Analysis of Circulating Forms of proBNP and NT-proBNP in Patients with Severe Heart Failure

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BACKGROUND: The specific forms of pro–B-type natriuretic peptide (proBNP) that occur in human blood are not yet clear. We demonstrated the presence of several proBNP forms in human plasma with a new affinity chromatography method that can be used in combination with nano–liquid chromatography electrospray ionization tandem mass spectrometry (nano-LC–ESI–MS/MS).

METHODS: For affinity chromatography, we coupled Fab′ fragments of polyclonal sheep antibodies specific for N-terminal proBNP (NT-proBNP) epitope 1–21 to silica beads. We connected a column (10 mm × 0.8 mm inner diameter) packed with these beads to a trypsin reactor and used a preconcentrator in combination with a fritless nanospray column to perform MS analyses of proBNP forms in preextracted and non-preextracted samples of plasma from patients with severe heart failure (HF). We used Western blotting in deglycosylation experiments to confirm the shifts in proBNP and NT-proBNP masses.

RESULTS: Tandem MS experiments demonstrated the presence of both NT-proBNP and circulating proBNP in preextracted samples of plasma from patients with severe HF, and Western blotting analyses revealed 2 bands of approximately 23 kDa and 13 kDa that shifted after deglycosylation to positions that corresponded to the locations of recombinant proBNP and synthetic NT-proBNP.

CONCLUSIONS: We obtained clear evidence for circulating proBNP in patients with severe HF and provided the first demonstration of O-glycosylation of NT-proBNP. The higher molecular masses for NT-proBNP and proBNP observed in the Western blotting analyses than those expected from calculations can be explained by O-glycosylation of these peptides in vivo.

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B-type natriuretic peptide (BNP)4 and N-terminal proBNP (NT-proBNP) are now established heart failure (HF) markers that have been incorporated into the guidelines for HF management (1, 2). These markers are becoming increasingly accepted by clinicians and laboratorians for the exclusion of HF, a disease with a high morbidity that currently affects 5 × 10⁶ people in the US and 10 × 10⁶ people in Europe and shows a high prevalence (>10%) in the elderly population (2, 3). Recent studies have also indicated that BNP and NT-proBNP may be useful as markers in other clinical settings, such as risk assessment in patients with acute coronary syndromes (4, 5); however, full assessment of several factors, such as the biological variation in BNP (6–8), its limited cardiac specificity, the various circulating forms of proBNP, and the specification of BNP and NT-proBNP assays, will be required to avoid confusion regarding interpreting results for these markers in daily practice (9).

The specific forms of the proBNP protein (amino acid residues 1–108) that occur in human blood are not yet clear. proBNP is the precursor hormone synthesized in cardiomyocytes after ventricular overload or an increase in wall tension, and the mechanism for its cleavage is still not fully understood. At the time of secretion into the blood or later, a serine protease is thought to cleave proBNP on an equimolar basis into

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the physiologically active BNP form [proBNP residues 77–108 (BNP 32)] and a form believed to be physiologically inactive, NT-proBNP (residues 1–76) (10). Previous studies of proBNP forms in human blood have yielded controversial results. Besides BNP 32 and NT-proBNP, researchers have also reported the presence of various other truncated forms of these peptides in human blood (11–13). One study did not find BNP 32 at all (14). Other studies suggested that high molecular weight BNP forms (10–36 kDa) and low molecular weight forms (3.7–4 kDa) were monomers and trimers of proBNP and monomeric BNP, respectively (11, 12, 15). Another study found NT-proBNP forms truncated at the C- and N-terminal ends but found no evidence for the presence of proBNP (13). The drawback of most of these studies is that they used indirect assay methods, such as immunoassays after HPLC separation, to demonstrate the different forms of circulating proBNP.

A recent study (16) demonstrated recombinant proBNP of Chinese hamster ovary cells to be O-glycosylated at 7 specific sites, and a Western blotting analysis has shown the presence of O-glycosylated proBNP in the plasma of HF patients. Little is known about the biological function of glycosylated proBNP. Glycosylation could be important in several areas that are related to the biology of BNP, including its proteolytic processing, the half-life, and its variation across disease states. Furthermore, because the commercially available assays currently use synthetic or recombinant calibrant peptides that are not glycosylated, the glycosylation of proBNP forms in vivo may adversely influence the performance of these assays.

