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Synthetic Phosphatidylethanolamines as Renin Inhibitors (39832)

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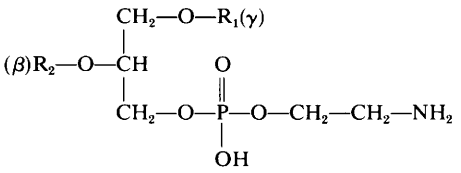
There is considerable evidence in the literature that the renin angiotensin system plays an important role in the pathogenesis of hypertension. Availability of a specific inhibitor of this system would be useful not only to clarify the physiological role of the renin angiotensin system, but also to treat patients with high levels of plasma renin.

Several authors have described renin inhibitory substances: heparin (1), synthetic tetrapeptide (2), deoxycholic acid (3), and pepstatin (4, 5). A phospholipid, isolated from dog kidney by Sen *et al.* (6, 7), also has renin inhibitory properties. Discovery of this substance was the result of observations that a constant amount of renin added to different human plasma samples generated varying amounts of angiotensin. This phospholipid was also isolated from plasma and erythrocytes of humans and dogs, respectively. The chemical structure of this substance is reportedly phosphatidylethanolamine(s) (PE) containing a large amount of unsaturated fatty acids (9). In the present experiment, 18 PE compounds were newly synthesized and renin inhibitory effects on high levels of dog plasma renin as well as the hypotensive actions were determined.

Materials and methods. Preparation of synthetic PE. The fundamental structure of the PE used herein is as follows:



Arachidonic acid ($C_{20}^{=4}$), linolenic acid ($C_{18}^{=3}$), and stearic acid (C_{18}) were substituted at the positions of β, γ, or both. A di-*O*-acyl-3-iodo-D(L or DL)-propane-1,2-diol prepared according to the method reported by DeHaas and VanDeenen (10) was condensed with silver benzyl-2-tritylaminoethylphosphate to give *N*-tritylphosphatidylethanolamine benzyl ester and was successively subjected to stepwise hydrolysis to give PE (11). Eighteen different synthetic PEs including optical isomers (six fundamental combinations of fatty acid and their D, DL, and L forms) were prepared (12).

Isolation of natural PE. Natural PE was isolated from porcine kidney following the method described by Sen *et al.* (6) with slight modification. A porcine kidney was extracted with chloroform–methanol (2:1), and the crude phospholipid was successively chromatographed through silicic acid columns to obtain a phosphatidylethanolamine fraction. The purity of this PE was confirmed on thin-layer chromatography. From 1 kg of the kidney, 315 mg of PE was obtained.

Preparation of lyso-PE. Lyso-PE was prepared according to the description of Rakhit (9).

High-renin plasma. High-renin plasma was taken from nembutal-anesthetized (30 mg/kg) dogs. To elevate the plasma renin activity, an adjustable clamp was placed on the aorta proximal to the renal arteries. Renal arterial pressure was considered equal to the aortic pressure measured by the indwelling polyethylene catheter from the left femoral artery at the level of the renal artery. The mean renal arterial pressure was decreased to approximately 70 mm Hg and was maintained at this level by adjustment of the clamp. After 30–40 min, the blood sample was collected through a catheter inserted into the right femoral artery or left renal vein. The blood was centrifuged at 4° with EDTA (3 m*M*) to obtain plasma. Renin activity of the plasma was determined by radioimmunoassay using a CEA-IRESORIN kit. Five different pools of plasma were used, and these plasma samples

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yielded 40–120 ng of angiotensin I/ml of the incubation mixture after 6 hr of incubation.

Experimental animals. Renal hypertension was produced in rats by clipping the left renal artery of male Wistar rats weighing 180–200 g (7 weeks). The clearance of the silver clips was 0.2 mm. The contralateral kidney was not disturbed. Systolic blood pressure was monitored daily using a tail sphygmographic method. Blood pressure stabilized at about 200 mm Hg (average pressure was 208 mm Hg, range: 199–211 mm Hg) 8 weeks after clipping. Spontaneously hypertensive rats (SHR, 16–20 weeks old; average blood pressure, 201 mm Hg; range, 194–206 mm Hg) and normotensive Wistar rats (NR; 15 weeks old) were employed as controls.

