Biochemical and Biophysical Research Communications xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

CNP signal peptide fragments are present in the human circulation

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ARTICLE INFO

Article history:
Received 6 May 2014
Available online xxxx

Keywords:
CNP
Myocardial infarction
Biomarkers
Signal peptide
Mass spectrometry

ABSTRACT

Background: Signal peptides may be novel biomarkers in cardiovascular diseases.
Methods: We developed a novel immunoassay to the signal peptide of preproCNP (CNPsp) and used this to document circulating venous concentrations of CNPsp in normal healthy volunteers ($n = 109$), regional plasma CNPsp concentrations in patients undergoing clinically indicated catheterisation ($n = 24$) and temporal CNPsp concentrations in patients with ST-elevation myocardial infarction (STEMI) <4 h after symptom onset ($n = 8$). The structure/sequence of circulating CNPsp was confirmed by tandem mass spectrometry (MS/MS).
Results: In normal human plasma, CNPsp was detectable at levels higher than NT-proCNP (74 ± 17 vs. 20 ± 5.5 pmol/L). There was no correlation between NTproCNP and CNPsp, but plasma concentrations of sibling signal peptides – CNPsp and BNPsp – were strongly correlated ($r = 0.532$, $P < 0.001$). In patients undergoing catheterisation, there were significant arterio-venous step-ups in CNPsp concentrations across the heart ($P < 0.01$) and kidney ($P < 0.01$). Arterial concentrations of CNPsp significantly correlated with heart rate ($r = 0.446$, $P < 0.05$). In STEMI patients, plasma concentrations of CNPsp showed a biphasic elevation pattern between 6 and 12 h after symptom onset, with 12 h values significantly elevated (~3-fold) compared with levels at presentation ($P < 0.05$). MS/MS verified circulating CNPsp to be preproCNP(14–23) and preproCNP(16–23) peptides.
Conclusions: This is the first report of a circulating preproCNP derived signal peptide. Given the clear cardiac and renal secretion profiles of CNPsp and its response in STEMI patients, further studies on potential biological functions and biomarker applications of CNPsp in cardiovascular disease are warranted.

1. Introduction

The identification of biomarkers that can potentially assist in the diagnosis of cardiovascular diseases is an important area of research. Whilst protein markers such as cardiac troponin [1] and B-type natriuretic peptide (BNP) [2] have a high degree of evidence and clinical utility, there are limitations to their use and markers that could supplant or supplement them are an unmet need. Signal peptides (SP) encoded from prepro-proteins are well described arbiters of protein transport and secretion [3,4] and it was commonly viewed that after translation and release from the endoplasmic reticulum (ER), SP are degraded and recycled and do not appear in the peripheral circulation [5]. As a consequence, they have not been considered as measurable circulating targets. However, a growing body of evidence indicates that fragments of SP survive within cells after protein translation, a proportion of which reaches the circulation, raising the possibility that they may be measurable entities in disease states [6–8].

We recently provided the first demonstration of a circulating SP fragment, derived from preproBNP (BNPsp) [9]. BNPsp concentrations in plasma fragment rise very early after the onset of ST-elevation MI (STEMI) [9] and after a provocative dobutamine cardiac stress test [10]. Following this, we then reported that an SP fragment prepro A-type natriuretic peptide (ANPsp) was also present in human circulation and that plasma levels of ANPsp also rise rapidly after STEMI [11]. We now document the presence in human plasma of an SP fragment derived from the third member of the natriuretic peptide family, C-type natriuretic peptide (CNPsp). We document normal circulating levels, possible secretory sources of and the response of plasma CNPsp levels during ST-elevation myocardial infarction (STEMI). In doing so, we provide the first evidence for circulating SP from an entire peptide family as being present in the human circulation and identify their consistently modified biochemistry.

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http://dx.doi.org/10.1016/j.bbrc.2014.05.020
0006-291X/

2. Methods

2.1. Chemicals

Synthetic human CNPsp(14–23), (*Tyr*)CNPsp(14–23) and (*Cys*)CNPsp(14–23) were synthesised by Mimotopes (Melbourne, Australia) and were confirmed as greater than 95% pure by mass spectrometry.

2.2. CNPsp(14–23) assay development

Residues 14–23 of preproCNP(1–126) were chosen for antibody generation based on our previous work with BNPsp [9] and ANPsp [11]. Specific polyclonal antibodies to CNPsp(14–23) for immunoassay use were raised in sheep. Briefly, synthetic (*Cys*)CNPsp(14–23) was coupled to malemide derivatised bovine BSA and injected s.c. into three sheep every four weeks until adequate titre and sensitivity levels were obtained. Sera were screened for assay characteristics and the optimal candidate chosen (S9-B8) after 5 bleeds.

2.3. CNPsp and cardiac marker assays

For the CNPsp assay, all sample extracts, radioactive trace, standard and antiserum solutions were diluted in phosphate based immunoassay buffer [9,11]. Sample extracts were concentrated 3–5-fold, depending on the source. The primary assay incubate consisted of 50 μL of extracted sample concentrate, or standard (0–3630 pmol/L of CNPsp(14–23) peptide) combined with 50 μL of antiserum S9-B8 (1:8000 primary dilution). Antiserum S9-B8 had negligible cross-reactivity (<0.01%) with the following peptides: ANP, BNP, CNP, NTproCNP(1–15), angiotensin I, angiotensin II, endothelin I, BNPsp, ANPsp, NTproBNP(1–21), NTproANP(1–30), nor did it cross react with aspirin, clopidogrel, dobutamine or morphine medications.

