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# ELEVATION OF RAT PULMONARY, HEPATIC AND LUNG SURFACTANT LIPIDS BY FLY ASH INHALATION

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**Abstract**—Fly ash contains many polycyclic aromatic hydrocarbons and genotoxic trace elements. In rats, fly ash exposure profoundly affects lung and liver histology. In the present study, the effect of fly ash inhalation on lung and liver lipids of rats was examined. Male Wistar strain rats were exposed daily to fly ash (0.27 ± 0.01 mg/L air) in an inhalation chamber, 6 hr daily over a period of 15 days, and were killed on various days, i.e. 16, 30, 60, and 120. Fly ash inhalation significantly ($P < 0.05$) increased total phospholipids (PL), phosphatidylcholine (PC) and phosphatidylethanolamine (PE) in lungs. PC and dipalmitoylphosphatidylcholine (DPPC) contents in microsomes and lung surfactant also were significantly ($P < 0.05$) higher in rats exposed to fly ash compared to control group animals. Radiolabeled precursor incorporation studies indicated that fly ash induced the synthesis of PC and DPPC by both CDP-choline pathway and N-methylation of PE in lung microsomes and enhanced their secretion into lung surfactant. In liver, PC and PE contents were elevated significantly ($P < 0.05$) by fly ash exposure on days 16 and 30 respectively. A similar elevation of PC was observed in hepatic microsomes; this increase was due to its increased synthesis. However, the increased synthesis of PC in liver occurred to a greater extent by the N-methylation pathway than by the CDP-choline pathway.

Biological membranes are composed primarily of proteins and lipids, and their ratio varies from membrane to membrane. Apart from being the architectural component of membranes, lipids serve specific functions such as modification of enzyme activity, electron transport and signal transduction [1].

Fly ash is an environmental pollutant that is emitted into the atmosphere during coal burning. It contains many cytotoxic and genotoxic metals and polycyclic aromatic hydrocarbons [2, 3]. Both *in vivo* and *in vitro* studies have demonstrated the cytotoxicity of fly ash [4–6]. Lung is the first target organ for environmental pollutants. Fly ash inhalation profoundly affects lung histology, causing septal thickening, alveolar dilatation and alveolar lipoproteinosis [6]. Fly ash can elicit these changes either by entering the cell or by modifying the cellular events through the plasma membrane. In both cases this pollutant interacts with architectural components of the cell membrane. Lipids account for 40% of the plasma membrane by weight. However, information on the effect of fly ash on membrane lipids is scanty.

Lung surfactant is responsible for maintaining stability of alveolar spaces and retaining the physical elasticity of lungs to air. Surfactant may serve the additional functions of preventing lung edema and fluid transudation into alveoli, aiding in the removal of foreign particles from airways, and assisting in the digestion of bacteria [7]. Lipids constitute 80% of the surfactant by weight. The majority of the lipids in surfactant are phospholipids (60%) especially dipalmitoylphosphatidylcholine (DPPC†). Many pollutants are known to alter the lipid level of lung surfactant in experimental animals [8, 9]; however, very little is known about the effect of fly ash on surfactant lipids.

Several particulate pollutants after deposition in the lungs are translocated to the extrapulmonary organs [10, 11]. Our laboratory observed the translocation of metals [12], polycyclic aromatic hydrocarbons [13], and $^{89}Sr$-enriched fly ash [14] from lungs to liver in rats. These results were corroborated by the alterations of liver histology and the detection of fly ash particles in livers of rats exposed to fly ash by inhalation [6] or intratracheal instillation [15]. Fly ash inhalation also decreases vitamin A levels and induces mixed-function oxidases in rat liver [16, 17]. All these observations suggest the translocations of fly ash or its chemical species from lungs to liver.

In view of these observations, the present study was planned to investigate the effect of fly ash inhalation on lung, liver and lung surfactant lipids in rats. To get insight into the effect of fly ash on phospholipid synthesis, radiolabeled precursor incorporation studies were carried out.

## MATERIALS AND METHODS

*Chemicals*. Acetyl acetone, 1-amino-2,4-napthol sulfonic acid (ANSA), 1,4-bis-[2(4-methyl-5-phenyl-oxazolyl)]benzene (POPOP), butylated hydroxy-toluene, chromotropic acid, 2,5-diphenyloxazole (PPO), and phospholipid standards were procured from the Sigma Chemical Co., St. Louis, MO, U.S.A.

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† Abbreviations: DPPC, dipalmitoylphosphatidylcholine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PMME, phosphatidylmonomethylethanolamine; PDME, phosphatidyldimethylethanolamine; and PL, total phospholipids.