Assay of renin inhibitor in high-renin plasma. Ten milligrams of synthetic PE or natural PE was added to 0.5 ml of isotonic saline and 0.1 ml of chloroform. This mixture was shaken vigorously, and the chloroform was evaporated off under reduced pressure. Three and two-tenths milliliters of high-renin plasma and 0.3 ml of 1/3 *M* phosphate buffer (pH 7.4) containing Neomycin (0.1%) were added to this mixture which was shaken again. The mixture was then incubated at 37° for 6 hr. After 4 and 6 hr of incubation, 1 ml of sample was taken from the incubation mixture and was immersed in boiling water to stop the reaction. One milliliter of distilled water was then added. As a control, 0.5 ml of isotonic saline was added instead of PE solution.

Angiotensin I formed after incubation of the mixture was determined by radioimmunoassay (CEA-IRE-SORIN kit). The renin inhibitory activity was expressed as a percentage of the reduction of the angiotensin I formation.

Hypotensive effect. Hypertensive and normotensive rats were given daily injections of PE in a dose of 10 mg/animal for 6 days. The different synthetic compounds were dissolved in 0.3 ml of peanut oil, and each compound was injected im into three rats.

Results. The inhibitory actions of the 18 different synthetic PEs, natural PE, and the lyso form of natural PE are summarized in Table 1. The inhibitory action on renin activity is expressed as a percentage of the reduction of angiotensin I formation during 4 or 6 hr of incubation. The final concentration of these compounds was 2.5 mg/ml. The inhibitory effect of natural PE was 31.5% at 6 hr of incubation. The lyso form of natural PE was more potent than the original natural one; the angiotensin formation was decreased by about 65.9%. The synthetic PE also showed an inhibitory effect. The β-linolenyl-γ-stearoyl-α-phosphatidyl-E; PE(β-$C_{18}^{=3}$, γ-C_{18}) series were the most potent inhibitors, and effects were more potent than those of natural PE. In the PE(β)-$C_{20}^{=4}$, γ-C_{18}) series, D and DL forms showed 41.2 and 58.5% inhibition, but the L form showed only 16.7% at 6 hr of incubation. On the contrary, in the PE(β-C_{18}, γ-$C_{20}^{=4}$) series, L and DL forms were more potent than the D form. PE(β-C_{18}, γ-$C_{20}^{=4}$) and PE(β-C_{18}, γ-$C_{18}^{=3}$) series were similar to the natural PE. PE(β-C_{18}, γ-C_{18}) and PE(β-$C_{20}^{=4}$, γ-$C_{20}^{=4}$) series were almost the same or slightly less potent than the natural one. Of these two series, the D form was weaker in the former, but the L form was weaker in the latter.

The hypotensive effects of natural PE, D-PE(β-$C_{18}^{=3}$, γ-C_{18}), and D-PE(β-C_{18}, γ-$C_{20}^{=4}$) on renal hypertensive rats are shown in Fig. 1. After injection, blood pressure decreased gradually and reached a maximum after 5–7 hr. The blood pressure tended to return to the original level the following day, but the hypotensive effect was maintained by daily injections and reached a maximum after 5–6 days. After cessation of injections, blood pressure returned to the original level within 2–3 days. No gross pathological signs were observed during the injection period. The maximum hypotensive effects of synthetic PE in the renal hypertensive rats are summarized in Table 2. Six out of ten tested compounds lowered blood pressure by more than 30 mm Hg. D- and DL-PE(β-$C_{18}^{=3}$, γ-C_{18}), DL-PE(β-C_{18}, γ-$C_{18}^{=3}$), and DL-PE(β-C_{18}, γ-$C_{20}^{=4}$) lowered the pressure by more than 40 mm Hg. The maximum hypotensive effect of natural PE was 29 mm Hg. Generally, the hypotensive effect of the D and DL forms was stronger than that of the L form. There is a linear relationship between the renin inhibitory effect and the hypotensive effect in renal hypertensive rats (r = 0.681,

TABLE 1. EFFECTS OF SYNTHETIC PHOSPHATIDYLETHANOLAMINES (PE), NATURAL PE ISOLATED FROM PORCINE KIDNEY, AND THE LYSO FORM OF NATURAL PE ON RENIN ACTIVITY.

