

Stimulation of surfactant phospholipid biosynthesis in the lungs of rats treated with silica

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The effects of intratracheally instilled silica (10 mg/rat) on the biosynthesis of surfactant phospholipids was investigated in the lungs of rats. The sizes of the intracellular and extracellular pools of surfactant phospholipids were measured 7, 14 and 28 days after silica exposure. The ability of lung slices to incorporate [¹⁴C]choline and [³H]palmitate into surfactant phosphatidylcholine (PC) and disaturated phosphatidylcholine (DSPC) was also investigated. Both intra- and extra-cellular pools of surfactant phospholipids were increased by silica treatment. The intracellular pool increased linearly over the 28-day time period, ultimately reaching a size 62-fold greater than controls. The extracellular pool also increased, but showed a pattern different from that of the intracellular pool. The extracellular pool increased non-linearly up to 14 days, and then declined. At its maximum, the extracellular pool was increased 16-fold over the control. The ability of lung slices to incorporate phospholipid precursors into surfactant-associated PC and DSPC was elevated at all time periods. The rate of incorporation of [¹⁴C]choline into surfactant PC and DSPC was maximal at 14 days and was nearly 3-fold greater than the rate in controls. The rate of incorporation of [³H]palmitate was also maximal at 14 days, approx. 5-fold above controls for PC and 3-fold for DSPC. At this same time point, the microsomal activity of cholinephosphate cytidyltransferase was increased 4.5-fold above controls, but cytosolic activity was not significantly affected by silica treatment. These data indicate that biosynthesis of surfactant PC is elevated after treatment of lungs with silica and that this increased biosynthesis probably underlies the expansion of the intra- and extra-cellular pools of surfactant phospholipids.

INTRODUCTION

A number of investigators have demonstrated that surfactant phospholipids accumulate in the lungs of animals exposed to silica (Gabor *et al.*, 1978; Richards & Curtis, 1984; Richards & Lewis, 1985; Dethloff *et al.*, 1986*a,b,c*). The significance of this accumulation and the mechanism responsible for it are not known. Heppleston *et al.* (1974) reported that the incorporation of [³H]-palmitate into lung PCs was increased approx. 3-fold in the lungs of silica-treated rats. More recently, Richards & Lewis (1985) reported that lung slices from silica-treated rats showed increased incorporation of [¹⁴C]-choline into total PC. Both of these investigators studied total phospholipid synthesis; neither specifically examined phospholipids of the surfactant system. Data presented in a recent paper by Lewis *et al.* (1986) indicated that incorporation of intravenously administered [¹⁴C]choline into extracellular surfactant was increased approx. 25% after silica treatment. The latter study is the only published report examining phospholipid precursor incorporation into surfactant phospholipid after silica exposure.

Dethloff *et al.* (1986*a*) have shown that at least 80% of the increase in phospholipids in silica-treated lungs was associated with the surfactant system and it is, therefore, reasonable to expect the rate of surfactant phospholipid

biosynthesis in the lungs of silica-treated rats to be elevated. Alternatively, expansion of the intracellular pool of surfactant phospholipids could be achieved by inhibition of the secretory process without an increase in the biosynthetic rate. Neither of these suppositions has been directly investigated.

To understand better the responses of the surfactant system to silica, we have examined the rates at which lung slices incorporate radiolabelled precursors into intracellular PC and DSPC associated with the intracellular pool of pulmonary surfactant. These studies were an attempt to test the hypothesis that one of the underlying causes for the expansion of the intracellular pool after intratracheal injection of silica is the enhanced biosynthesis of surfactant PCs.

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MATERIALS AND METHODS

Chemicals

The following chemicals were used: [9,10-³H(n)]-palmitic acid (30 Ci/mmol), [*methyl*-¹⁴C]choline chloride (52 mCi/mmol) (New England Nuclear, Boston, MA, U.S.A.), and silica in the form of Berkeley Min-U-Sil (< 5 μm particle size) (Pennsylvania Glass Sand Corp.,

Abbreviations used: PC, phosphatidylcholine; DSPC, disaturated phosphatidylcholine.

