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Alterations in rat alveolar surfactant phospholipids and proteins induced by administration of chlorphentermine

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Chlorphentermine is a cationic amphiphilic drug which produces a phospholipid storage disorder in rat lungs. Experiments were carried out to characterize changes in the composition of acellular alveolar lavage materials and to study possible mechanisms by which pulmonary surfactant phospholipidosis is produced by administration of the drug. Following ten daily injections of chlorphentermine (25 mg/kg body weight), there are 12.2- and 13.6-fold increases of pulmonary lavage total phospholipids and disaturated phosphatidylcholines (disaturated PC), respectively. In addition, there is a 2.8-fold increase in total protein and a 12.7-fold increase in the surfactant apoprotein group with molecular weights from 28000 to 32000. We measured incorporation of labeled palmitate, choline and glycerol into disaturated PC in type II cells and alveolar macrophages isolated from control and chlorphentermine-treated animals. The drug does not affect the incorporation of labeled substrates into disaturated PC in either cell type. However, in alveolar macrophages there is a decrease in the rate of intracellular degradation of recently synthesized disaturated PC in chlorphentermine-treated animals. The drug also inhibits the phospholipase-induced catabolism of rat surfactant disaturated PC which occurs during incubation of alveolar lavage fluid in vitro at 37°C. When the lavage fluid is divided into subfractions by differential centrifugation, a larger percentage of the phospholipid is distributed in the less sedimentable subfractions in chlorphentermine-treated animals relative to controls, suggesting the accumulation of older surfactant materials. These results suggest that chlorphentermine-induced phospholipidosis of pulmonary surfactant materials is due to decreased rates of phospholipid degradation.

Introduction

Chlorphentermine, an anorectic agent, is one of a group of cationic amphiphilic drugs which produces a phospholipid storage disorder in the lungs of laboratory animals [1–3]. Following repeated administration of the drug to rats, there is a decrease in body weight and a significant increase in lung weight, due mostly to accumulation of phospholipids [4]. The most striking changes in the lungs are an increase in the number of alveolar macrophages [3,5] and particulate debris [6] in the alveoli. Although the basic mechanism(s) responsible for the drug-induced phospholipidosis remains unknown, there is some evidence to indicate that phospholipid degradation is impaired [7,8].

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Recently, it has been reported that administration of chlorphentermine to rats causes an increase in the phospholipid levels of pulmonary surfactant materials [9]. Pulmonary surfactant is a mixture of lipids, protein and carbohydrate components which lines the alveoli and prevents their collapse by lowering surface tension forces. The major component is dipalmitoylphosphatidylcholine [10,11]. It is now generally accepted that the synthesis of surfactant occurs primarily in alveolar type II cells. There have been many studies directed at determining the mechanism involved in the removal, degradation and reutilization of surfactant materials. Some investigators have suggested that alveolar macrophages may be involved in these processes [12,13]. However, up to this point, the exact mechanisms remain unknown.

Since the mechanisms involved in the regulation of pulmonary surfactant levels are not clear and since administration of chlorphentermine results in an increase in surfactant phospholipids, the drug may be useful as a probe to study the metabolism of alveolar surface-active materials. The objectives of these experiments were: (1) to characterize changes in the composition of rat alveolar lavage fluid following administration of chlorphentermine, and (2) to study possible mechanisms by which the drug causes phospholipidosis in pulmonary lavage materials. A preliminary report of this work has appeared previously [14].

Methods

Isolation of pulmonary lavage materials, alveolar macrophages and type II cells. Male Sprague-Dawley rats (200–350 g) obtained from Charles River Laboratories, Inc. (Wilmington, MA) were used in all experiments. The animals were given daily injections of chlorphentermine hydrochloride dissolved in saline (25 mg/kg body wt., intraperitoneally, PreSate, Warner-Chilcott, Morris Plains, NJ) or equal volumes of saline for either 3 or 10 days. Since chlorphentermine is an anorectic agent, in some experiments (3-day treatment) a group of saline injected animals was pair-fed to make food intake equal to the drug-injected rats. These two groups of animals ate an amount of food equal to approx. 10% of their body weights each day and each lost an identical amount of weight during the 3-day treatment period (approx. 45 g). Control animals were fed ad lib and gained weight during this time (approx. 30 g). Following this treatment, pulmonary lavage materials, alveolar macrophages and alveolar type II cells were obtained from the animals.

The animals were anesthetized with sodium pentobarbital (0.2 g/kg body weight), exsanguinated by cutting the abdominal aorta, and the heart and lungs removed. Alveolar lavage materials were obtained by tracheal lavage (5 ml/g lung weight; three lavages) with Ca^{2+}-free phosphate-buffered medium (145 mM NaCl/5 mM KCl/9.35 mM Na_2HPO_4/1.9 mM NaH_2PO_4/5 mM glucose, pH 7.4). Alveolar macrophages were removed by centrifugation at $300 \times g$ for 5 min and the lavage fluid stored at 2°C until used for experiments. In some experiments, subfractions of alveolar lavage materials were obtained by differential centrifugation [15,16]. In order to obtain more alveolar macrophages from each animal, the lungs were lavaged nine more times with a total volume of 80 ml of Ca^{2+}-free phosphate-buffered medium according to the method of Myrvik et al. [17]. The alveolar macrophages were then separated from the fluid by centrifugation, washed twice in phosphate-buffered medium (containing 1.8 mM $CaCl_2$ and 1.0 mM $MgCl_2$), and resuspended in the same medium for use in all experiments.

