Oral Human Brain Natriuretic Peptide Activates Cyclic Guanosine 3′,5′-Monophosphate and Decreases Mean Arterial Pressure

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Background—The objective of this study was to address the feasibility and the biological activity of orally administered human brain natriuretic peptide (hBNP). Proprietary technology has been developed in which short, amphiphilic oligomers are covalently attached to peptides. The conjugated peptides are intended to have an improved pharmacokinetic profile and to enable oral administration. We hypothesized that novel oral conjugated hBNP (CONJ-hBNP) increases plasma hBNP, activates cGMP, and reduces mean arterial pressure (MAP).

Methods and Results—This randomized crossover-designed study tested the biological activity of oral CONJ-hBNP compared with oral native hBNP in normal conscious dogs. Measurements of MAP, plasma hBNP, and cGMP were made at baseline (BL) and repeated at 10, 30, 60, 120, 180, and 240 minutes after oral administration. Plasma hBNP was not detectable in dogs at BL. Plasma hBNP was detected after native hBNP and CONJ-hBNP administration. However, plasma hBNP concentration was significantly higher after CONJ-hBNP than after native hBNP administration ($P = 0.0374$ between groups). Plasma cGMP increased after CONJ-hBNP for 60 minutes (from $10.8 \pm 3$ to $36.8 \pm 26$ pmol/mL; $P < 0.05$), whereas it did not change after native hBNP ($P = 0.001$ between groups). MAP decreased at 10 minutes and remained decreased for 60 minutes after CONJ-hBNP (from $113 \pm 8$ to $101 \pm 12$ mm Hg after 10 minutes to $97.5 \pm 10$ mm Hg after 30 minutes to $99 \pm 13$ mm Hg after 60 minutes) while remaining unchanged after native hBNP ($P = 0.0387$ between groups).

Conclusions—This study reports for the first time that novel conjugated oral BNP activates cGMP and significantly reduces MAP, thus implying an efficacious coupling of CONJ-hBNP to the natriuretic receptor-A. These data advance a new concept of orally administered chronic BNP therapy for cardiovascular diseases. (Circulation. 2005;112:836-840.)

Key Words: blood pressure ■ cyclic GMP ■ natriuretic peptides

Oral administration of intact and biologically active peptides has long been an unsolved therapeutic challenge. Today, new technologies have been developed that may be instrumental in achieving this goal. In the present study, we applied these new technologies to human brain natriuretic peptide (hBNP).

BNP is an endogenous peptide produced by the heart as a nonactive 108–amino acid hormone.1–3 It is cleaved and activated into its 32–amino acid mature form by the transmembrane enzyme corin.4–6 BNP has natriuretic, diuretic, vasorelaxant, lusitropic, and antialdosterone properties, as well as direct and indirect antifibrotic actions.7 BNP binds to the natriuretic peptide receptor-A (NPR-A), which is a membrane-bound receptor located on cardiomyocytes, vascular endothelium, smooth muscle, kidneys, and lungs, resulting in activation of its second messenger, cGMP.

We recently reported that exogenous administration of BNP has favorable effects in experimental congestive heart failure (CHF).8 Furthermore, recent studies have demonstrated the efficacy of intravenous administration of recombinant hBNP in decreasing cardiac filling pressures and improving symptoms in the setting of acute decompensated CHF.9–12 In experimental hypertension, administration of long-acting BNP synthesized as a fusion peptide with albumin sustained blood pressure–lowering actions, supporting a strategy for longer-term BNP therapy in cardiovascular dis-
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cGMP generation after subcutaneous dosing in dogs. Its favorable profiles in reducing blood pressure and enhancing performance in oral absorption studies in rats (data not shown) and hBNP-021 for this oral in vivo study in dogs because of its powder after rotary evaporation and lyophilization.

The conjugate was obtained as a white elsewhere.14 The resultant conjugate was purified by reverse-phase and the conditions for conjugation have been reported in detail to the Lys3 of hBNP. The synthesis of the monodispersed oligomer regioselective attachment of a branched, amphiphilic PEG oligomer to peptides. In contrast to standard PEGylation technology, this technique uses comparatively small, amphiphilic oligomers that are monodisperse and comprise both a hydrophobic (alkyl) moiety and a hydrophilic polyethylene glycol (PEG) moiety. The oligomers are intended to improve the pharmacokinetic and pharmacodynamic profiles of the peptide and potentially to enable oral administration. This technology, however, by modifying the native structure of the hormone, could potentially result in a loss of biological action.

