The effect of colchicine and vinblastine on the release of pulmonary surface active material

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Abstract The secretion of phosphatidylcholine was studied by incubating hamster lung slices which had been prelabeled by the in vivo administration of ^{14}C-labeled choline. The release of ^{14}C-labeled phospholipid into the medium continued for 2 hr. The specific activity of phosphatidylcholine in the medium was one-third that found in the tissue, and the fatty acid composition of the released phosphatidylcholine corresponded to that of surfactant. The prior injection of colchicine resulted in a 60% inhibition of phosphatidylcholine release into the incubation medium. A similar effect was obtained when vinblastine was administered. Colchicine demonstrated no inhibiting effect on the release of protein from lung slices which had been prelabeled by the administration of ^{14}C-labeled leucine. The possible function of the microtubular system in the secretion of surface active components is discussed.

Supplementary key words secretion • phosphatidylcholine • surfactant protein • lung slices

The biosynthesis of disaturated phosphatidylcholine, the major component of the pulmonary surface active material (1, 2) has been investigated in numerous laboratories (3–5). Studies of the turnover rate have shown that the lipid and protein are continuously secreted (4, 6). However, the mechanism by which they are released into the alveolar space remains to be defined. The rate of release of plasma lipoprotein phospholipid from liver slices has been studied to assess the transport mechanism(s) in this tissue (7). Recent investigations have suggested that the microtubular system may function in a number of secretory processes including the release of lipoprotein from the liver (8). The purpose of this study was to investigate the mechanism(s) involved in the secretion of surfactant by lung tissue and to ascertain the possible role of the microtubular system in this process. An abstract of these results has already appeared[1].

METHODS

Male golden hamsters, weighing 105–115 g, which had been fasted overnight were employed in this study. The animals were pretreated by the intraperitoneal injection of 0.15 M NaCl containing various concentrations of colchicine or vinblastine. The total dose was given in three separate, equal injections at 4, 3, and 1.5 hr prior to killing the animals. The control animals were injected with 0.15 M NaCl. Three hr before killing [1,2-^{14}C]choline was injected intraperitoneally in 0.1 ml portions of 0.15 M NaCl. In the experiments in which the release of protein was demonstrated, the [1-^{14}C]-leucine was injected into the jugular vein 3 hr before removal of the lungs. The hamsters were anesthetized by intraperitoneal injection of 15 mg of Nembutal. The lungs were perfused with 30 ml of 0.15 M NaCl through the pulmonary artery to remove the remaining blood from the vascular bed. The lungs were then excised and lavaged with 0.15 M NaCl. For the in vitro experiments, the tissue was sliced with a Stadie-Riggs microtome and 90 mg tissue aliquots were transferred to 1 ml of oxygenated Krebs-Ringer bicarbonate buffer, pH 7.4. In the time course experiments this procedure was modified by the incubation of 200 mg of tissue in 2 ml of buffer. The slices obtained from the colchicine treated animals were incubated in buffer containing colchicine at the concentrations indicated (**Table 2**). After incubation, the medium was removed and centrifuged for 5 min at 700 *g* to remove tissue fragments. The slices were homogenized in 1 ml of 0.15 M NaCl. The homogenates, media, and lavages were separately extracted by the method of Folch, Lees and Sloan-Stanley (9). The lipid extracts were evaporated under N_2 and redissolved in 5 ml of chloroform–methanol 2:1. Aliquots were assayed for radioactivity by liquid scintillation spectrometry. The lipids were fractionated by thin-layer chromatography on silica gel H plates employing chloroform–methanol–acetic acid–water 75:25:1:3 as the developing solvent. The different molecular species of phosphatidylcholine were separated by argentation chromatography (10). The fatty acid pattern of the phosphatidylcholine in the tissue and medium was determined using a Packard Model 565 gas

[1] Delahunty, T. J., and J. M. Johnston, 1975. *J. Amer. Oil Chem. Soc.* **52**: Abstract No. 111.

