AACC Annual Meeting, July 21, 2009, Chicago, IL, Poster B-67

New insights into human proBNP processing: the evidence for furin-mediated proBNP processing *in vitro*

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Introduction

Brain natriuretic peptide (BNP) is a peptide hormone that acts to decrease systemic vascular resistance and central venous pressure and to increase natriuresis. BNP is secreted into the circulation by cardiomyocytes. Active BNP-32 hormone (32 amino acid residues, aar) is formed from the precursor molecule, proBNP (108 aar), along with N-terminal fragment (NT-proBNP; 76 aar), by specific enzyme cleavage. The cleavage site of the proBNP molecule is located between amino acid residues Arg76 and Ser77 (Fig. 1).

Recently it was shown that in HF patients unprocessed proBNP can be found in circulation in considerable amounts. The reason for incomplete processing of proBNP is still unknown.

The investigations of proBNP processing are important as processing impairment could be associated with HF development. It is not clear which enzyme cleaves proBNP *in vivo*. At present 2 proprotein convertases, furin and corin, are discussed as possible candidates responsible for proBNP processing. The aim of the present study was to investigate the role of furin in proBNP processing.

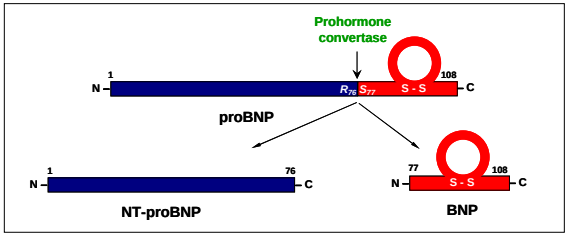

Figure 1. Schematic representation of proBNP processing.
Convertase-dependent cleavage of proBNP gives rise to N-terminal fragment (NT-proBNP, 76 aar) and biologically active C-terminal part (BNP, 32 aar).

Materials and methods

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

Human recombinant proBNP expressed in *E.coli* (originally non-glycosylated polypeptide) was also from HyTest (Finland). The antigen was used as calibrator in immunoassays.

Sandwich immunofluorescent assay (IFA). Capture antibodies, 1 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.

ProBNP and furin expressing plasmids. cDNA encoding full-length human precursor proBNP was cloned into mammalian expression vector pCMV/myc/cyto (Invitrogen) without tags or any other modifications. Plasmid for expression of human proBNP T71A variant was constructed by site-directed mutagenesis using full proBNP sequence as a template. Plasmid for furin (human) expression was purchased from Genecopeia (USA).

Estimation of proBNP processing level in LoVo and 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 analytes glycosylation, using MAb $21E3_{13\text{-}24}$ as capture and MAb $29D12_{5\text{-}12}$ as detection. NT-proBNP concentration was measured by the same assay in the samples after removal of proBNP (passed through ant-BNP affinity matrix; 99% of proBNP was extracted).

Mass spectrometry analysis. MALDI-TOF-MS was performed using the Ultraflex II TOF/TOF mass spectrometer (Bruker Daltonics, Germany).

Results and discussion

Recently we have demonstrated that processing of human proBNP is suppressed by O-glycosylation (Semenov *et al.* 2009). Being expressed in HEK 293 cells, only the proBNP variant with substitution Thr71→Ala71 (***T71A variant***) was effectively processed in cells due to the lack of O-glycosylation at Thr71, located close to the cleavage-site region, as substitution of alanine for serine prevents O-glycosylation. The fact that proBNP processing is inhibited by O-glycosylation enables us to use T71A variant of proBNP in further experiments on proBNP processing.

To elucidate the role of furin in proBNP processing, we expressed proBNP (T71A variant) in ***furin-deficient*** cell line LoVo by means of transient transfection and estimated the level of proBNP processing. We demonstrated that processing of recombinant proBNP (T71A variant) expressed in LoVo cells was inefficient. However, when proBNP (T71A variant) was coexpressed in LoVo cells with furin, it was effectively cleaved to form NT-proBNP and BNP-32 (**Fig. 2**).

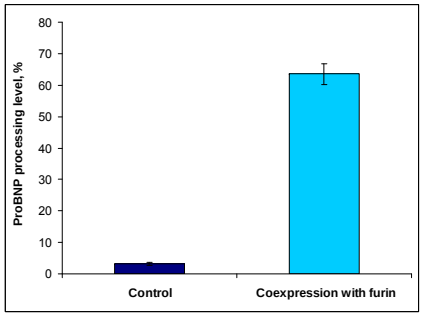

Figure 2. Processing of proBNP (T71A variant) in furin-deficient LoVo cells.
The level of proBNP processing is estimated as ratio of NT-proBNP molar concentration to total (proBNP + NT-proBNP) molar concentration in samples of conditioned media from LoVo cells transiently transfected with proBNP ("Control") or cotransfected with human furin and proBNP ("Coexpression with furin"). Results are expressed as means ± SD (n = 3).

