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A Specific Competitive Inhibitor of Angiotensin II

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Abstract. A peptide analog, [4-phenylalanine, 8-tyrosine]-angiotensin II, was prepared by solid phase peptide synthesis and its structure confirmed. The compound was found to be a potent inhibitor of angiotensin II *in vitro* (isolated rat uterus strips) and *in vivo* (rat blood pressure). The analog was found to be highly specific and did not inhibit the action of a number of other peptides and spasmogenic compounds.

Numerous analogs of peptide hormones have been synthesized in an attempt to delineate their structure–activity relationship. Analogs of different peptide hormones that compete for the active site and inhibit the action of the native hormone have been sought in order to separate receptor binding characteristics from biological activity. Analogs of vasopressin that antagonize its antidiuretic action have been found,[1] and one has been recently shown[2] to inhibit the vasopressin-induced stimulation of kidney adenyl cyclase. Synthetic D-histidyl-D-phenylalanyl-D-arginyl-D-tryptophanylglycine, the optical antipode of an active fragment of α-melanocyte-stimulating hormone, inhibited the action of the corresponding pentapeptide of the L-configuration.[3] This observation led to the synthesis of the optical antipodes of bradykinin,[4] angiotensin,[5] and oxytocin,[6] none of which showed activity either as agonist or antagonist. Although these latter studies have not yielded antagonists, they have important theoretical implications on the nature of the groups involved in binding.

Stewart and Woolley[7] have reported a bradykinin inhibitor; however, the intrinsic agonistic activity of this compound, as well as its lability as an inhibitor, limited its utility as an antagonist. Recently, Khairallah *et al.*[8] have reported an analog of angiotensin II ([Ile^5, Ala^8]-angiotensin II) which antagonized (at 500 ng/ml) the action of angiotensin on strips of guinea pig ileum, but had no antagonistic activity *in vivo*. Paradoxically, when this compound was assayed in lower doses (less than 500 ng/ml), it exhibited an agonistic activity equivalent to as much as 0.1% angiotensin II both *in vitro*[9] and *in vivo*.[8]

This investigation describes an analog of angiotensin II in which the phenylalanyl and tyrosyl residues have been transposed, [Phe^4, Tyr^8]-angiotensin II, which was prepared by solid-phase peptide synthesis and which has been found to be a potent specific inhibitor of angiotensin II both in isolated uterine muscle and in the intact animal.

Methods. Synthesis of [Phe^4, Tyr^8]-angiotensin II: The basic solid-phase procedure of Marshall and Merrifield[10] was followed with two major modifications. First,

the imidazole (*im*) ring was protected with the dinitrophenyl group,[11] which was removed prior to cleavage[12] of the covalent link to the polymer with anhydrous HF. 1.0 g of 2% divinylbenzene–polystyrene resin was substituted with 0.25 mmol of *t*-butyloxycarbonyl (Boc)-*O*-benzyl-L-tyrosine per g. The peptide chain was extended with the appropriate amino acid derivatives by the following protocol: wash 3× glacial acetic acid, deprotect 20 min with 1 N HCl in acetic acid, wash 3× glacial acetic acid, wash 3× ethanol, wash 3× chloroform, neutralize 10 min with triethylamine–chloroform 1:9, wash 3× chloroform, wash 3× methylene chloride, couple with 0.75 mmol of Boc-amino acid and 0.75 mmol of dicyclohexylcarbodiimide for at least 2 hr. In this manner (Boc-β-benzyl-L-aspartyl)-(nitro-L-arginyl)-L-valyl-L-phenylalanyl-L-valyl-(*im*-dinitrophenyl-L-histidyl)-L-prolyl-(*O*-benzyl-L-tyrosyl) polymer was synthesized. The dinitrophenyl group was removed by reaction with 6.25 mmol of thiophenol in dimethylformamide for 1 hr (J. M. Stewart, personal communication). The peptide polymer was then washed 3× with dimethylformamide and 3× with dichloromethane and dried under vacuum. The polymer was placed in a polypropylene reaction vessel with 2 ml of anisole and cleaved with 10 ml of anhydrous HF, in an apparatus similar to that described by Pourchot and Johnson[13], for 1 hr at room temperature. The HF and anisole were removed first with a stream of N_2 and then under reduced pressure. The dry material was washed with ethyl acetate, extracted with 1% acetic acid, and lyophilized to yield 0.475 g of slightly yellow crude product. 0.355 g was purified by partition chromatography on Sephadex G-25 according to Smeby *et al.*[14] with elution by the upper phase of butanol–acetic acid–water 4:1:5 on a 49 × 2.5-cm column. Fractions of 2.8 ml were collected. Tubes 63–110 were combined; water was added and evaporated under reduced pressure to remove butanol. The white product obtained by lyophilization weighed 159 mg, a yield of 69% based on the original tyrosine on the polymer.

