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PEPTIDE INHIBITORS OF RENIN ANGIOTENSINOGEN REACTION SYSTEM

TATSUO KOKUBU, KUNIO HIWADA, TAKETOSHI ITO, EINOSUKE UEDA, YUICHI YAMAMURA, TOMISHIGE MIZOGUCHI* and KEISUKE SHIGEZANE*

The Third Department of Medicine, Osaka University Medical School, Osaka, Japan and Organic Chemistry Research Laboratory, Tanabe Pharmaceutical Co., Ltd., Tokyo, Japan

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Abstract—Methyl or ethyl esters of synthetic tetrapeptides, Leu-Leu-Val-Tyr and Leu-Leu-Val-Phe, acted as competitive inhibitors in the renin renin–substrate reaction system. The chemical structures needed for the anti-renin activity were presumed to be as follows: (1) a Leu-Leu bond at the *N*-terminal end of the tetrapeptide; (2) a Leu-Leu bond with L-Leu at the *N*-terminal end; (3) the presence of Tyr or Phe at the *C*-terminal end; (4) the replacement of Val in position 3 by Leu did not reduce the activity; (5) the amide formation of the carboxylic acid of the *C*-terminal amino acid reduced the activity, but deoxidation produced little change in the activity; (6) the coupling of benzyloxycarbonyl or sodium metasulfonic acid group to the *N*-terminal end markedly reduced the activity; and (7) the addition of His to the *N*-terminal end caused almost no change in activity. The pressor response produced by renin injected intravenously into a rabbit being treated with an infusion of the tetrapeptide solution was inhibited compared with that in the control animals.

RENIN IS an endopeptidase which splits a Leu-Leu bond in an alpha-2-globulin substrate, angiotensinogen, to yield a decapeptide, angiotensin I. Angiotensin I is subsequently converted into an octapeptide, angiotensin II, by a converting enzyme. Angiotensin II has potent pressor activity.

A preliminary study from our laboratory[1] of synthetic tetrapeptide renin inhibitors has already been reported. In the present paper we report the effects of synthetic peptides with a Leu-Leu bond and their derivatives on the activity of renin in the formation of angiotensin in experiments *in vitro* and *in vivo*.

METHODS AND MATERIALS

Synthetic peptides studied. Di-, tri-, tetra-, penta- and octapeptides having mainly Leu-Leu bonds in the peptide chain were synthesized[2,3] as shown in Table 1. The peptides were confirmed to be pure by means of thin-layer chromatography and elemental analysis.

Preparation of renin and renin substrate. Renin was prepared from rabbit renal cortex using the method of Haas *et al.*,[4] followed by ammonium sulfate fractionation between 30 and 60 per cent saturation, and dialysis against physiological saline containing 2×10^{-3} M EDTA. The activity of renin was equivalent of 17·3 μg angiotensin II amide (CIBA) per ml of the preparation (protein amount, 10·8 mg/ml), according to a direct method[5] based on the pressor response produced by the intravenous injection into a rat preparation as described below. The renin preparation was diluted 20-fold with physiological saline prior to use.

* Tanabe Pharmaceutical Co., Ltd., Tokyo, Japan.

