Deconvolution Analysis of Cardiac Natriuretic Peptides During Acute Volume Overload

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Abstract—Cardiac natriuretic peptides, especially amino terminal pro–Brain Natriuretic Peptide (NT-proBNP), are emerging as powerful circulating markers of cardiac function. However, the in vivo secretion and elimination (t1⁄2) of these peptides during acute volume overload have not been studied. We present the first report of the secretion and elimination of cardiac natriuretic peptides, based on deconvolution analysis of endogenous ovine plasma levels measured by specific radioimmunoassay. Four normal, conscious sheep underwent rapid right ventricular pacing (225 bpm) for 1 hour to stimulate acute cardiac natriuretic peptide release. Plasma samples and right atrial pressure measurements were taken at regular intervals 30 minutes before, during, and 4 hours after pacing. Baseline right atrial pressure significantly increased (P=0.02) during the 1 hour of pacing in association with a prompt increase in plasma BNP (t1⁄2=0.03), atrial natriuretic peptide (P=0.01), and NT-proBNP (P=0.02). Deconvolution analysis showed that the t1⁄2 of NT-proBNP (69.6±10.8 minutes) was 15-fold longer than BNP (4.8±1.0 minutes). Despite sustained increases in atrial pressure, cardiac secretion of natriuretic peptides (particularly atrial natriuretic peptide) fell during the pacing period, suggesting a finite source of peptide for secretion. Size-exclusion high-performance liquid chromatography revealed NT-proBNP to be a single immunoreactive peak, whereas BNP comprised at least 2 immunoreactive forms. These findings, especially the prompt secretion of BNP and the prolonged t1⁄2 of NT-proBNP, clarify the metabolism of BNP forms and help to explain the diagnostic value of NT-proBNP measurement as a sensitive marker of ventricular function. (Hypertension. 2000;36:355-359.)

Key Words: heart failure ■ natriuretic peptides ■ myocytes ■ metabolism

Increases in circulating volume and cardiac filling pressures promote increased secretion of atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) from mammalian cardiac myocytes. ANP is synthesized and stored in the atria, whereas BNP is released from the ventricle.1,2 ProANP is the dominant storage form in mammalian cardiac extracts3 and is cleaved during secretion into 2 fragments, ANP4–6 and NT-proANP.7–9 In contrast, myocytes contain either proBNP alone or a complex ratio of proBNP, BNP, and NT-proBNP,3 suggesting that the mechanism responsible for cardiac processing of proBNP differs from that of proANP. Circulating ANP is rapidly cleared by specific clearance receptor (NPR-C) and enzymatic (neutral endopeptidase, NEP) pathways10–12 in contrast to NT-proANP,8,9,13 which is slowly metabolized and accumulates in plasma at concentrations 10- to 50-fold those of ANP.13 Whereas BNP is cleared from the circulation by NPR-C and NEP at variable rates across species,1,14 the absolute and proportional increment of NT-proBNP in clinical and experimental heart failure15–18 exceeds that of BNP.

The growing recognition of the value of plasma levels of BNP and NT-proBNP as markers of left ventricular function17 and prognosis after myocardial infarction18 has increased the need for a more detailed understanding of their secretion and metabolism in vivo. Indeed, recent findings suggest that increases in plasma BNP are a late response to cardiac decompensation,19,20 thus raising questions about the role of BNP as an early marker of heart failure. Accordingly, we have used deconvolution analysis,21,22 a well-described technique allowing the separation of in vivo secretion and elimination characteristics underlying a temporal series of plasma hormone levels, to compare the dynamic response of cardiac hormones during acute cardiac overload. Reported here is the first application of these techniques to the study of secretion and elimination of ANP, BNP, and NT-proBNP forms in sheep with acute cardiac overload.

Methods

Stimulation of Natriuretic Peptide Secretion and Blood Collection

The study protocol was approved by the Animal Ethics Committee of the Christchurch School of Medicine and was performed in accordance with the Guide for the Care and Use of Laboratory Animals.