Phosphatidylethanolamine	Optical activity	N^a	Percentage of inhibition after 6 hr of incubation
PE(β-$C_{18}^{=3}$, γ-C_{18})	D	5	61.0 ± 15.1**
	DL	7	66.0 ± 13.2*
	L	4	52.3 ± 2.3*
PE(β-$C_{20}^{=4}$, γ-C_{18})	D	3	41.2 ± 17.6
	DL	4	58.5 ± 20.9*
	L	6	16.7 ± 2.5**
PE(β-C_{18}, γ-$C_{20}^{=4}$)	D	4	20.4 ± 1.6*
	DL	3	43.3 ± 7.2*
	L	4	39.9 ± 0.9*
PE(β-C_{18}, γ-$C_{18}^{=3}$)	D	2	34.5 ± 3.0* (4 hr)[b]
	DL	5	35.3 ± 8.2*
	L	2	23.0 ± 0.2* (4 hr)
PE(β-C_{18}, γ-C_{18})	D	4	15.0 ± 6.7
	DL	4	25.0 ± 7.5*
	L	4	32.0 ± 11.1*
PE(β-$C_{20}^{=4}$, γ-$C_{20}^{=4}$)	D	4	23.8 ± 3.0**
	DL	5	33.3 ± 9.2*
	L	4	14.7 ± 2.2**
Natural phosphatidylethanolamine (isolated from porcine kidney)		9	31.5 ± 12.4*
Lyso form of natural phosphatidylethanolamine		4	65.9 ± 13.4*

[a] Numbers of experiments.
[b] Four-hour incubation value.
* $P < 0.05$.
** $P < 0.01$.

$p < 0.05$) with the exception of DL-PE(β-$C_{20}^{=4}$, γ-C_{18}) and D-PE(β-C_{18}, γ-$C_{20}^{=4}$).

Repeated injections of DL-PE(β-C_{18}, γ-$C_{20}^{=4}$) at a dose of 10 mg/animal/day lowered the blood pressure of both renal and spontaneously hypertensive rats (Fig. 2). The hypotensive effect of the present PE series was more remarkable in the renal hypertensive rats than in the SHR.

Discussion. Compounds containing a 1-adamantyl moiety and an ethanolamine side chain showed a renin inhibitory effect *in vitro* (13, 14). Pfeiffer *et al.* (15) also synthesized lyso-PE derivatives but did not obtain a more potent inhibitor than natural phospholipids from hog kidney. Turcotte *et al.* (16) reported the renin inhibitory effect of synthetic lyso-PE derivatives containing arachidonic acid.

Our group has been investigating renin inhibitory compounds, and, as a first step, we examined synthetic PE-containing arachidonic acid, linolenic acid, or stearic acid as esterified fatty acids. It is known that a high percentage of arachidonic acid is contained in the natural PE (6, 9).

Our newly synthesized PEs had an inhibitory effect on renin activity similar to that seen with natural PE isolated from porcine kidney, however, these activities were less potent than the lyso form of natural PE. PE(β-$C_{18}^{=3}$, γ-C_{18}) series were the most potent inhibitors among our derivatives and almost equipotent to the lyso form of natural PE. A relationship between the inhibitory action and chemical structure of synthetic PE cannot be determined from our limited number of derivatives, however, an even more effective inhibitor of renin may be found among the synthetic lyso form of PE, rather than in synthetic PE derivatives.

