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Effects of Smoke Inhalation on Alveolar Surfactant Subtypes in Mice

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The effects of smoke inhalation on alveolar surfactant subtypes were examined in mice exposed for 30 minutes to smoke generated from the burning of a flexible polyurethane foam. At 4 or 12 hours after the exposure, three surfactant pellets, P10, P60, and P100, and a supernatant, S100, were prepared by sequential centrifugation of lavage fluids at 10,000 g for 30 minutes (P10), 60,000 g for 60 minutes (P60), and 100,000 g for 15 hours (P100 and S100). Phospholipid analysis and electron microscopy were performed on each fraction. Smoke exposure dramatically altered the normal distributions of these fractions: it significantly increased the phospholipid content of the heavier subtype, P10, which is thought to represent newly secreted surfactant; had no effect on the intermediate form, P60; and dramatically increased the phospholipid content (approximately fivefold) of the lighter subtypes, P100 and S100, which are believed to represent catabolic end-products of alveolar surfactant. Only P100 was structurally altered by the smoke. These results represent alterations of the normal metabolic processing of alveolar surfactant. Whereas the mechanism is yet to be defined, it seems to involve a small but significant increase in the newly secreted surfactant, as well as an excessively high accumulation of the structurally altered catabolic forms of the secreted surfactant. (Am J Pathol 1994, 145:941–950)

Lung surfactant is a phospholipid-rich material that lines the mammalian lung where it functions to promote alveolar stability by decreasing the surface tension at the air-alveolar interface.[1,2] Lung surfactant is synthesized in the alveolar type II cell and stored in membrane-bound vesicles (lamellar bodies) for ultimate release to the alveoli.[1,2] Once released, the lamellar body complex undergoes a series of structural rearrangements to produce the active monolayer and to provide a readily available pool for the continuous replenishment of material forming the monolayer.[3] There is considerable evidence to indicate that used alveolar surfactant constituents are removed from the alveoli and recycled by the type II cell into new lamellar bodies.[4] In fact, alveolar surfactant can be separated by techniques involving centrifugation into a series of subfractions that have been shown to be in metabolic sequence such that the heavy, easy-to-sediment subfractions constitute newly secreted material, whereas the lighter, difficult to sediment fractions, containing much smaller vesicular structures represent the used alveolar surfactant constituents.[5] Fractions intermediate between the two have also been identified.

There is considerable evidence to indicate that many noxious agents and toxic substances cause derangement, or at least a perturbation of the pulmonary surfactant system by interruption of each or any of the processes of biosynthesis,[6,7] secretion,[8,9] alveolar processing,[10–13] or clearance of the used surfactant from the alveoli.[14–16] The processes involving biosynthesis, secretion, and clearance of surfactant have been somewhat extensively studied.[4] Recent attention has focused on intraalveolar processing, or metabolism, particularly as it pertains to the subtypes fractionated from alveolar lavage fluid. There is ample evidence to suggest that the proportions of these subtypes change, not only in response to certain physiological stimuli, such as birth,[17] exercise,[18] and deep breathing,[19] but also

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in a number of pathological conditions that are characterized by a dysfunctional surfactant system and the development of pulmonary edema.[7–15,19,20] An improved understanding of the mechanisms regulating these metabolic processes would have important implications for the development of surfactant treatments to alleviate these problems.

We have been studying a mouse model of lung injury, induced by exposure to smoke generated from the combustion of a flexible polyurethane foam. Lung injury from fire smoke inhalation is a major prognostic factor in the survival of victims rescued from accidental fires,[21,22] and it is a more common cause of death than burns alone.[23] About 80% of all fire-related deaths occur in residential fires,[24] which are commonly started by accidental contact of lighted cigarettes with sofas or bed materials containing flexible polyurethane foam.[25] The foam used in our model is of the type used in the manufacture of upholstered furniture, and mice exposed from smoke to it develop a sublethal pulmonary edema by at least 4 hours after a 30-minute exposure. We also observed significant effects on the surfactant system, affecting both the intracellular (lamellar body) and extracellular surfactant pool, the most dramatic effect being an almost twofold increase in the phospholipid content of the extracellular pool.[20] We subsequently found that the changes in the surfactant system were first evident at 4 hours after the exposure and that they were sustained for at least 12 hours. As we did not fractionate the lavage pool into individual surfactant subtypes in either of these studies, we were not able to determine which step in the surfactant life cycle was altered. Recently, we developed a fractionation scheme involving differential centrifugation for isolating individual alveolar surfactant subtypes in healthy mice.[26] In the present study, we used this fractionation scheme to study the effect of the smoke exposure on the individual subtypes.

Materials and Methods

Random-bred, pathogen-free, Carworth Farms White, Swiss-Webster male mice, 8 to 9 weeks old, were housed (five per cage) in a controlled environment with food and water available *ad libitum*. The model used for studying smoke toxicity incorporated the guidelines of the National Bureau of Standards[27,28] and the National Research Council[29] and was described elsewhere.[30,31] Briefly, the fuel was flexible polyurethane foam, synthesized from toluene diisocyanate and free of fire retardants and pigments. It was thermally decomposed, without flaming in a tube furnace, preheated, and maintained at 400 ± 8 C. Air was made to flow through the furnace and to carry the diluted smoke into a 20-l volume exposure chamber, where the smoke was uniformly dispersed by an internal fan, and allowed to escape. Nine hundred mg of foam were decomposed, based on the ratio of 45 mg per l of chamber volume. The exposure duration was 30 minutes. Usually 12 to 13 mice and, rarely, up to 16 mice were exposed, and in all instances, the body mass of the mice never exceeded 5% of the chamber volume; usually it was less than 3%. Throughout the exposures, the chamber temperature never exceeded 26 C, and the chamber oxygen levels did not fall below 18.5%, ie, there was neither heat stress nor a significant hypoxic atmosphere. Although not consistently monitored, chamber humidity occasional rose above 50%, depending on the ambient humidity. At either 4 or 12 hours after the exposure the mice were killed by an intraperitoneal injection of sodium pentobarbital for isolation and analysis of alveolar surfactant subtypes. These time intervals were selected as they represent 1) the earliest time at which changes in unfractionated lavage fluid phospholipids were observed (4 hours) and 2) a later time period in the injury for which the changes seemed to persist (12 hours). Unexposed mice served as controls.