The assay mixture was vortexed and incubated at 4 °C for 22 h. Following this, 50 μL of iodinated (*Tyr*)CNPsp(14–23) (3000–4000 cpm) was then added, the tubes vortexed and re-incubated for a further 24 h at 4 °C. Free and bound CNPsp were separated by solid phase second antibody method (donkey anti-sheep Sac-Cel, Immunodiagnostic Systems, Boldon, UK). Sac-Cel (1 mL) diluted in 5% dextran solution (final Sac-Cel concentration 5%) was added to each tube, the solution vortexed and incubated at room temperature for 30 min. Tubes were centrifuged at 2800*g* for 10 min at 20 °C and decanted, with the pellet counted in a Gammamaster (LKB, Uppsala, Sweden). The observed extraction efficiency of synthetic CNPsp(14–23) from plasma was ~90%. Sample assessment indicated that CNPsp immunoreactivity in assay was not altered by haemoglobin up to 8 g/L, nor by plasma lipid content up to 15 g/L.

BNPsp [9] and NT-proCNP [12] were assayed as previously described. CK-MB, myoglobin and troponin I measurements were all determined by late generation commercial assays (Abbott) in the core biochemistry lab of Canterbury Health Laboratories, Christchurch Hospital, New Zealand. Troponin I had a limit of detection of 0.01 μg/L, 99th percentile 0.028 μg/L and a co-efficient of variation <10% at 0.032 μg/L.

2.4. Human plasma sample collection

Human plasma samples were obtained from three patient groups; normal, healthy volunteers ($n = 109$), patients undergoing clinically indicated cardiac catheterisation ($n = 24$) and patients presenting to Christchurch Hospital with documented STEMI ($n = 8$). All sample collections had ethical approval from the New Zealand Health and Disability Ethics Committee and all participants gave informed consent before recruitment. All patient investigations described herein conform to the principles of the Declaration of Helsinki. Extracts of acidified human plasma (1:1 vol with 0.25 M HCl) were prepared for the measurement of CNPsp immunoreactivity using solid phase C_{18} cartridges as previously reported for BNPsp and ANPsp [9–11].

2.5. Purification, HPLC and tandem MS/MS identification of endogenous circulating human CNPsp

Immunoreactive CNPsp was purified from approximately 0.3 L of pooled EDTA plasma drawn from 5 patients with documented acute STEMI. Samples were centrifuged at 4 °C and plasma stored at −80 °C until it was slowly thawed, extracted on C_{18} cartridges and evaporated to dryness. Extracts were then reconstituted in a minimal volume of immunoaffinity buffer (20 mM Tris–HCl/0.5 M NaCl, pH 7.4), centrifuged at 10,000*g* to pellet solid debri and then combined to a single solution. This solution was run under gravity at 4 °C through an anti-CNPsp IgG (S9-B8) coupled AminoLink™ gel prepared according to the manufacturer's instructions (Pierce Biotechnology, IL). The column was then washed with a 5× volume of immunoaffinity buffer and eluted with 0.1 M glycine (pH 2.5). Elution fractions containing immunoreactive CNPsp were then submitted to liquid chromatography-coupled nanospray LTQ Orbitrap mass spectrometry (LC-LTQ Orbitrap MS), as previously described [9,11]. Briefly peptides were separated by a reversed phase gradient from 97% to 55% solvent A (0.2% formic acid water) in solvent B (0.2% formic acid in acetonitrile) over 20 min. Followed by an increase of solvent B to 99% over 5 min. using an Ultimate 3000 RSLC system (Dionex-Thermo Fisher Scientific, San Jose, CA) at a flow rate of 400 nL/min. Full MS spectra were acquired in the Orbitrap analyser (Thermo Fisher Scientific, San Jose, CA) at a resolution of 100,000 FWHM (Full Width at Half Maximum) at m/z 400. The strongest three precursor ions per scan set were used for collision induced dissociation (CID)-based MS/MS in the LTQ analyser followed by high energy CID of the same three precursors for high resolution fragment ion measurement in the Orbitrap analyser. Dynamic exclusion was enabled allowing two MS/MS acquisitions of each precursor during an exclusion period of 60 s. Raw data were processed through the Proteome Discoverer software (Thermos Scientific, San Jose, CA) and peak lists searched against the UniProt/SwissProt amino acid sequence database (downloaded November 2013, 541762 sequence entries) using the Mascot (http://www.matrixscience.com) and SEQUEST search engines. Unassigned spectra of potentially modified CNPsp were searched by screening the raw spectra for the presence of consistent fragment ions of CNPsp(14–23) containing the C-terminus (y-ion series) using the Qual Browser tool of the Xcalibur software (Thermo Fisher Scientific, San Jose, CA).

2.6. Statistics

Results are presented as mean ± SD and all statistical analysis was carried out using SPSS (IBM/SPSS, v19). Assessment of within individual and regional vascular CNPsp measurements was carried out using paired, two tailed Students *t*-test where appropriate. Relational analysis of plasma protein/peptide concentrations was done using Spearman rank order correlation testing and linear regression analysis. In all analyses, a *P*-value <0.05 was considered significant.

3. Results

3.1. Identification of endogenous CNP signal peptide immunoreactivity in human plasma

preproCNP has a signal peptide length of 23 amino acids (Fig. 1). We generated an antibody (S9-B8) directed towards the C-terminal