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Extraction and RIA of ANP, BNP, and NT-proBNP
Plasma (3 mL) was extracted on solid-phase C<sub>4</sub> cartridges (SepPak, Waters) as described previously. The recovery of synthetic ANP and BNP added to plasma with the use of this method is >85%. To estimate the recovery of ovine NT-proBNP, ~15 pmol of immunoactive NT-proBNP (purified from ovine plasma by high-performance liquid chromatography, HPLC) was added to charcoal-stripped plasma and extracted. The recovery of NT-proBNP (based on immunoreactive levels) was 47±5% (n=5). Immunoreactive levels of ovine ANP<sup>5</sup> and BNP<sub>26</sub> (identical to porcine BNP<sub>26</sub>) were measured as previously described. Immunoreactive NT-proBNP was measured as previously reported<sup>28</sup> but with a later antiserum bleed. The cross-reactivity was: human (h) BNP<sub>32</sub>, hNT-proBNP<sub>6(2-76)</sub>, hNT-proANP<sub>(1-30)</sub>, ovine NT-proANP<sub>(1-30)</sub>, porcine (p) BNP<sub>26</sub>, pBNP<sub>32</sub>, hANP<sub>6</sub>, hCNP<sub>22</sub>, and endothelin-1, all <0.1%. The assay had a mean zero binding of 40.5±0.6%, nonspecific binding (with the use of assay buffer) of 3.0±0.1%, a detection limit of 2.3±0.1 fmol/tube (7.4 pmol/L), and an IC<sub>50</sub> (concentration displacing 50% of tracer) of 82.1±1.4 fmol/tube (273 pmol/L) over 16 consecutive assays. Within-assay coefficients of variation over 15 consecutive assays were 5% at 1360 pmol/L, 10% at 448 pmol/L, and 9% at 173 pmol/L. The interassay coefficient of variation was 9% at 428 pmol/L.

Analysis of immunoreactive forms was undertaken by size exclusion HPLC (SEHPLC) with the use of a TSK G3000SW column (Toya Soda), as previously described.<sup>14</sup>

Deconvolution Analysis of Plasma Hormone Measurements
We used a well-described model of hormone secretion and elimination<sup>21,22</sup> defined by the following convolution integral: $C(t) = \int S(z) \cdot E(t-z) \, dz$, where $C(t)$ is the concentration of hormone at any instant in time $t$, $S(z)$ is the amount of hormone secreted per unit distribution volume per unit time at time $z$, and $E(t-z)$ is the amount of hormone elimination ($t/2$) that occurs in the time interval ($t-z$). Natriuretic peptide concentrations were subjected to a least-squares fit to the convolution integral described above and the half-life calculated from this. In the present study, secretion was approximated as a gaussian distribution with a continuous “baseline” component; elimination proceeded through a 1-compartment model. Secretion of BNP and NT-proBNP were simultaneously computed because previous research from our laboratory<sup>14,16</sup> indicates that ovine cardiac tissue contains proBNP only, that is, there should be a 1:1 formation of BNP and NT-proBNP at any secretion time $t$, but each fragment will have a different circulating $t/2$. ANP was analyzed alone because it derives from a separate gene product<sup>2</sup> and does not display the same plasma concentration profile as BNP in experimental cardiac overload.<sup>23</sup>

Statistical Analysis
All data derived from deconvolution analysis are presented as mean±SEM (n=4). Increases in plasma hormone levels and RAP from baseline (mean of 30-minute and 0-minute values) achieved during pacing (mean of 15-, 30-, 45-, and 60-minute values) were tested for significance by means of a paired $t$ test.

Results
Stimulation of Cardiac Natriuretic Peptide Release
Basal plasma levels of immunoreactive NT-proBNP were 20-fold those of mature BNP (Figure 1A) and 3-fold those of mature ANP (Figure 1B). During the pacing period, RAP increased significantly ($P<0.02$) (Figure 1C). In accordance with the increase in RAP, there were prompt and significant increases in immunoreactive BNP ($P=0.03$), ANP ($P=0.01$), and NT-proBNP ($P=0.02$) compared with basal levels before pacing. During pacing, the maximal concentrations of BNP

Figure 1. NT-proBNP and BNP (A), ANP (B), and hemodynamic (C) measurements observed before, during, and after pacing. Data in all panels are shown as mean±SEM (n=4). Symbols in A and B indicate actual measured values; curve indicates line of fit described by convolution analysis.

General anesthesia (20 mg/kg thiopentone sodium maintained by halothane, nitrous oxide, and oxygen) was induced in 4 Coopworth ewes (Lincoln University Farm, Christchurch, New Zealand), and 2 polyethylene catheters were inserted into the left jugular vein for blood collection and monitoring of right atrial pressure (RAP). A 7F His-bundle pacing wire for rapid pacing was inserted through the jugular vein and advanced to the right ventricle. The wire position and effective pacing were confirmed under x-ray. Animals were given 50 mg IM pethidine, transferred to metabolic crates, and allowed to recover for 48 hours before initiation of the study.

