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Synthetic inhibitors of endopeptidase EC 3.4.24.15: potency and stability *in vitro* and *in vivo*

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1 The role of the metalloendopeptidase EC 3.4.24.15 (EP 24.15) in peptide metabolism *in vivo* is unknown, in part reflecting the lack of a stable enzyme inhibitor. The most commonly used inhibitor, N-[1-(**R,S**)-carboxy-3-phenylpropyl]-Ala-Ala-Tyr-*p*-aminobenzoate (cFP-AAY-pAB, $K_i = 16$ nM), although selective *in vitro*, is rapidly degraded in the circulation to cFP-Ala-Ala, an angiotensin converting enzyme (ACE) inhibitor. This metabolite is thought to be generated by neutral endopeptidase (NEP; EC 3.4.24.11), as the Ala-Tyr bond of cFP-AAY-pAB is cleaved by NEP *in vitro*. In the present study, we have examined the role of NEP in the metabolism of cFP-AAY-pAB *in vivo*, and have tested a series of inhibitor analogues, substituted at the second alanine, for both potency and stability relative to the parent compound.

2 Analogues were screened for inhibition of fluorescent substrate cleavage by recombinant rat testes EP 24.15. D-Ala or Asp substitution abolished inhibitory activity, while Val-, Ser- and Leu-substituted analogues retained activity, albeit at a reduced potency. A relative potency order of Ala (1) > Val (0.3) > Ser (0.16) > Leu (0.06) was observed. Resistance to cleavage by NEP was assessed by incubation of the analogues with rabbit kidney membranes. The parent compound was readily degraded, but the analogues were twice (Ser) and greater than 10 fold (Leu and Val) more resistant to cleavage.

3 Metabolism of cFP-AAY-pAB and the Val-substituted analogue was also examined in conscious rabbits. A bolus injection of cFP-AAY-pAB (5 mg kg^{-1}, i.v.) significantly reduced the blood pressure response to angiotensin I, indicating ACE inhibition. Pretreatment with NEP inhibitors, SCH 39370 or phosphoramidon, slowed the loss of cFP-AAY-pAB from the plasma, but did not prevent inhibition of ACE. Injection of 1 mg kg^{-1} inhibitor resulted in plasma concentrations at 10 s of 23.5 μM (cFP-AAY-pAB) and 18.0 μM (cFP-AVY-pAB), which fell 100 fold over 5 min. Co-injection of ^{125}I-labelled inhibitor revealed that 80–85% of the radioactivity had disappeared from the circulation within 5 min, and h.p.l.c. analysis demonstrated that only 25–30% of the radiolabel remained as intact inhibitor at this time. Both analogues were cleared from the circulation at the same rate, and both inhibitors blunted the pressor response to angiotensin I, indicative of ACE inhibition.

4 These results suggest that both NEP and other clearance/degradation mechanisms severely limit the usefulness of peptide-based inhibitors such as cFP-AAY-pAB. To examine further EP 24.15 function *in vivo*, more stable inhibitors, preferably non-peptide, must be developed, for which these peptide-based inhibitors may serve as useful molecular templates.

Keywords: Metalloendopeptidase 24.15; angiotensin converting enzyme; neutral endopeptidase; phosphoramidon; bradykinin; angiotensin; pharmacokinetics

Introduction

The role of peptidases in the metabolism of vasoactive peptides such as angiotensin and bradykinin has been the focus of much research over the last two decades. In particular, the angiotensin converting enzyme (ACE; EC 3.4.15.1), which generates the vasoconstrictor peptide angiotensin II (AII) from angiotensin I (AI), as well as inactivating the vasodilator, bradykinin, has been extensively studied as a therapeutic target. Specific, orally active inhibitors of ACE, such as captopril and enalapril, have been developed and have revolutionized the treatment of certain forms of hypertension. More recently, inhibitors of another well-characterized metallopeptidase, namely neutral endopeptidase (NEP; EC 3.4.24.11), have been designed. These inhibitors, which lower blood pressure primarily by blocking metabolism of atrial natriuretic peptide, but also by reducing bradykinin breakdown, are currently being evaluated for use in hypertension, particularly in conjunction with ACE inhibitors (Schwartz *et al.*, 1990; Sybertz *et al.*, 1990).

Recently, a third metallopeptidase, endopeptidase 24.15 (EP 24.15; EC 3.4.24.15), has been proposed to contribute to bradykinin breakdown in the vasculature, as administration of an EP 24.15 inhibitor to rats reduced resting arterial blood pressure, and potentiated the depressor effects of exogenous bradykinin (Genden & Molineaux, 1991). First isolated from rat brain by Orlowski *et al.* (1983), EP 24.15 has generally been studied as a neuropeptidase, although the enzyme displays a relatively wide tissue distribution (for review, see Barrett *et al.*, 1995). The exact physiological role of EP 24.15 in either the brain or the periphery is still unknown, due in part to the lack of stable, specific enzyme inhibitors. Indeed, the compound used by Genden & Molineaux (1991), N-[1-(**R,S**)-carboxy-3-phenylpropyl]-Ala-Ala-Phe-*p*-aminobenzoate (cFP-AAF-pAB), although both potent and specific for EP 24.15 *in vitro* (Orlowski *et al.*, 1988), has subsequently been shown to be cleaved *in vitro* by NEP to generate N-[1-(**R,S**)-carboxy-3-phenylpropyl]-Ala-Ala (cFP-AA), a potent inhibitor of ACE (Cardozo & Orlowski, 1993; Williams *et al.*, 1993). Moreover, the bradykinin-potentiating effect of the EP 24.15 inhibitor *in vivo* has subsequently been shown by ourselves (Telford *et al.*, 1995) and by others (Yang *et al.*, 1994) to be due solely to

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inhibition of ACE by the metabolite. However, studies examining the role of NEP in the degradation of cFP-AAY-pAB *in vivo* are limited.

In the present study, we have directly examined the effect of NEP inhibition on the metabolism of the EP 24.15 inhibitor N-[1-(**R,S**)]-carboxy-3-phenylpropyl]-Ala-Ala-Tyr-*p*-aminobenzoate (cFP-AAY-pAB) in the circulation of conscious rabbits. Furthermore, as a first step toward the development of more stable EP 24.15 inhibitors, we have synthesized a series of analogues of cFP-AAY-pAB in which the second alanine residue was substituted in order to reduce susceptibility to NEP at the Ala-Tyr bond without compromising EP 24.15 inhibition. The analogues were tested for both potency against recombinant EP 24.15 and resistance to degradation by NEP *in vitro*. Finally, the metabolic fates of both the parent compound and the most promising analogue were evaluated *in vivo*.

Methods

Synthesis of EP 24.15 inhibitors

The EP 24.15 inhibitor N-[1-(**R,S**)-carboxy-3-phenylpropyl]-Ala-Ala-Tyr-*p*-aminobenzoate (cFP-AAY-pAB) and a series of analogues in which the second alanine residue was substituted by D-Ala, Asp, Val, Ser or Leu were synthesized by the method of Chu & Orlowski (1984), purified by reversed-phase h.p.l.c., and verified by mass spectrometry. For the most part, the inhibitors were used either as a racemic mix (approximately equal proportions of each stereoisomer) *in vivo* and for assessment of stability *in vitro*, or further purified to individual stereoisomers for inhibitory constant (K_i) determination. However, only one stereoisomer of the Leu-substituted analogue was used throughout. The configuration of the stereoisomers (i.e., **R** or **S**) was not determined.