TABLE 1. Effect of colchicine on the in vivo incorporation of [^{14}C]choline into lung, liver, and lavage saturated phosphatidylcholine

Treatment	Lung	Liver	Lavage	No. of Animals
	cpm/mg Phosphatidylcholine × 10^3			
Saline	12 ± 3	28 ± 6	10.6 ± 2.6	6
Colchicine (9 mg)	47 ± 13[a]	27 ± 9	8.1 ± 3.4	6

25 μCi of [^{14}C]choline was administered intraperitoneally to each hamster. After 3 hr the animals were killed and the specific activity of the saturated phosphatidylcholine was determined. The results are expressed as the mean ± SD. Colchicine was administered as described in the Methods section.

[a] Statistically different from controls ($P < .01$).

chromatograph (Packard Instrument Co., Downers Grove, Ill.) employing a column coated with 10% Supelco SP-222-P-S at 185°C (Supelco, Inc., Bellefonte, Pa.). The protein radioactivity was determined by precipitation of the protein of the media and tissue homogenate with 10% trichloroacetic acid followed by centrifugation at 700 *g* for 10 min. The precipitate was suspended in 0.5 ml of 1 N NaOH and the protein was quantified by the method of Lowry et al. (11). The protein associated with the phosphatidylcholine was obtained by differential centrifugation. The medium was adjusted to 1.1 g/ml with sodium bromide and centrifuged for 16 hr at 100,000 *g* in a total volume of 15 ml. Since the density of pulmonary surface active material has been reported to be approximately 1.09 g/ml (12), the upper 2 ml was considered to contain the d < 1.1 lipoprotein complex, and the lower 2 ml fraction in each tube the secretory products of d > 1.1. The phospholipid to protein ratio of the d < 1.1 fraction was found to be 5 times higher than the d < 1.1 fraction, indicating that this procedure removed contaminating protein from the lipoprotein complex in the medium. A similar ultracentrifugation technique is utilized for isolating the plasma lipoproteins (13). Protein radioactivity and total lipid phosphorus were determined by methods previously described (14, 15). Liquid scintillation spectrometry was performed in a Beckman LS-250 (Beckman Instruments, Fullerton, Cal.). The [1,2-^{14}C]choline (10 mCi/mmole) and [1-^{14}C]L-leucine (55 mCi/mmole) were from Schwarz-Mann, Orangeburg, N.Y. Colchicine and vinblastine were obtained from Sigma Chemical Corp., St. Louis, Mo. Lactate dehydrogenase was assayed using the kit supplied by Worthington Biochemical Corp., Freehold, N.Y.

RESULTS

In **Table 1** are shown the specific activities of saturated phosphatidylcholine isolated from the lungs and livers of control and colchicine-treated hamsters. In this experiment the ^{14}C-labeled choline was administered 3 hr before killing and a total of 9 mg of colchicine was given as described in the Methods section. The table clearly shows that the specific activity of the saturated phosphatidylcholine in the lungs of colchicine-treated animals was significantly greater than that

TABLE 2. Effect of colchicine and vinblastine on the release of [^{14}C]-labeled phosphatidylcholine from lung slices

Treatment	Injected Dose mg	Conc. in Medium	No. of Incubations	^{14}C-Labeled Phosphatidylcholine in Medium	Significance	Inhibition
		μM		*cpm*		%
Saline			19	537 ± 100		
Colchicine[a]	3	0.25	19	437 ± 114	$P < 0.01$	18.6
Saline			24	544 ± 75		
Colchicine[a]	6	0.5	23	405 ± 100	$P < 0.01$	25.5
Saline			7	442 ± 40		
Colchicine	12	1.0	8	181 ± 24	$P < 0.01$	59.0
Saline			7	471 ± 74		
Colchicine		1.25	7	411 ± 87	N.S.	12.7
Saline			8	556 ± 98		
Vinblastine	12	0.44	8	415 ± 68	$P < 0.01$	25.3

Each incubation consisted of 90 mg of hamster lung slices containing prelabeled ^{14}C-labeled phosphatidylcholine in 1 ml of Krebs-Ringer bicarbonate buffer, pH 7.4. The incubation time was 2 hr at 37°C. The radioactivity in the medium was corrected for variations in total lung tissue radioactivity between individual hamsters and is presented as the mean cpm ± SD. The incubations with control lung tissue were performed simultaneously with those of the treated animals.

