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The Role of Furin and Corin in Processing of Human B-type Natriuretic Peptide Precursor

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Introduction

B-type natriuretic peptide (BNP) and its N-terminal fragment (NT-proBNP) are the products of the enzyme-mediated cleavage of their precursor molecule, proBNP (Fig. 1). The clinical significance of proBNP-derived peptides as biomarkers of heart failure (HF) has been explored thoroughly, whereas little is known about the mechanisms of proBNP processing itself. It is still unknown what protease(s) is responsible for proBNP processing. However, the comprehensive assessment of proBNP processing mechanisms could be of value for better understanding of HF development and reliable interpretation of the results of BNP, NT-proBNP and proBNP measurements.

The aim of the present study was to analyze the role of two candidate convertases, furin and corin, in human proBNP processing and determine the specificity of the proBNP cleavage by the designated convertases.

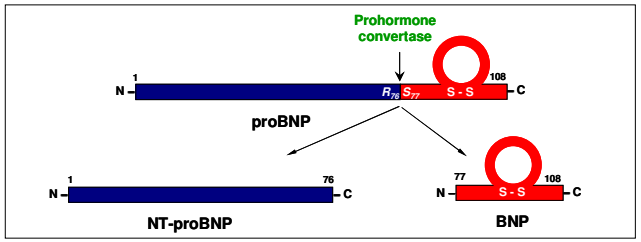

Figure 1. Schematic representation of proBNP processing.
Convertase-dependent cleavage of proBNP gives rise to N-terminal fragment (NT-proBNP, 76 AAR) and biologically active C-terminal part (BNP, 32 AAR).

Materials and methods

Monoclonal antibodies (MAbs) specific to human proBNP were from HyTest (Finland). The MAbs' epitopes, corresponding to different fragments of proBNP, are indicated by subscripts (e.g. $24C5_{87\text{-}93}$).

ProBNP, furin and corin-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. Plasmids for human furin and corin expression were purchased from Genecopeia (USA).

Sandwich immunofluorescent assay (IFA). For protein immunodetection several in-house sandwich IFAs, utilizing MAbs with different epitope specificity, were developed. Human recombinant proBNP expressed in *E. coli* (HyTest) was used as calibrator in all IFAs.

Estimation of proBNP processing level in HEK 293 cells. The level of proBNP processing was calculated as the 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 anti-BNP affinity matrix).

Extraction and fractionation of endogenous proBNP from plasma of HF patients. Extraction of endogenious proBNP from pooled plasma of HF patients (12 samples) was performed using affinity matrix containing immobilized MAbs specific to the BNP-part of proBNP molecule (MAbs $24C5_{87\text{-}93}$ and $50E1_{102\text{-}108}$). Fraction of endogenous proBNP non-glycosylated in 67-76 AAR region (cleavage-site region) was obtained by using affinity matrix containing immobilized MAbs specific to 67-76 AAR region of proBNP molecule ($24E11_{67\text{-}76}$), whereas proBNP glycosylated in 67-76 AAR region was purified from flow-through fraction.

ProBNP cleavage by recombinant furin. Human recombinant furin (1 U; Sigma–Aldrich) was added to the sample containing 4 ng of proBNP. The cleavage reaction was performed for 3.5 h at 37 °C. The reaction was stopped by freezing the samples at -70 °C. The level of furin-mediated cleavage was estimated as the ratio of the proBNP concentration in furin-treated samples to the concentration in control samples. The proBNP concentration was measured by the proBNP-specific sandwich-IFA ($50E1_{102\text{–}108}$-$16F3_{13\text{–}20}$).

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

Results and discussion

O-glycosylation and proBNP processing. As we have recently demonstrated (***Semenov et al., 2009***), the processing of human proBNP is inhibited by O-glycosylation of the threonine 71 (T71) residue located close to the cleavage site. So, in the current study we used nonglycosylated proBNP (expressed in *E. coli*) and the proBNP-T71A variant, along with the recombinant wild type (proBNP-WT), in the experiments on proBNP processing. Of note, the proBNP-T71A variant has no O-glycans linked to threonine 71, because the substitution of alanine for threonine prevents O-glycosylation at this site.

ProBNP-WT processing in HEK 293 cells. proBNP-WT was coexpressed in HEK 293 cells either with human furin or corin by means of transient transfection and the rate of proBNP processing was analysed. As follows from Fig. 2, coexpression of proBNP with furin or corin increased the rate of proBNP processing up to 3.1- and 2.2-fold respectively. The obtained results indicate that both furin and corin could be involved in the proBNP processing pathway. However due to the suppression action of O-glycans bound to T71 residue the increase in the rate of proBNP processing was not prominent.

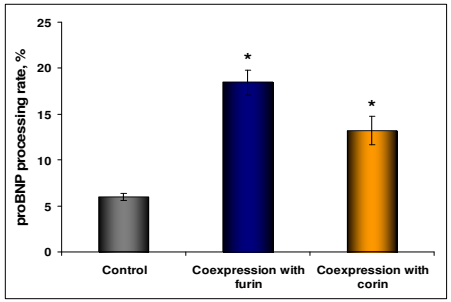

Figure 2. Processing of proBNP in HEK 293 cells.
Significant differences (*P<0.001) in proBNP-WT processing for the cells cotransfected with the furin or corin-expressing plasmid compared to noncotransfected cells. Data are presented as mean±SD (n=3).