The inhibitors, cFP-AAY-pAB and cFP-AVY-pAB, were radiolabelled with ^{125}I using Iodogen (Salacinski *et al.*, 1981), and purified by extraction on a Sep-Pak C18 column (Waters, Milford, MA, U.S.A.). In addition, 100 μg of each inhibitor was iodinated with the non-radioactive isotope of iodine and used to assess degradation of the iodinated inhibitor by kidney membranes. Comparison of the h.p.l.c. retention times of the radioactive and non-radioactive forms, in conjunction with mass spectrometry, confirmed that the inhibitors were predominantly di-iodinated.

In vitro *studies*

Inhibition of EP 24.15 by cFP-AAY-pAB analogues. Initial screen Racemic mixtures of each analogue were screened for inhibitory activity against the cleavage of synthetic gonadotropin-releasing hormone$_{1-9}$ ($GnRH_{1-9}$) by recombinant rat testes EP 24.15. This peptide is readily cleaved by the recombinant enzyme, which was prepared as previously described (Lew *et al.*, 1995). Inhibitors (0, 0.3, 1 and 3 μM) were pre-incubated with 100 ng EP 24.15 in 250 μl 0.2 M N-tris[hydroxymethyl]-methyl-2-aminoethanesulphonic acid (TES) buffer, pH 7.4, for 10 min at room temperature before addition of 5 μg $GnRH_{1-9}$ and incubation at 37°C for 60 min. The enzymatic reaction was stopped by addition of 400 μl methanol/1% trifluoroacetic acid (TFA) to precipitate protein, samples were centrifuged in a table-top Eppendorf microfuge (14,000 r.p.m., 5 min), and the supernatants dried by vacuum centrifugation (Speed-Vac, Savant) before analysis by h.p.l.c. (see below).

Kinetic analysis CFP-AAY-pAB analogues which could inhibit cleavage of $GnRH_{1-9}$ by EP 24.15 were further characterized by a specific quenched fluorescent substrate (QFS), 7-methoxycoumarin-4-acetyl-Pro-Leu-Gly-Pro-D-Lys(2,4-dinitrophenyl), similar to that described by Tisljar *et al.* (1990). The two stereoisomers of each analogue were separated by h.p.l.c., and quantitated by absorbance at 254 nm, relative to a known quantity of cFP-AAY-pAB. Each isomer (0.003–10 μM) was pre-incubated for 15 min on ice with 500 ng (2.67 nM) EP 24.15 in 2.5 ml Tris-buffered saline (25 mM Tris, 125 mM NaCl, pH 7.4) containing 0.3 mM dithiothreitol. Substrate (0.56 mM in dimethyl sulphoxide) was added to 4.5 or 9 μM final concentration, and the assay tubes incubated at 37°C for 30 min. Reactions were stopped by addition of 25 μl 100 mM $ZnCl_2$, and the tubes allowed to reach room temperature before reading fluorescence on a Perkin-Elmer LS-5 luminescence spectrometer ($E_x = 314$ nm, $E_m = 418$ nm). Enzymatic cleavage of the QFS was quantitated by comparison with a standard curve (50–3200 pmol) of the fluorescent product, 7-methoxycoumarin-4-acetyl-Pro-Leu (Novabiochem, La Jolla, CA, U.S.A.). The K_i for each inhibitor was determined from the IC_{50} at each substrate concentration, using the relationship $IC_{50} = K_i(1 + [S/K_m])$. The K_m for the QFS was determined to be 19.8 μM by Lineweaver-Burk analysis of initial velocities measured in triplicate at a range of QFS concentrations (1.1–140 μM).

Inhibitor degradation by kidney membranes Rabbit kidney membranes, a rich source of NEP (Schwartz *et al.*, 1990), were prepared by homogenization in 0.2 M TES buffer, pH 7.4, at 4°C, followed by ultracentrifugation (100,000 *g*, 60 min, 4°C). The resultant pellet was resuspended in fresh TES buffer, and re-centrifuged. The second pellet was again resuspended in TES buffer at a concentration of 30 mg ml^{-1}, and frozen in aliquots at −70°C. Twenty μg of each cFP-AAY-pAB analogue (racemic mix) was incubated at 37°C with 12 μg kidney membrane protein in 450 μl TES buffer. Aliquots (100 μl) were removed at times 0, 15, 30 and 60 min, diluted in 400 μl methanol/1% TFA, and processed for h.p.l.c. In a separate experiment, the degradation of cFP-AAY-pAB, cFP-AVY-pAB, cFP-AA-$[I]_2$Y-pAB, and cFP=AV-$[I]_2$Y-pAB (10 μg each) was examined in the same manner.

In vivo *studies*

Animals Sixteen rabbits of cross-bred English strain were used, weighing 2.0–2.8 kg (mean 2.3). The experiments were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (1990), and were approved in advance by the Alfred Hospital/Baker Medical Research Institute Animal Experimentation Ethics Committee. Experiments 1 and 2 were designed in a within-subject fashion, so that within each protocol, each rabbit was studied on 3 separate occasions at 7–14 day intervals. The order of these studies was randomized; however, due to the use of radiolabelled inhibitors in Experiment 3, each rabbit ($n = 4$ per group) was used only once.

Preparations for experiments Rabbits were acutely equipped with ear artery, ear vein, and right atrial catheters, and arterial pressure was recorded as described previously (Telford *et al.*, 1995).

Experimental protocols

(1) Effects of phosphoramidon and SCH 39370 on the ACE-inhibiting activity of cFP-AAY-pAB The aim of this experiment was to determine indirectly whether inhibition of NEP could prevent or diminish the metabolism of cFP-AAY-pAB to cFP-AA by testing the effects of SCH 39370 and phosphoramidon on the efficacy of cFP-AAY-pAB as an ACE inhibitor *in vivo*. The profile of effects of cFP-AAY-pAB on haemodynamic responses to the vasoactive peptides bradykinin, AI and AII was used as an index of ACE-inhibitory activity. Each of four rabbits was studied on three separate occasions. The effects on arterial pressure of right atrial administration of bradykinin (10 and 100 ng kg^{-1}), I (10 and 100 ng kg^{-1}) and II (10 and 100 ng kg^{-1}) were tested. The six vasoactive peptide treatments were administered in random order at 5 min intervals (25 min total). The rabbits were then

treated with either SCH 39370 (3 mg kg^{-1} plus 3 mg kg^{-1} h^{-1}), phosphoramidon (5 mg kg^{-1} plus 5 mg kg^{-1} h^{-1}) or vehicle (0.115 M phosphate buffer, pH 7.4; 1 ml kg^{-1} plus 1 ml kg^{-1} h^{-1}). Twenty minutes later, each rabbit was treated with cFP-AAY-pAB (5 mg kg^{-1}, i.v. in 1 ml kg^{-1} 10% w/v 2-hydroxypropyl-β-cyclodextrin), and following a 5 min equilibration period, the effects of the vasoactive peptides were re-tested. Arterial blood samples (2 ml) were collected immediately before administration of cFP-AAY-pAB, and immediately after the effects of the final vasoactive peptide were tested. These blood samples were processed for estimation of plasma levels of SCH 39370 and phosphoramidon (see below).