[a] The data from three separate experiments were averaged at the 3 and 6 mg dose level.

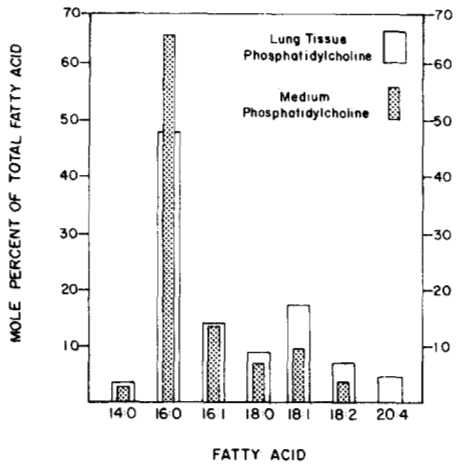

Fig. 1. Fatty acid composition of phosphatidylcholine isolated from hamster lung slices and from the medium following 2 hr of incubation. After methanolysis, the fatty acid methyl esters were quantified by gas–liquid chromatography. Each block represents the mole percentage of the fatty acid in tissue or medium.

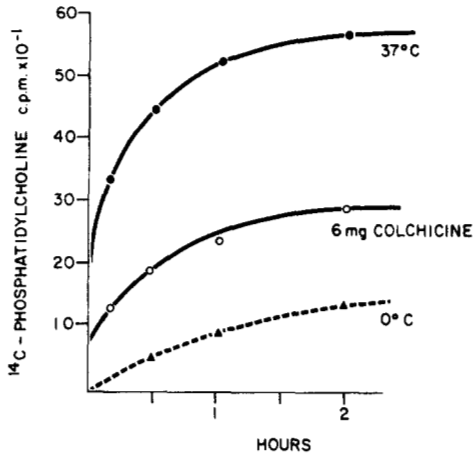

Fig. 2. The time course of the release of ^{14}C-labeled phosphatidylcholine from 200 mg of lung slices incubated in 2 ml of Krebs-Ringer buffer, pH 7.4. The solid and dashed lines represent secretion at 37°C and 0°C, respectively. Open circles signify the release of the labeled phospholipid from lung slices following treatment of the animals with 6 mg of colchicine. The radioactivity measured at each time point was adjusted for the concentration effect of removal of the aliquots taken at earlier times (0.2 ml each). The ^{14}C-labeled phosphatidylcholine in the medium at 2 hr was 5% of the total labeled phosphatidylcholine in the tissue slice. The radioactivity represented by the points on the control and colchicine curves is the mean of four separate analyses from two experiments, both of which showed the same pattern.

in the controls. In contrast, colchicine had no effect on the labeling of this phospholipid class in the liver. The observed effect in the lung would therefore not appear to be attributable to an effect of the drug on phospholipid biosynthesis. The ^{14}C-labeled phosphatidylcholine in the alveolar lavages was also analyzed, but no significant difference in the specific activity was found between control and colchicine treated animals.

The fatty acid composition of phosphatidylcholine in the medium and lung slices after a 2 hr incubation is illustrated in **Fig. 1.** The phosphatidylcholine of the medium had a relatively high content of palmitic acid (16:0) and a distinctly different fatty acid profile from that observed in the tissue phosphatidylcholine. These results are consistent with the conclusion that the lung slices were releasing phosphatidylcholine of a type found in the pulmonary surface active material (16). Lactate dehydrogenase was also assayed in the incubation medium as an index of tissue autolysis (17). The maximum release of the enzyme was 4% of the total tissue homogenate content, indicating that minimal breakdown of the tissue occurred during the incubation.

