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Transport and Secretion of Lung Surfactant Phospholipids

— Effect of Colchicine —

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The roles of the microtubular system in the transport and secretion of surfactant phospholipids in the lung were studied using colchicine-treated rats, who were administered *in vivo* by (methyl-^{3}H) choline and (2-^{3}H) glycerol. The radioactivities of phosphatidylcholine, disaturated phosphatidylcholine and phosphatidylglycerol were measured in four lung fractions, i.e., lung parenchyma, microsomes, lamellar bodies and alveolar wash, of colchicine-treated animals. The prior injection of colchicine resulted in marked increases in the labels of the phospholipids in microsomes as well as in lamellar bodies, and in the decrease in alveolar wash. These findings suggest that the microtubular system participates not only in the secretion of lamellar bodies into the alveolar space, but also in the translocation of surfactant phospholipids from the synthetic site, i.e., microsomes to lamellar bodies of the store house.

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Key words : Dipalmitoyl phosphatidylcholine, Lung surfactant, Microtubulus, Colchicine

1 Introduction

Dipalmitoyl phosphatidylcholine and phosphatidylglycerol are the main constituents of pulmonary surfactant[1], which has been implicated in the maintenance of lung compliance[2]. It is known that the pulmonary surfactant phospholipids are synthesized in the endoplasmic reticulum of alveolar type II cells, transferred to lamellar inclusion bodies and then secreted into alveolar spaces[3]. However, the mechanisms of the intracellular transport and secretion process of surfactant phospholipids remain unsolved. Phospholipid exchange proteins which present in cytosol and catalyze in the intracellular transfer of phospholipids, have been demonstrated in several mammalian species[4-6]. In the lung, the occurrences of such soluble proteins have been described for phosphatidylcholine[7,8] and phosphatidylglycerol[9,10]. On the other hand, Chevalier and Collet[3] showed the presence of a small lamellar body form as the vehicle of phosphatidylcholine transport between the Golgi complex and the lamellar body in studies by electron microscopic autoradiography. This small lamellar body appears to be small vesicles derived from the Golgi apparatus, indicating that the membrane flow mechanism, which has been shown in other tissues[11,12], may also be involved in the transport of surfactant phospholipids. Delahunty and Johnston[13] and Massaro[14] reported that microtubulus may be involved in the secretory process of the lung surfactant. There seems little doubt that the microtubular system functions for the secretion of lamellar bodies. However, the mechanism of the intracellular translocation of surfactant phospholipids from the synthetic sites to lamellar bodies is still unknown. In this communication, we describe that the microtubular system may also participate in the intracellular transport.

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2 Materials and Methods

2.1 Animal experiments

20 μCi of [methyl-^{3}H] choline (sp. act., 60 Ci/mmol, Amersham, UK) or 250 μCi of [2-^{3}H] glycerol (sp. act., 500 mCi/mol, Amersham, UK) dissolved in 0.25 ml of 0.9% saline was used.

Male Wistar rats were fasted overnight before the day of the experiment. The body weight of the animals was 240–250 g. The animals were pretreated by an intraperitoneal injection of colchicine (10 mg) in 0.9% saline (0.5 ml). The control animals were injected with 0.9% saline. After 3 h, the isotopes were injected into the femoral vein of the rats under light ether anesthesia. After different time intervals, the rats were killed by bleeding through the abdominal aorta.

2.2 Isolation of lung fractions

Immediately after sacrifice, the chests of the animals were opened, the tracheae cannulated, and the lungs were washed five times with each 5 ml of saline. The five washes were pooled. This fraction is referred to as the alveolar wash and contains more than 90% of the recoverable surfactant by alveolar washing[15]. The isolation procedure for rat lung fractions was carried out generally according to the method described for rabbit lung fractions[16,17].