Table 1. Effect of fly ash inhalation on lung phospholipids after various periods of exposure

| Period from first exposure in days | Group | PL | PC | PE |
|---|---|---|---|---|
| 16 | C | 0.30 ± 0.04 | 0.19 ± 0.03 | 0.10 ± 0.01 |
| | E | 0.86 ± 0.04* | 0.47 ± 0.06* | 0.16 ± 0.01* |
| 30 | C | 0.48 ± 0.02 | 0.24 ± 0.01 | 0.12 ± 0.01 |
| | E | 0.83 ± 0.05* | 0.46 ± 0.07* | 0.11 ± 0.01 |
| 60 | C | 0.48 ± 0.09 | 0.27 ± 0.02 | 0.11 ± 0.01 |
| | E | 0.84 ± 0.07* | 0.54 ± 0.02* | 0.13 ± 0.01 |
| 120 | C | 0.71 ± 0.03 | 0.34 ± 0.02 | 0.17 ± 0.02 |
| | E | 1.10 ± 0.05* | 0.43 ± 0.04 | 0.22 ± 0.04 |

Rats were exposed 6 hr daily for 15 consecutive days in a 624-L capacity inhalation chamber to a fly ash concentration of 0.27 ± 0.01 mg/L air. Controls were exposed to filtered clean air simultaneously for the same period of time under identical conditions. Six rats from each group were killed on days 16, 30, 60 and 120 from first exposure. Values (means ± SE from six animals in each group) are expressed in mg/lung and represent the inorganic phosphorus ($P_i$) contents of the respective lipid fraction. Abbreviations: C, control; and E, fly ash exposed.

* Significantly different from control, $P < 0.05$.

Ammonium molybdate, neutral alumina, perchloric acid and silica gel G were purchased from E. Merck, Darmstadt, F.R.G. Organic solvents were obtained from local companies and used after distillation.

*Radiochemicals.* $NaH_2{}^{32}PO_4$ (carrier free), [*methyl*-$^{14}$C]-L-methionine hydrochloride (sp. act. 30 mCi/mmol) and [*methyl*-$^{14}$C]choline (sp. act. 5 mCi/mmol) were purchased from BARC, Trombay, Bombay, India.

*Fly ash sample.* Fly ash was collected in bulk from the electrostatic precipitator of the Inderprastha Thermal Power Station, New Delhi, during its operation. It was passed through a 400 mesh (40 μm) stainless steel sieve before using it for generation of fly ash aerosols. The metal ion composition, particle size distribution, and particle shape of the resulting fly ash have been described previously [6].

*Maintenance and exposure of animals to fly ash.* Male Wistar strain rats weighing from 200 to 250 g were used in the present study. They were housed individually in aluminium cages with raised perforated floors in a temperature-controlled dust-free room. The rats were randomly divided into two groups of 32 each. One group was exposed to fly ash 6 hr daily for 15 consecutive days in a 624-L capacity stainless steel inhalation chamber. The fly ash concentration was maintained at 0.27 ± 0.01 mg/L of air (mean ± SE). The details of the inhalation chamber and the generation of fly ash aerosols have been described elsewhere [6]. Another group of rats was exposed to clean filtered air for the same period under identical conditions and served as the control. Both groups were fed Hind Lever diet for rats (Hindustan Lever Ltd., Bombay) *ad lib.* and had free access to water all the time except during exposure.

After 15 days of exposure, the rats were allowed to inhale clean air until they were killed. Six rats from each group were killed on days 16, 30, 60 or 120 from the first day of exposure to fly ash. Overnight-fasted rats were anesthetized with pentobarbital (60 mg/kg body wt), and the thoracic and abdominal cavities were opened. Lungs and livers were removed after *in situ* perfusion with ice-cold normal saline and dipped in cold normal saline. Weighed portions of the tissues were used for the isolation of lipids by the method of Folch *et al.* [18]. Lipid isolates were assayed for total phospholipids (PL) by estimating the inorganic phosphorus ($P_i$) contents [19].

*Radiolabeled precursor incorporation studies.* These studies were conducted to determine whether or not the increased levels of phospholipid fractions were due to their increased synthesis. All the radioincorporation experiments were carried out on day 16 (a period at which the most significant increase in phospholipids was observed after fly ash inhalation). After 15 days of exposure, control and fly ash exposed rats were fasted overnight. On the next day, rats of both groups were injected intraperitoneally with $NaH_2{}^{32}PO_4$ (300 μCi/kg body wt) in sterile normal saline. After 3 hr the animals were killed. Lungs and livers were removed after *in situ* perfusion. The lung surfactant was isolated with ice-cold physiological saline. Microsomes were prepared from lungs and liver by the method of Zannoni *et al.* [20]. Microsomal and surfactant lipids were isolated as described by Bligh and Dyer [21]. Phospholipids were fractionated by TLC and various fractions were subjected to radioactivity and phosphorus estimation. The radioactive counts were corrected for background and half-life decay.