The compound was characterized by electrophoresis at 2.5 kV for 90 min with pyridine acetate buffer (pyridine–acetic acid–water 1:10:289), pH 3.75, and gave one spot by Clorox–starch spray.[15] Thin-layer chromatography was performed on 0.25-mm Silica Gel G plates obtained from Analtech, Inc. In butanol–pyridine–acetic acid–water (BPAW) 50:10:3:12 the R_f of angiotensin was 0.16, that of the [Phe^4, Tyr^8] analog 0.07; in *sec*-butanol–3% ammonium hydroxide 100:44 the R_f of angiotensin was 0.34, that of the analog 0.32.

Amino acid analyses were performed on a Spinco 120C analyzer after digestion with 6 N HCl for 60 hr at 110°C or after enzymatic digestion in 0.067 M phosphate buffer, pH 7.5, at 37°C for 3 hr with aminopeptidase M obtained from Henley & Co., N.Y. (Table 1).

The carboxyl-terminal residue, tyrosine, was then removed by enzymatic digestion. 20 mg of the peptide was dissolved in 4 ml of water and adjusted to pH 7.2 with 2 N LiOH. This was digested for 2.5 hr at 37°C with 2.5 mg of carboxypeptidase A (treated with di-isopropyl phosphofluoridate, DFP) obtained from Sigma Chemical Co. The reaction was stopped by heating at 60°C for 10 min. The mixture was lyophilized and a portion chromatographed on thin-layer plates in BPAW. A new spot with the R_f of tyrosine was detected. The spot seen before carboxypeptidase digestion disappeared and was replaced by one with R_f 0.67 of that of the original spot. The material in the new spot was purified by thin-layer chromatography and hydrolyzed in 12 N HCl–propionic acid 1:1 for 2 hr at 130°C.[16] The amino acid analysis (Table 1) showed complete removal of the C-terminal residue by the absence of tyrosine in the hydrolysate.

Isolated uterus: The uterus was removed from a decapitated albino rat (Holtzman, 150–200 g), freed of adhering fat, and divided into four pieces (2-cm each). The tissue was suspended in a 5-ml tissue bath at room temperature in deJalon's solution[17] and aerated with 95% O_2–5% CO_2. The quiescent uterus preparations were equilibrated for 10 min before drug addition under 1 g of initial tension. Contractions were measured with a myograph (F-50 linear-core transducer, Physiograph, E & M Instrument Co., Houston, Texas). Agonists dissolved in water (5 μl) were added directly to the bath, which was drained after each response was completed. However, when the

TABLE 1. *Amino acid analysis of [Phe,[4] Tyr[8]]-angiotensin II and carboxypeptidase derivative.*

	Angiotensin analog		Carboxypeptidase derivative; acid hydrolysis
	Acid hydrolysis	Peptidase hydrolysis	
Asp	1.10	1.10	0.94
Arg	1.00	0.91	0.82
Val	1.70	1.80	2.13
Phe	1.00	1.00	1.00
His	0.87	0.69	0.75
Pro	1.10	0.67	0.85
Tyr	0.96	0.95	0.00

antagonist was studied the bath was not drained prior to addition of the agonist. Angiotensin standard curves were determined both before and after the antagonist experiments, so that each strip served as its own control.

