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INHIBITOR OF ANGIOTENSIN I CONVERTING ENZYME: (4R)-3-[(2S)-3-MERCAPTO-2-METHYLPROPANOYL]-4-THIAZOLIDINECARBOXYLIC ACID (YS-980)

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Abstract—Pharmacological studies on a new angiotensin I converting enzyme (ACE) inhibitor (4R)-3-[(2S)-3-mercapto-2-methylpropanoyl]-4-thiazolidinecarboxylic acid (YS-980), were carried out *in vitro* and *in vivo*. The activity of ACE prepared from rabbit lung by DEAE-Sepharose chromatography was determined using angiotensin I (Ang I) or hippuryl-L-histidyl-L-leucine (HHL) as a substrate. The concentrations of YS-980 producing 50 per cent inhibition of ACE activity (ID_{50}) when HHL or Ang I was used as a substrate were 9.5 and 13 nM, respectively. The mode of inhibition was competitive, with a K_i of 6 nM. Renin activity was not affected by YS-980. Arterial blood pressure was decreased slightly by intravenous injection of YS-980 (0.1 or 1.0 mg/kg) to anesthetized rats with or without pretreatment with pentolinium. Pretreatment with YS-980 suppressed the pressor response to Ang I but not to norepinephrine. Intravenous injection of YS-980 (1 mg/kg) caused a 21 per cent increase in the pressor response to angiotensin II. These results indicate that YS-980 is a potent and specific inhibitor of ACE.

Specific inhibitors of the renin angiotensin system may contribute to an understanding of the role of renin in hypertension, and to the diagnostic and therapeutic management of hypertensive diseases. Many potent renin inhibitors [1–3] and angiotensin II antagonists have been reported. Recently, Ondetti *et al.* [4] and Cushman *et al.* [5] found a new type of potent angiotensin I converting enzyme (ACE) inhibitor.

It is well known that ACE (peptidyldipeptide hydrolase, EC 3.4.15.1), which converts angiotensin I (Ang I) to angiotensin II (Ang II) by cleaving the C-terminal residue (His–Leu–OH), is a zinc-containing metaloenzyme [6]. This enzyme is inhibited by chelating agents such as EDTA, 8-hydroxy-quinoline and sulfhydryl compounds (2,3-mercapto-1-propanol, glutathione, 2-mercaptoethanol and dithiothreitol) [7–10], but these agents have little specificity of action. It was reported earlier that the sulfhydryl compound 2-mercaptopropionylglycine has the ability to complex with zinc [11] and also produces a hypotensive effect when administrated to rats [12]. More recently, we reported on the ACE inhibitory activity of derivatives of 2-mercaptopropionylglycine. Among them, (4R)-3-[(2S)-3-mercapto-2-methylpropanoyl]-4-thiazolidinecarboxylic acid (YS-980) was found to be an orally effective potent ACE inhibitor [13, 14]. The present study was conducted to elucidate the mode of inhibition of YS-980 on rabbit ACE activity, the specificity of inhibition, and the effects of YS-980 on blood pressure in anesthetized rats.

MATERIALS AND METHODS

Preparation of ACE. ACE was prepared from rabbit lung according to the modified method of Dorer *et al.* [15]. Rabbit lung was homogenized and extracted with 50 mM phosphate buffer, pH 7.0, then fractionated with ammonium sulfate (1.6–2.5 M) and further purified by DEAE-Sepharose CL-6B chromatography with 20 mM Tris–HCl buffer, pH 8.4. ACE was eluted by a linear gradient of NaCl (0→0.4 M). The activity of the ACE fraction was assayed using Ang I as a substrate. Prepared ACE activity was assayed using hippuryl-L-histidyl-L-leucine (HHL) as a substrate. One unit of ACE activity is defined as the amount of enzyme that hydrolyzes 1 μmole of HHL per min at 37°.Specific activity is expressed as units per mg of protein.

Chemicals. YS-980 (Fig. 1) was obtained from the Santen Pharmaceutical Co., Ltd., Osaka, Japan. Synthetic Ang I (Asp^1–$ILeu^5$), HHL and tetradecapeptide were purchased from the Protein Research Foundation, Osaka, Japan. The Ang I radioimmunoassay (RIA) kit was obtained from CEA-IRESOLIN, Italy. Dog renal renin and dog renin substrate were prepared by the method described previously by Funae *et al.* [16]. Hog renin substrate was obtained from Miles Laboratories, Inc., Elkhert, IN, U.S.A.