TABLE 1. SYNTHETIC PEPTIDES WITH LEU-LEU BOND EXAMINED*

Sp		Sp	
2	z.Leu-Leu	217	z.Leu-Leu-Glu
3	Leu-Leu	219	z.Leu-Leu-His
4	Leu-Leu.oMe	220	z.Leu-Leu-D-Val
7	CHO.Leu-Leu	154	Leu-Val-Tyr.oMe
51	CHO.Leu-D-Leu	105	Leu-Leu-Val-Tyr.oMe†
52	CHO.D-Leu-Leu	223	Leu-Leu-Val-Phe.oMe†
53	CHO.D-Leu-D-Leu	263	Leu-Leu-Val-Tyr.oEt†
59	Acetyl.Leu-Leu	242	Leu-Leu-Val-Phe.oEt†
60	Benzoyl-Leu-Leu	281	Leu-Leu-Leu-Phe.oMe†
101	z.Leu-Leu.NH.NH_2	299	Leu-Leu-Leu-Tyr.oMe†
15	Leu-Val	243	D-Leu-Leu-Val-Phe.oMe
54	Acetyl.Leu-Val	261	D-Leu-Leu-Val-Tyr.oMe
155	z.Leu-Val	227	Leu-Leu-Val-His.oMe
21	His-Leu	339	Leu-Leu-Val-Pro.oMe
8	z.Phe-His	221	Leu-Leu-Val-Gly.oMe
10	Phe-His	294	Leu-Leu-Val-NH$(CH_2)_2$M  OH
75	Benzoyl-Phe-His		
34	z.Ile-His.NH.NH_2	311	Leu-Leu-Val-Phe.oC_8H_{17}
76	z.Tyr-Set	224	z.Leu-Leu-Val-Phe
82	z.Val-Tyr	226	z.Leu-Leu-Val-His
135	z.Pro-Phe	340	z.Leu-Leu-Val-Pro
143	z.Val-His	332	Leu-Leu-Val-Phe
153	z.Pro-Phe-His-Leu	333	Leu-Leu-Val-Tyr
381	Phe-Phe-oMe	372	Leu-Leu-Val-Phe.NH_2†
24	His-Leu-Leu	384	Leu-Leu-Val-Phe-ol†
25	His-Leu-Leu	417	Leu-Leu-Val-Tyr-ol†
113	z.Leu-Leu-Val	501	$NaSO_3CH_2$-Leu-Leu-Val-Tyr.oEt†
117	Leu-Leu-Val.oMe	502	$NaSO_3CH_2$-Leu-Leu-Val-Tyr-ol
178	Tos.Leu-Leu-Val	244	Ile-Leu-Val-Phe.Me
184	CHO.Leu-Leu-Val	245	Val-Leu-Val-Phe.oMe
186	Benzoyl.Leu-Leu-Val	265	Val-Leu-Val-Tyr.oMe
187	$CH_3(CH_2)_{10}$Co.Leu-Leu-Val	283	His-Leu-Leu-Val-Phe.oMe†
188	C_2H_5OCO Leu-Leu-Val	291	His-Leu-Leu-Val-Tyr.oEt†
215	z.Leu-Leu-Leu	699	Pro-Phe-His-Leu-Leu-Val-Tyr-Ser-ol†

* Z, Benzyloxycarbonyl; D, D-form; oMe, methyl ester; oEt, ethyl esters; -ol, deoxidized form.
† These peptides are effective as inhibitors.

Renin substrate was prepared from the heparinized plasma of rabbit nephrectomized bilaterally 24 hr before use, according to the method of Sen *et al.*[5] This preparation contained renin substrate equivalent to 18·0 μg angiotensin/ml (protein amount, 9·3 mg/ml), according to the indirect method of Pickens *et al.*,[6] based on the estimation of angiotensin formed, which was produced by incubation of the renin-substrate preparation with an excess of rabbit renin in the presence of EDTA and DFP. The substrate for experiments was diluted five-fold with physiological saline prior to use.

Experiment in vitro. The reaction mixture consisted of 0·2 ml of renin solution, 0·5 ml of renin substrate, 2·3 ml of physiological saline or 0·06 M phosphate buffer, pH 6·4, containing the test material. The mixture was incubated at 37° for 5–10 min and the reaction was stopped by heating in a boiling water bath for 5 min. The control assay system produced usually 0·15 to 0·16 μg/ml of angiotensin. The renin renin-substrate reaction in the assay condition was a first-order reaction, as described previously.[7]

The angiotensin formed was assayed by means of its pressor response in rats weighing 170–180 g, anesthetized by intraperitoneal injection of pentobarbitone sodium (50 μg/kg), vagotomized, and treated subcutaneously with pentolinium tartrate (10 mg/kg). All assays were performed in comparison with Val-angiotensin II amide. The inhibitory effect was expressed as a percentage reduction in the amount of angiotensin formation of the control assay system, thereby giving an inhibitory per cent.

Experiment in vivo. The experimental animal (rabbit) was treated with an infusion of 10 ml of the peptide solution, containing 50 mg of the peptide sample, at a constant rate (between 1 and 2 ml/min) via the auricular vein. After a few ml (3–5 ml) of the peptide solution had been infused, the animal was simultaneously injected with 0·1 ml renin via the other auricular vein. The pressor response produced by the renin injection was compared to that produced in the control animal, which was treated with the same volume of physiological saline instead of the peptide solution. The pressor response produced by renin in each animal, control and experimental, was compared with that produced by 0·2 μg of synthetic angiotensin injected just prior to and immediately after the infusion of the peptide solution (experimental) or saline solution (control).

