Induction of intra- and extra-cellular phospholipids in the lungs of rats exposed to silica

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Intracellular and extracellular compartments of phospholipids in the lungs of rats were examined 28 days after intratracheal injection of silica (200 mg/kg). All compartments containing phospholipids were elevated, but the largest increases were seen in the intracellular and extracellular pulmonary surfactant. Intracellular pulmonary surfactant increased 123-fold from 1.18 ± 0.65 to 144.9 ± 53.8 and the extracellular surfactant increased 22-fold from 1.17 ± 0.04 to 25.1 ± 7.1 mg per pair of rat lungs respectively. The phospholipid composition of intracellular and extracellular surfactant did not change in response to silica, except for an almost 2-fold increase in the percentage of total phosphatidylinositol in both compartments. The phospholipid content of the lungs increased from 24.9 ± 4.6 to 268.6 ± 20.8 mg, with the intracellular and extracellular surfactant accounting for 59.1 and 24.6% of this total increase respectively. These data demonstrate that the major increases in the phospholipid content of the lungs induced by silica is associated with the pulmonary-surfactant system.

INTRODUCTION

The path that leads from inhalation of silica dust to silicosis is obscure. Fibrogenesis is a mandatory step, but events leading to, and subsequent to, fibrogenesis are not known. Early events could be especially significant, because reversal of the disease may be possible only in the initial stages. One of the early events after exposure of animals to silica dust is a marked stimulation of phospholipid biosynthesis and accumulation of phospholipids within the lungs (Kaw *et al.*, 1971; Grunspan *et al.*, 1973; Heppleston *et al.*, 1974). The reasons for this stimulation are not known, although it has been speculated that increased phospholipids could represent a defensive action of the lungs against the cytotoxic and fibrogenic effects of the silica (Richards & Curtis, 1984; Heppleston, 1969; Desai & Richards, 1975). For whatever reason, there seems little doubt that stimulation of phospholipid biosynthesis is an event that occurs very early after exposure to silica and as such could prove of primary significance in understanding the processes that ultimately contribute to the disease state.

Very little is known about the stimulation of phospholipid biosynthesis in the lungs of animals exposed to silica, and even less is known about the mechanisms through which this stimulation is achieved. The total content of phospholipids in the lungs has been shown to increase (Fallon, 1937; Kaw *et al.*, 1971; Grunspan *et al.*, 1973; Heppleston *et al.*, 1974) in response to silica, and at least part of this increase is due to increases in extracellular surfactant (Gabor *et al.*, 1978; Richards & Curtis, 1984). However, the extent to which the extracellular surfactant may contribute to total increases in pulmonary phospholipid is not known. The

phospholipid content of the lungs could increase through numerous processes involving not only the surfactant system but proliferation of subcellular organelles such as mitochondria and endoplasmic reticula.

We have attempted to locate the phospholipid response within the lungs by examining the phospholipid content of subcellular fractions and surfactant compartments. If the increase in phospholipids represents a generalized cellular reaction of the lungs to silica, then the phospholipid content of all subcellular fractions and surfactant compartments should be elevated. If, however, the response is focused within the surfactant system, then only the intracellular and extracellular compartments of surfactant would be expected to be increased.

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EXPERIMENTAL PROCEDURES

Chemicals

The chemicals used were as follows: sodium cacodylate, uranyl acetate, lead citrate (Fisher Scientific Co., Fairlawn, NJ, U.S.A.); OsO_4 (Colonial Metals, Elkton, MD, U.S.A.); NaCl (J. T. Baker Chemical Co., Phillipsburg, NJ, U.S.A.); paraformaldehyde, Hepes (Sigma Chemical Co., St. Louis, MO, U.S.A.); glutaraldehyde (Ladd Research Industries, Burlington, VT, U.S.A.); silica in the form of Berkeley Min-U-Sil ($< 5 \mu\text{m}$ particle size) (Pennsylvania Glass Sand Corporation, Pittsburgh, PA, U.S.A.); density-gradient-grade sucrose (Schwarz/Mann, Orangeburg, NY, U.S.A.); phospholipid standards (Applied Science, State College, PA, U.S.A.); 1-palmitoyl-

Abbreviations used: DPPC, dipalmitoyl phosphatidylcholine; [^3H]DPPC, 1-palmitoyl-2-[9,10- ^3H]palmitoyl phosphatidylcholine; LPC, lyso-phosphatidylcholine; SPH, sphingomyelin; PC, phosphatidylcholine; USPC, unsaturated phosphatidylcholine; DSPC, disaturated phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol.