In the present work, the inhibitory effect

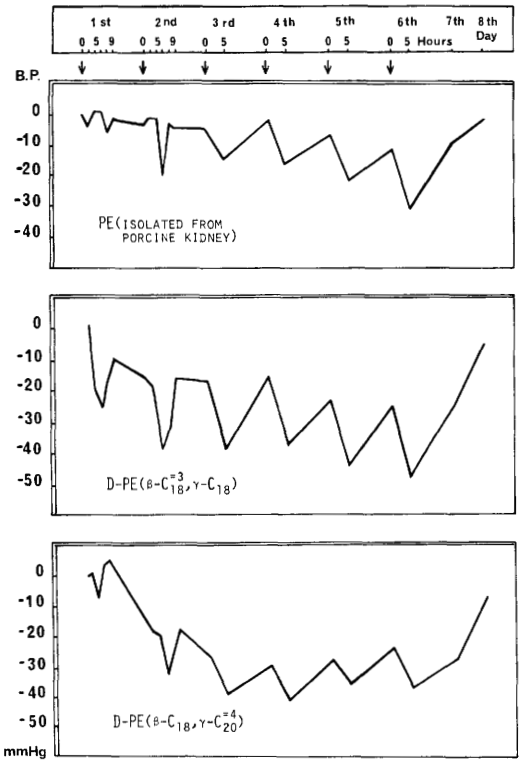

FIG. 1. Hypotensive effect of synthetic phosphatidylethanolamines (PE) and natural PE isolated from porcine kidney (10 mg/rat/day) given im to renal hypertensive rats. Data are the average of three animals. The arrows at 0 hr indicate the injection time.

of lyso-PE was greater than that of natural PE. Sen *et al.* (6) and Smeby *et al.* (7) reported anti-renin and hypotensive actions of natural PE and stated that the true renin inhibitor was the lyso form which was converted from the original phospholipid during the incubation procedure or by treatment of phospholipase A. Recently, Baggio *et al.* (17) using the same procedure of Ostovsky *et al.* (8) reported that a phospholipid isolated from human plasma failed to reduce the rate of angiotensin production in the renin and angiotensinogen systems, but the addition of phospholipase A to the incubation system resulted in a significant decrease in the production of angiotensin.

If the lyso form of PE is indeed a true renin inhibitor as mentioned by these investigators, the fatty acid which is substituted at the β position of synthetic PE should not influence the inhibitory action, because the lyso-form is generated by removing a fatty

TABLE 2. HYPOTENSIVE EFFECT OF SYNTHETIC PHOSPHATIDYLETHANOLAMINE (PE) AND NATURAL PE ISOLATED FROM PORCINE KIDNEY IN RENAL HYPERTENSIVE RATS.

Phosphatidylethanolamine	Optical activity	Maximum hypotensive response in renal hypertensive rats ($-\Delta$ blood pressure: mm Hg)
PE(β-$C_{18}^{=3}$, γ-C_{18})	D	46
	DL	41
	L	–
PE(β-$C_{20}^{=4}$, γ-C_{18})	D	–
	DL	16
	L	5
PE(β-C_{18}, γ-$C_{20}^{=4}$)	D	39
	DL	43
	L	22
PE(β-C_{18}, γ-$C_{18}^{=3}$)	D	36
	DL	43
	L	22
PE(β-$C_{20}^{=4}$, γ-$C_{20}^{=4}$)	D	–
	DL	12
	L	–
Natural phosphatidylethanolamine (isolated from porcine kidney)		29

