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THE BIOLOGICAL ACTIVITY OF ATRIAL NATRIURETIC FACTOR CLEAVED BY ENDOPROTEASE 3.4.24.11

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Ring cleavage of atrial natriuretic peptide (ANF) between Cys7 and Phe8 by endoprotease 3.4.24.11 yields X-ANF. Since endoprotease 3.4.24.11 may contribute to ANF metabolism *in vivo*, the present study determined if X-ANF exhibits reduced biological activity in comparison to the parent molecule.

KEY WORDS: Atrial Natrieuretic Factor (ANF), Endoprotease 3.4.24.11, Radioreceptor Assay.

INTRODUCTION

Atrial natriuretic factor (ANF) derived from pre-pro-ANF is synthesized in the atria of the heart. Since it's initial discovery[1] several ANF peptides have been isolated and sequenced.[2-4] The 28 residue peptide used in this study (corresponding to residues 99–126 of pro-ANF) is the major form of ANF in both rat and human plasma.[5,6] ANF contains a disulfide loop formed by 2 cysteine residues at position 7 and 23. In addition to its potent diuretic and natriuretic effects, ANF displays a variety of other biological activities, including smooth muscle relaxation, lowering of blood pressure and inhibition of aldosterone secretion by adrenal zona-glomerulosa cells. ANF receptors have been identified in a variety of potential target tissues, including renal and vascular tissue,[7] adrenal zona-glomerulosa,[8] lung[9] and various areas of the brain[10].

Several groups have demonstrated a rapid turnover of ANF *in vivo*[11-13] but the nature of the peptidases involved has only recently been elucidated. An early report described an atrial enzyme which converted atriopeptin II (ANF, Ser_5–Arg_{27}) to atriopeptin I (ANF, Ser_5–Ser_{25}) by removal of the C-terminal dipeptide Phe-Arg.[14] Later it was observed that atriopeptin III (ANF, Ser_5–Tyr_{28}) was degraded by rabbit brush border membranes with hydrolysis at the Ser_5–Ser_6, Cys_7–Phe_8 and Ser_{25}–Phe_{26} peptide bonds.[15] More detailed studies on the mode of attack by renal brush border membranes have now led to the identification of the peptidase responsible for the degradation of ANF as protease 3.4.24.11.[16] Recently two ANF peptides have been identified in plasma obtained from human coronary sinus.[17] The amino acid sequence of one of the peptides was identical to ANF while the second peptide, which we refer to as X-ANF was identified as the ring opened form of ANF. Protease 3.4.24.11 is known to cleave ANF between Cys_7 and Phe_8 and has been detected in heart tissue,

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albeit in very low quantities.[17] However, the role of this enzyme in generating X-ANF in coronary sinus *in vivo* has yet to be established. In view of the fact that X-ANF has been identified *in vivo* and that protease 3.4.24.11 is known to degrade ANF, we investigated the biological properties of X-ANF.

MATERIALS AND METHODS

ANF (99–126) was purchased from Peninsula Laboratories. Rat protease 3.4.24.11 was purified according to the method of Sonnenberg *et al.*[16]

Preparation of X-ANF

X-ANF was prepared according to the method of Sonnenberg *et al.*[16] by incubating purified rat protease 3.4.24.11 with ANF in a ratio of 1 to 150. X-ANF was purified and characterized according to the method of Koehn *et al.*[19]

Radioimmunoassay of X-ANF

The immunoreactive ANF and X-ANF were assayed using a radioiodinated ligand ANF and specific antibody developed by Peninsular Laboratories-RIK #9103 (Belmont, CA). This assay, in our hands, has an intra-assay variability of 6.1% and an interassay variability of 7.0%. The sensitivity of the assay is 8 pg and the antibody crossreacts 100% with atriopeptin I, suggesting that the antiserum recognizes the carboxy terminus of the ANF molecule.

Radioligand receptor binding

ANF and X−ANF were assayed using the instant radioreceptor assay kit developed by ANAWA Laboratories AG (P-Version, Product #A3-210) Wangen, Switzerland. The membranes used in this assay were prepared from bovine adrenal cortex. The measurement of ANF utilizes the competitive interaction between radioiodinated ANF (ligand) and unlabelled ANF and X−ANF for the receptor. The sensitivity of this assay system has been optimized for 1 ml of normal human plasma measured in the optimal range of the standard curve (i.e., close to EC_{50}). Furthermore, the ANF-RARA (ANAWA) is highly specific for physiologically active species of ANF.