The aims of this work were to determine what proBNP forms circulate in the blood of patients with severe HF and to investigate whether circulating NT-proBNP is O-glycosylated. To overcome the difficulties of previous studies that used indirect methods, we developed a new affinity chromatography method that can be used in combination with nano–liquid chromatography electrospray ionization tandem mass spectrometry (nano-LC–ESI–MS/MS).

Materials and Methods

SOLID-PHASE EXTRACTION OF PLASMA SAMPLES

The study was conducted in accordance with the Declaration of Helsinki. Heparin-treated plasma samples were obtained from HF patients with high concentrations of NT-proBNP [>10 000 ng/L, as assayed with the E 170 module for MODULAR ANALYTICS (Roche Diagnostics)] and were leftover from routine NT-proBNP measurements in the hospital’s central laboratory. Thawed plasma samples were centrifuged at 15 000 g for 15 min at 4 °C. We used a polymeric sorbent solid-phase extraction (SPE) column (strata™-X, cat. no. 8B-S100-FBJ; Phenomenex) at room temperature according to the manufacturer’s protocol for SPE of plasma samples. In brief, the column was activated with 4 mL of pure methanol and rinsed with 4 mL of HPLC-grade water. Then, 4 mL of plasma diluted with 8 mL of phosphate buffer (50 mmol/L, 5 mmol/L EDTA) was loaded onto the column. The column was subsequently washed with 4 mL of a solvent containing 50 mL/L methanol and 1 mL/L trifluoroacetic acid in water and eluted into 2-mL polyethylene vials with 2 mL of a solution of 950 mL/L methanol and 1 mL/L trifluoroacetic acid in water. The eluate was concentrated to <500 μL by evaporation at room temperature. The concentrated samples were diluted with an equal volume of phosphate buffer (100 mmol/L, pH 7.2) and subjected to affinity chromatography. We monitored recovery after SPE by measuring NT-proBNP solutes diluted 1:50 in plasma after each step (plasma sample, flow-through, wash, eluate).

AFFINITY CHROMATOGRAPHY

To capture proBNP and its N-terminal forms from samples of human plasma, we developed an affinity chromatography method that used Fab’ fragments of polyclonal sheep 1–21 antibodies [i.e., specific for epitope 1–21; Roche Diagnostics (see package insert)] (17–19). First, F(ab’)_2 1–21 antibodies dissolved in buffer (20 mmol/L phosphate buffer, 20 mmol/L NaCl, and 56 g/L saccharose, pH 7.4) were reduced to Fab’ 1–21 fragments with 2-mercaptoethylamine hydrochloride (final concentration, 100 mmol/L; final pH, 6.0; Sigma-Aldrich Handels) under final buffer conditions (15 mmol/L phosphate, pH 6, 15 mmol/L NaCl, and 50 g/L saccharose) for 1 h at 37 °C. Purified Fab’ 1–21 was isolated by size-exclusion chromatography (see Fig. 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol?issue?) on a G3000SW_XL column (300 mm × 7.8 mm inner diameter; Tosoh Bioscience). We eluted Fab’ fragments isocratically with 50 mmol/L phosphate buffer (pH 7.2) at a flow rate of 0.5 mL/min and collected the fractions. The antigen-binding properties of the isolated Fab’ 1–21 fragments were verified by antibody competition in an NT-proBNP assay (Elecsys 1010; Roche Diagnostics) with NT-proBNP samples spiked with purified Fab’ 1–21 fragments. After an equilibration time of 10 min to ensure antigen binding, we measured the concentration of the remaining NT-proBNP. Secondly, the purified Fab’ 1–21 fragments were coupled to beads following the approach of Guzman et al. (20, 21), with the exception that amino-derivatized silica beads (bead size, 7 μm; pore size, 30 nm) were used. After washing the silica beads (16 g/L) 3 times with phosphate buffer (50 mmol/L, pH 5.0), the Fab’ 1–21 fragments were captured on the beads.
7.2), we covalently bound 5 g/L sulfo-succinimidyl 4-((N-maleimidomethyl)cyclohexane-1-carboxylate (S-SMCC) (Sigma-Aldrich) to the free amino groups for 1 h at 30 °C. After removing excess reagent by washing with the phosphate buffer, we added 3–5 mg of purified Fab’ dissolved in phosphate buffer (50 mmol/L, pH 7.2) and incubated the solution for 40 h at room temperature. The beads were centrifuged and washed with the phosphate buffer, and the remaining binding sites of bound S-SMCC were blocked by incubating with 10 mmol/L 2-mercaptoethanol for 1 h at room temperature. Finally, the beads were washed and resuspended in the phosphate buffer. A polyethmaletherketone (PEEK®) column (10 mm × 0.8 mm inner diameter) constructed in house was packed with this slurry, equilibrated at 4 °C in 50 mmol/L phosphate buffer (pH 7.2) containing 0.01% sodium azide and stored at this temperature. The column was regenerated for 30 min after each run with 150 mmol/L NaCl and then equilibrated with 50 mmol/L phosphate buffer (pH 7.2). We evaluated the quality of the affinity chromatography column by measuring the NT-proBNP concentration in the flow-through, wash, and eluate of plasma extracts produced by SPE. These solutes were diluted 1:50 in plasma for measurements of NT-proBNP.

