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C-type natriuretic peptide (CNP) signal peptide fragments are present in the human circulation

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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

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evidence for circulating SP from an entire peptide family as being present in the human circulation and identify their consistently modified biochemistry.

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.

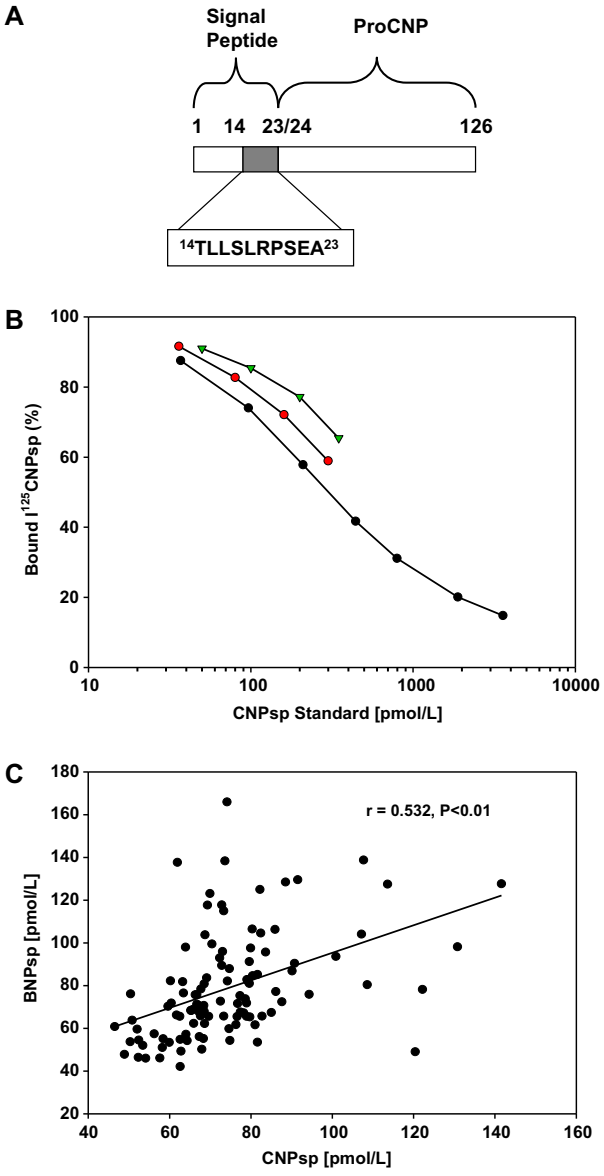

Fig. 1. (A) Schematic outlining CNPsp antibody epitope selection from preproCNP. Residues 14–23 from preproCNP(1–126) were synthesised and used to generate polyclonal antisera in sheep. Sequence given in single amino acid notation. (B) Representative standard curve (black line and circles) with comparative serial dilution of endogenous CNPsp immunoreactivity in plasma from a healthy volunteer (red circles) and patient with cardiovascular disease (green circles). (C) In normal, healthy human plasma CNPsp and BNPsp immunoreactivity displayed a moderately string positive correlation ($r = 0.532$, $P < 0.01$ on linear regression analysis, $n = 109$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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 10 amino acids of CNPsp, i.e. CNPsp(14–23) and used it to establish a sensitive, specific radioimmunoassay (Fig. 1A). This assay had a mean zero binding of 34 ± 3%, sample detection limit of 5 pmol/L, ED_{50} of 284 pmol/L and a working range of 35–1800 pmol/L in which the intra-assay CV was less than 7%. Inter-assay CVs were 22% at 200 pmol/L and 11% at 780 pmol/L. Initial assessment confirmed that CNPsp immunoreactivity was present in human plasma from peripheral blood collected into Na^3-EDTA and that it diluted in parallel with the synthetic standard curve (Fig. 1B). Mean immunoreactive CNPsp concentrations present in normal human venous plasma obtained from 109 healthy volunteers was 74 ± 17 pmol/L (range 47–142 pmol/L, 99th percentile = 130 pmol/L). Correlation analysis of plasma CNPsp with concomitant NT-proCNP levels (mean = 20 ± 5 pmol/L), age, gender and BMI revealed no significant associations, but there was a significant positive correlation with matching BNPsp concentrations ($r = 0.532$, $P < 0.01$, Fig. 1C).