The mechanism of secretion of phosphatidylcholine from lung slices was investigated. The tissue phosphatidylcholine was prelabeled by the injection of 25 μCi of ^{14}C-labeled choline 3 hr prior to killing the animal. The release of labeled phosphatidylcholine into the incubation medium was monitored for 2 hr. The rate of secretion of the phospholipid at 37°C and 0°C is illustrated in **Fig. 2** by the data depicted by the closed circles and dashed line, respectively. The temperature dependence of the release process is evident. The administration of 6 mg of colchicine, divided into three equal doses beginning 4 hr prior to killing, resulted in a decreased rate of release of phosphatidylcholine compared to controls (open circles, Fig. 2).

The effect of various doses of colchicine on the secretion of labeled phosphatidylcholine is presented in Table 2. It is apparent that an increased inhibition of secretion occurred with increasing amounts of colchicine administration. Moreover, it is clear that prior treatment of the animals with colchicine was required to effect a reduction in secretion. As can be seen in Table 2, on a molar basis, vinblastine was equally as effective as colchicine. The former compound is representative of a second class of microtubule inactivators. In addition to the observed effect on phosphatidylcholine release, an increase in the isotope labeling of phosphatidylcholine was noted in the lung tissue slices obtained from colchicine-treated animals (cf. Table 1). This finding further supports the concept that the drug was affecting the release phenomenon.

The protein in the lung slices was labeled by pretreating the animals with ^{14}C-labeled leucine and the radioactivity of the released ^{14}C-labeled protein in the incubation medium was determined. No difference was found between control and colchicine-treated (6 mg) animals (989 $\pm$ 26 cpm compared to 986 $\pm$ 23 cpm in the medium of the two groups, respectively).

Table 3 shows that in the ^{14}C-labeled choline pretreatment experiments, the specific activity of phosphatidylcholine in the medium was less than that in the tissue. In contrast, the specific activity of the total ^{14}C-labeled protein in the medium was not less than that in the tissue. Because of the apparent heterogeneity of the proteins released into the medium, the specific activity of the protein component of the $d < 1.1$ fraction was assayed and found to be 2.5 times greater than the tissue protein (**Table 4**). Colchicine treatment had no effect on the protein specific activity.

TABLE 3. Phosphatidylcholine and protein specific activities in the medium and lung slices prelabeled with [^{14}C]choline or [^{14}C]leucine

	Specific Activity	
	Medium	Tissue
	cpm/μg	
^{14}C-Labeled phosphatidylcholine	8.6 ± 1.65	24.5 ± 4.35[a]
^{14}C-Labeled protein	1.7 ± 0.23	1.4 ± 0.12

The incubation conditions were the same as described in Table 2, except that for the specific activity analysis of the protein the slices were prelabeled with 25 μCi of [^{14}C]leucine rather than [^{14}C]choline. The results given in the table are an average of 16 experiments.

[a] Significantly different from the medium specific activity ($P < 0.01$).

TABLE 4. Protein specific activities of the medium and slices prelabeled with [^{3}H]leucine

	Protein in Medium		Protein in Tissue
	Density < 1.1	Density > 1.1	
	cpm/μg		
Control	1.28 ± 0.11[a]	0.66 ± 0.12	0.53 ± 0.05
Colchicine (12 mg)	1.27 ± 0.30[a]	0.75 ± 0.07	0.50 ± 0.08

Lung slices (400 mg) prelabeled with 100 μCi of [^{3}H]leucine were incubated for 2 hr. The medium was subfractionated by ultracentrifugation at density of 1.1 gm/ml. The specific activities were averaged from a total of ten separate incubations. The quantities of protein in the d < 1.1 and d > 1.1 fractions were 190 μg and 1400 μg respectively.

[a] Significantly different from the tissue protein specific activity ($P < 0.01$).