Alveolar type II cells were isolated from both treated and control animals according to a method which we have described elsewhere [18]. Briefly, rats were anesthetized, the heart and lungs were removed en bloc, lungs were perfused with 0.9% NaCl to remove blood cells, and free alveolar macrophages were removed by tracheal lavage. The lungs were then filled with phosphate-buffered medium containing elastase (40 U/ml; type I; Sigma Chemical Co., St. Louis, MO) and DNAse (0.006%; Sigma) and incubated at 37°C for 30 min to free lung cells. Following enzymatic digestion, the lungs were minced with a tissue chopper, enzyme activity was arrested with fetal calf serum, cells were strained through nylon mesh of progressively smaller pore sizes (150, 330 and 440 mesh; New York Silk Stencil, New York, NY), and a population of cells enriched in alveolar type II cells was obtained by centrifugal elutriation. The

type II cell fraction was suspended in phosphate-buffered medium (with 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$ and 0.006% DNAse) for all experiments. The cells used in this study contained 88($\pm$1)% type II cells. Contaminating cells were leukocytes and alveolar macrophages. The number of cells in all suspensions was determined by using a Coulter Model Z_B electronic cell counter (Coulter Instrument Co., Hialeah, FL).

Incubation of cells and preparation of cells for analysis. Incorporation of [^{3}H]palmitate, [^{3}H]choline, or [^{14}C]glycerol into disaturated phosphatidylcholines in either type II cells or alveolar macrophages was measured. After isolation of the cells as described above, the cells were suspended in phoshate-buffered medium containing 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 0.1 mM palmitate (complexed with bovine serum albumin [19]), 0.05 mM choline chloride and 0.1 mM glycerol. In most experiments, the number of cells used was $1 \cdot 10^6$ per ml. At the start of the experiment, one of the following three radiolabeled substrates was added to the cell suspension; 1 μCi of 9,10-[^{3}H]palmitic acid (specific activity = 11.8 Ci/mmol), 1 μCi of [*methyl*-^{3}H]choline chloride (specific activity = 80 Ci/mmol), or 1 μCi of [1,3-^{14}C]glycerol (specific activity = 55.1 mCi/mmol). All labeled substrates were obtained from New England Nuclear Corp. (Boston, MA). Following incubation of these samples for 2–4 h at 37°C, lipids were extracted from samples of the cell suspensions and saved for analysis.

Measurement of disaturated PC and total phospholipids. The amount of total phospholipids in alveolar lavage fluid was measured as the phosphorus present in lipid extracts of the material [20]. Phospholipid content was obtained by multiplying lipid phosphorus values by 25 [21]. Disaturated PC was isolated from samples of lavage fluid according to the method of Mason et al. [22]. Both the total phospholipid and disaturated PC content of lavage materials were expressed as mg lipid per g of lung. Disaturated PC was also isolated from samples of alveolar macrophages and type II cells after incubation with the various labeled substrates. After isolation of the disaturated PC from the cell suspensions, Aquasol (10 ml; New England Nuclear Corp.; Boston, MA) was added to each sample and the samples were counted in a liquid-scintillation spectrometer (Model 3380; Packard Instrument Co.; Downers Grove, IL). The results of these experiments were expressed as nmol of substrate incorporated into disaturated PC.

Protein analysis. Total protein was determined by the method of Lowry et al. [23] with 1% sodium dodecyl sulfate added to reduce interference by lipids [24]. Sodium dodecyl sulfate (SDS) polyacrylamide slab gel electrophoresis [25] was used to analyze the protein composition of lavage materials. Two fractions of cell-free lavage fluid were analyzed: (1) the pellet from a 100 000 $\times$ *g*, 1 h centrifugation and (2) the resultant supernatant. Aliquots containing 25 μg of protein were dried under nitrogen in order to concentrate the samples. The residue was dissolved in electrophoresis sample buffer (0.0625 M Tris-HCl (pH 6.8)/10% SDS/10% glycerol). All the samples were reduced with 50 mM dithiothreitol.

The gels were 1.5 mm thick, the stacking gel was 2.5 cm long, and the separating gel was 6 cm long (Mighty Small Slab Gel Electrophoresis Unit, Hoefer Scientific Instruments, San Francisco, CA). The stacking gel contained 4% polyacrylamide in 0.125 M Tris-HCl (pH 6.8). The separating gel was 12% acrylamide in 0.375 M Tris-HCl (pH 8.8). Electrophoresis was carried out at a constant current of 30 mA/gel. The gels were stained with Coomassie blue.