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Pittsburgh, PA, U.S.A.). Fatty-acid-poor albumin was prepared from bovine serum albumin (Fraction V; Sigma Chemical Co., St. Louis, MO, U.S.A.) as described by Chen (1967). Palmitic acid was complexed to fatty-acid-poor albumin as described by Smith & Kikkawa (1978).

Animals

Adult male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA, U.S.A.), weighing 225–250 g, were used. Animals were allowed free access to food and water. Animals received either 10 mg of silica in 0.5 ml of sterile saline (0.9% NaCl), or 0.5 ml of sterile saline only, by intratracheal instillation as described by Dethloff *et al.* (1986a). At appropriate times, animals were killed with an overdose of sodium pentobarbital.

Isolation of pulmonary surfactant

For isolation of extracellular surfactant, lungs were excised and lavaged eight times with ice-cold 0.9% NaCl. Lavage effluents were combined and centrifuged at 580 *g* for 10 min to sediment cells. The resulting supernatant was centrifuged at 198000 *g* for 30 min to obtain a surfactant pellet. The intracellular pool of surfactant was isolated on sucrose density gradients as described by Dethloff *et al.* (1986a).

Incorporation of [³H]palmitate and [¹⁴C]choline into intracellular surfactant

After pentobarbital overdose of rats, lungs were rapidly excised and kept at 4 °C. Lung slices (1 mm thick) were prepared with a McIlwain tissue chopper (Brinkmann Instruments, Westbury, NY, U.S.A.) and approx. 100 mg portions were placed in beakers containing 1.5 ml of Krebs-Ringer bicarbonate buffer containing 3% fatty-acid-poor bovine serum albumin. Tissue slices were incubated at 37 °C under an atmosphere of O₂/CO₂ (19:1) in a shaking water bath, essentially as described by O'Neil *et al.* (1977). After allowing 15 min for temperature equilibration, 0.5 ml of a solution containing [³H]palmitate complexed to fatty-acid-poor bovine serum albumin and [¹⁴C]choline was added. Final concentrations were 100 μM-palmitate and 20 μM-choline; final specific radioactivities were 33 μCi/μmol for palmitate and 20 μCi/μmol for choline respectively. At various time intervals, portions of tissue were removed, washed several times with ice-cold phosphate-buffered saline (10 mM-sodium phosphate/0.15 M-NaCl, pH 7.4), and stored at –20 °C until processed for surfactant analysis.

Intracellular surfactant was isolated by homogenizing the tissue slices in 1.0 M-sucrose (12 passes of a Teflon pestle in a Potter-Elvehjem homogenizer). A 2.0 ml portion of the homogenate was overlaid with 0.25 ml each of 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3 and 0.2 M-sucrose and the gradients centrifuged at 200 *g* for 15 min and then at 116000 *g* for 180 min (Beckman SW60 rotor; Beckman Instruments, Palo Alto, CA, U.S.A.). This procedure is similar to that used for isolation of intracellular surfactant from whole lung (Dethloff *et al.*, 1986a), but was scaled down to accommodate the smaller amount of tissue in lung slices.

Phospholipid analysis

Lipids were extracted from the surfactant fractions by the method of Folch *et al.* (1957). DSPC was isolated

from the lipid extracts by the method of Mason *et al.* (1976). PC was isolated by t.l.c. (silica-gel 60 high-performance t.l.c. plates; E. Merck, Darmstadt, Germany) using the method of Gilfillan *et al.* (1983). Phospholipid content was determined by measuring phospholipid phosphorus (Shin, 1962). For incorporation studies, radioactivity associated with PC was determined by scraping thin-layer spots corresponding to PC into scintillation vials and then adding 0.5 ml of water and 10 ml of Aquasol (New England Nuclear Corp., Boston, MA, U.S.A.). Radioactivity associated with DSPC was determined by collecting the DSPC eluate directly into scintillation vials, evaporating the organic solvents under N₂ and then adding 10 ml of Aquasol.