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2-[9,10-³H]palmitoyl phosphatidylcholine, 60 Ci/mmol, and [*methyl*-¹⁴C]choline chloride (50.5 mCi/mmol) (New England Nuclear, Boston, MA, U.S.A.).

Animals

Adult male rats of the Sprague-Dawley strain (Charles River, Wilmington, MA, U.S.A.) weighing 225–250 g were used. Animals were allowed free access to food and water.

Preparation of silica

A stock of silica was prepared by boiling 30 g of silica in 1 litre of 1.0 M-HCl until the green colour disappeared. This was done to remove Fe₂O₃, which is known to contaminate the dust (Allison, 1976; Dauber *et al.*, 1980). The silica was then washed four times with deionized water to remove the acid and chloride. The preparation was dried in an oven at 100 °C. Prepared silica was sterilized by dry heat (200 °C, 2 h) and diluted to the appropriate concentration with sterile 0.9% NaCl. Suspensions for intratracheal injection were sonicated for 10 min before use (Branson Sonifier, output setting 5, 30% duty cycle).

Dosing of animals

Rats were anaesthetized by injecting intramuscularly mixtures of ketamine (8.6 mg; Parke-Davis, Morris Plains, NJ, U.S.A.) and xylazine (0.28 mg; Miles Laboratories, Shawnee, KS, U.S.A.) in a volume of 100 µl. The animals were held in a vertical position and intratracheal injections of silica suspensions were performed by threading a curved cannula [18 gauge, 3 in (7.6 cm), 2.25 mm-diameter ball] into the trachea until just above the tracheal bifurcation. Injections were made and the animals were returned immediately to their cages to recover.

Lobar distribution of silica

A 500 µg portion of 1-palmitoyl-2-[9,10-³H]palmitoyl phosphatidylcholine ([³H]DPPC) (12.5 µCi/mg of DPPC) in 1 ml of chloroform was added to 1.0 g of silica and mixed thoroughly. The silica was stirred and the chloroform allowed to evaporate at room temperature. Additional silica (1.5 g) was then mixed with the DPPC-coated silica. The stock silica contained 200 µg of DPPC and 2.5 µCi of ³H/g. The specific radioactivity of the adsorbed phospholipid was 12.5 µCi/g of DPPC.

The phospholipid-adsorbed silica was washed six times with 20 ml volumes of 0.9% NaCl. Removal of radiolabel decreased exponentially. Phospholipid was bound very strongly to the silica, because after six washes 92.6% of the initial radioactivity remained and the sixth wash removed only 0.25% of the initial radioactivity. These results indicated that the adsorbed phospholipid was unlikely to be released immediately from the silica upon its injection into the lungs.

Rats were injected intratracheally with silica (200 mg of silica/kg body wt; 0.2 µg of DPPC/mg of silica; 2.5 nCi of ³H/mg of silica) suspended in 0.5 ml of 0.9% NaCl. The rats were immediately killed by intraperitoneal injections of sodium pentobarbital (50 mg/rat). The lungs were removed intact and divided into constituent lobes. The left lung was divided laterally into two approximately equal parts. The lobes were weighed and homogenized in 2.0 ml of 0.9% NaCl with a Potter-

Elvehjem homogenizer (15 passes of the pestle). The homogenate was diluted to 5.0 ml with 0.9% NaCl and analysed for radioactivity and protein.

Isolation of extracellular pulmonary surfactant

Rats were killed with an overdose of sodium pentobarbital. Lungs were removed quickly with their tracheae intact. After removing extraneous tissue, the tracheae were cannulated and lungs filled to capacity with ice-cold 0.9% saline. Lavage effluents were then collected. This procedure was repeated nine times with fresh 0.9% NaCl. The combined effluents were centrifuged at 4 °C at 580 g for 10 min (Sorvall RT 6000 refrigerated centrifuge, H1000B rotor) to sediment cells. Lavage fluid was stored frozen at -20 °C until extracted for phospholipid analysis.

Isolation of intracellular pulmonary surfactant

The intracellular pool of pulmonary surfactant was isolated by centrifugation on sucrose density gradients by the method of Duck-Chong (1978) as modified by Young *et al.* (1981). Briefly, lavaged lungs were homogenized in 1.0 M-sucrose by using a Potter-Elvehjem homogenizer with a Teflon pestle [0.10–0.15 mm (0.004–0.006 in) clearance]. Twenty passes of the pestle were made and the resulting homogenate was filtered through one layer of monofilament nylon cloth (160 µm mesh size) (New York Stencil Silk Co., Halesite, NY, U.S.A.). The filtrate was adjusted to 9.0 ml with 1.0 M-sucrose. A 1 ml portion of 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3 and 0.2 M-sucrose was layered on top of the filtered homogenate and the gradients centrifuged for 15 min at 1000 rev./min (200 g) (Beckman L3-50 ultracentrifuge; SW 28.1 rotor). Without interruption the gradients were then accelerated to 25000 rev./min (116000 g) for 180 min. The lamellar bodies appeared as an opaque band at a density of approx. 1.054. This fraction was aspirated and stored frozen at -20 °C until extracted for phospholipid analysis.

