Fate of Bradykinin-Potentiating Peptide 9a after Intravenous Injection

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The fate of $<Glu^1\text{-}^3\text{H}$ -labelled bradykinin-potentiating peptide 9a [BPP_{9a}; $<Glu\text{-}Trp\text{-}Pro\text{-}Arg\text{-}Pro\text{-}Gln\text{-}Ile\text{-}Pro\text{-}Pro$, an inhibitor of angiotensin-converting enzyme (peptidyl dipeptidase)] was studied in the rabbit. After intravenous injection, BPP_{9a} was rapidly removed from blood and much of the associated radioactivity was excreted in urine. Approx. 8% of the radioactivity in urine collected 2 h after drug administration occurred in the form of BPP_{9a} itself, the remainder occurring in three lower homologues: $<Glu\text{-}Trp$ (60%), $<Glu\text{-}Trp\text{-}Pro\text{-}Arg\text{-}Pro\text{-}Gln$ (20%) and $<Glu\text{-}Trp\text{-}Pro\text{-}Arg\text{-}Pro\text{-}Gln\text{-}Ile$ (12%). Hydrolysis was not accounted for by enzymes in blood or urine. Apparently hydrolysis occurred within the kidney, as $<Glu\text{-}Trp$ was obtained in 60% yield in urine of isolated rat kidney perfused with [$<Glu^1\text{-}^3\text{H}$]BPP_{9a}.

Bradykinin-potentiating peptide 9a ($<Glu\text{-}Trp\text{-}Pro\text{-}Arg\text{-}Pro\text{-}Gln\text{-}Ile\text{-}Pro\text{-}Pro$, known also as BPP_{9a} and compound SQ 20881) is a potent inhibitor of angiotensin-converting enzyme (peptidyl dipeptidase, EC 3.4.15.1) (Greene *et al.*, 1970; Ondetti *et al.*, 1971, 1972; Cushman & Cheung, 1972; Cheung & Cushman, 1973). BPP_{9a} lowers blood pressure in animal models of renovascular hypertension (Engle *et al.*, 1973; Muirhead *et al.*, 1974) and has been used to treat patients with renovascular or malignant hypertension (Gavras *et al.*, 1974, 1975; Johnson *et al.*, 1975; Case *et al.*, 1976, 1977). However, the effects of BPP_{9a} are relatively short-lasting, a surprising point in view of the resistance of BPP_{9a} to hydrolysis by well-characterized proteinase and peptidase enzymes, including angiotensin-converting enzyme itself (Ondetti *et al.*, 1971; Dorer *et al.*, 1974). Thus the present study was begun to improve understanding of the factors that limit the activity of BPP_{9a} after intravenous injection.

Materials and Methods

Synthesis of [$<Glu^1\text{-}^3\text{H}$]BPP_{9a}

L-[3- ^3H]Pyroglutamic acid (pyrrolid-2-one-5-carboxylic acid) was prepared from L-[3- ^3H]glutamic acid (ICN, Waltham, MA, U.S.A.) by the method of Wilson & Cannon (1937). [$<Glu^1\text{-}^3\text{H}$]BPP_{9a} was synthesized by the Merrifield solid-phase technique (Merrifield, 1964; Stewart & Young, 1969) such that L-[3- ^3H]pyroglutamic acid, with carrier pyroglutamic acid, was incorporated as the *N*-terminal

residue at 10mCi/mmol. The synthetic peptide was purified by chromatography on a column (5cm × 45cm) of Bio-Gel P-6 equilibrated and developed with 0.1M-NaHCO₃ followed by chromatography on a column (1.2cm × 100cm) of Bio-Gel P-2 equilibrated and developed with 0.1M-acetic acid adjusted to pH 5.0 with pyridine.

The purified material behaved as a single substance in five t.l.c. systems [silica-gel plates; butan-1-ol/ethyl acetate/acetic acid/water (1:1:1:1, by vol.); ethyl acetate/pyridine/acetic acid/water (5:5:1:3, by vol.); benzene/acetic acid/water (9:9:1, by vol.); butan-1-ol/acetic acid/water (75:13:12, by vol.); butan-1-ol/acetic acid/water (4:1:5, by vol.)] and on paper electrophoresis at pH 2.0 and 5.0 (Ryan *et al.*, 1971). It had a specific radioactivity of 10mCi/mmol (Chiu *et al.*, 1976) and was equipotent with unlabelled BPP_{9a} as an inhibitor of angiotensin-converting enzyme of lung, serum and urine (*I*₅₀ = 30nM) (Fisher *et al.*, 1979).

