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Processing of brain natriuretic peptide precursor (proBNP) is affected by threonine 71 glycosylation

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Introduction

Brain natriuretic peptide (BNP) is a peptide hormone that promotes vascular relaxation, lowers blood pressure, increases natriuresis and diuresis. Active BNP is released into circulation in the process of enzymatic cleavage of the precursor molecule proBNP. Cleavage site is located between amino acid residues (aar) 76 and 77 of proBNP sequence. N-terminal part of proBNP (NT-proBNP) is produced together with BNP at equimolar amounts (Fig. 1).

BNP concentration is markedly elevated in the blood of patients with heart failure (HF). Recently it was shown that in HF patients some portion of synthesized proBNP is not processed and can be found in circulation in considerable amounts. The reason for incomplete processing of proBNP is still unknown. Investigations of proBNP processing are important as processing impairment could be associated with HF development. The aim of the present study was to investigate the factors that influence proBNP processing.

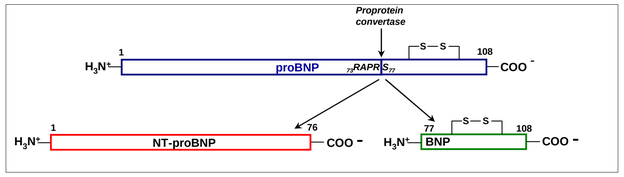

Figure 1. Schematic representation of proBNP processing. Convertase-dependent cleavage of proBNP gives rise to N-terminal fragment (NT-proBNP, 76 aar) and biologically active C-terminal part (BNP, 32 aar).

Materials and methods

Human recombinant NT-proBNP and proBNP expressed in *E. coli* (originally non-glycosylated polypeptides) were from HyTest. The antigens were used as calibrators in immunoassays.

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

Sandwich immunofluorescent assay (IFA). Capture antibodies, 2 µg per well in 100 µL of PBS, were incubated in immunoassay 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 incubation for 30 min at room temperature, 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 and NT-proBNP from conditioned media (HEK 293 cells transiently transfected with human proBNP cDNA) were characterised 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.
For NT-proBNP measurements conditioned media was passed through affinity matrix to remove proBNP. The same panel 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.

ProBNP expression plasmids. The full-length human proBNP was expressed in mammalian cells without tags and any other modifications. Plasmids expressing human proBNP mutants T71A, S77A, S84A were constructed by site-directed mutagenesis using full proBNP sequence.

Cell culturing and transfection. HEK 293 (human embryonic kidney cell line) was obtained from the American Type Culture Collection. Cells were cultured in DMEM supplemented with 10% FBS. Transient transfection of cells was performed using cationic lipid-based transfection reagent.

Estimation of proBNP processing level in HEK 293 cells. We calculated the level of proBNP processing as ratio of NT-proBNP molar concentration to total (proBNP + NT-proBNP) molar concentration measured in samples of 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 in the samples after removal of proBNP (passed through affinity matrix; 99% of proBNP was extracted).

Gel filtration studies. Conditioned media of HEK 293 cells transfected with WT or mutated proBNP (T71A variant) were loaded onto Superdex 75 10/300 GL column. The column was equilibrated with 0.1 mol/L potassium phosphate buffer, pH 7.4, containing 0.7 mol/L NaCl, 5 mmol/L EDTA, 0.1% Tween 40. BNP immunoreactivity in samples and fractions was analyzed using $24C5_{87-98}$ – Ab-BNP2 assay, which enables to determine both proBNP and BNP with high sensitivity.

Results and discussion

As it has been recently demonstrated by our group, the fragment of endogenous proBNP from 61 to 76 aar in contrast to the same region of NT-proBNP, is not recognized by region-specific MAbs. Since proBNP was shown to be an O-glycoprotein (Schellenberger et al. 2006), we hypothesized that different levels of glycosylation of this particular region in endogenous proBNP and NT-proBNP molecules could be the reason for difference in immunoreactivity. To examine this hypothesis, proBNP purified by affinity chromatography 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 epitopes located in the region of cleavage site (61-76) (data not shown).

When proBNP was expressed in HEK 293 cells by means of transient transfection, both proBNP and products of its processing – BNP and NT-proBNP – were detected in conditioned media. We have compared the immunoreactivity profiles of recombinant proBNP and NT-proBNP using MAbs, specific to different regions of proBNP molecule. Similarly to endogenous peptides, in recombinant NT-proBNP C-terminal part of the molecule was not glycosylated (the region was accessible for region-specific MAbs), whereas the same region of proBNP molecule was heavily glycosylated (the region was inaccessible to region-specific MAbs) (Fig. 2).

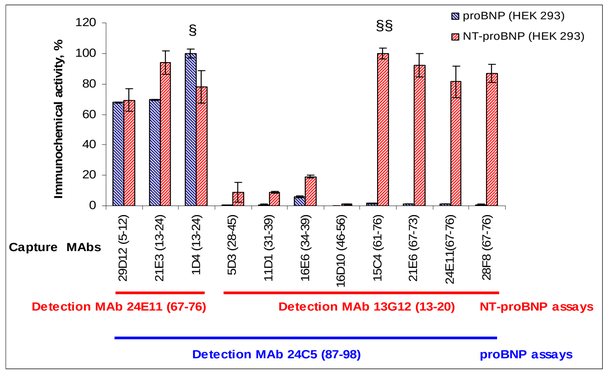

Figure 2. Immunochemical activity profiles of recombinant (expressed in eukaryotic cell line HEK 293) proBNP and NT-proBNP. Concentrations of proBNP measured by reference assay $1D4_{13-24}$-$24C5_{87-98}$ (§) was accepted as 100%. Concentrations of NT-proBNP measured by reference assay $15C4_{63-71}$-$13G12_{13-20}$ (§§) was accepted as 100%. Results are expressed as mean ± SD (n = 3).

