

## Airborne agricultural particulate matter induces inflammatory cytokine secretion by respiratory epithelial cells: Mechanisms of regulation by eicosanoid lipid signal mediators

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The purpose of this study was to elucidate the mechanism of the airborne poultry dust (particulate matter, PM)-induced respiratory tract inflammation, a common symptom in agricultural respiratory diseases. The study was based on the hypothesis that poultry PM would induce the release of inflammatory cytokine interleukin-8 (IL-8) by respiratory epithelial cells under the upstream regulation by cytosolic phospholipase A<sub>2</sub> (cPLA<sub>2</sub>) activation and subsequent formation of cyclooxygenase (COX)- and lipoxygenase (LOX)-catalyzed arachidonic acid (AA) metabolites (eicosanoids). Human lung epithelial cells (A549) in culture were treated with the poultry PM (0.1-1.0 mg) for different lengths of time, following which PLA<sub>2</sub> activity, release of eicosanoids and secretion of IL-8 in cells were determined. Poultry PM (1.0 mg/ml) caused a significant activation of PLA<sub>2</sub> in a time-dependent manner (15-60 min), which was significantly attenuated by the calcium-chelating agents, cPLA<sub>2</sub>-specific inhibitor (AACOCF<sub>3</sub>) and antioxidant (vitamin C) in A549 cells. Poultry PM also significantly induced the release of COX- and LOX-catalyzed eicosanoids (prostaglandins, thromboxane A<sub>2</sub> and leukotrienes B<sub>4</sub> and C<sub>4</sub>) and upstream activation of AA LOX in the cells. Poultry PM also significantly induced release of IL-8 by the cells in a dose- and time-dependent manner, which was significantly attenuated by the calcium chelating agents, antioxidants and COX- and LOX-specific inhibitors. The current study for the first time revealed that the poultry PM-induced IL-8 release from the respiratory epithelial cells was regulated upstream by reactive oxygen species, cPLA<sub>2</sub>-, COX- and LOX-derived eicosanoid lipid signal mediators.

**Keywords:** Organic poultry dust, Phospholipase A<sub>2</sub>, Interleukin-8, Lung epithelial cell, Prostaglandins, Leukotrienes, Occupational farm respiratory diseases

Organic and inorganic air pollutants, noxious among them being the ambient airborne particulate matter (PM), have adverse effects on the respiratory health<sup>1,2</sup>. Recent studies have linked the PM air contaminants,

originating from vehicle exhausts, industrial emissions and construction sites to the cardiovascular and cardiopulmonary diseases in urban environments<sup>3-5</sup>. Agricultural operations such as harvesting, primary processing of crops and animal farming are not exceptions in contributing to air pollution. Poultry farms are significant sources of the airborne organic dust (PM), endotoxins, microbes, fungi, molds and ammonia in concentrations that induce cellular and immunological responses, resulting in lung diseases<sup>6,7</sup>. Animal farming in enclosed areas results in growing number of farm workers and families with agricultural PM-mediated respiratory disorders<sup>8</sup>. Widely known agricultural lung diseases are the farmer's lung, agricultural chronic bronchitis, allergic and/or non-allergic asthma and rhinitis, and the most common organic dust syndrome<sup>9</sup>. Studies have shown that exposure to organic dusts results in inflammatory response that can result in the chronic respiratory disorders among humans<sup>10,11</sup>.

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**Abbreviations:** A549, adenocarcinomic human alveolar basal epithelial cells; AA, arachidonic acid; AACOCF<sub>3</sub>, arachidonyl trifluoromethyl ketone; BAPTA-AM, 1,2-bis-(o-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid tetra-(acetoxymethyl) ester; COX, cyclooxygenase; CSMC, cervical smooth muscle cell; CFM, cubic foot per minute; CSMC, cervical smooth muscle cell; DMSO, dimethyl sulfoxide; EGTA, ethylene glycol-bis (β-aminoethylether)-*N,N,N',N'*-tetraacetic acid; IL-8, interleukin-8; LT<sub>B4</sub>, leukotriene B<sub>4</sub>; LTC<sub>4</sub>, leukotriene C<sub>4</sub>; LAL, *Limulus* amoebocyte lysate; LPS, lipopolysaccharide; LOX, lipoxygenase; MAPK, mitogen-activated protein kinase; NAC, N-acetyl-L-cysteine; NDGA, nordihydroguaeretic acid; PBS, phosphate buffered saline; PM, particulate matter; PLA<sub>2</sub>, phospholipase A<sub>2</sub>; PG, prostaglandin; ROFA, residual oil fly ash; ROS, reactive oxygen species; TXB<sub>2</sub>, thromboxane B<sub>2</sub>.