Cholinephosphate cytidyltransferase (EC 2.7.7.15) activity

After sodium pentobarbital overdose, lungs were perfused with 0.9% NaCl (37 °C) until they were free of blood and then lavaged five times with 10 ml of ice-cold 0.9% NaCl. A 20% (w/v) homogenate was prepared in 0.25 M-sucrose/10 mM-Tris/1 mM-EDTA (pH 7.4). The homogenate was centrifuged at 10000 *g* for 20 min, and the resulting supernatant was centrifuged at 100000 *g* for 60 min. Enzyme activity was measured in the supernatant (cytosolic fraction) and in the pellet (microsomal fraction). Fractions were stored at –70 °C until assayed.

Cholinephosphate cytidyltransferase activity was determined in a reaction mixture consisting of 8 mM-CTP, 10 mM-MgCl₂, 4 mM-[methyl-¹⁴C]choline phosphate (0.81 nCi/nmol), 20 mM-Tris/succinate, pH 7.8, and appropriate portions of subcellular fractions. The total volume was 100 μl. Samples were incubated for 30 min at 37 °C. Reactions were stopped by the addition of 10 μl of ice-cold 55% (w/v) trichloroacetic acid (Rooney & Brehier, 1982). The product, CDP-choline, was isolated from portions of the stopped reaction mixture essentially as described by Rooney & Brehier (1982), except that descending paper chromatography was used. After chromatography, CDP-choline was located under u.v. light, and the spot corresponding to CDP-choline was scraped off and counted for radioactivity in Aquasol/water (9:1, v/v). Reactions performed in parallel without protein were used to determine background values. Under the conditions used, reaction rates were linear with time up to at least 30 min and with protein up to at least 40 μg. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Statistics

For incorporation data, overall statistical significance was assessed by one-way analysis of variance. Pairwise comparisons were made by using Fisher's least-significance-difference test. Cholinephosphate cytidyltransferase activities were compared by using Student's *t* test for unpaired samples (Steel & Torrie, 1980). Significance was set at *P* < 0.05.

RESULTS

Effect of silica on body weights and lung weights

Table 1 shows the effect of silica treatment on body-weight gain and lung weights. Over the time period of the experiment, there was no apparent effect of silica on

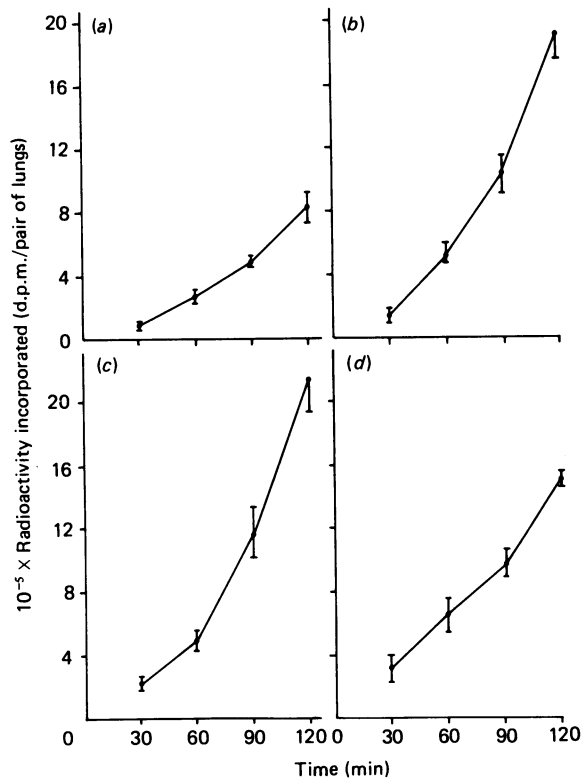


Fig. 3. Effect of silica on the incorporation of [methyl-¹⁴C]-choline into intracellular surfactant-associated PC by lung slices

Because control values did not change significantly over the time course, all control data were pooled. (a) Control; each point is the mean \pm S.E.M. for 12 animals; (b) 7 days after exposure to silica; (c) 14 days; (d) 28 days. For 'silica' results, each point is the mean \pm S.E.M. for four animals.

equilibrated with labelled precursor. Incorporation of [¹⁴C]choline by rat lung slices in a non-linear manner has been observed by others (Young & Silbajoris, 1986). Incorporation of [³H]palmitate into PC and DSPC was also non-linear with time (Figs. 5 and 6).

