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PHARMACOLOGICAL EFFECTS OF PHOSPHATIDYLSERINE LIPOSOMES: THE ROLE OF LYSOPHOSPHATIDYLSERINE

E. BIGON, E. BOARATO*, A. BRUNI, A. LEON* & G. TOFFANO*

Institute of Pharmacology, University of Padova, Largo Meneghetti 2, 35100 Padova, Italy
and Fidia Research Laboratories*, Abano Terme, Italy

1 Unique among the phospholipids, phosphatidylserine depresses brain energy metabolism when injected intravenously into mice in the form of sonicated liposomes. The possibility that this effect results from a metabolic transformation of phosphatidylserine is examined in this paper.

2 A strong enhancement of the phosphatidylserine effect is induced by the incubation of liposomes with rat serum. Similar phosphatidylserine activation is observed after the incubation of the phospholipid with purified phospholipase A_2 from pancreas. In both cases phosphatidylserine is split into the deacylated derivative, lysophosphatidylserine.

3 Lysophosphatidylserine reproduces with greater efficacy the effect of phosphatidylserine on brain energy metabolism. Other lysophospholipids are not effective.

4 It is concluded that the pharmacological effects of phosphatidylserine liposomes is due to the generation of lysophosphatidylserine.

Introduction

As reported recently (Bruni, Leon & Boarato, 1976a; Bruni, Toffano, Leon & Boarato, 1976b; Bigon, Boarato, Bruni, Leon & Toffano, 1979), the administration of phosphatidylserine liposomes to mice is followed by a marked decrease of brain energy metabolism. This effect is associated with an increase of catecholamine turnover in the hypothalamus (Toffano, Leon, Mazzari, Savoini, Teolato & Orlando, 1978) and a stimulation of acetylcholine release from the brain cortex (Mantovani, Pepeu & Amaducci, 1976). The effect of phosphatidylserine is not reproduced by other natural phospholipids, including phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol and diphosphatidylglycerol.

Since it is unlikely that liposomes cross the blood brain barrier, it has been supposed that the effect at the cerebral level is secondary to a metabolic transformation of the liposomes. Such a possibility has also been indicated by previous findings showing that the incubation of phosphatidylserine liposomes with rat blood serum is followed by a strong increase in activity (Bigon *et al.*, 1979) and that a protein with phospholipase A activity has been isolated from rat blood serum (Paysant, Bitran, Etienne & Polonovski, 1969). Accordingly, it will be shown that lysophosphatidylserine is formed from phosphatidylserine liposomes and that this compound exhibits high pharmacological activity.

Methods

Preparation of serum fraction

Blood serum was collected from male albino rats and supplemented with solid potassium bromide to reach a density of 1.284. The preparation was centrifuged for 24 h at 45,000 rev/min (150,000 *g*) at 15°C and the upper layer of chylomicrons and lipoprotein was discarded. The residue was dialyzed and then fractionated at 0°C with solid ammonium sulphate. The fraction between 30 and 60% saturation was collected and dialyzed for 20 h at 4°C against 400 volumes of 50 mM Tris–HCl pH 7.9 with a change at 2 h. The preparation had a phospholipid content of 0.2 μmol/mg protein and was stable for several weeks at −25°C. Liposomes of phosphatidylserine were incubated with this preparation by two procedures. First, the dry phospholipid was swollen in 50 mM Tris–HCl pH 7.9 containing 50 mg/ml serum factor and then sonicated. Second, the liposomes were sonicated in Tris buffer and subsequently incubated 10 min at 37°C with 50 mg/ml serum fraction. Since identical results were obtained, the first procedure was routinely employed for its simplicity.

Animals

Male albino mice were used to test the effectiveness of the phospholipids on brain metabolism. Approxi-

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mately 0.15 ml of the dispersions of phosphatidylserine vesicles or lysophospholipids were injected in the tail vein and the animals killed 30 min later by immersion in liquid nitrogen. The brain was removed then pulverized together with frozen 0.5 ml 0.66 N perchloric acid. After weight determination, centrifugation, and neutralization, glucose and lactate were determined by enzymatic procedures. When desired, blood was collected after decapitation and the glucose content measured after denaturation with perchloric acid. A detailed description of the procedures and reagents has been given previously (Bigon *et al.*, 1979).