Intravenous injection and monitoring of labelled peptides

Unanaesthetized female rabbits, each weighing 1–2 kg, were injected via an ear vein with [$<Glu^1\text{-}^3\text{H}$]BPP_{9a} in doses of 1–2mg/kg, dissolved in 0.5 ml of 0.9% NaCl. To determine the apparent volume of distribution of BPP_{9a}, [1- ^{14}C]inulin (0.7 μmol at 7.8mCi/mmol; New England Nuclear Corp., Boston, MA, U.S.A.) was injected simultaneously with [$<Glu^1\text{-}^3\text{H}$]BPP_{9a}. Blood samples were collected from a vein of the ear not used for injection. Serum was collected after clot retraction and centrifugation at 1600g for 15 min. Serum (50 μl) was mixed with

Abbreviation used: BPP_{9a}, bradykinin-potentiating peptide 9a ($<Glu\text{-}Trp\text{-}Pro\text{-}Arg\text{-}Pro\text{-}Gln\text{-}Ile\text{-}Pro\text{-}Pro$).

10ml of Hydromix (Yorktown Research, South Hackensack, NJ, U.S.A.). Radioactivity was measured by liquid-scintillation counting in a two-channel Searle Isocap 300 instrument (Searle Analytic, Middleburg Heights, OH, U.S.A.).

Blood samples were collected before and after injection of BPP_{9a}, and inhibitory effects of BPP_{9a} were measured by following the disappearance and then reappearance of intrinsic plasma angiotensin-converting enzyme activity. Each serum sample (3 μ l) was incubated for 20 min at 37°C in a 100 μ l solution containing 100 μ M-*p*-[³H]benzoyl-Gly-His-Leu (³H-labelled hippuryl-His-Leu; 10mCi/mmol) in 0.05M-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] buffer, pH8.0, containing 0.1M-NaCl and 0.75M-Na₂SO₄ (Ryan *et al.*, 1978). Reactions were stopped by adding 1.0ml of 0.1M-HCl, and ³H-labelled hippuric acid was extracted into 1.0ml of ethyl acetate. A sample (usually 500 μ l) of the ethyl acetate layer was transferred to a scintillation vial containing 10ml of Hydromix, and ³H was measured by liquid-scintillation counting.

Identification of metabolites in urine

After injection of the radiolabelled BPP_{9a}, urine was collected in 5% (w/v) trichloroacetic acid to prevent degradation by urinary enzymes. The trichloroacetic acid was removed by extraction five times with 2vol. of ether. No radioactivity was extracted by ether.

The volume of urine was decreased (to 1–2ml) by rotary evaporation at 40°C, and the entire sample was applied to a Bio-Gel P-2 column (1.2cm $\times$ 100cm) developed with 0.1M-acetic acid adjusted to pH5.0 with pyridine. Fractions (2.2ml) were collected, and 50 μ l samples of each were removed for liquid-scintillation counting. Fractions corresponding to radioactive peaks were combined and decreased in volume such that 10 μ l of each concentrate contained approx. 3000 c.p.m.

Procedures for identifying metabolites included both t.l.c. and paper electrophoresis. T.l.c. was performed with LDQ silica-gel plates (Quantum Industries, Fairfield, NJ, U.S.A.) in the following systems (Table 1): I, butan-1-ol/ethyl acetate/acetic acid/water (1:1:1:1, by vol.); II, ethyl acetate/pyridine/acetic acid/water (5:5:1:3, by vol.); III, benzene/acetic acid/water (9:9:1, by vol.); IV, butan-1-ol/acetic acid/water (75:13:12, by vol.).

Electrophoresis on Whatman 3MM paper at pH5.0 (0.1M-acetic acid adjusted to pH5.0 with pyridine) (system V) sufficed to separate all possible acidic radioactive metabolites (Table 1). The radioactive metabolites that were neutral at pH5.0 (those containing arginine) were identified in terms of co-chromatography with authentic *N*-terminal lower homologues of BPP_{9a}, synthesized as described previously (Fisher *et al.*, 1979).

Perfusion of isolated kidney with [³H]BPP_{9a}

Perfusion of blood-free rat kidney was performed by the method of Nishiitsutsuji-Uwo *et al.* (1967) as modified by Epstein and colleagues (Perez *et al.*, 1978). [³H]BPP_{9a} (0.15mg) was perfused via the renal artery, and urine (approx. 2.2ml) was collected into 20ml of chilled (0°C) ethanol over a period of 60 min. The volume of the perfusion solution was 100ml. The urine/ethanol solution was decreased in volume by rotary evaporation to approx. 1 ml and was applied to the Bio-Gel P-2 column. The identification of metabolites was accomplished by the methods described above.

