Post-heparin phospholipase and fatty acid transesterification in human plasma

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SUMMARY The incubation of post-heparin plasma and egg phosphatidyl ethanolamine in the presence of methanol, ethanol, or glycerol resulted in the synthesis of fatty acid esters of these alcohols. This synthesis requires fatty acids derived from the degradation of phosphatidyl ethanolamine. The fatty acid transesterification activity and the post-heparin phospholipase activity were found to involve the fatty acid at the 1-position of egg phosphatidyl ethanolamine.

KEY WORDS post-heparin plasma · man · phospholipase · transesterification · phosphatidyl ethanolamine · α-fatty acid transferase · monoglyceride formation

In a previous study (1) a human post-heparin plasma phospholipase was described which readily degrades egg phosphatidyl ethanolamine (PE) to lysophosphatidyl ethanolamine (LyPE) and fatty acids (FA). In the present study the site of action of this enzyme on the PE molecule has been explored in conjunction with the finding of a fatty acid transesterification activity in post-heparin plasma. Fatty acid esters of methanol, ethanol, or glycerol were formed when these alcohols were appropriately added to incubation mixtures of PE and post-heparin plasma. The fatty acids of this transesterification reaction were derived from the degradation of PE to LyPE. Our evidence shows that, in contrast to the site of action of venom or human pancreatic phospholipase A, the post-heparin plasma phospholipase and transesterification activity involve the fatty acid at the 1-position (α-position) of egg PE.

METHODS AND MATERIALS

Methods for the preparation of egg PE, for the quantification of post-heparin phospholipase activity, and for thin-layer chromatography (TLC) were described previously (1, 2). Lipid components were located on chromatoplates by spraying them with 0.05% (w/v) 2′,7′-dichlorofluorescein in ethanol.

Preparation of Post-Heparin Plasma

Five minutes after the intravenous administration of 50 mg of heparin to a donor, approximately 450 ml of blood was collected in a plastic bag without added anticoagulant and the plasma was separated and stored as previously described (2). The phospholipase of this plasma degraded PE in the incubation system previously described (1) at the rate of 30–35 μmoles /hr per ml of plasma.

Incubation Systems

All incubations with post-heparin plasma and PE were carried out at 38° in a total volume of 9.0 ml in systems similar to the one originally described (1). Each of three different incubation systems contained the same amount of PE: 1612 μg of PE phosphorus (52 μmoles of phospholipid).

In incubation system A the PE was emulsified in 5.0 ml of a solution, adjusted to pH 9.2 with NaOH, containing 400 mg of albumin (bovine Fraction V, Armour) and 0.45 ml of 1 M ammonium sulfate. The source of enzyme was 4.0 ml of a 1:1 mixture of post-heparin plasma and water.

Incubation system B was identical with A except that the 4.0 ml of enzyme source was taken from an alcohol mixture made in the following way: 2.4 ml of post-heparin plasma and 2.4 ml of water were mixed in a test tube and, while the mixture was agitated by a tube mixer, 1.0 ml of either methanol or ethanol was slowly added.

Incubation system C contained the phospholipid emulsified in 2.5 ml of the albumin–ammonium sulfate

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solution. Glycerol (2.5 ml), water (3.0 ml), and post-heparin plasma (1.0 ml) were added in that order.

Products obtained for methylation were: Monoglyceride (MG) and LyPE formed in incubation systems C and A respectively; LyPE formed using snake venom (3 mg/4 ml water) as the enzyme in aqueous system A; and LyPE and FA formed using venom phospholipase A action on PE in diethyl ether (3). In the latter system a volume of ethanol equal to the volume of ether was added prior to TLC, which showed about 5% unchanged PE. (The *Crotalus adamanteus* venom was obtained from Ross Allen's Reptile Institute, Inc., Silver Springs, Fla.)

Preparation and Thin-Layer Chromatography (TLC) of Lipid Extracts

Incubation systems A and B, 0.9 ml each, were extracted with 15.0 ml of ethanol–diethyl ether 3:1 v/v. The ethanol–ether extract was evaporated and the lipids were redissolved in 0.9 ml of chloroform–methanol 1:2. A 20 μl aliquot of this extract was applied to the chromatoplate.

