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Catabolism of rat surfactant disaturated phosphatidylcholines during incubation of alveolar lavage materials in vitro at 37°C

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Incubation of rat alveolar lavage materials in vitro at 37°C results in degradation of the endogenous surfactant disaturated phosphatidylcholines (disaturated PC). When exogenous dipalmitoylphosphatidylcholine (DPPC) vesicles are incubated with lavage materials, there is catabolism of the DPPC. The degradation process is temperature- and Ca^{2+}-dependent and has a pH optimum of 6.5–7.0. The products formed during catabolism of [^{3}H]palmitate- and [^{14}C]choline-labeled disaturated PC are free palmitate and water-soluble choline products. No lysophosphatidylcholines are formed. Measurements of lactate dehydrogenase levels in lavage fluid, use of more gentle lavage techniques, use of Ca^{2+}, Mg^{2+} and protein in the lavage medium, and measurements of bacterial contamination all suggest that enzymes are neither released into pulmonary lavage fluid via cellular damage nor are bacterial in origin. Degradation of surfactant disaturated PC does not occur during incubation of lavage materials from rabbits, mice or guinea pigs. These results suggest that phospholipases and/or lysophospholipases are present in rat alveolar lavage materials. Possible origins of these enzymes are discussed.

Introduction

It is now generally accepted that pulmonary surfactant materials are synthesized in alveolar type II cells and then released onto the alveolar surface. One major function of these materials is to reduce the surface tension at the air-liquid interface in the lungs and, thus, prevent alveolar collapse. The component of surfactant which is present in the greatest amount and which is the major surface active material is dipalmitoylphosphatidylcholine [1,2]. The most widely used technique for obtaining surfactant materials has been lung lavage. In some cases, additional procedures are used to isolate pulmonary surfactant from the lavage [3,4]. In other cases, experiments, such as those designed to study mechanisms involved in the life cycle of surfactant [5,6], are carried out with alveolar lavage fluid itself. Recently, we found that when pulmonary lavage fluid from rats is incubated in vitro at 37°C, the disaturated phosphatidylcholine (disaturated PC) content of the fluid is decreased. The objective of the experiments reported in this study was to characterize this catabolism of rat surfactant disaturated PC. A preliminary report of this work has appeared previously [7].

Methods

Male Sprague-Dawley rats (200–300 g) were anesthetized with sodium methohexital (35 mg/kg

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body weight) and given intravenous injections (via the penile vein) of either 30 μCi [^{3}H]palmitate (specific activity, 11.8 Ci/mmol; New England Nuclear Corp., Boston, MA) or 30 μCi [^{3}H]choline (specific activity 80 Ci/mmol, New England Nuclear) in saline (0.2 ml total volume). To prepare the palmitate for injection, palmitic acid was complexed with bovine serum albumin in a molar ratio of 5.3:1 (fatty acid/bovine serum albumin) as described by Hendry and Possmayer [8]. 22 h after injection of the labeled substrates, the animals were anesthetized with sodium pentobarbital (200 mg/kg body weight) and exsanguinated by cutting the abdominal aorta. Some animals which were not previously injected with labeled substrates were also killed. The heart, trachea and lungs were removed from the rats intact and alveolar lavage fluid was obtained by repeated injections of the same 3 ml of Ca^{2+}-free phosphate-buffered medium (145 mM NaCl/5 mMKCl/9.35 mM Na_2HPO_4/1.9 mM NaH_2PO_4 (pH 7.4)) into the right and left lungs. The total amount of fluid recovered was 2.2–2.7 ml per animal. Alveolar macrophages were removed by centrifugation at $300 \times g$, for 5 min. Absence of cells in the lavage fluid was verified by cell counts. For most experiments, Ca^{2+} (final concentration, 1.8 mM) and Mg^{2+} (final concentration, 1.0 mM) were added to the lavage fluid as chloride salts and this suspension was then incubated for various periods of time at 37°C.