DISCUSSION

The present study confirms the utility of using lung slices to study the secretion of pulmonary phosphatidylcholine. The fatty acid composition of the phosphatidylcholine released into the medium was similar to that reported for pulmonary surface active material (16). This finding, together with the results of the lactate dehydrogenase assay and the time and temperature dependence of the release, suggests that the appearance of phospholipid in the medium is not the result of cellular damage. The pattern of release was similar to that obtained by other investigators who have studied the secretion of plasma low density lipoprotein from liver slices (7, 18). The use of liver slices containing prelabeled phospholipid in the earlier study (7) afforded the opportunity to study the rate of phosphatidylcholine release directly. Recently Redman et al. (19) have shown that colchicine can inhibit the release of plasma proteins from liver slices. Lung slices have also been used to demonstrate secretion of labeled protein (20).

The increased specific activity of the saturated phosphatidylcholine in the lungs of colchicine-treated animals (Table 1) could be the result of an inhibited secretion of the phospholipid from the pneumocyte caused by the drug. It is unlikely that the effect was due to an increased synthesis of phospholipid since the specific activity in the livers of the same animals was unaffected, a finding which is consistent with the results of Stein and Stein (8). Since the phosphatidylcholine in the alveolar spaces is most likely subject to reutilization by the epithelial cells or macrophages (21, 22) and since there appeared to be considerable variation between individual animals with regard to the recovery of lavage material (Table 1), the effect of colchicine on the secretion process was investigated in the in vitro slice system. The results confirm that colchicine pretreatment caused an inhibition of phosphatidylcholine release from the pneumocyte.

The function of microtubules in many different secretory cells has been described (8, 19, 23–25). Although there is as yet no published evidence that microtubules are associated with pneumocyte granules, tubular structures have been observed in electron micrographs of pulmonary Type II cells[2]. The present report suggests that the pneumocyte secretes its complement of surfactant lecithin via the microtubular system. This seems likely since two known inactivators of microtubular function, vis. colchicine and vinblastine (26) inhibit this process. A preliminary study with a related compound, Colcemid, showed an accumulation of dipalmitoyl phosphatidylcholine in rat lung and lavage which was apparently due to a depletion of macrophages (22).

The finding that the secretion of the protein associated with the d < 1.1 fraction of the medium was not affected by colchicine treatment suggests that the release of phospholipid and its associated protein may occur by different mechanisms. This concept is further supported by a comparison of the specific activities of the phospholipid and protein. The phosphatidylcholine released into the medium appeared to come from a sequestered compartment with a relatively low specific activity compared to the total intracellular phosphatidylcholine. This finding is in agreement with the observation that discrete lamellar bodies containing phospholipid exist within the pneumocyte (27). On the other hand, the finding that the protein associated with the d < 1.1 fraction had a higher specific activity than the total tissue protein suggests that the protein of surfactant was derived from an intracellular compartment in which the turnover was more rapid than that of the total intracellular pool. This concept is in agreement with the findings of Gil and Reiss (27) who suggested that the protein component of alveolar surfactant did not originate solely in the lamellar bodies. The protein in the medium probably corresponds to that normally secreted into the alveolus since earlier experiments indicated that lung slices retained metabolic control over protein secretion during this period (20).

The recent suggestions by Shelley, L'Heureux, and Balis (28) that there may be no specific protein associated with the phosphatidylcholine in surface active material is not inconsistent with the concept that phospholipid is secreted independently of protein release. However, further experiments on the purification and characterization of the alveolar lipoproteins may show that small amounts of a specific peptide

[2] Personal Communication (Dr. Gloria Massaro).

are bound to the phosphatidylcholine. The concept that the protein and lipid components of surfactant are made in different types of alveolar cells (29) is also not excluded by the findings presented here. Further experiments with isolated cell types are currently in progress to delineate the role of the Type II cell in the synthesis and secretion of surfactant.

Note added in proof

It was recently observed that the ingestion of cholchicine to commit suicide resulted in respiratory distress syndrome and death in an adult woman [Annals of Internal Medicine **83**: 523–524, 1975]. The respiratory distress preceded cardiovascular *collapse* lending credence to the concept reported here in that the microtubular system is involved in the secretion of surface active material in the human lung.

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