After killing the mice, their lungs were lavaged with isotonic saline in 4 × 1.0 ml aliquots as described previously.[6] The lavage washings from two to three mice were pooled and following a 5-minute centrifugation at 140 *g* to remove a cellular pellet, surfactant subfractions were prepared by sequential centrifugation at 10,000 *g* for 30 minutes, 60,000 *g* for 60 minutes, and 100,000 *g* for 15 hours. The resultant subfractions are respectively referred to as P10 (10,000 *g* pellet), P60 (60,000 *g* pellet), P100 (100,000 *g* pellet), and S100 (100,000 *g* supernatant). The cellular pellet is designated as Pc. The details of this isolation procedure were described in a previous communication.[26] For comparative purposes, lamellar bodies were isolated from the postlavage lung tissue using differential and density gradient centrifugation as described previously.[32,33]

Aliquots of each lavage fraction and lamellar body preparation were extracted with chloroform/methanol (2:1, v/v) for phosphorus analysis using the procedure of Bartlett[34] as described previously.[29] Individual phospholipids were separated by two-dimensional, thin-layer chromatography, and the spots were visualized and their phosphorus content

determined as described elsewhere.[33] The disaturated species of phosphatidylcholine (DSPC) was isolated for analysis as described by Mason et al.[35]

In some experiments, samples of the alveolar surfactant subfractions, P10, P60, and P100 were fixed in 0.1 mol/L cacodylate buffered 2.5% glutaraldehyde (pH 7.3) and processed for electron microscopy as described previously.[33] In other experiments, aliquots of each alveolar surfactant fraction and lamellar body preparation were removed for assessment of surface activity using the pulsating bubble surfactometer as described by Enhorning.[36]

Statistical comparison of the results were performed using Student's *t*-test[37] or Duncan's New Multiple Range Test.[38] For the Duncan's, a one way analysis of variance was performed prior to the test. The F value was significant to 5%.

Results

As described elsewhere,[30,31] exposed mice manifested labored respirations by the end of the exposures, and this persisted for the entire 12 hours. The histopathology of the lung[31] revealed spotty necrosis of airway epithelium, mainly in the trachea, and marked parenchymal vascular congestion, collapse, and some edema, notably around bronchovascular trunks. Neutrophil leukocyte infiltration of parenchyma was not evident or significant until about 4 hours after exposure, an observation that correlated with the absolute and relative numbers of neutrophils counted in bronchoalveolar lavage fluid recovered from the lungs. On the other hand, there were no increases in macrophage numbers observed by histology, even by 8 and 12 hours after exposure. In fact, bronchoalveolar lavage showed significant decreases in macrophage recoveries. These decreased recoveries were shown to be mainly related to destruction of macrophages after phagocytosis of polymeric smoke particles.[30,31]

Also in agreement with previously reported results,[20] no significant differences were observed in the mean lung weight (grams wet lung per mouse) in the individual groups (untreated: 0.231 ± 0.027, n = 8; 4 hours after smoke exposure: 0.293 ± 0.025, n = 8 and 12 hours after smoke exposure: 0.241 ± 0.038, n = 20; $P > 0.05$ for each pair by Duncan's Multiple Range Test). Therefore, where relevant, data are expressed on the basis of wet lung weight.

The total phospholipid and total DSPC content of unfractionated lavage fluid is shown in Figure 1A. For comparative purposes, the results obtained for the isolated lamellar bodies are included (Figure 1B). In agreement with previous findings,[20] the total phospholipid content of the alveolar lavage fluid was significantly increased by the smoke exposure. This increase, which was almost twofold, was observed at 4 hours postexposure and persisted for 12 hours. Similar increases were observed in the DSPC content of

