LUNG SURFACTANT SYNTHESIS: A Ca^{++}-DEPENDENT MICROSOMAL PHOSPHOLIPASE A_2 IN THE LUNG

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Summary - We present the first direct evidence for a highly active, Ca^{++}-dependent phospholipase A_2 in the microsomal fraction of rat lung homogenate. Several previously reported studies from other laboratories strongly implicate this enzyme as a key metabolic step in the biosynthesis of dipalmitoyl lecithin, the primary component of pulmonary surfactant. In the present study, stoichiometric amounts of [^{3}H]lysophosphatidylethanolamine and [^{14}C]fatty acid were released during incubation of 1-[9,10-^{3}H]palmitoyl-2-sn-[1'-^{14}C]linoleoyl phosphatidylethanolamine with the lung microsomal fraction. Marker enzyme measurements showed that the microsomal activity cannot be due to contamination with mitochondria, which also show phospholipase A_2 in both lung and liver. In contrast, liver microsomes show predominantly a phospholipase A_1 activity.

Abnormally low levels of pulmonary surfactant have been implicated as the cause of respiratory distress syndrome in the newborn (1). Dipalmitoyl lecithin is the major component of pulmonary surfactant (for a review, see ref. 2) and synthesis of this lipid is believed to occur in the granular pneumocytes of lung tissue (Alveolar type II cells) (3). Since phospholipids synthesized by other tissues contain saturated fatty acids predominantly esterified to the 1-position of the glycerol backbone and unsaturated fatty acids at the 2-position (4), it would seem likely that lung tissue contains a unique enzymatic pathway responsible for incorporation of only saturated fatty acids into a large portion of synthesized lecithin. Several studies in lung tissue with radiolabeled lipid precursors have suggested that de novo lipid synthesis by way of the specific acylation of glycerol-3-phosphate by palmitoyl-coenzyme A plays only a minor role in the incorporation of large amounts of palmitate into lung lecithin (5-9)

Abbreviations: PE, phosphatidylethanolamine; LPE, lysophosphatidylethanolamine; FA, fatty acid; BSA, bovine serum albumin; PPO, 2,5-diphenyloxazole; bis-MSB, p-bis-(O-methylstyryl)-benzene.

Instead, remodeling of pre-formed lipid appeared to be the predominant pathway of production of large amounts of disaturated lipid in the lung (5-7, 9-11), either by a deacylation-reacylation cycle, or the transacylation between two monoacylphosphoglyceride molecules. Either of these pathways would include a phospholipase A_2 activity catalyzing the initial hydrolysis of a fatty acid from position 2 of a preformed phospholipid which would presumably contain unsaturated fatty acyl groups at that position.

Kyei-Aboagye et al. (9) have recently shown that rabbit lung homogenates catalyze a Ca^{++}-dependent release of [^{3}H]oleate from [^{3}H]oleoyl phosphatidylcholine and the concomitant incorporation of [^{14}C]palmitate into phosphatidylcholine. Radioautography of lung tissue radiolabelled with lipid precursors (12) indicates that the lipid content of lung lamellar bodies, the putative site of surfactant lipid storage, is derived from radioactive material which is first seen in the endoplasmic reticulum and which appears to migrate via Golgi complexes into the lamellar bodies.

Thus several lines of indirect evidence from different laboratories suggest that a Ca^{++}-dependent phospholipase A_2, associated with the endoplasmic reticulum of lung, may be the initial step in the remodeling of asymmetrical lipids into the uniquely disaturated lipid active as pulmonary surfactant. We present in this report the first direct evidence for such an enzyme in the microsomal fraction of rat lung.