3.2. Immunoreactive CNPsp is released from the human heart into the circulation

In 24 patients undergoing clinically indicated cardiac catheterisation, regional plasma sampling revealed that drainage from the heart (cardiac coronary sinus, 113.9 ± 43.9 pmol/L, $P < 0.01$) and kidney (renal vein, 104.9 ± 56.1 pmol/L, $P < 0.01$) contained significantly higher concentrations of immunoreactive CNPsp than simultaneously drawn arterial plasma samples (66.0 ± 38.3 pmol/L, Fig. 2A). There was no obvious evidence of organ based clearance of CNPsp. Both arterial and cardiac coronary sinus CNPsp

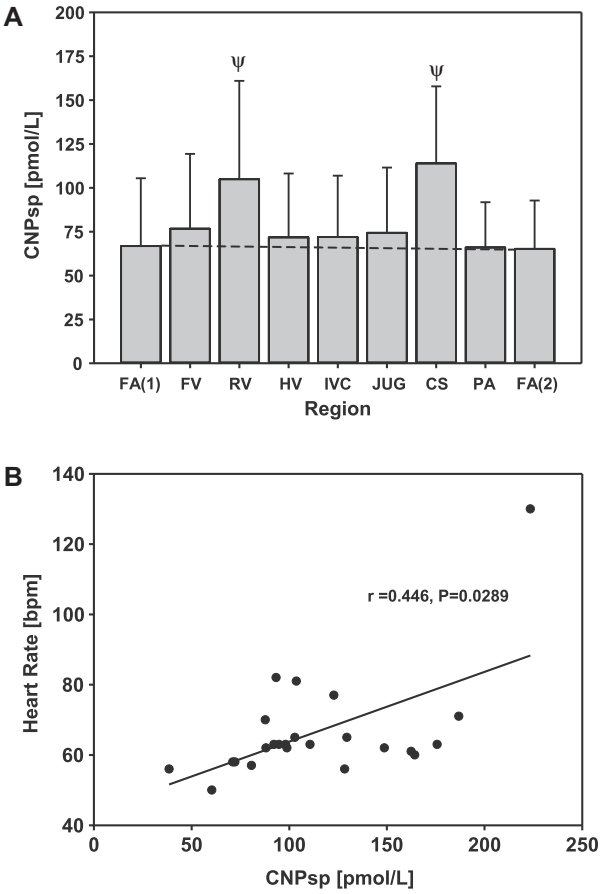

Fig. 2. (A) Regional vascular concentrations of CNPsp in 24 patients undergoing clinically indicated cardiac catheterisation. Vascular regions sampled are indicated as follows: FA1 = initial femoral artery sample, FV = femoral vein time matched to FA1, RV = renal vein, HV = hepatic vein, IVC = inferior vena cava, JUG = jugular vein, CS = cardiac coronary sinus, PA = pulmonary artery, FA2 = second (exit) femoral artery sample. Only CS and RV (ψ, $P < 0.01$) plasma had significantly elevated CNPsp concentrations above matched arterial samples. (B) Arterial CNPsp concentrations had a significant positive correlation with heart rate ($r = 0.446$, $P < 0.05$, $n = 24$) on linear regression analysis. Cardiac coronary sinus plasma CNPsp also correlated significantly with heart rate (data not shown).

concentrations in these catheterisation patients correlated positively with heart rate (both $P < 0.05$, Fig. 2B). No other significant correlations, such as ejection fraction, were observed.

3.3. Plasma CNPsp concentrations in STEMI patients

In 8 patients with documented STEMI, with chest pain onset less than 4 h duration, peripheral venous plasma concentrations of CNPsp tended to be elevated with 6 h of symptom onset (Fig. 3). Unlike BNPsp [9] and ANPsp [11], CNPsp tended to display a biphasic response during the course of STEMI, with the second peak at ~12 h after symptom onset significantly elevated compared with presentation levels ($P < 0.05$). Average peak levels (146 ± 18 pmol/L) were ~2-fold higher than the average observed in normal health, but only just beyond the 99th percentile upper limit of normal (130 pmol/L). Individual patient peak levels of CNPsp had no correlation with simultaneous CK-MB, myoglobin or troponin I (Fig. 3).