Assay of ACE activity. ACE activity was assayed

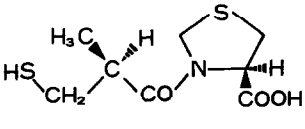

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Fig. 1. Chemical structure of YS-980, (4R)-3-[(2S)-3-mercapto-2-methylpropanoyl]-4-thiazolidinecarboxylic acid.

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by the spectrophotometric method of Cushman and Cheung [8], which monitored the conversion of HHL to hippuric acid, or by RIA of Ang I. The spectrophotometric assay was carried out in 0.25 ml containing the following constituents: 5 mM HHL, 300 mM NaCl, 100 mM phosphate buffer (pH 8.3), and 2 mU ACE. Incubation was carried out at 37° for 30 min. The RIA of Ang I was used to measure the amounts of substrate remaining after incubation with ACE. Assays were carried out using 0.1 ml of Ang I (100 ng), 0.1 ml of ACE (4 mU) and 0.8 ml of 0.1 M phosphate buffer, pH 8.3, containing 0.3 M NaCl and 5 mg/ml bovine serum albumin. Samples were then incubated at 37° for 50 min.

Kinetic studies. The K_i and the mode of inhibition were determined by measuring ACE activity using HHL (0.25 and 0.5 mM) as substrate in the presence of 2 mU ACE/assay. The K_i was determined by the method of Dixon and Webb [17].

Effect of YS-980 on the reaction of renin and renin-substrate. Renin activity was determined by measuring the amount of Ang I formed by the action of renin on renin-substrate in the presence or absence of YS-980. Dog or hog renin-substrate and synthetic tetradecapeptide were used as renin-substrate. The reaction mixture contained the following components: 0.1 ml of YS-980 (final concn 0.1 mM), 0.1 ml renin-substrate (2000 ng Ang I equiv./ml), 0.1 ml of renin solution (500 ng/ml/hr), and 0.7 ml of 0.1 M phosphate buffer, pH 7.2, containing 0.04% neomycin. Following incubation at 37° for 1 hr, the amount of Ang I formed was measured by the RIA method described above.

Effects of YS-980 on the pressor responses to Ang I, Ang II and norepinephrine. Wistar rats weighing 200–300 g were anesthetized with nembutal (50 mg/kg, i.p.). The right femoral vein was cannulated for intravenous injections, and the right femoral artery was cannulated for the recording of arterial pressure via a pressure transducer. Autonomic nerve reflexes were blocked with pentolinium tartrate (5 mg/kg, i.v.). When the blood pressure had stabilized, Ang I (30–200 ng/kg) was injected intravenously, and the blood pressure was monitored. After administration of YS-980 (0.1 or 1.0 mg/kg, i.v.), Ang I was again injected. Both Ang II (20 ng/kg) and norepinephrine (200 ng/kg) were tested for pressor responses as described above for Ang I.

RESULTS

Preparation of ACE. Two peaks of ACE activity were detected following DEAE-Sepharose chromatography. One peak appeared in the void volume and the other was eluted at a NaCl concentration between 0.15 and 0.23 M. The latter fraction was collected and lyophilized. The specific activity of this preparation was 0.14 units/mg protein when HHL was used as a substrate. The enzyme activity was stable for at least 6 months when stored at −20°. Slightly different pH optima were found with Ang I or HHL as substrate; pH optimum of 8.3 was found with Ang I and a pH optimum of 8.4 with HHL.

Inhibition studies in vitro. YS-980 inhibited ACE activity when Ang I or HHL was the substrate. The per cent inhibition and the ID_{50} value which represents the concentration of YS-980 producing 50 per cent inhibition of ACE activity are summarized in Table 1. When HHL was used as a substrate at a concentration of 2 mU of ACE/assay (0.25 ml), the ID_{50} value was 9.5 nM. When Ang I was used at a concentration of 4 mU/ml of ACE, the ID_{50} value was 13.0 nM. Thus, there was nearly the same ID_{50} value with HHL or Ang I as a substrate. YS-980 at a concentration of 1 μM completely inhibited the activity of ACE using either substrate. By means of Dixon–Webb plotting (Fig. 2), a K_i value of 6 nM was obtained, and the data show a competitive type of inhibition. This result further confirms the competitive inhibition demonstrated by a Lineweaver–Burk plot in a previous paper [13].

