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Research paper

Measurement of the total proANP product in mammals by processing independent analysis

Ingrid Hunter [a,b], Jens F. Rehfeld [a], Jens P. Goetze [a,*]

[a] Department of Clinical Biochemistry, Rigshospitalet, Faculty of Health Science University of Copenhagen, Denmark
[b] Department of Small Animal Clinical Sciences, KU-LIFE, University of Copenhagen, Denmark

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ABSTRACT

The cellular processing of natriuretic propeptides is attenuated in heart disease, resulting in release of a mixture of unprocessed precursor, partially processed fragments, and the bioactive hormone. Here, we report a species-independent method for quantification of pro-atrial natriuretic peptide (proANP) and its products irrespective of variable post-translational processing. The processing-independent assay (PIA) was developed raising mono-specific antibodies against the C-terminus of sequence 1–16 in proANP. The assay procedure included plasma extraction followed by tryptic cleavage, which releases the assay epitope from the N-terminal region. The PIA was tested in elderly patients with symptoms of heart failure ($n=450$), in pigs with acute myocardial infarction ($n=21$), and in normal dogs and dogs with heart failure ($n=77$). The epitope specificity permitted reliable measurement in man, dog, cat and pig. In human plasma, the PIA correlated well with an established proANP analysis ($r=0.86$, $P<0.0001$) but with a 5.5-fold difference in plasma level ($P<0.0001$). In pigs, the PIA measured 9.2-fold higher concentrations compared to a human assay (804 versus 87 pmol/L, $P<0.0001$). The basal proANP concentration was 396 pmol/L in dogs: a dramatic increase was seen in canine heart failure. Our new processing- and species-independent proANP assay allows for the measurement of the total proANP product, irrespective of changes in post-translational maturation. We suggest that this tool should be used for comparative studies between human patients and porcine and canine models of human cardiac disease.

1. Introduction

Measurement of natriuretic peptides in plasma has gained considerable use in cardiovascular research and pathology. Peptide measurement serves as rule-out markers in heart failure and has a prognostic role in patient assessment after myocardial infarction. Although the concept of natriuretic peptides as biomarkers was first reported for atrial natriuretic peptide (ANP) (Burnett et al., 1986), the most frequently measured peptides are derived from the B-type natriuretic peptide (BNP) precursor (Goetze, 2004; Richards, 2007; Daniels and Maisel, 2007). This is often attributed to the dominant ventricular expression of the BNP gene in mammalian heart disease, even though clinical studies have established that the ANP gene products are equally useful markers (Maisel et al., 2010). Both the ANP and the BNP genes are mostly of atrial origin in normal mammalian hearts from larger animals, whereas the ventricular expression is grossly activated by disease affecting ventricular function and metabolism (Hystad et al., 2000; Christoffersen et al., 2002; Goetze et al., 2010).

Both ANP (a 28-mer) and BNP (a 32-mer) are bioactive peptides cleaved from their respective prohormones prior to cellular secretion. Like other bioactive peptides in circulation, they are rapidly eliminated. They are also chemically unstable *in vitro*, which introduces difficulties in obtaining and storing plasma. In contrast to the BNP gene, the ANP gene is structurally well conserved during evolution. The ANP precursor thus

* Corresponding author at: Department of Clinical Biochemistry, KB 3-01-1, University of Copenhagen, Rigshospitalet, 9 Blegdamsvej, DK-2100 Copenhagen, Denmark. Tel.: +45 3545 2202; fax: +45 3545 2524.
E-mail address: JPG@dadlnet.dk (J.P. Goetze).

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constitutes a suitable target for design of immunoassays that measure proANP-derived peptides across mammalian species (Fig. 1). The concept of using precursor fragments for plasma measurement is well established for proinsulin-derived peptides, *i.e.* the C-peptide (Steiner, 2004), and was later suggested for progastrin and human proANP (Bardram and Rehfeld, 1988; Chen et al., 1990). This strategy has been most successfully adapted for proBNP in routine diagnostics (Goetze, 2004; Richards, 2007; Daniels and Maisel, 2007; Maisel et al., 2008).

Recently, insight into the pathophysiology of natriuretic peptides has changed our perception of cardiac peptide synthesis and secretion. While genetically modified mice have provided new perspectives on the translational phase of gene expression for ANP, BNP, and their common receptor (Nishikimi et al., 2006), the post-translational prohormone maturation is still incompletely elucidated. Notably, the molecular forms in human plasma are currently an object of new surprises, with the latest showing the BNP precursor as an O-linked glycoprotein (Schellenberger et al., 2006; Goetze, 2010). Nevertheless, plasma measurement in experimental animals is often used interchangeably without taking into account species-dependent differences in expression and post-translational processing, as well as variable elimination of the fragments.

