Hydrolysis of iodine labelled urodilatin and ANP by recombinant neutral endopeptidase EC. 3.4.24.11

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1 Urodilatin is a 32 amino-acid peptide of similar sequence to atrial natriuretic peptide (ANP), with four additional amino-acids at the N-terminus. Although ANP and urodilatin bind to the same receptors with similar affinities, urodilatin is more active than ANP as a natriuretic agent. Previous studies, using neutral endopeptidase EC 3.4.24.11 (NEP) derived from crude membrane preparations, were inconclusive, but suggested that urodilatin was more resistant than ANP to degradation by this enzyme. In the present study, we compared the degradation rates of [¹²⁵I]-urodilatin and [¹²⁵I]-ANP by pure recombinant NEP (rNEP).

2 Incubation of radioactively labelled ANP with rNEP resulted in a much more rapid degradation of the peptide than that for labelled urodilatin.

3 Both phosphoramidon and SQ-28,603, potent inhibitors of NEP, completely protected both peptides from metabolism by rNEP.

4 The circular dichroism spectra of the two peptides indicate that they are very similar and exist largely in unordered or flexible conformations.

5 These results support the relative resistance of urodilatin to NEP, and indicate that urodilatin may be of use as a therapeutic agent, in conditions in which ANP is ineffective.

Keywords: Atrial natriuretic peptide; urodilatin; neutral endopeptidase; h.p.l.c.; phosphoramidon; SQ-28,603

Introduction

Urodilatin (URO) is a 32 amino-acid peptide, first isolated from human urine (Schulz-Knappe *et al.*, 1988). It has the same amino-acid sequence as atrial natriuretic peptide (ANP_{99–126}) with four additional amino-acids at the amino-terminus. These amino-acids are the same ones that immediately precede ANP in its precursor molecule, suggesting that URO is derived from pre-pro-ANP. Like ANP, URO has a 17 amino-acids loop structure closed by a disulphide bridge between cysteine-105 and cysteine-121. While ANP is abundant in cardiac atria and the circulation, URO is found only in human urine, and has not been detected in blood (Schulz-Knappe *et al.*, 1988; Goetz, 1992; Abassi *et al.*, 1992a; Valentin & Humphreys, 1993).

URO and ANP exert similar renal and cardiovascular effects. In general, both peptides increase sodium excretion and urine flow (Goetz *et al.*, 1990; Abassi *et al.*, 1992b; Valentin & Humphreys, 1993). Infusion of either ANP or URO into experimental animals (Villarreal *et al.*, 1991; Abassi *et al.*, 1992b; Hildebrandt *et al.*, 1992) and man (Saxenhofer *et al.*, 1990; Kentsch *et al.*, 1992) induces significant natriuresis, diuresis and a reduction of blood pressure. In some studies, URO was found to be more potent than ANP (Riegger *et al.*, 1990; Saxenhofer *et al.*, 1990; Abassi *et al.*, 1992b; Hildebrandt *et al.*, 1992; Bestle & Bie, 1993). In normal subjects, bolus injections of URO induced natriuresis and diuresis, which were twice as large as those obtained with similar doses of ANP (Saxenhofer *et al.*, 1990). Similar findings were documented in rats (Abassi *et al.*, 1992b) and dogs (Riegger *et al.*, 1990; Villarreal *et al.*, 1991) with experimental congestive heart failure (CHF).

Since ANP and URO exert their effect by binding to the same receptors, with similar affinities *in vitro* (Heim *et al.*, 1989; Saxenhofer *et al.*, 1993; Valentin *et al.*, 1993; Koike *et al.*, 1993), the difference in potency between these peptides is either due to different conformations of the peptides *in vivo*, or, more likely, due to higher availability of URO at the

medullary collecting duct, resulting from its resistance to proteolytic degradation. URO was found to be less susceptible to biological inactivation by neutral endopeptidase EC 3.4.24.11 (NEP) in renal membrane preparations (Gagelmann *et al.*, 1988; Kenny *et al.*, 1993) and *in vivo* (Abassi *et al.*, 1992c). NEP is a zinc-containing metalloendopeptidase widely distributed in brain, lung and in the brush border of the kidney and intestines (Booth & Kenny, 1974; Johnson *et al.*, 1985). Several studies demonstrated that NEP is the main enzyme that degrades ANP, through a single cleavage between cysteine-105 and phenylalanine-106 (Olins *et al.*, 1987; Stephenson & Kenny, 1987). The product of the initial proteolytic attack is subject to further cleavage within the loop, and at the amino- and carboxy-termini (Olins *et al.*, 1986; Stephenson & Kenny, 1987; Vanneste *et al.*, 1988). The different stability of ANP and URO to degradation may account for the differences in the intensity of their renal effects. Data concerning the relative resistance of URO to degradation are based on work in crude membrane preparations. These preparations probably contain other enzymes, besides NEP 24.11. The present study was designed to assess directly the relative stability of [¹²⁵I]-ANP and [¹²⁵I]-URO to pure recombinant NEP 24.11 (rNEP).