Table 1. Inhibition of angiotensin I converting enzyme*

Substrate	Concn of YS-980 (nM)	Inhibition (%)	ID_{50} (nM)
HHL†	4	31.3 ± 4.3	
HHL	20	70.0 ± 2.8	9.5
HHL	40	84.1 ± 2.0	
Ang I‡	5	33.7 ± 3.8	
Ang I	10	47.7 ± 5.9	13.0
Ang I	50	68.7 ± 4.3	

* ID_{50} represents the concentration of YS-980 producing 50 per cent inhibition of angiotensin I converting enzyme activity. Abbreviations: HHL, hippuryl-L-histidyl-L-leucine, and Ang I, angiotensin I. The values of per cent inhibition are means ± S.E.M. of six experiments.
† Two mU of ACE/assay (0.25 ml).
‡ Four mU/ml of ACE.

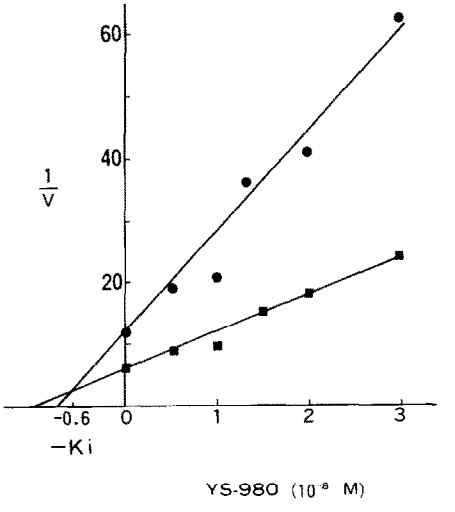

Fig. 2. Inhibitory constant of YS-980 against angiotensin I converting enzyme (ACE). Effect of YS-980 on ACE activity is illustrated by a Dixon–Webb plot. The ordinate shows the reciprocal value of maximum velocity at an optical density of 228 nm, in two separate experiments with two concentrations of hippuryl-L-histidyl-L-leucine (● = 0.25 mM and ■ = 0.5 mM). The abscissa shows the concentration of YS-980. A K_i value of 6 nM YS-980 was obtained.

Table 2. Effect of YS-980 on dog renin activity using different renin-substrates*

Renin-substrate	Generated angiotensin I (ng/ml/hr)	
	Control	YS-980 (0.1 mM)
Dog plasma	16.7 ± 0.1	16.3 ± 0.7
Hog plasma	20.9 ± 0.7	20.4 ± 0.7
Tetradecapeptide	46.1 ± 1.7	46.8 ± 0.3

* Values are means ± S.E.M. of five experiments.

Effects of YS-980 on renin activity. The amounts of Ang I generated by the reaction of dog renin with the natural renin-substrate (dog and hog) and with synthetic tetradecapeptide were measured (Table 2). Since the amounts of Ang I generated were not changed with YS-980 (0.1 mM), this compound did not affect renin activity at a concentration which completely inhibited ACE activity.

Inhibition studies in vivo. A typical pattern of pressor responses to Ang I and Ang II before and after intravenous injection of YS-980 (1.0 mg/kg) is shown in Fig. 3. Intravenous injections of Ang I at concentrations of 30, 60, 100 and 200 ng/kg caused dose-related increases in blood pressure of 29.6 ± 1.8, 39.6 ± 2.2, 50.7 ± 2.1 and 60.0 ± 2.7 mm Hg, respectively (Fig. 4). After administration of YS-980, the pressor response to Ang I resulted in a parallel shift to the right, indicating that YS-980 inhibited the pressor response to Ang I. After YS-980 administration in doses of 0.1 and 1.0 mg/kg, 5- and 13-fold increases in the dose of Ang I were required to exert the same increase in blood pressure.

