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Evidence for processing of human pro-B-type natriuretic peptide in the circulation.

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Introduction

B-type Natriuretic Peptide (BNP) is a circulating hormone primarily produced by the myocardium in response to volume overload and increased filling pressure. BNP is produced as a 108-amino acid residue (AAR) precursor molecule, proBNP. Cleavage of proBNP gives rise to the active BNP hormone (32 AAR) and N-terminal fragment, NT-proBNP (76 AAR). Increased plasma concentrations of BNP and NT-proBNP as well as proBNP have been shown to be associated with the onset of heart failure (HF). In contrast to the clinical utility of proBNP-derived peptides, the data regarding proBNP processing is rather limited. So still it is no clear where proBNP processing takes place. The presence of proBNP in plasma led us to hypothesize that proBNP is processed in circulation. The goal of the present study was to examine this hypothesis.

Materials and methods

Monoclonal antibodies (MAbs) specific to human proBNP were from HyTest (Finland). The MAb epitopes are indicated by subscripts (e.g., 24C5$_{87–93}$).

Human recombinant proBNP expressed in *E. coli* and in HEK 293 cells were from HyTest.

ProBNP infusion. Male Wistar rats weighed 300-400 g at the time of the study. Animals (4 rats for each peptide) were injected with 60–80 µL proBNP (concentration 60 mol/L) into the femoral vein at an initial dose of 12 nmol/kg. Blood samples (150 µL) were collected from the femoral artery at 0.5, 2, 4, 6, 8, 12, 18, 25, and 40 min after peptide injections. Samples were immediately placed into tubes containing EDTA and the protease inhibitor cocktail (Sigma, cat. No. P8340) and centrifuged at 1300 g for 10 min at 4°C. To evaluate the possible impact of coagulation on proBNP cleavage during blood collection, plasma samples obtained from the femoral artery of noninjected rats (n=4) were collected into a plastic syringe containing non-glycosylated proBNP, EDTA, and the protease inhibitor cocktail.

Sandwich immunofluorescent assay (IFA). Capture antibodies, 2 µg per well in 100 µL of PBS, were incubated in immunoassay plates for 30 min at RT. After washing, 50 µL of tested sample or calibrator and 50 µL of detection antibodies labeled with stable Eu3+ chelate in assay buffer were added. After incubation for 30 min at RT, the plates were washed and fluorescence was measured.

Gel filtration studies. Superdex Peptide column (GE Healthcare) was used to separate proteins from the rat plasma samples obtained after the injections of proBNP. BNP immunoreactivity in fractions was analyzed using single epitope sandwich (SES) IFA 24C5$_{87–93}$-Ab-BNP2, which equally recognizes both BNP and proBNP (**Fig. 1A**) (Tamm *et al.*, Clin Chem, 2008).

Mass-spectrometry analysis was performed using an Ultraflex II MALDI-TOF mass spectrometer (Bruker Daltonik). BNP-related peptides were extracted from the samples using the BNP-specific affinity matrix utilizing MAbs 24C5$_{87–93}$ and 50E1$_{102–108}$.

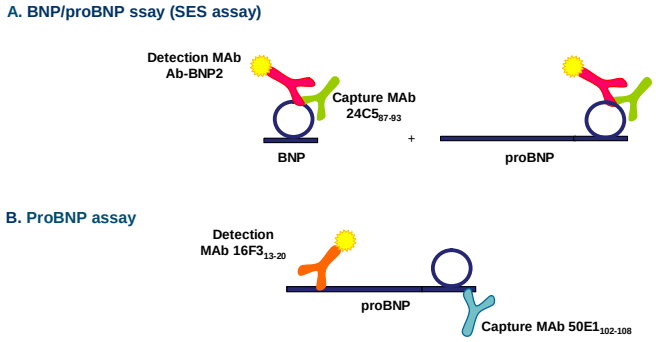

Figure 1. Schematic representation of SES BNP/proBNP (24C5$_{87–93}$–Ab-BNP2) assay **(A)** and proBNP (50E1$_{102–108}$–16F3$_{13–20}$) assay **(B)**. ProBNP assay enables specific determination of proBNP form, whereas total [BNP + proBNP] concentration is measured by the BNP/proBNP SES assay.

Results and discussion

Processing of proBNP in the circulation. As we have recently demonstrated, processing of human proBNP is inhibited by O-glycosylation of amino acid residues located close to the proBNP cleavage site. Both forms of proBNP, glycosylated and non-glycosylated in this region, are present in the blood of HF patients. Considering this finding non-glycosylated proBNP (expressed in *E. coli*) and glycosylated proBNP (expressed in HEK 293 cells) were used in this study. ProBNP produced in HEK 293 cells has been shown to be O-glycosylated in the central region and near the cleavage site. Non-glycosylated proBNP was used as a model protein which does not bear any oligosaccharides that may interfere with processing efficiency.

Glycosylated or non-glycosylated proBNP were injected intravenously in rats and plasma samples were analyzed using 2 sandwich IFAs (**Figs. 1A and 1B**).