Relaxation of Rat Aorta

Male Sprague Dawley rats (Tac:N(SD) fBR, Taconic Farms, Germantown, NY) weighing 300–350 g were killed by cervical dislocation and the aorta was rapidly removed and placed in physiological salt solution (PSS). The composition of PSS was as follows (mM): NaCl 112.6, KCl 5.0, KH_2PO_4 1.0, $MgSO_4$ 1.25, $NaHCO_3$ 25.0, glucose 12.6. Aortic strips, 2 or 3 mm wide, were cut open and suspended in 20 ml organ baths containing PSS at 37 ± 1°C and aerated with 95% O_2 and 5% CO_2. The aortic strips were connected to force displacement transducers (Grass FT .03) and equilibrated for 60 min. The resting tension on the muscle was maintained at 1.5 g during the equilibration period. Initially, each muscle strip was isometrically contracted twice with norepinephrine (3×10^{-7} M). At this concentration norepinephrine

produced a contractile response which was 80% of the maximal response. Once the contraction reached a plateau, ANF or X-ANF was added and the maximum relaxation for each concentration was measured. After the relaxation reached a plateau a higher concentration of the peptide under investigation was added and a cumulative dose response curve to the peptide determined.

Diuretic and Natriuretic Assay in Anesthetized Rats

Normally hydrated male Sprague Dawley rats (Tac:N(SD) fBR, Taconic Farms, Germantown, NY) weighing 225–275 g were anesthetized with an intraperitoneal injection of sodium pentobarbital (65 mg/kg). Following anesthesia, polyethylene (PE 50) catheters were implanted in the right fermoral vein for drug administration and in the left femoral vein for continuous infusion of isotonic sodium chloride. Both ureters were exposed through a midline abdominal incision and PE 10 catheters were placed for collection of urine. Determination of urine volume and sodium output were made according to the previously reported method of Webb *et al.*[20] Urinary cyclic GMP concentrations were determined using the commercially available cGMP [^{125}I] RIA kit from Advanced Magnetics Inc. (Cambridge, MA).

Immediately following two 10 min control urine collection periods, an intravenous bolus injection of X-ANF (2 nmole/kg) was administered in a volume of 0.1 ml of isotonic saline containing 0.001 N HCl and 0.1% albumin. The catheter was then flushed with 0.2 ml of isotonic saline. Renal function responses to X-ANF were followed over five consecutive 10 min collection periods. In the same animals, ANF (2 nmole/kg) was then injected and the renal functional responses were again followed over five consecutive collection periods. Previously we have shown that renal functional responses to ANF were identical following repeated administration of ANF[21] and that basal urine volume (UV) urinary sodium excretion (UNaV) and urinary cGMP (UcGMP) excretion (UNaV) and urinary cGMP (UcGMP) excretion remained stable over time.[20] The renal responses to X-ANF and ANF were compared to baseline values using student's *t*-test. Difference from baseline were considered significant if $p < 0.05$.

RESULTS AND DISCUSSION

The preparation, purification and characterization of X-ANF has been reported previously.[16,19] The immunoreactivity of X-ANF and ANF were determined using a Peninsula RIA kit. No differences were found in immunoreactivities using this array, because of the specificity of the antiserum, which is directed towards the carboxy terminus of the ANF molecule. Both ANF and X-ANF have the same carboxy terminus.

As shown in Figure 1, the receptor binding activity of X-ANF was about three orders of magnitude lower than ANF, and the dose response showed a parallel shift to the right. The EC_{50} of X-ANF was 5×10^{-8} M, whereas, that for intact ANF was 9×10^{-11} M. These results indicate that the cleavage of the peptide between residues 7 and 8, yields a peptide much less potent than the intact peptide ANF.

ANF relaxed norepinephrine contracted vascular strips in a concentration-dependent manner (Figure 2) with an EC_{50} of 4 nM. In contrast, the relaxation response to X-ANF was markedly reduced relative to ANF. The X-ANF concentra-