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Airborne contaminants, including the organic dusts (PM) have been shown to induce the synthesis and release of the inflammatory cytokines by the respiratory epithelium<sup>12</sup>. One such cytokine is interleukin-8 (IL-8), which plays a pivotal role in recruiting the inflammatory cells, including neutrophils and macrophages at the site of injury and inflammation. Secretion of IL-8 by the respiratory tract and lung epithelial cells upon exposure to the PM and metallic dusts has been reported<sup>13</sup>. Agricultural organic PM tends to contain considerable amounts of endotoxins, which trigger IL-8 secretion by human cervical smooth muscle (CSMCs) cells<sup>14</sup>. Human peripheral monocytes have been shown to modulate inflammatory mediators upon repeated exposures to the swine dust<sup>15,16</sup>. Thus, the induction of synthesis and release of inflammatory cytokines, such as IL-8 are crucial in mechanisms of agricultural PM-induced respiratory diseases.

Formation of inflammatory lipid mediators at the site of inflammation and injury is a common pathophysiological response<sup>17</sup>. Phospholipase A<sub>2</sub> (PLA<sub>2</sub>), belonging to a specific family of phospholipid hydrolases plays an active role in the inflammatory cascades<sup>18</sup>. PLA<sub>2</sub> catalyzes the hydrolysis and release of unsaturated fatty acid (typically arachidonic acid, AA) esterified at the *sn*-2 position of the membrane phospholipids<sup>19</sup>. Thus, the released AA from PLA<sub>2</sub>-catalyzed hydrolysis of membrane phospholipids is utilized by the cyclooxygenases (COX) and lipoxygenases (LOX) towards the formation of potent bioactive eicosanoids, which are active players in the inflammatory process<sup>17</sup>.

Role of eicosanoids in conditions of respiratory and pulmonary pathophysiology has been shown<sup>20</sup>. However, the activation of PLA<sub>2</sub>, induction of formation of eicosanoids (PGs and LTs) and the downstream release of the inflammatory cytokine IL-8 caused by the poultry PM in the respiratory epithelium either *in vitro* or *in vivo* have not been studied to date. Also, the mechanism of regulation of IL-8 release by respiratory epithelium exposed to the poultry PM is yet to be established. Therefore, it is highly critical to dissect out the early lipid signaling events that regulate the poultry PM-induced secretion of IL-8 by the respiratory tract and lung epithelial cells.

Hence, the current study has been based on the hypothesis that the poultry PM-induces PLA<sub>2</sub> activation upstream of inflammatory cytokine (IL-8) release, involving the AA, COX- and LOX-catalyzed

eicosanoid signaling in the lung epithelial cells *in vitro* (**Schema**). Accordingly, we have investigated the activation of PLA<sub>2</sub>, formation of eicosanoids, induction and secretion of IL-8 and the involvement of AA signaling in IL-8 secretion in the human lung epithelial cells (A549) exposed to the airborne poultry PM collected from the Central Ohio poultry farms.

Our results have indicated that the poultry PM (i) activates cytosolic PLA<sub>2</sub> (cPLA<sub>2</sub>) in a dose-dependent manner at very early periods of exposure (1-2 h) that is calcium-dependent and reactive oxygen species (ROS)-regulated, (ii) induces IL-8 release 6-8 h after exposure, and (iii) that cPLA<sub>2</sub> activation, ROS involvement and associated eicosanoid formation are upstream of IL-8 release in A549 cells. Our study has demonstrated for the first time the role of cPLA<sub>2</sub> and COX- and LOX-catalyzed AA metabolites in the poultry PM-induced IL-8 release by the respiratory epithelium and has suggested a crucial mechanism of the agricultural PM-induced respiratory inflammation.