Blood pressure: Blood pressure was measured on albino rats (Holtzman, 150–200 g), anesthetized with pentobarbital sodium (30 mg/kg, intraperitoneal). The rats were treated with phenoxybenzamine (10 mg/kg, intraperitoneal) 1 hr prior to surgery. The animals were bilaterally nephrectomized by ligation of the renal arteries and renal veins. The blood pressure was recorded from the carotid artery (linear-core transducer, Physiograph). Test materials (50 μl) were injected into the cannulated right jugular vein. An angiotensin II standard curve was determined before each test of the antagonist so that each animal served as its own control. Genetically hypertensive rats were obtained from Manor Research (East Brunswick, N.J.).

Results. ***In vitro* angiotensin antagonism:** The dose–response curve for contraction of isolated rat uterus by angiotensin (Hypertensin, Ciba) is illustrated in Fig. 1. The angiotensin analog, [Phe[4], Tyr[8]]-angiotensin II, was inactive at 10 μg/ml, but elicited a detectable oxytocic response at 20 μg/ml; this activity is less than 0.005% that of angiotensin II. The analog at 10 μg/ml completely inhibited uterine contractions produced by angiotensin at doses of 10 ng/ml or less. In the presence of the analog at this level, about 200 times as much angiotensin was required to produce a 0.5 g contraction as in controls. The antagonistic effect of [Phe[4], Tyr[8]]-angiotensin II was completely reversed by washing and there was no significant difference between the angiotensin dose–response curves before and after treatment of the uterine strips with the analog.

***In vivo* Angiotensin antagonism:** The ability of the analog to inhibit the vasopressor action of angiotensin was tested in nephrectomized, phenoxybenzamine-treated rats (Fig. 2). Similar doses of angiotensin were required *in vivo* to elicit changes in blood pressure and *in vitro* to cause contraction of uterine strips. Thus, 0.1 ng/g body weight produced about a 30% increase in mean blood pressure, and 0.1 ng/ml produced about a 30% increase in uterine tension. [Phe[4], Tyr[8]]-angiotensin II at a dosage of 10 ng/g increased mean blood pressure from 106 $\pm$ 6 to 128 $\pm$ 12 mm Hg, indicating that the activity is only 0.0025% that of angiotensin. (Lower doses produced no response.) The antagonistic effect of the analog (10 ng/g) was manifested by a 100-fold shift in the blood pressure dose–response curve to angiotensin, both in normotensive and hypertensive rats (Fig. 2).

The response of genetically hypertensive rats to angiotensin II and the antagonist was evaluated (Fig. 2). The initial mean blood pressure was almost twice

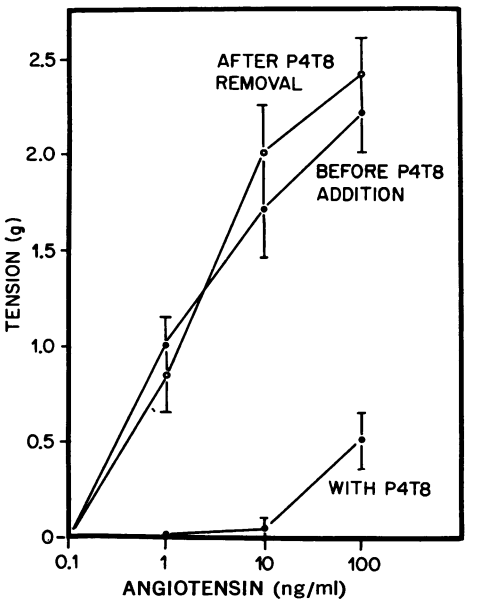

FIG. 1. Effect of [Phe^4, Tyr^8]-angiotensin II on the dose–response relationship for angiotensin II-induced contraction of isolated rat uterus strips. The values are reported as the average (±SE) for 12 uterine strips removed from four animals. The concentration of the analog (P4T8) was 10 μg/ml. Each strip was treated three times (since angiotensin II standard curves were performed before and after the experiment with the analog).