MASS SPECTROMETRY
Plasma samples (n = 7) from patients with severe HF were either preextracted via SPE or simply diluted 1:2 before injection with 50 mmol/L phosphate buffer (pH 7.2) without any preextraction steps. One milliliter of SPE sample or 2 mL of the diluted plasma was loaded onto the affinity chromatography column (flow rate, 13 μL/min at room temperature). Samples were injected with the mobile phase (50 mmol/L phosphate buffer, pH 7.2) for 75 min for SPE extracts and 170 min for the diluted plasma. During this phase, proBNP and its N-terminal fragment were allowed to bind to the covalently immobilized Fab’ 1–21, and the noninteracting material was washed out of the column and discarded. To further remove nonspecifically bound material, we rinsed the column with a high-salt buffer (50 mmol/L sodium acetate buffer (pH 5.0) containing 0.01% sodium azide) and stored at this temperature. The column was regenerated for 30 min after each run with 150 mmol/L NaCl and then equilibrated with 50 mmol/L phosphate buffer (pH 7.2). We evaluated the quality of the affinity chromatography column by measuring the NT-proBNP concentration in the flow-through, wash, and eluate of plasma extracts produced by SPE. These solutes were diluted 1:50 in plasma for measurements of NT-proBNP.

DEGLYCOSYLATION
For Western blot analysis (n = 5), peptide fractions obtained from affinity chromatography were incubated overnight at 37 °C with 0.25 mU O-glycosidase, 0.25 mU N-acetylneuraminidase, and 0.25 mU β-galactosidase in 50 mmol/L phosphate buffer, pH 6.0. All enzymes were obtained from Roche Diagnostics.

WESTERN BLOT ANALYSIS
Deglycosylated and non-deglycosylated peptide fractions, Biotinylated SDS-PAGE Standards (cat. no. 161-0319, Bio-Rad Laboratories), synthetic NT-proBNP (Roche Diagnostics), and recombinant proBNP (cat. no. 8PRO8, HyTest) were loaded onto 15% SDS-PAGE gels and electrophoresed at 120 V and 15 °C. Peptides were transferred onto a nitrocellulose membrane for Western blotting for 75 min at 100 V and 4 °C. The nitrocellulose membrane was blocked overnight with Roti® (Block (cat. no. A151.1, Carl Roth), washed with 5 mL/L polysorbate 20 (Twee 20; cat. no. 822184, Merck) in Tris-buffered saline (pH 7.5), and then incubated with polyclonal sheep antibody specific for NT-proBNP 1–21 (1:9000 dilution; Roche Diagnostics) for 1 h at room temperature. After washing the nitrocellulose membrane with 10 mL/L Tween 20 in

