Renin Inhibition by Synthetic Phosphatidyl and Phosphorylethanolamines

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MANY evidences suggesting that the renin angiotensin system may play an important role in the pathogenesis of hypertension have been accumulated. However, the exact role of this system has not been completely clear. It has been realized that the availability of effective inhibitors for renin angiotensin system might be useful to make clear the physiological and patholozical role of this system and to treat the hypertensive patients with high renin plasma.

Since *Khairalla et al*¹ have reported an analogue of angiotensin II (Ile⁵, Ala⁸-Ang II) which antagonizes the action of angiotensin II on a strip of guinea pig ileum, many angiotensin II analogues have been reported and used practically^{2,3}. Converting enzyme inhibitor which blocks the conversion of angiotensin I to II has been reported^{4,5} and one of these synthetic inhibitors has been used experimentally⁶.

On the other hand, several authors have described about the renin inhibitory substances; heparin⁷, synthetic tetrapeptide⁸, sodium deoxycholate⁹ and pepstatin¹⁰⁻¹⁴. *Sen et al*^{15,16} have developed the phospholipid renin inhibitor. This substance isolated from a dog or hog kidney originally has been named the preinhibitor as it has been considered that a lyso-phospholipid, formed by the treatment of phospholipase A, showed stronger effect than the original phospholipid. The chemical structure of this phospholipid, so called prerenininhibitor, has been assigned as phosphatidylethanolamine (s)[phos-

phatidyl-E] containing a large amount of unsaturated fatty acids.¹⁷ This study was carried out to find much potent synthetic renin inhibitor. About 30 kinds of Phosphatidyl-E were newly synthesized and evaluated the renin inhibitory effect in vitro and their hypotensive effect in vivo.

SYNTHETIC PHOSPHATIDYL-E

As a first attempt, 18 kinds of Phosphatidyl-E including optical isomers (6 fundamental combination of fatty acids and their D, DL and L form) were newly synthesized. The fundamental structure of Phosphatidyl-E used here is shown in Fig. 1. Arachidonic acid (C₂₀⁴), linolenic acid (C₁₈³), and stearic acid (C₁₈) were substituted at the position of β , γ or both. Synthesis of Phosphatidyl-E was performed according to the methods by *DeHaas et al*¹⁸ and *Billimoria and Lewis*¹⁹. Natural Phosphatidyl-E was isolated from porcine kidney following the partially modified method described by *Sen et al*¹⁵. Lyso-Phosphatidyl-E was prepared by the method according to *Rakhit*¹⁷ using phospholipase A.

Evaluation of phosphatidyl-E to inhibit renin catalyzed conversion of renin substrate to angiotensin I was examined in high renin dog plasma. High renin plasma was taken from Nembutal anesthetized dogs. To obtain the high renin activity plasma, an aortic clamp was placed on the aorta just above the renal arteries, and the renal arterial pressure was reduced about 70 mmHg by manipulation of the clamp for 40 min. The plasma taken by this procedure yielded 40 to 120 ng angiotensin I per ml of the incubation mixture after a 6 hr incubation. A

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Fatty Acid						
R ₁ (γ)	⁼⁴ C ₂₀	C ₁₈	C ₁₈	⁼⁴ C ₂₀	⁼³ C ₁₈	C ₁₈
R ₂ (β)	⁼⁴ C ₂₀	⁼⁴ C ₂₀	⁼³ C ₁₈	C ₁₈	C ₁₈	C ₁₈

Fig.1. Fundamental structure of phosphatidylethanolamine and combination of fatty acids substituted in R₁(γ) and R₂(β) positions. C₁₈; Stearic acid, C₁₈³; Linolenic acid, C₂₀⁴; Arachidonic acid.

mixture of test compound and high renin plasma was incubated for 6 hr at 37°C. After 2,4,6 hr incubation, generated angiotensin I was determined by radioimmunoassay (Fig. 2). The renin inhibitory effect was expressed as percent reduction in the production of angiotensin I. Table I is the summarized result at a dose of 5 mM. The inhibitory effect of natural Phosphatidyl-E was 31%. The lyso-form of natural Phosphatidyl-E was stronger than the original natural one.

The synthetic Phosphatidyl-E also showed inhibitory effect. The β-linolenyl-γ-stearoyl-α-phosphatidylethanolamine; PE(β-C₁₈³, γ-C₁₈) series were the most potent inhibitors among newly synthesized Phosphatidyl-E. The effect was stronger than that of the natural Phosphatidyl-E and almost equipotent to the lyso-form of natural Phosphatidyl-E. In the PE (β-C₂₀⁴, γ-C₁₈) series, D and DL form showed 41.2 and 58.5% inhibitions but L form showed only 16.7% at a 6 hr incubation. On the contrary, in the PE (β-C₁₈, γ-C₂₀⁴) series, L and DL form were stronger than D form.