Results

Fate after intravenous injection

After intravenous injection of [³H]BPP_{9a}, blood concentrations of radioactivity increased abruptly and then decreased asymptotically (Fig. 1). When [³H]BPP_{9a} (1 mg/kg) and [1-¹⁴C]inulin

Fig. 1. Disappearance of radioactivity from blood after intravenous injection of [³H]BPP_{9a} and [1-¹⁴C]inulin

Rabbits were injected as described in the text. Venous blood samples were collected at timed intervals, and samples of serum were assayed for ³H and ¹⁴C. Erythrocytes did not take up ³H or ¹⁴C. The maximum radioactivity (c.p.m.) achieved in blood (20s after injection) was taken as 100%. ³H is indicated as ●, ¹⁴C as ▲. Some serum samples were assayed for their intrinsic angiotensin-converting enzyme activity (cf. Ryan *et al.*, 1977, 1978) to estimate plasma concentrations of active inhibitor. Before injection of inhibitor, serum angiotensin-converting enzyme activity was 17.8 nmol/min per ml. Inhibitor activity after injection of BPP_{9a} was expressed in terms of units: one unit was defined as the amount of BPP_{9a} required to decrease serum angiotensin-converting enzyme activity by 50%. A curve was constructed [enzyme activity (% of control) versus log(inhibitor concentration)] from the assumed relationship $E+I \rightleftharpoons E \cdot I$ (where E represents enzyme and I inhibitor). Inhibitor concentration was determined by interpolation and is indicated by ○.

(3 mg/kg) were injected together, ^{14}C - and ^3H -labelled materials disappeared from serum at comparable rates, a result that shows that BPP_{9a} and/or its metabolites are not restricted to the plasma space. BPP_{9a} , 3.5 min after injection, was distributed in an apparent volume of 247 ml/kg. Approx. 3% of the ^3H was found in urine collected 5 min after injection. The rate of appearance of ^3H in urine was variable: 25–80% ($52.5 \pm 19.6\%$, $n = 5$) of the radioactivity was excreted within 2 h of injection. Little more radioactivity was excreted over the next 22 h (approx. 5% of the total injected). In one experiment, urine was collected at timed intervals: 3% of the total radioactivity was excreted within 5 min of injection. Another 29% was excreted over the next 35 min and 20% more was excreted over the next 80 min.

Plasma angiotensin-converting enzyme activities remained at low values even after blood concentrations of radioactivity decreased to near base-line values (Fig. 1), a finding that suggests that the residual radioactivity is bound to the plasma enzyme. Presumably, immediately after injection of [^3H]- BPP_{9a} , the inhibitor in plasma is in at least two states or compartments: bound and unbound to plasma angiotensin-converting enzyme.

Radioactive material in urine collected 2 h after injection was separated into two peaks [fractions 22–33 (A) and 34–41 (B)] by molecular-sieve chromatography on Bio-Gel P-2. The labelled metabolites in these fractions were identified from their electrophoretic and chromatographic behaviours by comparison with authentic synthetic lower homologues (summarized in Table 1). The material designated

metabolite 1 was intact BPP_{9a} and accounted for approx. 8% of the total radioactivity excreted. Metabolite 2 (accounting for 12% of the excreted radioactivity) was identical with the heptapeptide <Glu-Trp-Pro-Arg-Pro-Gln-Ile and was clearly distinguished from the *N*-terminal octapeptide and hexapeptide (system II). Metabolite 3 (20% of the excreted radioactivity) co-chromatographed with the *N*-terminal hexapeptide <Glu-Trp-Pro-Arg-Pro-Gln and was distinguished from the pentapeptide <Glu-Trp-Pro-Arg-Pro. Metabolite 4, the only compound found in the second fraction from Bio-Gel P-2, behaved like <Glu-Trp in all five systems and was well separated from the other possible acidic metabolites on electrophoresis at pH 5.0 (system V). The dipeptide <Glu-Trp accounted for 60% of the total excreted radioactivity.

Metabolism by isolated perfused kidney

Perfusion of [^3H]- BPP_{9a} through isolated rat kidney yielded results similar to those obtained in experiments with intact rabbits. [^3H]-<Glu-Trp constituted 60% of the radioactivity excreted within 60 min of injection. The excreted radioactivity was 41% of the total injected. The other metabolites were not identified. The radioactivity in the perfusion solution remained in the form of BPP_{9a} ; there were no degradation products. [^3H]- BPP_{9a} was not degraded by incubation with rat or rabbit serum nor by rat urinary proteins (the latter concentrated 15-fold by ultrafiltration as described by Ryan *et al.*, 1978). In these experiments 10–50 μg of BPP_{9a} was incubated with 50 μl of serum or a solution of urinary

Table 1. Chromatographic and electrophoretic behaviours of BPP_{9a} , its lower homologues and its metabolites in urine. In t.l.c. systems I–IV the values given are calculated as R_F . In electrophoretic system V the values are given as R_{Arg} or ($R_{\text{Pieric acid}}$). Solvent systems are described in the text.