The amounts of either the [^{3}H]palmitate or [^{3}H]choline label in the endogenous surfactant disaturated PC in the lavage fluid were measured prior to and after various periods of incubation at 37°C. Disaturated PC was isolated from 0.5 ml aliquots of the surfactant materials according to the method of Mason et al. [9]. Briefly, total lipids were extracted with chloroform/methanol (2:1, v/v), the solvent was evaporated, and the lipids were reacted with osmium tetroxide dissolved in carbon tetrachloride. Following evaporation of the CCl_4, the samples were dissolved in chloroform/methanol (20:1, v/v) and placed on a column of neutral alumina (100-200 mesh, Bio-Rad Laboratories; Richmond, CA). The disaturated PC was eluted from the columns with chloroform/methanol/7 M ammonium hydroxide (70:30:2, v/v). After evaporation of the solvent, 10 ml of Aquasol (New England Nuclear) were added to each sample of disaturated PC and the samples were counted in the tritium channel of a liquid scintillation spectrometer (Model 3380; Packard Instrument Co., Downers Grove, IL). In general, the amount of labeled disaturated PC was expressed as a percent of that present prior to incubation. In some experiments, the products of disaturated PC catabolism were isolated by thin-layer chromatography [10], the spots were scraped from the plates, and the radioactivity associated with each spot was counted.

The amount of labeled exogenous dipalmitoylphosphatidylcholine (DPPC) present prior to and after incubation with lavage fluid was measured in some experiments. DPPC vesicles labeled with [^{14}C]DPPC were prepared according to a method published by King and MacBeth [11]. Briefly, DPPC (5 mg) and [1-^{14}C]DPPC (1 μCi; specific activity, 115 mCi/mmol; Amersham Corp., Arlington Heights, IL) were dissolved in 100 μl of ethanol. This solution was rapidly injected into 5 ml of warm (48°C) phosphate-buffered medium with a Hamilton syringe and then sonicated for 30 s. Various amounts of this stock solution were added to pulmonary lavage fluid. The DPPC was isolated as described above prior to and after incubation at 37°C. The amount of labeled DPPC present was measured with a liquid scintillation counter. Finally, in some experiments, the level of lactate dehydrogenase in samples of alveolar lavage fluid was monitored as an indicator of cell damage which may have occurred during the lavage process. Lactate dehydrogenase was determined by measuring the production of NADH at 340 nm during the conversion of L-lactate to pyruvate [12].

Results

Time-courses for catabolism and temperature dependence

Pulmonary lavage fluid obtained from rats injected with labeled choline 22 h prior to killing was incubated at 37°C, and the labeled disaturated PC in the fluid was measured as a function of time. The results are shown in Fig. 1A. The endogenous labeled disaturated PC disappears along a time-course which is linear for approx. 10