Electrophoretic transfer of proteins from SDS-polyacryalmide gels to nitrocellulose paper was performed according to the method of Burnette [26] with modifications which are described by Wright et al. [27]. After electrophoretic transfer, the nitrocellulose paper was washed and treated as previously described. The primary antibody was polyclonal antisera raised in rabbits against isolated surfactant apoproteins. Surfactant apoproteins were isolated as described in Hawgood et al. [28] and consisted of a triplet of peptides of 38, 32 and 26 kDa [29–34]. We call this group of proteins pulmonary surfactant apoproteins 32 (pulmonary surfactant apoprotein). The rabbit anti-rat surfactant apoprotein antibody was fully absorbed against rat serum and showed no reactivity against rat serum on either rocket immunoelectrophoresis or on immunoblots. The secondary antibody, used to visualize the bound primary

antibody, was horseradish peroxidase-conjugated anti-rabbit IgG (Cappel Laboratories, Cochranville, PA). The horseradish peroxidase color was developed with the Bio-Rad Immunoblot Assay Kit (Bio-Rad Laboratories, Richmond CA).

Rocket immunoelectrophoresis [35] was used to quantitate the amount of antigenic surfactant apoprotein in the samples. Agarose gels containing 0.16% (v/v) rabbit anti-rat surfactant apoprotein antibody, 1% (w/v) agarose and 0.5% Triton X-100 (v/v) were poured 1.5-mm thick onto an 8.3 cm × 10.2 cm glass plates. Electrophoresis was carried out overnight at 2 V/cm at 15°C. The plates were dried and stained with Coomassie blue [35]. The rat surfactant apoprotein was purified as described by Hawgood et al. [28] and was used as a standard. The rocket heights were linear over the concentrations tested.

Results

Effects of chlorphentermine on total phospholipid and disaturated PC content of alveolar lavage materials

In order to determine the effects of chlorphentermine administration on the phospholipid content of pulmonary surfactant, the amounts of total phospholipid and disaturated PC in alveolar lavage fluid from drug-injected (10 daily injections) and control rats were measured. The results are shown in Table I. Administration of chlorphentermine leads to 12.2- and 13.6-fold increases over control in surfactant total phospholipids and disaturated PC, respectively. Thus, there is an increase in the disaturated PC content when expressed as a percent of the total phospholipids. Lung weights are also greater in chlorphentermine-treated animals, as has been previously reported [4].

Effects of chlorphentermine on total protein and surfactant apoprotein in alveolar lavage materials

The effects of chlorphentermine treatment on alveolar lavage proteins were examined in another group of animals (Table II). Chlorphentermine treatment results in a 2.8-fold increase in total protein in the lavage. In this treatment group, the drug caused only a 4.5-fold increase in total phospholipid of pulmonary lavage materials. We do not know why the increase in phospholipid in this group was less than in the other group (Table I). However, the protein levels in this group may have been even higher if the degree of drug-induced phospholipidosis had been as great as that shown in Table I.

The protein composition of the lavage material from chlorphentermine-treated and control animals is shown in the electrophoretogram in Fig. 1A. We divided the lavage material into a pellet from a 100000 × *g*, 1 h centrifugation and the resulting supernatant. Although the patterns of the proteins in the control and treated animals are similar, it appears that the pellets from the lavages of chlorphentermine-treated animals contain more material with molecular weight comparable to rat pulmonary surfactant apoprotein. Immunoblots with polyclonal antibody against rat

TABLE I

EFFECTS OF CHLORPHENTERMINE ON TOTAL PHOSPHOLIPID AND DISATURATED PC CONTENT OF ALVEOLAR LAVAGE MATERIALS

Total phospholipids were determined by measuring the phosphorus content of lipid extracts from alveolar lavage fluid and multiplying the values by 25. Disaturated PC was measured as the phosphorus levels (×25) in the disaturated PC fraction. Chlorphentermine-treated animals were given ten daily injections of chlorphentermine (25 mg/kg body wt.) and controls were injected with saline. The numbers shown are mean values ± S.E. for six experiments.

	Treatment of animals	
	control	chlorphentermine
Total phospholipid (mg/g lung)	0.92 (±0.07)	11.24 (±0.67)
Disaturated PC (mg/g lung)	0.42 (±0.04)	5.71 (±0.41)
Disaturated PC (% total phospholipid)	44 (±1)	51 (±1)
Lung wt. (g)	1.55 (±0.03)	1.99 (±0.06)

TABLE II

EFFECTS OF CHLORPHENTERMINE ON TOTAL PROTEIN AND SURFACTANT APOPROTEIN (PSAP 32) CONTENT OF ALVEOLAR LAVAGE MATERIALS

Pellets were obtained by centrifugation of cell-free alveolar lavage fluid at 100000 × *g* for 1 h. The supernatant was decanted and analyzed separately. Total protein was measured by the method of Lowry et al. [23]. Surfactant apoprotein was measured by rocket immunoelectrophoresis using isolated surfactant apoprotein as a standard. Chlorphentermine-treated animals were given ten daily injections of chlorphentermine (25 mg/kg body wt.) and controls were injected with saline. The numbers shown are mean values ± S.E. for three (control) or seven (chlorphentermine) different experiments. n.d., not detectable.

	Control	Chlorphentermine
Protein (mg/g lung)		
Pellet	0.36 (±0.03)	0.98 (±0.08)
Supernatant	1.96 (±0.22)	5.45 (±0.41)
PSAP 32 (mg/g lung)		
Pellet	0.03 (±0.01)	0.38 (±0.06)
Supernatant	n.d.	n.d.

surfactant apoprotein also suggest that lavage materials from chlorphentermine-treated animals contain an increased amount of pulmonary surfactant apoprotein (Fig. 1B). Pulmonary surfactant apoprotein 32 was not detectable in the supernatant fractions from either control or treated animals.