(2) Effects of phosphoramidon and SCH 39370 on blood levels of cFP-AAY-pAB The aim of this experiment was to test directly whether inhibition of NEP could retard, or even prevent the metabolism of cFP-AAY-pAB to cFP-AA *in vivo*. The design of this experiment was identical to protocol 1, except that the effects of vasoactive peptides were not tested. Rather, blood samples were collected for measurement of blood levels of intact cFP-AAY-pAB. Arterial blood samples (2 ml) were collected immediately before administration of cFP-AAY-pAB, and 10 s, 1 min, 5 min, and 30 min after cFP-AAY-pAB administration. In two other untreated rabbits, arterial blood samples were collected into tubes containing standard concentrations of cFP-AAY-pAB. All of these samples were processed for measurement of blood levels of intact cFP-AAY-pAB (see below).

(3) Metabolic fate of cFP-AAY-pAB and cFP-AVY-pAB This study was designed to determine the fate of intravenously administered cFP-AAY-pAB, and to compare its metabolism with cFP-AVY-pAB, which was more resistant to degradation by kidney membranes. In each rabbit ($n=4$ per analogue), haemodynamic responses to AI (1, 10 and 100 ng kg^{-1}) were measured as in the above experiments, prior to injection of cFP-AAY-pAB or cFP-AVY-pAB, spiked with 2×10^7 c.p.m. radiolabelled inhibitor. Due to limited supplies of cFP-AVY-pAB, a lower dose (1 mg kg^{-1}) of inhibitor was used; previous studies have shown that this dose of cFP-AAY-pAB is still effective in inhibiting ACE (Telford *et al.*, 1995). Arterial blood samples (2 ml) were withdrawn just prior to and at 10 s, 30 s, 1 min, 5 min, 30 min and 60 min after injection. Aliquots of blood were removed to determine radioactivity and haematocrit prior to centrifugation and processing of plasma (see below). The dose-response curve to AI was repeated and extended (additional doses of 1000 and 3000 ng kg^{-1}) between the 5 min and 30 min blood samples to assess the inhibition of ACE.

Processing of blood samples

Blood samples (2 ml) were collected via an ear artery catheter into chilled syringes, and immediately transferred into chilled 5 ml heparinized tubes. Each sample was centrifuged at 3000 r.p.m. for 10 min at 4°C (Minifuge G.L., Heraeus, Germany).

Determination of plasma levels of NEP inhibitors These were determined by assessing the ability of plasma samples collected in experiment 1 to inhibit the degradation of cFP-AAY-pAB by kidney membranes. An aliquot (100 μl) of each plasma sample was added to 6 μg of kidney membranes and 5 μg of cFP-AAY-pAB in a final volume of 150 μl (100 μl plasma and 50 μl TES buffer), and incubated at 37°C for 60 min. Standard curves for phosphoramidon and SCH 39370 were constructed by adding a known amount of each inhibitor to 100 μl blank plasma to achieve final concentrations of 0, 0.03, 0.1, 0.3, 1 and 3 μM; these standards were then assayed in parallel with the experimental samples. Following incubation, the enzymatic reactions were stopped by addition of 600 μl methanol/1% TFA and processed for h.p.l.c. analysis.

The extent of cFP-AAY-pAB degradation by kidney membranes, and in turn, its protection by inhibitors of NEP, was assessed by summation of the areas of the two u.v. absorbance peaks representing the intact cFP-AAY-pAB stereoisomers. Standard curves relating the total cFP-AAY-pAB peak area to the NEP inhibitor concentration were constructed for both phosphoramidon and SCH 39370, and the inhibitor concentrations present in the experimental samples were determined from these curves. The levels of detection of these assays were 8 nM (SCH 39370) and 16 nM (phosphoramidon).

Measurement of blood levels of EP 24.15 inhibitors Plasma samples from experiments 2 and 3 were analysed for intact cFP-AAY-pAB and cFP-AVY-pAB content. Plasma (1 ml) was added to 5 ml methanol to precipitate protein, and centrifuged at 3000 r.p.m. for 10 min at 4°C. The supernatant was collected into 5 ml tubes and partially evaporated (to remove the organic component) in a Speedvac concentrator. The remaining aqueous phase ($\sim$ 0.5 ml) was then transferred to Eppendorf tubes.

For experiment 2, the volume of the 10 s, 1 min and 5 min samples were restored to 1 ml with water, and 50, 100 or 200 μl, respectively, was then analysed by h.p.l.c., with absorbance monitored at 254 nm to minimize interference by plasma components. For the 30 min samples, the volume was adjusted to 500 μl, of which 200 μl was analysed. Blood samples containing standard concentrations of cFP-AAY-pAB (0.01, 0.1, 1.0 and 10 μM) were assayed in the same manner. The level of detection was approximately 0.05 μM.

For experiment 3, samples were dried completely, reconstituted in 0.08% TFA, and the total radioactivity determined prior to analysis. When total radioactivity exceeded 100,000 c.p.m., only half the sample was injected on to the column. Levels of unlabelled cFP-AAY-pAB or cFP-AVY-pAB were measured as in experiment 2, and were undetectable at the 30 and 60 min time points. In addition, the metabolism of [^{125}I]-cFP-AAY-pAB and [^{125}I]-cFP-AVY-pAB was assessed by counting radioactivity (Packard Riastar γ-counter) in 70 fractions (0.5 ml) collected from each h.p.l.c. run.

The level of cFP-AAY-pAB or cFP-AVY-pAB in plasma samples was determined by relating peak area to concentration using the standards, followed by estimation of levels in the experimental samples. The extent of degradation of [^{125}I]-cFP-AAY-pAB and [^{125}I]-cFP-AVY-pAB was quantitated by summing the counts present in fractions identified as 'intact inhibitor' (based on h.p.l.c. analysis of iodinated inhibitor standards), 'free ^{125}I' (non-retained counts), or 'intermediate metabolites' (other clearly identifiable peaks), and expressing as a percentage of the total radioactivity (summation of all fractions).

High performance liquid chromatography (h.p.l.c.)

Samples were injected on to a Novapak C18 column (8.00 mm i.d. $\times$ 100 mm) contained within a radial compression module (Waters Associates), and constituents were eluted from the column by a linear (30 min) gradient from 3% – 70% solvent B (70% CH_3CN/0.08% TFA; solvent A = 0.08% TFA) at a flow rate of 1 ml min^{-1}. For analysis of iodinated inhibitors, a gradient from 3% – 100% B over 30 min was used. Chromatography was performed with a Waters h.p.l.c. system, consisting of an automated gradient controller (Model 680), two Model 510 pumps, a Model 712 (WISP) autosampler, and a Model 441 detector (214 or 254 nm), and the data analysed by Waters Maxima computer software.

Statistical analysis

The statistical computer software package SYSTAT (Wilkinson, 1990) was used for statistical analyses. P values ≤ 0.05 were considered to be significant. In most cases, repeated measures analysis of variance was utilized, P values being conservatively adjusted using the Greenhouse-Geiser correc-

tion (Ludbrook, 1994). Conventional analysis of variance followed by specific multiple comparisons was performed in some cases. When more than one P value was used from a particular analysis, the Dunn-Sidak correction was applied to protect against the increased risk of type I error (Ludbrook, 1991). All data are expressed as the mean ± 1 s.e.mean, except for estimated plasma levels of the endopeptidase inhibitors cFP-AAY-pAB, cFP-AVY-pAB, SCH 39370 and phosphoramidon. These are expressed as the geometric mean followed by the 95% confidence intervals calculated from the logarithms (to base 10) of the estimated values.