Methods

Recombinant human neutral endopeptidase EC.3.4.24.11 was produced by Genentech, Inc. (San Francisco, CA, U.S.A.). The full length cDNA coding for human neutral endopeptidase EC.3.4.24.11 was cloned originally from a human placental cDNA library (Malfroy *et al.*, 1988) and transfection of mammalian cells with an expression plasmid containing this cDNA resulted in the production of the enzymatically active protein (Gorman *et al.*, 1989), which was purified by a modification of a standard procedure and quantified in terms of protein catalytic activity. The active enzyme is a glycoprotein, of approximately 90 kilodaltons.

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One ng of either [125 I]-Tyr $_{126}$ -human α -ANP $_{99-126}$ or [125 I]-Tyr $_{126}$ -URO $_{95-126}$ (both 2000 Ci mmol $^{-1}$, NEN, Boston, MA, U.S.A.), were incubated with either 1 or 10 μ g of the rNEP at 37°C, in 1 ml solution of 50 mM HEPES, pH = 7.4, 50 mM NaCl, 10% acetonitrile, for different times (0.5–120 min). This concentration of acetonitrile was necessary to obtain a homogeneous solution of [125 I]-URO and prevent its adherence to the reaction tubes, because [125 I]-URO is less water-soluble than [125 I]-ANP. It had been confirmed in preliminary experiments that this concentration of acetonitrile did not interfere with NEP activity upon either [125 I]-ANP or [125 I]-URO. In some experiments either phosphoramidon (Peptides International, Louisville, KY, U.S.A.) or SQ-28,603 (N-[2-(mercaptomethyl)-1-o x o-3-phenylpropyl]-L-alanine; Bristol-Myers Squibb Pharmaceuticals, Princeton, NJ, U.S.A.) was added to the solution at a final concentration of 10 μ M prior to the addition of the enzyme. Each reaction was terminated by the addition of 50 μ l of 70% acetic acid, followed by heating at 60°C for 3 min. Fifty microliters of each reaction mixture were analysed by reverse phase high performance liquid chromatography (h.p.l.c.) (C $_{18}$ μ Bondapak, 3.9 x 300 mm column, Waters, Milford, MA, U.S.A.), using a linear gradient of 10–60% acetonitrile in 0.1% trifluoroacetic acid over 35 min, at a flow rate of 1 ml min $^{-1}$. The h.p.l.c. system was calibrated with 125 I-labelled ANP or 125 I-labelled URO. Radioactivity eluting from the column was continuously monitored with a gamma-counter (Beckman Instruments, Irvine, CA, U.S.A.). In addition, 50 μ l of each reaction mixture ($n = 6$ for each time period) were treated with 250 μ l of 10% trichloroacetic acid (TCA) at 0°C and centrifuged at 2200 g. The pellets were resuspended in 250 μ l of TCA at 0°C and recentrifuged. The radioactivity in either the combined supernatants (TCA-soluble) or in the pellet (TCA-precipitate) was determined. The TCA-precipitable 125 I-radioactivity was shown by h.p.l.c. to be mainly intact [125 I]-ANP or [125 I]-URO, while the soluble 125 I-radioactivity represents mainly free 125 I and some hydrolytic products. This agrees with previous reports in the literature (Almeida *et al.*, 1989; Chiu *et al.*, 1991; Abassi *et al.*, 1992c).

Circular dichroism (CD) spectra were measured on a JASCO model J-500A/DP-501N spectropolarimeter using 1 mm path Hellma quartz cells. Spectra were scanned from 190 to 260 nm, and the spectral amplitudes are expressed as mean residue ellipticities $[\theta]$. Spectra were measured on 16 μ M solutions in pH 7.4, 50 mM phosphate buffer containing 10% acetonitrile, as well as in the same buffer containing 10% acetonitrile and 40% trifluoroethanol.

The disappearance rate of [125 I]-ANP was compared to that of [125 I]-urodilatin by using two-way analysis of variance (ANOVA), followed by an unpaired t test; a value of $P < 0.05$ was considered significant.

Results

Radioactively labelled URO and ANP eluted from the h.p.l.c. with retention times of 23 and 24 min, respectively. Incubation of [125 I]-ANP with 1 μ g ml $^{-1}$ rNEP at 37°C resulted in rapid degradation of the peptide (Figure 1). After a short incubation with the enzyme, most of the radioactivity eluted at 22 min, whereas increasing the incubation time to more than 30 min resulted in the appearance of an additional major peak at 16 min (Figure 2a). Increasing the concentration of rNEP to 10 μ g ml $^{-1}$, resulted in total degradation of the 125 I-ANP within 2 min (Figure 2a).

