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DOES DE NOVO SYNTHESIS OF LYSOPHOSPHATIDYLCHOLINE OCCUR IN RAT LUNG MICROSOMES?

A.J. AARSMAN and H. VAN DEN BOSCH

Laboratory of Biochemistry, Padualaan 8, 3584 CH Utrecht (The Netherlands)

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Summary

Incubation of rat lung microsomes with CDP[*Me*-^{14}C]choline resulted in formation of radioactive lysophosphatidylcholine and phosphatidylcholine. Evidence is provided which suggests that lysophosphatidylcholine formation cannot be ascribed completely to phospholipase A degradation of phosphatidylcholine. Lysophosphatidylcholine production can be stimulated by addition of monoacylglycerol or diacylglycerol. It is suggested that diacylglycerol is partly hydrolyzed to monoacylglycerol and subsequently converted to lysophosphatidylcholine. A direct transfer of phosphocholine from CDPcholine to monoacylglycerol is demonstrated by equimolar incorporation of 1(3)-[9,10-$^{3}H_2$]palmitoylglycerol and phospho[*Me*-^{14}C]choline into lysophosphatidylcholine.

Introduction

Lung function depends upon the presence in the alveolar lumen of a unique lipid-protein complex known as pulmonary surfactant. The major surface active component in this surfactant is 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine [1,2], the synthesis of which has attracted much attention (see Refs. 3—5 for reviews). It is generally believed that de novo synthesis of phosphatidylcholine in lung produces monoenoic- and dienoic species with primarily palmitate at the *sn*-1-position [6,7]. Two pathways are thought to contribute to the remodelling of these species into fully saturated phosphatidylcholine, i.e. acyl-CoA dependent acylation of lysophosphatidylcholine [8] and transacylation between two molecules of lysophosphatidylcholine [9,10]. Both pathways require lysophosphatidylcholine as substrate. This compound can be formed by the action of phospholipases A_2 reported to be present in lung [11—13] or can

be taken up from the circulation [9]. Phospholipase action on diacylglyerophospholipids has long been thought to represent the only pathway for lysoglycerophospholipid formation. A novel pathway involving the direct transfer of phosphocholine from CDPcholine to monoacylglycerol was recently proposed to occur in microsomes of chicken embryo fibroblasts [14]. The alternative possibility, that the lysophosphatidylcholine was formed by the action of phospholipases A was discounted by these investigators, but no experimental data were shown to support this conclusion. In addition, no data were provided to show increased lysophosphatidylcholine synthesis upon addition of monoacylglycerol or a direct conversion of labelled monoacylglycerol into lysophosphatidylcholine. In the present paper we provide evidence which indicates that lung microsomes can indeed synthesize lysophosphatidylcholine from monoacylglycerol and CDPcholine.

Materials and Methods

Materials

Cytidine 5'-diphospho[*Me*-^{14}C]choline (spec. radioact. 60.9 Ci/mol) was obtained from Radiochemical Centre, Amersham, U.K. Unlabelled CDPcholine was purchased from Sigma, St. Louis, MO, U.S.A. Palmitic acid was obtained from J.T. Baker Chemicals N.V., Deventer, Holland, whereas [9,10-$^{3}H_2$]palmitic acid was bought from NEN Chemicals, Dreieich, F.R.G. Diisopropylfluorophosphate was a product from Aldrich Europe, Beerse, Belgium.

Synthesis of substrates

Palmitoylchloride was prepared according to Daubert et al. [15]. 1(3)-Monopalmitoylglycerol was synthesized by acylation of excess glycerol with palmitoylchloride in dry tetrahydrofuran and pyridine. The monoacylglycerol was purified on silica G thin-layer plates which were developed in diethyl ether/hexane (75 : 25, v/v). The radioactive monopalmitoylglycerol had a specific radioactivity of 14 400 dpm/nmol.