In separate sets of experiments, overnight fasted control and fly ash exposed rats were injected with [*methyl*-$^{14}$C]choline (50 μCi/kg body wt) or [*methyl*-$^{14}$C]-L-methionine (50 μCi/kg body wt) 60 or 90 min, respectively, before being killed. After sacrifice, tissues were processed as described for $^{32}$P incorporation. The PMME (phosphatidylmonomethylethanolamine), PDME (phosphatidyldimethylethanolamine) and PC were fractionated on TLC by the method of Hirata *et al.* [22]. PMME

Table 2. Effect of fly ash inhalation on hepatic phospholipids of rats at various periods of exposure

| Days from first exposure | Group | PL | PC | PE |
|---|---|---|---|---|
| 16 | C | 2.40 ± 0.27 | 1.20 ± 0.21 | 0.63 ± 0.06 |
| | E | 2.20 ± 0.13 | 2.36 ± 0.13* | 0.58 ± 0.06 |
| 30 | C | 3.07 ± 0.37 | 1.91 ± 0.45 | 0.56 ± 0.09 |
| | E | 2.57 ± 0.15 | 1.52 ± 0.13 | 0.82 ± 0.05* |
| 60 | C | 4.21 ± 0.45 | 2.10 ± 0.27 | 0.97 ± 0.13 |
| | E | 3.69 ± 0.19 | 1.94 ± 0.13 | 1.01 ± 0.10 |
| 120 | C | 8.57 ± 0.34 | 4.76 ± 0.06 | 2.81 ± 0.21 |
| | E | 10.63 ± 0.88 | 5.33 ± 0.61 | 3.23 ± 0.37 |

Experimental details are given in Table 1. Values (means ± SE from six animals in each group) are expressed in mg/liver.
* Significantly different from control, $P < 0.05$.

and PDME could not be quantitated due to their small amounts. However, radioactivity was determined in all these fractions as described above. DPPC was isolated from the total lipid extracts of microsomes and surfactant by the procedure of Mason *et al.* [23]. All the radioactive determinations were done using a Beckman liquid scintillation counter, model LS 2800. The values for all phospholipids have been expressed in terms of their $P_i$ contents.

The results were statistically analyzed by Student's *t*-test.

## RESULTS

Fly ash inhalation for 15 consecutive days does not have any effect on the body weight and liver weight of the animals but significantly increases the lung weight with respect to controls [6].

Table 1 presents the effect of fly ash inhalation on lung phospholipids of rats at various periods from the first day of exposure. A significant increase in the levels of PL in lungs of fly ash exposed rats as compared to respective controls was noticed throughout the period of study. PC levels in lungs of the fly ash exposed group showed significant increases over control values at 16, 30 and 60 days. However, fly ash inhalation did not have any effect on PE content at any period except on day 16 when a significant increase was noticed in the fly ash treated group.

The effects of fly ash inhalation on hepatic phospholipids at various periods of exposure are shown in Table 2. Fly ash inhalation significantly increased PC and PE contents on days 16 and 30 respectively. Various phospholipids of control and fly ash exposed groups were comparable on days 60 and 120. Microsomes, being the site of PC synthesis, were estimated for their PC contents on day 16 only (a period at which PC contents of the whole organs were affected).

Fly ash exposure significantly elevated the levels of PL, PC and DPPC in pulmonary microsomes (Table 3). The microsomal PE contents of fly ash exposed and control rats were comparable (Table 3). In lung surfactant, inhaled fly ash also brought about significant increases in PL, PC and DPPC (Table 3). However, in hepatic microsomes fly ash inhalation increased PC without affecting PL or PE (Table 4). To elucidate whether the increased microsomal phospholipid contents were because of increased synthesis or decreased breakdown, radiolabeled incorporation studies were carried out.

*Effect of fly ash on incorporation of* $NaH_2{}^{32}PO_4$ *into hepatic and pulmonary microsomes and surfactant phospholipids.* Incorporation of $NaH_2{}^{32}PO_4$ into various phospholipids of pulmonary microsomes, lung surfactant and hepatic microsomes of control and fly ash exposed rats is shown in Tables 5–7 respectively. Fly ash inhalation significantly increased the total radioactivity (cpm/lung) of PL and PC in lung microsomes (Table 5). In spite of the significant increase in the pool size of PC, the specific activities of radiolabeled orthophosphate incorporation in control and fly ash exposed lung microsomes were comparable. The total radioactivities in lung microsomal PE of control and fly ash exposed groups were comparable. However, the total radioactivity (cpm/lung) and the specific radioactivity (cpm/mg $P_i$) of microsomal DPPC in fly ash treated rats were significantly higher than those of control rats (Table 5). These results indicate that fly ash inhalation enhanced the synthesis of PC and DPPC in lungs. However, the possibility of their slow breakdown cannot be ruled out.

Incorporation of $NaH_2{}^{32}PO_4$ into phospholipids of lung surfactant was studied to evaluate the secretion of these phospholipids into surfactant; the results are given in Table 6. The fly ash inhalation significantly enhanced the incorporation of $NaH_2{}^{32}PO_4$ into PL, PC and DPPC of surfactant (Table 6). A nearly 2-fold increase in the amounts of PC and DPPC was observed in the lung surfactant of fly ash exposed rats as compared to control rats. Therefore, it can be concluded that the increase observed in surfactant PC and DPPC was due to their increased synthesis in microsomes and enhanced secretion into surfactant (Tables 5 and 6).