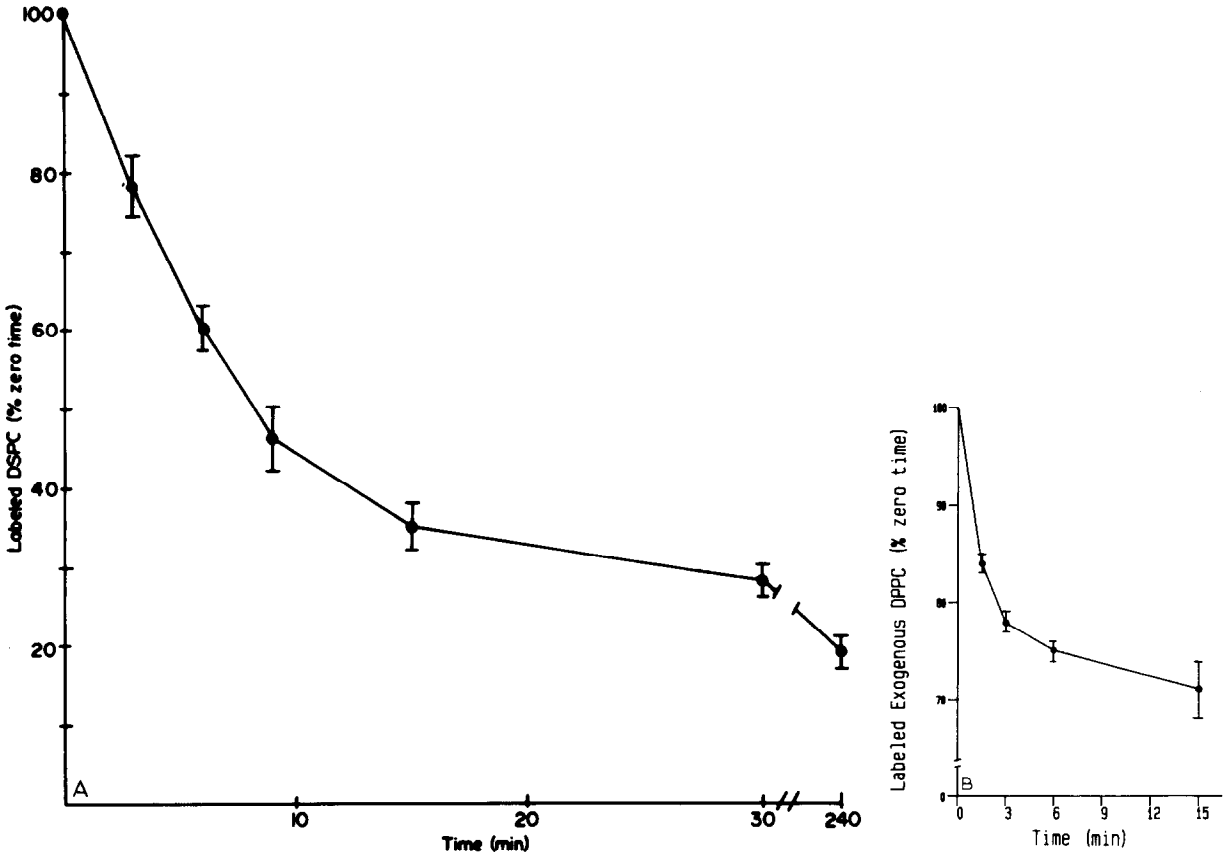

Fig. 1. A. Time-course for catabolism of endogenous surfactant disaturated PC (DSPC). Pulmonary lavage fluid obtained from animals injected with [^{3}H]choline 22 h prior to killing was incubated at 37°C. Measurements of labeled disaturated PC were made from 0.5 ml samples obtained after varying incubation periods. The points are mean values for six experiments and the bars represent the S.E. of the means. B. Time-course for catabolism of exogenous DPPC vesicles in alveolar lavage fluid. Vesicles of DPPC (containing [^{14}C]DPPC) were added to pulmonary lavage fluid and incubated at 37°C. The final concentration of DPPC was 0.6 mg/ml. Measurements of labeled DPPC were made from 0.5 ml samples obtained after varying incubation periods. The points are mean values for four experiments and the bars represent the S.E. of the means.

min and then slows considerably. In fact, after 4 h of incubation, about 80% of the labeled disaturated PC is no longer present. In a separate set of experiments, we determined that the apparent disappearance of the phospholipid is not due to adherence of the material to the incubation vessels.

Other experiments were performed to determine if exogenous DPPC disappears when it is incubated with alveolar lavage fluid. The results are shown in Fig. 1B. The exogenous DPPC also disappears with time, although the rate is linear for only 3 min. In other experiments (data not shown) we found: (1) when equal amounts of endogenous disaturated PC and exogenous DPPC are included in the lavage fluid, the amount of catabolism which occurs is identical for each ; (2) catabolism of exogenous DPPC is linear with respect to the amount of lavage fluid used; (3) the $K_{1/2}$ value (amount of DPPC at which one-half maximal catabolism occurs) when 1 ml of lavage fluid is used is 0.6 mg/ml (lavage fluid contains approx. 0.18 mg endogenous disaturated PC per ml).

The temperature dependence for catabolism of disaturated PC in pulmonary lavage fluid was studied. While incubation of the fluid at 37°C for 4 h results in 76 ± 2% of the disaturated PC disappearing, there is only 8 ± 2% catabolism at 2°C.

Furthermore, when the alveolar lavage fluid is preincubated at 60°C for 20 min in order to inactive enzymes and then incubated at 37°C for 4 h, there is only 26 ± 3% catabolism. Thus, the disappearance of disaturated PC is temperature dependent. All of these data taken together suggest that this catabolism is an enzymatic process.

Products of catabolism

In order to attempt to determine the products of disaturated PC degradation, we incubated [^{3}H]palmitate- and [^{3}H]choline-labeled alveolar lavage fluid at 37°C for 30 min and measured the appearance of the label in some of the lipids identified with thin-layer chromatography. The results are shown in Table I. In unincubated lavage fluid from choline-labeled animals, 59% of the label is found as water-soluble products and 41% is disaturated PC. During the incubation period, disappearance of the label from disaturated PC coincides with its appearance in the fraction containing water-soluble products. In unincubated lavage materials from palmitate-labeled animals, the label appears to be distributed equally between disaturated PC and the free palmitate fraction. Disappearance of the label from disaturated PC during incubation coincides with its appearance in the palmitate fraction. There is virtually no lysophophatidylcholine (lysoPC) present in the lavage either before or after incubation. Some other experiments, for which data are not shown, were also performed. We found that the degradation of disaturated PC is enhanced by 50% with inclusion of 1.8 mM Ca^{2+} in the medium, and the pH optimum is 6.5 7.0. These results are consistent with the presence of phospholipases and/or lysophospholipases in rat pulmonary lavage fluid.