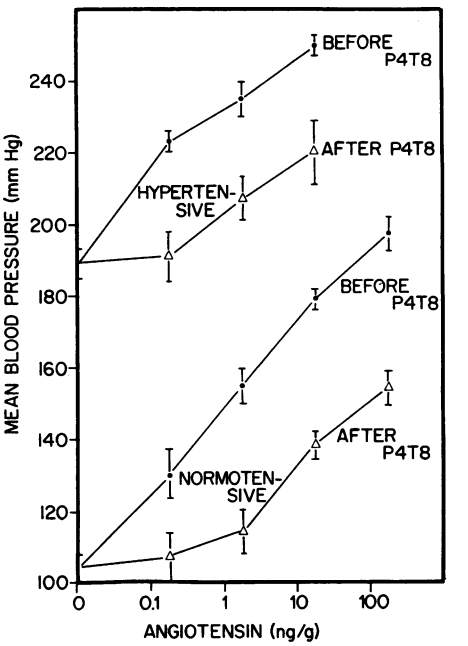

FIG. 2. Effect of [Phe^4, Tyr^8]-angiotensin II on the dose–response relationship for the angiotensin II-induced pressor effect in normotensive and hypertensive rats. The starting point of each curve indicates the average (±SE) mean blood pressure of the anesthetized, nephrectomized, phenoxybenzamine-treated rats. The upper two curves are the results obtained from genetically hypertensive rats (n = 4) and the lower two curves are from normotensive rats (n = 10). An angiotensin standard curve was performed in each animal before the antagonist was tested. The dosage of the analog (P4T8) was 10 ng/g.

normal (190 mm Hg). Angiotensin caused a further elevation of blood pressure, but the net increase was only about half that exhibited in normotensive animals. Administration of the antagonist (up to 20 ng/g) did not lower the high baseline blood pressure of the hypertensive rats. In contrast to what happens with normal rats, high levels of the analog (10 ng/g) did not produce a rise in blood pressure; but its antagonistic effect was similar to that in normal rats.

Specificity of antagonist: Isolated uterine strips were used to determine if the analog was merely a physiological antagonist which produced generalized inhibition of smooth muscle contractility, or whether it was a specific antagonist of angiotensin. One such experiment is illustrated in Fig. 3. The angiotensin analog at a concentration of 10 μg/ml completely inhibited the response to angiotensin without affecting the contraction produced by bradykinin (Fig. 3*A*). A mixture of angiotensin and bradykinin produced a contraction which approxi-

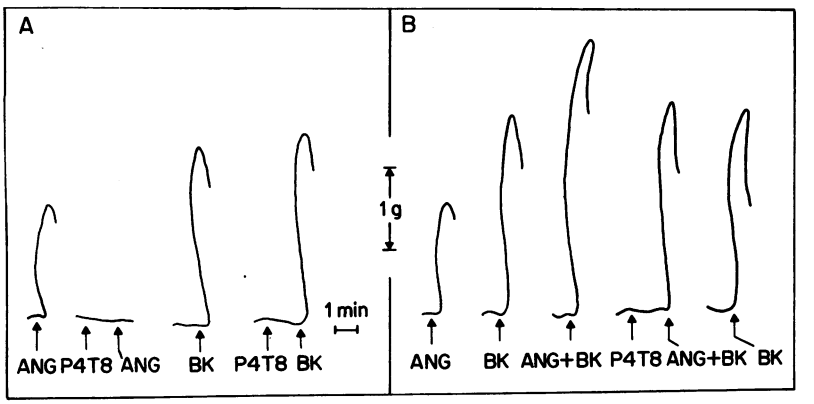

FIG. 3. Comparison of the effect of [Phe^4, Tyr-^{8}]angiotensin II on uterine contraction induced by angiotensin II and bradykinin. The compounds were employed in the following concentrations: angiotensin II (ANG) 5 ng/ml, [Phe^4, Tyr^8]-angiotensin II (P4T8) 10 μg/ml, bradykinin (BK) 0.5 ng/ml. Recordings illustrate an actual tracing of the contractions of a uterine strip. The myograph was calibrated so that a 2-cm contraction equaled 1 g of tension.

mated the sum of their two individual contractions (Fig. 3*B*). When the mixture of the two peptides was added to a strip in the presence of the analog, the angiotensin component was inhibited and the increase in tone was equivalent to that of the bradykinin alone.

In other experiments in rat uterine strips, the analog exhibited a high degree of specificity and did not alter any portion of the dose–response curve for bradykinin, oxytocin, vasopressin, or serotonin. In blood pressure experiments in rats, the analog did not change the vasopressor response to epinephrine nor the vasodilator response of nitroglycerin.