The previously washed lung tissues were homogenized with 0.32 M sucrose, 10 mM Tris-HCl, 0.15 M NaCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$ and 0.1 mM EDTA at pH 7.4. Aliquots of the homogenates were saved to measure the amount of radioactivity in phospholipids. This crude homogenate fraction is referred to as the lung parenchyma. Lamellar bodies were then isolated from the lung homogenates by discontinuous sucrose density gradient centrifugation steps. The lamellar body fraction was recovered between 0.45 M and 0.65 M sucrose buffer. Microsomal fractions were isolated as organelles centrifuged at 105,000 $\times$g through 0.8 M sucrose buffer.

The fraction of lamellar bodies was electronmicroscopically identified[18], and the marker enzymes for mitochondria, succinate: cytochrome c reductase [EC 1.3.99.1] and for plasma membranes, 5'-nucleotidase [EC 2.7.8.2], were not detectable in the lamellar body fraction. The recovery of phospholipids in the lamellar body fraction and the microsomes isolated from rat lung was 1.3% and 10.7% of the total lung phospholipids, respectively. The ratio of phospholipid (μmole) to protein (mg) was 0.33 for the microsomes and 2.15 for the lamellar body fraction[19].

2.3 Lipid analysis

Lipids in lung parenchyma, microsomes, lamellar bodies and alveolar wash were extracted according to the method of Bligh and Dyer[20] and concentrated under N_2 at less than 50℃. Individual lipid classes were separated by two-dimensional thin-layer chromatography as described by Poorthuis *et al.*[21], which was specifically improved for distinct separation of phosphatidylglycerol. After chromatography, the spots were detected by a fluorescein spray and each lipid was recovered from the gel by the method of Arvidson[22]. The lipid-phosphorus and radioactivity in each spot were determined. The phosphatidylcholine, isolated by two-dimensional thin-layer chromatography was also subjected to permanganate/periodate oxidation to isolate the disaturated phosphatidylcholine species according to the method of Shimojo *et al.*[23]. Phosphorus was determined by the method of Bartlett[24]. Radioactivity was determined with a Packard liquid scintillation spectrometer using a toluene based scintillator.

2.4 Enzyme assays

Pulmonary CDPcholine: 1,2-diacylglycerol cholinephosphotransferase [EC 2.7.8.2] was assayed in the microsomes according to the method of Oldenborg and van Golde[25]. Acyl CoA: 1-acyl-sn-glycero-3-phosphocholine acyltransferase [EC 2.3.1.23] was assayed with palmitoyl CoA by the method of Hasegawa-Sasaki and Ohno[26]. Succinate: cytochrome c reductase [EC 1.3.99.1] and NADPH:

cytochrome c reductase were assayed according to Sottocasa *et al.*[27], and 5′-nucleotidase was assayed by the method of Gentry and Olsson[28].

3 Results

Table 1 shows the contents and labelings with (^{3}H) choline of the total phospholipids in the lung fractions isolated from the control and colchichine-treated rats. The phospholipid contents are expressed as μmole/rat lung, because it was impossible to exactly determine lung weights after alveolar washing. The phospholipid contents in the lung fractions were not significantly changed by colchicine treatment, whereas there were marked differences in the labelings with (^{3}H) choline between the control and colchicine-treated rats. Marked increases of the labelings in parenchyma, microsome and lamellar body fractions by colchicine treatment were observed.

Table 2 shows the results of phosphatidylcholine and its disaturated species in the lung fractions. The contents of phosphatidylcholine and disaturated phosphatidylcholine in each lung fraction were not significantly changed by colchicine treatment. However, the specific activities in the microsomes and lamellar bodies were significantly greater than those in the controls. In alveolar wash, their specific activities seemed to decrease in the colchicine-treated animals, though it was not statistically significant. The accumulation of the labeled phospholipids in the lamellar bodies and the decrease in alveolar wash indicate that the secretion of the surfactant phospholipids from lamellar bodies to alveolar spaces may be inhibited by the colchicine treatment. Moreover, the significant accumulation of newly-synthesized phospholipids in microsomes was also noted, indicating that the surfactant-directed phospholipid in microsomes may not be sufficiently transported to lamellar bodies in the colchicine-treated animals.

