Introduction

NT-proBNP and BNP are established plasma markers of heart failure (HF). Maturon BNP is formed in the process of specific cleavage of its precursor proBNP in the site R108/NT-proBNP is produced along with BNP at the equimolar amounts. Proprotein converter 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 phenomenon is still unknown. It has been demonstrated that proBNP from HF patients’ plasma is oligo- glycoprotein. In the current study we investigate glycosylation level of proBNP in the region located close to the cleavage 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. MAb epitope corresponding to proBNP amino acid sequence was designated as subscript (24C5-60, 13G12-20, 5D3-28, 13G12-45, 1D4-13, 1D1-24, 6D3-34, 16D10-46).

Human recombinant NT-proBNP and proBNP expressed in E. coli were used as controls in immunoblot analysis. Both proteins were obtained from HyTest.

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

To prepare affinity matrix for proBNP extraction two MAb specific to the C-terminal part of proBNP (24C5-60 and 16D10-60) were coupled with Sepharose CL 4B.

Enzymatic deglycosylation of proBNP

Endogenous proBNP from plasma was incubated with either an enzyme mixture (endo-N-acetylgalactosaminidase, N-acetylneuraminidase, β-N-acetylhexosaminidase, β-N-acetylgalactosaminidase) or without enzymes for 1.5 hours at 37°C.

Sandwich immunofluorescent assay (IFA)

Capture antibodies (2 µg per 100 µL of PBS) were incubated in 96-well plates for 30 min at room temperature. After washing, 50 µL of tested sample or control and 50 µL of detection antibodies (1:200, diluted in stable europium (III) chelate in assay buffer) were added. After 30 min incubation the plates were washed, the enhacement solution was added, and fluorescence was measured.

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

ProBNP from HF patients’ plasma or conditioned media was characterized using eleven two-site MAb combinations. Capture antibodies were specific to different epitopes covering whole NT-proBNP molecule (29D5-12, 1D1-12, 21E6-10, 15C4-25), NT-proBNP (61E6-61, 11E6-61, 61E6-10, 11E6-10, 21E6-241E6), proBNP (24C5-60, 13G12-20, 5D3-28) specific to the C-terminal part of proBNP. The same set of capture antibodies was used to detect proBNP in the same plasma or conditioned media from transfected cells were used as a source of endogenous (recombinant) proBNP. The presence or absence of endogenous glycose sequence were designated as subscript (24C5-60, 13G12-20, 5D3-28).

Detection antibodies were used for detection of proBNP or NT-proBNP concentrations in plasma samples by direct and sandwich assay. Detection antibodies were used in combinations with capture antibodies specific to region 28-76 of NT-proBNP (Fig. 1B).

We have compared the immunoreactivity profiles of proBNP and NT- proBNP from HF patients’ plasma using MAb specific to different regions of NT-proBNP molecule. The profile of immunoreactivity activity for both molecules was similar with the exception for the region 61-76 located close to the cleavage site (Fig. 2A). The region 61-76 of proBNP from HF patients’ plasma was more sensitive to all used antibodies, with proBNP treatment with glycosidases NT-proBNP was not glycosylated (Fig. 3B). The level of proBNP processing in CHO-K1 media was estimated as ratio of proBNP concentrations in samples after and before furin treatment.

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 glycosylate 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. The level of proBNP processing for the protein expressed in NT-proBNP was studied for three mammalian cell lines (HEK 293, CHO and NIH 3T3). Level of proBNP glycosylation was estimated as ratio of the level of cleavage site varied significantly the protein expressed in different cell lines (Fig. 3A). The highest level of processing 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. The level of proBNP processing in HEK 293 media was estimated as ratio of proBNP concentrations in samples before and after furin treatment. ProBNP concentration in the samples was measured by assay 5B6 (recombinant) proBNP expressed in HEK 293 and chemically deglycosylated proBNP expressed in CHO-B and proBNP expressed in CHO-A.

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 vitro and in vivo.