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Tris-buffered saline, we added secondary antibody (peroxidase-conjugated rabbit antisheep IgG; cat. no. 313-035-003, Jackson ImmunoResearch Europe) to a final dilution of 1:5000 and streptavidin–horseradish peroxidase conjugate (Streptavidin-POD; cat. no. 11089153001, Roche Diagnostics) at a final dilution of 1:6000 dilution. We incubated the membrane for 1 h at room temperature, and washed the membrane again with 5 mL/L Tween 20 in Tris-buffered saline. We used Amersham ECL™ Western Blotting Detection Reagents (cat. no. RPN2106, GE Healthcare/Amersham Biosciences) for electrochemiluminescent visualization of bands on x-ray films.

Results

We achieved high recovery of NT-proBNP from the plasma of patients after SPE (median, 69.6%; range, 62.3%–71.7%; n = 3) (Fig. 1A). The recovery of NT-proBNP from SPE-treated plasma samples, which were used to evaluate the quality of the affinity chromatography column, was also high (median, 86.4%; range, 70.0%–111.0%; n = 6) (Fig. 1B).

This affinity-based nano-LC–ESI–MS/MS approach to the assay of preextracted plasma samples enabled us to demonstrate the presence of proBNP in the circulation. The base peak chromatogram reveals the presence of several different peptide fragments obtained after tryptic digestion of the affinity chromatography–trapped plasma sample (Fig. 2A). We found proBNP peptide fragments in the MS/MS spectra that covered 59.2% of the amino acid sequence of NT-proBNP 1–76 (Fig. 2D; see Fig. 2, A–H, in the online Data Supplement); however, we also identified 2 additional peptide fragments in the BNP 32 region (80–89 and 94–103) (Fig. 2, B and C). We calculated a coverage of 60.2% of the proBNP 1–108 sequence (i.e., NT-proBNP 1–76 plus BNP 32). When we applied a non-preextracted plasma sample directly, we also identified NT-proBNP, with 47% coverage of the NT-proBNP sequence.

The MS results were corroborated in the Western blotting analysis. By extracting plasma with the SPE method, we achieved an effective preconcentration of NT-proBNP in plasma samples, which is mandatory for Western blotting analysis. After trapping proBNP forms by the affinity chromatography method and subsequent eluting the forms, we identified 2 major bands (approximately 23 kDa and 13 kDa) by Western blotting of samples from HF patients (n = 5; Fig. 3). After deglycosylation, these bands shifted to molecular weights of approximately 13 kDa and 6.5 kDa, corresponding to the positions where we detected recombinant proBNP and synthetic NT-proBNP in our SDS-PAGE gels. These marked shifts in peptide mass of 10 kDa and 6.5 kDa, respectively, and the MS results for circulating proBNP and NT-proBNP indicate that both proBNP and NT-proBNP occur as glycosylated monomers in human blood. Additionally, the MS results in combination with the Western blotting results demonstrate that proBNP is present in the circulation of patients with severe HF.

Discussion

We have developed a new affinity chromatography method for efficiently capturing low-abundance peptides, such as proBNP forms in human plasma, for further analysis by different analytical methods. This af-
finity chromatography method can be combined with nanospray MS. Our new affinity-based nano-LC–ESI–MS/MS approach has provided the first clear evidence of the presence of intact proBNP in circulating human plasma. We also have demonstrated that NT-proBNP is O-glycosylated in the plasma of patients with severe HF.