1,2-Diacyl-*sn*-glycerol was prepared from egg phosphatidylcholine with phospholipase C from *Clostridium welchii* (enzyme kindly donated by Dr. B. Roelofsen). The ^{14}C-labelled diacylglycerol was prepared in the same way from 1-acyl-2-[1-^{14}C]oleoyl-*sn*-glycero-3-phosphocholine (spec. radioact. 3500 dpm/nmol), which was prepared as described by Waite and van Deenen [16].

Isolation of microsomes

Lungs from two rats were homogenized with a Potter-Elvehjem homogenizer in 0.25 M sucrose/10 mM Tris/1 mM EDTA (pH 7.4). Microsomes were isolated according to established procedures. The microsomal fraction was resuspended in the same buffer. Microsomes treated with diisopropylfluorophosphate (3 μmol/mg protein) for 30 min at 30° C are designated DP-microsomes. Protein was determined as described by Lowry et al. [17].

Incubation and product analysis

Incubation conditions are described in the legends to the respective figures and tables. Optimal diacylglycerol (8 mM) and CDPcholine (0.26 mM) con-

centrations for cholinephosphotransferase assays were taken from reported studies on rat lung microsomes [23]. The initial experiments described in table I used sub-optimal amounts of CDPcholine. The latter was used at the highest specific radioactivity available to establish that formation of radioactive lysophosphatidylcholine took place. Reactions were stopped by addition of 3 vols. of $CHCl_3/CH_3OH$ (1 : 2, v/v), to initiate extraction of the lipids according to Bligh and Dyer [18].

Neutral lipids were separated on silica G plates by development with light petroleum ether/diethyl ether/formic acid (60 : 40 : 1.5, v/v). Phospholipids were fractionated on silica H thin-layer plates which were run in chloroform/methanol/acetic acid/water (100 : 50 : 16 : 8, v/v).

Radioactive spots in single-labelled experiments were scraped immediately into scintillation vials containing toluene scintillation fluid/Triton X-100/water (2 : 1 : 0.2, v/v). In double-labelled experiments the radioactive compounds were eluted from the silica gel with $CHCl_3/CH_3OH$ (20 : 80, v/v). The organic solvent was evaporated, the residue dissolved in a small volume of CH_3OH and transferred into scintillation vials containing toluene scintillation fluid (5 g PPO and 0.25 g dimethyl POPOP per liter). Radioactivity measurements were carried out in a Packard Tricarb model 3320 liquid scintillation spectrometer.

Results and Discussion

Table I shows that lung microsomes, when incubated with CDP[*Me*-^{14}C]choline, produce both phosphatidylcholine and lysophosphatidylcholine. The formation of these products is not inhibited by pretreatment of the microsomes with diisopropylfluorophosphate. In contrast, addition of 10 mM Ca^{2+} completely abolishes formation of radioactive phospholipids. For phosphatidylcholine synthesis this result is in agreement with findings of Kennedy and Weiss obtained with rat liver microsomes [19]. Addition of monoacylglycerol results in an approx. 5-fold increase in lysophosphatidylcholine production and at the same time seems to inhibit phosphatidylcholine synthesis somewhat. As expected a large increase in phosphatidylcholine synthesis is observed upon addition of 1,2-diacylglycerol. However, an increased synthesis of lysophosphatidylcholine is also observed under these conditions. Several possibilities can be raised to explain these findings. First, the 1,2-diacylglycerol either contains sufficient monoacylglycerol to stimulate lysophosphatidylcholine formation or is partly converted by microsomal lipase(s) into monoacylglycerol during the course of the incubation. Secondly, increased formation of phosphatidylcholine could give rise to enhanced lysophosphatidylcholine production through the action of microsomal phospholipase(s) A. The following experiments were disigned to differentiate between these possibilities.