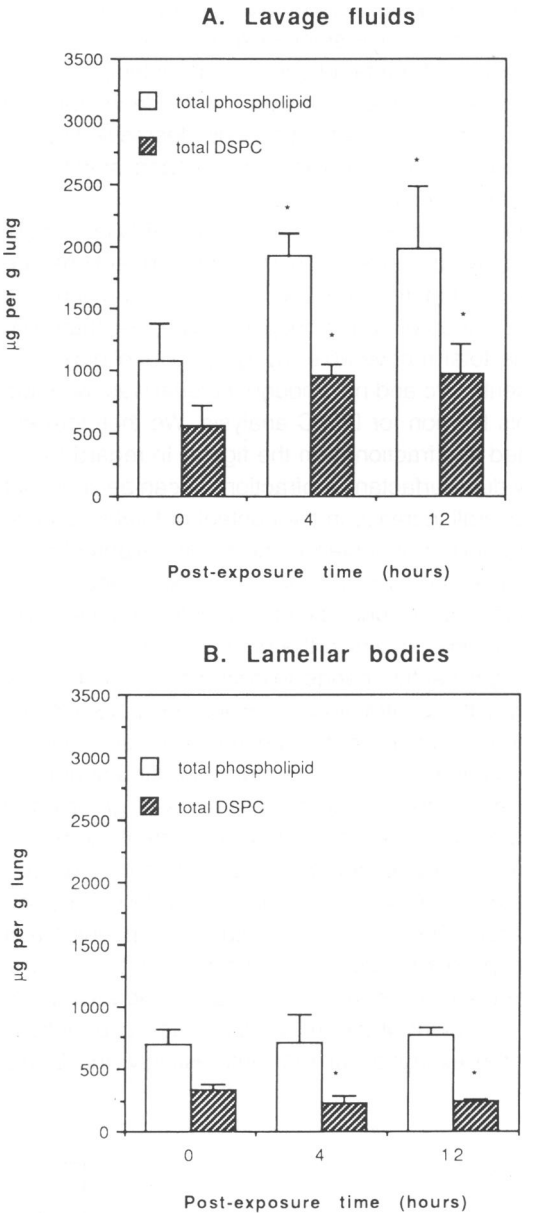

Figure 1. *Effect of smoke exposure on the total phospholipid (open bars) and total disaturated phosphatidylcholine (closed bars) content of alveolar lavage fluids* (**A**) *and lamellar bodies* (**B**) *obtained from mice. Lamellar bodies were isolated from the postlavaged lung tissue by procedures involving differential and density gradient centrifugation. Each value represents the mean ± 1 SD for nine (control), eight (4 hours postexposure), or six (12 hours postexposure) determinations.*

the alveolar lavage fluids. Also in agreement with previous studies,[20] the smoke exposure did not significantly alter the total phospholipid content of the lamellar body fraction but significantly decreased the DSPC content of this fraction. This decrease was observed at 4 hours postexposure and persisted for the 12-hour observation period.

The total phospholipid and total DSPC content of the individual alveolar surfactant subfractions are shown in Figure 2. For each time period examined, very small amounts of phospholipid (ie, less than 10% of the total recoverable lavage phospholipid) were present in Pc and not enough material was available in this fraction for DSPC analysis. We therefore excluded this fraction from the figure. In regard to the individual surfactant subfractions, it can be seen that the overall increase in the content of total lavagable phospholipid observed in the smoke-exposed mice was reflected by significant increases in each of the lavage subfractions, except the intermediate pellet fraction, P60, in which the phospholipid content did not significantly change following smoke exposure. Among the subfractions in which the phospholipid content did increase, the greatest increases seemed to occur in P100 (which contains the largest amount of phospholipid in the smoke-exposed group) and S100 in which the phospholipid content increased nearly fivefold, ie, from 60.1 ± 25.4 in the unexposed controls to 291.4 ± 158.5 μg/g lung at 12 hours postexposure. This pattern of change was observed at 4 hours postexposure and persisted for up to 12 hours. Smoke exposure also significantly increased the DSPC content of the individual lavage subfractions with the exception of P60. Interestingly, the DSPC content of this subfraction was significantly decreased following the smoke exposure but only after 12 hours.

The effect of smoke exposure on the distribution of total alveolar surfactant phospholipid over the individual subtypes is shown in Table 1. The results are expressed as relative percent of total alveolar surfactant (P10 + P60 + P100 + S100) present in each subfraction. The cellular pellet, Pc, comprised less than 10% of total lavage phospholipid for each study group and was not included in the calculation. As shown, smoke exposure significantly altered the distribution of phospholipid among the subfractions. The most dramatic changes were in P60, which decreased from 30 to 10% of the total alveolar pool, and P100, which increased almost twofold following the smoke exposure. As a consequence of these changes, P60, which comprised the second largest subfraction in the unexposed mice, constituted a minor component in the smoke-exposed mice, and P100, which comprised only 20% of the alveolar surfactant in the unexposed mice, represented the major constituent in the smoke-exposed group. As a percent of total alveolar phospholipid, changes were also observed in the other two subtypes, such that the relative proportion of P10 slightly but significantly decreased, whereas that of S100 significantly increased following the smoke exposure. It is also noteworthy that the pattern of change in the distribution of alveolar surfactant phospholipids was observed as early as 4 hours after the exposure.

Despite these dramatic changes in the phospholipid content of the alveolar surfactant subfractions, smoke exposure had very little effect on the overall

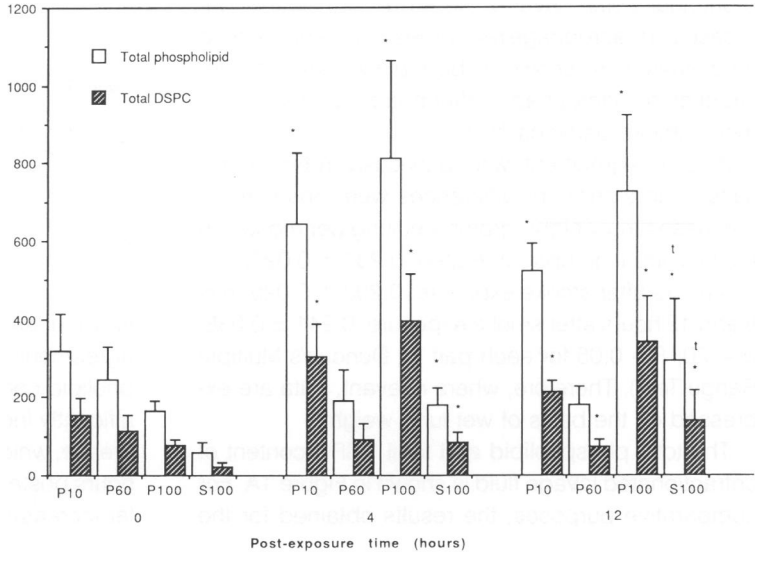

Figure 2. *Effect of smoke exposure on the total phospholipid* (open bars) *and total disaturated phosphatidylcholine* (closed bars) *content of alveolar surfactant subfractions. Fractions were prepared by sequential centrifugation of lavage returns at 10,000 g for 30 minutes (P10), 60,000 g for 60 minutes (P60), and 100,000 g for 15 hours (P100-pellet and S100-supernatant) after an initial 5-minute centrifugation at 140 g to remove a cellular pellet, Pc. Data representation are as for Figure 1. *Significantly different from control* (P>< *0.01*), t *significantly different from 4-hour postexposure value* (P < *0.05*) *by Duncan's Multiple Range Test.*