Earlier studies (23–27) reported the presence of BNP 32 in the circulation, together with an ill-defined high molecular weight BNP species of 12–15 kDa, possibly proBNP 1–108. These studies required the extraction of considerable volumes of human plasma (up to 19 mL) with Vycor glass powder or C18 cartridges before the BNP species could be separated by size-

Fig. 2. Nano-LC–ESI–MS/MS analysis following SPE of a representative sample from a patient with severe HF. (A), Base peak chromatogram of the trypsin-digested protein fraction obtained after affinity chromatography. Arrows indicate the positions of the peptide fragments shown in (B) and (C). (B), Nano-LC–ESI–MS/MS spectra of proBNP amino acid residues 80–89. (C), Nano-LC–ESI–MS/MS spectra of proBNP amino acid residues 94–103. The ions at m/z 549.8 and 498.3 corresponding to [M+2H]^2+ were subjected to fragmentation. Fragment masses were assigned with the aid of the SEQUEST algorithm in the Bioworks 3.3 software package, and the b- and y-ions so obtained were identified as peptide fragments 80–89 and 94–103. Asterisk indicates carboxymethyl-modified cysteine due to the iodoacetamide treatment. (D), The identified peptides (in gray) of proBNP are illustrated schematically in the graph; the amino acid sequences of the peptides are presented as the 1-letter codes. The specific amino acids detected by nano-LC–ESI–MS/MS are in boldface. The table presents the observed amino acid sequences, the singly protonated molecular ions ([M+H]^+) of the proBNP fragments, the charge state (z), the cross-correlation score (XC), the percentage of the mass (% Mass), the amino acid residues (AA), the percentage of the proBNP 1–108 amino acid sequence of the specific fragments (% AA), and the retention time of the peptide fragments in minutes (RT).
exclusion HPLC. The investigators then studied the different fractions with self-developed competitive RIAs by using calibrator peptides corresponding to the N terminus of proBNP (amino acid residues 1–13) to demonstrate the presence of proBNP in specific fractions. In addition, a competitive RIA that used antibodies directed against the middle region of proBNP (residues 62–76) detected a third form, approximately 13 kDa, which is the same position where we found a shift of approximately 10 kDa to a molecular mass of 3.7 kDa in the plasma of HF patients; healthy individuals had BNP concentrations that were not sufficiently high for HPLC-RIA analysis. In more recent studies, Shimizu et al. (11, 12) also reported both high molecular weight and low molecular weight BNP species in congestive HF patients. In contrast to Yandle et al., the high molecular weight form found by Shimizu et al. was much larger, 36 kDa—3 times the size of proBNP (12 kDa). Both peaks found by gel-filtration HPLC showed immunoreactivity in a more sensitive IRMA (Shionoria BNP assay with monoclonal antibodies against BNP 14–21 and BNP 27–32). Interestingly, after analyzing the smaller peak by reversed-phase HPLC and IRMA, the investigators detected immunoreactive peaks only for species smaller than BNP 32, which were assumed to be digests of proBNP or BNP (11). The high molecular weight peak was thought to be a trimer of proBNP (12). A recent study that used plasma samples from HF patients that were not preextracted and were applied directly to gel-filtration fast protein liquid chromatography columns confirmed the presence of a major peptide of 37 kDa, a minor peptide of 4–6 kDa, and a 25-kDa peptide (28). Sensitive sandwich immunofluorescence assays for proBNP, BNP, or NT-proBNP developed by the investigators recognized these peptides. In contrast to earlier studies, in which only nonspecific immunoassays could be used to infer the molecular forms, we have used affinity-based nano-LC-ESI-MS/MS to directly identify the amino acid sequences of the proBNP forms occurring in the blood of HF patients. In particular, we have demonstrated the presence of proBNP and NT-proBNP in the blood on the basis of the combined results of 2 different methods. The MS analysis identified the amino acid sequences of the proBNP fragments obtained after trypsinic digestion, and the Western blotting analysis permitted the immunologic detection of proBNP. In the MS analysis, we found the 2 peptide fragments of BNP 32 (80–89 and 94–103) and located 8 additional fragments in the NT-proBNP 1–76 region. This remarkable finding can be explained in 2 ways: It can arise from the trypsic digestion of circulating proBNP or from the cleavage of circulating BNP 32 itself. Because the polyclonal sheep 1–21 antibodies from Roche Diagnostics are specific for the 1–21 epitope of NT-proBNP 1–76 and do not cross-react with BNP 32 (according to the manufacturer’s package insert) (17), this small peptide fragment is evidence for the occurrence of circulating proBNP in human plasma. In a very recent study (29), proBNP 1–108 was found in healthy individuals and HF patients by means of a very specific ELISA that uses a monoclonal antibody against the cleavage site of proBNP 1–108 (Hinge76, against amino acid residues 75–80) and exhibits no cross-reactivity with either BNP 32 or NT-proBNP 1–76.