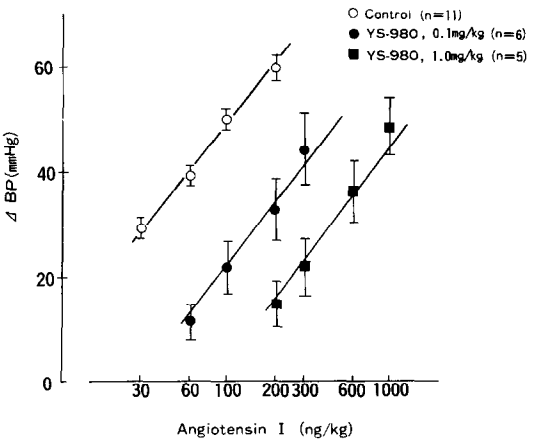

Fig. 4. Effect of YS-980 on the pressor response to angiotensin I in pentobarbital- and pentolinium tartrate-treated rats. The pressor response to angiotensin I was investigated before (○) and after intravenous administration of YS-980 [0.1 mg/kg (●); 1.0 mg/kg (■)]. Values are means ± S.E.M.

The pressor response to Ang II (20 ng/kg, i.v.) was significantly enhanced, by 21.6 per cent (from 33.1 to 40.0 mm Hg), after 1.0 mg/kg of YS-980, but not after 0.1 mg/kg. The pressor response to norepinephrine (200 ng/kg, i.v.) was unaffected by YS-980 (0.1 or 1.0 mg/kg) (Fig. 5).

After intravenous injection of YS-980 in doses of 0.1 mg/kg and 1.0 mg/kg, mean arterial blood pressure was decreased by 9–13 mm Hg, with recovery within 10–15 min. The extent of the decrease of blood pressure was not dose dependent, while the

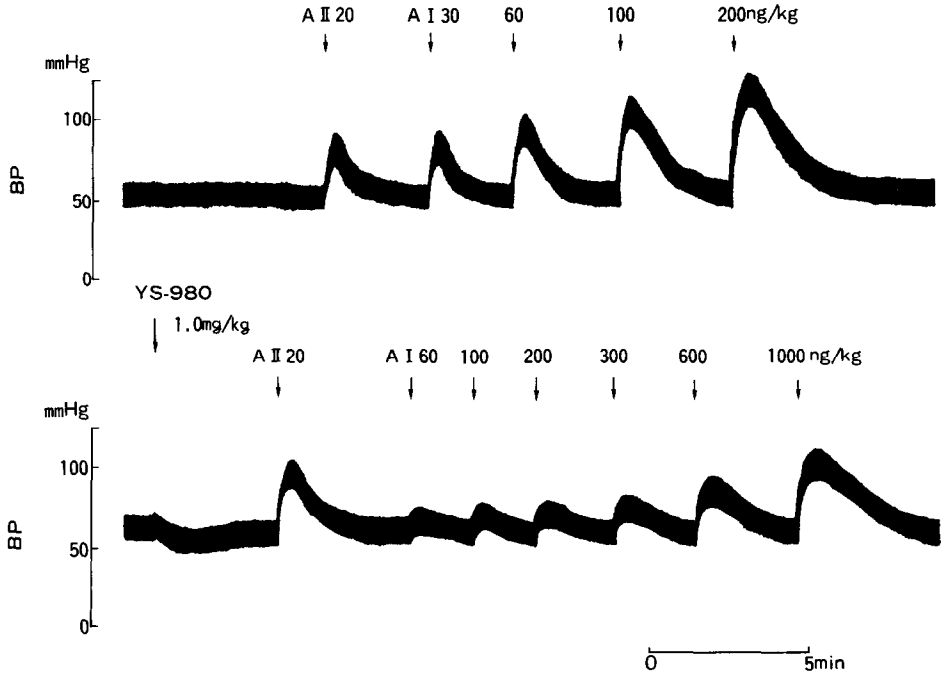

Fig. 3. Effect of intravenous administration of angiotensin I (30–1000 ng/kg), angiotensin II (20 ng/kg) and YS-980 (1.0 mg/kg) on blood pressure in a Wistar rat. The rat was anesthetized with pentobarbital (50 mg/kg, i.p.) and treated with pentolinium tartrate (5 mg/kg, i.v.).