## Materials and Methods

### Materials

Human lung epithelial cells (A549) (passage 1-4) were procured from Clonetics, Cambrex Corporation (San Diego, CA). RPMI 1640 medium, EDTA, sodium pyruvate, fetal bovine serum (FBS), L-glutamine, antibiotic-antimycotic and Dulbecco's phosphate buffer (PBS) were obtained from Gibco (Grand Island, NY). [<sup>3</sup>H]Arachidonic acid (AA) was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Aspirin, N-acetylcysteine (NAC), L-ascorbic acid, dimethyl sulfoxide (DMSO), *E. coli* lipopolysaccharide (LPS), 1,2-bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid tetra-(acetoxymethyl) ester (BAPTA-AM), nordihydroguareric acid (NDGA) and ethylene glycol-bis (β-aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA) were procured from Sigma Chemical Co. (St Louis, MO, USA). Arachidonyl trifluoromethylketone (AACOCF<sub>3</sub>), ibuprofen, enzyme immunoassay kits (EIA) for the determination of total prostaglandins, thromboxane B<sub>2</sub> (TXB<sub>2</sub>), 8-isoprostane, leukotriene B<sub>4</sub>, and leukotriene C<sub>4</sub> (LTB<sub>4</sub>) were obtained from Cayman Chemical Co. (Ann Arbor, MI).

### Collection and size determination of poultry PM

The agricultural (poultry) PM were collected from the Wooster (Ohio) poultry farms by the Department of Agricultural Engineering, The Ohio State University, Columbus, OH. A six-stage Anderson air

sampler<sup>21</sup> was used to collect the poultry PM (dust) samples at a poultry farm. The inertial cascade sampler was used to collect the poultry PM and determine size of the poultry PM in six different size ranges from 0.65-7 and above. Dust (PM)-laden air was passed through the sampler at a rate of 1 CFM (ft<sup>3</sup>/min). Poultry PM impacted on the glass petri dishes present in the sampler according to their size differences during collection were used in the current study.

#### Cell Culture

A549 cells were cultured in the RPMI 1640 medium supplemented with 10% FBS, antibiotics, pyruvate and L-glutamine in an environment of 95% air–5% CO<sub>2</sub> at 37°C in sterile T-75 cm<sup>2</sup> flasks. A549 cells at passages 6-8 were used in the experiments. Confluent A549 human lung epithelial cells were trypsinized (0.05% trypsin), resuspended in fresh RPMI 1640 medium and subcultured in sterile 35-mm or 60-mm dishes to ~70% confluence under controlled conditions of 95% air–5% CO<sub>2</sub> at 37°C for treatment with the chosen pharmacological agent and/or the poultry PM.

#### Aqueous extraction

Appropriate amount of the poultry PM was weighed out on an analytical balance and transferred into a 50 ml conical vial. The corresponding amount of liquid (RPMI 1640 medium for experiments or sterile water for determination of endotoxin) was added to the conical vial. The vial was capped and vortexed at the high speed setting for 30 min.

#### Organic solvent extraction

Appropriate amount of the poultry PM was weighed out, transferred in to a 16 ml glass test tube and 1 ml of distilled water was added to the tube. The test tube was covered with parafilm and vortexed at the maximum speed setting for 5 min, followed by the addition of 2 ml chloroform and 1 ml methanol to the aqueous mixture. The tube was again covered and vortexed three times for 5 min. The layers were separated upon centrifugation for 5 min at 1000 × *g* and the bottom organic layer was recovered using a glass pipette and dried under the stream of nitrogen. The resulting dried film of the organic solvent extract was reconstituted in a desired amount of the basal RPMI medium upon sonication for 10 min with a probe-type sonicator for three times at a setting of 2 with 1 min intermittent

cooling periods. Sonication, a routine procedure to disperse lipophilic molecules in aqueous medium, was used to facilitate the dispersion of the hydrophobic molecules (extracted from the poultry PM) in the aqueous medium used for the treatment of cells. Amounts of the poultry PM used for the organic solvent extraction were same as those used to prepare the aqueous poultry PM suspensions for studies to treat the cells.

#### Interleukin-8 (IL-8) assay

Cells were grown to 70% confluence in 35-mm dishes at 37°C under 95% air–5%CO<sub>2</sub> environment, treated with 1 ml basal RPMI medium or 1 ml basal RPMI medium containing various concentrations of chosen poultry PM aqueous suspension, without or with the chosen pharmacological inhibitors for 4, 8 and 12 h. After treatment, 200 µl of the medium was withdrawn and analyzed for IL-8 that was released by the cells utilizing the enzyme-linked immunosorbent assay (ELISA) method.

#### Determination of endotoxin

Levels of endotoxins in the chosen agricultural PM (poultry and swine) were determined utilizing the *Limulus* amoebocyte lysate (LAL) endotoxins assay kit (Associates of Cape Cod Inc., East Falmouth, MA) according to the manufacturer's recommendations.