MATERIALS AND METHODS

Male Sprague-Dawley rats weighing 250-275 g were stunned and decapitated. The lungs were rapidly removed and chilled in ice-cold tris-sucrose buffer (0.25 M sucrose, 0.01 M tris-HCl, pH 7.4). The lobes of the lungs were separated and all major visible bronchii and blood vessels were removed. The tissue was minced with a razor blade, then homogenized in 5 volumes of tris-sucrose buffer. A teflon pestle (0.017 inch clearance) was used in a Potter-Elvehjeim type homogenizer for 4-5 strokes, followed by 7-8 strokes with a second pestle (0.007 inch clearance). Pestles were mechanically driven at 280 RPM. The homogenate was filtered through 6 layers of cheese cloth and centrifuged at 600 x g for 10 min to remove debris. The supernatant was centrifuged at 6,000 x g for 15 min. The resulting pellet was resuspended in tris-sucrose buffer, and designated mitochondria I. The supernatant was centrifuged at 20,000 x g for 15 min to produce a pellet containing a mixture of mitochondria and microsomes, which was discarded, and the supernatant was centrifuged at 100,000 x g for 45 min. The resulting pellet was resuspended in tris-sucrose buffer and designated microsomes I.

A more highly purified fraction of mitochondria was obtained by centrifuging a portion of mitochondria I at 600 x g for 10 min, discarding the pellet and centrifuging the supernatant at 6,000 x g for 15 min. The resulting pellet, resuspended in tris-sucrose buffer was designated mitochondria II.

A more highly purified fraction of microsomes was obtained by centrifuging a portion of microsomes I at 20,000 x g for 15 min and discarding the pellet. The supernatant was centrifuged at 100,000 x g for 45 min. The pellet was resuspended in tris-sucrose buffer and designated microsomes II.

Protein concentration of the resulting fractions was determined according to Lowry et al. (13) using bovine serum albumin as a standard.

Two radiolabelled substrates were used to assay for phospholipase A. 1-[9,10-^{3}H]palmitoyl-2-sn-acyl phosphatidylethanolamine was synthesized according to Robertson and Lands (14). 1 acyl-2-sn-[1'-^{14}C]linoleoyl phosphatidylethanolamine was prepared according to Waite and Van Deenen (15). Hydrolysis by Crotalus adamanteus snake venom (Sigma) revealed that 99% of each isotope was located at the indicated positions.

Lysophospholipase assays were performed using [^{3}H]lysophosphatidylethanolamine as substrate, prepared by treatment of 1-[9,10-^{3}H]palmitoyl-2-sn-acyl phosphatidylethanolamine with Crotalus adamanteus snake venom.

Lipase assays were performed using tri-[1'-^{14}C]oleoyl glycerol (Research Products International) as substrate.

Incubation mixtures contained 100mM tris-HCl, pH 9, 10 mM $CaCl_2$, and 5-10 µg of protein together with the appropriate labeled substrate, in a total volume of 1 ml. Phosphatidylethanolamine was added as an ultrasonicated suspension in water. Triolein was emulsified by sonication in the presence of bovine serum albumin (0.5 mg BSA/200 nmoles triolein). Enzyme assays were performed in duplicate. Mixtures were incubated for 20 min at 37°. Reactions were stopped by adding 3 ml of methanol: chloroform (2:1). Mixtures were acidified by addition of 0.1 ml of 1 M acetic acid and the lipids were extracted by the method of Bligh and Dyer (16), followed by 2 additional extractions of the aqueous phase with 2 ml of chloroform per extraction. No significant radioactivity remained in the aqueous phase. The chloroform extracts were evaporated to dryness under a stream of N_2 and redissolved in 0.2 ml of chloroform: methanol (2:1). The redissolved lipids were chromatographed on glass thin layer plates coated with 0.5 mm of silica gel H applied as a slurry of 50 gm of gel in 135 ml of 10mM Na_2CO_3 or on pre-coated commercial plates of silica gel G (Analtech). Solvent systems were chloroform: methanol: water: acetic acid (70:30:4:2 v/v) for products of phospholipid hydrolysis and petroleum ether: diethyl ether: acetic acid (80:20:2 v/v) when triolein was the substrate. Appropriate standards were co-chromatographed with the extracted lipids and detected by iodine vapor. Radioactivity migrating with the standard compounds was detected by scraping the gel into scintillation vials and counting in 10 ml of toluene: triton X-100: water (2:1:0.2) containing 5 mg PPO and 0.5 mg bis-MSB per liter of toluene. Control incubations with boiled enzyme showed 2-3% hydrolysis of the added substrate. About 90% of radioactivity originally present in incubations was recovered from the thin layer chromatography.