The lipids of incubation system C were obtained by first heating 0.9 ml of the incubation mixture with 1.5 ml of methanol briefly to boiling, cooling, and adding 3.0 ml of chloroform and then 5.0 ml of water. After centrifugation, 100 μl of the lower phase was applied to the chromatoplate. A 25 μl volume of methanol added to the applied spot spread the lipid more uniformly.

Monoglyceride was separated by developing the plates in chloroform–methanol-water 80:15:1 for 4 min and then with petroleum ether–ethyl ether–acetic acid 90:10:1 for 6 min. FA were separated by developing the plates successively in these two solvents for intervals of 2 and 6 min respectively. The LyPE was obtained from plates developed for 20 min with chloroform–methanol–water 80:15:1. With incubation system A this development separated the endogenous plasma lecithin from the LyPE, but the plasma sphingomyelin was within the LyPE area.

The desired lipid areas were located by spraying portions along each side of the plate. The unsprayed area was removed from the plate and the lipid was ex-

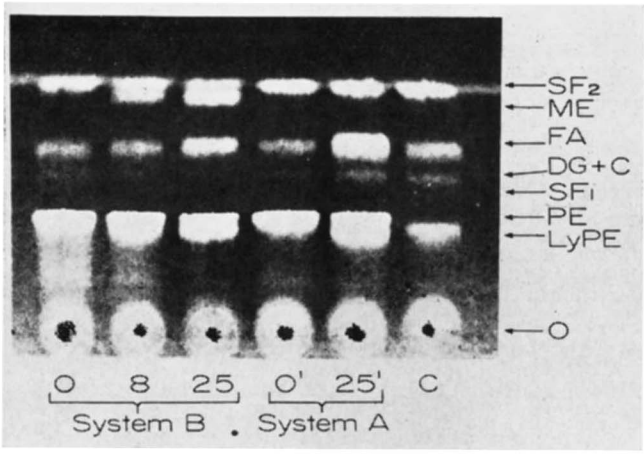

FIG. 1. Thin-layer chromatography of the reaction products of the incubation of PE and human post-heparin plasma with or without added methanol.

Post-heparin plasma was incubated with PE in incubation system A (without methanol) and aliquots of lipid extracts of the incubation mixture initially and after 25 min were applied at origin areas 0′ and 25′ respectively. Methanol-treated post-heparin plasma was incubated with PE in system B and aliquots of lipid extracts initially and after 8 and 25 min were applied at origin areas 0, 8, and 25 respectively. An aliquot of a lipid extract of system A without added PE was applied at origin area C. The chromatoplate was developed for 2 min with chloroform–methanol–water 80:35:2 and then with petroleum ether–ethyl ether–acetic acid 80:15:1 for 3 min.

Abbreviations: O, origin; LyPE, lysophosphatidyl ethanolamine; PE, phosphatidyl ethanolamine; SF_1, first solvent front; DG + C, isomeric diglycerides and free cholesterol; FA, fatty acids; ME, methyl esters of fatty acids; SF_2, second solvent front containing esterified cholesterol. Triglycerides, when detectable, appear just below ME.

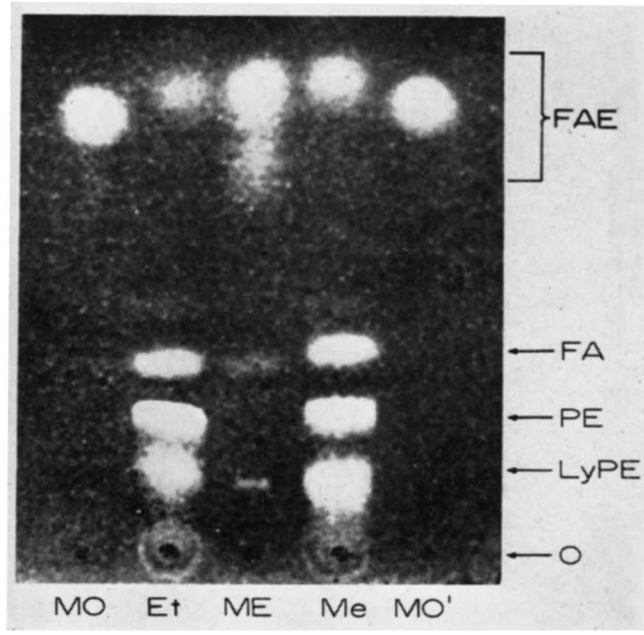

FIG. 2. Chromatogram comparing mobilities of fatty acid esters formed.