Smeby *et al*^{15,16} studied the reaction of renin and its substrate with natural phospholipid and noticed that no renin inhibition was observed during the first hour of a 4 hr incubation and after the first hour definite renin inhibition was observed. Thus, they suggested that the true renin inhibitor was the lyso-form which was

converted from the original phospholipid by phospholipase A during the incubation procedure. Recently, Baggio *et al*²⁰ have reported a similar observation that the phospholipid isolated from human plasma failed to reduce the rate of angiotensin I production in renin and renin substituted at β-position of synthetic Phosphatidyl-E should not have influence on the a significant decrease of angiotensin production. In our experiment, the inhibitory effect of lyso-Phosphatidyl-E was indeed stronger than that of natural one. If lyso-form of Phosphatidyl-E is true renin inhibitor as mentioned by these investigators, the fatty acid which is substituted at β-position of synthetic Phosphatidyl-E should not have influence on the inhibitory effect, because lyso-form is generated by removing of fatty acid at β-position by phospholipase A. However, as shown in Table 1, Phosphatidyl-E possessing the same fatty acid at the γ position and different fatty acid at the β position showed different inhibitory effect. Additionally, it is noticeable that D-form of Phosphatidyl-E showed a stronger activity than that of L-form. With regard to optical isomers, it has been known that phospholipase A acts only on L-form but not on D-form of lecithin and phosphatidylserine²¹ The present result suggested that Phosphatidyl-E itself could inhibit renin activity directly without conversion to lyso-form. Therefore, a conversion from the

Fig.2. Flowsheet of assay system in high renin dog plasma.

original Phosphatidyl-E, so called prerenininhibitor, to lyso-form was not essential.

Hypotensive effects of synthetic Phosphatidyl-E were also studied in vivo. Ten mg of Phosphatidyl-E was given daily intra muscularly for 6 days to two kidney renal hypertensive rats (8 weeks after clipping, average pressure was 208 mmHg; range 199–211 mmHg), spontaneously hypertensive rats (SHR, 16–20 weeks old; average blood pressure was 201 mmHg, range 194–206 mmHg) and normotensive rats (15 weeks old). Figure 3 shows the hypotensive effect of DL-PE (β -C₁₈, γ -C₂₀⁴) in renal hypertensive rat. Blood pressure monitored using a tail sphygmographic method, decreased gradually and reached maximum after 5 to 7 hrs. The blood pressure then tended to almost recover its

original level on the next day, but the hypotensive effect was strengthened by the daily repeated injections. After stopping the injections, the pressure recovered to the original level within 2 to 3 days. Phosphatidyl-E was less effective in spontaneously hypertensive rats and noneffective in normotensive rats. The hypotensive effects of Phosphatidyl-E are summarized in the Table I. Six out of 10 synthesized compounds lowered blood pressure more than 30 mmHg. The hypotensive pattern was similar to that of natural Phosphatidyl-E. Generally, the hypotensive effect of D and DL-form was stronger than L-form. This result also supported the direct effect of Phosphatidyl-E without conversion to lyso-form on account of the resistance of phospholipase A to D-form. A linear relationship

TABLE I THE RENIN INHIBITORY EFFECT AND HYPOTENSIVE RESPONSE OF SYNTHETIC PHOSPHATIDYLETHANOLAMINES, NATURAL PHOSPHATIDYL-ETHANOLAMINE (PE) ISOLATED FROM PORCINE KIDNEY AND LYSOFORM OF NATURAL PE (LYSO-PE).