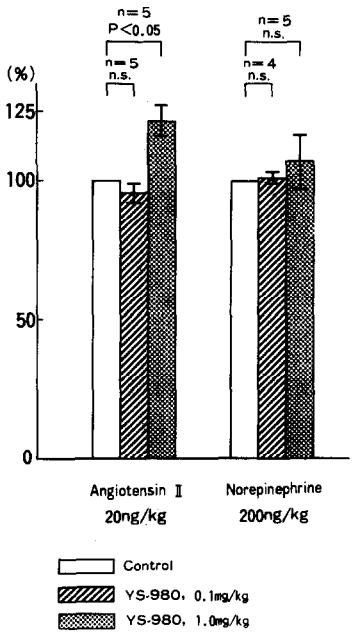

Fig. 5. Effect of YS-980 (0.1 or 1.0 mg/kg, i.v.) on the pressor responses to angiotensin II (20 ng/kg) and norepinephrine (200 ng/kg).

duration of hypotension was dependent upon the dose of YS-980 administrated.

DISCUSSION

It is well known that certain sulfhydryl compounds (e.g. glutathione and cysteine) inhibit ACE. However, their inhibitory specificity toward ACE is very low. We newly synthetized (4R)-3-[(2S)-3-mercapto-2-methylpropanoyl]-4-thiazolidinecarboxylic acid (YS-980). In experiments with partially purified rabbit lung ACE *in vitro*, YS-980 has been found to be a potent competitive inhibitor (K_i = 6 nM) of ACE (Dixon–Webb plots). The concentrations that produced 50 per cent inhibition (ID_{50}) were 9.5 and 13 nM for HHL and Ang I as substrates of ACE, respectively.

In vivo experiments showed that YS-980 inhibited the pressor response to Ang I. It caused inhibition of ACE, as indicated by the ID_{50} and the K_i values derived from *in vitro* experiments. It is a matter of concern, however, that YS-980 might affect the reaction of renin with renin-substrate, because certain sulfhydryl compounds affect that reaction. Dithiothreitol augments the generation of Ang I [16, 18, 19], while cysteine and thioglycollate inhibit renin activity [20]. In the present experiment, the effects of YS-980 on renin activity were determined. At a concentration of 0.1 mM, which inhibited ACE activity completely, YS-980 did not affect the reaction of dog renin with dog, hog or tetradecapeptide substrates, indicating that YS-980 specifically inhibited the ACE activity in the renin–angiotensin system.

Intravenous administration of YS-980 to anesthetized rats resulted in an inhibition of the pressor response to Ang I but not to Ang II. The observations of this study indicate that YS-980 has a strong specificity for the inhibition of ACE. In anesthetized rats, YS-980 caused a slight decrease in arterial blood pressure. A similar decrease of arterial blood pressure was detected after intravenous administration of SQ 14,225 to normotensive rabbits [21] and rats [22]. Lazar *et al.* [23] reported that a slight decrease in blood pressure was observed after intravenous injection of the renin inhibitor pepstatin in anesthetized rats. These findings suggested that inhibition of the renin–angiotensin system would result in hypotension. This interpretation is supported by the observation that a decrease in arterial blood pressure does not occur in anephric rabbits after administration of SQ 14,225 [21]. On the other hand, the slight decrease of arterial blood pressure may be due to the inhibition of degradation of vasodepressor bradykinin. It has been suggested that ACE and kininase II are identical [24, 25]. Thus, YS-980 may have decreased the vasoactive Ang I and increased the vasodepressor bradykinin simultaneously, resulting in a decrease of arterial blood pressure. On the other hand, since the slight decrease of blood pressure was detected with or without treatment of pentolinium, it can not be excluded that the slight decrease of blood pressure was caused by directly affecting the vascular smooth muscle or dilating the capillary network; that is, dithiothreitol, belonging to the same sulfhydryl compounds as YS-980, prevents angiotensin-induced contractions when incubated with rabbit and guinea pig aortas [26].

The pressor response to Ang II was enhanced significantly at a dose of 1.0 mg/kg, but not at 0.1 ml/kg. Such observations were reported with SQ 14,225 in conscious rabbits [21]. The enhancement effect of Ang II after the administration of YS-980 (1 mg/kg, i.v.) seemed to be related to the lack of endogenous Ang II and the modification of Ang II receptors by the sulfhydryl compound (YS-980) mentioned by Fleisch *et al.* [26]. Additionally, the susceptibility of angiotensinases to YS-980 should also be considered. Angiotensinase is a general name for Ang II degrading enzymes. Some of them are inhibited by conventional chelating agents, including SH compounds [27]. Therefore, it is possible that YS-980 inhibits some angiotensinases as it does ACE, resulting in the increase of Ang II.

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