In view of the non-linearity of incorporation of [¹⁴C]-choline and [³H]palmitate, the interval between 90 and 120 min was used to calculate an incorporation rate as a means for comparing the various groups. Rates of incorporation of [¹⁴C]choline into DSPC were significantly elevated at days 7 and 14, but not at day 28, after silica treatment (Table 2). Statistically significant elevations in the incorporation rate of [³H]palmitate into surfactant PC was seen at 14 days and 28 days. For DSPC, only the 14-day point was significantly elevated.

Cholinephosphate cytidyltransferase

The activity of cholinephosphate cytidyltransferase was examined at the time of maximal incorporation of the radiolabelled precursors, i.e. 14 days after silica treatment (Fig. 7). Silica treatment had no effect on the total activity recovered in the cytosolic fraction. However, total enzyme activity recovered in the microsomal fraction was increased approx. 4.5-fold above control values. In addition, 38% of the enzyme activity was microsomal in silica-treated lungs, whereas only 13% was microsomal in control lungs.

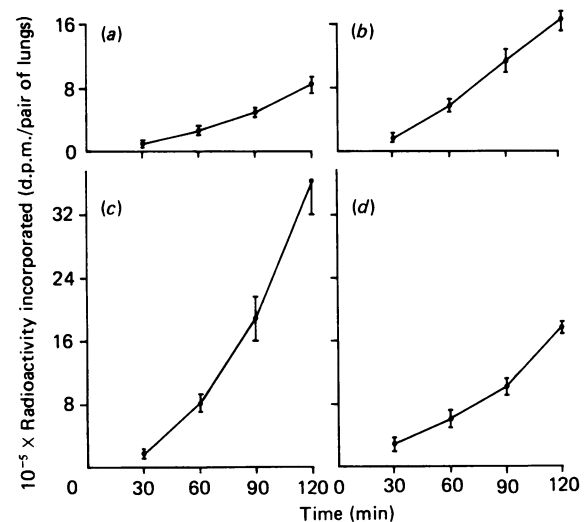


Fig. 4. Effect of silica on the incorporation of [methyl-¹⁴C]-choline into intracellular surfactant-associated DSPC by lung slices

For details, see the legend to Fig. 3.

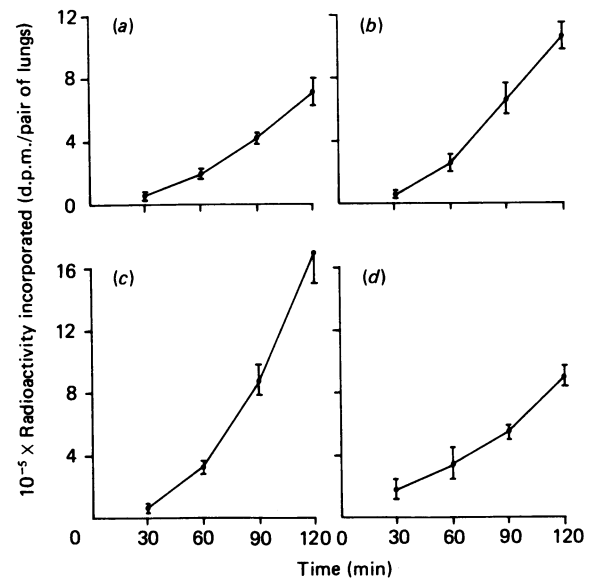


Fig. 5. Effect of silica on the incorporation by lung slices of [³H]-palmitate into intracellular surfactant-associated PC

Because control values did not change significantly over the time course, all control data were pooled. (a) Control; each point is the mean \pm S.E.M. of 12 animals; (b) 7 days after exposure to silica; (c) 14 days; (d) 28 days. For silica, each point is the mean \pm S.E.M. for four animals.