In order to accurately measure the ANP gene translational products across mammalian species, we designed a processing-independent immunoassay (PIA) that measures the total proANP contents in selected animals, irrespective of species-related differences in exo- and endoproteolytical cleavages and possible amino acid modifications (Rehfeld and Goetze, 2003). Moreover, the assay is calibrated against species-specific peptides, which, for the first time, allows for molar comparison of concentrations between the species.

2. Materials and methods

2.1. Peptides

ProANP-derived peptides for immunisation, tracer and calibration curves were custom synthesized (Cambridge Research Biochemicals, Cleveland, UK). For immunisation, 10 mg of porcine proANP 1–16 extended N-terminally with Cys was coupled to 25 mg bovine serum albumin (BSA) using m-Maleimido-benzoic acid N-Hydroxysuccinimide (MBS) ester conjugation method to give an N-terminally coupled conjugate. The immunisation against a porcine fragment was chosen because of the close homology to all included species (identical to feline proANP 1–16, Fig. 1). The immunisation procedure and immunoassay are hereafter referred to as a *C-terminal proANP 1–16* assay. The assay used porcine proANP 1–16 as tracer, where the Tyr residue in position 4 was used for iodination. Calibration peptides were human, feline/porcine, canine or mouse proANP 1–16, respectively. The identity and purity of all peptides was determined by mass spectrometry and amino acid analysis.

2.2. Antiserum

Eight New Zealand white rabbits were immunised with porcine proANP 1–16 extended N-terminally with Cys and coupled to BSA using a standard protocol in the department (Goetze et al., 2006). Briefly, 5 μg conjugate was dissolved in 4 mL sterile H_2O and mixed with 4 mL Freund's complete adjuvant (Statens Serum Institut (SSI), Copenhagen). A 0.5 mL sample was injected subcutaneously bilaterally in the groin of the rabbits. The animals were thereafter boosted at 8-week intervals, exchanging complete adjuvant to incomplete. Approximately 40 mL blood was collected from an ear vein 10–14 days after every boost, and the serum was stored at −20 °C for later evaluation.

2.3. Tracer preparation

Porcine proANP 1–16 was iodinated using a mild chloramine T protocol as previously described (Stadil and Rehfeld, 1972). The tracer was purified on a reversed phase high-performance liquid chromatography (RP-300 C8 column; 4,6×220 mm; Pierce/Thermo Fisher Scientific, Denmark) eluted by a linear ethanol gradient in 1% trifluoroacetic

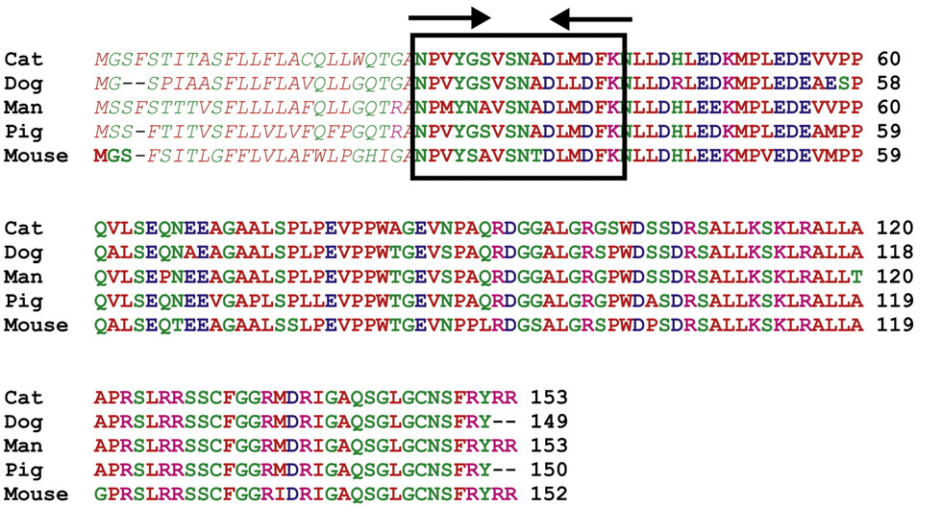

Fig. 1. Primary structure of preproANP in five mammalian species. The prepeptide/signal peptide is marked by italics, the left arrow shows the start of the prostructure, and the right arrow indicates the cleavage site for the processing-independent assay, which exposes the C-terminal region for quantification.