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The difference in glycosylation between human proBNP and NT-proBNP suggests a new regulatory level in proBNP processing

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Introduction

NT-proBNP and BNP are established plasma markers of heart failure (HF). Mature BNP is formed in the process of specific cleavage of its precursor proBNP in the site $_{76}R\downarrow S_{77}$. NT-proBNP is produced along with BNP at the equimolar amounts. Proprotein convertase furin is described in literature as possible enzyme responsible for proBNP processing.

Recently it was reported that significant portion of proBNP is secreted in unprocessed form and can be found in circulation. The reason(s) for this incomplete processing is still unknown. It has been demonstrated that proBNP from HF patients' plasma is O-glycoprotein. In the current study we investigate glycosylation level of proBNP in the region located close to the cleavage site and the influence of glycosylation on proBNP processing.

Materials and Methods

Monoclonal antibodies (MAbs) specific to different regions of human proBNP molecules were from HyTest. MAbs epitopes corresponding to proBNP amino acid sequence are designated as subscript ($24C5_{87-98}$).

Human recombinant NT-proBNP and proBNP expressed in *E. coli* were used as calibrators in immunoassays. Both peptides were from HyTest.

Expression of recombinant proBNP in HEK 293, CHO-K1 and NIH 3T3 cell lines

HEK 293, CHO-K1 and NIH 3T3 cells were transiently transfected with plasmid containing gene encoding human proBNP using cationic lipid-based transfection reagent.

Extraction of endogenous proBNP from HF patients' plasma and from conditioned media of transfected cells

To prepare affinity matrix for proBNP extraction two MAbs specific to the C-terminal part of proBNP (MAbs $24C5_{87-98}$ and $50E1_{102-108}$) were coupled with Sepharose CL 4B.

Enzymatic deglycosylation of proBNP

Endogenous proBNP extracted from plasma was incubated with either an enzyme mixture (endo-α-N-acetylgalactosaminidase, N-acetylneuraminate glycohydrolase, β-N-acetylhexosaminidase, β(1-4) galactosidase) or without enzymes for 1.5 hours at 37°C.

Sandwich immunofluorescent assay (IFA)

Capture antibodies, 2 µg per well in 100 µL of PBS, were incubated in 96-well plates for 30 min at room temperature. After washing, 50 µL of tested sample or calibrator and 50 µL of detection antibodies labeled with stable europium (III) chelate in assay buffer were added. After 30 min incubation the plates were washed, then enhancement solution was added, and fluorescence was measured.

Studies of immunochemical properties of proBNP and NT-proBNP using sandwich IFAs

ProBNP from HF patients' plasma or conditioned media was characterized using eleven two-site MAbs combinations. Capture antibodies were specific to different epitopes covering whole NT-proBNP molecule ($29D12_{5-12}$, $21E3_{13-20}$, $1D4_{13-24}$, $5D3_{28-45}$, $11D1_{31-39}$, $16E6_{34-39}$, $16D10_{46-56}$, $15C4_{63-71}$, $21E6_{67-73}$, $24E11_{67-76}$, $28F8_{67-76}$). MAb $24C5_{87-98}$ specific to the C-terminal part of proBNP was used as detection (Fig. 1A). Pooled plasma from 12 HF patients or conditioned media from transfected cells were used as a source of endogenous (recombinant) proBNP. ProBNP treated and non-treated with glycosidases mixture was analyzed by IFAs described above.

For NT-proBNP measurements pooled plasma (or conditioned media) was passed through affinity matrix to remove proBNP. The same set of capture antibodies was used for NT-proBNP measurements. Detection antibody $24E11_{67-76}$ was used to form pairs with capture MAbs specific to region 5-24 of NT-proBNP, whereas detection MAb $13G12_{13-20}$ was used in combinations with capture antibodies specific to region 28-76 of NT-proBNP (Fig. 1B).