Recovery of intracellular surfactant

Recovery of intracellular surfactant was estimated as follows. Four rats were each injected intravenously with 5 µCi of [¹⁴C]choline (50.5 mCi/mmol) and killed 3.5 h later. Lamellar bodies were isolated by using the procedure of Duck-Chong (1978) as modified by Young *et al.* (1981). The lamellar-body fraction was further purified by resuspending in 1.0 M-sucrose and re-centrifuging on sucrose density gradients as used previously for isolation of the lamellar bodies. The radiolabelled lamellar bodies were homogenized with rat lungs and the intracellular surfactant pool isolated as described above. Recoveries of surfactant were estimated from the recovery of radiolabelled phosphatidylcholine. In addition, recoveries were estimated in the presence of increasing quantities of intracellular surfactant previously isolated from silica-treated rats.

Preparation of subcellular fractions

Rats treated with silica 28 days beforehand were killed by intraperitoneal injection of sodium pentobarbital (50 mg/rat). Lungs from silica-treated and from control rats were removed and dissected free of all extraneous tissue. All subsequent steps were performed at 4 °C. Extracellular surfactant was removed from the lungs by bronchoalveolar lavage and intracellular surfactant was

isolated by density-gradient centrifugation as described above. Intracellular surfactant was removed from the gradients and the remaining material mixed and diluted with water to give a final sucrose concentration 0.25 M. A 5 ml portion of each tissue suspension was fractionated by differential centrifugation by the procedure of Hook *et al.* (1972). A crude plasma-membrane fraction was obtained by centrifuging the suspensions at 600 *g* for 10 min (Sorvall RC-5B refrigerated superspeed centrifuge, SS-34 rotor). Centrifugation of the 600 *g* supernatant at 3300 *g* for 10 min yielded a crude heavy-mitochondrial fraction. The light-mitochondrial fraction was obtained by centrifugation of the 3300 *g* supernatant at 10000 *g* for 20 min and microsomes were isolated from the 10000 *g* supernatant by centrifugation at 178000 *g* for 45 min (Beckman L3-50 ultracentrifuge; 50 Ti rotor). All pellets were resuspended in 0.9% NaCl and adjusted to a final volume of 5.0 ml. The fractions were stored frozen at -20 °C until protein, phospholipid and DNA analyses were performed.

Phospholipid analyses

Total lipid from the various lung fractions was extracted by the method of Folch *et al.* (1957). Disaturated phosphatidylcholine was isolated by the method of Mason *et al.* (1976). Phospholipid extracts were separated into component species by thin-layer chromatography (silica-gel 60; HPTLC plates; E. Merck, Darmstadt, Germany) with chloroform/methanol/light petroleum (b.p. 35–60 °C)/acetic acid/boric acid [200:100:150:50:9 (v/v/v/v/w)] by the single-plate method of Gilfillan *et al.* (1983). Phospholipid content was determined by measuring phospholipid phosphorus (Shin, 1962).

Biochemical analyses

Protein was estimated by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. DNA content was quantified by the method of Schneider (1957).

Electron microscopy

Samples were fixed as suspensions in 2% glutaraldehyde containing 1% paraformaldehyde, buffered with 0.1 M-sodium/potassium phosphate, pH 7.4, at 4 °C. After 2 h the fixed suspensions were centrifuged at 25500 *g* (Beckman L3-50 ultracentrifuge; 50 Ti rotor) for 10 min. All subsequent processing of the pellets was performed essentially by the procedure of Williams (1977). The pellets were post-fixed for 18 h in 1.5% OsO₄ buffered with 0.1 M-sodium cacodylate, pH 7.4, at 4 °C, and then rinsed in three 10 min changes of 50 mM-sodium maleate buffer, pH 5.2. The pellets were stained for 90 min in 1.5% uranyl acetate in 25 mM-sodium maleate, pH 6.0, at 4 °C and then rapidly dehydrated in cold, graded acetone solutions and infiltrated with Epon 812 (Luft, 1961). Ultrathin sections were cut on a Porter-Blum ultramicrotome MT-1 by using a du Pont (E. I. du Pont de Nemours and Co., Wilmington, DE, U.S.A.) diamond knife. The sections were stained with 5% uranyl acetate (Watson, 1958) for 10 min and with lead citrate (Reynolds, 1963) for 6 min; thin sections were examined in a Phillips EM-400 electron microscope.

