either with inhibition of the angiotensin-converting enzyme (18) or angiotensin receptor blockade (ARNi, LCZ696 (19)). Importantly, although the former strategies aim to simply leverage the biological effect of the NPs, the latter approach (i.e., combined agents) may have broader effects because of the broader biological benefits of neprilysin inhibition plus the presence of vasodilator therapy.

Neprilysin is a membrane-bound and circulating protease that has been involved in the catabolism of numerous vasoactive peptides, including A-type NP (ANP), bradykinins, angiotensin I, substance P, and endothelin-1 (20,21). In many animals, neprilysin is also responsible for the degradation of the BNP (22). In particular, mouse BNP is successively cleaved by meprinA and neprilysin (22). MeprinA cleaves BNP₁₋₃₂ into still-bioactive BNP₇₋₃₂, which makes mouse BNP amenable to neprilysin cleavage within the ring structure. By contrast, human BNP is poorly sensitive to neprilysin degradation in vitro (23–25) possibly as it is not cleaved by meprinA (26). However, a shorter version of human BNP, that is, BNP₉₋₃₂, is also poorly sensitive to degradation by neprilysin in vitro (27), suggesting that human BNP is susceptible to neither meprinA nor neprilysin degradation. Instead, human BNP appears to be degraded by leupeptin-sensitive proteases (28,29), possibly from the kallikrein family (28). Nevertheless, human BNP retains affinity to bind neprilysin (23,27) and has been shown to interfere with ANP cleavage in vitro (27). Because proBNP is also detected by proBNP assays and differs from BNP by a longer N-terminal extremity, proBNP may also interact with neprilysin. We therefore hypothesized that human irBNP—that is, BNP and proBNP—besides being effectors of the cardiac response to cardiomyocytes stretching in ADHF, could also act as an endogenous neprilysin inhibitor in vivo.

**METHODS**

This study was approved by local ethic committees and written consent was obtained from patients or next of kin. This study using human samples was performed according to the current revision of the Helsinki Declaration and registered at ClinicalTrials.gov under the NCT01374880 identifier.

**STUDY POPULATION.** The studied population (n = 684) has been previously described (30). In brief, the cohort consisted of 638 patients presenting to the emergency department with shortness of breath as
their primary complaint: 469 were diagnosed with ADHF and 169 suffered from noncardiac dyspnea (non-ADHF). The diagnosis of cardiac or noncardiac origin of the dyspnea was performed by the emergency physicians according to clinical practice guidelines (1). All plasma samples were collected within 4 h after admission to the emergency department; 30-day and 1-year mortality was available for 507 of these patients. Plasma was also obtained from 46 patients with stable CHF in the cardiology outpatient center of Lariboisière Hospital (Paris). Lastly, plasma samples were obtained from 10 age-matched healthy controls with no recorded history of cardiac or neurological disease.

**BIOMARKER QUANTIFICATION.** Venous blood samples were collected in tubes containing EDTA (ethylenediaminetetraacetic acid). Blood samples were immediately centrifuged at 3,500 rpm for 15 min at 4°C. BNP plasma levels were immediately measured on an Abbott Architect system (Abbott Laboratories, Abbott Park, Illinois); the remaining of the plasma samples was immediately stored at −80°C until further use. Neprilysin (EC 3.4.24.11) concentration was measured using the SEB785Hu ELISA kit from Uscn Life Science (Wuhan, China). The analytical parameters for the assay were intra-assay coefficient of variation (CV): 4.6%, interassay CV: 7.3%, limit of detection: 5 pg/ml, and linearity: 12 to 350 pg/ml. Neprilysin activity was determined by fluorometry as previously described (31). The analytical parameters for the assay were intra-assay CV: 5.9%, interassay CV: 8.7%, limit of detection: 0.012 nmol/ml/min, and linearity: 0.025 to 0.700 nmol/ml/min. Substance P plasma concentrations were determined by a radioimmunoassay (Phoenix Pharmaceuticals, Belmont, California) according to the manufacturer’s instructions.

**IN VITRO ADDITION OF HUMAN RECOMBINANT BNP TO PLASMA SAMPLES.** Human recombinant BNP (MolBio, Lohmar, Germany) or human synthetic nonglycosylated proBNP (Roche, Basel, Switzerland) were reconstituted in 0.1% Bovine Serum Albumin (Pierce, Life Technologies, Carlsbad, California) in saline to a 100 μg/ml concentration. BNP and proBNP were serially diluted into saline before being added to the plasma of age-matched healthy controls at final concentrations of 300 and 1,600 pg/ml. The volume of recombinant BNP solution added to the samples was 10% of the final volume. Plasma samples were incubated for 2 h at 37°C before neprilysin concentration and activity measurements.