Analytical procedures

Phospholipid phosphorus was determined as described by Bartlett (1959) and protein according to Lowry, Rosebrough, Farr & Randall, (1951). After thin layer chromatography (Skipski & Barclay, 1969) the phosphorus content of phospholipid spots was determined without elution.

Drugs and reagents

Purified phospholipase A_2 from porcine pancreas (Sigma) was selected for its low toxicity in mice. Polyacrylamide gel electrophoresis under dissociating conditions (Weber & Osborn, 1969) yielded one main and two faint contaminant bands of high molecular weight. Phosphatidylserine was prepared from bovine brain as described by Lees (1957). The purity of the preparation was tested by thin layer chromatography. Liposomes were formed in 50 mM Tris–HCl pH 7.9 and dispersed by sonication at 0°C with a Branson sonifier (bursts of 2 min with cooling periods for a total time of 8 min). Lysophosphatidylserine in an amount sufficient for the pharmacological tests was prepared by dissolving 30 μmol phosphatidylserine in 20 ml moist diethylether; 1.0 ml of 10 mM Tricine(N-tris[hydroxymethyl]-methylglycine)–KOH pH 8.0 containing 0.1 mM $CaCl_2$ and 100 μg phospholipase A_2 was added and the mixture incubated 16 h at 20°C in the dark with continuous stirring. The gelatinous suspension was centrifuged and the upper layer discarded. The lower layer was extracted twice with 20 ml dry diethylether, dissolved with 20 vol. chloroform–methanol 2:1 (v/v), filtered through paper and taken to dryness. The dry residue was dispersed in 5 ml chloroform–methanol–water (13:7:1, by vol.). The slightly turbid suspension clarified after washing with 0.2 vol. of 0.4% $MgCl_2$ according to Folch, Lees & Sloane-Stanley (1957). Residual phosphatidylserine was eliminated by a silicic acid column (Silica gel 60, Merck) prepared and equilibrated with the chloroform–methanol–water solution. During the elution with the same solvent, phosphatidylserine appeared in the first fractions followed later by the lysoderivative. The fractions containing pure lysophosphatidylserine were pooled, taken to dryness, dissolved in 5 ml of the chloroform–methanol–water solution and washed with 0.2 vol. of 0.4% $MgCl_2$. The yield was 50 to 60% of the initial amount of phosphatidylserine. A sample of commercial lysophosphatidylserine (Supelco) was used as comparison. Lysophosphatidylcholine (Sigma) and lysophosphatidylethanolamine (General Biochemicals) each yielded a single spot in the chromatographic control. When needed the dry lysophospholipids were dispersed in 50 mM Tris–HCl pH 7.9 by Vortex mixing followed by 2 min sonication.

Results

The influence of phosphatidylserine on brain energy metabolism was conveniently assessed by the determination of the glucose accumulated in the brain tissues. As shown previously (Bigon *et al.*, 1979) this originated from the inhibition of glycolytic flux induced by the elevated level of the adenylate energy charge. From Table 1 it is seen that phosphatidylserine at 50 μmol/kg produced more than 100% increase of brain glucose content. At 20 μmol/kg the effect was considerably lower but could be increased to a maximum by previous incubation with rat serum or rat serum rendered free of lipoprotein by ultracentrifugation and further purified by ammonium sulphate fractionation. Since the phospholipid content of this latter preparation was negligible (0.2 μmol/mg protein), the possibility that the activation was due to a concerted effect of phosphatidylserine and blood lipids was excluded. The activation of phosphatidylserine liposomes by the sera of various animal species was not identical. Relative to the effect in mice, the serum from rat, rabbit, horse and sheep produced activation whereas sera from pig, ox and guinea-pig were inactive. The incubation of liposomes with mouse serum generally did not increase the activity. Significant activation was observed with some preparations which, however, lost their activity upon storage at 0°C or −25°C.

Table 2 shows that the activation of phosphatidylserine liposomes by the purified rat serum preparation was inhibited by EGTA indicating a calcium-dependent reaction. This suggested the operation of a phospholipase. A protein with phospholipase A activity has been reported to be present in rat serum (Paysant *et al.*, 1969). To test this possibility, phosphatidylserine was sonicated with the purified rat serum and then re-isolated by chloroform–methanol extraction. A comparison was made with a sample in which purified phospholipase A_2 from porcine pancreas substituted for the rat serum fraction. In both cases strongly activated lipid preparations were

obtained. Appropriate control tests excluded the possibility that the phospholipase preparation influenced the brain metabolism when used alone. Figure 1 presents conclusive evidence indicating that the activation of phosphatidylserine was associated with the generation of lysophosphatidylserine. Thin layer chromatography of the chloroform–methanol extract after incubation with rat serum revealed that 34% (range 23 to 47% in 5 determinations) of the added phosphatidylserine was split into more polar, ninhydrin positive compound. This compound migrated in the same way as lysophosphatidylserine obtained by digestion with purified phospholipase A_2. The mobility of lysophosphatidylethanolamine was distinct from that of lysophosphatidylserine.