RESULTS

Distribution of silica

A measure of the lobar distribution of silica immediately after intratracheal injection was obtained by using silica to which a small amount of radiolabelled DPPC had been adsorbed (0.02 µg of DPPC/mg of silica; 2.5 nCi of ³H/mg of silica). This [³H]DPPC could not be removed by washing the silica with 0.9% NaCl, indicating that the phospholipid was firmly adsorbed and unlikely to redistribute immediately after dosing of the animals. The lobar distribution of silica as indicated by the distribution of radioactivity was the same as the distribution of pulmonary tissue as measured by wet weight or protein (Table 1). Intratracheal injection resulted in none of the pulmonary lobes being exposed to concentrations of silica higher than that of any other lobe.

Effect of silica on pulmonary weight, DNA, protein and phospholipid

At 28 days after a single intratracheal injection of silica (50 mg), lung weights were increased 4.6-fold (Table 2). The fluid content of the lungs accounted for approx. 80% of the increase in lung weight; however, this fluid was not entirely oedema fluid, as indicated by the increase in the ratio of dry weight to wet weight of the lungs. The number of cells increased, as demonstrated by the 3.4-fold increase in DNA content of the lungs. The protein content of the lungs was affected to the same extent as the DNA content. The total phospholipid content of the lungs increased 16.1-fold in response to silica, an increase that was much larger than that of DNA or protein. The concentration of phospholipid in the tissue also increased significantly (3.5-fold), a response that was not seen with either DNA or protein.

Extracellular surfactant phospholipids

Extracellular surfactant phospholipids were isolated from the lungs of silica-treated and control rats by pulmonary lavage. Lungs were lavaged 10 times and the extracellular phospholipid measured in each lavage effluent. Removal of surfactant phospholipids from the

Table 1. Lobar distribution of silica after intratracheal injection

Silica was treated with 1-palmitoyl-2-[9,10-³H]palmitoyl phosphatidylcholine as described in the Experimental procedures section. Male rats (225–250 g) were injected intratracheally with a 50 mg dose of silica (0.2 µg of DPPC/mg of silica; 2.5 nCi/mg of silica in 0.5 ml 0.9% NaCl). Immediately after dosing, the rats were killed with an overdose of sodium pentobarbital injected intraperitoneally. The lungs were divided as indicated. Results are means ± S.E.M (*n* = 12).

Lobe	Distribution (% of total recovered)		
	Silica	Wet weight	Protein
Cephalic	11.4 ± 2.8	12.0 ± 1.4	12.9 ± 1.0
Medial	12.9 ± 2.9	13.2 ± 0.9	13.4 ± 0.7
Caudal	27.7 ± 9.5	25.6 ± 1.2	27.2 ± 2.4
Left upper half	15.9 ± 4.2	18.8 ± 1.5	16.6 ± 1.4
Left lower half	19.9 ± 7.6	18.4 ± 1.9	16.5 ± 1.1
Post caval	10.8 ± 2.1	12.1 ± 1.9	13.4 ± 1.3

Table 2. Effect of silica on DNA, protein, phospholipid and weight of the lungs

Male rats (225–250 g) were injected intratracheally with a single 50 mg dose of silica on day 0 and killed on day 28. Results are means $\pm$ S.D. ($n = 4$). Significance: ** $P < 0.01$; NS, not significant.

	Control	Silica	Increase (fold)
Lung weight			
(g wet wt.)	1.22 $\pm$ 0.14	5.65 $\pm$ 0.25**	4.6
(g dry wt.)	0.12 $\pm$ 0.01	1.05 $\pm$ 0.04**	8.8
Fluid (g/pair of lungs)	1.13 $\pm$ 0.13	4.66 $\pm$ 0.31**	4.1
Dry weight/wet wt.	0.096 $\pm$ 0.006	0.184 $\pm$ 0.011**	1.9
DNA			
(mg/pair of lungs)	7.0 $\pm$ 0.4	23.9 $\pm$ 6.0**	3.4
(mg/g wet wt.)	5.7 $\pm$ 0.8	4.2 $\pm$ 0.9 NS	0.7
Protein			
(mg/pair of lungs)	238.8 $\pm$ 44.1	840.8 $\pm$ 148.3**	3.5
(mg/g wet wt.)	190.5 $\pm$ 20.1	147.2 $\pm$ 24.6	0.8
Phospholipid			
(mg/pair of lungs)	29.6 $\pm$ 4.4	476.1 $\pm$ 129.2**	16.1
(mg/g wet wt.)	23.7 $\pm$ 1.2	83.1 $\pm$ 21.0**	3.5

Table 3. Recovery of extracellular surfactant by sequential bronchoalveolar lavage

Rats were injected intratracheally with silica (50 mg) on day 0 and killed on day 28. Controls were injected with saline only. Lungs were lavaged and extracellular surfactant phospholipids were measured as described in the Experimental procedures section. Results are percentages of total phospholipid recovered in ten lavages; results are means $\pm$ S.D. ($n = 3$).

