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DIPALMITOYLPHOSPHATIDYLCHOLINE (L-α-LECITHIN) STIMULATES PHOSPHOLIPASE A_2 ACTIVITY IN HUMAN AMNION

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SUMMARY : In order to investigate the mechanism of dipalmitoylphosphatidylcholine (DPPC, L-α-lecithin) stimulation of the prostaglandin E (PGE) production of the amniotic membrane, effects of DPPC (50-800 μg/ml) on phospholipase A_2 (PLA_2), phospholipase C (PLC), PG endoperoxide synthase, and PGE synthase activities of human amniotic membrane were studied. Only PLA_2 activity was increased by DPPC, suggesting that lecithin, the major surfactant component, increases the PGE production of the amniotic membrane by activating PLA_2. © 1990 Academic Press, Inc.

It has been reported that the increase of PGE_2 production by the amniotic membrane is essential in the involvement of prostaglandins (PGs) in the mechanism of the onset of human parturition (1, 2, 3). Among the many substances reported to increase amniotic membrane PGE production, we have been interested in amniotic fluid surfactant phospholipid, which was reported by López Bernal et al. (4), because its stimulating effect was observed under physiological concentrations. Surfactant phospholipids are secreted by the alveolar type II cells, and lack of these phospholipids induces respiratory distress syndrome of the newborn. Lecithin composes 80-90% of surfactant phospholipids and 60-90% of its fatty acid is made up of palmitic acid. Although López Bernal's group concluded that surfactant is a major source of arachidonic acid for PGE production, we have shown that lecithin (dipalmitoylphosphatidylcholine, DPPC) increases PGE output as a stimulator, not as a substrate (5). However, the site of the stimulatory effect of DPPC on PGE production remains unknown. The purpose of this study was to investigate the mechanism of the stimulating effect of DPPC on amniotic membrane PGE production.

Abbreviations: PE, 2-[^{14}C] AA, 1-acyl-2-[1-^{14}C] arachidonyl-L-3-phosphatidyl-ethanolamine; [^{3}H] AA, [5, 6, 8, 9, 11, 12, 14, 15-^{3}H] arachidonic acid; [^{3}H] PI, L-3-phosphatidyl-[2-^{3}H] inositol; [^{14}C] AA, [1-^{14}C] arachidonic acid; [^{3}H] PGE_2, [5, 6, 8, 11, 12, 14, 15 (n)-^{3}H] PGE_2; lysoPE, 1-palmitoyl-lysophosphatidylethanolamine; AA, arachidonic acid; PE, 2-AA, 1-palmitoyl-2-arachidonylphosphatidylethanolamine; PI, phosphatidylinositol; DPPC, dipalmitoylphosphatidylcholine; lysoPC, lysophosphatidyl-choline; FFA-free BSA, fatty acid free bovine serum albumin.

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We studied the effects of DPPC on phospholipase A_2 (PLA_2, 3.1.1.4), phospholipase C (PLC, 3.1.4.3), PG endoperoxide synthase (1.14.99.1), and PGE synthase (5.3.99.3) activities of human amniotic membrane.

MATERIALS AND METHODS

Eight placentas were obtained from pregnant women who had term deliveries without complications; each of the women gave birth to a single, healthy baby. Informed consent was obtained from all the women. Five placentas were used for PLA_2 assay, two for PLC assay, and the remaining one for PG endoperoxide synthase and PGE synthase assay.

All chemicals and solvents used were of reagent or analytical grade. 1-Acyl-2-[1-^{14}C] arachidonyl-L-3-phosphatidylethanolamine (PE, 2-[^{14}C] AA, 2.07 GBq/mmol), [5, 6, 8, 9, 11, 12, 14, 15 - ^{3}H] arachidonic acid ([^{3}H] AA, 7.67 TBq/mmol), L-3-phosphatidyl-[2-^{3}H] inositol ([^{3}H] PI, 644 GBq/mmol), [1-^{14}C] arachidonic acid ([^{14}C] AA, 2.07 GBq/mmol), [5, 6, 8, 11, 12, 14, 15 (n) - ^{3}H] PGE_2 ([^{3}H] PGE_2, 7.67 TBq/mmol) were purchased from Amersham Japan (Tokyo, Japan). Arachidonic acid (AA) was obtained from Nu-Chek Prep, Inc. (Elysian, Minnesota, USA). Adenosine triphosphate (ATP) and fatty acid free bovine serum albumin (FFA-free BSA) were from Sigma (St. Louis, USA). 1-Palmitoyl-lysophosphatidylethanolamine (lysoPE) and phosphatidylinositol (PI) were products of Serdary Research Laboratories (Ontario, Canada). Coenzyme A (CoA) was obtained from Wakamoto Pharmaceutical Co., Ltd. (Tokyo, Japan). Dipalmitoyl-phosphatidylcholine (DPPC) was from Funakoshi Pharmaceutical Co., Ltd. (Tokyo, Japan). Wistar strain male rats (30 weeks old, about 400 g) were used.