Fly ash exposure significantly increased the incorporation of $NaH_2{}^{32}PO_4$ (cpm/liver) into microsomal PL and PC (Table 7). However, $^{32}P$ incorporation into hepatic microsomal PE of control

Table 3. Effect of fly ash inhalation on pulmonary microsomal and surfactant phospholipids of rats

| | Control | Fly ash exposed | % Change over control |
|---|---|---|---|
| | | Microsomes | |
| PL | 96.24 ± 11.24 | 178.45 ± 17.3* | +85.42 |
| PC | 42.84 ± 0.98 | 74.12 ± 7.12* | +73.10 |
| PE | 22.16 ± 1.60 | 28.56 ± 3.43 | +22.86 |
| DPPC | 20.06 ± 2.16 | 37.41 ± 4.09* | +86.49 |
| | | Lung surfactant | |
| PL | 88.38 ± 8.51 | 257.41 ± 66.18* | +191.25 |
| PC | 63.13 ± 1.96 | 113.07 ± 16.30* | +79.11 |
| PE | 15.52 ± 0.78 | 19.51 ± 1.01 | +25.71 |
| DPPC | 46.13 ± 6.13 | 98.18 ± 15.20* | +112.83 |

Rats were exposed to fly ash in an inhalation chamber as described in Table 1. On day 16, rats of both groups were killed after overnight fasting. Lungs were removed after perfusion. Lavage was isolated. Weighed amount of tissues were processed for the isolation of microsomes. The microsomal and surfactant phospholipids were isolated, fractionated and estimated. Values (means ± SE from six animals in each group) are expressed in μg/lung.

* Significantly different from control, $P < 0.05$.

Table 4. Effect of fly ash inhalation on rat hepatic microsomal phospholipids

| | Control | Fly ash exposed | % Change over control |
|---|---|---|---|
| PL | 2289.70 ± 174.57 | 2631.72 ± 200.65 | +14.96 |
| PC | 990.88 ± 51.06 | 1316.69 ± 76.40* | +32.88 |
| PE | 486.71 ± 17.55 | 541.66 ± 52.17 | +11.32 |

Details of fly ash exposure are given in Table 1. Livers were perfused and processed for microsomal lipid isolation and phospholipid fractionation. Values (means ± SE from six animals in each group) are expressed in μg/liver.

* Significantly different from control, $P < 0.05$.

Table 5. Effect of fly ash inhalation on $NaH_2{}^{32}PO_4$ incorporation into microsomal phospholipids of rat lung

| | | Control | Fly ash exposed | % Change over control |
|---|---|---|---|---|
| PL | (a) | 331 ± 40 | 675 ± 67* | +103.93 |
| | (b) | 353 ± 32 | 412 ± 41 | +16.71 |
| PC | (a) | 242 ± 35 | 685 ± 131* | +183.06 |
| | (b) | 605 ± 126 | 1280 ± 469 | +111.57 |
| PE | (a) | 45 ± 3 | 49 ± 7 | +8.89 |
| | (b) | 205 ± 10 | 242 ± 16 | +15.29 |
| DPPC | (a) | 70 ± 9 | 144 ± 24* | +105.71 |
| | (b) | 350 ± 30 | 455 ± 29* | +30.00 |

Rats were exposed to fly ash as described in Table 1. On day 16 from the first day of exposure overnight-fasted rats were injected i.p. with $NaH_2{}^{32}PO_4$ (300 μCi/kg body wt) in sterile physiological saline and killed 3 hr after radioisotope administration. Lungs were removed after perfusion. Lung microsomes were isolated and processed for lipid isolation. Phospholipids were fractionated on TLC and subjected to quantitation and radioactivity determination. Values (means ± SE from six animals in each group) for (a) are expressed in cpm × $10^{-2}$/lung, and for (b) in cpm × $10^{-3}$/mg $P_i$.

* Significantly different from control, $P < 0.05$.

Table 6. Effect of fly ash inhalation on incorporation of $NaH_2{}^{32}PO_4$ into lung surfactant phospholipids

| | | Control | Fly ash exposed | % Change over control |
|---|---|---|---|---|
| PL | (a) | 93 ± 7 | 151 ± 10* | +62.08 |
| | (b) | 98 ± 11 | 97 ± 15 | −0.08 |
| PC | (a) | 80 ± 7 | 147 ± 28* | +82.89 |
| | (b) | 116 ± 10 | 132 ± 34 | +14.37 |
| PE | (a) | 14 ± 2 | 15 ± 2 | +3.45 |
| | (b) | 82 ± 5 | 80 ± 7 | −2.17 |
| DPPC | (a) | 37 ± 5 | 71 ± 7* | +90.26 |
| | (b) | 88 ± 20 | 101 ± 31 | +15.01 |

Details of fly ash exposure and radioisotope administration are given in Tables 1 and 5 respectively. Values (means ± SE from six animals in each group) for (a) are expressed in dpm × $10^{-2}$/lung, and for (b) in dpm × $10^{-3}$/mg $P_i$.

* Significantly different from control, $P < 0.05$.