Lavage number	Percentage recovered	
	Control	Silica
1	40.1 $\pm$ 11.4	48.7 $\pm$ 5.6
2	25.2 $\pm$ 3.7	29.3 $\pm$ 4.0
3	12.8 $\pm$ 2.0	8.4 $\pm$ 2.5
4	11.3 $\pm$ 4.1	5.9 $\pm$ 1.2
5	4.6 $\pm$ 4.0	2.4 $\pm$ 0.3
6	3.0 $\pm$ 2.6	1.5 $\pm$ 0.2
7	1.1 $\pm$ 1.9	1.2 $\pm$ 0.2
8	1.0 $\pm$ 1.9	0.8 $\pm$ 0.4
9	0.4 $\pm$ 0.7	0.9 $\pm$ 0.3
10	0.4 $\pm$ 0.7	0.8 $\pm$ 0.4

lungs of silica-treated and untreated rats occurred in an exponential manner, as shown in Table 3. The exponential function that describes the removal of surfactant phospholipids from the lungs is a characteristic of the pulmonary extracellular compartment that contains surfactant (Hook, 1978). The curves generated by connecting mean values of percentages of surfactant phospholipids recovered for silica-treated and untreated rats were indistinguishable from each other, suggesting that the nature of the extracellular compartments containing surfactant phospholipids was unchanged by silica, in spite of large increases in total phospholipids. In addition, recovery of extracellular phospholipid from the lungs was not affected by silica. Extrapolation of the

Table 4. Effect of silica on intracellular and extracellular compartments of pulmonary-surfactant-associated phospholipids

Male rats (225–250 g) were injected intratracheally with a single 50 mg dose of silica on day 0 and killed on day 28. Intracellular and extracellular compartments of surfactant phospholipids were determined as described in the Experimental procedures section. Recovery of extracellular surfactant was 98.4 $\pm$ 0.6% and 99.6 $\pm$ 0.4% for control and silica-treated rats respectively. Recovery of intracellular surfactant was 73.7 $\pm$ 0.3% and 74.4 $\pm$ 4.3% for control and treated rats respectively. The data are not corrected for recoveries. Results are means $\pm$ S.D. (n); significance: ** $P < 0.01$.

	Phospholipid		Increase (fold)
	Control	Silica	
Extracellular			
Total (mg)	1.17 $\pm$ 0.04 (3)	25.1 $\pm$ 7.1 (5)**	22
mg/g of lung	0.90 $\pm$ 0.08 (3)	4.3 $\pm$ 1.4 (5)**	5
Intracellular			
Total (mg)	1.18 $\pm$ 0.65 (3)	144.9 $\pm$ 53.8 (5)**	123
mg/g of lung	1.36 $\pm$ 0.32 (3)	24.5 $\pm$ 9.2 (5)**	18

exponential functions indicated that lavaging the lungs 10 times resulted in removal of 99.6 $\pm$ 0.4 and 98.4 $\pm$ 0.6% of the total available extracellular phospholipid from the lungs of silica-treated and untreated rats respectively.

Treatment of rats with silica resulted in a 22-fold expansion of the extracellular compartment of surfactant phospholipids from 1.17 $\pm$ 0.04 mg to 25.1 $\pm$ 7.1 mg (Table 4). The concentration of extracellular surfactant phospholipids in the tissue (mg of phospholipid/g of lung) also increased (5-fold).

Intracellular surfactant phospholipids

Treatment of lungs with silica increased the lamellar-body phospholipid fraction (intracellular surfactant phospholipid compartment) 123-fold from 1.18 $\pm$ 0.65 mg to 144.9 $\pm$ 53.8 mg of phospholipid. The tissue concentration of intracellular surfactant phospholipids increased 18-fold (Table 4). The recovery of lamellar bodies from rat lung homogenates was not quantitative when the procedure of Duck-Chong (1978), as modified by Young *et al.* (1981), was used. Radiolabelled lamellar bodies were isolated as described in the Experimental procedures section and added to non-radiolabelled rat lung homogenates. Recovery of radiolabelled lamellar bodies from the sucrose gradient was consistent and independent of the presence of rat lung homogenates. Recovery of lamellar bodies was also independent of the ratio between pulmonary surfactant and rat lung tissue. Recoveries were measured by using rat lung homogenates to which various quantities of intracellular surfactant had been added. Although the ratios between surfactant and tissue ranged from 1.0 mg of surfactant phospholipid/g of lung to 50 mg/g of lung, the recovery did not change. These data indicate that the recovery of intracellular surfactant was not affected by the expansion of the intracellular compartment of surfactant. We estimated the recoveries of lamellar bodies to be 73.7 $\pm$ 0.3% ($n = 3$) and

