Mismeasure of C-Type Natriuretic Peptide

To the Editor:

It has been difficult to establish an endocrine role for C-type natriuretic peptide (CNP). Release of CNP from the heart remains to be convincingly demonstrated (1–5), and data concerning CNP in cardiac disease have been conflicting, possibly because of specificity problems in measurement of CNP. All natriuretic peptides (NPs) display strong homology, and antibodies raised against one NP may consequently bind other NPs. With the relatively high plasma concentrations of A- and B-type NPs (ANP and BNP), a CNP assay with even a small degree of ANP or BNP cross-reactivity may produce falsely high CNP concentrations. This is especially relevant in heart failure with augmented cardiac ANP and BNP expression. We examined the selectivity of a widely used CNP RIA.

To evaluate cross-reactivity in the CNP assay, we measured serial dilutions of human CNP-22 (4.5–582 pmol/L), ANP-28 (5–100 000 pmol/L), and BNP-32 (4–81 000 pmol/L) against the calibrators of the CNP assay. We obtained human cardiac tissue samples from a heart failure patient undergoing cardiac transplantation. The local ethics committee approved the use of human tissue, and written informed consent was obtained from the patient before surgery. The extracts were subjected to measurements of CNP, BNP, NT-proBNP, and NT-proCNP using a commercial CNP RIA (Phoenix), a BNP assay (Shionoria), and in-house RIAs directed against the N-terminus of proBNP and proCNP, respectively.

Results are expressed as mean (SE). We used nonlinear regression analysis to fit 4-parameter logistic models to the data from the buffer experiments. Based on the model, we calculated the cross-reactivity as a function of BNP-32 concentration using a 1-phase exponential decay model. Likewise, we calculated cross-reactivity as a function of the BNP concentration by fitting data from the heart extract experiments to a 1-phase exponential decay model. Cross-reactivity was calculated as measured CNP concentration divided by measured BNP concentration.

Serial dilutions of human CNP-22, BNP-32, and ANP-28 in barbital buffer measured with the CNP RIA revealed cross-reactivity for BNP-32 in physiological concentrations (Fig. 1A). The dilution curves for CNP-22 and BNP-32 were not parallel; cross-reactivity increased with decreasing BNP-32 concentrations, being 5.1% at a BNP-32 concentration of 100 pmol/L and 1.6% at 1000 pmol/L (Fig. 1B). As expected, BNP and NT-proBNP concentrations were high in the atria and lower in the ventricles (Fig. 1C). NT-proCNP and particularly CNP concentrations were very low in both atria and ventricles (Fig. 1D). With only trace amounts of NT-proCNP in the extracts, most of the measured CNP represents cross-reacting BNP. Calculating cross-reactivity as CNP concentrations divided by BNP concentrations in the extracts, we fitted a curve parallel to that obtained in the buffer experiment (Fig. 1B). Cross-reactivity was estimated to be 3.2% at a BNP concentration of 100 pmol/L and 1.1% at 1000 pmol/L.

Nearly all reports on CNP measurement in plasma are based on the same RIA. According to the manufacturer, the assay does not exhibit any cross-reactivity with ANP or BNP. Nevertheless, our data indicate substantial BNP cross-reactivity of the CNP assay in buffer with added BNP-32 and in heart extracts (Fig. 1B). Ideally, chromatographic evaluation of the extracts could resolve the BNP and CNP forms, as coelution of CNP and BNP immunoreactivity would have provided further proof of BNP cross-reactivity. However, the CNP concentration in the heart extracts did not exceed 15 pmol/L, rendering chromatography very difficult. The difference in the degree of cross-reactivity between the buffer with added BNP-32 and the unmodified heart extracts may be partly because immunoreactive BNP in the extracts comprises both BNP-32 and intact proBNP, which may have another cross-reactivity profile. Alternatively, BNP measured by the Shionoria assay may partly have been cross-reacting ANP, which is highly expressed in the cardiac atria. Because N-terminal proCNP, proANP, and proBNP have virtually no sequence resemblance, cross-reactivity of the NT-proCNP assay with proANP and proBNP is highly unlikely. If intact proCNP(1-103) is present in the heart, the NT-proCNP assay would probably also measure it.

The CNP assay may still be valuable for CNP measurement in samples with low BNP concentrations; however, measurements done in samples with a high BNP concentration—such as heart tissue extracts or plasma samples from patients with heart failure—should be interpreted cautiously.