Analysis of corin-mediated proBNP processing. In the current study the influence of proBNP glycosylation on corin-mediated processing was analysed. Non-glycosylated (*E. coli*) and two glycosylated (proBNP-T71A and proBNP-WT expressed in HEK 293 cells) forms were added respectively to the culture media of HEK 293 cells transfected or not with the plasmid expressing human corin and incubated for 3 hours. As follows from Fig. 3, corin was able to proces nonglycosylated proBNP (40.7±1.1%) and proBNP-T71A (11.7±1.3%), whereas proBNP-WT *completely* glycosylated in the cleavage site region was unsusceptible to corin-mediated cleavage. These results are in good agreement with those that were previously demonstrated for furin (***Semenov et al., 2009).***

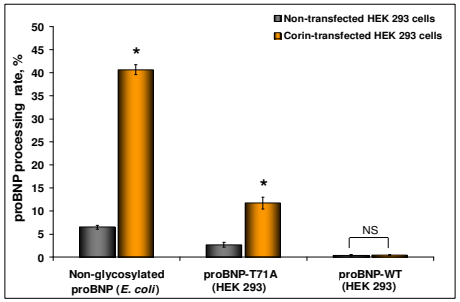

Figure 3. Corin-mediated cleavage of recombinant proBNPs.
The rate of proBNP processing was measured in the samples of conditioned media from HEK 293 cells. Significant differences in proBNP processing in transfected and non-transfected cells are indicated (*P<0.001). Data are presented as mean±SD (n=3). NS indicates difference is not statistically significant.

Gel filtration and mass-spectrometry studies. Gel filtration and mass-spectrometry (MS) were applied to analyze BNP forms obtained after corin and furin-mediated cleavage of nonglycosylated proBNP (*E. coli*). The presence of the truncated BNP form in the case of the corin-mediated processing of recombinant proBNP was revealed by GF studies (Fig. 4). MS analysis showed that this form corresponds to BNP 4-32. Furin-expressing HEK 293 cells were also able to process non-glycosylated proBNP (*E. coli*) at the cell surface resulting in BNP 1-32 formation. The ability of corin to produce BNP 4–32 from BNP 1–32 molecule was also tested. No detectable BNP 4 –32 formation was observed in this case.

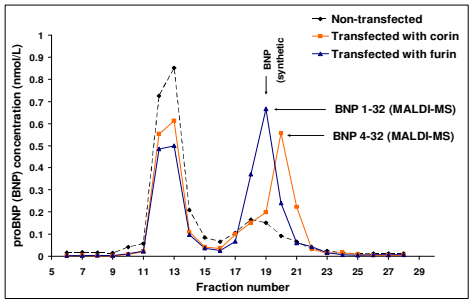

Figure 4. Analysis of corin and furin-mediated cleavage of recombinant proBNP (*E. coli*) by gel-filtration and MALDI-mass-spectrometry.
Proteins from the culture media samples obtained after the incubation of proBNP (*E. coli*) with HEK 293 cells transfected as indicated were separated on Superdex Peptide colum. BNP-immunoreactivity in fractions was measured by assay $24C5_{87\text{-}93}$-Ab-BNP2 (***Tamm et al. 2008***).

Furin-mediated processing of endogenous proBNP. Affinity chromatography was used to purify two forms of endogenous proBNP: glycosylated and nonglycosylated (~70% and ~30% of total proBNP, respectively) in the region, located close to the cleavage site. The ability of these proBNP fractions to be processed by furin was tested. Endogenous proBNP glycosylated in the cleavage site region was hardly susceptible to furin-mediated cleavage, whereas up to 91±1.5% of molecules were cleaved by furin in case when proBNP was not glycosylated in the designated region (Fig. 5). About 30% of proBNP were processed when total fraction of endogenous proBNP was incubated with furin. That is in good agreement with the content of nonglycosylated proBNP in the cleavage site region. MS analysis revealed that the furin-mediated cleavage of endogenous proBNP resulted in the BNP 1-32 formation.

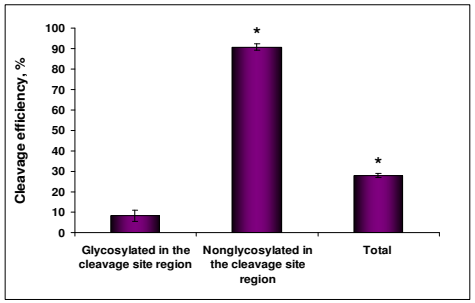

Figure 5. Furin-mediated processing of endogenous proBNP from plasma of HF patients.
Significant differences was observed (*P<0.001) for the proBNP nonglycosylated in the cleavage site region and total proBNP compared to the proBNP glycosylated in the cleavage site region. Data are presented as mean±SD (n=3).

Conclusions

Both furin and corin could be involved in the proBNP processing pathway, giving rise to distinct BNP forms: BNP 1-32 (furin) and BNP 4-32 (corin). The phenomenon of the existance in circulation of unprocessed proBNP that could be processed by the endogenous convertase(s) should be further investigated for better understanding the BNP physiology.

References:

Semenov et al. Processing of pro-brain natriuretic peptide is suppressed by O-glycosylation in the region close to the cleavage site. Clin Chem 2009;55:489-98.

Tamm et al. Novel immunoassay for quantification of brain natriuretic peptide and its precursor in human blood. Clin Chem 2008;54:1511-8.

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HyTest