Incubation of [125 I]-URO with rNEP, at the same concentration as [125 I]-ANP, resulted in a much slower degradation of this peptide. After incubation with rNEP 1 μ g ml $^{-1}$, degradation of [125 I]-URO was first detected at 5 min of incubation (Figure 1a). Even after incubation of labelled URO with rNEP at a concentration of 10 μ g ml $^{-1}$, about half of the radioactivity in the reaction mixture eluted as intact [125 I]-URO after 15 min (Figure 1b and Figure 2b).

These results are further confirmed by the use of TCA precipitation. The rate of disappearance of the TCA precipitable (TCA-ppt) radioactivity, which represents the intact peptide, is shown in Figure 3. The disappearance of [125 I]-URO was significantly slower than that of [125 I]-ANP, at both concentrations of rNEP. Addition of either of the NEP inhibitors, SQ-28,603 or phosphoramidon, completely inhibited the degradation of both [125 I]-ANP and [125 I]-URO by rNEP (data not shown).

The CD spectral features of the two peptides are very similar in buffered solutions (pH 7.4) containing 10% acetonitrile, i.e., showing single negative ellipticity maxima at 197 nm, with mean residue ellipticity values of $-7300 \text{ deg} \times \text{cm}^2 \times \text{dmol}^{-1}$ for ANP and $-8700 \text{ deg} \times \text{cm}^2 \times \text{dmol}^{-1}$ for urodilatin. On addition of 40% trifluoroethanol to solutions of the peptides the spectra became identical with negative ellipticity maximum shifted to 205 nm, $[\theta] = -8000 \text{ deg} \times \text{cm}^2 \times \text{dmol}^{-1}$ and the appearance of a broad shoulder in the 217–222 nm region $[\theta] = -(3799 \text{ to } 3200) \text{ deg} \times \text{cm}^2 \times \text{dmol}^{-1}$. The concentrations of ANP and URO in the solutions were confirmed by u.v. absorption maximum at 275 nm with extinction coefficient, $\epsilon = 2000$ for both peptides.

Discussion

The present study confirms that [125 I]-URO is relatively resistant to degradation by NEP. This finding is in agreement with previous *in vitro* studies. Gagelmann *et al.* (1988) demonstrated that ANP, but not URO, was proteolytically deg-

Figure 1 Disappearance of intact [125 I]-atrial natriuretic peptide (□) and [125 I]-urodilatin (●) incubated with recombinant neutral endopeptidase EC.3.4.24.11, (1 μ g (a) and 10 μ g (b)) for different time periods (0.5–120 min). The intact [125 I]-atrial natriuretic peptide and [125 I]-urodilatin were separated from their hydrolytic products by h.p.l.c. (see Figure 2).

Figure 2 Reversed-phase h.p.l.c. radioactive flow monitoring analysis of the fragmentation of (a) [125 I]-atrial natriuretic peptide ([125 I]-ANP) and (b) [125 I]-urodilatin ([125 I]-URO) by human recombinant neutral endopeptidase. The figure depicts typical radiochromatograms of reaction mixture of 1 ng of [125 I]-ANP or [125 I]-URO with $10 \mu\text{g ml}^{-1}$ of recombinant neutral endopeptidase incubated for different time periods (0.5–30 min). The arrow indicates the elution times of intact [125 I]-ANP (a) and [125 I]-URO (b).

raded within 5 min by crude preparations of dog kidney cortex. Similarly, Kenny *et al.* (1993), reported that URO was relatively resistant to metabolism by NEP derived from porcine choroid plexus membranes, whereas other members of the natriuretic peptide family, namely, ANP, brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP), were degraded rapidly by the enzyme. In these studies, enzymes were extracted from membranes that are rich in NEP 24.11, as well as other enzymes. Use of the recombinant enzyme enabled us to determine specifically the metabolism of ANP and URO by a pure preparation. However, a reservation should be pointed out, that the iodine labelling of URO and ANP might cause steric changes in these peptides,

that might interfere with their interactions with the enzyme. Even though the site of labelling (Tyr 126) is far from the site of hydrolysis (Cys105-Phe106), the possibility of such interference cannot be ruled out.