Rocket immunoelectrophoresis was used to quantitate the amount of pulmonary surfactant apoprotein in the lavage material. Although chlorphentermine treatment results in a 2.8-fold increase in total protein in the lavage, pulmonary surfactant apoprotein is increased by 12.7-fold (Table II). All of the antigenic material is found in the pellet obtained from a 100000 × *g*, 1 h centrifugation of lavage material. In control animals, the pulmonary surfactant apoprotein comprises approx. 10(+3)% (mean ± S.E., $n = 3$) of the total protein in the pellet. On the other hand, pulmonary surfactant apoprotein is 38(±5)% (mean ± S.E., $n = 7$) of the total protein in the pellet from lavage materials of chlorphentermine-treated animals. Thus, chlorphentermine treatment results in both an increase in the percentage and total amount of pulmonary surfactant apoprotein in lavage material. In addition, the increase in pulmonary surfactant apoprotein is greater than the increase in total protein. The remainder of the experiments

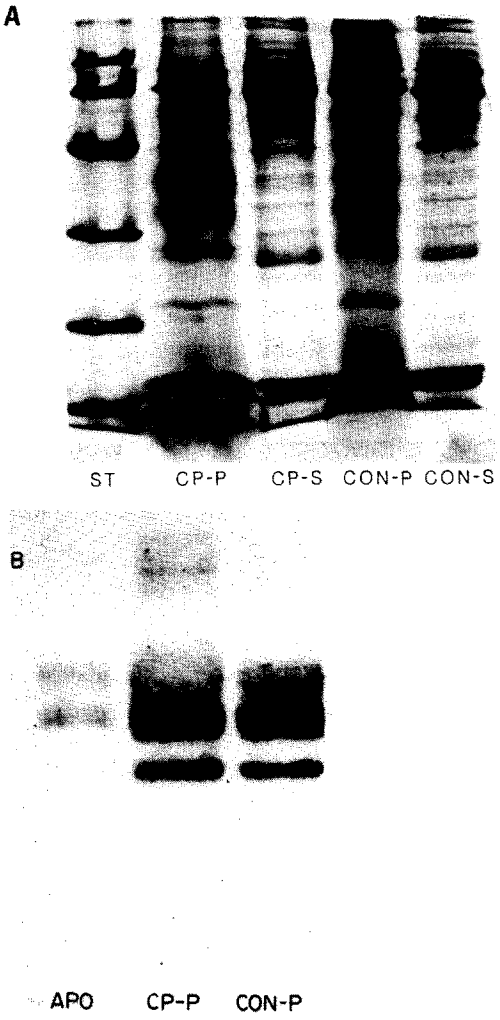

Fig. 1(A) Polyacrylamide slab gel electrophoretogram, stained with Coomassie blue, of pellets (100000 × g, 1 h) and supernatants from control and chlorphentermine-treated animals. ST, standards, from top to bottom: phosphorylase B, 94 kDa; albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor, 20.1 kDa; α-lactalbumin, 14.4 kDa. CP-P, pellet from lavage fluid of chlorphentermine-treated animals. CP-S, supernatant from lavage fluid of chlorphentermine-treated animals. CON-P, pellet from lavage fluid of control animals. CON-S, supernatant from lavage fluid of control animals. (B) Immunoblot stained with antibody against surfactant apoproteins. APO, isolated surfactant apoprotein. CP-P, pellet from lavage fluid of chlorphentermine-treated animals. CON-P, pellet from lavage fluid of control animals. The isolated apoprotein also contained a band of 28 kDa, which did not reproduce well after photography.

reported in this paper are designed to study possible mechanisms responsible for the drug-induced phospholipidosis.

Effects of chlorphentermine on incorporation of substrates into disaturated PC in alveolar macrophages and type II cells

One way in which chlorphentermine may increase the amount of surfactant disaturated PC is to accelerate its synthesis. Therefore, we measured the effects of the drug on incorporation of three substrates, palmitate, choline and glycerol, into disaturated PC in alveolar macrophages and type II cells. The effects of incubating the cells from untreated animals with chlorphentermine in vitro are shown in Table III. In these experiments, 0.1 mM chlorphentermine was used. At concentrations of drug greater than 0.1 mM, the viability of both cell types (as measured by oxygen consumption) was impaired. The results indicate that short-term incubation of either alveolar macrophages or type II cells with chlorphentermine has very little effect on the incorporation of labeled substrates into disaturated PC.

The effects of chlorphentermine administration to rats (three daily injections) on incorporation of substrates into disaturated PC in type II cells and alveolar macrophages isolated from drug-treated animals are shown in Table IV. (3-day treatment is sufficient to produce a substantial amount of phospholipidosis.) Since chlorphentermine has an anorectic effect, especially during the first 3 days of injections [36], a pair-fed group of rats was also included. Both the drug-injected and pair-fed groups lost approx. 45 g over the 3-day period, while control animals gained about 30 g. As a result of decreased food intake, incorporation of all three labeled substrates into disaturated PC is increased in type II cells. However, chlorphentermine has no effect beyond that of food deprivation. In alveolar macrophages, only labeled choline incorporation is increased due to starvation. In these cells, however, the drug produces an increase in incorporation of all three labels into disaturated PC beyond that produced by food deprivation. These results indicate that administration of chlorphentermine leads to an increase in net incorporation of labeled substrates into disaturated PC in alveolar macrophages but has no effect in type II cells.