Phosphatidyl-ethanolamine (PE)	O.A.	N	Percent Inhibition after 6hr Incubation	Maximum Hypotensive Response in Renal Hypertensive Rats - Δ Blood Pressure (mmHg)
PE (β -C ₁₈ , γ -C ₂₀ ⁼⁴)	D	4	20.4 $\pm$ 1.6*	39
	DL	3	43.3 $\pm$ 7.2*	43
	L	4	39.9 $\pm$ 0.9*	22
PE (β -C ₁₈ , γ -C ₁₈ ⁼³)	D	2	34.5 $\pm$ 3.0* (4 hr)	36
	DL	5	35.3 $\pm$ 8.2*	47
	L	2	23.0 $\pm$ 0.2* (4 hr)	24
PE (β -C ₁₈ , γ -C ₁₈)	D	4	15.0 $\pm$ 6.7	—
	DL	4	25.0 $\pm$ 7.5*	—
	L	4	32.0 $\pm$ 11.1*	—
PE (β -C ₁₈ ⁼³ , γ -C ₁₈)	D	5	61.0 $\pm$ 15.1**	46
	DL	7	66.0 $\pm$ 13.2*	41
	L	4	52.3 $\pm$ 2.3*	—
PE (β -C ₂₀ ⁼⁴ , γ -C ₁₈)	D	3	41.2 $\pm$ 17.6	—
	DL	4	58.5 $\pm$ 20.9*	16
	L	6	16.7 $\pm$ 2.5**	5
PE (β -C ₂₀ ⁼⁴ , γ -C ₂₀ ⁼⁴)	D	4	23.8 $\pm$ 3.9**	—
	DL	5	33.3 $\pm$ 9.2*	12
	L	4	14.7 $\pm$ 2.2**	—
Natural PE (Isolated from porcine kidney)		9	31.5 $\pm$ 12.4*	29
Lyso-PE		4	65.9 $\pm$ 13.4*	—

O.A.; Optical Activity, N; Number of Experiment, (4 hr); 4hr Incubation Value, * $p < 0.05$, ** $p < 0.01$

between renin inhibitory effect in high renin plasma system and hypotensive effect on renal hypertensive rats was observed ($p < 0.05$) with the exception of DL-PE(β -C₂₀⁼⁴, γ -C₁₈) and D-PE(β -C₁₈, γ -C₂₀⁼⁴).

Previous studies have shown a significant elevation of renin activity in the kidney and the plasma in renal hypertensive rats²²⁻²⁴ and the increase of plasma renin activity in spontaneously hypertensive rat of 12 to 20 weeks of age²⁵. Although, we did not measure plasma renin activity after repeated administration of Phosphatidyl-E, the present data strongly suggested a participation of renin angiotensin system

in the hypotensive mechanism of Phosphatidyl-E.

STUDY OF SYNTHETIC PHOSPHORYLETHANOLAMINE

Renin inhibitory effect of synthetic Phosphatidyl-E was confirmed in previous study but their inhibitory effect did not exceed the effect of lyso-form of natural Phosphatidyl-E. In the second series, we newly prepared synthetic lyso-Phosphatidyl-E and several Phosphoryl-E compounds (Table II) and examined their renin inhibitory effect in vitro and in vivo. First, evaluation of these compounds was performed in high renin plasma.

Fig.3. Comparison of hypotensive effect of LD-PE (β -C₁₈, γ -C₂₀⁼⁴) on renal hypertensive rat (RHR), spontaneously hypertensive rat (SHR) and normotensive rat (N.R.).

Table II shows the result of a 4 hr incubation at a concentration of 2.5 mM. Among these compounds, C₁₇H₂₉ CONH(CH₂)₃-O-PO(OH)-O-(CH₂)₂NH₂ (PE-72) showed the most potent inhibition (41.6%). The inhibitory rate was stronger than synthetic lyso-form,

C₁₇H₂₉COOCH₂-CH(OH)-CH₂-O-PO(OH)-O-(CH₂)₂NH₂ (PE-69DL). More detail kinetic study of PE-72 on the inhibitory effects were carried out in the reaction system of partially purified dog renin and renin substrate. Lineweaver-Burke representation of dog renin inhibi-

TABLE II PERCENT INHIBITION OF SYNTHETIC LYSO-PHOSPHATIDYLETHANOLAMINE (DL-PE-69) AND PHOSPHORYL-ETHANOLAMINES IN A CONCENTRATION OF 2.5 mM, AFTER A 4 HR INCUBATION

$ \begin{array}{c} \text{O} \\ \parallel \\ \text{R}-\text{O}-\text{P}-\text{O}(\text{CH}_2)_m\text{NH}_2 \\ \\ \text{OH} \end{array} $			
R	m	Series Number	Inhibition Rate (%) 4 hr Incubation
$\text{C}_{17}\text{H}_{29}\text{COOCH}_2-\text{CH}(\text{OH})-\text{CH}_2-$	2	DL-PE-69	14.0
$\text{C}_{17}\text{H}_{29}\text{COOCH}_2-\text{CH}(\text{CH}_3)-\text{CH}_2-$	2	PE-70	22.6
$\text{C}_{17}\text{H}_{29}\text{CH}_2-$	2	PE-68	26.2
$\text{C}_{17}\text{H}_{29}\text{CONH}(\text{CH}_2)_3-$	2	PE-72	41.6
$\text{C}_{17}\text{H}_{31}\text{CONH}(\text{CH}_2)_3-$	2	PE-83	15.7
$\text{C}_{17}\text{H}_{31}\text{CONH}(\text{CH}_2)_2-$	2	PE-82	14.0
$\text{C}_{17}\text{H}_{31}\text{CONH}(\text{CH}_2)_2-$	3	PE-84	26.6
$\text{C}_{17}\text{H}_{31}\text{CONH}(\text{CH}_2)_2-$	6	PE-87	17.1
$\text{C}_{10}\text{H}_{15}(\text{CH}_2)_2-$	2	PE-76	0