Corroborating this assumption, we supposed that O-glycosylation of amino aside residues located close to the cleavage site of proBNP could be involved in the regulation of proBNP processing. To confirm this hypothesis, we consequently substituted possible sites of O-glycosylation – T71, S77 and S84 residues, located close to site of proteolysis, to alanine by the method of site-directed mutagenesis (T71A, S77A and S84A variants, respectively). HEK 293 cells were transiently transfected with constructs containing WT or mutated proBNP and proBNP processing rate was determined (Fig. 3).

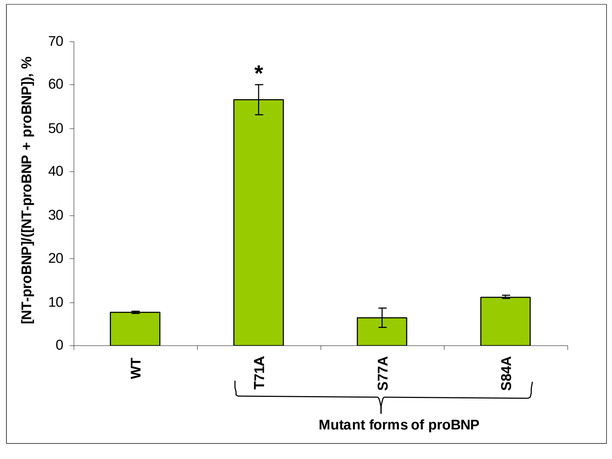

Figure 3. Processing of mutant forms of proBNP in HEK 293. The level of processing is estimated as ratio of NT-proBNP molar concentration to total (proBNP + NT-proBNP) molar concentration in samples of conditioned media from HEK 293 transiently transfected with WT, T71A, S77A and S84A mutant forms of proBNP. Measured by immunoassay $5B6_{1-12}$-$13G12_{13-20}$. Results are expressed as means ± SD (n = 3).
*- Significant difference, P<0.01, T71A *vs.* WT.

In comparison with WT proBNP (processing rate 7.6%), T71A variant exhibited markedly increased processing level (processing rate 56.7%). There was no significant difference between WT, S77A, and S84A variants (processing rate 7.6, 6.4 and 11.2%, respectively). Importance of T71 O-glycosylation for proBNP processing was confirmed in gel filtration studies. Proteins from the conditioned media after cell transfection with WT or T71A proBNP were separated by gel filtration fractions were analyzed for BNP immunoreactivity using assay $24C5_{87-98}$ – Ab-BNP2 (Fig. 4).

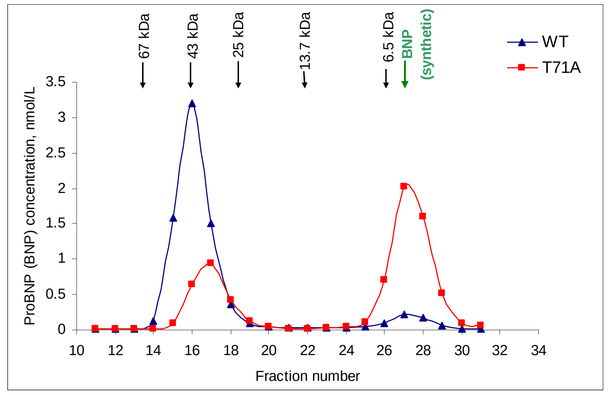

Figure 4. BNP immunoreactivity in fractions. Proteins from conditioned media of HEK 293 cells transiently transfected with WT proBNP and T71A variant were separated by gel filtration on Superdex 75 10/300 GL column. Immunoreactivity was measured by assay $24C5_{87-98}$-Ab-BNP2. Arrows mark molecular weight standards and peak immunoreactivity of synthetic BNP.

WT and T71A proBNP processing levels measured by BNP formation in gel filtration studies, 8.2 and 69%, respectively, were very similar to the corresponding processing levels measured by NT-proBNP formation (7.6 and 56.7%, respectively) (Fig. 3). Light shift of immunoreactivity peak maximum (T71A variant regarding to WT) towards proteins with lower molecular weights was observed. This difference corresponding to ~7 kDa could reflect the size of O-glycan attached to T71 distinguishing WT from T71A proBNP.
So, results of experiments with proBNP mutants confirm our hypothesis that O-glycosylation of T71 residue of proBNP negatively influence proBNP processing. We suggest that proprotein convertase is able to process proBNP non-glycosylated at T71 but it fails to process T71-glycosylated proBNP effectively.

Conclusions

We conclude here that O-glycosylation of T71 residue of proBNP is a considerable barrier in the natural proBNP processing. We suppose that O-glycosylation-dependent inhibition of proBNP processing can be of great clinical value and should be comprehensively investigated in future studies.

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