Phospholipase A_2 activity has been described in rat lung microsomes [11,13]. Although the enzyme was reported to have an absolute requirement for Ca^{2+} when assayed with exogenous phosphatidylethanolamine [11] and Ca^{2+} was not present in the incubations in which we found lysophosphatidylcholine formation, it was felt necessary to investigate whether lysophosphatidylcholine production could be ascribed to endogenous microsomal phospholipase activity. Microsomes, pretreated with diisopropylfluorophosphate were labelled by

TABLE I

SYNTHESIS OF ACYLGLYCEROPHOSPHOCHOLINES IN RAT LUNG MICROSOMES

The incubation mixture contained 15 mM Tris-HCl buffer (pH 7.4), 20 mM $MgCl_2$, 1 mM EDTA, 17 μM CDP[*Me*-^{14}C]choline (spec. act. 125 000 dpm/nmol) and 250 μg microsomal protein. Additions were present in the following final concentrations: Ca^{2+}, 10 mM; monoacylglycerol (MG), 1 mM or diacylglycerol (DG), 8 mM in 0.01% (w/v) Tween 20. Incubation was done for 30 min at 37°C in a total volume of 0.5 ml. Data from one experiment, representative of three experiments showing similar results, are given. Abbreviations: n.d., not detectable; DP-microsomes, microsomes treated with diisopropylfluorophosphate.

Enzyme	Addition	Lysophosphatidylcholine (pmol)	Phosphatidylcholine (pmol)
Microsomes	none	12	304
	Ca^{2+}	n.d.	n.d.
	MG	80	220
	DG	80	2440
DP-microsomes	none	22	312
	MG	100	246
	DG	118	2340

incubation with CDP[*Me*-^{14}C]choline in the presence or absence of added 1,2-diacylglycerol and reisolated by centrifugation. The prelabelled microsomes were resuspended in the same media in which the labelling was carried out, except that non-radioactive CDPcholine was used. Further incubation was done either without addition, with 20 mM Ca^{2+} or with 50 mM EDTA. As can be seen in Table II lysophosphatidylcholine contained 4.7—4.9% of the ^{14}C-radioactivity in the lipid extract of microsomes incubated with 1,2-diacylglycerol. In microsomes prelabelled in the absence of added 1,2-diacylglycerol this value amounted to 6.2—6.6%. These values increase somewhat during further incubation, especially when Ca^{2+} is present. This suggests that phospholipases may indeed contribute to lysophosphatidylcholine formation. However, increases

TABLE II

INCUBATION OF PRELABELLED MICROSOMES

Microsomes, pretreated with diisopropylfluorophosphate, were incubated with CDP[*Me*-^{14}C]choline in the presence or absence of 1,2-diacylglycerol as described in Table I. The microsomes were isolated by centrifugation (30 min at 150 000 × *g*) and resuspended in the same media in which the labelling was done, except that CDP[*Me*-^{14}C]choline was replaced by CDPcholine. The prelabelled microsomes were incubated for the indicated time periods at 37°C either with no addition, with 20 mM Ca^{2+} or with 50 mM EDTA.

Incubation time (min)	Percentage ^{14}C label in lysophosphatidylcholine					
	without 1,2-diacylglycerol			with 1,2-diacylglycerol		
	no addition	Ca^{2+}	EDTA	no addition	Ca^{2+}	EDTA
0	6.6	6.6	6.2	4.9	4.7	4.7
30	6.6	7.4	6.8	5.9	6.6	5.4
60	6.4	7.7	6.8	6.5	7.6	5.9
100	6.6	8.6	7.2	6.9	8.8	6.8

observed in the first 30 min of the incubations without additions, i.e. exactly the same conditions as during the prelabelling, are much lower than the lysophosphatidylcholine labelling attained during the prelabelling. This suggests that formation of lysophosphatidylcholine during the prelabelling experiment cannot be explained completely by hydrolysis of phosphatidylcholine by phospholipases.