It should be emphasized, that the resistance of URO to NEP is not absolute and URO is degraded by high concentrations of the enzyme, or by exposure to low concentrations for long periods, as indicated by Kenny *et al.* (1993). In addition, these investigators noted, that the relative resistance of URO to NEP is not merely due to its larger size, since BNP, which is also a 32 amino acids peptide, is susceptible to degradation by NEP. The large difference between the sensitivity of ANP and URO in the present study supports this

Figure 3 Disappearance of trichloroacetic acid-precipitable (TCA-ppt) radioactivity, i.e., intact [¹²⁵I]-atrial natriuretic peptide ([¹²⁵I]-ANP) (□) or intact [¹²⁵I]-urodilatin ([¹²⁵I]-urodilatin) (●) in the presence of 1 µg ml⁻¹ (a) or 10 µg ml⁻¹ (b) of recombinant neutral endopeptidase. Radioactivity in intact peptide is expressed as counts per min (c.p.m.) in 50 µl of reaction mixture. Results are means ± s.e.; n = 5–6 in each group. The degradation of ANP by neutral endopeptidase was much faster than that of urodilatin; P < 0.001 by analysis of variance (ANOVA).

view. It is therefore suggested, that the four N-terminal amino acids of URO (Thr-Ala-Pro-Arg) make a specific contribution to the relative resistance of URO to NEP. On the other hand, when shorter natriuretic peptides, such as: CNP, ANP_{4–23} and ANP_{5–28}, were incubated with NEP, their degradation was faster than that of ANP_{1–28} (Kenny *et al.*, 1993). In addition, incubation of ANP_{103–126} (atriopeptin III) with NEP resulted in rapid inactivation due to attacks on several cleavage sites, compared to ANP, in which the initial attack is restricted to a single site between Cys₁₀₅ and Phe₁₀₆ (Olins *et al.*, 1986). These findings led to the controversial assumption, that the susceptibility of natriuretic peptides to NEP depends primarily on their length, so that elongated peptides are relatively more resistant to NEP inactivation than shorter forms or fragments of the peptides. Our findings that the circular dichroism spectra of ANP and URO are very similar, tends to exclude the possibility that differences

in configuration contribute to the relative resistance of URO to NEP. The spectral features of ANP and URO indicate that both peptides have random or flexible conformations, with a notable lack of α-helical or β-sheet conformation components (Woody *et al.*, 1985). The structure of ANP in dimethylsulphoxide was examined earlier with n.m.r. by others, and was shown to have a very flexible N-terminal segment (Kobayashi *et al.*, 1988). It appears that elongation of the N-terminal region of ANP by four amino acids (URO) does not fix the conformation of that region to any extent measurable by CD spectroscopy. Addition of a model lipophilic solvent, such as trifluoroethanol, induces an increased order in the structure, as evidenced by the red-shift of the 197 nm spectral band, and by the appearance of the board shoulder in the 217–222 nm spectral region. However, these solvents induced parallel conformational changes in both peptides.

The relative resistance of URO, compared with ANP, to NEP is of special interest, since it suggests a potential clinical application of URO in pathophysiological conditions, in which beneficial effects of ANP are still doubtful. For instance, the renal response to infusion of pharmacological doses of ANP into patients (Cody *et al.*, 1986) or experimental animals (Riegger *et al.*, 1988; Hoffman *et al.*, 1988; Abassi *et al.*, 1990; 1991) with congestive heart failure (CHF) is attenuated, and even blunted in severe stages of the disease. In contrast, infusion of equimolar doses of URO into rats with CHF (Abassi *et al.*, 1992b) or dogs with CHF (Riegger *et al.*, 1990; Villarreal *et al.*, 1991), and into healthy individuals and animals (Saxenhofer *et al.*, 1990; Hildebrandt *et al.*, 1992; Bestle & Bie, 1993), increased sodium excretion more than ANP. These differences in the potency of URO and ANP at the renal level may be explained partially by the relative resistance of URO to NEP, so that it reaches its target, namely, the distal segment of the nephron, in greater amounts, and thereby induces greater sodium excretion. Support for this view comes from several studies, in which inhibition of NEP increased longevity and augmented the natriuretic and diuretic effects of exogenous and endogenous ANP in animals (Koepke *et al.*, 1989; Wilkins *et al.*, 1990) and in man (Jardine *et al.*, 1989; Northridge *et al.*, 1989), possibly due to protection of ANP from NEP inactivation (see review by Roques *et al.*, 1993). However, we found that the specific NEP inhibitor SQ-28,603 also potentiated the renal effects of URO in rats with CHF (Abassi *et al.*, 1992b). The finding that URO is not completely resistant to NEP, may explain the stimulatory influence of SQ-28,603 on the renal effects of the peptide. But, we cannot exclude other possibilities, such as protection of other peptides with natriuretic activity, e.g., bradykinin (Ura *et al.*, 1987) and BNP (Kirk & Wilkins, 1993; Mark Richards *et al.*, 1993) and the inhibitory effects of the drug on sodium reabsorption in the proximal tubule (Cavero *et al.*, 1990).

In summary, using purified recombinant NEP the present study confirms previous reports that suggested that URO was less susceptible than ANP to degradation by NEP. This advantage of URO over ANP may make the former a better potential candidate for the treatment of a variety of diseases characterized by attenuated or blunted renal response to ANP.

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