In order to establish whether lysophosphatidylserine increased the effect of phosphatidylserine or was active itself, a sufficient amount of this compound was prepared by phospholipase A_2 digestion and purified by silicic acid column chromatography. As shown in Figure 2, lysophosphatidylserine used alone was about 8 times more active than was phosphatidylserine. Separate experiments with lysophosphatidylserine at 5 μmol/kg showed that the effect on brain glucose started 5 min after the injection and declined after 1 h. The peak effect was at 30 min. During this time

Table 1 The influence of blood serum from various animals on the effect of phosphatidylserine

Preparation injected	n	*Dose (μmol/kg)*	*Brain glucose (μmol/g wet wt.)*	*Difference*
None	16	—	1.96 ± 0.09	—
PS in Tris buffer	9	50	4.28 ± 0.23	+2.32
PS in Tris buffer	17	20	2.46 ± 0.10	+0.50
PS in rat serum	17	20	5.56 ± 0.19	+3.60*
PS in rat serum fraction	16	20	5.52 ± 0.20	+3.56*
PS in rabbit serum	13	20	6.09 ± 0.22	+4.13*
PS in horse serum	9	20	4.28 ± 0.35	+2.32*
PS in sheep serum	9	20	3.48 ± 0.28	+1.52*
PS in pig serum	9	20	2.22 ± 0.12	+0.26
PS in ox serum	9	20	2.24 ± 0.14	+0.28
PS in guinea-pig serum	5	20	2.91 ± 0.30	+0.95

Phosphatidylserine (PS) was dispersed by sonication in 50 mM Tris–HCl pH 7.9 with or without 50 mg/ml of blood serum prepared from the animals indicated. Rat serum fraction was obtained as described in Methods. The phospholipid dispersion was injected intravenously into mice. Separate controls showed the ineffectiveness of blood sera used alone. Mean ± standard error. n = the number of determinations. The significance of the difference between the preparations of phosphatidylserine in the absence or in the presence of blood sera was assessed by Student's t test: $*P < 0.01$.

Table 2 Similar activation by rat serum fraction and phospholipase A_2

Preparation injected	n	*Brain glucose (μmol/g wet wt.)*	*Difference*
None	8	2.12 ± 0.19	—
PS in Tris buffer	15	3.15 ± 0.18	+1.03
PS sonicated with rat serum fraction	12	5.87 ± 0.25	+3.75*
PS sonicated with rat serum fraction plus EGTA	5	3.38 ± 0.32	+1.26
PS sonicated with phospholipase A_2	5	6.28 ± 0.20	+4.16*
PS extracted after the sonication with serum fraction	5	6.68 ± 0.67	+4.56*
PS extracted after the sonication with phospholipase A_2	5	5.74 ± 0.39	+3.62*

Phosphatidylserine (PS) was dispersed and injected (20 μmol/kg) as described in Table 1. Where indicated 0.1 mM EGTA was present during the preparation of liposomes. After sonication in the presence of rat serum fraction (50 mg/ml) or phospholipase A_2 from pancreas (0.01 mg/ml) the phospholipid was extracted in chloroform–methanol 2:1 (v/v). The extract was filtered, taken to dryness and used for a new preparation of liposomes in Tris buffer. $*P < 0.01$ for the difference between the untreated phosphatidylserine and the phosphatidylserine treated with rat serum fraction or phospholipase A_2. n = number of determinations.