Treatment of the antagonist with carboxypeptidase, which removed the C-terminal amino acid (tyrosine), eliminated antagonistic activity when measured at 10 μg/ml in the rat uterus assay.

Analysis of inhibition: Competition was studied in uterine strips by determination of angiotensin dose–response curves in the presence of different concentrations of the angiotensin analog. Reciprocal plots of contraction against angiotensin were linear within experimental limits (Fig. 4). All the lines intersected the vertical axis at the same point, which indicates that the antagonist did not decrease the maximal response to angiotensin (6.7 cm, equivalent to an increase of 3.4 g in tension of the uterine strip). In the absence of the antagonist, half-maximal response was produced by 6.7 ng of angiotensin per ml. The data are consistent with the hypothesis that the antagonist competes with angiotensin for an active site, and that its dissociation constant is 240 ng/ml. This would indicate a ratio of 36:1 between affinities of the agonist and antagonist for the uterine receptors.

Discussion. The close structural relationship between angiotensin and the antagonistic peptide suggests the possibility of competition for the same receptor site. This idea is supported by the kinetic data in Fig. 4 and by the fact that at very high concentrations, the analog causes contraction of uterine strips and

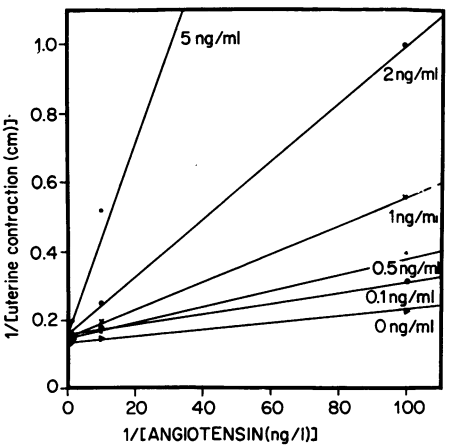

FIG. 4. Reciprocal plot of uterine strip response versus angiotensin concentration in the presence of different concentrations of [Phe^4, Tyr^8]-angiotensin II. Response is expressed as cm of uterine contraction (with 1 g of tension equal to 2 cm). Each curve represents the average value obtained from eight uterine strips. The number on each line represents the concentration of antagonist present in the bath.

produces a vasopressor response in normotensive rats. Angiotensin inhibition was still present after the analog induced an agonistic effect of its own. The immediate relaxation of contracted uterine strips upon addition of the analog would be consistent with rapid displacement of the agonist from the receptor site. Removal of the C-terminal amino acid by carboxypeptidase digestion resulted in loss of antagonistic activity. This is analogous to the loss of activity seen in angiotensin II when the C-terminal residue is removed,[19] and is supportive evidence for similar binding requirements. The quantitative dependence of the extent of inhibition on the ratio of antagonist to agonist, as well as the reversibility of the inhibition with high doses of agonist, tends to support the hypothesis of competitive inhibition.

It should be noted that both antagonistic and agonistic activities correlated closely in both *in vivo* pressor measurements and *in vitro* myotropic assays. This contrasts with the [Ile^5, Ala^8]-angiotensin which has been reported to antagonize angiotensin II in guinea pig ileum, but not in pressor assay nor in the inhibition of norepinephrine uptake in rabbit heart.[9] Guinea pig ileum appears to be a poor smooth muscle preparation for evaluating angiotensin analogs and for correlating with their biological properties. For example, the guinea pig ileum has been shown to respond to a cyclic hexapeptide which has essentially no activity in the rat pressor assay.[18]

The ability of [Phe^4, Tyr^8]-angiotensin II to inhibit angiotensin II is somewhat surprising since similar analogs have been synthesized and biologically evaluated without a report of antagonist activity.[19] In particular, the report of [Asn^1, Phe^4, Tyr^8]-angiotensin II having 0.2% biological activity bears further investigation. The other structurally related analogs, [Phe^4]-angiotensin II and [Tyr^8]-angiotensin II, have relatively high biological activities, 10 and 83% of angiotensin II respectively, and no antagonistic activity. It is clear that molecular requirements for competitive antagonistic activity of angiotensin II analogs await further structure–activity studies.