Drugs

Drugs used in this study were SCH 39370 (N-[N-[1-(s)-carboxyl-3-phenylpropyl]-(s)-phenylalanyl]-(s)-isoserine) (Sybertz *et al.*, 1989) (a gift from Schering-Plough Pty Ltd, Baulkham Hills, Australia); phosphoramidon (Peptide Institute Inc., Osaka, Japan); 7-methoxycoumarin-4-acetyl-Pro-Leu-Gly-Pro-D-Lys(2,4-dinitrophenyl), angiotensin I, angiotensin II and bradykinin (Auspep, Parkville, Australia); gonadotropin-releasing hormone$_{1-9}$ (gift of Dr Paul Schober, Peptide Technology, Sydney, Australia) and 2-hydroxypropyl-β-cyclodextrin (Research Biochemicals Incorporated, U.S.A.).

Results

In vitro *studies*

Inhibition of EP 24.15 by analogues of cFP-AAY-pAB In the initial screening of EP 24.15 inhibition, the Val-, Leu- and Ser-substituted cFP-AAY-pAB analogues were 6.5, 8.3 and 20 fold less potent than the parent compound, respectively (Table 1); analogues containing a D-Ala or an Asp substitution were at least 4000 fold less potent than the parent compound and were not studied further. Kinetic analysis of individual stereoisomers of the active analogues was then performed using a specific quenched fluorescence substrate. This revealed that inhibition of EP 24.15 is highly stereospecific, with a 15–50 fold difference in the inhibitory constants between stereoisomers (Table 1); this is greater than the 2 fold difference in stereoisomer potency observed previously for cFP-AAY-pAB (Orlowski *et al.*, 1988) and the 9 fold difference seen for the related inhibitor cFP-AAF-pAB (Chu & Orlowski, 1984). The rank order of potency among the more active stereoisomers of the analogues was Ala > Val > Ser > Leu, with a 3 fold difference between the parent compound and the Val-substituted analogue.

Table 1 Potency and stability of cFP-AAY-pAB analogues *in vitro*

Compound	*Initial screen* IC_{50} (μM)	K_i (μM)	t_{50} (min)
cFP-AAY-pAB	0.23		
stereoisomer 1		0.141	33.4
stereoisomer 2		0.009	23.1
cFP-AVY-pAB	1.5		
stereoisomer 1		0.030	>300
stereoisomer 2		1.44	>300
cFP-ALY-pAB			
stereoisomer 1	1.9	0.147	>300
cFP-ASY-pAB	4.5		
stereoisomer 1		1.62	75.9
stereoisomer 2		0.055	87.6
cFP-AdAY-pAB	>1000	ND	ND
cFP-ADY-pAB	>1000	ND	ND

Shown are IC_{50} values for each analogue (racemic mix) determined by the inhibition of $GnRH_{1-9}$ degradation by recombinant EP 24.15; K_i values for each stereoisomer of each analogue determined by the inhibition of fluorescent substrate cleavage by recombinant EP 24.15; and the time taken for 50% of the stereoisomer to be degraded by rabbit kidney membranes (t_{50}). cFP-AAY-pAB = N-[1-(**RS**)-carboxy-3-phenylpropyl]-Ala-Ala-Tyr-*p*-aminobenzoate. The single letter code for amino acid residues is used, such that A = alanine, V = valine, Y = tyrosine, L = leucine, S = serine, D = aspartate, and dA = the D-isomer of alanine. ND = not determined.

Inhibitor degradation by kidney membranes The parent compound was readily degraded by kidney membranes, consistent with previous evidence that NEP can cleave the inhibitor (Cardozo & Orlowski, 1993; Williams *et al.*, 1993; Telford *et al.*, 1995). Degradation of cFP-AAY-pAB in this system can be ascribed chiefly to NEP, as cleavage was >85% inhibited by 1 μM phosphoramidon or SCH 39370 (data not shown). Of the analogues, only the Ser-substituted inhibitor was significantly degraded in this assay, although it too was more stable than cFP-AAY-pAB (Table 1). Cleavage of both cFP-AAY-pAB and cFP-ASY-pAB displayed some stereospecificity, with the

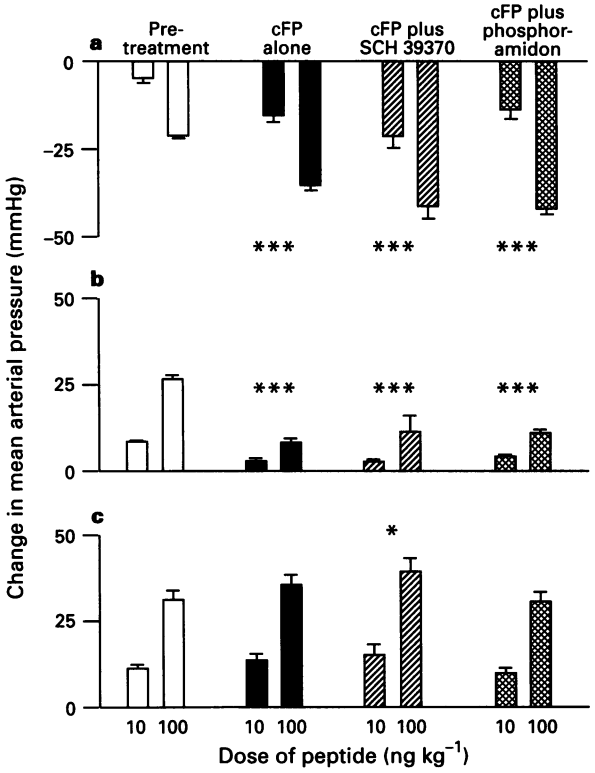

Figure 1 Effects of right atrial administration of (a) bradykinin, (b) AI and (c) AII (10 and 100 ng kg^{-1} of each) on mean arterial pressure in conscious rabbits. The open columns show the average maximum responses before treatment, while the solid columns show the maximum responses after cFP-AAY-pAB (5 mg kg^{-1}). The hatched columns show the maximum responses when cFP-AAY-pAB (abbreviated cFP in figure) was administered after pretreatment with SCH 39370 (3 mg kg^{-1} plus 3 mg kg^{-1} min^{-1}) and (cross-hatched columns) with phosphoramidon (5 mg kg^{-1} plus 5 mg kg^{-1} min^{-1}), respectively. The columns represent means and s.e.means of four observations. These data were analysed by analysis of variance, the factors being peptide dose (10 and 100 ng kg^{-1}), time (before and after the enzyme inhibitor treatments), and treatment (SCH 39370, phosphoramidon or vehicle). Each analysis was then partitioned by treatment, and the test statistic used was the main effect of time. Thus, $*P \leq 0.05$ and $***P \leq 0.001$ for the effect of each treatment on the dose-dependent effects of bradykinin, AI and AII. Since three comparisons were made within each analysis of variance, the Dunn-Sidak correction was applied to protect against the risk of type I errors.

half-times of degradation differing between isomers by approximately 10 min. Interestingly, the stereospecificity of inhibitor stability did not correlate with potency, i.e. the more potent isomer of cFP-AAY-pAB was also the more susceptible to cleavage, whereas the more potent cFP-ASY-pAB isomer was more resistant. Iodination of cFP-AAY-pAB and cFP-AVY-pAB increased the half-times of degradation 7 and 34 fold, respectively (data not shown).