Table 7. Effect of fly ash inhalation on incorporation of $NaH_2{}^{32}PO_4$ into rat hepatic microsomal phospholipids

| | | Control | Fly ash exposed | % Change over control |
|---|---|---|---|---|
| PL | (a) | 1716 ± 129 | 2585 ± 145* | +49.07 |
| | (b) | 755 ± 18 | 1001 ± 88* | +32.58 |
| PC | (a) | 1093 ± 69 | 1476 ± 129* | +35.04 |
| | (b) | 1096 ± 47 | 1167 ± 32 | +6.48 |
| PE | (a) | 451 ± 52 | 647 ± 76 | +43.46 |
| | (b) | 1034 ± 87 | 1185 ± 59 | +14.60 |

Rats were exposed to fly ash as described in Table 1. On day 16 overnight-fasted rats were injected i.p. with $NaH_2{}^{32}PO_4$ (300 μCi/kg body wt) in sterile physiological saline. Three hours later rats were killed and livers were removed after perfusion. Weighed portions of tissues were processed for isolation of microsomes. Other details are given in Table 5. Values (means ± SE from six animals in each group) for (a) are expressed in cpm × $10^{-3}$/liver, and for (b) in cpm × $10^{-3}$ mg $P_i$.

* Significantly different from control, $P < 0.05$.

and fly ash group was comparable. It can be inferred from these results that fly ash inhalation increased the synthesis of hepatic microsomal PC (Table 7).

PC is synthesized by several pathways but the CDP-choline pathway and sequential methylation of PE are the major routes of its synthesis. It is now known that PC synthesized by different pathways is used for different purposes by the cell. Therefore, the *de novo* synthesis of PC by CDP-choline and N-methylation pathways was evaluated in lung and liver of control and fly ash exposed rats.

*Effect of fly ash inhalation on incorporation of* [methyl-$^{14}$C]*choline into microsomal and surfactant PC.* In the present study, the *de novo* synthesis of PC and DPPC by the CDP-choline pathway was evaluated by studying the incorporation of [*methyl*-$^{14}$C]choline into PC of liver microsomes and PC and DPPC of lung microsomes. The incorporation of the radiolabeled precursor into PC and DPPC of lung surfactant was used as a parameter to study the secretion of these phospholipids synthesized by the CDP-choline pathway into lung surfactant. The animals of both the control and the fly ash group were maintained on the same diet; therefore, the choline pools of these two groups were the same. Fly ash inhalation significantly increased the incorporation of [*methyl*-$^{14}$C]choline into PC and DPPC of microsomes and surfactant (dpm/lung) (Table 8). In addition, the specific radioactivities of PC or DPPC in the fly ash exposed and control groups were comparable in both surfactant and microsomes in lung (Table 8). From these results it can be concluded that fly ash inhalation increased the synthesis of PC and DPPC by the CDP-choline pathway in lung microsomes and enhanced their secretion into surfactant.

However, in liver, although the incorporation of [*methyl*-$^{14}$C]choline (dpm/liver) was comparable in the two groups, the specific radioactivity (dpm/mg $P_i$ of PC) was increased significantly by fly ash inhalation (Table 9).

*Effect of fly ash inhalation on incorporation of* [methyl-$^{14}$C]*methionine into PC and its precursors.* The sequential N-methylation of PE is another pathway for the synthesis of PC. It involves three methylation steps from PE to PC. The intermediates in the conversion of PE to PC are PMME and PDME. Therefore, in the present investigation, the synthesis of PC from PE by the N-methylation pathway was evaluated by studying the incorporation

Table 8. Effect of fly ash inhalation on incorporation of [*methyl*-$^{14}$C]choline into pulmonary microsomal and surfactant phosphatidylcholine in rats

| | | Control | Fly ash exposed | % Change over control |
|---|---|---|---|---|
| | | | Microsomes | |
| PC | (a) | 278 ± 27 | 376 ± 36* | +35.24 |
| | (b) | 539 ± 44 | 556 ± 71 | +3.15 |
| DPPC | (a) | 119 ± 5 | 196 ± 20* | +64.71 |
| | (b) | 180 ± 13 | 168 ± 15 | −6.67 |
| | | | Lung surfactant | |
| PC | (a) | 27 ± 4 | 49 ± 7* | +83.00 |
| | (b) | 28 ± 4 | 29 ± 2 | +2.96 |
| DPPC | (a) | 11 ± 2 | 34 ± 7* | +220.91 |
| | (b) | 88 ± 8 | 84 ± 8 | −4.90 |

Rats were exposed to fly ash for 15 days, 6 hr daily. On day 16 overnight-fasted rats were injected i.p. with [*methyl*-$^{14}$C]choline chloride (50 $\mu$Ci/kg body wt) in sterile physiological saline 1 hr before being killed. Lung microsomes and surfactant were isolated and processed for lipid isolation. The phospholipids were fractionated and subjected to determination of radioactivity and phosphorus. Values (means ± SE from six animals in each group) for (a) are expressed in dpm × $10^{-2}$/lung, and for (b) in dpm × $10^{-3}$/mg $P_i$.

* Significantly different from control, $P < 0.05$.