RESULTS AND DISCUSSION

When lung microsomes or mitochondria were incubated with the doubly labeled substrate, 1-[^{3}H]palmitoyl-2-[^{14}C]linoleoyl phosphatidylethanolamine, the products of the reaction were [^{3}H]LPE and [^{14}C]FA, released in stoichiometric amounts (Table I), at rates of about 20 and 34nmoles per min per mg of protein for microsomes and mitochondria, respectively. No significant amounts of either [^{3}H]FA

Table I. Phospholipase A_2 activity of lung microsomes and mitochondria

	nmoles per minute per mg protein	
	[^{14}C]FA	[^{3}H]LPE
Microsomes II	22.3 ± 2.5	19.0 ± 0.8
Mitochondria II	36.4 ± 1.7	32.2 ± 0.8

Incubation mixture described in Materials and Methods. Substrate was 144 μM 1-[9,10-^{3}H]palmitoyl-2-sn-[1'-^{14}C]linoleoyl PE (170 ^{3}H-CPM per nmole, 160 ^{14}C-CPM per nmole). The average of duplicate determinations and range is shown.

or [^{14}C]LPE appeared under the given assay conditions. These results conclusively demonstrate a phospholipase A_2 in both of these fractions, that is, a phospholipase specific for hydrolysis of the fatty acid at position 2 of a phospholipid. This specificity has also been observed for phospholipase A in liver mitochondria (17, 18). Liver microsomes, however, show predominantly a phospholipase A_1 activity, that is, specific for hydrolysis at position 1 (18, 19). In the present study, rat liver microsomes assayed in the standard incubation mixture with 2-[^{14}C]linoleoyl PE as substrate, showed a specific activity of 4.2 nmoles of labeled LPE released per min per mg protein.* Phospholipase A_2 activity was relatively low in the same assay, as indicated by the release of only 1.5 nmoles of [^{14}C]FA per min per mg protein.

The difference in specificity between the lung microsomal phospholipase A and the liver microsomal phospholipase A is consistent with the involvement of the lung enzyme in a metabolic pathway unique to lung, such as synthesis of large amounts of disaturated phospholipid. Further characterization of this enzyme is thus of interest in understanding the synthesis of pulmonary surfactant.

* A Ca^{++} concentration of 2 mM, reported to be optimal for liver microsomes (19), was used.

The high activity of phospholipase A_2 in lung mitochondria made it necessary to establish that the phospholipase A_2 activity in the microsomal fraction was not due to mitochondrial contamination. Three marker enzymes therefore were assayed in the fractions obtained from lung homogenate. The results are shown in Table II.

Table II. Distribution of phospholipase A_2 and marker enzymes in lung mitochondria and microsomes. NADPH-cytochrome c reductase (20), succinate cychrome c reductase (21) and monoamine oxidase (22) were assayed according to published procedures. Phospholipase A_2 was assayed as described in the Materials and Methods, using 123 μM 2-[^{14}C]linoleoyl PE (120 CPM per nmole) as substrate. Specific activities are expressed as nmoles per min per mg protein.

Fraction	Specific Activity (or Ratios)			
	NADPH Cytochrome c Reductase	Succinate Cytochrome c Reductase	Monoamine Oxidase	Phospholipase A_2
Homogenate	11	3.4	-	-(*)
Mitochondria I	22	19	3.3	14
Mitochondria II	19	31	6.4	26
Microsomes I	48	1.6	0.83	17
Microsomes II	59	1.2	0.80	14
$\frac{\text{Microsomes II}}{\text{Mitochondria II}} \times 100$	-	3.8	13	54
$\frac{\text{Mitochondria II}}{\text{Microsomes II}} \times 100$	32			

(*) The presence in lung homogenate of an active lysophospholipase (23) precludes determination of phospholipase A_2 activity in that fraction.