In vivo *studies*

(1) Effect of NEP inhibition by phosphoramidon and SCH 39370 on the ACE-inhibiting activity of cFP-AAY-pAB Right atrial administration of bradykinin (10 and 100 ng kg^{-1}) was followed by transient reductions in mean arterial pressure of 5 ± 1 and 21 ± 2 mmHg respectively (Figure 1a). Following cFP-AAY-pAB (5 mg kg^{-1}, i.v.), the magnitude (Figure 1a) and duration (data not shown) of these responses was increased (P always ≤ 0.001), as we have shown previously (Telford *et al.*, 1995). The depressor effect of bradykinin was also potentiated when cFP-AAY-pAB was administered after pretreatment with SCH 39370 (3 mg kg^{-1} plus 3 mg kg^{-1} h^{-1}) or phosphoramidon (5 mg kg^{-1} plus 5 mg kg^{-1} h^{-1}) (P always ≤ 0.001) (Figure 1a).

Administration of AI (10 and 100 ng kg^{-1}) was followed by transient increases in mean arterial pressure of 9 ± 1 and 27 ± 1 mmHg, respectively (Figure 1b). The magnitude of the responses to AI was reduced to 3 ± 1 and 8 ± 1 mmHg following cFP-AAY-pAB-treatment, suggesting inhibition of ACE. The pressor response to AI was similarly inhibited when cFP-AAY-pAB was administered after pretreatment with SCH 39370 or phosphoramidon (P always ≤ 0.001).

Administration of AII (10 and 100 ng kg^{-1}) was followed by transient increases in mean arterial pressure of 11 ± 1 and 30 ± 3 mmHg, respectively (Figure 1c). CFP-AAY-pAB treatment alone had no effect on the response to AII ($P=0.33$). In contrast, when cFP-AAY-pAB was administered following pretreatment with SCH 39370 (but not phosphoramidon), the magnitude of the responses to AII was increased to 15 ± 3 and 39 ± 4 mmHg ($P=0.05$).

Following the start of SCH 39370 treatment, the estimated plasma levels of the NEP inhibitor averaged 2.7 (1.5–5.0) μM (expressed as mean value with 95% confidence limits in parentheses) at 5 min. Twenty min after the start of SCH 39370 treatment, at the time of cFP-AAY-pAB administration, plasma levels had increased still further to average 3.4 (1.7–6.8) μM. By the time the effects of the vasoactive peptides had been re-tested (50 min after starting SCH 39370 infusion), plasma levels were reduced, averaging 0.31 (0.08–1.3) μM. Similarly, infusion of phosphoramidon resulted in estimated plasma levels of 0.85 (0.26–2.8) μM at 5 min, 1.6 (0.37–6.6) μM at 20 min, and 0.74 (0.21–2.6) μM at 50 min. Thus throughout the experimental period, plasma levels of the NEP inhibitors were well above the K_i for the enzyme (11 nM for SCH 39370, Sybertz *et al.*, 1989; 2 nM for phosphoramidon, Thorsett & Wyvratt, 1987).

(2) Effects of SCH 39370 and phosphoramidon on plasma levels of cFP-AAY-pAB When cFP-AAY-pAB (5 mg kg^{-1}) was administered without prior treatment with either of the NEP inhibitors, plasma levels fell rapidly from a peak of 125 (52–298) μM 10 s after bolus administration, to be 34 (14–79), 2.9 (1.2–6.9) and 0.32 (0.11–0.97) μM at 1, 5 and 30 min respectively. Prior treatment with SCH 39370 or phosphoramidon slowed the disappearance of cFP-AAY-pAB from the circulation slightly, but statistically significantly ($P=0.02$ and 0.002 respectively). This effect was most apparent 5 min after cFP-AAY-pAB administration, when levels of cFP-AAY-pAB after phosphoramidon or SCH 39370 treatment averaged 8.0 (3.6–18) μM and 8.8 (7.2–11) μM respectively.

(3) Metabolic fate of cFP-AAY-pAB and cFP-AVY-pAB As seen in experiment 1, AI administration was followed by transient and dose-dependent increases in mean arterial pressure (Figure 2). The dose of AI which increased mean arterial pressure by 20 mmHg ($EC_{20\ mmHg}$) was increased 10.7 (0.6–186) fold (expressed as mean value with 95% confidence limits in parentheses, $P=0.015$) by 1 mg kg^{-1} cFP-AAY-pAB. Treatment with cFP-AVY-pAB also increased the $ED_{20\ mmHg}$ (by 2.6 (1.1–7.2) fold; $P=0.015$). Although there was no significant difference between the dose-ratios (i.e., the ratio of the

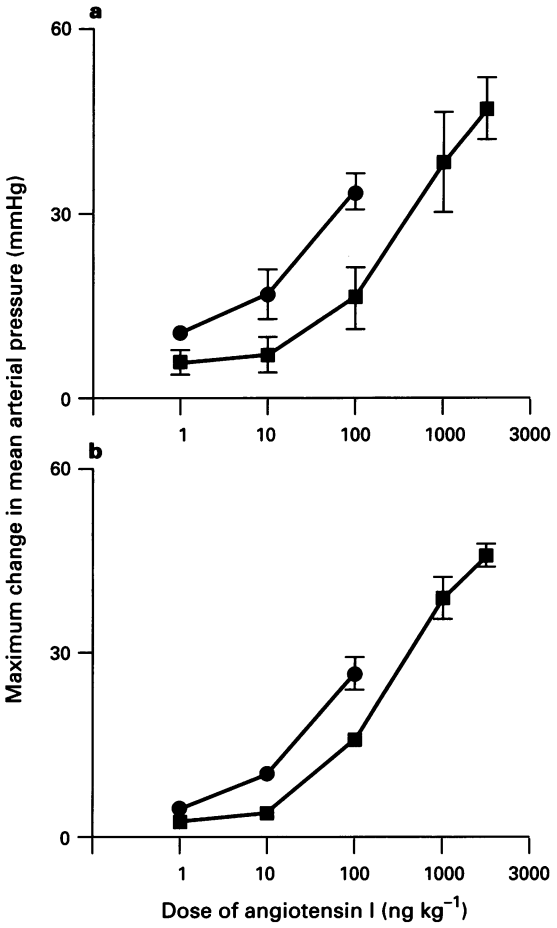

Figure 2 Effects of right atrial administration of AI on mean arterial pressure in conscious rabbits before (●) and after (■) administration of 1 mg kg^{-1} cFP-AAY-pAB (a) or cFP-AVY-pAB (b). Each point represents the mean maximum change in arterial pressure ± s.e.mean of four observations.

Table 2 Plasma cFP-AAY-pAB and cFP-AVY-pAB concentrations following bolus administration (1 mg kg^{-1})

Time after injection	*Mean plasma inhibitor concentration (μM) (95% confidence interval)* *cFP-AAY-pAB*		*cFP-AVY-pAB*	
10 s	23.5	(16.7-32.9)	18.0	(14.4-22.4)
30 s	11.3	(9.2-13.9)	8.4	(6.8-10.4)
1 min	5.5	(4.5-6.7)	2.6	(1.8-3.9)
5 min	0.21	(0.17-0.24)	0.17	(0.15-0.20)
30 min		<0.05		<0.05
60 min		<0.05		<0.05

Inhibitor concentrations were measured in plasma samples by u.v. absorbance (254 nm) during h.p.l.c. analysis. Values represent the geometric mean (μM) of 4 observations at each time point; 95% confidence intervals are indicated in parentheses. The rates of disappearance of cFP-AAY-pAB and cFP-AVP-pAB were not different ($P=0.215$).