Table 9. Effect of fly ash inhalation on incorporation of [*methyl*-$^{14}$C]choline into hepatic microsomal phosphatidylcholine of rats

| | Control | Fly ash exposed | % Change over control |
|---|---|---|---|
| (a) | 368 ± 39 | 429 ± 67 | +16.58 |
| (b) | 266 ± 26 | 345 ± 30* | +29.70 |

Details of fly ash inhalation are given in Table 1. On day 16 overnight-fasted rats of both the groups were injected i.p. with [*methyl*-$^{14}$C]choline chloride (50 $\mu$Ci/kg body wt) in sterile physiological saline. One hour later rats were killed, and livers were removed after perfusion. Other details are given in Table 8. Values (means ± SE from six animals in each group) for (a) are expressed in dpm × $10^{-3}$/liver, and for (b) in dpm × $10^{-3}$/mg $P_i$.

* Significantly different from control, $P < 0.05$.

of [*methyl*-$^{14}$C]methionine into PC, PMME and PDME.

Fly ash inhalation significantly increased the incorporation of [*methyl*-$^{14}$C]methionine (dpm/lung) into PC and PMME of lung microsomes and into PC of lung surfactant. The radiolabeled precursor incorporation into microsomal PDME was unaffected by fly ash inhalation (Table 10). However, in lung surfactant, incorporation of radiolabeled methionine into both PMME and PDME was unaffected by fly ash. In the liver also fly ash inhalation significantly increased the incorporation of [*methyl*-$^{14}$C]methionine into microsomal PC (dpm/liver and dpm/mg $P_i$), PDME and PMME (dpm/liver) (Table 11). These results suggest that in lung fly ash inhalation enhanced the synthesis of PC by the N-methylation pathway to the same extent as by the CDP-choline pathway (Tables 6 and 8), whereas in the liver the N-methylation pathway was induced to a greater extent than the CDP-choline pathway (Tables 9 and 11).

## DISCUSSION

The results of the present study demonstrate the alteration of lung and hepatic membrane lipids by fly ash inhalation (Tables 1 and 2). The lipid contents have been expressed on a per organ basis in the above-mentioned tables to display the effect of fly ash on the total pool of the lipid species. However, the pattern of the lipid profile remained the same even when the data were expressed on a per unit organ weight basis (data not given). The alteration in the profiles of various lipid species after exposing the animals to particulate pollutants has been observed by several workers. Inhalation of particulate materials like silica or quartz has been reported to increase lung cholesterol and phospholipids [2, 8]. Metallic dust inhalation also increases phospholipids and volume density of, type II cells in lung [24]. Srivastava and Misra [25] also noticed similar changes in lung lipids by intratracheal administration of fly ash after grinding. Lipids account for 40% of the membrane by weight and fly ash inhalation increased the membrane lipids (Tables 1–4); therefore, the results of the present study corroborate our finding of alveolar septal thickening by fly ash inhalation [6].

The alterations in the levels of hepatic phospholipids observed in the present study (Tables 2 and 4) suggest the translocation of fly ash and/or its chemical species from lungs to liver. This observation is in accord with our earlier reports [6, 12–14].

Fly ash inhalation also increased the PC and DPPC contents of lung microsomes (Table 3). Radiolabeled precursors incorporation studies suggest that these changes were due to an increase in the synthesis of these phospholipids (Tables 5, 8 and 10). Since the total incorporation of both [*methyl*-$^{14}$C]choline and [*methyl*-$^{14}$C]methionine was increased in PC of microsomes by fly ash, it can be concluded that the observed increase in PC was due to its increased synthesis by both CDP-choline and N-methylation

Table 10. Effect of fly ash inhalation on methylation of phosphatidylethanolamine rat pulmonary microsomes and surfactant

| | | Control | Fly ash exposed | % Change over control |
|---|---|---|---|---|
| | | | Lung microsomes | |
| PC | (a) | 563 ± 51 | 919 ± 82* | +63.23 |
| | (b) | 11,912 ± 2784 | 11,058 ± 1165 | −7.17 |
| PDME | (a) | 335 ± 17 | 381 ± 58 | +13.73 |
| PMME | (a) | 249 ± 8 | 318 ± 18* | +27.71 |
| | | | Lung surfactant | |
| PC | (a) | 403 ± 25 | 593 ± 73* | +47.15 |
| | (b) | 4885 ± 594 | 4856 ± 188 | −0.06 |
| PDME | (a) | 215 ± 22 | 226 ± 17 | +5.12 |
| PMME | (a) | 266 ± 5 | 255 ± 2 | −4.14 |

Details of exposure of rats to fly ash are given in Table 1. On day 16 overnight-fasted rats from both groups were injected i.p. with [*methyl*-$^{14}$C]-L-methionine (50 $\mu$Ci/kg body wt) in saline solution 1.5 hr before being killed. Lung microsomes and surfactant were isolated and processed for the isolation of lipids. PC, PDME and PMME were fractionated by TLC and subjected to quantitation of radioactivity and $P_i$. Values (means ± SE from six animals in each group) for (a) are expressed in dpm/lung, and for (b) in dpm/mg $P_i$.

* Significantly different from control, $P < 0.05$.