Fig. 1 shows time-dependent labeling of (2-^{3}H) glycerol into phosphatidylcholine and phosphatidylglycerol in lung fractions of colchicine-treated animals. The prior injection of colchicine resulted in marked increases in labelings with the (^{3}H) glycerol of the two phospholipids in microsomes and lamellar bodies. These colchicine effects were found as early as 30 min after the label injection. In contrast, the decrease in the labelings of phosphatidylcholine and phosphatidylglycerol was found in the alveolar wash of colchicine-treated rats.

Table 1 *Effect of prior injected colchicine on in vivo incorporation of (^{3}H) choline into total phospholipids of rat lung fraction.*
Saline or cholchicine (10 mg) in saline was intraperitoneally injected into rats at 3 h prior to the isotope injection. [methyl-^{3}H] Choline (200 μCi) was administered intrafemorally to each rat. After 2 h, the animals were killed. The phospholipid content and the isotope incorporation into total phospholipids of rat lung fractions were determined.

Lung fraction	Treatment	Total phospholipid content (μmole/rat lung)	(^{3}H) Choline incorporation (‰ to injected dose)
Parenchyma	Saline	20.5 ±0.7	19.7 ±3.1
	Colchicine	22.4 ±1.8	38.0 ±2.6a
Microsomes	Saline	1.08±0.14	1.17±0.11
	Colchicine	1.22±0.25	2.38±0.39a
Lamellar bodies	Saline	0.32±0.04	0.62±0.17
	Colchicine	0.46±0.07	2.02±0.17a
Alveolar wash	Saline	2.13±0.42	0.53±0.25
	Colchicine	1.87±0.70	0.37±0.18

Values are means ± S.D.(n=4).
a: significant ($p<0.01$) compared to saline treatment.

Table 2 *Effect of prior injected colchicine on the content and labelings with (^3H) choline of phosphatidylcholine and disaturated phosphatidylcholine of rat lung fractions.*
Saline or colchicine (10 mg) in saline was intraperitoneally injected into rats at 3 h prior to the isotope injection. [methyl-^3H] Choline (200 μCi) was administered intrafemorally to each rat. After 2 h, the animals were killed and the specific activities of phosphatidylcholine and its disatrated species of rat lung fractions were determined.

Lung fraction	Treatment	Phosphatidylcholine		Disaturated Phosphatidylcholine	
		Content*	Relative specific activity**	Content*	Relative specific activity**
Parenchyma	Saline	11.9 ±2.0	100±27	3.37±0.43	60±14
	Colchicine	16.3 ±2.7[a]	148±30[a]	4.06±0.26	114±23[a]
Microsomes	Saline	0.62±0.07	108±18	0.18±0.04	73±18
	Colchicine	0.73±0.20	191±13[a]	0.22±0.05	132±34[a]
Lamellar bodies	Saline	0.31±0.05	91±41	0.14±0.05	78±26
	Colchicine	0.39±0.08	269±23[a]	0.18±0.06	194±58[a]
Alveolar wash	Saline	2.15±0.48	16± 4	0.90±0.19	14± 5
	Colchicine	1.80±0.70	13± 2	0.80±0.39	9± 2

* The content of phospholipids is expressed as μmole/rat lung.
** Relative specific activity was calculated on the basis of specific activity of phosphatidylcholine isolated from lung parenchyma at 2 h after the injection of saline, which was 8.50±2.29 dpm. 10^4/μmole. Values are means±S. D. (n=4).
a: Significant ($p<0.01$) compared to saline treatment.