Table 1. *Effect of Smoke Exposure on Distribution of Phospholipid over Individual Alveolar Surfactant Subfractions in Mice*

Treatment	*n*	Relative percent of total alveolar surfactant phospholipid			
		P10	P60	P100	S100
Control	9	39.4 ± 3.3	31.1 ± 3.9	21.9 ± 4.4	7.7 ± 2.8
4 hours postexposure	8	34.9 ± 4.6*	10.3 ± 3.0‡	44.6 ± 7.1‡	10.2 ± 3.7
12 hours postexposure	6	31.0 ± 5.1†	10.4 ± 11.2‡	41.7 ± 11.2‡	16.6 ± 7.6†§

Mice were killed either 4 or 12 hours after a 30-minute exposure. Alveolar surfactant subfractions, P10, P60, P100, and S100 were prepared as described in the legend to Figure 2.

PC, which contributed less than 10% of total lavage phospholipid for each group, was not included.

* Significantly different from control ($P < 0.05$); † significantly different from control ($P < 0.01$); ‡ significantly different from control ($P < 0.001$), § significantly different from 4-hour postexposure group ($P < 0.05$) by Duncan's Multiple Range Test.

phospholipid composition of these fractions. Representative results are shown for subfractions obtained from mice killed 12 hours after the exposure (Table 2). In comparing the compositions of the individual subfractions, only one slight difference was observed and that was a slight decrease in the relative proportion of phosphatidylcholine (PC) in S100 in comparison to P100. These phospholipid profiles are almost identical to those obtained for untreated controls and mice examined 4 hours after the exposure (results not shown but reported in previous publications[20]). In agreement with previous reports,[20] smoke exposure had no effect on the overall phospholipid composition of lamellar bodies. The overall phospholipid profile of these structures was very similar to those of the alveolar surfactant subfractions (results not shown).

Interestingly, however, changes were observed in the DSPC/PC ratio of some of the alveolar surfactant subfractions and the lamellar bodies as well (Table 3). In agreement with previous reports,[20] the DSPC/PC ratio of lamellar bodies progressively and significantly decreased following the smoke exposure. This decrease was observed as early as 4 hours following the exposure. In the alveolar surfactant subfractions, decreases were also observed in the DSPC/PC ratio following smoke exposure, but only in the P10 and P60 subfractions and only at 12 hours following the exposure.

In a previous study,[26] we demonstrated ultrastructural differences in P10, P60, and P100 isolated from normal healthy mice. P10 consisted mainly of large multilamellated structures typical of newly secreted surfactant; P100 consisted of small unilamellar vesicles typical of catabolic forms of secreted surfactant, and P60, though less homogeneous than P10 and P100, contained mainly large unilamellar vesicles with somewhat diffuse membranes. Electron micrographs of these fractions obtained following smoke exposure are shown in Figure 3. There seemed to be no loss of structural integrity of the isoforms present in P10 and P60. In addition to the large multilamellated structures, tubular myelin was also present in P10. A dramatic finding, however, was the virtual absence of vesicular structures from P100. This fraction seemed to consist almost entirely of amorphous material with occasional disclike structures appearing in each field examined. We did not examine the isolated lamellar bodies by electron microscopy in the present study as we had done so previously and found no obvious effects of smoke exposure on the isolated structures.[20]

In a previous study,[26] we reported that P10, P60, and isolated lamellar bodies demonstrated surface activity in that they were able to reduce the surface tension to less than 10 mN/m when compressed to minimum bubble radius in a pulsating surfactometer when examined at concentrations at 5 mg phospholipid/ml. We also reported that P100 did not exhibit this property. In the present study, only a few samples from the smoke-exposed mice (4-hour postexposure group) were available for surface tension studies. We found that smoke exposure did not seem to alter the

Table 2. *Phospholipid Composition of Alveolar Lavage Subfractions Obtained from Smoke-Exposed Mice*

Fraction	*n*	% of total lipid phosphorus						
		PC	PG	PI	PS	SM	PE	X
P10	4	82.2 ± 2.7	10.4 ± 0.6	1.6 ± 1.0	1.7 ± 2.1	1.6 ± 0.4	2.5 ± 2.4	0.1 ± 0.1
P60	4	84.3 ± 0.8	10.3 ± 0.4	1.4 ± 0.7	0.8 ± 0.4	0.6 ± 0.6	2.3 ± 1.0	0.2 ± 0.3
P100	5	83.8 ± 1.6[a]	10.3 ± 1.4	1.6 ± 0.8	0.8 ± 0.8	0.7 ± 0.6	2.4 ± 0.6	0.5 ± 1.0
S100	4	79.9 ± 2.0*	9.4 ± 0.4	2.0 ± 0.2	1.5 ± 1.3	2.3 ± 2.5	1.8 ± 0.4	3.1 ± 2.1

Mice were killed 12 hours after a 30-minute exposure. Each value represents the mean ± 1 SD for the number of determinations shown. Fractions were prepared as described in the legend to Figure 2. Not enough material was present in Pc for analysis.

Phospholipids include: PC, phosphatidylcholine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin; PE, phosphatidylethanolamine; X, unidentified phospholipid.

* Significantly different from P100 ($P < 0.05$ by Duncan's Multiple Range Test).