TABLE III

EFFECTS OF CHLORPHENTERMINE ON INCORPORATION OF PALMITATE, CHOLINE AND GLYCEROL INTO DISATURATED PC (DSPC) IN ALVEOLAR MACROPHAGES AND TYPE II CELLS FROM CONTROL ANIMALS

Experiments were performed with the cells suspended in phosphate-buffered medium containing 0.1 mM palmitate, 0.05 mM choline, 0.1 mM glycerol, and the appropriate radiolabeled substrate. The concentration of chlorphentermine used was 0.1 mM. Measurements were made from samples of cell suspension (0.5 ml total volume) which contained $5 \cdot 10^5$ cells after an incubation period of 2 h (for incorporation of palmitate and choline) or 4 h (glycerol incorporation). The effects of chlorphentermine are expressed as percent control. The control values (nmol of labeled substrate incorporated/10^6 cells per h) are: palmitate, 1.13 ($\pm$0.15) for type II cells and 0.63 ($\pm$0.10) for alveolar macrophages; choline, 0.45 ($\pm$0.06) for type II cells and 0.19 ($\pm$0.04) for alveolar macrophages; glycerol, 0.011 ($\pm$0.002) for type II cells and 0.002 ($\pm$0.001) for alveolar macrophages. The numbers shown are mean values $\pm$ S.E. for five different experiments.

Substrate	Incorporation of labeled substrate into DSPC for each cell type (% control)	
	type II cells	alveolar macrophages
Palmitate	92 ($\pm$5)	104 ($\pm$6)
Choline	89 ($\pm$6)	120 ($\pm$8)
Glycerol	93 ($\pm$14)	104 ($\pm$11)

Effects of chlorphentermine on catabolism of disaturated PC

We have demonstrated in a prior paper [37] that both synthesis and catabolism of disaturated PC seem to occur simultaneously in alveolar macrophages. Therefore, it is possible that the increase in the net incorporation of labels into disaturated PC shown in Table IV is due to a chlorphentermine-induced decrease in the rate of disaturated PC catabolism in these cells. Experiments were performed to test this possibility. Alveolar macrophages from control, drug-treated, or pair-fed rats (six experiments each) were incubated with [^{14}C]choline for 2 h, centrifuged, washed free of choline, and resuspended in substrate-free medium. The amounts of [^{14}C]choline-labeled disaturated PC in the intra- and extracellular compartments were measured before and after a 3-h incubation period. During this period of time, none of the labeled disaturated PC appears in the

TABLE IV

EFFECTS OF CHLORPHENTERMINE ADMINISTRATION AND DECREASED FOOD INTAKE ON INCORPORATION OF PALMITATE, CHOLINE AND GLYCEROL INTO DISATURATED PC (DSPC) IN ALVEOLAR MACROPHAGES AND TYPE II CELLS

Rats were divided into three groups: control (fed ad. lib.), chlorphentermine-treated, and pair-fed animals. Chlorphentermine-treated animals were given three daily injections of chlorphentermine (25 mg/kg body wt.) and the other two groups were injected with saline. Alveolar macrophages and type II cells were isolated from the animals and incubated in phosphate-buffered medium containing 0.1 mM palmitate, 0.05 mM choline, 0.1 mM glycerol, and the appropriate radiolabeled substrate. Measurements were made from samples of cell suspension (0.5 ml total volume) which contained $5 \cdot 10^5$ cells after an incubation period of 2 h (for incorporation of palmitate and choline) or 4 h (glycerol incorporation). Results are expressed as a percent of incorporation in cells from control animals. The control values (nmol of labeled substrate incorporated/10^6 cells per h) are: palmitate, 1.00 ($\pm$0.07) for type II cells and 0.43 ($\pm$0.03) for alveolar macrophages; choline, 0.47 ($\pm$0.01) for type II cells and 0.33 ($\pm$0.09) for alveolar macrophages; glycerol, 0.010 ($\pm$0.003) for type II cells and 0.004 ($\pm$0.002) for alveolar macrophages. The numbers shown are mean values $\pm$ S.E. for six different experiments.

Cell type and substrate	Incorporation of labeled substrate into DSPC (% control)	
	chlorphentermine treatment	pair-feeding
Type II cells		
palmitate	137 ($\pm$22)	163 ($\pm$42)
choline	169 ($\pm$35)	159 ($\pm$25)
glycerol	158 ($\pm$20)	161 ($\pm$33)
Alveolar macrophages		
palmitate	232 ($\pm$12)	99 ($\pm$14)
choline	254 ($\pm$22)	172 ($\pm$13)
glycerol	148 ($\pm$16)	105 ($\pm$10)

extracellular compartment. In cells from control animals, $58 \pm 2\%$ of the label in intracellular labeled disaturated PC is lost. Incubation of alveolar macrophages from control animals with 0.1 mM chlorphentermine in vitro has no effect on disaturated PC catabolism ($59 \pm 3\%$ loss). On the other hand, there is a diminished amount of intracellular disaturated PC catabolism (only $25 \pm 5\%$ of label lost) in cells from drug-treated animals. Pair-feeding has no effect ($55 \pm 5\%$ loss). Therefore, the effect of chlorphentermine administration on alveolar macrophages may be a decrease in the rate of intracellular disaturated PC degradation.