3.4. Mass spectrometric characterisation of CNPsp in human plasma

After solid phase extraction and immunoaffinity purification, analysis of the CNPsp plasma component by LC-LTQ Orbitrap MS/MS and subsequent interpretation of fragment spectra by Mascot and SEQUEST revealed a significant identification of the CNPsp(14–23) sequence TLLSLRPSEA with a Mascot ion score of 28 and a SEQUEST score (XCorr) of 2.27. We also detected the shorter sequence LSLRPSEA corresponding to CNPsp(16–23) with a Mascot ion score of 37 and a SEQUEST XCorr of 2.49. Further manual interpretation of unassigned raw spectra that contain a signature of consistent fragment ions carrying the C-terminus (y-ion series) revealed that CNPsp(14–23) was modified at or near the N-terminus (Fig. 4) with mass adducts ranging between 163 and 385 mass units. Taken together, these results confirm that our immunoassay detects authentic endogenous preproCNP(14–23) containing N-terminal modifications and partially degraded preproCNP(16–23).

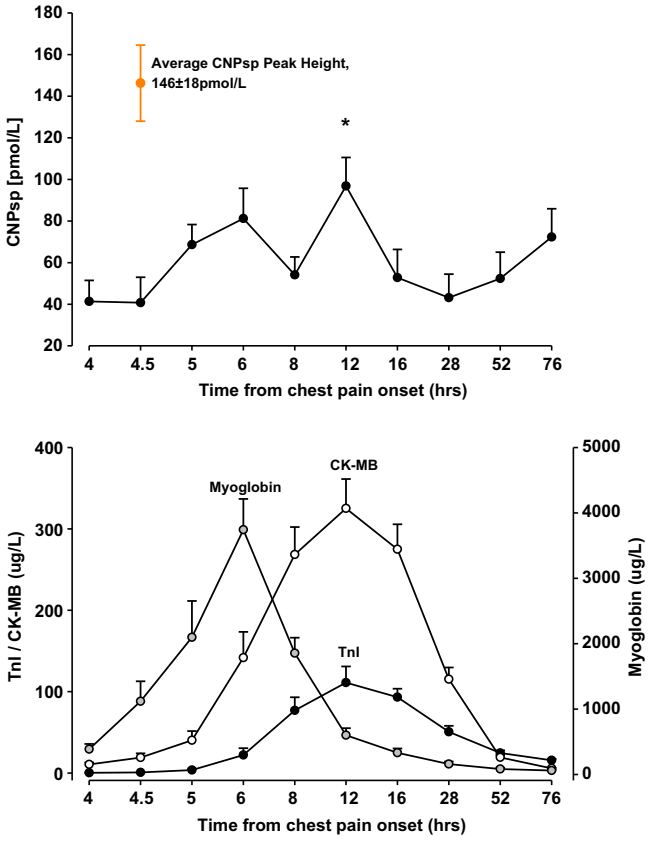

Fig. 3. Temporal, average profiles of plasma biomarkers in patients with documented ST-elevation myocardial infarction. CNPsp concentrations (upper panel) displayed a moderate bi-phasic increase with peak levels at 12 h significantly higher than those at 4 h post-symptom onset (* = $P < 0.05$). The average peak CNPsp concentration achieved in these patients was 146 ± 18 pmol/L. In contrast, myoglobin, CK-MB and cardiac troponin I (TnI, all lower panel) displayed dynamic and large increases in plasma concentrations, peaking between 6 and 12 h post-symptom onset, with peak levels between 30 and 300× outside the healthy normal range.

4. Discussion

The generation of CNPsp(14–23) and CNPsp(16–23) species from the 23 amino acid preproCNP signal peptide is consistent with our previous reports on the formation of both BNPsp and ANPsp [8,11]. Thus, distinct, 10 amino acid C-terminal entities are generated from the SP of all three natriuretic peptides with their first residue located immediately after the hydrophobic core of the full length SP. ANPsp and CNPsp share some similarity in that they can be cleaved into shorter 8 amino acid species, whereas we only detected one BNPsp species, BNPsp(17–26) [8]. The commonality of their amino acid start position suggests the C-terminus fragments of natriuretic peptide SP are cleaved from the intact SP by a common factor, most likely signal peptide peptidase (SPPase) [13]. Further cleavage of the amino terminus of ANPsp and CNPsp could be achieved by multiple candidates such as aminopeptidase but the precise enzymes responsible remain undetermined. A notable feature of all three SP fragments is that regardless of the identity of their amino terminus residue (Leu, Thr and Ala), they are all modified by different sized adducts. The modifications identified here on CNPsp(14–23) differ in mass from those identified on BNPsp and ANPsp, which in turn differ from each other [8,11]. This variation suggests either subtle differences in the mechanism of adduct processing or the source of adduct derivation and might therefore hold information about the site and mechanism of release.