$ED_{20\ mmHg}$ after inhibitor to the $ED_{20\ mmHg}$ before inhibitor) for the two inhibitors ($P=0.20$), it is interesting that the fragment cFP-AA (generated by incubation with kidney membranes) was also approximately 4 fold more potent than cFP-AV in inhibiting plasma ACE *in vitro* ($IC_{50}=0.2$ μM vs. 0.8 μM) (C. Murone & F.A.O. Mendelsohn, personal communication).

Table 3 Disappearance of radioactivity from the circulation following bolus administration of cFP-AA-[^{125}I]$_2$Y-pAB and cFP-AV-[^{125}I]$_2$Y-pAB

	Plasma ^{125}I (% total injected)	
Time	*cFP-AA-[^{125}I]$_2$Y-pAB*	*cFP-AV-[^{125}I]$_2$Y-pAB*
10 s	88.0±8.0	71.4±11.4
30 s	63.0±8.1	59.3±11.2
1 min	37.6±2.4	37.9±7.9
5 min	13.2±1.2	18.8±6.0
30 min	14.6±0.8	9.9±0.9
60 min	12.5±0.7	10.1±1.1

The radioactivity in the plasma compartment at each time point was determined by extrapolating the counts in a 50 μl aliquot of blood to the estimated blood volume of each rabbit (65 ml kg^{-1}); this was then expressed as a percentage of the total radioactivity injected. Each value represents the mean±s.e.mean of 4 observations. The rates of disappearance of cFP-AA-[^{125}I]$_2$Y-pAB and cFP-AV-[^{125}I]$_2$Y-pAB from the plasma were not different ($P=0.365$).

Administration of 1 mg kg^{-1} cFP-AAY-pAB resulted in a peak plasma concentration of 23.5 (16.7–32.9) μM at 10 s (Table 2). As would be expected, this level is approximately 5 fold less than that seen after injection of 5 mg kg^{-1} cFP-AAY-pAB. Injection of cFP-AVY-pAB (1 mg kg^{-1}) resulted in initial plasma concentrations very similar to cFP-AAY-pAB (18.0 (14.4–22.4) μM), and the disappearance rate of the inhibitors was indistinguishable (interaction term from repeated measures analysis of variance, $P=0.2$). Neither inhibitor could be detected by u.v. absorbance in the 30 or 60 min samples.

Following administration of cFP-AA-[^{125}I]$_2$Y-pAB or cFP-AV-[^{125}I]$_2$Y-pAB, total radioactivity disappeared rapidly from the circulation, with less than 20% of the injected label still present 5 min later (Table 3). After this initial redistribution, the level of radioactivity in the plasma stabilized and remained at 10–15% of the amount injected for the next hour. H.p.l.c. analysis revealed that 5 min after injection, only 25–30% of the remaining radioactivity in the plasma was in the form of intact inhibitor, be it cFP-AAY-pAB or cFP-AVY-pAB (Figure 3a). At this same time point, much of the radiolabel was not retained on the C18 column, and based on our previous observations, represented either free ^{125}I or a small, non-hydrophobic metabolite of the iodinated tyrosine residue. The rate of liberation of this non-retained material was greater for cFP-AV-[^{125}I]$_2$Y-pAB than for cFP-AA-[^{125}I]$_2$Y-pAB (Figure 3b; $P=0.02$, interaction term from repeated measures analysis of variance). A significant proportion of radiolabel was also present in 4–5 other readily identifiable peaks (Figure 4), which represent a number of unknown metabolites of the in-

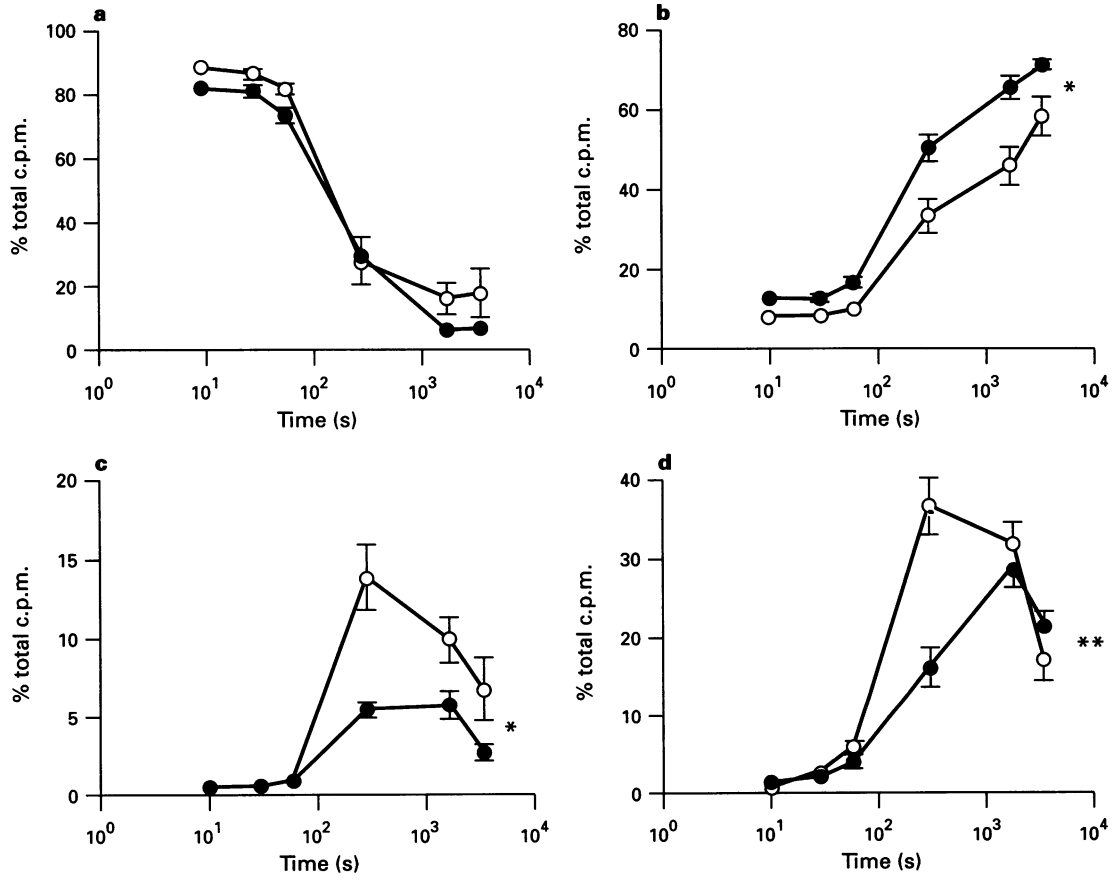

Figure 3 Metabolism of cFP-AA-[^{125}I]$_2$-Y-pAB (○) and cFP-AV-[^{125}I]$_2$-Y-pAB (●) in conscious rabbits. Plasma samples at each time point were analysed by h.p.l.c. and the collected fractions counted for radioactivity. Counts present in fractions identified as intact inhibitor (a), free ^{125}I (b), [^{125}I]$_2$-Y-pAB (c), and total intermediate metabolites (d), are expressed as a percentage of the total radioactivity (summation of all fractions). Each point represents the mean±s.e.mean of four observations. Levels of intact inhibitor, intermediate metabolites, and free ^{125}I in the iodinated inhibitor standards averaged 84.0±1.7%, 0.6±0.1%, and 10.7±2.7%, respectively, for cFP-AA-[^{125}I]$_2$-Y-pAB, and 70.2±3.1%, 2.8±0.9%, and 18.1±2.2%, respectively for cFP-AV-[^{125}I]$_2$-Y-pAB ($n=4$/inhibitor, 10,000–90,000 c.p.m./h.p.l.c. run). *$P\leq0.05$, **$P\leq0.01$, outcome of repeated measures analysis of variance testing of non-parallelism of the metabolite profiles of the two iodinated analogues.