In a previous communication [38], we reported that phospholipases are present in alveolar lavage fluid from rats so that when lavage fluid is incubated in vitro at 37°C, there is breakdown of the surfactant disaturated PC. Therefore, we studied the effects of chlorphentermine on this process. Incubation of lavage materials from control animals for 2 h results in disappearance of $57 \pm 7\%$ of the surfactant disaturated PC. When chlorphentermine is incubated in vitro with lavage fluid from control animals or when lavage materials from drug-treated rats is used, the amount of breakdown is greatly reduced (6 ± 1 and $8 \pm 3\%$, respectively). These results indicate that chlorphentermine can inhibit the phospholipase-induced degradation of surfactant disaturated PC.

Effects of chlorphentermine on the distribution of phospholipids in subfractions of alveolar lavage materials

It has been suggested by other investigators [16] that subfractions of alveolar lavage materials can be obtained by differential centrifugation such that smaller, less dense subfractions represent older materials while larger, more dense subfractions ar newer surfactant materials. We obtained pulmonary lavage fluid from control and chlorphentermine-treated rats and prepared four subfractions from each group of animals. Alveolar macrophages were removed from the lavage fluid by spinning at $300 \times g$ for 5 min. The cells were washed three times with phosphate-buffered medium and all supernatants from the washes were combined. A pellet, designated as P_1, was obtained by centrifuging the material from the cell washes. The cell-free lavage fluid was then spun at $1000 \times g$ for 20 min to obtain a pellet (P_2) and another supernatant. This supernatant was centrifuged at $60\,000 \times g$ for 60 min to obtain another pellet (P_3) and a supernatant (S_3). The order of the subfractions, from most to least sedimentable, is $P_1 > P_2 > P_3 > S_3$. The total phospholipid and disaturated PC content of each subfraction was measured and the results are shown in Table V. There is a larger percentage of total phospholipid and of disaturated PC distributed in the less sedi-

TABLE V

EFFECTS OF CHLORPHENTERMINE ON THE PERCENT DISTRIBUTION OF TOTAL PHOSPHOLIPIDS (PL) AND DISATURATED PC (DSPC) IN SUBFRACTIONS OF ALVEOLAR LAVAGE MATERIALS

Alveolar macrophages were removed from the lavage fluid by spinning at $300 \times g$ for 5 min. The cells were washed three times by alternate spinning and resuspension (at $300 \times g$) and all supernatants from the washes were combined. The material obtained from the washes was spun (at $12000 \times g$) to form a pellet (P_1). The cell-free lavage fluid was centrifuged at $1000 \times g$ for 20 min. The pellet obtained in this manner was designated P_2. Then, the supernatant from the $1000 \times g$ spin was centrifuged at $60000 \times g$ for 1 h in order to obtain another pellet, P_3, and supernatant, S_3. Thus, the order of materials from most to least sedimentable is $P_1 > P_2 > P_3 > S_3$. Total phospholipid and disaturated PC were determined by multiplying the lipid phosphorus values by 25. Total phospholipid is expressed as the percent in each subfraction for each group of animals. Disaturated PC is expressed as a percent of the total amount of phospholipids in each subfraction. The ratios of chlorphentermine to control total phospholipids and disaturated PC are also shown. Chlorphentermine-treated animals were given ten daily injections of drug (25 mg/kg body wt.) while the controls were given saline. The numbers shown are mean values ± S.E. for six different experiments.

Subfraction	Total PL (%)			DSPC (% total PL)		
	control	chlorphentermine	chlorphentermine/control	control	chlorphentermine	chlorphentermine/control
P_1	8 (±1)	11 (±1)	1.38	4 (±1)	6 (±1)	1.50
P_2	28 (±1)	8 (±1)	0.29	12 (±1)	4 (±1)	0.33
P_3	47 (±2)	29 (±5)	0.62	24 (±2)	18 (±5)	0.75
S_3	17 (±2)	52 (±4)	3.06	8 (±1)	30 (±2)	3.75