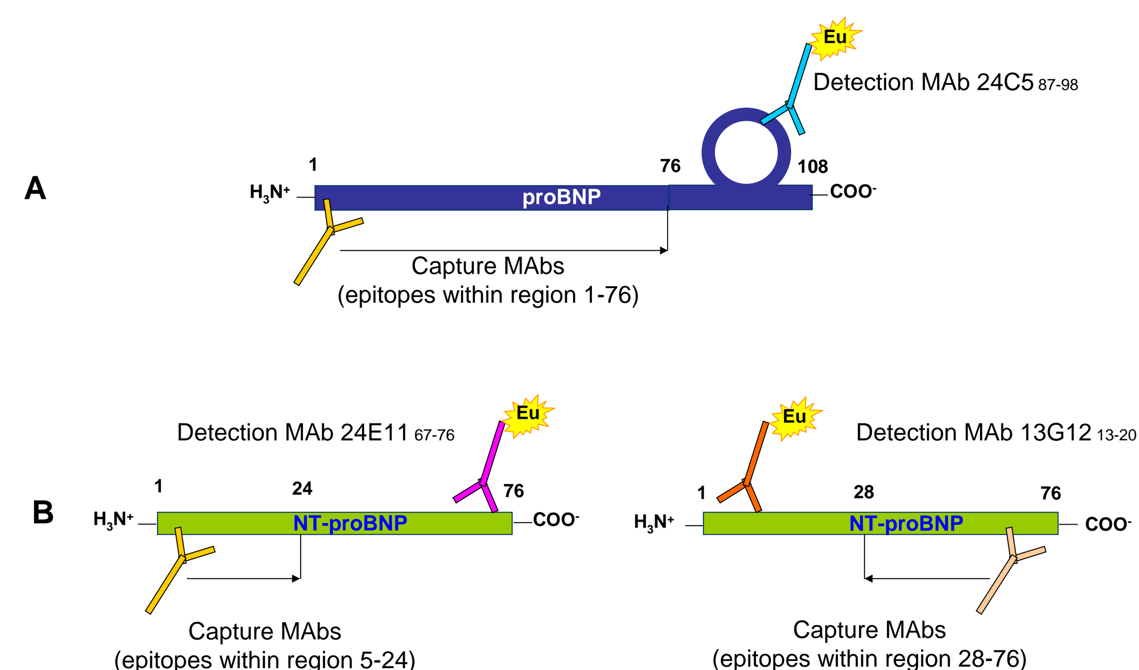

Figure 1. Schematic representation of sandwich IFAs used in the study for proBNP (**A**) and NT-proBNP analysis (**B**).

ProBNP processing rate in HEK 293, CHO-K1 and NIH 3T3 cells

Level of proBNP processing was determined as ratio [NT-proBNP]/[proBNP + NT-proBNP] in conditioned media. To quantify both proBNP and NT-proBNP, we used assay, non-sensitive to glycosylation, using MAb $5B6_{1-12}$ as capture and MAb $13G12_{13-20}$ as detection. NT-proBNP concentration was measured by the same assay in samples after proBNP removal.

Chemical deglycosylation and furin cleavage of recombinant proBNP

Affinity purified proBNP expressed in HEK 293 was deglycosylated by trifluoromethanesulfonic acid (TFMS) – method. ProBNP expressed in *E. coli*; deglycosylated or non-deglycosylated proBNP expressed in HEK 293 cells were then incubated with or without recombinant furin for 2.5 h at 37°C.

Results and discussion

We have compared the immunoreactivity profiles of proBNP and NT-proBNP from HF patients' plasma using MAbs, specific to different regions of NT-proBNP molecule. The profile of immunochemical activity for both molecules was similar with the exception for the region 61-76 located close to the cleavage site $_{76}R\downarrow S_{77}$ (Fig. 2A). The region 61-76 of endogenous proBNP was inaccessible to specific MAbs, whereas the same antibodies recognized endogenous NT-proBNP with high efficiency. Since proBNP was shown to be an O-glycoprotein (Schellenberger *et al*, 2006), we hypothesized that glycosylation could be the reason why antibodies are unable to recognize the region 61-76 of endogenous proBNP. To examine this hypothesis, proBNP extracted from HF patients' plasma was treated with mixture of O-specific glycosidases. Deglycosylation resulted in 2-2.3-fold increase of immunoreactivity of proBNP in the assays using MAbs specific to the region 61-76 (Fig. 2B). So it was concluded that region located close to the cleavage site is glycosylated in endogenous proBNP in comparison with NT-proBNP.