TABLE I

PRODUCTS OF CATABOLISM OF SURFACTANT DISATURATED PC

Experiments were performed with either [^{3}H]choline-labeled or [^{3}H]palmitate-labeled lavage materials suspended in phosphate-buffered medium. Samples were taken before and after 30 min of incubation at 37°C. The lipids were identified with thin-layer chromatography. Water-soluble products were those obtained in the aqueous phase after washing the chloroform-methanol extract of the materials. LPC, lysophosphatidylcholines; DSPC, disaturated PC. The numbers are mean values for five experiments ± S.E. of the means.

Lipid	Distribution of label (%)	
	prior to incubation	after 30 min of incubation
Choline-labeled lavage fluid		
DSPC	41 (±5)	8 (±2)
water-soluble products	59 (±5)	92 (±2)
LPC	0.1(±0.1)	0.1(±0.1)
Palmitate-labeled lavage fluid		
DSPC	53 (±10)	17 (±9)
palmitate	47 (±10)	83 (±9)
LPC	0.2(±0.1)	0.1(±0.1)

Relationship between cell damage and catabolism

To determine whether cell damage during the lavage procedure is responsible for the appearance of enzymes in lavage fluid, the relationship between catabolism of surfactant disaturated PC during incubation at 37°C and lactate dehydrogenase, a classical cytosolic marker for cell damage, was determined. The lactate dehydrogenase levels of freshly isolated lavage fluid and the amount of catabolism of [^{3}H]choline-labeled surfactant disaturated PC during a 4 hour incubation at 37°C were measured for the same samples. The results are shown in Fig. 2. The correlation coefficient (R) for these points is 0.269. In order for a significant correlation to exist for this number of points, R must be 0.414. Therefore, there is no correlation between the lactate dehydrogenase level and the amount of disaturated PC catabolism which occurs in lavage fluid, suggesting that enzymes are not present in alveolar lavage fluid due to cell damage.

Experiments were performed to measure activity of the degradative enzymes in two different subfractions of pulmonary lavage fluid. Alveolar lavage fluid was spun at 100000 × g for 2 h. Following the fractionation, 75% of the surfactant phospholipid is found in the pellet and 25% in the supernatant. Each fraction was incubated at 37°C for 30 min with medium containing [^{14}C]DPPC vesiscles. There is approx. 6-times more catabolism of the exogenous DPPC when material from the pellet is incubated than when the supernatant is used (170 ± 10 compared to 30 ± 10 μg/ml; n = 7). Furthermore, there is 4-times more endogenous phospholipid in the pellet than in the super-

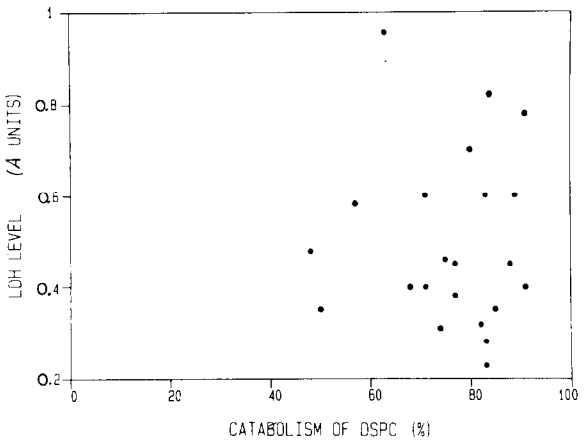

Fig. 2. Relationship between lactate dehydrogenase (LDH) level of alveolar lavage fluid and catabolism of endogenous disaturated PC (DSPC) in lavage fluid. Lactate dehydrogenase levels of pulmonary lavage fluid were measured immediately after removal of alveolar macrophages. Alveolar lavage fluid obtained from [^{3}H]choline-injected animals was incubated at 37°C for 4 h and the labeled disaturated PC present was measured before and after the incubation. The amount of disaturated PC catabolized was expressed as a percent of that present prior to the incubation. Each point represents a sample of lavage fluid from a different animal. The correlation coefficient (R) for these points is 0.269. In order for a significant correlation to exist, R must be 0.414 for this number of points.

natant (0.39 ± 0.01 and 0.09 ± 0.01 mg/ml; $n = 7$). Since the endogenous phospholipids also serve as substrates for the phospholipases, the difference between the enzyme activities in the two fractions was underestimated in these experiments. Therefore, these results indicate that most of the enzyme(s) in alveolar lavage fluid responsible for catabolism of disaturated PC is probably bound to the lipid and is not found free in the soluble phase.