Fig. 1. Morphological appearance of intracellular surfactant isolated from the lungs of (B) silica-treated and (A) untreated rats

A, control rats that received 0.9% NaCl only. The isolation procedure resulted in considerable disruption of lamellar bodies; fragmented lamellar bodies are evident from multilamellated appearance of the structures (arrows). Magnification $32\,600\times$. B, Silica-treated rats (50 mg of silica on day 0 killed on day 28). The intracellular surfactant contained more intact lamellar bodies than the controls. Magnification $32\,600\times$.

$74.4 \pm 4.3\%$ ($n = 3$) for controls and silica-treated rats respectively.

Under the electron microscope the morphological

appearance of the phospholipid-rich material from the intracellular compartments of pulmonary surfactant was as shown in Fig. 1. The fraction from untreated lungs

consisted primarily of multilamellated membranous structures consistent with their lamellar-body origins. The fraction from silica-treated lungs was similar but, in addition, contained many intact lamellar bodies. We attribute disruption of the lamellar bodies to the extensive homogenization of the tissue.

Phospholipid composition of intra- and extra-cellular compartments of surfactant

The effects of silica on the phospholipid composition of the intracellular and extracellular compartments of pulmonary surfactant are shown in Table 5. In the lungs of control animals the phospholipid compositions of the intracellular and extracellular compartments were very similar. The control values were obtained from surfactant pooled from four rats because insufficient material was available from individuals. Values obtained with silica are from four individuals.

The phospholipid composition of intracellular and extracellular surfactant compartments from silica-treated rats were very similar to each other as well as to the control values. Thus silica did not produce compositional changes in pulmonary surfactant, except for phosphatidylinositol, which increased as a percentage of the total in both intra- and extra-cellular pools. The low LPC content of the extracellular surfactant compartment indicated an absence of free extracellular phospholipase in the alveoli and airways that might be expected if cellular disintegration were a significant item to be considered.

Phospholipid and protein distributions among subcellular fractions

The protein content of all subcellular fractions increased in response to silica (Fig. 2). The largest increase was seen in the heavy mitochondrial fraction (17-fold). Phospholipid increased in each subcellular compartment, but the largest increases were seen in the two surfactant compartments (52.4-fold and 28.2-fold for the intracellular and extracellular compartments respectively) and the microsomal fractions (10.4-fold). Fig. 2 demonstrates that all compartments increased in their content of protein and phospholipid, but the intracellular and extracellular surfactant compartments were outstanding.

The protein contents of each subcellular fraction isolated from the lungs of silica-treated and untreated rats are shown in Table 6. The protein content of the lung increased by 657 mg after intratracheal injection of silica. Most of this increase was accounted for by increased protein content of the 600 g fraction, the heavy mitochondrial fraction and the soluble fraction. The surfactant compartments together accounted for only 3.7% of the total protein increase in the lungs. After intratracheal injection of silica, the phospholipid content of the lung increased by 243.7 mg (Table 7). Of this increase, 83.7% was accounted for by increases in the phospholipid content of the intracellular and extracellular compartments. The other subcellular fractions together accounted for only 14.4% of the total increase.

DISCUSSION

Stimulation of the phospholipid content of the lungs by silica has been known for many years (Fallon, 1937; Marks & Marasas, 1960), but the phenomenon has

Table 5. Effect of intratracheally injected silica on phospholipid composition of intra- and extra-cellular compartments of surfactant in the lungs of rats

Male rats (225–250 g) were injected intratracheally with a single 50 mg dose of silica on day 0 and killed on day 28. Control rats: analyses were performed on surfactant compartments pooled from four rats. Silica-treated rats: analyses were performed on surfactant compartments from individual rats. Results are means $\pm$ S.D. ($n = 4$).