An aliquot of methyl oleate was applied at origin areas MO and MO′. An aliquot of a solution of methyl esters obtained from egg PE (4) was applied at origin area ME. Aliquots of lipid extracts obtained from 30-min incubations of PE with ethanol- or methanol-treated post-heparin plasma were applied at origin areas Et and Me respectively. The chromatoplate was developed with chloroform–methanol–water 80:20:1 for 3 min and then with petroleum ether for 60 min.

Abbreviations: As in Fig. 1, except FA, mostly fatty acids, but also contains free plasma cholesterol and diglyceride and marks the height of the first solvent front; FAE, fatty acid esters of methanol or ethanol. The original chromatogram showed faint areas of plasma triglyceride just above the FA component of Et and Me. The second solvent front and plasma esterified cholesterol are not shown.

tracted from the silicic acid with two batchwise elutions with chloroform–methanol 1:1. The silicic acid was removed from the solvent by centrifugation and the chloroform–methanol was evaporated off at 40° under reduced pressure.

Preparation of Fatty Acid Methyl Esters for Gas–Liquid Chromatography (GLC)

The methyl esters formed in incubation system B with methanol-treated plasma were separated as in Fig. 2, recovered from chromatoplates as described above, and dissolved in hexane for GLC.

Lipids were methanolyzed by heating them for 15 hr in sealed tubes at 80° in 3.0 ml of either a 1:50 or a 1:20 dilution of concd HCl in methanol–benzene 9:1 (personal communication, Dr. E. C. Horning). Egg PE and LyPE were methanolyzed in the stronger acid solution. The methyl esters were recovered by adding 3.0 ml of water and 2.0 ml of hexane to each tube, mixing, centrifuging, and removing the hexane layer. The hexane was reduced in volume to 0.5 ml containing 4–12 μmoles of the methyl esters. The solutions derived from the methylation of PE and LyPE showed trace amounts of a component with the R_F of FA when subjected to TLC.

GLC was carried out on a 20% diethylene glycol succinate column at 160°. The percentage of each methyl ester in a sample was calculated from area measurements obtained by triangulation. The analysis of National Institutes of Health standards for GLC showed reliable linearity of response to the detector. There was no evidence of contaminating material in the silicic acid (Mallinckrodt) of the chromatoplates, as judged by blank runs.

RESULTS

Formation of Fatty Acid Esters of Methanol and Ethanol in Incubations of Post-Heparin Plasma

Figure 1 shows the formation of FA and LyPE in incubation system A. In system B, in which methanol had been added, methyl esters (ME) were found in addition to FA. An aliquot of system A without added PE was applied at position *C* and demonstrates the endogenous plasma lipid components plus FA of the Armour albumin.

The mobilities of the FA esters formed in incubation B are compared with those of reference compounds in the chromatogram of Fig. 2. The mobilities of these fatty acid esters (opposite FAE) are similar to those of pure methyl oleate applied at MO, and of the least polar components of total methyl esters of egg PE (4) applied at ME. The rather discrete areas of the esters above "Et" and "Me" compared to those above "ME" indicate a rather selective transfer of FA from PE to the alcohol.