The absence of glycosylation in C-terminal part of endogenous NT-proBNP enabled us to suggest that NT-proBNP was a product of processing of proBNP non- or slightly glycosylated in the region located close to the cleavage site. To confirm this hypothesis, we investigated the correlation between the level of glycosylation of this fragment and the level (degree) of proBNP processing for the protein expressed in three mammalian cell lines (HEK 293, CHO and NIH 3T3). Level of proBNP glycosylation in the region of cleavage site varied significantly in the protein expressed in different cell lines (Fig. 3A). The highest level of fragment 61-76 glycosylation was shown for proBNP expressed in HEK 293 cells, significantly lower for NIH 3T3- and CHO-derived proteins. But independently of the cell line type, this region in NT-proBNP molecules was not glycosylated (Fig. 3B). The level of proBNP processing in different cell lines negatively correlated with glycosylation level of the region 61-76. NT-proBNP/(NT-proBNP + proBNP) molar ratio in CHO-K1 and NIH 3T3 conditioned medium was found to be about 5 times higher than in HEK 293 media (Fig. 3C). These data strongly supports our hypothesis that glycosylation in the region of cleavage site limits proBNP processing.

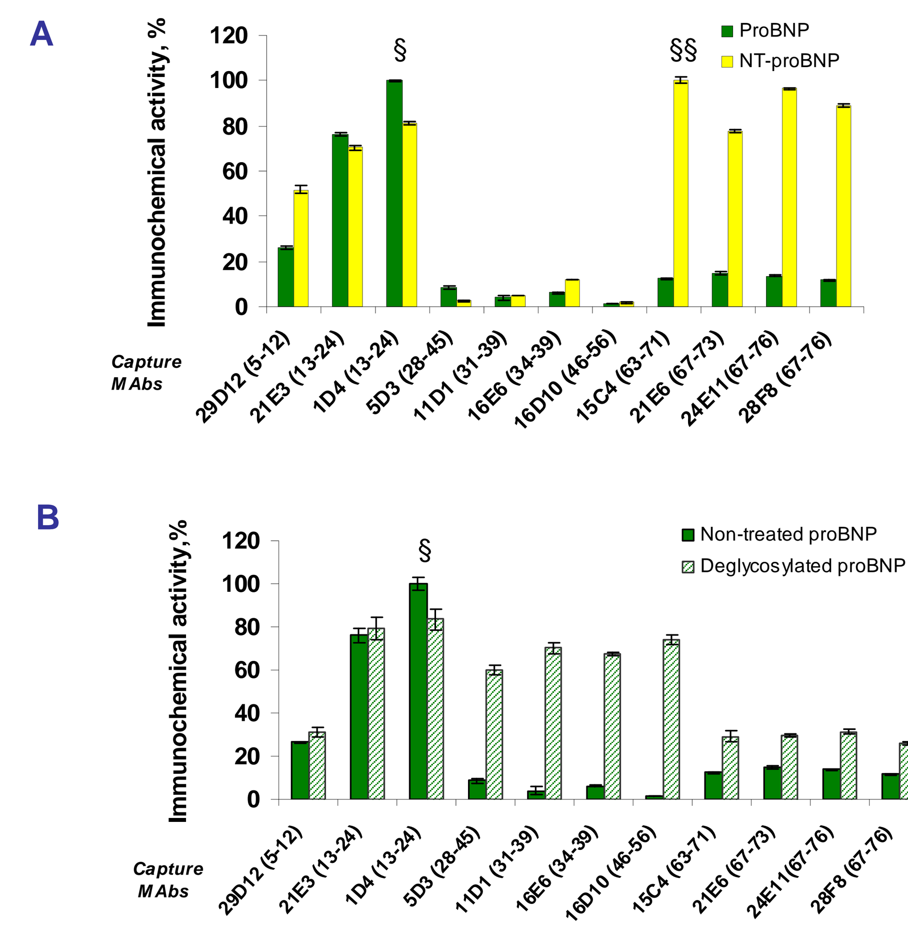

Figure 2. Immunochemical activity profiles of endogenous proBNP and NT-proBNP (**A**). Concentration of endogenous proBNP measured by reference assay $1D4_{13-24}$-$24C5_{87-98}$ (§) was accepted as 100%. Concentration of endogenous NT-proBNP measured by reference assay $15C4_{63-71}$-$13G12_{13-20}$ (§§) was accepted as 100%.

Comparison of endogenous proBNP immunoreactivity before and after enzymatic deglycosylation (**B**). Concentrations of endogenous proBNP in non-treated sample measured by reference assay $1D4_{13-24}$-$24C5_{87-98}$ (§) was accepted as 100%.

Results are expressed as mean ± SD (n = 3).