The aim of the present study was to address the feasibility and the biological activity of acute orally administered conjugated hBNP (CONJ-hBNP). First, we evaluated different forms of CONJ-hBNP to determine whether these modified peptides would maintain biological activity when administered subcutaneously. Second, from the subcutaneous studies, we evaluated for the first time a novel form of CONJ-hBNP (hBNP-021) through oral administration (Figure 1). Specifically, our goal was to demonstrate that acute subcutaneous and oral administration of CONJ-hBNP would result in an increase in plasma levels of hBNP and cGMP, together with a reduction in mean arterial pressure (MAP) in normal conscious dogs.

Methods
The present study was performed in accordance with the Animal Welfare Act and was approved by the Mayo Institutional Animal Care and Use Committee.

Synthesis of CONJ-hBNP
The hBNP monoclonal hBNP-021 (BN-021) was prepared by regioselective attachment of a branched, amphiphilic PEG oligomer to the Lys3 of hBNP. The synthesis of the monodisperse oligomer and the conditions for conjugation have been reported in detail elsewhere.14 The resultant conjugate was purified by reverse-phase high-performance liquid chromatography (Phenomenex; C18; 1.0-cm internal diameter, 25-cm length) using a gradient system (A=H2O with 0.1% TFA; B=acetonitrile with 0.1% TFA; %B=25 to 75 over 120 minutes). The conjugate was obtained as a white powder after rotary evaporation and lyophilization.

More than 50 different conjugates have been prepared using similar technology. Roughly one fourth of these conjugates had sufficient in vitro activity to be evaluated in vivo. We selected hBNP-021 for this oral in vivo study in dogs because of its performance in oral absorption studies in rats (data not shown) and its favorable profiles in reducing blood pressure and enhancing cGMP generation after subcutaneous dosing in dogs.

Study Protocol
Normal adult male mongrel dogs (weight, 15 to 20 kg) were used in these studies. Before experiments, an arterial port was placed in the femoral artery as previously reported.15 After recovery from port placement, the dogs were fed a fixed sodium diet of 100 mEq/d for 5 days before initiation of studies with ad libitum access to water and with acclimation to standing calm in a sling. On the day of the experiment, the dogs were fasted and placed in a sling in a quiet room for 30 minutes before the beginning of the experiment. Four different forms of CONJ-hBNP were administered (25 µg/kg SQ) in 8 conscious normal dogs. Specifically, each CONJ-hBNP was administered to 2 different dogs after baseline (BL) measurements of MAP and blood sampling for canine ANP (cANP), canine BNP (cBNP), hBNP, and cGMP. MAP and blood samplings were repeated at 10, 30, 60, 120, 180, and 240 minutes after subcutaneous administration.

Among the 4 different forms of CONJ-hBNP tested for subcutaneous administration, we chose for oral administration the hBNP conjugate hBNP-021, which showed the most favorable profile in inducing cGMP generation and blood pressure lowering after subcutaneous administration. Specifically, we compared the efficacy of oral hBNP-021 (350 µg/kg) to native hBNP (350 µg/kg) in 6 normal dogs. We designed a randomized crossover study in which hBNP-021 or native hBNP was administered orally in 1-week intervals after BL measurements of MAP and blood sampling for cANP, cBNP, hBNP, and cGMP. MAP and blood samplings were repeated at 10, 30, 60, 120, 180, and 240 minutes after oral administration.

Hormone Analysis
Blood samples were collected in chilled EDTA tubes, immediately placed on ice, and centrifuged at –4°C. Plasma was stored at –80°C until analysis. After extraction, plasma cANP, cBNP, hBNP, and cGMP were assessed by radioimmunoassay as previously described.16,17

Statistical Analysis
Results are expressed as mean±SEM. Data were assessed by 1-way ANOVA for comparisons within groups, followed by post hoc Dunnett’s test. Two-way ANOVA was used for comparisons between groups, followed by Bonferroni’s posttest. Student paired t test was performed for single comparisons between groups (Graph-Pad Prism software 4.0). Statistical significance was accepted at a value of P<0.05.

Results
Subcutaneous Administration
We tested 4 different forms of CONJ-hBNP. Although we observed slight differences among the 4 conjugates tested, all had similar significant biological actions. Therefore, the results are presented in aggregate (Figure 2). Plasma hBNP was not detectable in dogs at BL. After subcutaneous administration, plasma hBNP significantly increased compared with BL at 10 and 30 minutes (P<0.001). Plasma cGMP was elevated compared with BL at 10 minutes and remained elevated through 180 minutes (P<0.05). MAP decreased at 10 minutes and remained decreased through 180 minutes (P<0.05), with no change in heart rate for the duration of the study. Plasma cANP and cBNP were unchanged compared with BL throughout the protocol (data not shown).