Table 3. *Effect of Smoke Exposure on DSPC/PC Ratios of Lamellar Bodies and Alveolar Surfactant Subfractions*

Treatment	DSPC/PC Ratio				
	Lamellar bodies (*n*)	P10 (*n*)	P60 (*n*)	P100 (*n*)	S100 (*n*)
Control	0.593 ± 0.065 (9)	0.601 ± 0.041 (10)	0.583 ± 0.012 (4)	0.582 ± 0.050 (10)	0.592 ± 0.024 (4)
4 hours post-exposure	0.473 ± 0.064* (7)	0.586 ± 0.091 (9)	0.576 ± 0.024 (3)	0.586 ± 0.082 (3)	0.602 ± 0.037 (5)
12 hours post-exposure	0.434 ± 0.015* (3)	0.497 ± 0.101†‡ (10)	0.484 ± 0.043*§ (5)	0.561 ± 0.053 (5)	0.600 ± 0.066 (5)

Mice were killed either 4 or 12 hours after a 30-minute exposure. Lamellar bodies were prepared from postlavage tissue as described in the legend to Figure 1 and alveolar surfactant subfractions, P10, P60, P100, or S100 as described in the legend to Figure 2. Each value represents the mean ± 1 SD for the number of determinations shown.

* Significantly different from control ($P < 0.01$), † significantly different from control ($P < 0.05$), ‡ significantly different from 4-hour postexposure group ($P < 0.05$), § significantly different from 4-hour postexposure group ($P < 0.01$) by Duncan's Multiple Range Test.

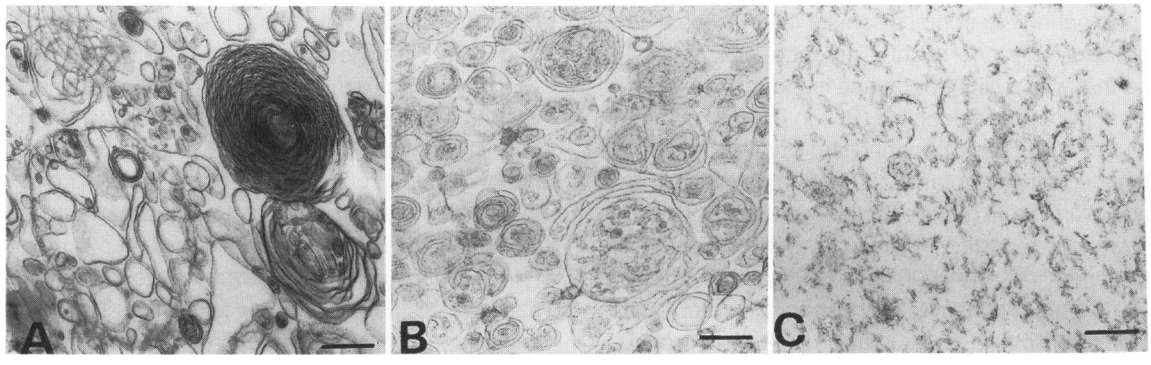

Figure 3. *Electron micrographs of mouse alveolar subfractions P10* (**A**); *P60* (**B**); *P100* (**C**) *prepared 4 hours after a 30-minute exposure to smoke by sequential centrifugation of lavage returns as described in the legend to Figure 2. Scale bar, 340 nm. Magnification 29,000×.*

surface properties of these fractions. Lamellar bodies, P10, and P60, were capable of reducing the surface tension to 1.0 to 8.8 mN/m, whereas for the P100 fraction, this value ranged from 14.7 to 24.5 mN/m in individual samples and remained at these high values for up to at least 20 minutes.

Discussion

In agreement with our previous report,[20] we found that the conditions of smoke exposure described in this study resulted in an almost twofold increase in the phospholipid content of the total alveolar surfactant pool. Elevated pools of alveolar phospholipids have been shown to result from experimental exposure to a variety of other injurious agents, including diesel fuel particles,[39] silica dust,[15] ozone,[10,40] and radiation.[11] Until recently, however, there have been few reports on the identification of the particular subfraction affected.

Using morphometric techniques, Balis et al[10] found that ozone exposure in rats resulted in an increased accumulation of the alveolar surfactant subtypes typical of newly released surfactant, ie, multilamellated structures, and a paucity of the much smaller unilamellar structures typical of the metabolized or end-product forms of alveolar surfactant. This result suggests that ozone in some way inhibited the unwinding of the newly secreted surfactant, which ordinarily progresses through the normal metabolic pathway with delivery of constituents to the active monolayer, possibly because ozone exposure impairs some of the functions of the major surfactant-associated protein, SP-A,[41] one of which may indeed be to initiate the metabolic conversion of secreted surfactant.[13]

Similarly Gross,[11] in a study of a mouse model of adult respiratory distress syndrome induced by irradiation, found an accumulation of the earlier released (or secreted) surfactant subtype and a decrease in the lighter metabolic end-product subtypes. This finding was attributed to an inhibitor, eg, α_1-antitrypsin, which prevents the catalytic conversion of secreted surfactant to the ultrastructural form, namely tubular myelin, which is the first produced in the metabolic sequence. In a previous report from this same group,[42] the metabolic conversion of the alveolar surfactant was attributed to a serine proteinase activity,

a convertase. A role for SP-A, possibly as a substrate for this convertase, has been implicated in this process as well.[13]