If the pressor responses (mm Hg) to the renin injection in the experimental and control animals are labeled as Y_1 and Y_2 respectively, and those to the angiotensin injections before and after the infusion X_1 and X_2 respectively, then the inhibitory effect in the experiment can be calculated from the difference in the pressor response ratios of the experimental and control animals. The pressor response ratio in each animal was expressed as follows:

$$\frac{(Y_1 \text{ [or } Y_2])/(X_1 + X_2)}{2}$$

All animals were anesthetized by intravenous injection of pentobarbitone sodium and treated with pentolinium tartrate before use.

RESULTS

Experiment in vitro. None of the di- or tripeptides had any inhibitory effect against renin activity. However, the methyl or ethyl esters of the tetrapeptides, Leu–Leu–Val–Tyr and Leu-Leu-Val-Phe, inhibited the formation of angiotensin in the experiment *in vitro*. The tetrapeptides without a Leu-Leu bond had no effect against the renin activity. The peptides that were effective are indicated in Table 1.

From the results, the chemical structures needed for anti-renin activity are presumed to be as follows: (1) the tetrapeptides having a Leu-Leu bond at the *N*-terminal end were effective; (2) a Leu-Leu bond with L-Leu at the *N*-terminal end was needed for anti-renin activity, that is, it was noted that no inhibitory effect was detected in the peptides when the *N*-terminal amino acid of the peptide was replaced with a D-form (Sp 243 and 261); (3) the replacement of Tyr or Phe at the *C*-terminal end by the other amino acids (Sp 227, 339 and 221) had no activity, that is, the presence of Tyr or Phe at the *C*-terminal end was essential for anti-renin activity; (4) the replacement of Val in position 3 by Leu (Sp 281 and 299) did not reduce the activity; and (5) amide formation or deoxidation of the carboxylic acid of the *C*-terminal amino acid (Sp 372, 384 and 417) did not completely destroy the activity.

TABLE 2. INHIBITORY EFFECTS OF SYNTHETIC PEPTIDES ON ANGIOTENSIN FORMATION OF RENIN

Sp	Synthetic peptides	Inhibitory per cent					
		0·1 mg	0·25 mg	0·5 mg	1·0 mg	2·0 mg	5·0 mg
105	Leu-Leu-Val-Tyr-oMe.HCl	4·6	13·6	27·3	40·9	50·0	77·3
263	Leu-Leu-Val-Tyr.oEt.HCl	0·0	2·3	24·5	44·5	57·5	81·5
223	Leu-Leu-Val-Phe.oMe.HCl	13·6	22·8	40·9	59·1	72·8	100·0
242	Leu-Leu-Val-Phe.oEt.HCl	13·4	33·4	46·7	66·7	80·0	93·4
299	Leu-Leu-Leu-Tyr.oMe.HCl	0·0	15·0	30·7	43·3	67·5	75·0
417	Leu-Leu-Val-Tyr.ol.HCl	0·0	21·3	41·5	34·4	64·7	76·8
384	Leu-Leu-Val-Phe.ol.HCl	33·0	26·0	39·7	52·6	63·6	87·1
291	His-Leu-Leu-Val-Tyr.oEt		19·2	27·8		63·5	90·1
501	$NaSO_3CH_2$-Leu-Leu-Val-Tyr.oEt	2·5	10·6	18·7	26·9	18·7	49·6
502	$NaSO_3CH_2$-Leu-Leu-Val-Tyr-ol	0·0	0·0	0·0	0·0	0·0	27·2
372	Leu-Leu-Val-Phe.NH_2HCl	0·0	0·0	0·0	22·0	39·0	55·0
699	Pro-Pre-His-Leu-Leu-Val-Tyr-Ser.ol	14·3	44·0	69·4	81·1	90·4	94·2

Table 2 indicates the results of a comparative study of inhibitory effects in the tetrapeptides and their derivatives. Amide formation of the carboxylic acid of the *C*-terminal amino acid (Sp 372) moderately reduced the activity as compared to that of Sp 223, which showed about 50 per cent inhibition. However, the deoxidation of the carboxylic acid of the *C*-terminal end (Sp 417 and 384) showed only a slight reduction in activity. The coupling of a metasulfonic acid sodium group to the *N*-terminal end (Sp 501) moderately reduced the activity (about 50 per cent inhibition compared to Sp 223).