Neither this recently described indirect method nor our present study can definitively exclude the presence of trimerized proBNP. Nevertheless, we doubt that proBNP circulates as a trimer, although our MS data cannot distinguish between monomers or multimers and although multimers would have been separated as monomers under the denaturing conditions of our SDS-PAGE system. We strongly suspect that the high molecular weight BNP described earlier may be due to O-glycosylation of proBNP, which would increase its molecular mass. In fact, we found this shift in molecular mass in each of the plasma samples we investigated by Western blotting after we had deglycosylated the proBNP and NT-proBNP trapped on the affinity chromatography column. Thus, proBNP showed a shift of approximately 10 kDa to a molecular mass of 13 kDa, which is the same position where we found
recombinant proBNP 1–108; NT-proBNP shifted approximately 6.5 kDa to a position where we found synthetic NT-proBNP 1–76. This O-glycosylation of human proBNP was also shown recently by Western blotting after the immunoprecipitation of plasma from HF patients with a monoclonal antibody specific for amino acid residues 81–91 of proBNP (16). The shift in proBNP molecular weight observed after deglycosylation in the latter study was comparable to our results. We have extended these findings by demonstrating that NT-proBNP is also O-glycosylated in human blood. Additionally, Crimmins (30) used sedimentation equilibrium ultracentrifugation and circular dichroism methods to demonstrate that synthetic NT-proBNP dissolved in phosphate-buffered saline cannot oligomerize by a coiled-coil reaction. Specifically, amino acid residues 17–38 were thought to form coiled-coil motifs (31). Because NT-proBNP and proBNP share these residues, Crimmins’ results may be extended to proBNP as well, further supporting the idea that proBNP is in a monomeric state. The molecular masses of the peptides detected in our Western blotting experiments provide strong evidence for, but do not prove, the occurrence of both monomeric NT-proBNP and proBNP in the blood. Of note is that the synthetic NT-proBNP used for the Western blot analysis showed the correct molecular mass of 8.4 kDa in the MS analysis, whereas synthetic NT-proBNP migrates faster in the SDS gel used for Western blot analysis, in the size range of approximately 6.5 kDa. This discrepancy is easily explained by the limited resolution of the SDS gel. Nevertheless, the decrease in molecular mass observed after deglycosylation was obvious, and MS unambiguously identified NT-proBNP and proBNP derived from the same sample.

We cannot draw conclusions about all of the possible BNP forms that occur in human blood, because we trapped proBNP forms with an antibody (NT-proBNP 1–21) incapable of capturing C-terminal forms of proBNP. A recent study with an analysis by (NT-proBNP 1–21) incapable of capturing C-terminal cause we trapped proBNP forms with an antibody possible BNP forms that occur in human blood, be-
The method we have described achieves highly specific coupling of Fab’ fragments to silica beads. We can assume a correct orientation of the active antibody side, which is crucial for optimal alignment to and recognition of the antigen, especially for capturing low-abundance peptides at sufficiently high concentrations for further analysis. Our results yielded an even a higher recovery than reported by Schellenberger et al. (16) (86% vs 75%), who used immunoprecipitation. The immunoprecipitation method has the disadvantage of the presence of some loose antibody-coupled beads, which can hinder analysis with MS methods.

In summary, our laboratory has used a new affinity-based nano-LC–ESI–MS/MS approach to provide the first direct demonstration of the presence of proBNP in the plasma of HF patients; truncated NT-proBNP was also found with this method. Furthermore, we corroborated that both proBNP and NT-proBNP are O-glycosylated in human blood. O-glycosylation is responsible for considerable shifts in mass to higher molecular weights that could be misinterpreted as oligomerized states of these peptides. These findings may be of particular relevance in clinical diagnosis and highlight the importance of using well-characterized and properly immobilized and oriented antibodies in immunoassays to achieve optimal diagnostic performance.

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