**STATISTICAL ANALYSES.** All statistical analyses were performed using the R-statistical software (R Foundation for Statistical Computing, Vienna, Austria). Data are expressed as median [interquartile range (IQR)]. Variables were tested with Wilcoxon rank sum test or Kruskal-Wallis test, as appropriate; p values for multiple comparisons were adjusted using the Holm’s method. Paired data were analyzed using paired Wilcoxon rank sum test. Relationships between variables were assessed using Spearman correlation coefficient. A 2-sided p value <0.05 was considered statistically significant.

Biomarkers cutoff values were determined as follows.

First, cutoff of neprilysin activity was determined using a multistep approach: 1) neprilysin activity distribution was centered and standardized using the R scale function; 2) because neprilysin activity distribution was found bimodal, 2 intersecting normal distributions were modeled using a 2-component mixture model (mixtools R package [32]); and 3) the cutoff was defined as the intersection of the 2 normal distributions. Thus, 2 groups were defined as either “high” or “low” neprilysin activity.
and stable CHF patients (210 pg/ml [IQR: 48 to 469 pg/ml]) (Figure 1A). Plasma neprilysin concentration was the lowest in non-ADHF and the highest in CHF patients: CHF (352 pg/ml [IQR: 325 to 380 pg/ml]) > ADHF (314 pg/ml [IQR: 257 to 377 pg/ml]) > non-ADHF (256 pg/ml [IQR: 58 to 339 pg/ml]) (Figure 1B).

Conversely, circulating neprilysin activity was the highest in non-ADHF and the lowest in ADHF patients: non-ADHF (0.37 nmol/ml/min [IQR: 0.3 to 0.5 nmol/ml/min]) > CHF (0.29 nmol/ml/min [IQR: 0.22 to 0.35]) > ADHF (0.22 nmol/ml/min [IQR: 0.13 to 0.34 nmol/ml/min]) (Figure 1C). There was no correlation between circulating neprilysin activity and concentration (p = 0.04; p = 0.27) (Figure 1D).

**RELATION BETWEEN PLASMA irBNP LEVELS AND CIRCULATING NEPRILYSIN ACTIVITY.** We next examined the distribution of circulating neprilysin activity with respect to the plasma concentrations of irBNP. Figure 2A shows an inverse relationship between plasma irBNP and circulating neprilysin activity. Notably, patients with irBNP ≥916 pg/ml exhibited an almost 3-fold reduction in circulating neprilysin activity compared with those with irBNP <916 pg/ml (0.13 nmol/ml/min [IQR: 0.11 to 0.14 nmol/ml/min] vs. 0.34 nmol/ml/min [IQR: 0.28 to 0.42 nmol/ml/min]; p < 0.0001) (Figure 2B), regardless of the origin of the acute dyspnea (Figure 2A). The circulating neprilysin activity cutoff of 0.21 nmol/ml/min was computed as the intersection of the 2 normal distributions modeled from the circulating neprilysin activity binomial distribution (Online Figure 1). The optimal irBNP threshold values that discriminated best patients with neprilysin activity < and ≥0.21 nmol/ml/min was calculated by ROC analysis. Furthermore, considering patients in 4 subgroups (irBNP < or ≥916 pg/ml and neprilysin activity < or ≥0.21 nmol/ml/min), remarkably, more than 95% of the studied population was distributed either in the top left (irBNP <916 pg/ml and neprilysin activity ≥0.21 nmol/ml/min) or in the bottom right (irBNP ≥916 pg/ml and neprilysin activity <0.21) quadrants of the irBNP versus circulating neprilysin activity distribution (Figure 2A). In addition, the hazard ratio between patients with irBNP < or ≥916 pg/ml was 1.58 (95% CI: 1.11 to 2.26) (p = 0.01) for 30-day and 1.98 (95% CI: 1.10 to 3.56) (p = 0.02) for 1-year all-cause mortality.