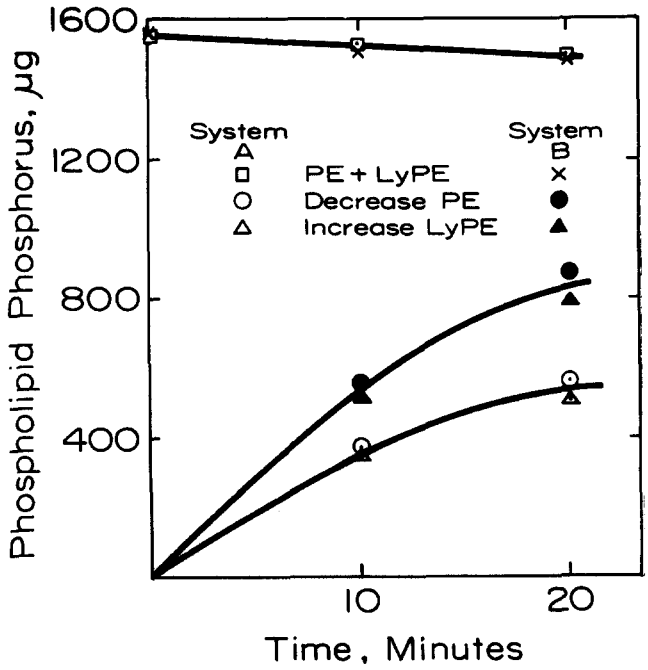

FIG. 3. The effect of methanol on the degradation of PE by human post-heparin plasma.

PE was incubated with post-heparin plasma in incubation system A (no methanol) and with methanol-treated plasma in incubation system B. The conversion of PE to LyPE during a 20 min incubation of each system was plotted. The total amounts of phospholipid phosphorus present in each system initially and at each incubation interval are also shown. The method for quantification of enzymatic activity was described previously (1, 2).

Figure 3 shows an apparent activation of post-heparin phospholipase activity in the presence of methanol. The greater decreases of PE and increases of LyPE in system B compared to the magnitudes of these changes in system A occurred even though less plasma was present in system B because of the dilution with methanol. The total amount of lipid phosphorus decreased slightly in each system during the incubation. In both systems decreases of PE were nearly balanced by the LyPE formed, which indicates that the FA of the methyl esters formed in incubation B are not derived from the LyPE, for glycerophosphoryl ethanolamine resulting from such a degradation would not be recovered in the LyPE area of the chromatograms and the decrease of PE would not be balanced by the recovery of an equal amount of LyPE.

Monoglyceride Formation in Incubations Containing Post-Heparin Plasma, PE, and Glycerol

The formation of monoglyceride (MG) during the degradation of PE in incubation system C containing glycerol is shown in Fig. 4. Increasing amounts of MG were

formed as the PE was degraded to LyPE and FA. The basis for identifying the new component as MG is as follows: the new component was not observed in the absence of added glycerol; there was no phosphorus in this component recovered from chromatoplates; and the component had the same R_F as MG produced in digests of triglyceride by lipase. MG have been observed to be formed in incubation mixtures containing 12% and 50% (v/v) glycerol as well as at the 27% concentration shown here.

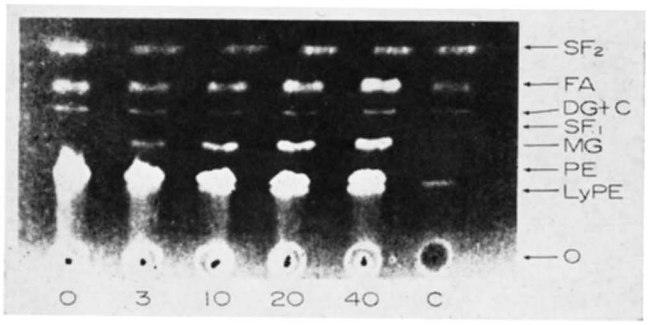

FIG. 4. Monoglyceride synthesis in an incubation system containing PE, human post-heparin plasma, and glycerol.

Aliquots of lipid extracts of incubation system C (containing 27% glycerol) initially and after intervals of 3, 10, 20, and 40 min were applied at origin areas 0, 3, 10, 20, and 40 respectively. An aliquot of a lipid extract of system C without added PE was applied at origin area C. The chromatoplate was developed for 2.5 min with chloroform–methanol–water 80:35:2 and then with petroleum ether–ethyl ether–acetic acid 80:15:1 for 3.5 min.

Abbreviations: As in Fig. 1, plus MG, monoglyceride.

Additional Studies of the Post-Heparin Transferase Activity

When palmitic, stearic, or linoleic acid (13, 26, or 52 μmoles) was added instead of PE to systems B and C there was no formation of methyl or ethyl esters of these acids. Thus, the esterification reaction apparently involved a direct transesterification rather than esterification of FA removed from the PE. No transesterification was observed with egg lecithin as substrate in incubation systems B and C. Our attempts to extend the observed reactions to include esterification of cholesterol, lysolecithin, glycerophosphoryl choline, or glycerophosphate were not successful. The transesterification activity reported has been observed in each of four other post-heparin plasmas which also contained phospholipase activity. Neither activity was observed with five normal (pre-heparin) human plasmas (two collected with citrate and three collected with heparin as the anticoagulants).