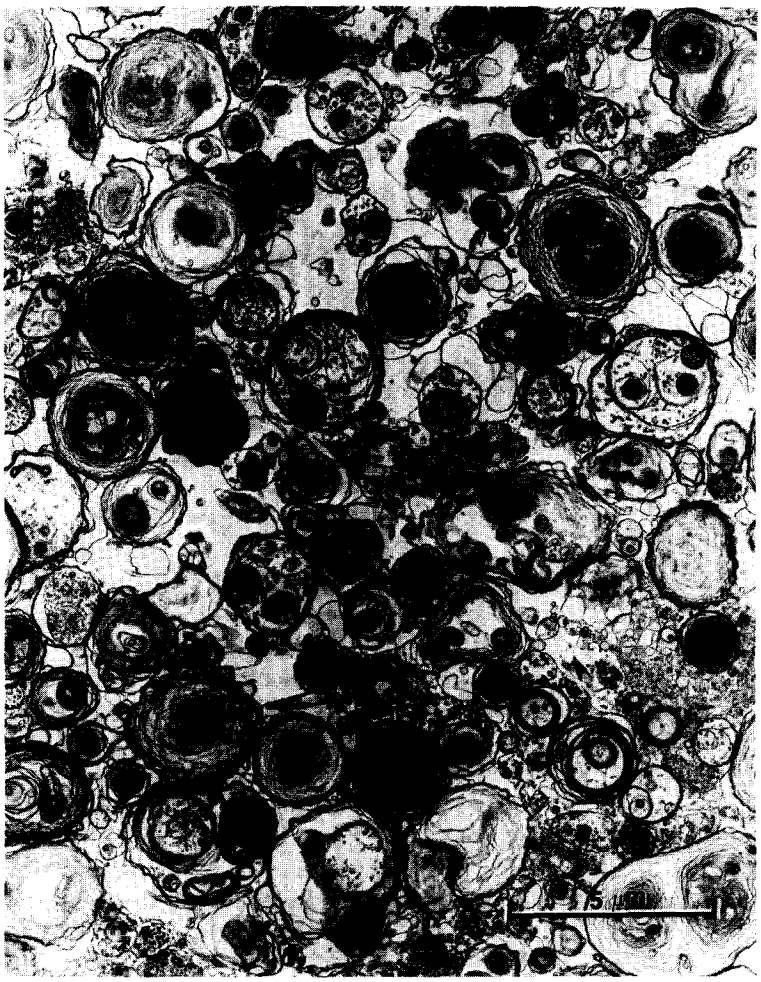

Fig. 2. Electron micrograph of subfraction P_1 from alveolar lavage materials from a chlorphentermine-treated rat. Alveolar lavage fluid was spun at $300 \times g$ for 5 min to remove alveolar macrophages. The cells were washed three times with phosphate-buffered medium and all supernatants were combined. This material was spun at $12000 \times g$ for 10 min to form a pellet. Small pieces (approx. 1 mm^3) of the pellet were fixed for 2 h in cold (4°C) buffered 2% osmium tetroxide, washed in four changes of cacodylate buffer, and treated for 2 h in 2% tannic acid. The specimens were then dehydrated in ethanol and embedded in Epon (LX-112). Ultrathin sections (700–900 Å) were cut and stained with uranyl acetate and lead citrate. The magnification is ×10000.

mentable subfractions in lavage materials from chlorphentermine-treated animals relative to controls. One possible explanation for these data is that there is an accumulation of older surfactant materials following administration of chlorphentermine.

We were interested in characterizing the morphologic appearance of materials obtained from washing alveolar macrophages (P_1). Because there is more of this material in lavage from chlorphentermine-treated animals, we used it. An electron micrograph is shown in Fig. 2. This subfraction of lavage fluid appears to contain lamellar-type structures, trace amounts of tubular myelin, and vesicular structures. The pellet is also relatively rich in disaturated PC; i.e., 55($\pm$1)% of the phospholipid found here is disaturated PC. A subfraction similar to this one can also be obtained from lavage materials of control rats, but in much smaller quantities (data not presented). Therefore, lavage fluid from several control rats must be used in order to have enough lipids for analysis and electron microscopy.

Discussion

The results of this paper demonstrate that administration of chlorphentermine to rats produces an increase in the phospholipid and protein content of alveolar lavage materials. There appears to be a greater relative increase in disaturated PC than in total phospholipids and a greater increase in pulmonary surfactant apoprotein than in total protein. The phospholipidosis does not seem to be caused by an increase in the incorporation of substrates into disaturated PC in either alveolar macrophages or type II cells. On the other hand, administration of chlorphentermine leads to a decrease in the rate of disaturated PC degradation in alveolar macrophages and in alveolar lavage fluid during incubation at 37°C in vitro. Treatment of the animals with this drug also leads to accumulation of phospholipids in less sedimentable subfractions of lavage materials relative to control. These data suggest that chlorphentermine-induced phospholipidosis of surfactant materials is due to decreased rates of phospholipid degradation.

Other investigators have suggested that an impairment in phospholipid degradation is involved in the production of phospholipidosis by cationic amphiphilic drugs in various tissues [7,8]. It has been shown that these drugs inhibit lysosomal phospholipases [39]. Furthermore, it has been proposed that cationic amphiphilic drugs bind to phospholipids and the resulting complex is resistant to phospholipase activity [2,8]. In this regard, we have demonstrated that following administration of chlorphentermine to rats, some of the drug is bound to surfactant phospholipids [40]. Exactly when and how chlorphentermine becomes bound to alveolar phospholipids is not known. In any event, these data, as well as data from our present paper, are consistent with the idea that chlorphentermine is bound to surfactant phospholipids and that this complex is resistant to pulmonary phospholipases.

Administration of chlorphentermine to rats partially inhibits degradation of disaturated PC in alveolar macrophages and in alveolar lavage fluid incubated at 37°C. The inhibition can be demonstrated by addition of the drug to lavage fluid from control animals in vitro, but incubation of chlorphentermine with alveolar macrophages in vitro has no effect on disaturated PC degradation. This is probably due to the fact that chlorphentermine binds rapidly to surfactant disaturated PC during incubation with alveolar lavage materials [40]. However, catabolism of disaturated PC by alveolar macrophages is an intracellular process and inhibition would require accumulation of chlorphentermine at the site of degradation. It is possible that the drug does not gain access to this site over the relatively short incubation periods used.