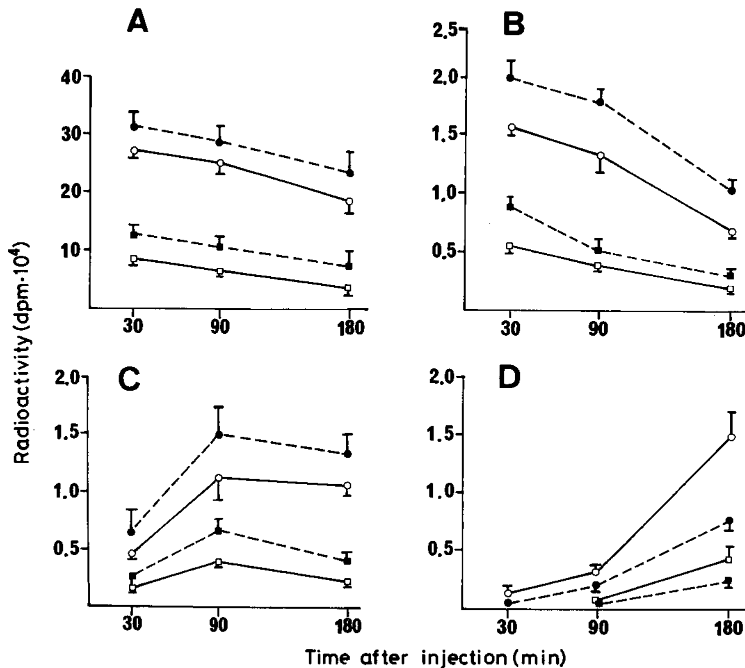

Fig. 1 Labeling of phosphatidylcholine and phosphatidylglycerol with (2-^{3}H) glycerol in lung fractions of control and colchicine-treated rats.
Saline or colchicine (10 mg) in saline was intraperitoneally injected into rats at 3 h prior to the isotope injection. 250 μCi of (2-^{3}H) glycerol was administered into the femoral vein of each rat. The animals were killed at different time intervals after the isotope injection. The radioactivity of phosphatidylcholine (○, ●) and phosphatidylglycerol (□, ■) in rat lung fractions was derermined. Each mark is the average from 4 animals and the vertical bar expresses the standard deviation.
A, lung parenchyma; B, lung microsomes; C, lamellar bodies; D, alveolar wash.
Solid line: control rats, dotted line: colchicine-treated rats.

Enzyme activities involved in phosphatidylcholine synthesis were examined in lung microsomes isolated from the colchicine-treated rats. The colchicine treatment *in vivo* did not affect the enzyme activities (Table 3), indicating that phosphatidylcholine synthesis in the lung microsomes is unaffected in the state of microtubular disruption. Therefore, it is unlikely that the colchicine effect mentioned above is due to an increased synthesis of phospholipids in the lung microsomes.

Table 3 *Effect of colchicine treatment on enzyme activities involved in phosphatidylcholine biosynthesis in rat lung microsomes.*

Treatment	Enzyme activity (n mole/min/mg protein)	
	CDP-choline : 1,2-diacylglycerol cholinephosphotransferase	Palmitoyl CoA : 1-acyl glycerophosphocholine acyltransferase
Saline	2.38±0.40	11.5±1.5
Colchicine	2.24±0.14	12.1±0.8
	N. S.	N. S.

Values are mean±S.D. (n=4). N. S.: not significant between saline and colchicine treatments. Saline or colchicine (10 mg) in saline was intraperitoneally injected into rats at 3 h prior to sacrifice.

4 Discussion

The present study clearly demonstrated that the prior treatment of colchicine resulted in marked decreases in the secretion of lamellar bodies into the alveolar space, as indicated by a significant increase in phosphatidylcholine, disaturated phosphatidylcholine and phosphatidylglycerol labeled with (^{3}H) choline or (^{3}H) glycerol in lamellar bodies. There was a decrease in the labelings in the alveolar wash as well. These findings confirm the idea previously presented by Delahunty and Johnston[13] and Massaro[14] that the lung microtubular system participates in the secretion of surfactant phospholipids.