Phospholipid	Composition (% of total)			
	Intracellular		Extracellular	
	Control	Silica	Control	Silica
LPC	1.5	0.5 $\pm$ 0.1	0.5	1.0 $\pm$ 0.4
SPH	3.8	1.3 $\pm$ 0.5	1.1	1.7 $\pm$ 0.4
PC				
USPC	29.5	32.8 $\pm$ 3.2	30.4	35.2 $\pm$ 1.4
DSPC	51.8	49.4 $\pm$ 2.9	54.5	49.2 $\pm$ 3.0
PI	2.4	6.2 $\pm$ 0.8	3.7	5.3 $\pm$ 0.5
PS	1.6	1.1 $\pm$ 0.5	1.1	1.2 $\pm$ 0.4
PE	5.0	4.2 $\pm$ 1.0	3.9	3.1 $\pm$ 1.0
PG	4.5	4.5 $\pm$ 1.0	4.9	3.4 $\pm$ 1.3

Fig. 2. Increases in protein and phospholipid in subcellular fractions of the rat lung in response to intratracheal injections of silica

Rats were injected intratracheally with 50 mg of silica on day 0 and killed on day 28. Subcellular fractions were prepared as described in the Experimental procedures section.

$$\text{Increase factor} = \frac{\text{Total/fraction from silica-treated lungs}}{\text{Total/fraction from control lungs}}$$

Abbreviations used: P, 600 g fraction; H, heavy mitochondrial fraction; L, light mitochondrial fraction; M, microsomal fraction; S, soluble fraction; IP, intracellular surfactant; EP, extracellular surfactant.

Table 6. Effect of silica on protein distribution among subcellular fractions of the lungs of rats

Male rats (225–250 g) were injected intratracheally with 50 mg of silica on day 0 and killed on day 28. Subcellular fractions were isolated as described in the Experimental procedures section. Results are means $\pm$ S.D. ($n = 4$). Significance: $**P < 0.01$. Intra- and extra-cellular surfactant values are not corrected for recoveries.

	Protein (mg)		Percentage of total increase due to compartment
	Control	Silica	
Unfractionated lung	230 $\pm$ 35	887 $\pm$ 66**	100
600 g	94.4 $\pm$ 4.0	252 $\pm$ 33**	24.0
Heavy mitochondrial fraction	8.9 $\pm$ 1.0	152 $\pm$ 36**	21.8
Light mitochondrial fraction	10.5 $\pm$ 1.8	16.0 $\pm$ 1.5**	0.8
Microsomal fraction	7.1 $\pm$ 0.9	37.0 $\pm$ 5.2**	4.6
Soluble fraction	68.2 $\pm$ 3.5	247.2 $\pm$ 33.6**	27.3
Intracellular surfactant	2.7 $\pm$ 0.4	15.9 $\pm$ 1.8**	2.0
Extracellular surfactant	2.4 $\pm$ 0.6	13.8 $\pm$ 2.4**	1.7

Table 7. Effect of silica on the distribution of phospholipids among subcellular fractions of the lungs of rats

Male rats (225–250 g) were injected intratracheally with 50 mg of silica on day 0 and killed on day 28. Subcellular fractions of the lungs were isolated as described in the Experimental procedures section. Results are * means $\pm$ S.D. ($n = 4$); significance: $**P < 0.01$. Intra- and extra-cellular surfactant values are not corrected for recoveries.

	Phospholipid (mg)		Percentage of total increase due to compartment
	Control	Silica	
Unfractionated lung	24.9 $\pm$ 4.6	268.6 $\pm$ 20.8**	100
600 g fraction	10.3 $\pm$ 1.6	11.6 $\pm$ 1.9	0.5
Heavy mitochondrial fraction	1.0 $\pm$ 0.2	2.7 $\pm$ 0.5**	0.7
Light mitochondrial fraction	2.2 $\pm$ 0.7	8.5 $\pm$ 1.0**	2.6
Microsomal fraction	2.4 $\pm$ 0.3	24.9 $\pm$ 4.5**	9.2
Soluble fraction	1.1 $\pm$ 0.1	4.6 $\pm$ 0.5**	1.4
Intracellular surfactant	2.8 $\pm$ 0.3	146.8 $\pm$ 23.4**	59.1
Extracellular surfactant	2.2 $\pm$ 0.6	62.1 $\pm$ 14.1**	24.6

received very little attention. Fallon (1937) believed that the increase in pulmonary phospholipids was due to their release from disintegrating monocytes that entered the lungs in response to silica. Heppleston *et al.* (1974) measured increases in phospholipid biosynthesis in the lungs and noted the similarity between alveolar accumulations in the lungs of rats exposed to silica and patients with pulmonary alveolar proteinosis (Heppleston, 1967, 1975; Heppleston *et al.*, 1970), thus providing strong circumstantial evidence that implicated the pulmonary surfactant system. Gabor *et al.* (1978) lavaged the lungs of rats exposed to silica and provided the first direct evidence that clearly demonstrated large increases in the phospholipid content of the extracellular compartment of pulmonary surfactant. This stimulatory effect of silica on phospholipids of extracellular surfactant has been confirmed by others (see, e.g., Richards & Curtis, 1984). We have extended these studies by demonstrating that the effect of silica on phospholipid accumulation in the lungs is associated not only with increases in the extracellular compartment of pulmonary surfactant but also, and to an even larger extent, with increases in the intracellular surfactant compartment. Although previous studies have reported increases in phospholipids of the extracellular compartment of pulmonary surfactant (Gabor *et al.*, 1978; Richards & Curtis, 1984), this is the first time that the intracellular compartment has been examined.