In contrast to the above studies, alveolar injury in rabbits induced by N-nitrosos-N-methylurethane was associated with a nearly fivefold increase in the quantity of the lighter surfactant subtypes with a concomitant decrease in the heavier subtype following the injury,[12] suggesting accelerated metabolic processing. An increased accumulation of the lighter subtypes was also shown to be responsible for the excessive amounts of alveolar surfactant found in rats exposed to the anorectic drug, chlorphentermine.[16] Though the end result seems to be the same, these compounds seem to operate through different pathways, N-nitrosos-N-methylurethane by affecting the actions of SP-A[13] either directly or indirectly by causing type II cell necrosis[12,13] and chlorphentermine by directly binding the alveolar surfactant phospholipids.[16] Chlorphentermine seems to have no adverse effects on the type II cell.[16]

In the present study, we found not only an increase in the phospholipid content of the heavy, easy-to-sediment alveolar surfactant subfraction following exposure to smoke, but also a substantial and significant increase in the light and final supernatant subfractions. We did not do metabolic labeling studies in our mouse model. However, the subfractions that we describe in this and a previous report on normal mouse lung[21] are very similar to those identified by Gross and Narine in both normal and injured mice.[11,43] These authors have also shown by both *in vivo*[43] and *in vitro*[44] studies the same metabolic progression from heavy to light subtype as has been shown for several other species, in both normal and pathological states.[5,12,13,16] It is thus reasonable to suggest that the increase in P10 that we found in this study may result from increased secretion of surfactant by the type II cell, as has been found on exposure to a variety of other noxious agents,[15,39,40] whereas the increased accumulation of material in the lighter subfractions may result from an accelerated conversion to the lighter subtypes and/or a decrease in the rate of removal of these products. We reported some preliminary work[45] that suggests an accelerated conversion of P10 to P100 or S100 may, in fact be the initial response. Further study would be necessary to confirm these preliminary findings.

Previous studies with our model[30,31] showed a significant decrease in bronchoalveolar lavage-recoverable alveolar macrophages following smoke exposure. Though the alveolar macrophages may not provide a significant route for trafficking or clearance of alveolar surfactant components,[4,46] it is not unreasonable to speculate that at least a part of the accumulation of surfactant constituents, and in particular DSPC, that we found in P100 and S100 may in fact be due to the reduced macrophage numbers, which would eliminate one possible route for clearance of these constituents under normal circumstances. Whereas the precise role of alveolar macrophages in this process is not known, our results consistently demonstrate that changes in surfactant and alveolar macrophages are early effects and not secondary to a later inflammatory reaction. Another possibility to consider, of course, in explaining the accumulation of the P100 subfraction, is an interruption of the clearance by type II cell. This could result from damage to the type II cell itself or to the alveolar surfactant constituents or to any wide variety of causes. These may be difficult to delineate because the toxicity of the smoke from flexible polyurethane foams seems to be significantly related to thermal breakdown of polymeric urethane bonds with release of isocyanates, highly reactive free radicals.[25]

In addition to the observed accumulation of phospholipid in P100 and S100, the slowed clearance of these fractions is suggested in the present study by the finding that the DSPC/PC ratios of these fractions, in contrast to those of P10 and P60 and the lamellar bodies, had not decreased even after 12 hours. This suggests that P100 and S100 may contain metabolized lamellar bodies that were released before the smoke insult, whereas P10 and P60 may contain the more recently released lamellar bodies, ie, those with a reduced DSPC/PC ratio.

The effects of smoke exposure or the structural integrity of the isoforms was also of interest. P10 and P60 seemed to be little affected; in fact, the presence of tubular myelin in P10 indicates that the formation of this particular isoform may not be perturbed. P100, on the other hand, lost its vesicular structure. Whether this is a primary effect of the smoke constituents, or secondary to some other effect, cannot be determined from the present study. Whatever the cause, the structural alteration could be a major contribution to their failure to be cleared from the alveoli. Smoke exposure did not affect the surface properties of the individual subfractions. We did not do a detailed protein analysis in this study, but we did observe large amounts of albumin associated with each of the subfractions. This would probably not affect the surface activity of P10 and/or P60, provided adequate levels of SP-A, SP-B, and/or SP-C were present.[47,48] How it might affect P100, which in the normal healthy animal is not surface active, is not known. Given that this

fraction accumulates in the smoke-exposed lung, such that by 12 hours it constitutes nearly 60% of the total alveolar surfactant phospholipid pool *versus* less than 30% for the normal unexposed lung, suggests that this may constitute the major factor contributing to the impairment of lung function associated with this model.[30,31] Further study would be required to confirm these findings.

Another interesting finding in the present study is the apparent lack of effect of the smoke exposure on the quantity of phospholipid in the intermediate subfraction, P60. Recently, Nicholas et al[49] reported that changes in breathing patterns altered subtype distribution but with essentially no change in a subtype they designated as PL alv-1. This is a heavy subtype, obtained by centrifugation at 1,000 *g* for 25 minutes, is tubular myelin-enriched, and they suggest that is the controlled variable in surfactant homeostasis, at least in their model system. In our study, the heavy subtype increased in response to smoke exposure, and it was a more intermediate fraction that was unchanged. Though the significance of this is not clear, it does suggest different mechanisms for controlling surfactant homeostasis in different situations. For our own purposes, we don't presently know if our P60 fraction constitutes a discrete metabolic intermediate of alveolar surfactant and if it does, what its precise role might be. That it contains structures not present in either P10 or P100[26] supports the concept of a discrete subfraction; as to a possible role, one can only speculate at this time. That it occupies an intermediate position between the heavy and light isoform and that it remains relatively unaltered by the smoke suggest that it may play a regulatory role in controlling the rate of progression from the heavy to the light subtype. Another possibility is that it may in some way stimulate the secretory process. That the alveolar milieu, eg, the degree of unsaturation of PC, dramatically affects surfactant secretion has been reported by several investigators.[50–52] In the present study, for example, one might speculate that the decreased DSPC in P60 might be the trigger that stimulates secretion of more lamellar bodies. If this were so, then it would ensure that adequate levels of P10 are present at the alveolar surface. Further study is underway to explore these concepts in more detail.