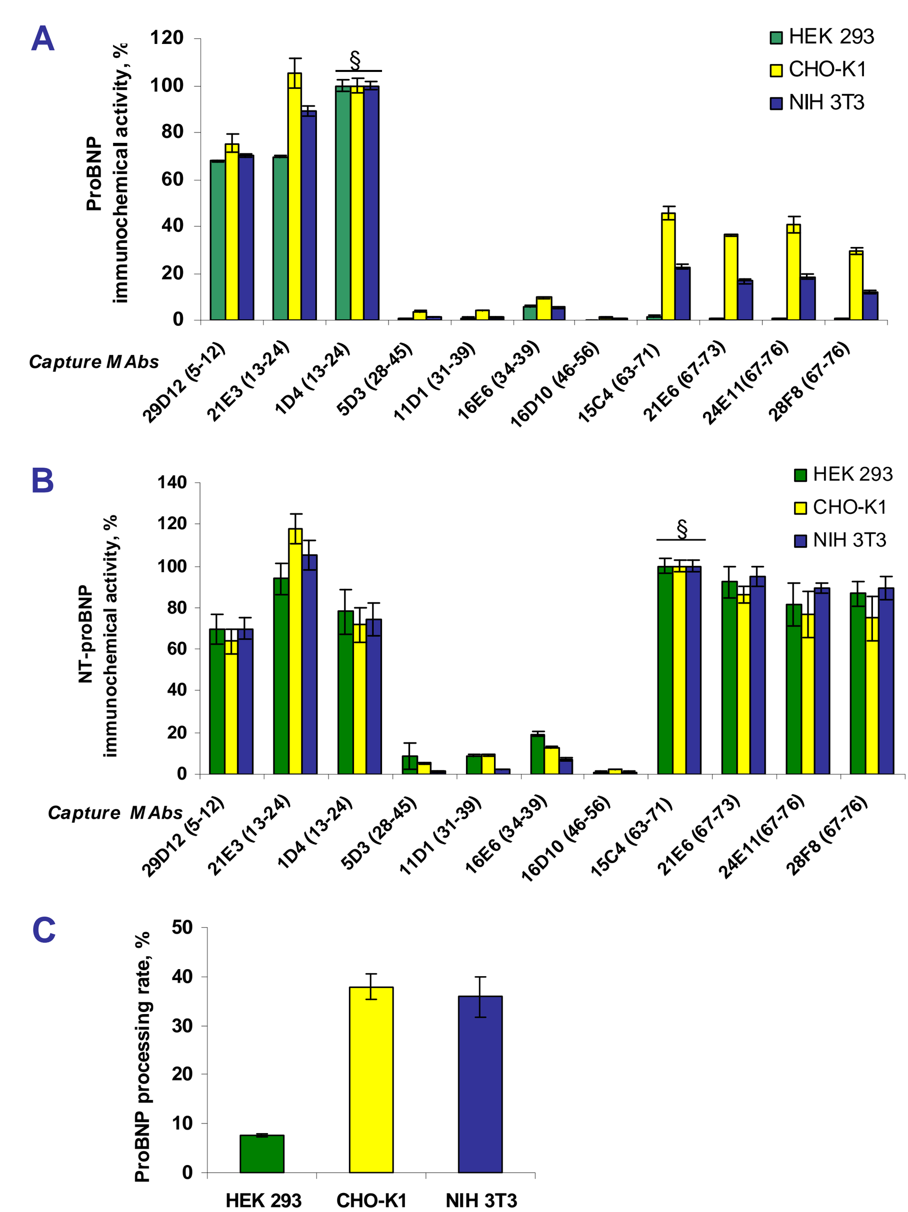

Figure 3. Immunochemical activity profiles of proBNP (**A**) and NT-proBNP (**B**) from conditioned media of HEK 293, CHO-K1, NIH 3T3 cells transiently transfected with human proBNP. Concentrations of proBNP measured by reference assay $1D4_{13-24}$-$24C5_{87-98}$ (§) were accepted as 100% for each cell line (**A**). Concentrations of NT-proBNP measured by reference assay $15C4_{63-71}$-$13G12_{13-20}$ (§) were accepted as 100% for each cell line (**B**).

Processing of proBNP expressed in HEK 293, CHO-K1 and NIH 3T3 cells (**C**). The level of processing was estimated as ratio of NT-proBNP molar concentration to total (proBNP + NT-proBNP) molar concentration in samples of conditioned media. Measured by immunoassay $5B6_{1-12}$-$13G12_{13-20}$.