DISCUSSION

Dethloff *et al.* (1986b) showed that, in the normal lung, the relationship between the intra- and extra-cellular pools of surfactant phospholipids was relatively constant, implying the existence of regulatory mechanisms maintaining a balance between both pools. Under normal circumstances a steady state must exist between the intracellular and extracellular pools of surfactant

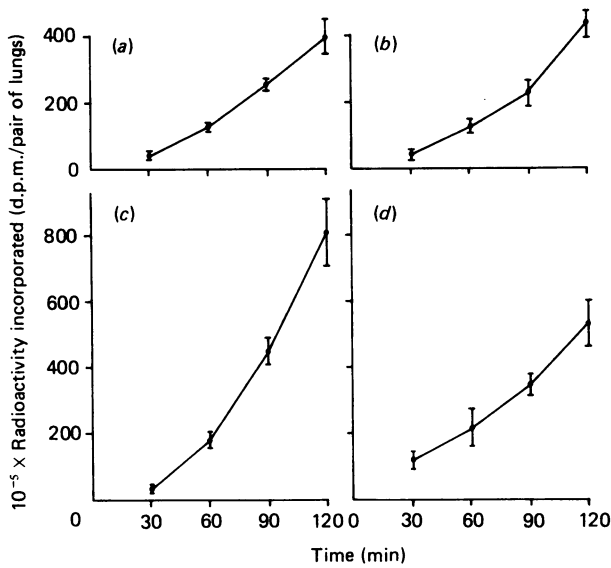


Fig. 6. Effect of silica on the incorporation of [³H]palmitate into intracellular surfactant-associated DSPC by lung slices

For details, see the legend to Fig. 3.

phospholipids. If this were not true, then the relationship between the intra- and extra-cellular pools of surfactant would change. Dethloff *et al.* (1986b) further demonstrated that the relationship between the two pools could be severely disturbed by silica. We have extended those findings by showing that both intra- and extra-cellular pools of surfactant increase markedly in a time-dependent manner after intratracheal instillation of silica. However, although both surfactant pools expanded, the responses of the intra- and extra-cellular pools were not identical. The intracellular pool of surfactant phospholipids increased linearly over the 28-day time period, whereas the extracellular pool increased for the first 14 days and then declined. In view of these differences in the responses of the two pools, where the extracellular or product pool did not follow passively the intracellular or precursor pool, it would appear that the regulatory mechanism governing the relationship between the intra- and extra-cellular surfactant pools is disturbed by silica.

Expansion of the intracellular pool of surfactant after silica exposure could arise as a result of several possible mechanisms; increased biosynthesis and/or decreased

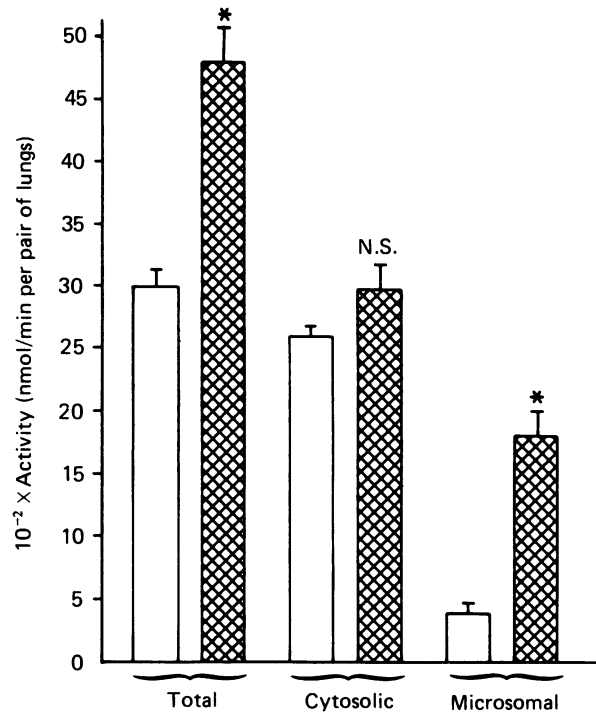


Fig. 7. Cholinephosphate cytidylyltransferase activity in lung subcellular fractions