			Relative migration				
Standard compounds	System	...	I	II	III	IV	V
<Glu			0.23	0.50	0.50	0.40	(1.27)
<Glu-Trp			0.43	0.64	0.53	0.40	(0.54)
<Glu-Trp-Pro			0.44	0.60	0.57	0.30	(0.26)
<Glu-Trp-Pro-Arg			0.42	0.50	0.14	0.10	0.23
<Glu-Trp-Pro-Arg-Pro			0.19	0.14	0.0	0.0	0.16
<Glu-Trp-Pro-Arg-Pro-Gln			0.23	0.31	0.0	0.0	0.12
<Glu-Trp-Pro-Arg-Pro-Gln-Ile			0.50	0.50	0.13	0.07	0.11
<Glu-Trp-Pro-Arg-Pro-Gln-Ile-Pro			0.53	0.62	0.30	0.21	0.10
<Glu-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro (BPP _{9a})			0.45	0.40	0.12	0.03	0.23

Metabolites P-2 fractions	Metabolite		Recovery from urine (%)					
	No.	Structure						
22-33 (A)	1	BPP _{9a}	8	0.45	0.41	—	—	0.23
	2	<Glu ¹ ...Ile ⁷	12	0.50	0.51	—	—	0.10
	3	<Glu ¹ ...Gln ⁶	20	0.23	0.30	—	0.0	0.13
34-41 (B)	4	<Glu-Trp	60	0.42	0.64	0.52	0.40	(0.53)

proteins and 50 μ l of 0.2M-Tris, adjusted with 1M-HCl to pH 7.0, 8.0 or 9.0, for 3 h at 37°C. The reactions were monitored by chromatography on Bio-Gel P-2 and by t.l.c.

Discussion

The rapid appearance of BPP_{9a} and its metabolites in urine leads one to suspect that a single passage through the kidney is sufficient to remove large amounts of the peptide. Presumably BPP_{9a} is excreted by glomerular filtration, as is inulin. The fact that 60% of recovered radioactivity occurs as [³H]<Glu-Trp in perfusions of isolated kidney suggests that metabolism occurs after filtration. In view of these findings, it seems unusual that no [³H]pyroglutamic acid was detected even though the kidney is known to contain relatively large amounts of pyroglutamate aminopeptidase (EC 3.4.11.8) (Szewczuk & Kwiatkowska, 1970). It is possible that pyroglutamate aminopeptidase does not have ready access to a peptide such as BPP_{9a} entering the nephron by glomerular filtration. Alternatively, if pyroglutamic acid is released, it may be reabsorbed via an active-transport system for amino acids.

Our results indicate that the activity of BPP_{9a} is limited in part by rapid excretion via the kidney. However, in none of our experiments was the excretion quantitative. Given the fact that BPP_{9a} is an oligopeptide comprising six common amino acids, it is possible that some of the drug is degraded to its constituent amino acids, which are, in turn, further degraded or incorporated into new protein synthesis.

BPP_{9a} acts as a competitive inhibitor but not as a substrate for angiotensin-converting enzyme (Cheung & Cushman, 1973; Dorer *et al.*, 1974). Thus none of the metabolites of BPP_{9a} found in urine can be attributed to angiotensin-converting enzyme activity. The dissimilarity of the three peptide bonds hydrolysed (Trp²-Pro³, Gln⁶-Ile⁷ and Ile⁷-Pro⁸) indicates that as many as three enzymes catalyse the hydrolytic reactions. Hydrolysis of the Gln⁶-Ile⁷ bond suggests the possibility that a neutral endopeptidase (EC 3.4.24.11) (see Kenny & Booth, 1978) may participate in the metabolism of BPP_{9a}. The neutral endopeptidase is strategically situated (along the brush border of the renal proximal tubule) for processing filtered peptides. Finally, our results suggest that three enzymes, in addition to angiotensin-converting enzyme, have the ability to bind BPP_{9a}. Whether any of the known pharmacological effects of BPP_{9a} can be accounted for by its binding to enzymes other than angiotensin-converting enzyme remains to be determined.

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