In addition to these findings, the present study also demonstrated marked increases in the labelings of the surfactant phospholipids in lung microsomes of colchicine-treated rats. It is unlikely that the high labels in the lung microsomes were due to an increased synthesis of phospholipids, since the enzyme activities involved in phosphatidylcholine synthesis in lung microsomes were unaffected. The labels in the microsomes may be increased by feed back control due to the accumulation of surfactant phospholipids in the lamellar bodies. However, this finding also suggests that the microtubular system may participate not only in the secretion but also in the transfer of surfactant phospholipids from microsomes to lamellar bodies.

Two mechanisms have been postulated regarding the transfer of surfactant phospholipids from the site of synthesis to lamellar bodies. Engle *et al.*[7] first showed the occurrence of phosphatidylcholine exchange proteins in lung cytosol which catalyze the transfer of phosphatidylcholine not only between microsomes and mitochondria but also between microsomes and lamellar bodies. Robinson *et al.*[8] partially purified phosphatidylcholine exchange proteins from rat lung cytosol that catalyzed the transfer of phosphatidylcholine between microsomes and lamellar bodies. More recently, the presence of specific phosphatidylglycerol transfer proteins have been reported[9,10]. These reports have emphasized that phospholipid exchange proteins may participate in the biogenesis of lamellar bodies by transporting surfactant phospholipids from the synthetic sites.

On the other hand, another mechanism by which surfactant phospholipids are selectively accumulated in lamellar bodies from the site of synthesis, has been postulated, which may involve the membrane flow mechanism[12,13]. Chevalier and Collet[3] found in electron microscopic autoradiography studies that (^{3}H) choline was initially localized in the endoplasmic reticulum of alveolar type II cells, and was then rapidly transferred through the Golgi complex and stored in lamellar bodies. In the transport from the Golgi

compex to lamellar bodies, they found the presence of a small lamellar body form which is the carrier of phospholipids between the two structures. It is well-established that newly synthesized phospholipid molecules form cytoplasmic vesicles derived from the Golgi complex[29,30], which are vectors specialized in the transport of precursors of plasma membranes. Furthermore, when secretory vesicles produced in the Golgi apparatus are released into the cytoplasma, they would migrate vectorically to the cell surface[31,32]. The microtubular network may participate in this vectorical transport of the cytoplasmic phospholipid vesicles or secretory vesicles from the Golgi complex. In the present study, the marked accumulation of surfactant phospholipids was found in lung microsomes as well as in lamellar bodies of colchicine-treated rats. This finding, which is newly presented, shows that microtubulus in alveolar type II cells may participate not only in the movement of lamellar bodies to cell surfaces, but also in the translocation of small vesicles including surfactant phospholipids from the Golgi complex to lamellar bodies.

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肺サーファクタントリン脂質の輸送と分泌

── コルヒチンの影響 ──

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コルヒチン処理ラットに(メチル-^{3}H)コリンおよび(2-^{3}H) グリセロールを*in vivo*投与し，肺サーファクタントリン脂質の輸送と分泌における微小管の役割を検討した．コルヒチン処理ラットの肺の4画分，つまり肺実質，ミクロソーム，ラメラ封入体，肺洗浄液におけるホスファチジルコリン，飽和ホスファチジルコリン，ホスファチジルグリセロールへの放射活性の取り込みを測定した．標識前にコルヒチンで処理したラットでは，これらのリン脂質への放射活性の取り込みは，ラメラ封入体，ミクロソームで著明に上昇したが，肺洗浄液では低下していた．この知見は，微小管がラメラ封入体の肺胞腔への分泌に関与しているのみならず，肺サーファクタントリン脂質の合成部位，つまりミクロソームからその蓄積部位のラメラ封入体への輸送にも関与していることを示唆している．

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