□, Control; ▨, silica-treated; **P* < 0.01; N.S., not significant.

secretion or increased recycling of extracellular surfactant could all lead to an eventual expansion of the intracellular surfactant compartment. Richards & Lewis (1985) reported that lung slices from silica-exposed rats showed enhanced incorporation of [¹⁴C]choline into total lung PC. This same group recently reported (Lewis *et al.*, 1987) that, in rats treated with silica, there were no significant alterations in the uptake by lung tissue of radiolabelled surfactant instilled into the lungs of rats. These observations and the data in the present experiments are consistent with enhanced biosynthesis as the cause of the expansion of the intracellular surfactant pool. Incorporation of [¹⁴C]choline and [³H]palmitate into phospholipids of the intracellular pool was elevated at all time periods studied up to 28 days after intratracheal injection of silica, strongly indicating increased bio-

Table 2. Rates of [¹⁴C]choline and [³H]palmitate incorporation into intracellular surfactant PC and DSPC

Incorporation rates were determined by taking the slope of the incorporation between 90 and 120 min (see Figs. 4–7). Results are means ± S.E.M. Significance: **P* < 0.05 compared with the corresponding control value.

Treatment	<i>n</i>	10 ⁻³ × Incorporation rate (d.p.m./min per pair of lungs)			
		[¹⁴ C]Choline		[³ H]Palmitate	
		PC	DSPC	PC	DSPC
Control	12	11.2 ± 1.8	9.6 ± 1.3	11.1 ± 2.0	4.5 ± 0.9
Silica					
7 days	4	29.5 ± 2.3*	13.4 ± 1.1	18.9 ± 1.9	7.0 ± 1.4
14 days	4	32.4 ± 4.4*	26.4 ± 3.3*	59.3 ± 5.1*	12.1 ± 0.3*
28 days	4	18.5 ± 1.6	13.6 ± 1.0	25.0 ± 0.7*	7.9 ± 0.8

synthesis of surfactant phospholipids. However, the rate at which [^{14}C]choline appears in intracellular surfactant phospholipids depends not only on the rate of biosynthesis, but also on the sizes of the precursor pools; that is, the sizes of the intracellular pools of choline, choline phosphate and CDP-choline. Consequently, unless pool sizes are known, the incorporation of [^{14}C]choline must be interpreted with caution. Post *et al.* (1984) measured the sizes of the precursor pools in Type II cells isolated from normal rats. However, it is not known to what extent silica treatment may alter these precursor pools. Additionally, silica treatment could stimulate choline uptake by Type II cells, which could then in turn result in increased biosynthesis of surfactant PC and DSPC. The rate at which [^3H]palmitate appears in surfactant phospholipids depends on the sizes of the intracellular pools of palmitate and numerous lipidic intermediates. With palmitate, determination of the appropriate intermediate pools would be technically difficult, if not impossible, at this time. Palmitate and choline enter the PC-biosynthetic pathway at quite different points, and together they give a more complete assessment of phospholipid biosynthetic activity than either alone. Increased incorporation rates could be obtained without actual increases in biosynthesis of phospholipids if the precursor pools were decreased in Type II cells in the lungs of silica-treated rats. However, because of the observed increases in size of many of the Type II cells in silica-treated lungs (Miller *et al.*, 1986), we think that it is unlikely that any of these precursor pools would be decreased after exposure of lungs to silica. Unequivocal proof of increased biosynthesis will require examination of phospholipid precursor pools in isolated Type II cells. Nevertheless, the observation that incorporation of both [^{14}C]choline and [^3H]palmitate into surfactant phosphatidylcholines were elevated after exposure to silica is good evidence that biosynthesis itself is also increased.