Can these effects of chlorphentermine be related to the normal metabolism of pulmonary surfactant disaturated PC? The steps involved in surfactant metabolism probably include synthesis, reutilization, and degradation or removal. It is well known that synthesis of disaturated PC occurs primarily in type II cells and then it is released on to the alveolar surface. Our data suggest that chlorphentermine has no effect on disaturated PC synthesis. However, it should be pointed out that the population of type II cells obtained from drug-treated animals may differ from that of control animals. Thus, data from experiments dealing with synthesis of disaturated

PC (Table IV) should be interpreted with this in mind. Following synthesis and release, much of the phospholipid is then recycled [41–43]. In fact, the disaturated PC seems to be reutilized intact with greater than 90% efficiency [42]. The effect that administration of chlorphentermine has on reutilization of surfactant disaturated PC is not known. However, since chlorphentermine binds to phospholipids, this process may be affected.

Although the mechanism(s) responsible for surfactant disaturated PC degradation has not been positively identified, there are at least two possibilities; (1) catabolism in alveolar macrophages and (2) enzymatic degradation on the alveolar surface. Several investigators have suggested a role for alveolar macrophages in surfactant catabolism [44,45]. In this paper, we have shown that chhlorphentermine inhibits the intracellular degradation of recently synthesized disaturated PC in alveolar macrophages. Furthermore, it has been reported that following administration of chlorphentermine to rats the drug is found in high concentrations in alveolar macrophages [46]. We reported previously the existence of phospholipases in rat alveolar lavage materials [38]. Others have reported phospholipase activity in lamellar bodies [47,48]. Thus, it is possible that some catabolism takes place on the alveolar surface and we have demonstrated here that chlorphentermine might inhibit this process. Therefore, chlorphentermine is an effective inhibitor at both of these two possible sites for disaturated PC degradation. Also, the average rate of surfactant disaturated PC accumulation in drug-treated animals is 20–30% of the steady-state turnover rate calculated from tracer studies [49]. Therefore, an inhibition in the degradation rate of only 20–30% can account for this accumulation, if the secretion rate remains constant.

Magoon et al. [16] have suggested that subfractions of alveolar lavage materials, which can be obtained by differential centrifugation, represent surfactant materials at different stages in the lifecycle. Larger, more sedimentable material may be newer surfactant while smaller, less sedimentable material may be older. Although administration of chlorphentermine increases the amounts of phospholipids in all subfractions, the percent distribution is altered. In chlorphentermine-treated animals, there is a larger percentage of phospholipids distributed in the non-sedimenting subfraction relative to control. One possible explanation for this is accumulation of older materials which are not degraded due to the presence of drug. Once the older materials accumulate, there may be a build up of phospholipids in all subfractions.

We obtained the most easily sedimented subfraction of the alveolar lavage materials by washing the alveolar macrophage pellet three times. It contains structures which look like lamellar bodies, trace amounts of tubular myelin, and vesicular structures. It may be enriched in the newest surfactant (Fig. 2). This subfraction is easily obtained from chlorphentermine-treated rats due to the phospholipidosis. We were also able to demonstrate this subfraction in untreated rats by using several animals in order to collect enough material for analysis. This finding supports the idea that the most sedimentable materials are the most recently released surfactant.

Chlorphentermine treatment also produces an increase in total protein and surfactant apoproteins in the lavage fluids of treated rats. The increase in total protein (2.7-fold) is much less proportionally than the increase in total phospholipid (12.2-fold). However, the increase in the surfactant apoprotein is 12.7-fold. These results suggest that surfactant apoprotein metabolism may also be affected by chlorphentermine treatment. Very little is known about apoprotein metabolism or the potential relationships between apoprotein and phospholipid metabolism. The mechanism of the effects of chlorphentermine on apoprotein and total protein metabolism will require further investigation. These results do suggest that chlorphentermine may have effects on surfactant apoprotein metabolism that are different from its effects on the metabolism of other proteins found in lavage fluid.

One interesting finding from our experiments is not related to the effects of chlorphentermine. Following 3 days of reduced food intake, the rates of incorporation of labeled palmitate, choline and glycerol into disaturated PC in isolated alveolar type II cells were increased. It has been reported that food deprivation for 2–3 days results in decreased amounts of surfactant phospholipids

[50,51]. We obtained a similar result with our pair-fed animals (data not presented). One possible explanation is that food deprivation results in decreased amounts of surfactant phospholipids due to limited substrate availability. The increased net incorporation of substrates into disaturated PC which we measured in isolated type II cells may represent a compensatory mechanism, i.e., an attempt by the cells to increase the amount of available surfactant. Alternatively, the experimental restoration of precursor pools following their depletion during food deprivation may result in greater specific activities in these cells and, thus, more label being incorporated into disaturated PC.

In summary, administration of chlorphentermine to rats results in increased amounts of surfactant total phospholipids, disaturated PC, total lavage protein, and surfactant apoproteins on the alveolar surface. The phospholipidosis does not appear to be due to increased rates of disaturated PC synthesis. However, the results do suggest that the drug-induced accumulation of surfactant phospholipids is due to decreased rates of degradation.

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