The combination of deoxidation of the carboxylic acid of the *C*-terminal amino acid and coupling of a metasulfonic acid sodium group to the *N*-terminal end almost completely reduced the activity (Sp 502). The deoxidized form of the octapeptide, Pro-Phe-His-Leu-Leu-Val-Tyr-Ser, had potent anti-renin activity (Sp 699).

Val-angiotensin II amide (0·3 μg) was mixed with 5·0 mg of the peptide (Sp 105, 223 and 417) and incubated at 37° for 30 min. The pressor response of these mixtures was the same as that of angiotensin II amide. The result indicated that the peptides having anti-renin activity did not modify the physiological pressor activity of angiotensin and had no hypotensive effect when injected intravenously. Renin substrate, 0·5 ml, was incubated with 5·0 mg of the peptides at pH 6·4, 37°, for 1 hr. After the incubation, the concentration of renin substrate was measured by a modification of the method of Pickens *et al.*[6] Incubation of renin substrate with the peptides was sustained without any reduction in the amount of angiotensin formed when compared to the control experiment. These peptides, therefore, do not directly inactivate renin substrate under the conditions of the experiment. Renin, 0·2 ml, was incubated with 5·0 mg of the peptide at pH 6·4, 37°, for 60 min. Preincubation of renin with the peptide showed no reduction in enzyme activity either through the pressor response in the experiment *in vivo* or angiotensin formation in the experiment *in vitro*. In these experiments, the peptides were removed from the incubation mixtures by dialysis against distilled water before estimation of the amount of renin substrate or renin activity. The results suggested that the peptides inhibited the reaction of renin with angiotensinogen.

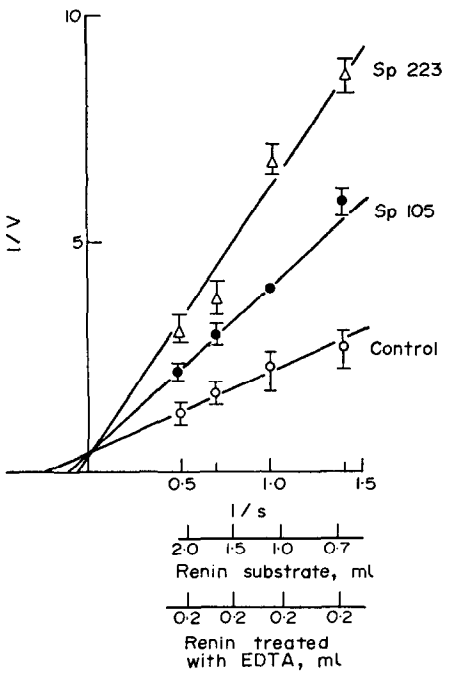

FIG. 1. Inhibitory effect of tetrapeptides (Sp 105 and 223). Lineweaver–Burk plots of the relationship between the reciprocals of substrate concentration and reaction velocity. Control: renin substrate (3·6 μg angiotensin/protein amount, 1·86 mg/ml), 0·7, 1·0, 1·5, 2·0 ml; rabbit renin (1·73 μg/protein amount, 1·08 mg/ml), 0·2 ml; experiment (with compound): renin substrate, 0·7, 1·0, 1·5, 2·0 ml; rabbit renin, 0·2 ml; compound (1 mg) in saline. Total volume 3·2 ml, 5 min, 37°. The methods for preparation of renin and renin-substrate were indicated in the text. Sp 105, Leu-Leu-Val-Tyr.oMe; Sp 223, Leu-Leu-Val-Phe.oMe.

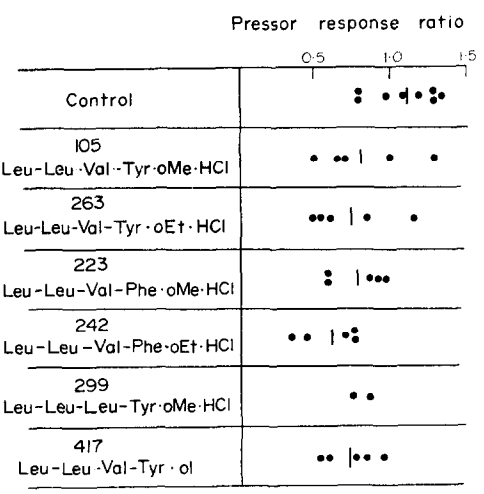

FIG. 2 Inhibitory effect of tetrapeptides on renin activity in the experiment *in vivo* using rabbits. The experimental method and pressor response ratio were mentioned in the text.