Quantification of the intracellular compartment of surfactant is difficult because of the absence of specific markers. The procedure that we used gave results very similar to those reported by Young *et al.* (1981) and others (Kikkawa *et al.*, 1975; Mason *et al.*, 1977). Our method for estimating recovery of intracellular surfactant phospholipids, by using radiolabelled lamellar bodies, has not been reported previously. Recovery of intracellular surfactant was not influenced by the presence of homogenized pulmonary tissue, indicating that the primary factors determining recovery must reside with the migration of the surfactant through the density gradient. Perhaps the low density of some lamellar bodies could be altered in the presence of high-density sucrose. Fortunately, the recovery of intracellular surfactant was highly reproducible and we considered the average value of 74.2 $\pm$ 3.7% to be satisfactory. Quantification of the extracellular compartment of surfactant gave values for the DSPC content only about 60% of those reported by Young *et al.* (1981). However, on the basis of the exponential nature of the lavage effluent curves we were able to estimate recoveries of at least 98% of the total recoverable extracellular surfactant. Reasons for these differences between our data and the data of Young *et al.* (1981) are not immediately obvious, but could reside with differences in lavage procedure and age of animals used.

The intracellular and extracellular compartments of pulmonary surfactant are closely linked, sharing a direct precursor/product relationship (Jacobs *et al.*, 1982). Surfactant phospholipids are stored in the intracellular compartment and subsequently secreted by the Type II cells into the extracellular compartment. Expansion of one compartment might be expected as a consequence of expansion of the other. In the normal lungs the intracellular and extracellular compartments appear to be in a steady state, with neither compartment undergoing noticeable changes in size. In the lungs of silica-treated rats, with both compartments enormously expanded, steady-state conditions must not be maintained. Heppleston *et al.* (1974) measured the rates of phospholipid biosynthesis and disappearance in whole lungs and concluded that accumulation of phospholipids in the lungs of silica-treated rats was due to an imbalance between the two rate processes. Biosynthesis was increased 4-fold, but disappearance was increased only 2-fold, thereby leading to accumulation of phospholipid. Unfortunately, the study of Heppleston *et al.* (1974) did not directly address the rate processes governing the presence of pulmonary surfactant.

Expansion of the intracellular compartment of pulmonary surfactant could be achieved through either proliferation of Type II cells and/or increased quantities of surfactant within the cytoplasm of the cells. If we assume that the lungs of rats contain about 20% Type II cells (Young *et al.*, 1981), then to account for our observed 3.5-fold increase in DNA content of the lungs, the Type II cells would have to increase about 20-fold. A 20-fold increase in Type II cells would be required to account for all of the increase in DNA, but because the lungs of silica-treated rats contain many migratory cells such as polymorphonuclear leukocytes and macrophages, the actual increase in Type II cells must be much less. However, the intracellular compartment of surfactant increased 123-fold and therefore the Type II cells from silica-treated lungs must contain more surfactant than Type II cells from untreated lungs. Type II cells in the lungs of rats treated with silica have been reported to contain more lamellar bodies (Heppleston & Young, 1971).

The relationship between the acute surfactant response and chronically induced silicosis is not known. Heppleston (1969) suggested that silicotic nodules were not produced as long as the phospholipid response was active, suggesting that perhaps surfactant served a protective role in the lungs. However, others have shown phospholipid accumulation in the lungs of silica-treated rats to be accompanied by increased collagen content, indicating that fibrogenesis and surfactant production might be linked. Current hypotheses regarding the pathogenesis of silicosis identify the alveolar macrophages as a primary target and source of inflammatory factors that stimulate other pulmonary cells. Alveolar macrophages have not been shown to affect directly Type II cells, although alveolar macrophages have been shown to stimulate fibroblasts in a number of ways, especially after exposure of those macrophages to silica (Heppleston & Styles, 1967). Fibroblasts have been shown to synthesize and release factors that directly or indirectly lead to the stimulation of surfactant biosynthesis in Type II cells

(Smith, 1979). Therefore it is conceivable that stimulation of alveolar macrophages by silica would result in the stimulation of fibroblasts that could in turn release factors that stimulate surfactant biosynthesis in the Type II cells.

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