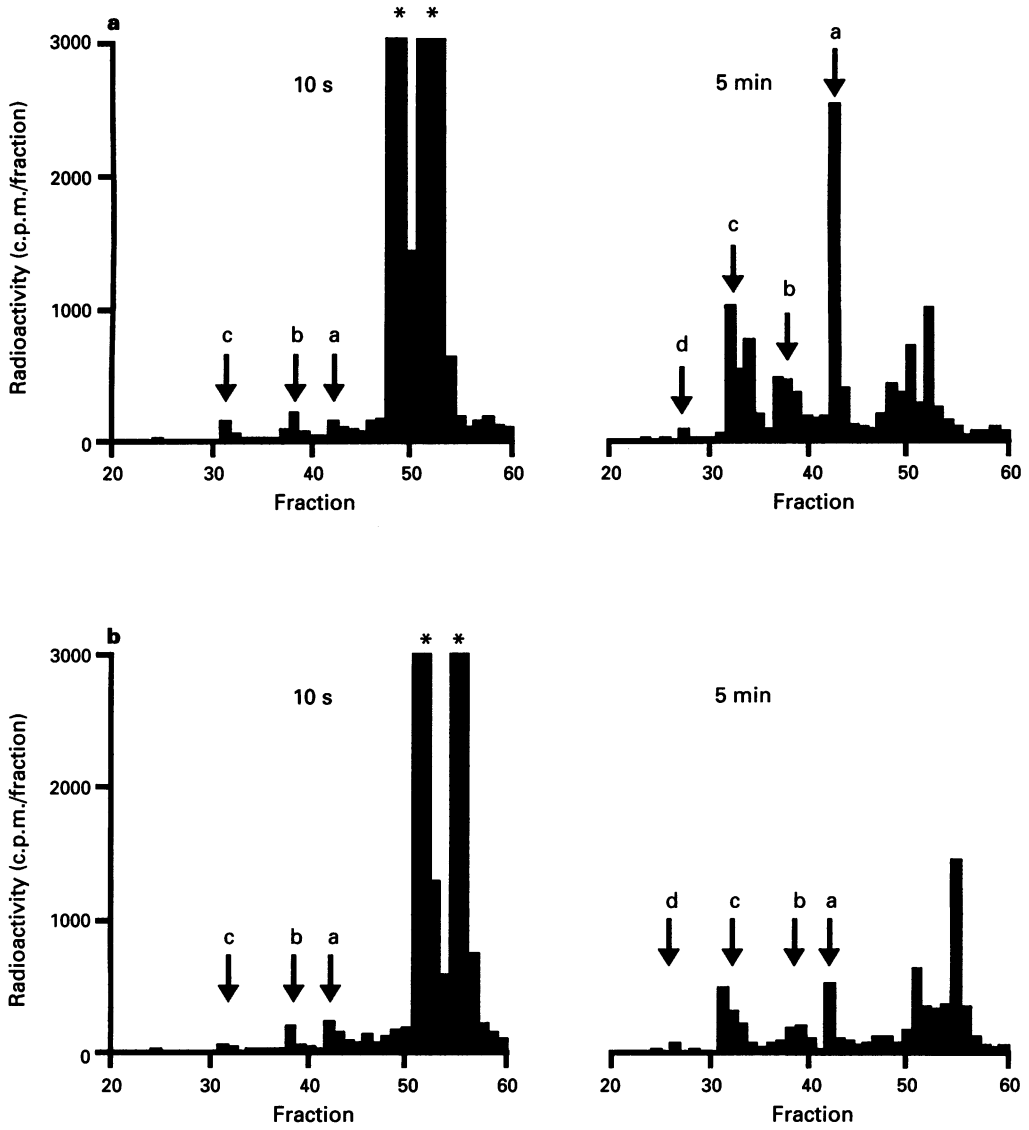

Figure 4 H.p.l.c. profiles of radioactivity in plasma samples from two rabbits at times 10 s and 5 min following administration of either cFP-AA-[^{125}I]$_2$-Y-pAB (a) or cFP-AV-[^{125}I]$_2$-Y-pAB (b). The asterisks represent the two stereoisomers of each inhibitor, which were >3000 c.p.m./fraction. Metabolites are indicated as peaks a–d; peak a corresponds to the NEP-generated fragment [^{125}I]$_2$-Y-pAB. Free ^{125}I was not retained on the column and eluted in fractions 7 and 8 (not shown).

hibitors. One of the peaks of radioactivity (peak 'a' in Figure 4) had a retention time identical to [I]$_2$-Tyr-pAB (generated *in vitro* and identified by mass spectrometry), and was formed from both radiolabelled analogues *in vivo*. The rate of accumulation of this metabolite was significantly greater for cFP-AA-[^{125}I]$_2$Y-pAB than for cFP-AV-[^{125}I]$_2$Y-pAB (Figure 3c; $P=0.01$). Indeed, the rate of accumulation of the total percentage of radioactivity in the form of intermediates was less for cFP-AV-[^{125}I]$_2$Y-pAB than for cFP-AA-[^{125}I]$_2$Y-pAB (Figure 3d; $P=0.002$); this probably reflects a slower rate of cleavage by membrane and/or plasma peptidases.

Discussion

Although cFP-AAY-pAB and other inhibitors of EP 24.15 have proved useful in the biochemical characterization of the enzyme, studies of its biological function *in vivo* have been hampered by the rapid metabolism of these inhibitors in the circulation. In particular, the ACE-inhibitory effect of a major metabolite has confounded the interpretation of experiments in which the compound is administered. For example, the potentiation of bradykinin-induced hypotension by cFP-AAF-pAB led Genden & Molineaux (1991) to propose a significant role for EP 24.15 in the turnover of the vasodilator peptide in the circulation. Subsequent studies have shown that cFP-AAY-pAB not only slows bradykinin breakdown, but also blocks the conversion of AI to AII (Yang *et al.*, 1994; Telford *et al.*, 1995), a peptide cleavage not catalyzed by EP 24.15 *in vitro* (Chu & Orlowski, 1985; Lew *et al.*, 1995). These effects of cFP-AAY-pAB were equivalent to, and not additive with, the effects of ACE inhibitors (Yang *et al.*, 1994; Telford *et al.*, 1995). This observation, coupled with the cleavage of cFP-AAY-pAB by NEP *in vitro* to generate an enalapril-like metabolite (Cardozo & Orlowski, 1993; Williams *et al.*, 1993), strongly suggested that inhibition of ACE, rather than EP 24.15, accounted for the effects of cFP-AAY-pAB on bradykinin metabolism.