Heating the plasma at 55° or prior incubation of the plasma with diethyl-*p*-nitrophenyl phosphate (paraoxon) completely inhibited the transesterification activity as well as the phospholipase activity (1). Although the optimal conditions for the transesterification activity are similar to those for phospholipase activity (1), i.e., in the presence of albumin and ammonium sulfate at pH 9.2, both activities were observed in phosphate buffer at pH 6.5 (without albumin or ammonium sulfate), but to a much lesser degree.

The FA esters formed in incubation systems B and C were recovered from chromatoplates. Each ester was used instead of PE as substrate and incubated in system A. Each ester decreased in amount and FA appeared. Thus, these esters also are acted on by enzymes in post-heparin plasma.

Molecular Site of Action of Post-Heparin Plasma Phospholipase and Transesterification Activity

The fatty acid compositions of products formed in the degradation of egg PE by snake venom phospholipase A and by post-heparin plasma were analyzed by GLC. The distribution of fatty acids in these lipids is shown in Table 1.

In incubation system B (experiment 6) large percentages of palmitic and stearic acid were found in the methyl esters. Hawke (5) calculated (experiment 1) that palmitic acid was esterified entirely at the α- or 1-position of egg PE. If our egg PE also contained all or nearly all of its palmitic acid at the 1-position, the transesterification activity involved the FA at the 1-position. Then, assuming that the plasma phospholipase activity involved the 2-position, a demonstration of two post-heparin activities was considered possible. Van Deenen and coworkers (6) have found that the site of action of human pancreatic phospholipase A is the same as the established site of action of snake venom phospholipase A, namely the 2-position of phospholipids.

Comparison of the FA composition of our egg PE (experiment 2) with that of the products of venom phospholipase A action (experiments 3 and 4), clearly shows that nearly all of the palmitic and stearic acids were esterified at the 1-position of our PE. There is a close similarity in the composition of FA in LyPE formed by venom phospholipase action (experiment 4) and the FA in LyPE formed in the aqueous system A (experiment 5). The FA of MG in experiment 7 are closely similar in composition to those obtained in experiments 4, 5, and 6. It follows that the FA of the methyl esters and of the MG formed in incubation systems B and C were derived from the 1-position of PE.

In experiment 8 the FA of the LyPE resulting from the action of plasma phospholipase on egg PE were closely similar in composition to the FA removed from the 2-position of egg PE by venom phospholipase A (experiment 3) in the production of LyPE (experiment 4).

TABLE 1. FATTY ACID COMPOSITION OF PRODUCTS OF ACTION OF SNAKE VENOM PHOSPHOLIPASE A AND OF POST-HEPARIN PLASMA ON EGG PE

Expt.	Enzyme	Incubation System (Time)	Study of Product	Fatty Acid Distribution 16:0	18:0	18:1	18:2	20:4
(1)			Hawke (5) egg PE					
			FA distribution α-	40	45	13		
			β-		34	(----------66*----------)		
(2)			Total FA of PE	20	38	17	11	14
(3)	Venom	Ether	FA released	2	2	39	28	29
(4)		Ether	FA of LyPE	38	58	4		
(5)		A (80 min)†	FA of LyPE	31	60	9		
(6)	Post-heparin plasma	B (30 min)†	FA of methyl esters	34	60	6		
(7)		C (40 min)†	FA of monoglyceride	38	57	5		
(8)		A (40 min)†	FA of LyPE	11	8	28	27	26

* Hawke (5) reported a distribution of 28, 18, and 20% of C_{18}, C_{20}, and C_{22} unsaturated fatty acids respectively at the β-position of egg PE. These fatty acids were collectively characterized as to content of unsaturated bonds. Myristic (<1%) and palmitoleic acid (<2%) were assigned to the α-position.

† Incubations in which 35–50% of the PE substrate was hydrolyzed.

Thus, it appears that the site of action of both plasma activities was the 1-position of egg PE.