A strain of spontaneously hypertensive rats has been selected by inbreeding techniques. The underlying cause of their elevated blood pressure is unknown.[20] The analog was capable of antagonizing the pressor response to exogenous angio-

tensin, and would therefore also be expected to inhibit endogenous angiotensin. The observation that [Phe^4, Tyr^8]-angiotensin II did not lower the blood pressure in these hypertensive rats implies that the elevated pressure is not a result of a direct pressor effect of increased angiotensin levels.

Vasoactive compounds in blood or tissue extracts are frequently analyzed by bioassay, because of the lack of an adequate chemical method. Such compounds as acetylcholine, catecholamines, histamine, and serotonin are readily characterized by means of specific antagonists (atropine, phenoxybenzamine, and propranolol, diphenhydramin, and lysergic acid diethylamide). The presence of polypeptides in extracts can be confirmed by loss of activity with proteolytic enzymes, but such treatment would not distinguish between different peptides (e.g., angiotensin, bradykinin, substance P). The specificity of the antagonist, [Phe^4, Tyr^8]-angiotensin II, therefore, would provide a qualitative test for angiotensin in extracts. Finally, this analog may prove to be useful in interfering with the physiological or pathological effects of endogenous angiotensin.

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[1] Rudinger, J., and I. Krejci, in *Handbuch der Pharmacologie*, ed. B. Berde (Heidelberg: Springer-Verlag, 1969), p. 748.

[2] Dousa, T., O. Hechter, R. Walter, and I. L. Schwartz, *Science*, **167**, 1134 (1970).

[3] Yajima, H., and K. Kubo, *J. Amer. Chem. Soc.*, **87**, 2039 (1965).

[4] Stewart, J. M., and D. W. Woolley, *Nature*, **206**, 619 (1965).

[5] Vogler, K., R. O. Studer, W. Lergier, and P. Lanz, *Helv. Chim. Acta*, **48**, 1407 (1965).

[6] Flouret, G., and V. duVigneaud, *J. Amer. Chem. Soc.*, **87**, 3775 (1965).

[7] Stewart, J. M., and D. W. Woolley, in *International Symposium on Vaso-Active Polypeptides: Bradykinin and Related Kinins*, eds. M. Rocha e Silva and H. A. Rothschild, (Sao Paulo: Ribeirao Preto, 1967), p. 7.

[8] Khairallah, P. A., A. Toth, and F. M. Bumpus, *J. Med. Chem.*, **13**, 181 (1970).

[9] Peach, M. J., F. M. Bumpus, and P. A. Khairallah, *J. Pharmacol. Exp. Ther.*, **167**, 291 (1969).

[10] Marshall, G. R., and R. B. Merrifield, *Biochemistry*, **4**, 2394 (1965).

[11] Shatiel, S., *Biochem. Biophys. Res. Commun.*, **29**, 178 (1967).

[12] Lenard, J., and A. B. Robinson, *J. Amer. Chem. Soc.*, **89**, 181 (1967).

[13] Pourchot, L. M., and J. J. Johnson, *Org. Prep. Proced.*, **1**, 121 (1969).

[14] Smeby, R. R., P. A. Khairallah, and F. M. Bumpus, *Nature*, **211**, 1193 (1966).

[15] Nitecki, D. E., and J. W. Goodman, *Biochemistry*, **5**, 665 (1966).

[16] Scotchler, J., R. Lozier, and A. B. Robinson, *J. Org. Chem.*, **35**, 3151 (1970).

[17] Burn, J. H., *Practical Pharmacology* (Oxford: Blackwell Scientific Publ., 1952), pp. 7–16.

[18] Jorgensen, E. C., and W. Patton, *J. Med. Chem.*, **12**, 935 (1969).

[19] Bumpus, F. M., and R. R. Smeby, in *Renal Hypertension*, eds. I. H. Page and J. W. McCubbin (Chicago: Year Book Medical Publishers, 1968), pp. 83–87.

[20] Clineschmidt, B. V., R. G. Geller, W. C. Govier, and A. Sjoerdsma, *Eur. J. Pharmacol.*, **10**, 45 (1970).