Fig.4. Lineweaver-Burke representation of dog renin (50ng angiotensin I Eq/hr) inhibition by synthetic phosphorylethanolamine (PE-72).

tion by PE-72 indicated competitive inhibition. K_m was 602 ng angiotensin I Eq/ml and K_i from Dixon-Webb plot was 5.1 mM.

Hypotensive effect of PE-72 was also studied in rats and dogs. In two kidney renal hypertensive and normotensive rats, 10 mg of PE-72 was given *Lm* daily for 6 days. The blood pressure monitored, using a tail sphygmographic method, decreased gradually and the hypotensive effect reached maximum (45 mmHg) after 6 days. After stopping the injection, blood pressure returned to the control level within 3 days. No pressure response was observed in the normotensive rats as well as in the case of Phosphatidyl-E. Intravenous infusion of partially purified dog renin in the anesthetized dogs resulted in an increase of plasma renin activity and angiotensin I concentration accompanied with a rise in blood pressure. The elevation of these parameters were significantly reduced by intravenous injection of 200 mg/kg of PE-72 (Fig. 5).

EFFECT OF PE-104 AS RENIN INHIBITOR

As mentioned above, synthetic Phosphatidyl-E and Phosphoryl-E showed renin inhibi-

Fig.5. Effects of phosphorylethanolamine (PE-72) on blood pressure and plasma angiotensin I concentration in Nembutal anesthetized dog with dog renin infusion.

tory effect in vitro and in vivo. In the third series, we tried to evaluate other synthetic Phosphoryl-E derivatives which excluded a long fatty acid chain from the already tried Phosphoryl-E. Among several derivatives, the following compound, PE-104, had the most potent

action.

The comparison of percent inhibition of PE-104 and Phosphoryl-E including a long fatty acid chain in dog renin and substrate incubation system was shown in Fig. 6. PE-104 showed almost 45% inhibition at a dose of 2 mM. This

2-[4-(4'-chlorophenoxy) phenoxyacetyl amino]-ethylphosphoryl ethanolamine (PE-104)

Fig.6. Inhibition of the partially purified dog renin and substrate reaction by varying concentrations of PE-72 and PE-104.

effect of PE-104 was twofold that of PE-72. By means of Lineweaver-Burke plot, competitive type of inhibition (K_m ; 609 ng angiotensin I Eq/ml) was observed and K_i value introduced by Dixon-Webb plot was 2.0 mM. The effect of PE-104 on other proteolytic enzymes, trypsin and papain, was examined on the inhibitory activity on the hydrolysis of casein. A concentration of 10^{-2} mM of PE-104, which inhibited renin activity more than 80%, inhibited trypsin only 14% and was non effective on papain. In vivo experiment was carried out using male Wistar rats weighing about 400g. Under Nembutal anesthesia, the left carotid artery, right femoral artery and bilateral femoral veins were cannulated. Blood pressure was measured from the carotid artery via pressure transducer. Prior to the experiment, pentrinium (5 mg/kg) was injected. After the blood pressure became stable, dog renin (400 ng angiotensin Eq) was injected. The blood pressure gradually increased by 15 to 20 mmHg and then decreased to the control level

Fig.7. Effects of dog renin injection of 400ng angiotensin I Eq/hr on blood pressure response and plasma angiotensin I concentration in pentrinium (5mg/kg) treated Nembutal anesthetized rats before (open circles) and after (solid circles) phosphorylethanolamine (PE-104) infusion (9mg/0.03ml/min.). Pressure response is suppressed significantly ($P < 0.01$, $n=6$) and plasma angiotensin I concentration does not increase ($P < 0.05$, $n=5$) after the infusion of PE-104.

within 10 min. When the pressure had stabilized, PE-104 was infused at a rate of 9 mg/min (0.03 ml/min) from unilateral femoral vein. After 10 to 15 min infusion, the same amount of renin was injected. One ml of blood sample was withdrawn from the femoral artery, just before the renin injections and when the blood pressure had reached maximum after the renin injection. To compensate for volume loss, 1 ml of 0.9% saline was injected from the contralateral femoral vein. Plasma angiotensin I concentration was determined by radioimmunoassay. Change of blood pressure response and plasma angiotensin I concentration by renin injection are shown in Figure 7. The infusion of PE-104 resulted in a significant decrease in pressor response and plasma angiotensin I concentration.