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References

Fig. 1. Dilution curves for human CNP-22, ANP-28, and BNP-32 measured with Phoenix CNP-22 assay. (A) Vertical broken lines mark ED_{50} of CNP-22 and BNP-32. (B) Fitted curves for cross-reactivity as a function of BNP-32 in the buffer experiment and BNP immunoreactivity in the cardiac tissue extract experiment. (C) BNP and N-terminal proBNP in cardiac extracts. (D) CNP and NT-proCNP in cardiac extracts. RA, right atrium; LA, left atrium; RV, right ventricle; LV, left ventricle.
Hexaprimer Amplification Refractory Mutation System PCR for Simultaneous Single-Tube Genotyping of 2 Close Polymorphisms

To the Editor:

Tetraprimer amplification refractory mutation system PCR (T-ARMS-PCR) is a simple and inexpensive genotyping method for differentiating both alleles of a polymorphism/mutation (both single-nucleotide polymorphisms and small insertions/deletions) with a single-tube PCR (1). In T-ARMS-PCR, a pair of common (outer) primers produces a non–allele-specific control amplicon and in combination with 2 allele-specific (inner) primers (designed to anneal in the opposite orientation) produces 2 allele-specific amplicons. These allele-specific amplicons have different sizes because the polymorphism/mutation is asymmetrical located with respect to the common primers. Thus, the amplicons can be separated by standard gel electrophoresis. T-ARMS-PCR has also been designed in a multiplex fashion to genotype more than one polymorphism/mutation by a single-tube PCR (2).

We describe a modified multiplex T-ARMS-PCR, the hexaprimer ARMS-PCR (H-ARMS-PCR), which is for when 2 polymorphisms are close in the sequence. H-ARMS-PCR uses only 6 primers and provides direct information about haplotype structure.

The CTLA4 gene (cytotoxic T-lymphocyte–associated protein 4; also known as CD152) is a negative regulator of T-cell function (3). The CTLA4 polymorphisms −318 C>T (rs5742909) and +49 A>G (rs231775) are associated with susceptibility to autoimmune diseases and cancer (3, 4). To genotype these 2 polymorphisms, which are only 365 bp apart in the 5′ region of the CTLA4 gene, we designed an H-ARMS-PCR that combines a single pair of common primers and 2 pairs of allele-specific primers in the same tube (Fig. 1A).

The PCR reaction (25 μL) contained 200 μmol/L deoxyxylulose triphosphates, 100 ng of genomic DNA, 1.25 U of HotStarTaq DNA Polymerase with its buffer (Qiagen), and the following primers at the indicated concentrations (deliberate mismatches from the reference sequence, GenBank no. M74363, are in boldface italics): −318fo, 5′-CAATGAATGAAATTGACTGGA TG-3′ (0.5 μmol/L); +49ro, 5′-TA CAGAGCCAGCCAAGCCAGATT 3′ (0.8 μmol/L); −318fi(c), 5′-CTC CACTTATATCCAGATCGTC-3′ (2.5 μmol/L); −318ri(t), 5′-AC TGAACGTTCAATGGACTCTTA-3′ (0.5 μmol/L); +49fi(a), 5′-GCACA AGGCTAGCTGAACCTTGAGTA-3′ (0.1 μmol/L); and +49ri(g), 5′-ACAGAAAGATGTCAGGCCAG GTCTACAG-3′ (0.5 μmol/L).

Cycling conditions were 12 min at 95 °C, followed by 5 cycles of 94 °C for 30 s, 62 °C for 30 s, and 72 °C for 45 s; and a final cycle at 72 °C for 10 min.

We genotyped both the −318 C>T and +49 A>G CTLA4 polymorphisms with this newly designed H-ARMS-PCR (Fig. 1A) for samples from 80 individuals that had previously been characterized with 3 established methods [restriction fragment length polymorphism (RFLP) PCR, direct DNA sequencing, and T-ARMS-PCR] (4) and observed no discrepancies. Moreover, H-ARMS-PCR provides both an additional internal control and direct information about the haplotype structure by generating an amplicon that is specific for 1 of the 4 haplotypes that 2 polymorphisms can theoretically have. In this case, the haplotype-specific amplicon is observed when the alleles −318C and +49G are on the same chromosome (Fig. 1A).

We further tested this approach by implementing H-ARMS-PCR in the genotyping of 2 polymorphisms that are 142 bp apart in the 3′ untranslated region (UTR) of the CYP19A1 gene (cytochrome P450, family 19, subfamily A, polypeptide 1): rs10046 (C>T) and rs4646 (G>T). CYP19A1 encodes aromatase, the enzyme responsible for the final step of estrogen biosynthesis. CYP19A1 polymorphisms have been associated with estrogen concentrations in woman and with susceptibility to breast and prostate cancers (5).

We used the PCR to analyze these 2 CYP19A1 polymorphisms in the 3′ UTR (Fig. 1B; see legend for PCR conditions) in 100 individuals already characterized by RFLP PCR and/or direct DNA sequencing (P.P. and R.N., unpublished data) and observed no discrepancies. A haplotype-specific amplicon is present when alleles rs10046C and rs4646T are on the same chromosome. This H-ARMS-PCR is currently being used in a mul-