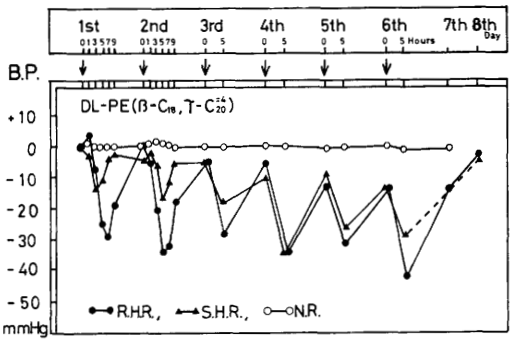

FIG. 2. Comparison of the hypotensive effect of DL-PE (β-C_{18}, γ-$C_{20}^{=4}$) in renal hypertensive rats (RHR), spontaneously hypertensive rats (SHR), and normotensive rats (NR). Each point is the mean value of three different animals. The arrows indicate the injection time.

acid at the β position by phospholipase A. But, as shown in Table 1, PE possessing the same fatty acid at the γ position and a different fatty acid at the β position showed different inhibitory actions. It is assumed that the fatty acid in the β position would alter

the rate of hydrolysis by phospholipase A, and, if so, the inhibitory activity would differ, even when the fatty acid in the γ position was the same.

We found that the D form of PE had a more potent activity than that of the L form. With regard to optical isomers, it is known that phospholipase A of snake venom acts on the L form, but not on the D form, of lecithin and phosphatidylserine (18). Additionally, Etienne *et al.* (19) and Osmond *et al.* (20) reported that 5 m*M* EDTA completely inhibited phospholipase A activity in rat serum and rat plasma, respectively. When we obtained high-renin plasma from dogs, 1 mg of EDTA was added to each milliliter of whole blood, so that the final concentration of EDTA in plasma would be over 6 m*M*. In the light of this data, the possibility that PE itself may inhibit renin without conversion must be considered.

In agreement with other investigators (21), the hypotensive effect was observed with repeated daily injections of natural PE to renal hypertensive rats. Antonello *et al.* (22) reported that natural PE did not reduce blood pressure in renal hypertensive rats, but here, only 2 mg was given in a single injection. We injected 10 mg a day for 6 days and a gradual hypotension was evident. The hypotensive effect was also observed in SHR. Other workers have reported significant elevations of renin activity in the kidney and plasma of renal hypertensive rats (23–25) and in the plasma of SHR of 12–20 weeks of age (26).

These newly synthesized PE as renin inhibitors, should be a useful tool in clarifying the actual role of the renin angiotensin system.

Summary. Anti-renin and hypotensive effects of synthetic PEs were examined.

(i) Eighteen PEs including optical isomers were newly synthesized. Arachidonic acid, linolenic acid, and stearic acid were substituted at the positions of β, γ, or both. Natural PE was extracted from porcine kidney, and the lyso form was prepared by treatment of phospholipase A.

(ii) Anti-renin activity of these compounds was determined using high-renin plasma obtained from the dog. The inhibition of renin activity was expressed as a percentage of the reduction in the production rate of angiotensin I as measured by radioimmunoassay.

(iii) The inhibitory effects of PE(β-$C_{18}^{=3}$, γ-C_{18}) series, D- and DL-PE(β-$C_{20}^{=4}$, γ-C_{18}), and DL- and L-PE(β-C_{18}, γ-$C_{20}^{=4}$) were greater than that of the natural PE, but did not exceed the effect of lyso-PE.

(iv) Hypotensive effect was evaluated in conscious normotensive, spontaneously hypertensive, and renal hypertensive rats by daily im injection of the test compounds.

(v) Following repeated administration of natural PE, D-PE(β-C_{18}, γ-$C_{20}^{=4}$), and D-PE(β-C_{18}, γ-$C_{18}^{=3}$) to renal hypertensive rats, blood pressure decreased by more than 30 mm Hg. D- and DL-PE(β-$C_{18}^{=3}$, γ-$C_{20}^{=4}$), DL-PE (β-C_{18}, γ-$C_{18}^{=3}$), and DL-PE(β-C_{18}, γ-$C_{20}^{=4}$) lowered pressure more than 40 mm Hg. DL-PE(β-C_{18}, γ-$C_{20}^{=4}$) showed hypotensive effects both in renal hypertensive and spontaneously hypertensive rats, but not in normotensive rats.

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