SUMMARY

Renin inhibitory effects of about 30 kinds of newly synthesized Phosphatidyl-E and Phosphoryl-E were studied in vitro and in vivo. Among the synthetic Phosphatidyl-E, PE (β -C₁₈, γ -C₁₈) series were the most potent and these were stronger than natural Phosphatidyl-E. We also confirmed that the conversion from the original Phosphatidyl-E, so called prerenin-inhibitor, to lyso-form to exhibit renin inhibition as mentioned by *Sen et al.*^{15,16} and *Baggio et al.*²⁰ was not essential. But a stronger inhibition to renin was observed in PE-72, one of synthetic Phosphoryl-E analogues. PE-104, Phosphoryl-E without long fatty acid chain, was the most potent inhibitor in this study. PE-72 and PE-104 inhibited the reaction between dog renin and substrate in a competitive way.

In dogs and rats, Phosphoryl-E decreased hypertensive response and increased angiotensin I concentration induced by the exogenous renin. Hypotensive effects of Phosphatidyl-E and Phosphoryl-E were also demonstrated in renal hypertensive rats and not in normotensive rats.

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Discussion:

Chairman: TATSUO KOKUBU, Ehime Univ.

Dr. T. KOKUBU (Chairman): May I ask two questions?

- 1) Did you check the contamination of phospholipase in high renin plasma which you used in the incubation system?
- 2) How do you think about the specificity of a synthetic phosphorylethanolamine (PE-104) on renin inhibition?

Dr. MIYAZAKI (Osaka City Univ.): We have not checked whether the high renin plasma is contaminated with phospholipase or not. There may occur phospholipase in high renin plasma.

PE-104 did not inhibit both papain and trypsin on the hydrolysis of casein. Thus I think that PE-104 has high specificity for renin.

Dr. KAWASHIMA (Jichi Medical Univ.): In routine measurement of plasma renin activity, pH of incubation system is 5.6. Why did you choose pH 7.4 in your incubation system?

Dr. MIYAZAKI: I believe that pH 7.4 is much better than pH 5.6, because the former is near to physiological condition.

Dr. UEDA (Ehime Univ.): You showed that in vitro experiments, the longer incubation time, the more increase in inhibitory percent. How do you explain the findings?

Dr. MIYAZAKI: I can not explain it clearly. There are two possibilities. One is low solubility of synthetic phosphorylethanolamine compounds. During the long incubation, these compounds dissolve in the incubation mixture. Another possibility is that these compounds might be converted into active forms by phospholipase during the incubation.

Dr. KANEKO (Yokohama City Medical Univ.): In your vivo experiments, you injected 10 mg of phosphatidylethanolamine intramuscularly in two-kidney renal hypertensive rats which were clipped 4 weeks previously. Did you study the hypotensive effects of phosphatidylethanolamine in the early stage that plasma renin activity was more higher than the time you studied?

Dr. MIYAZAKI: No, we did not study it in the early stage.

Dr. KANEKO: You have found the hypotensive effects of phosphatidylethanolamine in hypertensive rats. In that case is there any relationship between plasma renin activity and the hypotensive effects of phosphatidylethanolamine?

Dr. MIYAZAKI: We did not examine it.

Dr. KAWASHIMA: In the infusion experiments, the homologous renin was infused two times in a dog. Intravenous injection of phosphatidylethanolamine derivative (PE-72) was carried out during the second time infusion. The blood pressure elevation was significantly suppressed. Is the suppression of blood pressure elevation due to the effect of PE-72 or due to renin tachylaxis?

Dr. MIYAZAKI: In our control experiments, the blood pressure elevation caused by the second time renin infusion was same as that by the initial renin infusion. Therefore, I think that the suppression of blood pressure elevation is due to the effect of PE-72.

Dr. UEDA: How long did the hypotensive effect a single injection of PE-72 continue during renin infusion?

Dr. MIYAZAKI: I think that PE-72 might be metabolized rapidly in vivo, because the hypotensive effect of a single injection of PE-72 is short.