Cardiac secretion of natriuretic peptides was stimulated by rapid ventricular pacing for 1 hour at 225 bpm, after which pacing was discontinued. Jugular blood (5 mL) for specific radioimmunoassay (RIA) was collected at the intervals shown in Figure 1 into ice-chilled tubes containing 100 µL of 15 mg/mL Na<sub>2</sub>-EDTA, inverted for 30 seconds, and immediately centrifuged at 2500 rpm at 4°C for 15 minutes. Plasma was stored at −70°C until extraction and assay. The total volume of blood drawn from each sheep was ~200 mL. RAP measurements were made at intervals shown in Figure 1, with the use of Statham pressure transducers (Spectramed Medical Products) connected to online computer data-integration (Dataflow, Crystal Biotech).
Elimination curves (t½) for NT-proBNP, BNP, and ANP were calculated to be secreted in plasma ANP, NT-proBNP, and BNP in the present study. Calculated secretion and elimination profiles are given in Figure 2. During pacing in all 4 sheep, the time point of maximum NT-proBNP and BNP secretion (average 38.2±3.6 minutes; ~5.8 pmol · min⁻¹) was later but not significantly different from the time of maximal ANP secretion (average 30.5±4.5 minutes; ~45.0 pmol · min⁻¹, Figure 2A). Further, BNP and NT-proBNP were calculated to be secreted in equimolar amounts. By the end of the pacing period, BNP and NT-proBNP secretion had fallen to ~45% of the peak value, whereas ANP secretion had returned to baseline levels (Figure 2A). Elimination curves (t½) for NT-proBNP, BNP, and ANP are shown in Figure 2B. The calculated half-lives (mean±SEM) for each of the peptides were NT-proBNP, 69.6±10.8 minutes; BNP, 4.8±1.0 minutes; and ANP, 11.9±2.7 minutes. On the basis of these calculations, the t½ for NT-proBNP is ~15-fold longer than that of mature BNP and 6-fold longer than mature ANP.

**SEHPLC Analysis of Immunoreactive NT-proBNP, BNP, and ANP Forms**

Extracts of plasma drawn at peak secretion were pooled from all 4 sheep and subjected to size-exclusion HPLC. Immunoreactive NT-proBNP eluted as a single sharp peak consistent with the monomer (Mr ~8000), with no evidence of smaller forms (data not shown). In contrast, BNP was shown to comprise 2 distinct peaks consistent with BNP-26–like and BNP-29–like materials. A peak corresponding to authentic ANP was also seen with evidence of additional smaller molecular forms, possibly ANP₁₀₁–₁₂₆ or ANP₁₀₃–₁₂₆ (data not shown).

**Discussion**

Previous reports addressing the secretion and elimination mechanisms of the cardiac natriuretic peptides have used isolated perfused rat heart and/or single peptide infusion methodologies. However, no reports have focused on the relative in vivo contributions of secretion and elimination to plasma natriuretic peptide levels in the setting of acute cardiac overload. Thus, the current study is the first to use deconvolution analysis—separating hormone secretion and elimination—to analyze the heart’s endocrine response to overload in vivo and provide measurements of the circulating half-life of NT-proBNP.

The concentration curves (Figure 1) suggest that the time course of increases in plasma BNP and ANP in response to acute cardiac pacing is similar. However, deconvolution analysis (Figure 2) revealed that a prompt increase in ANP secretion had returned to baseline levels before the cessation of pacing despite sustained increases in RAP. This observation confirms previous reports on the time course of ANP secretion in vitro and is consistent with rapid depletion of a “stretch-sensitive pool” of ANP. ANP, BNP, and NT-proBNP have been colocalized in human, rat, and porcine cardiac myocytes, and it may be that some of the observed increases in plasma ANP, NT-proBNP, and BNP in the present study are derived from corelease from a single class of cardiac myocyte granule. Thus, it is possible that the concept of a readily releasable pool of atrial ANP for stretch-induced release may also apply to colocalized BNP within secretory granules.

In contrast with ANP, BNP secretion continued for longer during pacing and was ~45% peak maximum at the completion of pacing. The molar ratio of BNP to ANP at peak secretion (1:8) is higher than that found with respect to stored hormone content extracted from the normal ovine atrium (BNP/ANP ratio 1:30), raising the possibility that the BNP secretion observed here is augmented by enhanced BNP gene expression. It is of interest to note that atrial BNP mRNA transcripts increase within 1 hour of stimulus, whereas increases in ANP mRNA are not observed until 3 to 4 hours. The signaling mechanisms controlling this early response profile of cardiac BNP gene expression are unclear but may be related to the hypertrophic stabilization of BNP mRNA and its putative functional linkage to the protein kinase C.
signaling pathway.\textsuperscript{27,28} Such a differential response in gene expression could account for the longer duration of BNP secretion as well as the enhanced BNP-to-ANP ratio of peak secretion compared with that found in hormone stores. Similar analysis of secretion over a longer time period (eg, 3 to 4 hours of sustained pacing) coupled with sequential cardiac tissue sampling for natriuretic peptide transcript analysis is needed to clarify the underlying mechanism of these changes.