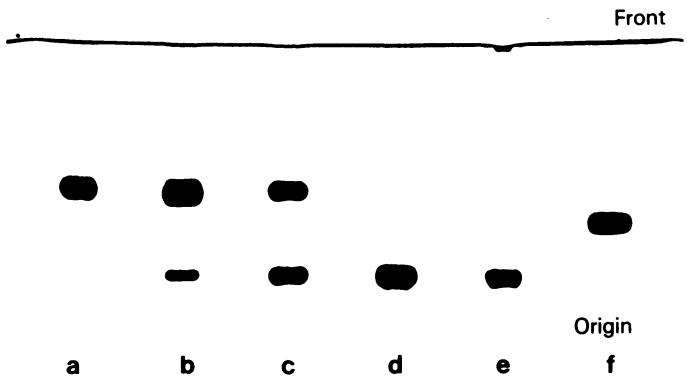

Figure 1 Hydrolysis of phosphatidylserine by rat serum fraction: 4 μmol phosphatidylserine was sonicated in 1 ml of 50 mM Tris–HCl buffer pH 7.9 with or without 50 mg rat serum fraction or 10 μg phospholipase A_2 from pancreas. After extraction with chloroform–methanol 2:1 (v/v) and washing with 0.2 vol. of 0.1% $MgCl_2$, an aliquot of the phospholipid extract (0.1 μmol) was separated by thin layer chromatography and identified with ninhydrin. (a) Phosphatidylserine sonicated alone; (b) phosphatidylserine sonicated with rat serum fraction; (c) phosphatidylserine sonicated with phosphclipase A_2; (d) lysophosphatidylserine prepared as described in Methods; (e) a commercial sample of lysophosphatidylserine; (f) lysophosphatidylethanolamine.

the mice were less active but no serious toxic effects were manifest. Figure 3 shows a decreased level of lactate in the brain, indicating no cerebral anoxia or hypoxia and confirming that the accumulation of glucose in the brain was concomitant with a reduced glycolytic flux. During the effect of lysophosphatidylserine an elevation of blood glucose content was observed. However, the brain to blood glucose ratio rose from a control value of 0.12 to 0.27 at 5 μmol/kg and 0.37 at 10 μmol/kg. This demonstrated that the effect at brain level was not secondary to the elevation of blood glucose. Similar observations were made with phosphatidylserine (Bigon *et al.*, 1979).

Since lysophospholipids are known to induce a specific damaging effect on cell membranes because of their detergent properties, a comparison was made with lysophosphatidylcholine and lysophosphatidylethanolamine which were expected to be similar to lysophosphatidylserine in this respect. As shown in

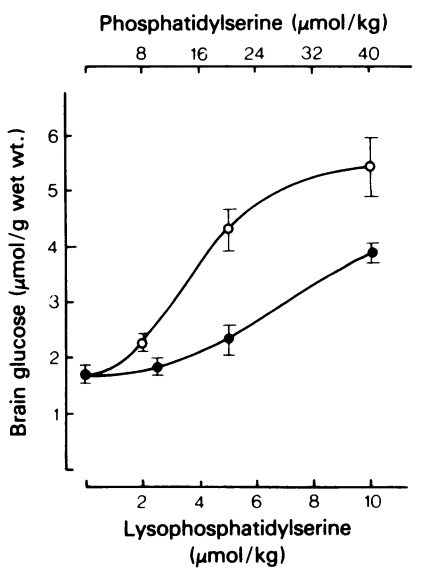

Figure 2 Activity of lysophosphatidylserine (○) and phosphatidylserine (●). The phospholipids were dispersed in 50 mM Tris–HCl pH 7.9 and injected into groups of 10 mice by the intravenous route at the doses indicated.

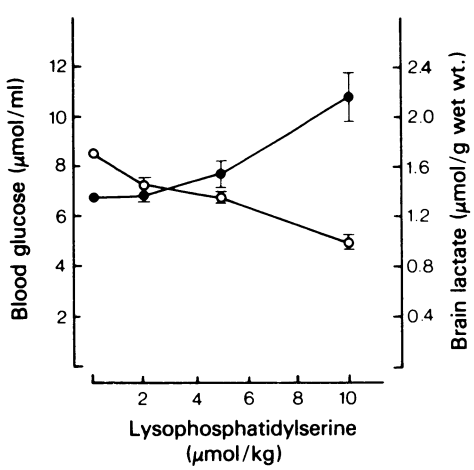

Figure 3 Effect of lysophosphatidylserine on brain lactate (○) and blood glucose (●). Experimental conditions as in Figure 2.

Table 3, the other lysophospholipids did not reproduce the effect of lysophosphatidylserine. Apparently the pharmacological activity is associated with specific properties of the phosphorylserine polar head.