PLA_2 assay

2-Arachidonylphosphatidylethanolamine (PE, 2-AA) was synthesized enzymatically by the method of Okazaki et al. (6). Briefly, 18 μmol of AA was incubated with 18 μmol of lysoPE, 40 μmol of CoA, 400 μmol of ATP, 200 μmol of magnesium chloride, 250 μmol of reduced glutathione, and rat liver microsomal preparation (7) (20 mg protein) in a total volume of 10 ml of 50 mM potassium phosphate buffer, pH 7.4. After incubation at 37°C for 3 hr, the reaction was terminated by adding 40 ml of chloroform : methanol (2:1). PE, 2-AA was isolated by silicic acid column chromatography (8). PE, 2-AA was quantified by determining phosphorus by Shibuya's method; that is, 1.0 ml of reagent A (10 N sulfuric acid : 0.1 M periodic acid : ethanol (3:1:6)) was added to 0.2 ml of sample; after heating for 10 min in boiling water, reagent B (25% ammonium molybdate : aminonaphthol sulfonic acid : water (3:5:32)) was added; then after heating again for 10 min in boiling water, the optic density at 820 nm was measured. Aminonaphthol sulfonic acid was composed of 0.1% 1-amino-2-naphthol-4-sulfonic acid, 0.2% sodium sulfite, and 6% sodium hydrogen sulfite. A standard curve was made by potassium phosphate. The purity of PE, 2-AA was confirmed by thin-layer chromatography (Merck Art. 5547 HPTLC plates, Darmstadt, F.R.G.) developed in chloroform : methanol : acetic acid : water (50:15:4:2) and chloroform : methanol : ammonia water (65:25:4).

The amniotic membrane was removed from the placenta and washed with cold saline. It was then homogenized with a polytron homogenizer for 40 sec at top speed in 4-5 vol of 0.25 M sucrose, 100 mM Tris-HCl buffer, pH 8.0. The homogenate was centrifuged at 750 g for 10 min. The supernatant was employed as an enzyme source.

Protein concentration was determined by the method of Lowry et al. (9) with human serum albumin as a standard.

PE, 2-AA (27-39 nmol/tube) and PE, 2-[^{14}C]AA (50,000-100,000 DPM/tube) were prepared in 100 mM Tris-HCl buffer, pH 8.0, containing 1 mM ethylenediaminetetraacetic acid (EDTA) and 0.3% FFA-free BSA and used as the substrate.

PLA_2 activity was measured in a reaction mixture containing 100 mM Tris-HCl buffer, pH 8.0, 2 mM calcium chloride, 100-200 μg of supernatant protein, and 100 μl of the substrate in a final volume of 0.4 ml. The incubation was carried out at 37℃ for 120 min and terminated by adding 4 ml of chloroform : methanol (2:1). After addition of 0.27 ml of hydrochloric acid, the upper layer was removed. The lower layer was evaporated, and the released AA was separated by HPTLC. The recovery rate was calculated by the addition of [^{3}H]AA. Radioactivities were counted by a liquid scintillation counter Aloka RT 3600 (Tokyo, Japan). The developing solvent system for HPTLC was diethylether : benzene : ethanol : acetic acid (40:50:2:0.2). PLA_2 activity was expressed as nmol/mg protein/hr.

PLC assay

For PLC assay, amniotic membrane was homogenized in 3-4 vol of 0.32 M sucrose, 20 mM Tris-HCl buffer, pH 7.0 (10). The homogenate was centrifuged at 105,000 g for 60 min. The supernatant was used as an enzyme source.