Results are expressed as means ± SD (n = 3).

To confirm the influence of glycosylation on proBNP processing, we studied cleavage of recombinant proBNP expressed in HEK 293 cells by recombinant convertase furin before and after chemical deglycosylation with TFMS-method. ProBNP expressed in HEK 293 cells was used as a model in our studies because of the highest level of its glycosylation in the fragment 61-76. Chemical method was selected because of its higher efficiency than enzymatic deglycosylation. Deglycosylation level of amino acid residues in the region of cleavage site was controlled using MAbs specific to this region.

Treatment of proBNP expressed in HEK 293 cells with TFMS led to significant growth (from 2.9 to 80.5% of reference assay value) of immunochemical activity in the region 61-76 (Fig. 4A). ProBNP expressed in HEK 293 cells was hardly susceptible to furin-mediated cleavage, whereas deglycosylated proBNP was cleaved by furin up to 89% (Fig. 4B). ProBNP expressed in *E. coli* was cleaved up to 95% in the same conditions. Thus we concluded that glycosylation of proBNP significantly suppressed furin-mediated processing in vitro.

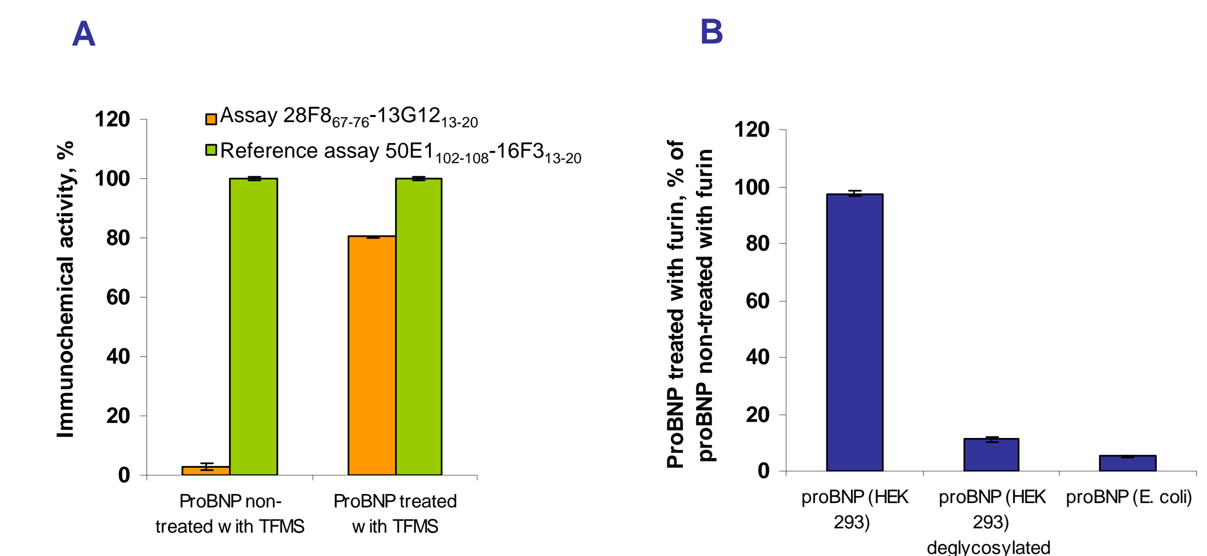

Figure 4. Chemical deglycosylation of proBNP expressed in HEK 293 cells by TFMS-method (**A**). Immunochemical activity was measured by assay $28F8_{67-76}$-$13G12_{13-20}$ specific to the region located close to the cleavage site and reference assay $50E1_{102-108}$-$16F3_{13-20}$. ProBNP concentration measured by reference assay was accepted as 100% in each sample (treated and non-treated with TFMS).

Furin-mediated cleavage of proBNP expressed in HEK 293, chemically deglycosylated proBNP expressed in HEK 293 and proBNP expressed in *E. coli* (**B**). The level of furin-mediated cleavage was estimated as ratio of proBNP concentrations in samples after and before furin treatment. ProBNP concentration in the samples was measured by assay $50E1_{102-108}$-$16F3_{13-20}$.

Results are expressed as means ± SD (n = 3).

Conclusions

We conclude here that glycosylation of amino acid residues in the region located close to the cleavage site of proBNP molecule suppresses proBNP processing *in vivo* and *in vitro*.

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