Additional evidence which indicates increased biosynthesis of surfactant phospholipids is the increased activity of cholinephosphate cytidyltransferase in the microsomal fraction from the lungs of silica-treated animals. Cholinephosphate cytidyltransferase is thought to be the rate-limiting enzyme in PC biosynthesis in a variety of systems, including the lung (Vance & Choy, 1979). Moreover, the microsomal form of this enzyme is believed to be the physiologically active form, with the cytoplasmic form being a storage or reserve pool of enzyme (Vance & Pelech, 1984). The approx.-4.5-fold increase in microsomal activity coupled with the observed increase in incorporation of [^{14}C]choline are indicative of enhanced biosynthetic activity in response to silica. Consistent with our findings, Richards & Lewis (1985) reported that, in tissue slices from silica-exposed lungs, increased incorporation of [^{14}C]choline into total lung PC was associated with increased cholinephosphate cytidyltransferase activity in tissue homogenates.

PC is a major constituent of membranous structures within the cell, including surfactant. No single phospholipid is unique to surfactant, and only the relatively high content of DSPC distinguishes surfactant PC from the PC of other membranous structures (Gilfillan *et al.*, 1983). Therefore we examined both total phospholipid and DSPC as an assurance that we were indeed dealing with surfactant phospholipids and not phospholipids of other membranous structures that conceivably might

contaminate our isolated surfactant phospholipids. Interestingly, we found that, although the percentage of DSPC in the intracellular pool of surfactant was not affected by silica and did not change throughout the time course of the experiment, the percentage of DSPC in the extracellular pool was markedly altered by silica. In the control animals the percentages of DSPC in the intra- and extra-cellular pools were similar, in agreement with results obtained in previous studies (Dethloff *et al.*, 1986a). However, with silica, the percentage of DSPC in the extracellular pool decreased markedly below that of the controls and the intracellular pool. Because of the methodology used to isolate extracellular surfactant, the extracellular pool may have been contaminated with membranous components from dead cells. Examination of lung tissue sections and lavage effluent from silica-treated lungs indicated the presence of cellular debris (Dethloff *et al.*, 1986c), suggesting that, during isolation, the extracellular pool could have become contaminated with the membrane fragments from damaged cells. Contamination with cell debris would result in an overestimation of extracellular surfactant, which could contribute to the apparent non-linear response of the extracellular pool to silica. Consequently, measurement of extracellular DSPC is likely to be a better indicator of the true size of the extracellular surfactant pool than is measurement of total phospholipid.

Increases in the size of the intracellular pool of surfactant phospholipids could be accounted for by either increased numbers of Type II cells and/or increased amounts of surfactant phospholipid per cell. Previously, we demonstrated that the number of Type II cells is increased in silica-treated lungs (Miller *et al.*, 1987a). On the basis of the number of Type II cells in the lungs and their estimated surfactant content (Young *et al.*, 1981) the expanded intracellular pool of surfactant cannot be accounted for simply by an increased number of Type II cells (Dethloff *et al.*, 1986a). We have recently shown that many Type II cells in the lungs of silica-treated rats are hypertrophic (Miller *et al.*, 1986), and it is to these cells that we must turn to account for the expanded intracellular pool of surfactant. Moreover, after treatment of the lungs with silica, the population of normal Type II cells remained relatively constant, but the hypertrophic Type II cells increased markedly (Miller *et al.*, 1987b). The elevations in incorporation of precursors into surfactant phospholipids will likely prove to be associated primarily with the hypertrophic Type II cells. Increased surfactant biosynthesis by hypertrophic Type II cells would indicate that this cell type may be very important in the regulation of surfactant biosynthesis, especially under conditions involving pulmonary damage and/or disease.

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