We then investigated whether the increased formation of lysophosphatidylcholine, observed upon addition of 1,2-diacylglycerol (Table I), could be ascribed to incorporation of phosphocholine into monoacylglycerol produced by microsomal lipases from the added 1,2-diacylglycerol. Lipases are known to be present in lung tissue [20—22]. When microsomes were incubated with 1-acyl-2[1-^{14}C]oleoylglycerol a rapid decrease in diacylglycerol was observed (Fig. 1). The label lost from diacylglycerol was quantitatively recovered in free fatty acid and monoacylglycerol, presumably as a result of the action of a diacylglycerol lipase and a monoacylglycerol lipase. The latter enzyme appears to be more sensitive to inhibition by diisopropyfluorophosphate. The limited hydrolysis of diacylglycerol observed in microsomes pretreated with diisopropylfluorophosphate gave rise to the exclusive formation of monoacylglycerol with no production of free [^{14}C]oleate. Due to the fact that monoacylglycerol formation could not be completely prevented in incubations with diacylglycerol, the stimulation of lysophosphatidylcholine production observed upon diacylglycerol addition could indeed be the result of this monoacylglycerol formation. This would require the direct transfer of phosphocholine from CDPcholine to monoacylglycerol. More direct evidence for the occurrence of this pathway was obtained in experiments with monoacylglycerol as substrate. Fig. 2 shows the effect of increasing monoacylglycerol concentrations on the incorporation of phosphocholine from CDPcholine into lysophosphati-

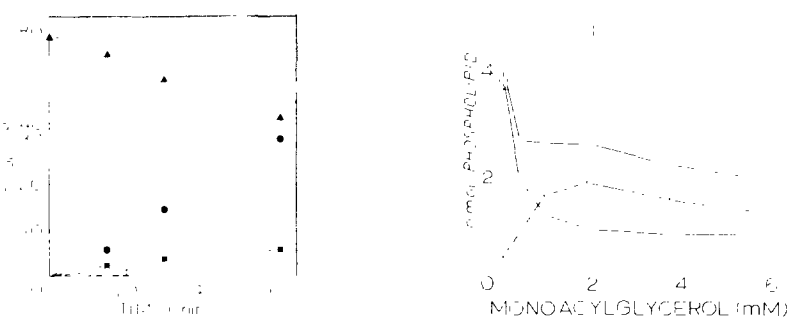

Fig. 1. Fate of diacylglycerol upon incubation with rat lung microsomes. 1-Acyl-2-[1-^{14}C]oleoyl-*sn*-glycerol (0.33 mM) was incubated with 200 μg microsomal protein in 25 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$ and 5 mM $CaCl_2$ in 0.01% (w/v) Tween 20 in a volume of 0.26 ml. Open symbols, diisopropylfluorophosphate-treated microsomes; closed symbols, untreated microsomes. △- - - - -△, ▲———▲ diacylglycerol; ○- - - - -○, ●———●, fatty acid; □- - - - -□, ■———■, monoacylglycerol.

Fig. 2. Effect of monoacylglycerol on formation of lysophosphatidylcholine and phosphatidylcholine in rat lung microsomes. Diisopropylfluorophosphate-treated microsomes (600 μg protein) were incubated in 25 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 0.03% (w/v) Tween 20 and 0.26 mM CDP[*Me*-^{14}C]choline (spec act. 10 000 dpm/nmol) in a total volume of 0.4 ml during 30 min at 37°C. □———□, total glycerophosphocholines; ○———○, phosphatidylcholine; X———X, lysophosphatidylcholine. Results given are the averages of two determinations.