Some discrepancies in the percentages of palmitic, stearic, and oleic acid in experiment 8 compared with those in experiment 3 may arise from the inclusion of endogenous plasma sphingomyelin in this LyPE area. Sweeley (7) has reported a plasma sphingomyelin FA distribution of palmitic, stearic, and oleic acid of 42, 9, and 1% respectively. However, it is possible that the discrepancy may also arise in part through transesterification reactions involving the LyPE and the other endogenous plasma lipids in a manner described by Marinetti (8). Such an exchange, however, would require a preferential exchange of oleic acid of LyPE for palmitic and stearic acid of the endogenous lipids.

DISCUSSION

The results indicate the presence of a fatty acid transferase activity in post-heparin plasma which utilizes the FA detached from PE during the formation of LyPE. The optimal conditions for this activity are similar to those for the phospholipase previously described (1). Similarly, those treatments of the plasma which abolish its phospholipase activity (heating at 55° or prior incubation with paraoxon) also abolish the transesterification reaction. A study of some of the products of the two activities by GLC shows that both involve FA at the 1-position of egg PE. This alpha site of action on a phospholipid is in contrast to the 2-position specificity of both snake venom phospholipase A (9) and human pancreatic phospholipase A (6). It has been predicted that a phospholipase showing a specificity for FA at the 1- rather than the 2-position of phospholipids might be found (6, 10).

Positive evidence for two separate enzymes in post-heparin plasma was not obtained. Since in all incubations in which transesterification of FA occurred an appreciable amount of unesterified FA (bound to protein) was also found, a number of mechanisms are possible: (*a*) concurrent action of a separate phospholipase and transferase; (*b*) transferase activity only with some FA as they are released by the phospholipase and not with others, which become bound to protein; (*c*) inhibition of the phospholipase by the alcohols, the transferase producing FA esters (substrates for post-heparin plasma enzymes) which are in part degraded; (*d*) transferase activity only in a medium containing the LyPE that result from phospholipase activity; (*e*) reversible esterolytic activity which utilizes FA released from PE by the phospholipase; (*f*) susceptibility of the FA, as they are released by the phospholipase and before they bind to protein, to ester formation by a nonenzymatic reaction.

The investigations of others support the existence of a FA transferase or have a bearing on the mechanisms considered above. Marinetti (8) has proposed enzymatic transesterification of FA between phospholipids and glycerides. The evidence was obtained from exchanges of FA occurring in plasma lipids after the addition of snake venom or Pancreatin. Labeled FA bound to protein were not involved in the reactions. Glomset (11) in studies of the mechanism of the plasma cholesterol esterification reaction has presented evidence for a FA transferase, the chief source of FA for the esterification of cholesterol being the FA at the 2-position of plasma lecithin. His evidence indicated that protein-bound FA did not become esterified with cholesterol. Borgström

(12), however, found labeled protein-bound oleic acid to be readily exchangeable with FA of chylomicron glycerides in an incubation system containing chylomicra, purified human clearing factor, and albumin.

Although our results may be taken to indicate transferase activity in the present study, the net synthesis of MG observed may have arisen from the heparin-released lipase also present in the medium. Additional pertinent findings include the report of Margolis and Vaughan (13) that various alcohols added to incubations containing adipose tissue microsomes were esterified with palmitate, and the work of McBride and Korn (14) who found an esterification of ethanol with palmitate in incubations containing homogenates of the lactating gland of guinea pigs. The reaction was enzymatic and required homogenate, ATP, and CoA.

There are two reports suggesting in vivo FA transesterification. Goodman and Deykin (15) have found that ethanol-C^{14} administered intravenously to rats was recovered from the total body lipids as long-chain FA ethyl esters. Dhopeshwarkar and Mead (16) observed methyl esters of FA as a normal component of body lipids of guinea pigs. These methyl esters contained 28, 58, and 7% of 16:0, 18:0, and 18:1 respectively. The proportions of these fatty acids in the esters were quite different from those in other body lipids and are indeed quite similar to the percentage distribution of these FA in experiment 6 of Table 1 with egg PE as substrate.

Further studies are obviously required to establish whether the post-heparin plasma phospholipase and FA transesterification activities demonstrated in vitro are important in vivo. Such studies may reveal additional activities in post-heparin plasma which involve glycerolipids.

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