PI (1 μmol/tube) and [^{3}H]PI (50,000-150,000 DPM/tube) were prepared in 20 mM Tris-HCl buffer, pH 7.0 and used as the substrate.

PLC activity was measured in a reaction mixture containing 20 mM Tris-HCl buffer, pH 7.0, 8 mM calcium chloride, 4 mM mercaptoethanol, 100-150 μg of supernatant protein, and 100 μl of PI and [^{3}H]PI in a final volume of 0.5 ml. The incubation was carried out at 37℃ for 30 min (or for 5 min in the case of kinetic study) and terminated by adding 2 ml of chloroform : methanol (1:2). After adding 0.6 ml of chloroform and 0.6 ml of 2 M potassium chloride, 1.5 ml of the upper phase was evaporated and counted for radioactivity. PLC activity was expressed as μmol/mg protein/hr.

PG endoperoxide synthase and PGE synthase assay

Amniotic membrane was homogenized in 5 vol of 0.25 M sucrose, 2 mM EDTA and 50 mM phosphate buffer, pH 8.0. The homogenate was centrifuged at 10,000 g for 1 hr, and the supernatant was recentrifuged at 100,000 g for 2 hr. The pellet was suspended in one twentieth volume of the same buffer and used as the microsome fraction.

AA (1 μg/tube) and [^{14}C]AA (150,000 DPM/tube) were prepared in 2 mM EDTA, 50 mM phosphate buffer and used as the substrate.

PG endoperoxide synthase and PGE synthase activity was measured in a reaction mixture containing 50 mM phosphate buffer, pH 8.0, 2 mM EDTA, 0.13 mM reduced glutathione, 0.1 μM bovine hemoglobin, 100 μl of the microsome fraction (260 μg protein), and 100 μl of AA and [^{14}C]AA in a final volume of 0.5 ml (11). Incubation was carried out for 10 min at 37℃ and then terminated by adding 2 ml of methanol.

For calculation of the recovery rate, [^{3}H]PGE_2 was added. Impurities were removed by extracting twice with petroleum ether, then the residue was acidified, and PGE_2 was extracted twice with ethyl acetate. Further purification was performed by HPTLC. The developing solvent system for HPTLC was ether acetate : acetic acid : 2, 2, 4-trimethylpentane : water (110:20:50:100). PG endoperoxide synthase and PGE synthase activity was expressed as ng PGE_2 produced/10 min.

Effect of DPPC

DPPC was dissolved in buffer by sonication, and its effect on each enzyme activity was evaluated.

Statistical analysis

Statistical analysis was performed by Student's t test.

RESULTS

PLA_2 assay

Chromatographic separation of PE, 2-AA from lysoPE is shown in Fig. 1. The first peak yielded a single spot on HPTLC by each of the two different developing solvent systems. Rf values of the spot were the same as those of PE, 2-[^{14}C]AA and different from those of lysoPE.

Arachidonic acid released by PLA_2 increased linearly up to 120 min under our incubation conditions. DPPC increased PLA_2 activity significantly in all five cases (Table 1, Fig. 2).

PLC assay

The rate of hydrolysis of PI was constant for 30 min under our incubation conditions. DPPC suppressed PLC activity dose-dependently (Fig. 3). The inhibition pattern was competitive (Fig. 4). The kinetic study showed that the Km value increased in the presence of 400 μg/ml of DPPC (from 250.0 to 953.8 μg/ml) without changing the Vmax value (4.55 vs. 4.76 μmol/mg protein/hr).

PG endoperoxide synthase and PGE synthase assay

PGE_2 produced by the microsome fraction of the amniotic membrane increased up to 10 min under our assay conditions. DPPC had no effect on PG endoperoxide synthase and PGE synthase (Fig. 5).