Neprilysin concentrations were moderately higher in patients with irBNP ≥916 pg/ml (317 pg/ml [IQR: 258 to 381 pg/ml]) than in patients with irBNP <916 pg/ml (305 pg/ml [IQR: 226 to 364 pg/ml]; p = 0.02), but no correlation was found between neprilysin and BNP plasma concentrations (p = 0.15).
NEPRILYSIN DIRECT INHIBITION BY BNP AND proBNP. To test a potential direct inhibitory effect of irBNP, that is, BNP and proBNP, on neprilysin activity, the plasma of 10 age-matched healthy controls (irBNP: 19 pg/ml [IQR: 14 to 28 pg/ml]) was spiked with human recombinant BNP or human synthetic proBNP. Both recombinant peptides were added independently to final concentrations of 300 pg/ml or 1,600 pg/ml to achieve levels < and ≥916 pg/ml, respectively.

Before addition of recombinant BNP, neprilysin activity was similar in both groups (300 pg/ml: 0.38 nmol/ml/min [IQR: 0.37 to 0.46 pg/ml], 1,600 pg/ml: 0.39 nmol/ml/min [IQR: 0.37 to 0.46 nmol/ml/min]; p = 0.68). After incubation for 2 h at 37°C, neprilysin activity was markedly reduced in the sample that contained 1,600 pg/ml BNP (0.06 nmol/ml/min [IQR: 0.05 to 0.08 nmol/ml/min]) compared with the samples containing 300 pg/ml BNP (0.32 nmol/ml/min [IQR: 0.23 to 0.39 nmol/ml/min]; p < 0.0001) (Figure 3A). Neprilysin concentration remained unchanged throughout the experiment (Figure 3B).

Before the addition of synthetic proBNP, neprilysin activity was similar in both groups (300 pg/ml: 0.39 nmol/ml/min [IQR: 0.35 to 0.45 nmol/ml/min], 1,600 pg/ml: 0.39 nmol/ml/min [IQR: 0.35 to 0.45 nmol/ml/min]; p = 1). After incubation for 2 h at 37°C, neprilysin activity was markedly reduced in the sample that contained 1,600 pg/ml proBNP (0.05 nmol/ml/min [IQR: 0.04 to 0.07 nmol/ml/min]) compared with the samples containing 300 pg/ml proBNP (0.40 nmol/ml/min [IQR: 0.37 to 0.41 nmol/ml/min]; p = 0.002) (Figure 3C). Again, neprilysin concentration remained unchanged throughout the experiment (Figure 3D).

CIRCULATING NEPRILYSIN ACTIVITY AND PLASMA SUBSTANCE P CONCENTRATION. We next investigated the relationship between circulating neprilysin activity and the plasma concentration of the vasoactive peptide substance P, a known substrate to neprilysin. Figure 4A shows a strong negative correlation between circulating neprilysin activity and plasma concentrations of substance P (ρ = −0.80; p < 0.0001): the lower the circulating neprilysin activity, the higher the plasma concentration of substance P. Figure 4B further shows that irBNP at 916 pg/ml could stratify more than 91% of the patients into 2 subpopulations across a threshold of substance P at 54 pg/ml: patients with irBNP ≥916 pg/ml had higher substance P concentrations (59 pg/ml [IQR: 57 to 64 pg/ml]) than those with irBNP <916 pg/ml (43 pg/ml [IQR: 37 to 49 pg/ml]; p < 0.0001) (Figure 4C), confirming irBNP-mediated inhibition of neprilysin also prevented substance P breakdown.

DISCUSSION

The present study indicates the marked elevation of BNP in response to clinical HF results in inhibition of endogenous circulating neprilysin activity. These novel results suggest a pivotal role for BNP and proBNP as “molecular switches” regulating the metabolism of major cardiovascular mediators and is of considerable importance, given the recent landmark results supporting neprilysin and angiotensin receptor inhibition to treat patients with HF (19,34); our data provide a possible mechanistic explanation for the benefits of this treatment.
When irBNP plasma levels—that is, BNP and proBNP—rose above 916 pg/ml (a value closely tied to prognosis in this population), we observed: 1) a marked reduction in circulating neprilysin activity in vivo; 2) a similar decrease in neprilysin activity in vitro after addition of recombinant BNP or synthetic proBNP to control plasma; and 3) an inverse correlation between circulating neprilysin activity and circulating substance P concentration in vivo. The similar inhibition of neprilysin by both BNP and proBNP is critical since irBNP consist of a mixture of both peptides (3), and in severe HF patients, proBNP is predominant (4–6). The biological significance of the 916 pg/ml irBNP threshold was further strengthened because the majority of patients could be segregated into 2 groups with respect to circulating neprilysin activity and substance P concentration: irBNP 400 to 916 pg/ml, neprilysin $\geq 0.21$ nmol/ml/min, substance P $< 54$ pg/ml; and irBNP $\geq 916$ pg/ml, neprilysin $< 0.21$ nmol/ml/min, substance P $\geq 54$ pg/ml. Interestingly, these data are reminiscent of those previously published on a limited number of patients with septic shock (35). This suggests that irBNP-mediated neprilysin inhibition occurs as soon as irBNP rises above a critical threshold, regardless of the etiology of cardiac stress.