In normal individuals, CNPsp concentrations showed no correlation with NT-proCNP. This finding is consistent with its related SP, BNPsp and ANPsp, [8,11] and indicates that circulating SP of the natriuretic peptides most likely enter the blood via a different mechanism from their congener propeptide siblings. It may be possible that natriuretic peptide SP enter the circulation via a similar mechanism because CNPsp had a moderate, statistically significant correlation with BNPsp. What this mechanism might be is unclear, but given that it is known that ~10% of the circulating proteome does not bear a classical SP motif and enters it via unconventional pathways [14], it will be important to identify potential mechanism related to cellular SP release.

CNP and NT-proCNP are synthesised and secreted from multiple organ sources with the heart featuring as the highest single contributing source [15]. In contrast, organ clearance of CNP may occur through the liver and kidney, whereas NT-proCNP is unlikely to rely on organ based clearance. In comparison, regional vein sampling in 24 patients indicated that CNPsp is likely to be secreted primarily from the heart and kidney, without obvious specific organ clearance. Further studies addressing this contrast would be of potential interest and importance. Arterial and cardiac coronary sinus concentrations of CNPsp had significant positive correlations with heart rate, but with no other haemodynamic parameters. It is known that CNP can stimulate cardiac rate and electrical conduction via its guanylate cyclase linked natriuretic peptide receptor-B (NPR-B) [16] and that it has overall negative inotropic action which is attenuated in hypertrophic cardiomyocytes [17]. Given the