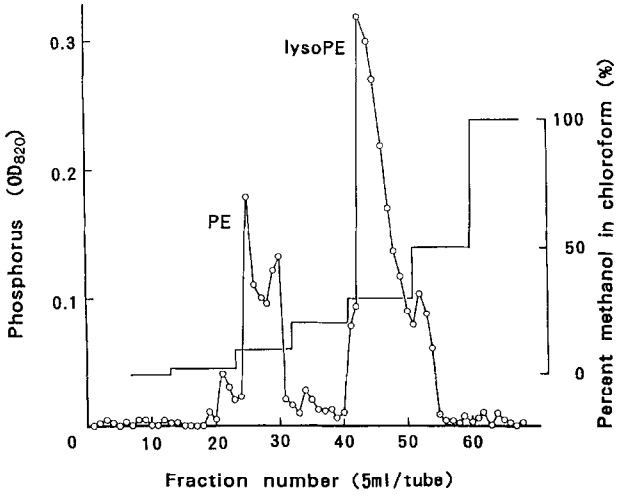


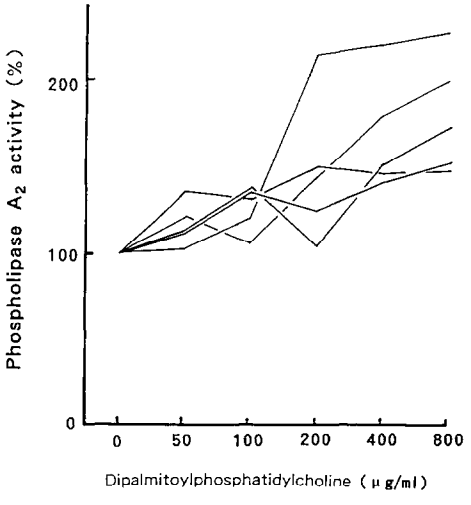
Fig. 1. Chromatographic separation of PE from lysoPE. The first peak eluted at 10% methanol and the second peak, at 30% methanol. The first peak was confirmed to be PE by HPTLC.

Table 1. The effect of DPPC on phospholipase A_2 activity. Mean value of phospholipase A_2 activity (nmol arachidonic acid release/mg protein/hr) for each amniotic membrane is shown. Values that were significantly increased from the basal values are underlined

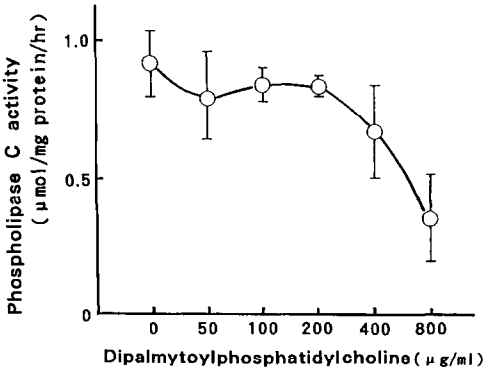
DPPC (μg/ml)	0	50	100	200	400	800
No. 1 n=4	5.60	**6.55**	**7.64**	**7.02**	**7.96**	8.58
(SD)	(0.28)	(**0.73**)	(**1.38**)	(**1.03**)	(**1.01**)	(3.96)
No. 2 n=4	4.32	4.82	5.98	4.51	6.51	**7.48**
(SD)	(0.33)	(1.58)	(1.88)	(0.83)	(1.94)	(**2.60**)
No. 3 n=4	2.60	**3.50**	3.47	**3.87**	**3.79**	**3.86**
(SD)	(0.49)	(**0.48**)	(0.93)	(**0.43**)	(**0.53**)	(**0.53**)
No. 4 n=3	3.32	3.43	4.04	**7.22**	**7.32**	**7.52**
(SD)	(0.49)	(0.64)	(0.69)	(**0.60**)	(**0.50**)	(**0.88**)
No. 5 n=3	8.15	9.91	8.60	**11.70**	**14.70**	**16.10**
(SD)	(0.73)	(2.05)	(0.15)	(**1.95**)	(**3.90**)	(**0.97**)

DISCUSSION

We have already reported that lecithin (DPPC) increases PGE production of the amnion as a stimulator. However, the site of the stimulatory action of DPPC remains to be clarified. In this study, activities of the enzymes involved in the



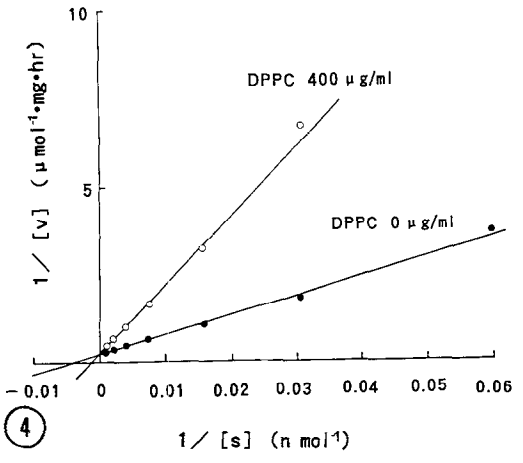
(2)



(3)

Fig. 2. DPPC increased PLA_2 activity significantly in all five cases.