NADPH cytochrome c reductase, an enzyme commonly used as a microsomal marker (24), was found to have highest specific activity in the most purified microsomal fraction, enriched 5-6 fold over the crude homogenate. Succinate cytochrome c reductase, a marker for inner mitochondrial membrane (25), was en-

riched nearly 10 fold over crude homogenate in the most purified mitochondrial fraction. Monoamine oxidase, considered an outer mitochondrial membrane enzyme (26), was likewise most active in the mitochondrial fraction of lung. Calculated from the specific activities of marker enzymes, contamination of microsomes by inner mitochondrial membrane was 3.8% and by outer mitochondrial membrane was 13%. These amounts of mitochondrial contamination clearly could not account for the high specific activity of phospholipase A_2 in microsomes, which was 54% of that in the mitochondrial fraction. Thus the microsomal association of this enzyme is established.

The microsomal phospholipase A_2 was absolutely dependent upon added Ca^{++}, with an apparent K_D of 1 mM, independent of phospholipid substrate concentration, as shown in Figure 1. The Ca^{++}-dependency is consistent with the involve-

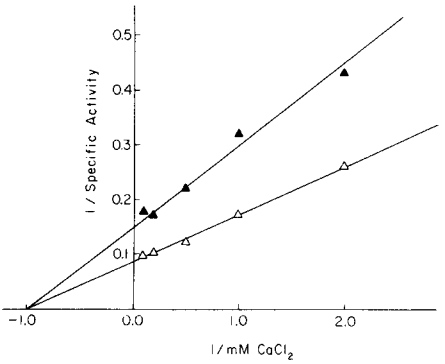

Figure 1. Ca^{++} concentration dependence of phospholipase A_2 of lung microsomes. Incubation mixture described in Materials and Methods. Substrate was 50 µM 2-[^{14}C]linoleoyl PE (300 CPM per nmole) ▲, and 150 µM 2-[^{14}C]linoleoyl PE (100 CPM per nmole) △. Specific activity is in nmoles [^{14}C]FA released per min per mg protein.

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ment of this enzyme in the Ca^{++}-dependent remodeling of phospholipid by lung homogenate observed by Kyei-Aboagye et al. (9).

A pH optimum of 9.0 was found for the enzyme. Hydrolysis of fatty acid from the 2 position of PE was linear with time during the 20 min incubation period and proportional to added protein up to 10-15 µg. When larger amounts

of lung microsomal protein were added, a small accumulation of [^{3}H]fatty acid was observed using 1-[^{3}H]palmitoyl-PE as substrate (Figure 2) which could be

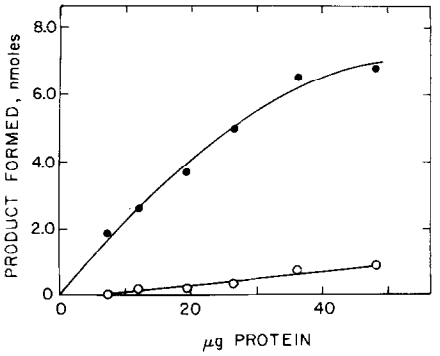

Figure 2. Protein concentration dependence of phospholipase A_2 of lung microsomes. Incubation mixture described in Materials and Methods. Substrate was 127 μM 1-[^{3}H]palmitoyl PE (120 CPM per nmole). Products formed are [^{3}H]LPE (●) or [^{3}H]FA (o).

attributed to the action of a lysophospholipase on the LPE product of the phospholipase A_2, since an accumulation of 4-6 nmoles of [^{3}H]LPE appears to be a pre-requisite for release of significant amounts of labeled fatty acid. Preliminary experiments with [^{3}H]LPE as substrate showed that, in fact, lung microsomes contain a lysophospholipase activity showing kinetic behavior similar to that described for a particulate lysophospholipase in rat brain by Leibovitz-Ben Gershon *et al.* (27).

No lipase activity was observed in lung microsomes using the standard incubation mixture and 150 μM tri-[1'-^{14}C]oleoylglycerol as substrate. Liver microsomes under the same conditions catalyzed the release of 7 nmoles of fatty acid per min per mg protein consistent with previous lipase determinations in liver (28).

Thus we report the first conclusive evidence for Ca^{++}-dependent phospholipase A_2 associated with the microsomal fraction of rat lung homogenate. Previous work in several other laboratories strongly suggests the role of such an enzyme in pulmonary surfactant synthesis via a remodeling of phosphoglycerides with an asymmetric fatty acid distribution.

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