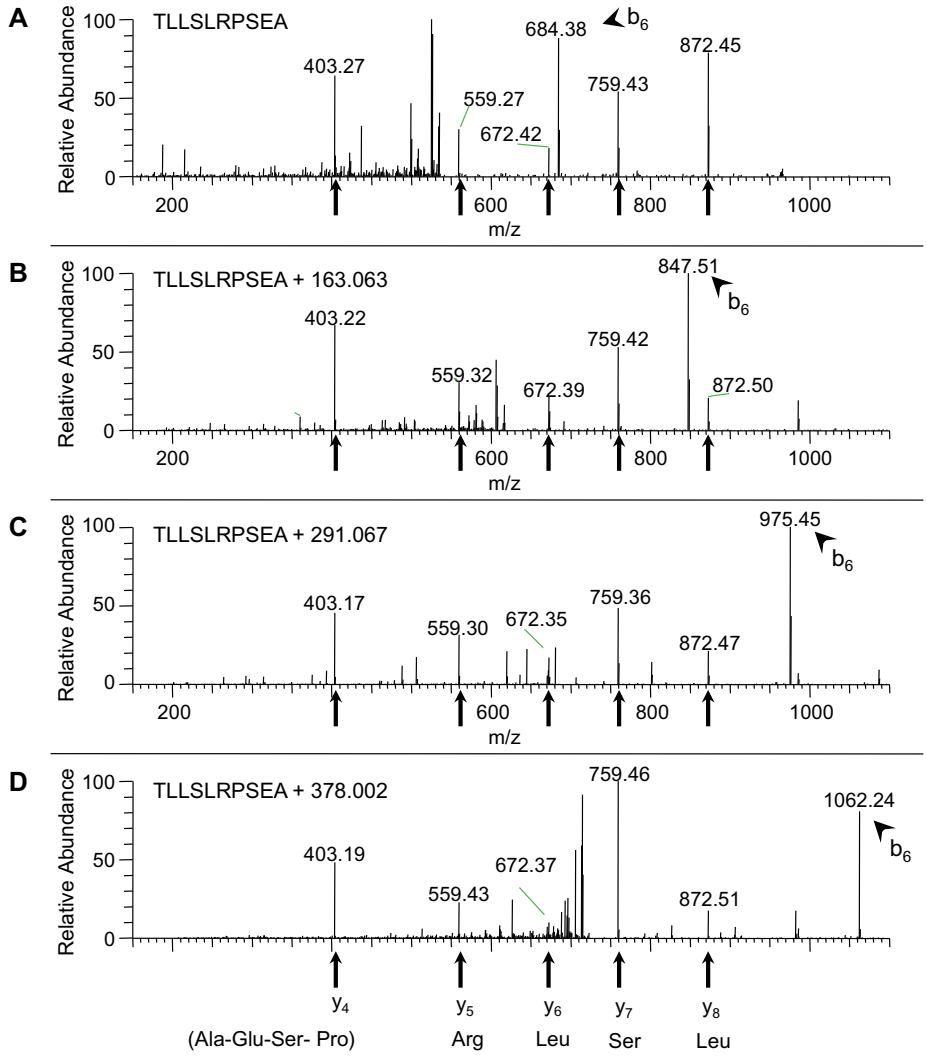

Fig. 4. Characterization of immunoaffinity purified CNPsp immunoreactivity from STEMI patient plasma by mass spectrometry. (A) The collision induced dissociation (CID) fragment spectrum of the signal at *m*/*z* 543.811 (M + H$^+$ of 1086.615) confirms the sequence *H*-TLLSLRPSEA-*OH* of CNPsp(14–23). The y-ion fragments (arrows) which carry the C-terminus cover the sequence LSLRPSEA-*OH*. The arrowhead points at the prominent b6-ion of the N-terminal fragment *H*-TLLSLR. (B)–(D) Representative examples of CID-fragment spectra of modified CNPsp(14–23) at *m*/*z* 625.343 (M + H$^+$ of 1249.678), 689.345 (M + H$^+$ of 1377.682) and 732.812 (M + H$^+$ of 1464.617). The constant y-ion series (arrows) indicates that the fragment LSLRPSEA-*OH* is unmodified. The prominent b6-ion (TLLSLR) signal however, is shifted by 163.13, 291.07 and 377.86 mass units corresponding to the mass adducts of 163.063, 291.067 and 378.002 on the respective modified CNPsp(14–23). The combination of b- and y-ion signals confines the site of modification to the N-terminus or side chains of the N-terminal residues of threonine or leucine.

comparable cardiac secretion profiles of CNP and CNPsp it will be of future interest to see if CNPsp will also have any effect upon cardiac rate or conduction.

The bi-phasic rise in plasma CNPsp in patients with documented STEMI was not as impressive or as rapid as that observed for BNPsp and ANPsp [8,11]. Like BNPsp and ANPsp, there was no correlation between peak CNPsp and concurrent troponin, myoglobin or CK-MB peaks in these patients. Taken together, it is apparent that – at least in STEMI patients – natriuretic peptide SP species are potential markers of cardiac ischaemia or dysfunction, with time courses that do not correlate with accepted necrosis markers. Further prospective recruitment studies in "real world" patients presenting with chest pain to emergency departments will be needed to identify a potential use for CNPsp measurement.

This report is limited by the fact we have studied relatively small groups of patients, especially the STEMI group. Thus, our data reporting no correlation of CNPsp with demographic parameters, the possibility of renal tubular production of CNPsp into urine and the observed relationships of CNPsp with haemodynamic and protein indices will all need to be confirmed in larger sample sets, as well as studies that determine mechanisms underlying these observations.

In conclusion, we provide the first evidence for the existence of two CNPsp fragments derived from preproCNP in the human circulation. CNPsp is potentially secreted from the heart and kidney. It is not significantly correlated to demographic parameters or to NT-proCNP. In providing this report, we have now classified an entire peptide family as producing measurable, circulating SP fragments from their prepro-peptides. All carboxyl terminus SP bear biochemical adducts to their amino terminus. Whether this reflects disease status or has any bearing upon putative biological function remains to be determined.

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Disclosures

The University of Otago, New Zealand has pending patent applications on the use of CNP signal peptide measurement in cardiovascular disorders. C.J.P and A.M.R. are listed as inventors on this application. All other authors have nothing to disclose.

Conflict of interest

None declared.

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