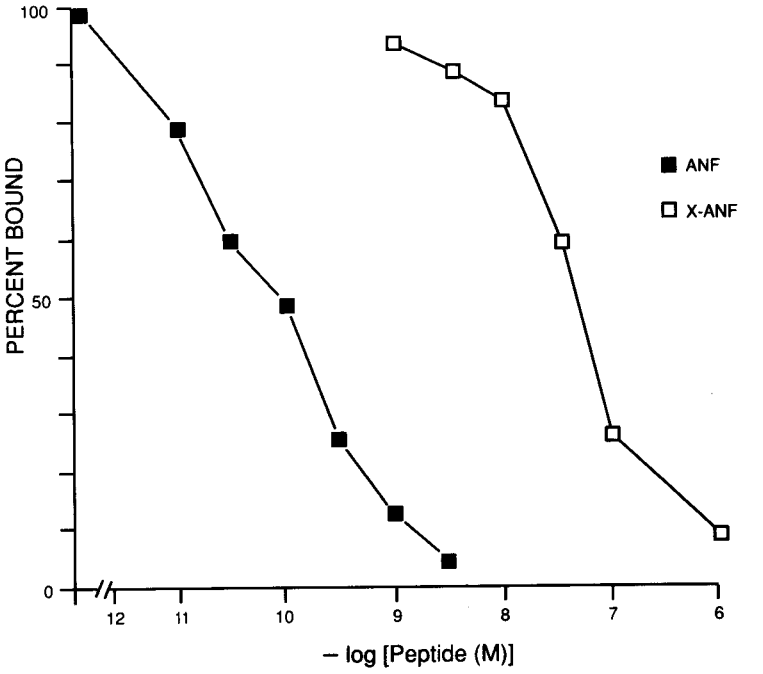

FIGURE 1 Radioreceptor assay of ANF and X-ANF. Results represent mean of three experiments.

tion response curve showed a parallel shift to the right (Figure 2). An EC_{50} value of 400 nM was obtained for the cleaved peptide in the above assay, indicating that cleavage between residue 7 and 8 of ANF results in the formation of a peptide which is 100 times less potent than the intact peptide. This reduced vasorelaxant property of X-ANF is consistent with the binding results, suggesting that the ring cleavage considerably reduces the biological activity.

As shown in Table I, there was a 3-fold increase in urine volume (UV) and sodium excretion (UNaV) in the anesthetized rat in response to a 2.0 nmole ANF/kg i.v. A 10-fold increase in cyclic GMP (UcGMP) was also observed. In contrast, renal functional parameters were not significantly affected by a similar dose of X-ANF. Thus, *in vivo* results correlate well with *in vitro* results.

We have previously shown that protease 3.4.24.11 cleaves ANF resulting in the formation of X-ANF.[16,19,22] The present results indicated that although X-ANF maintained immunological cross reactivity it was only weakly active in the *in vitro* and *in vivo* bioassays. *In vivo* data from Yandle[17] and Kreiter *et al.*[23] clearly demonstrates the existence of X-ANF in circulation. Recently it has been shown by several investigators[24–27] that inhibitors of protease 3.4.24.11 prevent the degradation of exogenously administered ANF *in vivo*. Therefore, administration of protease 3.4.24.11 inhibitors prolong the half life of endogenous ANF.

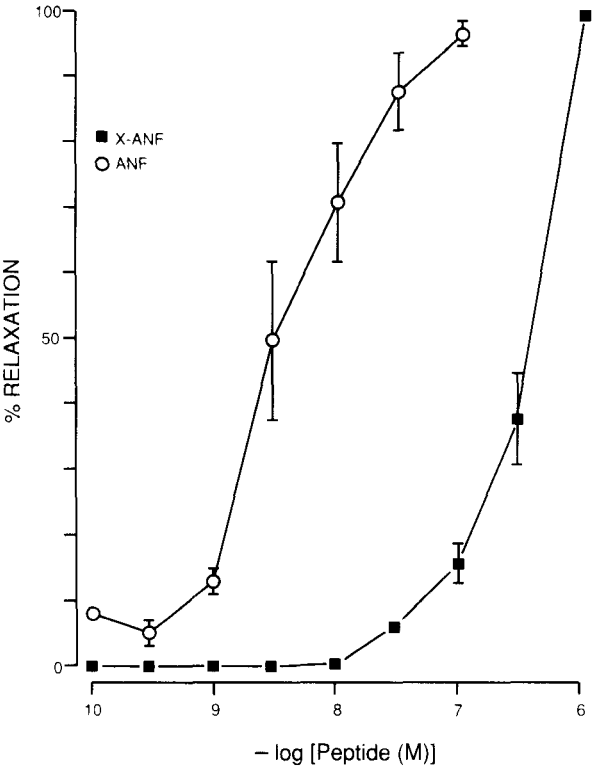

FIGURE 2 Concentration response curves of ANF and X-ANF in isolated rat aortic strips. Values are mean ± SEM; $n = 6$.

TABLE I
Diuretic, Natriuretic and Urinary cGMP Responses to ANF and X-ANF

	UV (μl/10 min)	UNaV (μEq/10 min)	UcGMP (pmol/10 min)
Control	342 ± 35	62 ± 8	286 ± 47
ANF	942 ± 151*	178 ± 30*	2996 ± 507*
X-ANF	497 ± 93	89 ± 16	368 ± 21

Values are mean ± SEM; $n = 5$
UV = Urine volume; UNaV = Sodium excretion; UcGMP = Urinary cGMP. Peptides are administered as an intravenous bolus (2 nmol/kg). *Significant difference from control response where $P < 0.05$.

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