Discussion

The findings described in this paper show that the generation of lysophosphatidylserine is an important step in the pharmacological effects of phosphatidylserine. This conclusion is supported by the phospholipase A_2-induced activation of phosphatidylserine and by the high activity of the purified lysoderivative. In the animal species possessing phospholipase A activity in the blood (e.g. rat) the deacylation of phosphatidylserine can readily be accomplished within the blood stream. In the others, the production of the minute amounts of lysophosphatidylserine needed for the pharmacological effects may follow the distribution of phosphatidylserine in the organs containing phospholipase A activity. In the liver where the injected liposomes predominantly accumulate (Tyrell, Heath, Colley & Ryman, 1976), phospholipase A_2 activity is present in mitochondria, lysosomes and plasma membrane. The association of liposomes with the lysosomal fraction after their assimilation has been documented and may be relevant in this respect (Tyrell *et al.*, 1976; Steger & Desnick, 1977). Indirect support for the possibility of phosphatidylserine activation in the organs is given by the low (approx. 4 min) plasma half-life of the negatively charged, sonicated liposomes injected intravenously in rats (Tyrell *et al.*, 1976; Steger & Desnick, 1977). We have confirmed this observation in mice as only 5 min after phosphatidylserine administration the phospholipid blood level is not detectable by the chromatographic method.

A further conclusion of this paper is that lysophosphatidylserine is a potent drug capable of decreasing the brain energy metabolism in mice. Since the energy requirement by the brain tissue is a consequence of neuronal activity, probably this activity is primarily affected. The possibility that lysophosphatidylserine acts by the aspecific detergent effect common to all lysophospholipids is in contrast with the ineffectiveness of lysophosphatidylcholine and lysophosphatidylethanolamine. Previous investigations (Rathbone, Magee & Thompson, 1962) have shown that lysophosphatidylserine has the same lytic effect as lysophosphatidylcholine on erythrocytes but it is less effective as a cytolytic agent on brain slices. Considering the low doses of lysophosphatidylserine needed to produce the pharmacological effects (2 μmol/kg), a maximal plasma concentration of 50 μM can be calculated. The concentration of free drug is expected to be much lower due to the binding of phospholipid to plasma protein and the distribution between tissue and blood. At this dilution, the lytic property of lysophospholipids is probably not very great whereas the possibility of high affinity binding to cellular membranes becomes more important. To this end the configuration of the phosphorylserine polar head may play a specific role.

Since mammalian tissues contain lysophospholipase activity it is of interest to establish whether the effect of lysophosphatidylserine is direct or whether it is the consequence of further metabolic conversion. A direct effect is indicated by the low dosage required for full effectiveness and by the rapid (5 min) onset of the action. The transfer across the blood–brain barrier is in this case ensured by the different mode of lysophospholipid aggregation in comparison to the diacylphospholipids (Helenius & Simons, 1975). Furthermore, investigations on the distribution of lysophosphatidylcholine and lysophosphatidylethanolamine after intravenous administration in rats, have shown that the plasma half-life of these compounds is 6 to 10 min and that degradation is not detectable in plasma (Stein & Stein, 1966). The lysophospholipids are taken up mainly by the liver, kidney, skeletal

Table 3 Specificity of lysophosphatidylserine

Phospholipid	*Dose* (*μmol/kg*)	*Brain glucose* (*μmol/g wet wt.*)	*Differences*
None	—	2.01 ± 0.08	—
Phosphatidylserine	20	2.86 ± 0.27	+0.85
Lysophosphatidylcholine	20	2.41 ± 0.09	+0.40
Lysophosphatidylethanolamine	20	2.64 ± 0.25	+0.63
Lysophosphatidylserine (prepared)	10	5.44 ± 0.56	+3.43*
Lysophosphatidylserine (commercial)	10	5.08 ± 0.75	+3.07*

The lysophospholipids were dispersed in 50 mM Tris–Hcl buffer pH 7.9 by vortex mixing followed by 2 min sonication and injected intravenously into groups of 5 mice. *$P < 0.01$. Lysophosphatidylserine was prepared as described in Methods. A commercial sample was tested for comparison.

muscles and small intestine. In these organs as well as in the brain (Illingworth & Portman, 1972) the major metabolic step for the injected lysophospholipids is found to be the direct acylation to the respective diacylphospholipids. Depending upon the organ and the phospholipid, the acylation is completed in 30 to 60 min and may constitute an efficient system for terminating the lysophosphatidylserine effect.

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