Fig. 3. DPPC suppressed PLC activity dose-dependently.



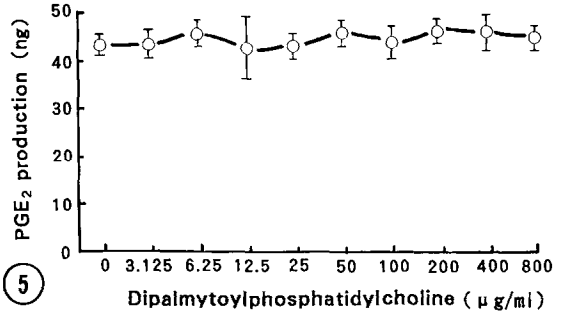


Fig. 4. Lineweaver-Burk plot showing competitive suppression of PLC by DPPC.

Fig. 5. DPPC had no effect on PG endoperoxide synthase and PGE synthase.

pathway from phospholipids to PGE were evaluated as functions of DPPC action. DPPC increased PLA_2 activity, decreased PLC activity, and had no effect on PG endoperoxide synthase and PGE synthase activities. The degree (150-200%) of PLA_2 stimulation agreed with the stimulating effect of DPPC on amniotic membrane PGE production, which we described previously (5).

PLA_2 is reported to be localized in the lysosomes and cell membrane (12). Since DPPC is too large to enter amnion cells through the cell membrane, the first possible site for PLA_2 stimulation is at the cell membrane. Romero et al. (13) reported that DPPC, which was used as the substrate, activated porcine pancreatic PLA_2 by the mechanism involving enzyme dimerization. It is possible that PLA_2 of the cell membrane was activated by DPPC at first, then hydrolysis of PE, 2-AA along with DPPC by this enzyme increased. Even though DPPC suppressed PLC in vitro, extracellular DPPC in vivo or in previous incubation experiments with amniotic membrane (5) could not suppress PLC activity in the cytosol fractions. The second possible mechanism for stimulation is through lysophosphatidylcholine (lysoPC). LysoPC is reported to increase the release of lysosomal enzymes, PLA_2, etc (14, 15). In our study, lysoPC, enzymatically or nonenzymatically converted from DPPC, possibly enters the cell and increases the release of lysosomal PLA_2. All the products of PLA_2 activity (lysoPC, lysoPE, AA, and PGs) have been reported to be potent lytic agents of lysosomes, a process that results in a positive feedback chain reaction (14). Variations in the response of PLA_2 activity to DPPC (Fig. 2) may be due to differences in the conversion rates of DPPC to lysoPC.

DPPC (and/or lysoPC) increased PLA_2 activity and decreased PLC activity. Although PLC activity (μ mol/mg protein/hr) is several hundreds times higher than

PLA_2 activity (nmol/mg protein/hr) at each optimal condition in vitro, the final product (PGE) was increased by DPPC (and/or lysoPC) under physiological conditions (5). These results suggest that PLA_2, and not PLC, is the key enzyme for PGE production in the amnion.

Among the many substances reported to increase amniotic membrane PGE production, the effect of surfactant phospholipids has been observed under physiological concentrations. Although López Bernal et al. reported surfactant to be a major source of arachidonic acid for PGE production, we have shown that lecithin increases PGE production as a stimulator. Furthermore, here we have clarified that PLA_2 is the site for stimulation of amniotic membrane PGE production. Because surfactant lecithin in amniotic fluid increases from 33-34 weeks gestation with fetal lung maturation and thereafter there is no marked change until before labor, lecithin does not seem to directly cause the rapid increase of amniotic membrane PGE production. Nevertheless, lecithin increases amniotic membrane PGE production with fetal lung maturation by stimulating PLA_2 and may play a role in the mechanism of human parturition.

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