Table 11. Effect of fly ash inhalation on incorporation of [*methyl*-$^{14}$C]methionine into hepatic microsomal phosphatidylcholine and its precursors in rats

| | | Control | Fly ash exposed | % Change over control |
|---|---|---|---|---|
| PC | (a) | 337 ± 22 | 428 ± 14* | +27.00 |
| | (b) | 244 ± 17 | 338 ± 21* | +38.52 |
| PDME | (c) | 4601 ± 341 | 6181 ± 383* | +34.34 |
| PMME | (c) | 3352 ± 442 | 4842 ± 494* | +44.45 |

Details of fly ash exposure are given in Table 1. On day 16 overnight-fasted rats were injected i.p. with [*methyl*-$^{14}$C]methionine (50 $\mu$Ci/kg body wt) in sterile physiological saline. After 90 min rats were killed and hepatic microsomal lipids were isolated as described earlier. PC, PDME and PMME were fractionated on TLC and subjected to the quantitation of phosphorus and radioactivity. Values (means ± SE from six animals in each group) for (a) are expressed in dpm × $10^{-3}$/liver, for (b) in dpm × $10^{-3}$/mg $P_i$, and for (c) in dpm/liver.

* Significantly different from control, $P < 0.05$.

pathways in lung. However, in liver only the N-methylation pathway was induced by fly ash to a significant extent (Table 11). The mechanism of this induction by fly ash is not understood. However, it is known that several signals acting on the cell surface regulate the synthesis of PC by N-methylation of PE [26]. Sastary *et al.* [27] observed the increase in PC synthesis by the N-methylation pathway in rat liver after 3-methylcholanthrene treatment. Polycyclic aromatic hydrocarbons present in fly ash are translocated to the liver from the lungs [13]; therefore, it is likely that translocated polycyclic aromatic hydrocarbons are responsible for the observed increase in hepatic PC synthesis by N-methylation of PE. Waku *et al.* [28] observed a several-fold increase in PC in rats after long-term exposure to cadmium. Fly ash exposure induces pulmonary and hepatic microsomal mixed-function oxidase system [17, 29]. Since PC is obligatory for their optimal activity, increased PC synthesis by fly ash is in accord with our earlier observations [17, 29].

The observed increase in the contents of surfactant PL, PC and DPPC by fly ash inhalation may be due to the secretion of newly synthesized phospholipids from microsomes (Table 3). Because the incorporation of both [*methyl*-$^{14}$C]choline and [*methyl*-$^{14}$C]methionine into surfactant PC was increased, it can be inferred that secretion of PC synthesized by both CDP-choline and N-methylation pathways into surfactant was enhanced by fly ash exposure (Tables 8 and 10). Exposure of rats to silica also increases phospholipid synthesis and enhances secretion of PC particularly DPPC into lung surfactant [8]. Inhalation of metallic nickel, cadmium, copper and cobalt dusts also has been found to increase the phospholipids, especially DPPC in lungs of experimental animals [24, 30]. Both *in vivo* and *in vitro* administration of benzo[*a*]pyrene have been reported to stimulate rat microsomal PC synthesis [31]. Since fly ash consists of metals, polycyclic aromatic hydrocarbons and aluminosilicates, it may be argued that each of its constituents elevates pulmonary membrane lipids.

However, the higher magnitude of increase observed in the present study in rats exposed to fly ash may be due to the synergistic effects of its constituents.

It has been proposed that surfactant covers the toxic dusts and thus decreases the degree of damage that these dusts can cause to the lungs [9]. Fly ash has been reported to increase the minimum surface tension of the lung surfactant [32]. DPPC is responsible for the majority of the surface active property of the lung surfactant [7]. Therefore, it appears that the enhanced synthesis of PC and DPPC in microsomes and their increased secretion into alveolar spaces is a defense mechanism which protects the tissue from further injury by fly ash and lung collapse due to fly ash caused increase in surface tension.