To show that reduction of furin level in HEK 293 cells (***normally expressing furin***) or inhibitor-dependent reduction of furin activity in the cells leads to the reduction in proBNP processing level, the cells expressing recombinant proBNP were transfected with furin-specific siRNA (Santa Cruz Biotechnology, USA) or treated with furin inhibitor Dec-RVKR-CMK (Enzo Life Sciences, Inc, USA) and the level of proBNP processing was estimated. We showed that siRNA-mediated furin suppression as well as inhibition of furin activity led to significantly impaired proBNP processing in HEK 293 cells (**Fig. 3**).

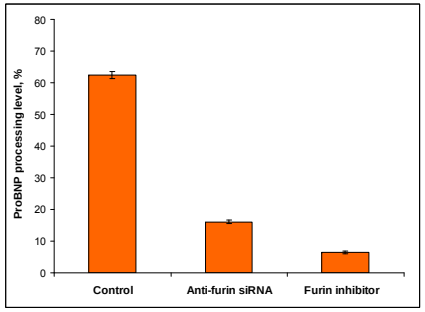

Figure 3. The influence of anti-furin siRNA and furin inhibitor on proBNP (T71A variant) processing in HEK 293 cells.
The level of proBNP processing is estimated as ratio of NT-proBNP molar concentration to total (proBNP + NT-proBNP) molar concentration in samples of conditioned media from HEK 293 transiently transfected with proBNP ("Control"), cotransfected with anti-furin siRNA (50nM) and proBNP ("anti-furin siRNA") or treated with furin inhibitor Dec-RVKR-CMK (60 µM) after proBNP transfection ("Furin inhibitor"). Results are expressed as means ± SD (n = 3).

Gel filtration studies. The negative influence of anti-furin siRNA or furin inhibitor on proBNP processing in HEK 293 cells was confirmed in gel filtration studies. Proteins from the conditioned media of cells transfected with proBNP (T71A variant), cotransfected with anti-furin siRNA or treated with furin inhibitor Dec-RVKR-CMK after proBNP transfection were separated by gel filtration and fractions were analyzed for BNP immunoreactivity (**Fig. 4**). ProBNP processing level measured by BNP formation in gel filtration studies was very similar to the corresponding processing levels measured by NT-proBNP formation (compare **Fig. 4** and **Fig. 3**).

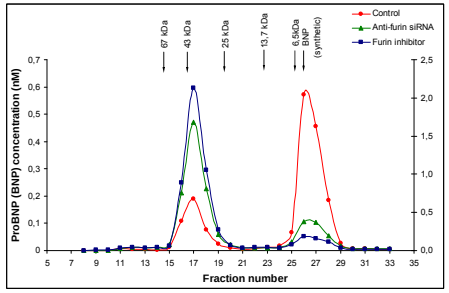

Figure 4. BNP immunoreactivity in fractions after gel filtration.
Separation was performed on Superdex 75 10/300 GL column. BNP-immunoreactivity was measured by assay $24C5_{87\text{-}98}$-Ab-BNP2 (Tamm *et al.* 2008). Arrows mark molecular weight standards and peak immunoreactivity of synthetic BNP. Left Y-axis – corresponds to "control" and "anti-furin siRNA"samples; righ Y-axis – corresponds to "furin-inhibitor" sample.

MALDI-MS analysis. To demonstrate that proBNP cleavage by furin is site-specific and results in formation of BNP-32 molecule, we analyzed the products of *in vitro* proBNP cleavage by mass-spectrometry. Recombinant proBNP expressed in *E.coli* and endogenous proBNP non-glycosylated in 63-76 a.a.r. region were treated with recombinant furin. MALDI-MS analysis revealed that furin-mediated cleavage of recombinant proBNP expressed in *E.coli* as well as of endogenous proBNP led to the specific formation of BNP-32 (**Fig. 5**).

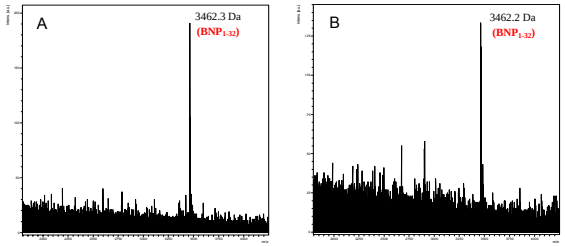

Figure 5. MALDI-TOF mass from samples of recombinant proBNP expressed in *E.coli* (A) or endogenious proBNP non-glycosylated in 63-76 aar region (B) treated with recombinant furin.

Conclusions

In the present study we demonstrated that furin is able to cleave recombinant and endogenious proBNP specifically with formation of NT-proBNP and BNP-32. We conclude here that the data obtained in the study confirm the hypothesis of furin-mediated proBNP processing.

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