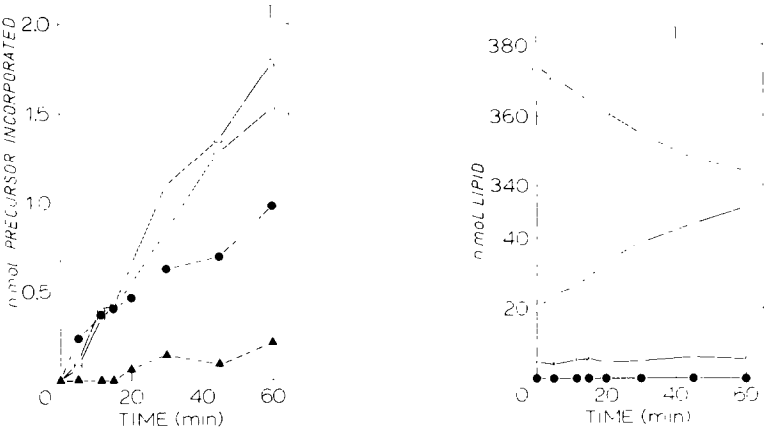

Fig. 3. Incorporation of [^{3}H]monoacylglycerol and phospho[*Me*-^{14}C]choline into phospholipids of rat lung microsomes. Diisopropylfluorophosphate-treated microsomes (300 μg protein) were incubated under the same conditions as described in Fig. 2, expect that 1 mM [^{3}H]monoacylglycerol was present. Open symbols, lysophosphatidylcholine; closed symbols, phosphatidylcholine. △———△, ▲- - - - -▲, ^{3}H label and ○———○, ●- - - - -●, ^{14}C label.

Fig. 4. Fate of monoacylglycerol upon incubation with rat lung microsomes. Incubation conditions as in Fig. 3. ●———●, triacylglycerol; X———X, diacylglycerol; ○———○, free fatty acid and △———△, monoacylglycerol.

dylcholine and phosphatidylcholine. Increasing concentrations of monoacylglycerol up to 2 mM stimulate lysophosphatidylcholine formation, while at the same time inhibiting phosphatidylcholine synthesis. One explanation for this observation could be that lysophosphatidylcholine and phosphatidylcholine are synthesized by a single cholinephosphotransferase. Although it is tempting to conclude from Fig. 2 that monoacylglycerol is a substrate for lysophosphatidylcholine synthesis alternative explanations can still be provided, e.g. inhibition of cholinephosphotransferase and stimulation of phospholipase activities by the added monoacylglycerol would yield similar results. To show that monoacylglycerol is indeed incorporated into lysophosphatidylcholine, experiments were carried out with 1(3)-[9,10-$^3H_2$]palmitoylglycerol in the presence of CDP[*Me*-^{14}C]choline. As can be seen in Fig. 3, within experimental error equimolar amounts of monoacylglycerol and phosphocholine were incorporated into lysophosphatidylcholine. The fact that phosphocholine incorporation is consistently 10—20% lower than monoacylglycerol incorporation is most likely due to either dilution of CDP[*Me*-^{14}C]choline with unlabelled CDPcholine adhering to the microsomes or to a systematic error introduced in the determinations of the specific radioactivity of the precursors. During the first 15 min of the incubation, no ^{3}H label appears in phosphatidylcholine. The slight incorporation observed thereafter is presumably due to either acylation of [^{3}H]lysophosphatidylcholine or endogenous lysophosphatidylcholine with [^{3}H]palmitate (compare Fig. 4). Acylation of [^{3}H]monoacylglycerol to diacylglycerol and subsequent conversion into phosphatidylcholine is less likely since no formation of diacylglycerol was detected (Fig. 4). Theoretically, the latter pathway (monoacylglycerol → diacylglycerol → phosphatidylcholine) could proceed without accumulation of the intermediary diacylglycerol and this pos-

sibility cannot be excluded completely. However, the appearance of ^{3}H label in lysophosphatidylcholine cannot be explained by the action of a phospholipase A on phosphatidylcholine. At all time points the $^{3}H/^{14}C$ ratio in lysophosphatidylcholine is much higher than in phosphatidylcholine. Phospholipase A action on phosphatidylcholine would yield lysophosphatidylcholine with a $^{3}H/^{14}C$ ratio equal to or lower than that in phosphatidylcholine. These considerations and the almost equimolar incorporation of [^{3}H]monoacylglycerol and phospho[*Me*-^{14}C]choline into lysophosphatidylcholine clearly demonstrated the feasibility of de novo synthesis of lysophosphatidylcholine.

Fig. 4 demonstrates that even in microsomes pretreated with diisopropylfluorophosphate some hydrolysis of [^{3}H]monoacylglycerol still takes places. As mentioned above incorporation of the released [^{3}H]palmitate into lysophosphatidylcholine could be partially responsible for the appearance of ^{3}H label in phosphatidylcholine. However, no formation of either diacylglycerol or triacylglycerol is observed.