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## REFERENCES

1. Akino T and Ohno K, Phospholipids of lung in normal, toxic and diseased states. *CRC Crit Rev Toxicol* **9**: 201–274, 1981.
2. Natusch DFS and Wallace JR, Urban aerosol toxicity: The influence of particle size. *Science* **184**: 695–699, 1974.
3. Davison RL, Natusch DFS and Wallace JR, Trace elements in fly ash. *Environ Sci Technol* **8**: 1107–1113, 1974.
4. Aranyi C, Miller FJ, Anders S, Ehrlich R, Fenters J, Gardner DE and Waters MD, Cytotoxicity to alveolar macrophages of trace metals adsorbed on fly ash. *Environ Res* **20**: 14–23, 1979.
5. Garrett NE, Campbell JA, Stack HF, Waters MD and Lewtas J, The utilization of the rabbit alveolar macrophage and Chinese hamster ovary cell for evaluation of the toxicity of particulate materials. II: Particles from coal-related processes. *Environ Res* **24**: 366–376, 1981.
6. Chauhan SS, Chaudhary VK, Narayan S and Misra UK, Cytotoxicity of inhaled coal fly ash in rats. *Environ Res* **43**: 1–12, 1987.
7. Hollingsworth M and Gilfillan AM, The pharmacology of lung surfactant secretion. *Pharmacol Rev* **36**: 69–90, 1984.
8. Heppleston AG, Pulmonary toxicology of silica, coal and asbestos. *Environ Health Perspect* **55**: 111–127, 1984.
9. Richards RJ and Curtis CG, Biochemical and cellular mechanisms of dust-induced lung fibrosis. *Environ Health Perspect* **55**: 396–416, 1984.
10. Holt PF, Transport of inhaled dusts to extrapulmonary sites. *J Pathol* **133**: 123–129, 1981.
11. Snipes MB, Muggenburg BA and Bice DE, Translocation of particles from lung lobes to the peritoneal cavity or the regional lymph nodes in beagle dogs. *J Toxicol Environ Health* **11**: 703–712, 1983.
12. Srivastava VK, Sengupta S, Kumar R and Misra UK, Distribution of metals of inhaled fly ash in various organs of rats at various periods after exposure. *J Environ Sci Health* (Part A) **19**: 663–677, 1984.
13. Srivastava VK, Chauhan SS, Srivastava PK, Kumar V and Misra UK, Fetal translocation and metabolism of PAH obtained from coal fly ash given intratracheally to pregnant rats. *J Toxicol Environ Health* **18**: 459–469, 1986.
14. Srivastava PK, Srivastava VK and Misra UK, Translocation of intratracheally administered $^{89}Sr$ enriched fly ash into extrapulmonary organs of rats. *J Environ Sci Health* **419**: 925–941, 1984.
15. Srivastava PK, Chaudhary VK, Chauhan SS, Srivastava VK and Misra UK, Biochemical and pathological effects of fly ash on lung, liver and blood of rats. *Arch Environ Contam Toxicol* **13**: 441–452, 1984.
16. Chauhan SS, Banerjee R and Misra UK, Effect of inhalation of coal fly ash on Vitamin A distribution in organs of rats. *J Toxicol Environ Health* **16**: 655–659, 1985.
17. Chauhan SS, Singh SK and Misra UK, Induction of pulmonary and hepatic cytochrome P-450 species by fly ash inhalation in rats. *Toxicology* **56**: 95–105, 1989.
18. Folch J, Lees M and Sloane Stanley GH, A simple method for the isolation of the total lipids from animal tissue. *J Biol Chem* **226**: 497–507, 1957.
19. Bartlett GR, Phosphorus assay in column chromatography. *J Biol Chem* **234**: 466–468, 1959.
20. Zannoni VG, Flynn EJ and Lynch M, Ascorbic acid and drug metabolism. *Biochem Pharmacol* **21**: 1377–1392, 1972.
21. Bligh EG and Dyer WJ, A direct method for total lipid isolation and purification. *Can J Biochem Physiol* **37**: 911–917, 1959.
22. Hirata F, Viveros OH, Diliberto J Jr and Axelrod J, Idenification and properties of two methyl transferases in the conversion of phosphatidylethanolamine to phosphatidylcholine. *Proc Natl Acad Sci USA* **75**: 281–284, 1976.
23. Mason RJ, Nellenbogen J and Clements JA, Isolation of disaturated phosphatidylcholine with osmium tetraoxide. *J Lipid Res* **17**: 281–284, 1976.
24. Curstedt T, Cassarett-Bruce M and Camner P, Changes in glycerophosphatides and their ether analogs in lung lavage of rabbits exposed to nickel dust. *Exp Mol Pathol* **41**: 26–34, 1984.
25. Srivastava PK and Misra UK, Cytotoxicity of intratracheally administered coal fly ash: Studies on lipids of lung in rats. *Arch Environ Contam Toxicol* **14**: 95–104, 1985.
26. Mato JM and Alemany S, What is the function of phospholipid N-methylation? *Biochem J* **213**: 1–10, 1983.
27. Sastary BVR, Stathum CN, Meeks RG and Axelrod J, Changes in the phospholipid methyltransferases and membrane microviscosity during induction of rat liver microsomal cytochrome P-450 by phenobarbital and 3-methylcholanthrene. *Pharmacology* **23**: 211–222, 1981.
28. Waku K, Shibhata T, Nakazawa H and Yamashina S, Acceleration of rat liver phospholipid metabolism after long term cadmium administration. *Arch Biochem Biophys* **238**: 509–516, 1985.
29. Chauhan SS, Srivastava PK, Srivastava VK, Kumar V and Misra UK, Inhibition of coal fly ash polycyclic aromatic hydrocarbon and metal induced mixed-function oxidase activity by Vitamin A and citrate. *J Toxicol Environ Health* **17**: 357–363, 1986.
30. Johansson A, Camner P, Jarstrand C and Wiernik A, Rabbit lungs after long-term exposure to low nickel dust concentration. II. Effect on morphology and function. *Environ Res* **30**: 142–151, 1983.
31. Baraud J and Maurice A, Phospholipid synthesis and exchange between rat liver microsomes and mitochondria in the presence of benzo[*a*]pyrene. *J Lipid Res* **21**: 347–353, 1980.
32. Wilkins E, Wilkins MG and Seodi O, Physico-chemical interaction of lung surfactant with fly ash: A preliminary study. *J Environ Sci Health* (Part A) **17**: 169–186, 1982.