Oral Administration
Figure 3 reports the responses to orally administered native hBNP and hBNP-021 in 6 normal conscious dogs. Plasma hBNP was not detectable in the dogs at BL. After oral administration of hBNP-021 and native hBNP, plasma hBNP
was detected throughout the time course of the study. Specifically, plasma hBNP was significantly higher after 10 minutes of hBNP-021 (2509 ± 370 pg/mL) compared with BL (P < 0.05), whereas hBNP at 10 minutes after native hBNP administration was not detected. During the time course of the study, plasma hBNP was overall significantly higher after hBNP-021 compared with native hBNP administration (P = 0.0374 between groups). Plasma cGMP significantly increased for 60 minutes after dosing of hBNP-021 (from 10.8 ± 3 pmol/mL at BL to 36.2 ± 16 pmol/mL after 10 minutes to 44.4 ± 26 pmol/mL after 30 minutes to 36.8 ± 26 pmol/mL after 60 minutes; P < 0.05). In contrast, cGMP levels were not statistically different from BL levels after native hBNP administration and remained significantly lower compared with hBNP-021 (P = 0.001 between groups). MAP decreased at 10 minutes and remained decreased through 60 minutes after hBNP-021 administration (from 113 ± 8 mm Hg at BL to 101 ± 12 mm Hg after 10 minutes to 97.5 ± 10 mm Hg after 30 minutes to 99 ± 13 mm Hg after 60 minutes). In contrast, MAP did not change after native hBNP administration throughout the time course of the study (P < 0.0387 between groups). Plasma cANP and cBNP were unchanged compared with BL throughout the protocol in both groups (data not shown).

Discussion

This study defines for the first time the acute actions of subcutaneously and orally administered CONJ-hBNP in normal conscious dogs. Specifically, hBNP-021 was absorbed and present in the plasma after subcutaneous and oral administration. More importantly, acute subcutaneous and oral CONJ-BNP activated cGMP generation and induced a significant reduction in MAP.

BNP is an endogenous peptide produced by the heart under physiological and pathological conditions. BNP has natriuretic, diuretic, vasorelaxant, lusitropic, antialdosterone, and antifibrotic actions that could mediate cardiorenal protection in cardiovascular diseases. Its concentration increases progressively in patients with CHF and has also been reported to be increased in hypertensive heart disease with left ventric-
ular hypertrophy.\textsuperscript{18,19} However, despite this increase in circulating BNP in cardiovascular disease states, exogenous administration in experimental and human hypertension and CHF has had favorable actions.\textsuperscript{8,11,12,20} On the basis of this evidence, the exogenous administration of recombinant hBNP has been recently approved for the treatment of acute CHF. Colucci et al\textsuperscript{11} have reported the beneficial actions of acute intravenous administration of BNP (nesiritide) in patients hospitalized with decompensated CHF. Furthermore, we have previously reported that repeated subcutaneous administration of native BNP for 10 days during the evolution of left ventricular dysfunction in a model of canine CHF resulted in an improvement of cardiac hemodynamics, thus supporting the beneficial effects of chronic administration of BNP in the treatment of CHF.\textsuperscript{21} More recently, Yancy and coworkers\textsuperscript{12} have reported the safety of intermittent chronic effects of chronic BNP therapy. More recently, Yancy et al\textsuperscript{22} confirmed and extended these data in human CHF patients, once again demonstrating the potential beneficial effects of chronic BNP therapy. More recently, Yancy and coworkers\textsuperscript{12} have reported the safety of intermittent chronic intravenous BNP in patients with more severe symptomatic CHF. Furthermore, in experimental hypertension, long-acting subcutaneous BNP fused to albumin induced a sustained blood pressure reduction in spontaneously hypertensive rats.\textsuperscript{13}

The therapeutic use of peptidic hormones continues to increase. Although these therapeutics are generally characterized by high potency and few adverse events, their administration has almost exclusively been relegated to either intravenous infusion or subcutaneous injection. Although oral administration would be preferred in many cases, potential denaturation in the stomach, proteolysis in the stomach and small intestine, and insufficient transport across the intestinal mucosa and epithelium have proven to be formidable barriers. Although many efforts are underway to deliver native peptides orally via novel formulation technologies,\textsuperscript{23} our approach is based on derivatization of the therapeutic peptide with small, amphiphilic oligomers that are monodisperse and comprise both a hydrophobic (alkyl) moiety and a hydrophilic polyethylene glycol (PEG) moiety. During the course of screening >50 BNP conjugates, in vitro activity assays had shown that many conjugates retained activity comparable to the native peptide (data not shown). Also, screening in rats had shown which conjugates were the most orally bioavailable (data not shown). Therefore, to test whether the hBNP conjugates maintained the affinity to NPR-A and the biological activities in vivo, we tested low doses of 4 different conjugates (25 \textmu g/kg) administered subcutaneously in 8 normal dogs. We observed that CONJ-hBNP increased cGMP concentration, thus implying activation of its receptor, in association with significantly reduced MAP. Thus, these data establish that these modified forms of hBNP are biologically active in vivo.