Although the possibility of de novo synthesis of lysophosphatidylcholine in lung microsomes has been proven in vitro, it cannot yet be stated to what extent this reaction contributes to lysophosphatidylcholine formation in vivo.

In experiments comparable to those described in Table I but in the presence of saturating levels of CDPcholine (0.26 mM) the addition of an equimolar mixture of monoacylglycerol and diacylglycerol (7 mM each) resulted in an about 20-fold enhanced synthesis of both lysophosphatidylcholine and phosphatidylcholine (data not shown). Lysophosphatidylcholine contained about 6% of the ^{14}C radioactivity, irrespective of whether monoacylglycerol plus diacylglycerol was present or not. Thus, when both monoacylglycerol and diacylglycerol are present in excess there is still a large preference for phosphocholine incorporation into diacylglycerol. De novo synthesis of lysophosphatidylcholine is thus a minor pathway compared with de novo synthesis of phosphatidylcholine (compare Table I). Whether and to what extent these syntheses are influenced by the relative concentrations of monoacylglycerol and diacylglycerol in microsomal membranes remains to be determined in future work. Also, the relative importance of de novo synthesis of lysophosphatidylcholine in comparison to lysophosphatidylcholine formation by the action of microsomal phospholipases A cannot be evaluated at present. The experiments described in Tables I and II suggest that de novo synthesis could contribute considerably to lysophosphatidylcholine formation.

References

1 Goerke, J. (1974) Biochim. Biophys. Acta 344, 241—261
2 King, R.J. and Clements, J.A. (1972) Am. J. Physiol. 223, 715—726
3 Van Golde, L.M.G. (1976) Am. Rev. Respir. Dis. 114, 977—1000
4 Frosolono, M.F. (1977) in Lipid Metabolism in Mammals (Snyder, F., ed.), Vol. 2, pp. 1—38, Plenum Press, New York
5 Ohno, K., Akino, T. and Fujiwara, T. (1978) in Reviews in Perinatal Medicine (Scarpelli, E.M. and Cosmi, E.V., eds.), Vol. 2, pp. 227—318, Raven Press, New York
6 Vereyken, J.M., Montfoort, A. and van Golde, L.M.G. (1971) Biochim. Biophys. Acta 260, 70—81
7 Sarzala, M.G. and van Golde, L.M.G. (1976) Biochim. Biophys. Acta 441, 423—432
8 Lands, W.E.M. (1960) J. Biol. Chem. 235, 2233—2237
9 Akino, T., Yamazaki, I. and Abe, M. (1972) Tohoku J. Exp. Med. 108, 133—139
10 Brumley, G. and van den Bosch, H. (1977) J. Lipid. Res. 18, 523—532

11 Garcia, A., Newkirk, J.D. and Mavis, R.D. (1975) Biochem. Biophys. Res. Commun. 64, 128—135
12 Ohta, M., Hasegawa, H. and Ohno, K. (1972) Biochim. Biophys. Acta 280, 552—558
13 Longmore, W.J., Oldenborg, V. and van Golde, L.M.G. (1979) Biochim. Biophys. Acta 572, 452—460
14 Edwards, R.G. and Blough, H.A. (1978) Virology 89, 314—317
15 Daubert, B.F., Fricke, H.H. and Longenecker, H.E. (1943) J. Am. Chem. Soc. 65, 2142—2144
16 Waite, M. and van Deenen, L.L.M. (1967) Biochim. Biophys. Acta 137, 498—517
17 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
18 Bligh, E.G. and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911—917
19 Kennedy, E.P. and Weiss, S.B. (1956) J. Biol. Chem. 222, 193—214
20 Hamosh, M. and Hamosh, P. (1975) Biochim. Biophys. Acta 380, 132—140
21 Wang, M.C. and Meng, H.C. (1975) Lipids 10, 721—725
22 Armstrong, H.J., Kuenzig, M.C. and Peltier, L.F. (1967) Proc. Soc. Exp. Biol. 124, 959—961
23 Possmayer, F., Duwe, G., Hahn, M. and Buchnea, D. (1977) Can. J. Biochem. 55, 609—617