The response of plasma natriuretic peptide levels to cardiac pacing, as previously reported,\textsuperscript{23,35,36} varies according to pacing rate, duration, and experimental design, but a proportionately greater increase of ANP than BNP is a consistent feature of short-term studies. Previous studies\textsuperscript{3} of the acute response of plasma BNP to acute pacing in anesthetized dogs is markedly delayed\textsuperscript{19,20} and apparently not increased compared with control animals despite the development of acute heart failure. The present study, in normal conscious sheep, with the use of a species-specific BNP RIA shows that peripheral plasma concentrations of both BNP and ANP increase (7- and 8-fold, respectively) during acute cardiac challenge. Associated with a 3-fold increase in RAP, deconvolution analysis indicates that secretion rates of ANP, and BNP or NT-proBNP, increase 15- and 6-fold, respectively, similar in proportion to previous measurements of secretion in the isolated rat heart.\textsuperscript{24} The failure to detect evidence of increased BNP secretion during cardiac pacing in dogs\textsuperscript{19} and humans\textsuperscript{1,2} may reflect variations in time of sampling, anesthetic use—and in the case of humans—the longer half-life of BNP, which will affect the kinetics of plasma hormone peak response to stimulation.

The higher concentration of ovine NT-proBNP (compared with BNP) seen in the basal and stimulated (pacing) states is likely to be the result of delayed clearance and the much longer half-life (15-fold that of BNP). Previous experiments from our laboratory\textsuperscript{16} have shown that NT-proBNP levels are significantly elevated within 3 hours of coronary artery ligation. Taken together with the present results, it appears that significant elevations in NP-proBNP levels can occur within 3 to 4 hours of sustained (\geq 1 hour) cardiac production. As reported previously,\textsuperscript{16} the much-prolonged half-life of NT-proBNP compared with ANP and BNP in sheep is likely to be related to low (if any) affinity for the 2 major natriuretic peptide degradation pathways—NPR-C and NEP. These findings, together with our current observations that NT-proBNP secretion in sheep closely parallels that of BNP in response to acute cardiac overload, underline the physiological basis for NT-proBNP assays as markers of left ventricular dysfunction\textsuperscript{17} and prognosis after acute myocardial infarction\textsuperscript{18} in humans.

The current study has confirmed that ovine plasma NT-proBNP is of a high molecular weight, possibly NT-proBNP 1-74,\textsuperscript{16} with no evidence of smaller forms. However, in the case of both BNP and ANP, smaller forms were observed when extracts were analyzed by HPLC. The precise identity of these forms is not yet clear but most likely represent BNP\textsubscript{(78-103)} (BNP 26) and BNP\textsubscript{(75-103)} (BNP 29) and ANP\textsubscript{101-126} or ANP\textsubscript{103-126}.\textsuperscript{6} Although it cannot be conclusively stated that these latter forms are products of secretion rather than metabolites, they may have different affinities for NEP and NPR-C. Infusion studies in sheep\textsuperscript{57} report half-lives of 2.5 and 4 minutes for BNP and ANP, respectively, yet half-lives calculated by deconvolution analysis in this report were \(\approx\) 2-fold (BNP, 4.8 minutes) and 3-fold (ANP, 11.9 minutes) higher. The discrepancy may arise from the fact that infusion studies use a single species of BNP or ANP, for example, BNP26 or \(\alpha\)ANP, whereas in the present study, multiple immunoreactive forms may contribute to the t\(\frac{1}{2}\) calculations for BNP and ANP. Unfortunately, the unavailability of purified or synthetic ovine NT-proBNP, sufficient for infusion studies, precludes direct measurement of its disappearance rate or metabolism in vivo.

In summary, we have provided the first report of the in vivo secretion and elimination characteristics of cardiac natriuretic peptides by using the powerful technique of deconvolution analysis. The analysis shows that both BNP and ANP secretion increase rapidly in response to acute cardiac overload but, in contrast to the progressive increase in RAP, this secretion—especially that of ANP—is not sustained. Whereas both BNP and NT-proBNP are secreted simultaneously and in equimolar amounts, the half-life of NT-proBNP is 15-fold greater than that of BNP. Taken together, the findings suggest a role for BNP early in the development of acute heart failure and support the use of plasma NT-proBNP measurements as a stable and sensitive marker of cardiac function, including early cardiac decompensation.

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