As indicated in Methods, we chose for the oral administration study the forms of CONJ-hBNP that in the subcutaneous studies possessed the most favorable profile in inducing cGMP activation and blood pressure lowering. Here, orally administered hBNP-021 induced a significant increase in plasma hBNP 10 minutes after its administration and remained detectable in the circulation for 4 hours. Importantly, such levels of hBNP were significantly greater after hBNP-021 than after native hBNP administration throughout the time course of the study. Although plasma hBNP was detected in very low levels after oral native hBNP administration, it did not lead to significant activation of cGMP. Therefore, it is possible that the native hBNP measured in the plasma was, at least in part, degraded by gastric enzymes and, although detected by radioimmunoassay, lacked the ability to bind to NPR-A. Alternately, if the native hBNP detected in circulation was intact, then it was not present in sufficient quantities to elicit a response. Indeed, only oral hBNP-021, not native BNP, significantly activated cGMP. Furthermore, the increased levels of hBNP and cGMP after oral hBNP-021 administration induced a significant and sustained reduction in MAP that was not observed after oral native hBNP administration. Thus, only modified hBNP maintained biological activities when administered orally.

As discussed, the barriers to oral delivery of a functional therapeutic peptide are significant. Although a rather high dose of CONJ-hBNP (350 \textmu g/kg) was required for oral administration, hBNP-021 induced a significant and sustained activation of cGMP, and a concomitant sustained reduction in MAP was not observed with native hBNP at the same dose. At present, however, we are generating new forms of CONJ-hBNP that may be even more amenable to oral delivery and have more prolonged actions so that lower oral doses can be pursued in the near future. Of note, this study was not designed to determine renal actions of oral conjugate hBNP. Data pertaining to urinary cGMP, sodium excretion, and plasma changes in antidiuretic hormones such as aldosterone and angiotensin II will be collected in future studies with oral hBNP conjugates.

In conclusion, these 4 novel forms of CONJ-hBNP were absorbed and present in the circulation when administered subcutaneously. Furthermore, they activated cGMP and induced a significant reduction in MAP, displaying a magnitude and duration of action that was equal to (or better than) that of native hBNP. In addition, a novel CONJ-hBNP was also absorbed and present in the circulation for the duration of the study when administered orally. More importantly, hBNP-021 activated cGMP and induced a significant reduction in MAP that was not observed with native hBNP. Thus, this study clearly demonstrates that hBNP-021 is absorbed intact and promotes sustained biological actions when administered orally. These data suggest the importance of pursuing further studies with subcutaneous and oral administration of conjugated forms of human BNP for the long-term treatment of cardiovascular diseases.

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References

Clinical Perspective

Human b-type natriuretic peptide (hBNP) has emerged as a new therapeutic for the treatment of acute heart failure as an intravenous drug. Extensive research by our group and others has established that this peptide of myocardial origin functions as a circulating hormone to maintain optimal cardiorenal homeostasis. Among its many actions, it counteracts the renin-angiotensin-aldosterone system with local effects on the heart, including inhibition of fibrosis and improvement in diastolic function. The current dosage of hBNP is limited to continuous infusion because of its short half-life and rapid gastrointestinal degradation. However, the recent development of alkylPEGylation (in which short, monodispersed, amphiphilic oligomers are covalently attached to specific sites on proteins) has made it possible to modify the hydrophilicity and hydrophobicity of hBNP, render the resultant conjugate suitable for oral delivery, and retain the activity inherent to the native peptide. In the present study, we found that a conjugated form of hBNP activates the same biochemical pathway of native hBNP and, most importantly, is biologically active after oral administration. Ultimately, this conjugation technology makes oral hBNP administration feasible and the long-term administration of hBNP very practical. Although hBNP is currently an important drug in treating hospitalized patients with acute decompensation, an oral dosage form may broaden the application of this therapy to patients at various stages in the progression of heart failure. Potentially, oral hBNP could become an important preventive strategy in evolving heart failure. These findings open new therapeutic options across the entire spectrum of heart failure and warrant further research.
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