Discussion

The data presented in this paper indicate tha there is catabolism of endogenous pulmonary surfactant disaturated PC when alveolar lavage fluid from rats is incubated at 37°C in vitro. Also, incubation of exogenous DPPC vesicles with the lavage fluid results in its degradation. The process of catabolism is temperature-dependent, Ca^{2+}-dependent, and has a pH optimum of 6.5–7.0. The products of degradation suggest that phospholipases and/or lysophospholipases are present in the lavage fluid. Furthermore, experiments with subfractions of the lavage obtained via differential centrifugation suggest that most of the enzyme(s) is bound to phospholipid.

Catabolism of both endogenous disaturated PC and exogenous DPPC is linear for 10 and 3 min, respectively, and then becomes slower. Two possible reasons for the nonlinearity are inhibition of the enzymes due to accumulation of products and inactivation of the enzymes. Addition of high levels of palmitate and choline to the lavage fluid does not affect catabolism of exogenous DPPC vesicles. Thus, product inhibition does not appear to be a factor. In other experiments (data not presented) we incubated one aliquot of lavage fluid at 37°C and another at 2°C for 15 min. Then, exogenous DPPC vesicles were added to each and catabolism of phospholipid was measured. Degradation was much greater in the sample which had been maintained at 2°C. These data suggest that the enzyme(s) becomes inactivated during incubation at 37°C and this may be the reason for the slowing of the catabolism. The factors which may be responsible for inactivation are not known, although various hydrolases, which are present in alveolar lavage materials [13], may be involved.

The origin of the enzymes in the alveolar lavage fluid responsible for degradation of disaturated PC is not known. One possibility is that the enzymes are released from cells due to damage during the lavage procedure. However, there are three lines of evidence to indicate that this is not the case: (1) there is no correlation between lactate dehydrogenase levels in the lavage and catabolism of disaturated PC (Fig. 2); (2) when more gentle lavage procedures, such as injection of 4 ml of phosphate-buffered medium into both lungs simultaneously, or using a standard technique of lavage with a volume of 5 ml/gm lung five times [14], there is still degradation of disaturated PC upon incubation of the fluid at 37°C; (3) if Ca^{2+}, Mg^{2+} and bovine serum albumin are included in the lavage medium or if a cell culture medium, such as Hank's balanced salt solution, is used, there is catabolism of disaturated PC during the incubation process. In the latter two instances, there is somewhat less disaturated PC degradation. Another possibility is that the phospholipases are derived from bacterial contamination. However,

we found no correlation between disaturated PC catabolism and the number of bacterial colonics obtained during culture of lavage materials. Also, degradation of disaturated PC occurs to the same extent during incubation of lavage fluid from pathogen-free animals. Thus, cell damage and/or bacterial contamination do not seem to be the sources of the enzymes.

Another possibility is that the enzymes are present in lamellar bodies and find their way on to the alveolar surface during surfactant release. Heath and Jacobson [15] found phospholipase A_1 and phospholipase A_2 activities in the soluble phase of disrupted rabbit lamellar bodies at acid pH. Also, Hook and Gilmore [16] reported the existence of the same two enzymes at acid pH in whole rabbit lamellar bodies. On the other hand, other investigators have looked for phospholipase activity in rat lamellar bodies at neutral pH and found none [17,18]. However, even if these enzymes are normally present on the alveolar surface, it is difficult to reconcile the rapid degradation rates reported in this paper with the published turnover rates of surfactant.

In summary, our data indicate that there is phospholipase activity in rat pulmonary surfactant materials obtained by tracheal lavage. Thus, when the surfactant materials are incubated in vitro at 37°C, there is catabolism of the disaturated PC. The degradation occurs in all rat species tested, i.e., Sprague-Dawley, pathogen-free, and Long-Evans hooded rats. However, we could find no evidence of disaturated PC catabolism when alveolar lavage fluid from rabbits, mice, or guinea pigs are incubated in vitro, even though the lactate dehydrogenase levels in lavage fluid from these animals are as great as those from rats. The reasons for the degradation remain unknown, but these results should be taken into account when rat alveolar lavage materials are used for experiments.

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