The inhibitory mechanism of the peptides (Sp 105 and 223) was investigated and the Lineweaver–Burk method of plotting was used for distinguishing types of inhibition. As shown in Fig. 1, inhibition of a competitive nature was observed.

Experiment in vivo. In the control experiment, the pressor response ratio ranged between 0·8 and 1·3, mean value 1·15, as shown in Fig. 2. However, in the experiment using an infusion of the peptides having anti-renin activity in the experiment *in vitro*, the pressor response ratio was reduced compared with that in the control experiment, that is: Sp 105, ranged between 0·5 and 1·25 (mean value 0·8); Sp 263, between 0·48 and 1·18 (0·7); Sp 223, between 0·55 and 0·98 (0·75); Sp 242, between 0·28 and 0·75 (0·6); and Sp 417, between 0·48 and 0·9 (0·7).

DISCUSSION

Five kinds of renin inhibitors have been reported. These are the naturally occurring renin pre-inhibitor, lysophospholipid, reported by Sen *et al.*,[5,8] heparin reported by Sealey *et al.*,[9] and our group has already reported methyl or ethyl esters of the synthetic tetrapeptides, Leu-Leu-Val-Tyr and Leu-Leu-Val-Phe,[1] and bile acids.[7,10]

This report concerns the investigation of the effect of methyl or ethyl esters of the tetrapeptides, Leu-Leu-Val-Tyr and Leu-Leu-Val-Phe, and their derivatives, which were synthesized by our group, on the angiotensin formation of renin in experiments *in vitro* and *in vivo* together with their corresponding anti-renin activities as compared with parent tetrapeptide esters.

None of the di- or tripeptides having a Leu-Leu bond had any anti-renin activity, demonstrating that the tetrapeptide is the minimum structure with anti-renin activity. The replacement of Leu in position 1 by Ile or Val (Sp 244, 245 and 265) did not inhibit the renin renin-substrate reaction system and the peptide having the D-form instead of the L-form of the Leu-Leu bonds (Sp 243 and 261) had no activity. The presence of Tyr or Phe at the *C*-terminal end was needed for anti-renin activity, because the replacement of Tyr or Phe at the *C*-terminal end by other amino acids, for instance, His, Pro or Gly (Sp 227, 239 or 221), showed no activity. This finding suggests that the phenyl group in the *C*-terminal amino acid might be essential. The replacement of Val in position 3 by Leu did not reduce the activity. Hence, it was supposed that the amino acid in position 3 is in some way essential for maintaining the length of the peptide chain as a tetrapeptide. Amide formation of the carboxylic acid of the *C*-terminal amino acid (Sp 372) reduced the activity to about 50 per cent and deoxidation (Sp 384 and 417) produced little change in activity. Consequently, esterification of the free carboxylic acid is not essential for anti-renin activity. However, the peptide with a free carboxyl group (Sp 332 and 333) could not be tested because these peptides were insoluble in saline or buffer solution. On coupling the metasulfonic acid sodium group to the *N*-terminal end (Sp 501), the activity was reduced by about 50 per cent. However, the addition of the amino acid, His (Sp 291) or Gly (Sp 331), showed activity similar to that of the parent peptide esters. The combination of deoxidation of the carboxylic acid of the *C*-terminal amino acid and coupling of a metasulfonic acid sodium group to the *N*-terminal end almost completely reduced the activity (Sp 502). Hence, it seems that the tetrapeptides having the *N*-terminal amino acid protected by any group except amino acid may lead to reduced anti-renin activity.

Previously we reported the competitive inhibitory effect of the same tetrapeptides using crude renin and plasma. The present studies showed the same competitive inhibitory effects using semipurified renin and renin substrate.

Furthermore, the experiment *in vivo* revealed that the pressor response, produced by renin injection in a rabbit being treated with an infusion of the tetrapeptide solution (Sp 263, 223, 242 and 417), was inhibited when compared to that in the control animal being treated with saline solution. This experiment was carried out using a different animal every time in order to avoid renin tachyphylaxis.

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