To our knowledge, this is the first demonstration that BNP is an endogenous neprilysin inhibitor in humans. A mechanism for BNP resistance to neprilysin degradation, involving the sequence of the C-terminal tail of the human BNP, has been proposed (27). Further studies are required to determine the mechanism whereby irBNP inhibits neprilysin.

Neprilysin is involved in the catabolism of numerous vasoactive peptides (21), including bradykinins, ANP, endothelin-1, and substance P. Given the results we obtained with substance P, one may anticipate that the irBNP-mediated neprilysin inhibition also promotes accumulation of other neprilysin substrates (36). Altogether, the data we presented strongly argue for a model whereby BNP acts as a “molecular switch” that participates in the accumulation of bioactive vasoactive peptides (Figure 5). Further in vitro and in vivo studies are required to evaluate the impact of BNP-mediated neprilysin inhibition, and how this impacts the cardiovascular system.

Our study has 2 major clinical implications. First, it defined a biologically significant cutoff for BNP that discriminates 2 populations of HF patients with different vasoactive peptide profiles and outcome. If confirmed, this may identify an important threshold for managing HF patients using our novel “metabolic” irBNP cutoff; this concept is in line with natriuretic peptide-guided HF management (37). Second, our results complement the recent PARADIGM-HF trial (Prospective Comparison of ARNI With ACEI to Determine Impact on Global Mortality and Morbidity in Heart Failure) (19). In this trial, HF patients had a left ventricular ejection fraction $\leq 35\%$, were mostly New York Heart Association functional class II and had median plasma irBNP at 255 pg/ml [IQR: 155 to 474 pg/ml]. On the basis of our results, neprilysin activity was likely high ($> 0.21$ nmol/ml/min) in the large majority of these patients. Moreover, LCZ696 seemed less effective for New York Heart Association functional class III and IV patients (19). Taken together, these results strongly suggest that neprilysin inhibition may be the most effective when neprilysin activity is high. Further trials should assess effects of ARNi in patients with plasma irBNP $> 916$ pg/ml for whom neprilysin activity is already low.
STUDY LIMITATIONS. We did not assess the plasma concentrations of other vasoactive peptides that are neprilysin substrates other than substance P, and they should be considered in further studies. Furthermore, the number of patients tested is limited. However, the relationships between plasma irBNP and circulating neprilysin activity, and between neprilysin activity and plasma substance P concentrations were unambiguous. Finally, we did not assess the effect of the truncated forms of BNP on neprilysin activity. However, because neprilysin-mediated BNP cleavage only occurs when the C-terminus is truncated (27), it is anticipated that most of BNP truncated forms would act as neprilysin inhibitors.

CONCLUSIONS

We showed that elevated irBNP acts as an endogenous inhibitor to neprilysin, with clear ramifications with respect to the pathophysiology of HF as well as providing insights into recent important clinical trials in HF. It is widely anticipated that neprilysin inhibition will become a primary treatment option for patients with HF, representing the first potential change to the usual HF care algorithm in a decade (34). Our data provide useful insights into the mechanism of the benefit of neprilysin inhibition, and inform important data regarding this novel class of HF therapeutics.

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PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: Immunoreactive BNP is a gold-standard biomarker in HF; a cutoff at 916 pg/ml discriminates patients with high and low neprilysin activity, hence potentially predicts responders to neprilysin inhibition.

TRANSLATIONAL OUTLOOK: We identified 916 pg/ml as a biologically relevant BNP cutoff that discriminated most of the HF patients with different outcomes and vasoactive profiles. Further analysis is needed to confirm and precisely identify the impact of BNP-mediated neprilysin inhibition, and to determine whether patients across this cutoff need different management.
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APPENDIX For a supplemental figure, please see the online version of this article.