In summary, exposure of mice to smoke generated from the combustion of a flexible polyurethane foam dramatically altered the distribution of the alveolar surfactant subtypes. These alterations were observed by 4 hours after the exposure and were sustained for at least 12 hours. In contrast to reports on other models of alveolar injury, in which either the heaviest or the lightest alveolar surfactant subtype accumulated at the expense of the other,[10–12] we found significant increases in both of these pools, the more dramatic increase being in the lightest subfraction, P100. The structural organization of this light subfraction was also dramatically altered by the smoke, and this could account for its failure to be cleared from the alveolar space. An intermediate fraction, designated as P60, seemed to be less affected by the smoke. These data indicate that the major effect on the surfactant system in the smoke inhalation injury is an excessive accumulation of structurally altered light subtype. Whereas increases were also observed in the heavy subtype, the magnitude of increase was not as great as that observed in the light subtype. Though the mechanisms responsible for these changes is not known, it seems that the increase in P10 might be due to an increase in surfactant secretion, whereas the increased accumulation of P100 could be due to decreased clearance by the alveolar macrophages and/or type II cells, which in turn might be due to damage or destruction of these cell types and/or the structural alteration of P100 itself. An accelerated conversion of secreted surfactant to P100 cannot be discounted, however, as being responsible for the increase in P100. Whatever the mechanism, it seems that the surfactant phospholipids, which undergo only minor changes, may play an insignificant role in the subtype distribution. The surfactant proteins, especially SP-A, which plays a major role in processing of alveolar surfactant, are more likely candidates. Studies are in progress to further probe these possibilities. Finally, it is important to note that differences, not only in the agent that induces the lung injury, but also the animal model used to study the response must be considered to elucidate fully the underlying mechanisms regulating alveolar surfactant metabolism in both the normal and diseased lung.

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References

1. Dobbs LG: Pulmonary surfactant. Annu Rev Med 1989, 40:431–446
2. Hawgood S, Clements JA: Pulmonary surfactant and its apoproteins. J Clin Invest 1990, 86:1–6
3. Williams MC: Conversion of lamellar body membranes into tubular myelin in alveoli of fetal rat lungs. J Cell Biol 1977, 74:1027–1031

4. Wright J, Dobbs LG: Regulation of pulmonary surfactant and clearance. Annu Rev Physiol 1991, 53:395–414
5. Magoon MW, Wright JR, Bartussio A, Williams MC, Benson BJ, Hamilton RL, Clements J: Subfractions of lung surfactant: implications for metabolism and surface activity. Biochim Biophys Acta 1983, 750:18–31
6. Scott JES, Forkert PG, Oulton M, Rasmusson MG, Temple S, Fraser MO, Whitefield S: Pulmonary toxicity of trichloroethyline: induction of changes in surfactant phospholipids and phospholipase A2 activity in the mouse lung. Exp Mol Pathol 1988, 49:141–150
7. Giri SN: Effects of intratracheal instillation of bleomycin on phospholipid synthesis in hamster lung tissue slices. Proc Soc Exp Biol Med 1987, 186:327–332
8. Tahvanainen J, Hallman M: Surfactant abnormality after endotoxin-induced lung injury in guinea pigs. Eur J Respir Dis 1987, 71:250–258
9. LeMesurier SM, Lykle WJ, Stewart BW: Reduced yields of pulmonary surfactant: patterns of response following administration of chemicals to rats by inhalation. Toxicol Lett 1980, 5:89–93
10. Balis JU, Paterson JF, Lundh JM, Haller EM, Shelley SA, Montgomery MR: Ozone stress initiates acute perturbations of secreted surfactant membranes. Am J Pathol 1991, 138:847–857
11. Gross N: Surfactant subtypes in experimental lung damage: radiation pneumonitis. Am J Physiol 1991, 260 (Lung Cell Mol Physiol 4):L302–L310
12. Lewis JF, Ikegami M, Jobe AH: Altered surfactant function and metabolism in rabbits with acute lung injury. J Appl Physiol 1990, 69:2303–2310
13. Higuchi R, Lewis J, Ikegami M: In vitro conversion of surfactant subtypes is altered in alveolar surfactant isolated from injured lungs. Am Rev Respir Dis 1992, 145:1416–1420
14. Amanuma K, Suzuki KT: Effects of intratracheal instillation of cadmium chloride on phospholipids in alveolar wash fluid. Toxicol 1987, 44:321–328
15. Dethloff LA, Gilmore LB, Hook GER: The relationship between intra- and extra-cellular surfactant phospholipids in the lungs of rabbits and the effects of silica-induced lung injury. Biochem J 1986, 239:59–67
16. Miles PR, Bowman L, Tucker J, Reasor MJ, Wright JR: Alterations in rat alveolar surfactant phospholipids and proteins induced by administration of chlorphentermine. Biochim Biophys Acta 1986, 877:167–178
17. Spain CL, Silbajoris R, Young SL: Alterations in surfactant pools in fetal and newborn rat lungs. Pediatr Res 1987, 21:5–9
18. Power JHT, Barr HA, Jones ME, Nicholas TE: Changes in surfactant pools after a physiologic increase in alveolar surfactant. J Appl Physiol 1987, 63:1902–1911
19. Nicholas TE, Power JHT, Barr HA: The pulmonary consequences of a deep breath. Respir Physiol 1982, 49: 315–324
20. Oulton M, Moores HK, Scott JE, Janigan DT, Hajela R: Effects of smoke inhalation on surfactant phospholipids and phospholipase A2 activity in mouse lung. Am J Pathol 1991, 138:195–202
21. Demling RH: Burns. N Engl J Med 1985, 313:1389–1398
22. Heimbach DM: Inhalation injuries. Ann Emerg Med 1988, 17:1316–1320
23. Harwood B, Hall JR: What kills in fires? Smoke inhalation or burns? Fire J 1989, 83:28–34
24. Karter M: Fire loss in the United States during 1990. NFPA Journal 1991, 85:36–48
25. Orzel RA, Womble SE, Ahmed F, Brasted HS: Flexible polyurethane foam: a literature review of thermal decomposition products and toxicity. J Am Coll Toxicol 1989, 8:1139–1175
26. Oulton M, MacDonald J, Janigan DT, Faulkner GT: Mouse alveolar surfactant subfractions: characterization of subtypes prepared by differential centrifugation. Lipids 1993, 28:715–720
27. Levin BC, Fowell AJ, Birky MM, Paabo M, Stolte A, Malek D: Further development of a test method for the assessment of the acute inhalation toxicity of combustion products. Nat Bur Stand Washington, DC, 1983
28. Levin BC, Paabo M, Fultz ML: An acute inhalation toxicological evaluation of combustion products from fire-retarded and non fire-retarded flexible polyurethane foam and polyester. Nat Bur Stand Washington, DC, 1983
29. Committee on Fire Toxicology: Fire and Smoke, Understanding the Hazards. National Research Council. Washington, DC, National Academy Press, 1986, p 8
30. Janigan DT, Moores H, Hajela R: Polyurethane foam smoke inhalation in mice: early decreases in lavagable lung macrophages. Chest 1989, (suppl)96: 2925
31. Moores HK, Janigan DT, Hajela RP: Lung injury after experimental smoke inhalation particle-associated changes in alveolar macrophages. Toxicol Pathol 1993, 21:521–527
32. Oulton M, Fraser M, Dolphin M, Yoon R, Faulkner G: Quantification of surfactant pool sizes in rabbit lung during perinatal development. J Lipid Res 1986, 27: 602–614
33. Oulton M, Martin TR, Faulkner GT, Stinson D, Johnson JP: Developmental study of a lamellar body fraction isolated from human amniotic fluid. Pediatr Res 1980, 14:722–728
34. Bartlett GR: Phosphorus assay in column chromatography. J Biol Chem 1959, 234:466–468
35. Mason RJ, Nallenbogen J, Clements JA: Isolation of disaturated phosphatidylcholine with osmium tetroxide. J Lipid Res 1976, 17:281–284
36. Enhorning G: Pulsating bubble technique for evaluating pulmonary surfactant. J Appl Physiol 1977, 43: 198–203
37. Mendenhall N: Introduction to Probability and Statistics, 4th edition. Belmont, CA, Duxbury Press, 1975
38. Ott L: An Introduction to Statistical Methods and Data

