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Pro-Atrial natriuretic peptide (proANP) as a stable circulating ANP form that is not affected by neprilysin-mediated cleavage

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Introduction

A novel heart failure drug named Entresto™ (LCZ696), which combines a neutral endopeptidase (neprilysin, NEP) inhibitor and an inhibitor of angiotensin II receptor, has been suggested to augment circulating natriuretic peptides concentrations, A-type and B-type natriuretic peptides (ANP and BNP), which are involved in maintaining cardiorenal homeostasis (see Figure 1). Both ANP and BNP are synthesized in the form of a prohormones, proANP and proBNP. Previously we have shown that, in contrast to BNP, its precursor, proBNP, is not cleaved by neprilysin. ANP is known to be a highly unstable peptide and it is degraded into smaller molecular fragments mainly by neprilysin. Currently the susceptibility of proANP (which is also present in the human circulation along with ANP and exhibits diuretic and natriuretic effects) to degradation by neprilysin is uknown.

The aim of this study was to determine whether the ANP precursor, proANP, is susceptible to neprilysin-mediated cleavage and establish whether plasma proANP can benefit from the inhibition of neprilysin activity by the therapy with neprilysin inhibitors (e.g. EntrestoTM).

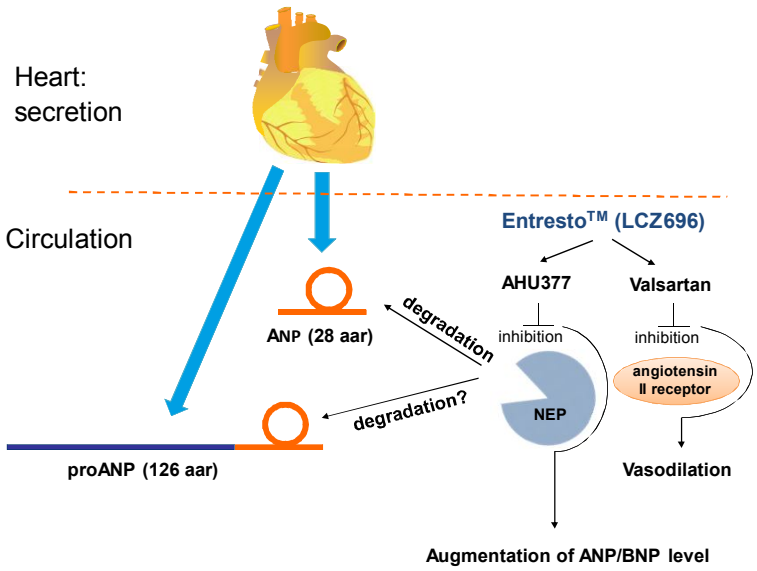


Figure 1. The mechanism of action of LCZ696 (Entresto™) and its influence on ANP/proANP degradation.

Methods

Recombinant human proANP with N-terminal SES-tag (FGRKMDR) was expressed in E. coli and purified by affinity chromatography on Sepharose coupled with 24C5 antibodies (HyTest), which are specific for the sequence of SES-tag.

Synthetic human ANP 1-28 (Bachem) and recombinant human proANP were incubated with human recombinant neprilysin (R&D Systems) at a substrate-enzyme ratio of 10:1 for different periods of time (up to 4 hours).

The susceptibility of ANP and proANP to neprilysin cleavage was analyzed using two immunoassays. In a competitive assay, an anti-ANP mouse monoclonal antibody (epitope within the ring structure, Bio-Rad) was used as a capture antibody and recombinant proANP that was labeled with europium chelate was used as a tracer. The second assay (proANP measurements only) was a sandwich-type assay with the same anti-ANP antibody used as a capture and the 24C5 antibody was used as a detection antibody. Mass-spectrometry (MS) (MALDI-TOF) was applied to determine the sites of ANP cleavage by neprilysin. A schematic representation of the two ANP/proANP assays used in this study is shown in Figure 2.

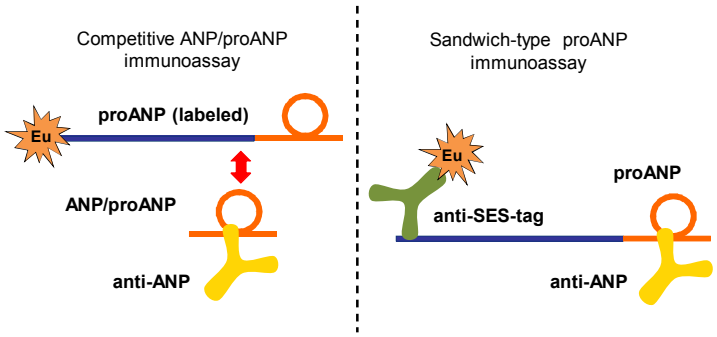


Figure 2. ANP/proANP immunoassays used in the study.

Results

As follows from the results of the immunoassay experiments (see Figure 3), proANP, in contrast to ANP, was not susceptible to neprilysin. In fact, proANP remained intact even after prolonged incubation with the enzyme (4 hours). These results were also confirmed by MS analysis (data is not shown).

The sites of in vitro ANP cleavage by neprilysin as determined by MS are shown in Figure 4. According to these data, the main sites of ANP cleavage are located within the ring structure of ANP. This explains the sensitivity of anti-ANP antibodies (which are specific to the ring structure) used in the study to the cleavage of ANP by neprilysin.

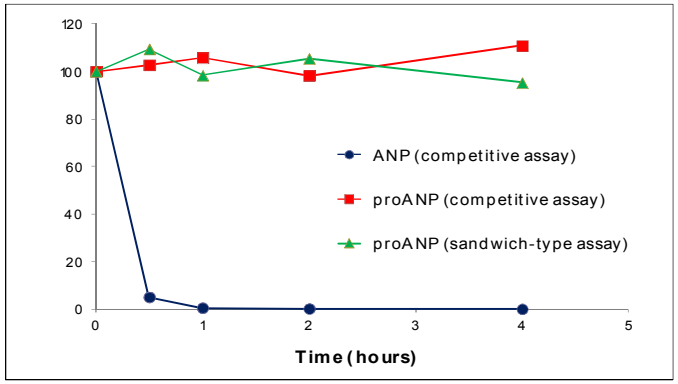
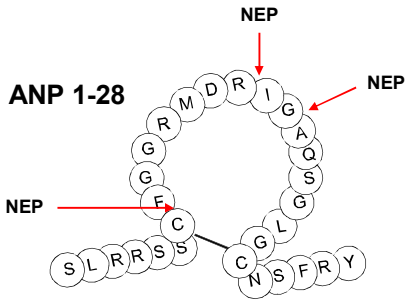


Figure 3. NEP-mediated proteolysis of ANP 1-28 and proANP 1-126.



Incubation time	Bonds hydrolyzed
0.5 h	Cys_7-Phe_8, Arg_{14}-Ile_{15}, Gly_{16}-Ala_{17}

Figure 4. Sites of ANP cleavage by NEP detected by MALDI-MS.

Conclusions

The present data suggest that proANP is not a substrate of neprilysin and, consequently, its plasma concentration should not be augmented by the inhibition of neprilysin activity. In light of this, proANP might represent a stable ANP form that is not affected by proteolytic degradation in the circulation. These results advance our understanding of the heart as an endocrine organ and the natriuretic peptide system with biological, diagnostic and therapeutic implications.

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