ProBNP-specific assay detected 2.1- and 6.2-fold less non-glycosylated proBNP compared with the BNP/proBNP assay 6 and 40 min after injection respectively, suggesting that non-glycosylated proBNP is cleaved in the bloodstream (**Fig. 2A,** red and black curves). The clearance rate curves (green and blue curves) for glycosylated proBNP obtained by measuring proBNP concentrations in the samples with both proBNP and BNP/proBNP assays were quite similar, i.e. glycosylated proBNP was stable. These results corresponds well with previously obtained data regarding the suppressive action of glycosylation on the efficiency of proBNP processing. **Fig. 2B** represents the apparent rate of proBNP processing in the circulation which was estimated as the ratio of the proBNP concentration to the total [proBNP+BNP] concentration.

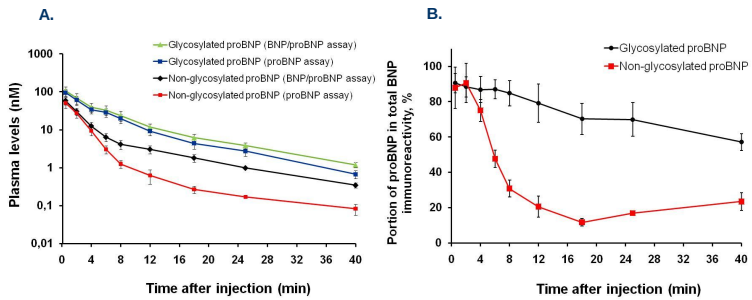

Figure 2. Clearance rate curves (semilog plot) of glycosylated and non-glycosylated proBNP after intravenous injection in rats at an initial dose of 12 nmol/kg **(A)**. Apparent rate of proBNP processing in the circulation **(B).**

When plasma samples collected at 0.5, 6, and 12 min after injection of glycosylated proBNP were analyzed by means of gel filtration, no BNP-related peptides were observed **(Fig. 3A)**; however, cleavage of non-glycosylated proBNP with formation of BNP-immunoreactive component in the approximately 3-kDa region, similar to synthetic BNP, was identified (**Fig. 3B**). No marked proBNP cleavage was observed in the control plasma samples spiked with proBNP expressed in *E. coli* (data not shown). This finding demonstrates that coagulation enzymes do not significantly contribute to proBNP cleavage during sample preparation.

Site-specificity of proBNP cleavage in the circulation. BNP forms in the samples obtained 8 min after injection of non-glycosylated proBNP were analyzed using MALDI-MS. Multiple BNP forms were detected: BNP (1-32), BNP (3-32), BNP (4-32), BNP (5-32), and BNP (5-31) (**Fig. 3C**). Importantly, these BNP forms were observed only in the samples from rats receiving injections and never in control samples from non-injected rats. MS analysis of NT-proBNP forms revealed that cleavage of proBNP occurred exclusively at the site –R76↓S77–, suggesting that the observed truncated BNP forms are the products of the proteolytic degradation of BNP (1–32) (data not shown).

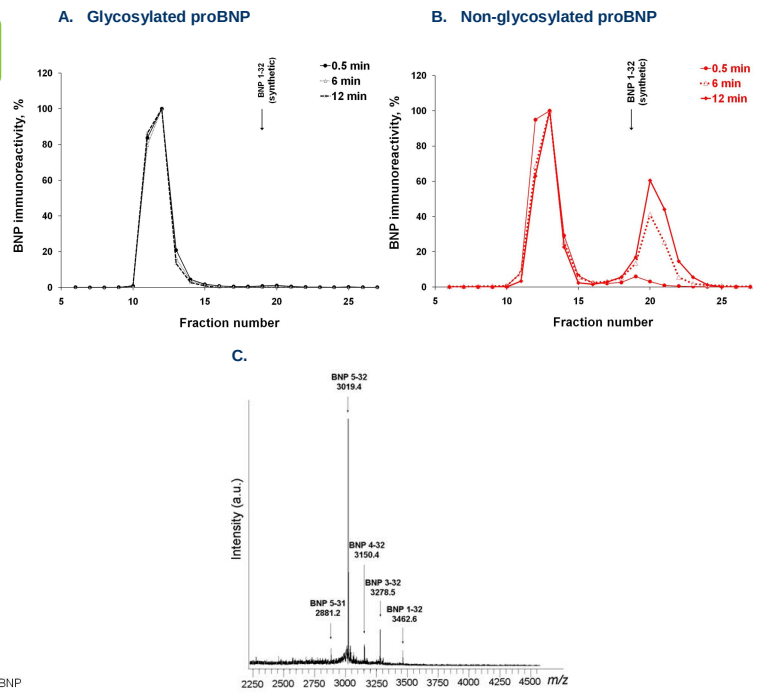

Figure 3. BNP-related peptides from plasma samples obtained at 0.5, 6, and 12 min after intravenous injection of glycosylated **(A)** or non-glycosylated proBNP **(B)**. The maximum value of BNP immunoreactivity displayed by proBNP was set as 100% for each sample. Results of MALDI-TOF MS analysis of BNP forms extracted from the samples collected 8 min after non-glycosylated proBNP injection in rats; a.u., arbitrary units **(C)**.

Conclusions

The present study shows that proBNP is processed in the circulation with formation of mature BNP 1-32. This observation suggests that the circulation can be considered as a place where proBNP processing occurs. Further studies are needed to demonstrate the relevance of these animal-based experiments to humans.

References

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