Similarly, the degradation of GnRH has also been ascribed to EP 24.15, as both intravenous and intracerebroventricular administration of cFP-AAF-pAB could retard the loss of exogenous GnRH (Molineaux *et al.*, 1988; Lasdun *et al.*, 1989; Lasdun & Orlowski, 1990). However, we have shown that this peptide is a poor substrate of EP 24.15, unless the C-terminal glycinamide has been removed by prolyl endopeptidase (Lew *et al.*, 1994; 1995). Given that GnRH is cleaved by ACE at

several peptide bonds (Skidgel & Erdos, 1985), the observations of Lasdun *et al.* (1989; 1990) may be the result of inhibition of that enzyme by the metabolite.

In the present study, we examined the effect of NEP inhibition, by two different inhibitors, on the metabolism of intravenously administered cFP-AAY-pAB, and the effect of these peptidase inhibitors on responses to angiotensin and bradykinin. Infusion of either phosphoramidon or the more specific SCH 39370, at levels which would significantly block NEP function, was ineffective in preventing the inhibition of ACE as assessed by pressor responses to AI (Figure 1). Interestingly, the magnitude of the response to AII was slightly, but statistically significantly, augmented by the combination of cFP-AAY-pAB and SCH 39370, but not phosphoramidon (Figure 1). The reason for this is unclear, but can be duplicated by SCH 39370 administration alone (unpublished observations), which can also boost AII levels in sheep receiving chronic AII infusions (Charles *et al.*, 1995), and in rats given AI acutely (Yamamoto *et al.*, 1992). These authors proposed a possible role for NEP in the degradation of AII, but it is unclear why the effect was seen only with SCH 39370 and not with phosphoramidon in our experiment.

Not only were phosphoramidon and SCH 39370 ineffective in preventing ACE inhibition by cFP-AAY-pAB, but these NEP inhibitors only marginally increased plasma cFP-AAY-pAB concentrations. This contrasts with the observations of Lasdun *et al.* (1989), who were the first to recognize the potential contribution of NEP to the clearance of cFP-AAF-pAB *in vivo*. These investigators sought to prevent metabolism of the EP 24.15 inhibitor by treatment with cFP-Phe-pAB, a NEP inhibitor (Pozsgay *et al.*, 1986). Eighty min after administration, plasma levels of cFP-AAF-pAB, as determined by inhibition of substrate cleavage by purified EP 24.15, were boosted 10 fold by co-administration of the NEP inhibitor. The relatively modest effect of NEP inhibition on cFP-AAY-pAB metabolism that we observed, compared to Lasdun *et al.* (1989), may be due to several factors, including: the time at which measurements were made (4 time points over 30 min vs. a single 80 min time point); the EP 24.15 inhibitor used (cFP-AAY-pAB vs. cFP-AAF-pAB), the latter compound being more susceptible to NEP (data not shown; Pozsgay *et al.*, 1986); the doses administered (8.3 μmol kg^{-1} in the present study vs. 156 μmol kg^{-1}); and the different species used.

Finally, in our study, an analogue of the EP 24.15 inhibitor, cFP-AVY-pAB, which was at least 10 fold more resistant to NEP degradation *in vitro* (Table 1), disappeared from the circulation at the same rate as cFP-AAY-pAB (Table 2) and also effectively blocked ACE (Figure 2). These results, together with the inability of the NEP inhibitors to prevent ACE inhibition, suggest that although NEP does degrade cFP-AAY-pAB, other mechanisms also contribute substantially to its metabolism and clearance.

One such mechanism is the very rapid re-distribution of cFP-AAY-pAB out of the plasma compartment, as shown by the disappearance of radiolabelled inhibitor (Table 2). Just 1 min after injection, more than 60% of the total radiolabel had been removed from the circulation, while very little degradation of the inhibitor had occurred. At later time points, the ^{125}I present in the plasma plateaued (Table 2), and degradation of the inhibitor became more apparent (Figures 3 and 4). Clearly, since iodination slows the cleavage of cFP-AAY-pAB by NEP, the degradation of the iodinated compounds followed here does not directly reflect the metabolism of the unlabelled inhibitor; however, a peak of radioactivity (peak 'a' in Figure 4) with a retention time identical to [^{125}I]$_2$-Tyr-pAB (generated *in vitro* and identified by mass spectrometry) was formed from both radiolabelled analogues *in vivo*, suggesting cleavage at the Ala/Val-Tyr bond. Furthermore, the magnitude of this peak was larger in plasma from rabbits receiving cFP-AA-[^{125}I]$_2$Y-pAB than from those receiving cFP-AV-[^{125}I]$_2$Y-pAB (Figure 3c; $P=0.01$), consistent with the relative resistance of the Val analogue to NEP. The presence of other radiolabelled intermediates (Figure 3) suggests pathways of degradation in addition to NEP; although these alternative cleavage events probably do not generate an ACE-inhibitory product, they would still contribute to the loss of EP 24.15 inhibitor from the circulation.

Although cFP-AAY-pAB and cFP-AVY-pAB behaved similarly *in vivo*, marked differences in the potency and stability of the EP 24.15 inhibitor analogues *in vitro* were observed. Introduction of the D-isomer of alanine or an acidic residue at the position of the second alanine resulted in a substantial reduction in the inhibition of recombinant EP 24.15, while Val-, Leu- and Ser-substitutions reduced potency more moderately (Table 1). Furthermore, we observed a significant difference in potency between the stereoisomers of each compound, as has been noted by Orlowski and co-workers (Chu & Orlowski, 1984; Orlowski *et al.*, 1988). Thus, the active site of EP 24.15 exhibits stereospecificity, and is sensitive to substitutions at the second alanine residue of cFP-AAY-pAB, particularly when a carboxyl (Asp) or hydroxyl group (Ser) is introduced.

Amino acid substitutions at this site also greatly affected the inhibitor's susceptibility to NEP cleavage. All the analogues were more resistant to degradation by kidney membranes than the parent compound (Table 1); indeed, the only analogue to be significantly degraded (>10% after 60 min) in this system was cFP-ASY-pAB. A bulky hydrophobic residue in the P_1 position apparently interferes with cleavage by NEP, as noted for phenylalanine by Pozsgay *et al.* (1986). Likewise, cleavage of the D-alanine analogue was also markedly reduced (data not shown), in keeping with the stereospecificity of NEP (Hersh & Morihara, 1986). Interestingly, both the potency and stability of a cFP-AAY-pAB analogue with a D-isomer in the first alanine position (D-Ala^1) were intermediate between the parent compound and the D-Ala^2 analogue (data not shown), suggesting that substitutions at this position are more readily tolerated by both EP 24.15 and NEP.

In summary, we have demonstrated that the EP 24.15 inhibitor cFP-AAY-pAB is cleared from the circulation by a number of mechanisms, including cleavage by NEP. However, administration of NEP inhibitors could protect cFP-AAY-pAB only marginally and could not adequately prevent the generation of an ACE inhibitor from the compound. Furthermore, an analogue of cFP-AAY-pAB which was relatively resistant to NEP *in vitro* also effectively inhibited ACE *in vivo*. These studies suggest that because of their rapid metabolism, the cFP-AAY-pAB-like inhibitors of EP 24.15 are of limited use in examining the role of this enzyme in the whole animal. Very recently, a series of phosphinic peptide inhibitors of EP 24.15 have been developed, which are both highly selective and very potent *in vitro* (Jiracek *et al.*, 1995). No data concerning their stability *in vivo* are yet available; however, as they are peptide-based, they may still be susceptible to proteolytic degradation. Ideally, more stable, preferably non-peptide-based inhibitors will be developed in the future which will allow us to assess more accurately the biological functions of EP 24.15.

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