Analysis. North Scituate, CA, Duxbury Press, 1975, pp 392–393
39. Eskelson CD, Chvapil M, Strom KA, Vostal JJ: Pulmonary phospholiposis in rats respiring air containing diesel particulates. Environ Res 1987, 44:260–271
40. Balis JU, Paterson JF, Haller EM, Shelley SA, Montgomery MR: Ozone-induced lamellar body responses in a rat model for alveolar injury and repair. Am J Pathol 1988, 132:330–344
41. Oosting RS, Van Iwaarden, Van Breel, Verhoff J, Van Golde LMG, Haagsman HP: Exposure of surfactant protein A to ozone in vitro and in vivo impairs its interactions with alveolar cells. Am J Physiol (Lung Cell Mol Physiol 6) 1992, 262:L63–L68
42. Gross NJ, Schultz RM: Serine proteinase requirement for the extracellular metabolism of pulmonary surfactant. Biochim Biophys Acta 1990, 1044:222–230
43. Gross NJ, Narine KR: Surfactant subtypes in mice: characterization and quantitation. J Appl Physiol 1989, 66:342–349
44. Gross NJ, Narine RK: Surfactant subtypes of mice: metabolic relationships and conversion in vitro. J Appl Physiol 1989, 67:414–421
45. Oulton M, Janigan DT, Scott JES: Early changes in alveolar surfactant subfractions following smoke inhalation. Prog Respir Res 1993, 27:160–164
46. Rider ED, Ikegami M, Jobe AH: Localization of alveolar surfactant clearance in rabbit lung cells. Am J Physiol 1992, 263:L201–L209
47. Cockshutt A, Absolom DR, Possmayer F: The role of palmitic acid in pulmonary surfactant: enhancement of surface activity and prevention of inhibition by blood proteins. Biochim Biophys Acta 1991, 1085:248–256
48. Seeger W, Gunther A, Thede C: Differential sensitivity to fibrinogen inhibition of SP-C vs. SP-B-based surfactants. Am J Physiol 1992, 261:L286–L291
49. Nicholas TE, Power JHT, Barr HA: Effect of pattern of breathing on subfractions of surfactant in tissue and alveolar compartments of the adult rat lung. Am J Respir Cell Mol Biol 1990, 3:251–258
50. Scott JES: Phosphatidylcholine synthesis, secretion and reutilization during differentiation of the surfactant-producing type II alveolar cell from fetal rabbit lungs. Exp Lung Res 1992, 18:563–580
51. Suwabe A, Mason RJ, Velker D: Pulmonary surfactant secretion is regulated by the physical state of extracellular phosphatidylcholine. Am Rev Respir Dis 1991, 143:A313
52. Pinkerton KE, Lewis J, Mulder AM, Ikegami M, Jobe AH: Surfactant treatment effects on alveolar type II cell morphology in rabbit lungs. J Appl Physiol 1993, 74: 1240–1247