#### [<sup>3</sup>H]Arachidonic acid (AA) labeling and assay of phospholipase A<sub>2</sub> activity

The activity of PLA<sub>2</sub> was assayed according to the published methods<sup>22-24</sup> by determining the release of AA from the cells. Cells (70% confluence) were labeled overnight with 1 ml of the medium containing 0.5 µCi/ml of [<sup>3</sup>H]AA. The radioactive medium was aspirated following labeling with [<sup>3</sup>H]AA and the cells were washed with 1 ml basal RPMI 1640 medium. Cells were then treated with 1 ml basal RPMI medium alone or 1 ml RPMI containing the poultry PM without and with different concentrations of the selected pharmacological inhibitors/agonists like calcium chelators BAPTA and EGTA, antioxidant vitamin C and cPLA<sub>2</sub>-specific inhibitor AACOCF<sub>3</sub> and incubated for different lengths of time at 37°C under the humidified environment of 95% air–5% CO<sub>2</sub>. Following incubations, radioactivity released in to the medium was measured in a liquid scintillation counter. PLA<sub>2</sub> activity was expressed as disintegrations per min (DPM) of [<sup>3</sup>H]AA released/dish.

**Determination of cyclooxygenase (COX)- and lipoxygenase (LOX)-catalyzed formation of arachidonic acid (AA) metabolites (Eicosanoids)**

The COX- and LOX-catalyzed formation of AA metabolites (eicosanoids) in A549 cells cultured in 35-mm dishes ( $5 \times 10^5$  cells/dish), following their exposure to different concentrations of the poultry PM in RPMI for 1 and 2 h, was determined using the commercially available EIA kit (Cayman Chemical Co, Ann Arbor, MI) as described previously<sup>25</sup>. Release of AA metabolites, including the total prostaglandins (PGs), thromboxane A<sub>2</sub> (TXA<sub>2</sub> measured as TXB<sub>2</sub>), 8-isoprostane and LTs, including the leukotrienes B<sub>4</sub> (LTB<sub>4</sub>) and C<sub>4</sub> (LTC<sub>4</sub>) by cells was determined according to the manufacturer's recommendations. The release of eicosanoids from the cells was expressed as pg/ml of incubation medium.

**LOX Assay**

The *in vitro* activity of AA LOX in A549 lung epithelial cells was assayed using the standard spectrophotometric method by determining the extent of formation of conjugated dienes in AA as the exogenous substrate according to the published method<sup>25</sup>. Following treatment of A549 cells in 35-mm dishes ( $5 \times 10^5$  cells/dish) with RPMI containing the chosen concentrations of poultry PM, cells were detached with a cell scraper and lysed in 100 mM Tris-HCl buffer (pH 7.4). The final assay mixture contained 10  $\mu$ M AA and cell lysate (500  $\mu$ g of protein) in 100 mM Tris-HCl (pH 7.4). After incubation of reaction mixture for 5 min at 37°C, the absorbance was measured at 234 nm against appropriate blanks. The activity of LOX was expressed as the conversion of AA into conjugated dienes by the enzyme in the cell lysate.

**Microscopic examination of poultry PM**

Freshly collected poultry PM from the Central Ohio poultry farms was subjected to the microscopic examination as dry PM and aqueous slurry. Dry poultry PM was placed on a clean slide glass, covered with a coverslip and examined under Olympus light microscope at 20X and 60X magnifications. Aqueous slurry (50% wt/vol) was prepared in PBS, placed on a clean slide glass, covered with a coverslip and examined under a light microscope at 20X and 60X magnifications. The images were captured digitally.

**Statistical analysis**

All experiments were carried out in triplicates. Standard deviation (SD) for each data point was calculated from three independent determinations. Data were subjected to one-way analysis of variance and pair-wise multiple comparisons were done by Dunnett's method with the significance set at  $P < 0.05$ .

**Results****Microscopic nature of poultry PM**

Poultry PM collected from a typical experimental poultry farm was subjected to light microscopic examination as dry dust immediately after collection and as hydrated dust (slurry prepared with phosphate-buffered saline, PBS). Aggregates of PM were evident under both dry and hydrated states. Microscopic examination also revealed the spherical nature and heterogeneous size distribution of the PM (Fig. 1A). The size of poultry PM used in the current study was determined to be in the 0.1-10  $\mu$  range during the PM collection by the Anderson air sampler (inertial cascade sampler) as described in the 'Materials and Methods'.

**Poultry PM contains endotoxin**

Endotoxins, such as lipopolysaccharides (LPS) as exogenous mediators of inflammation have been shown to be present in organic agricultural dusts<sup>20,26,27</sup>. Therefore, the presence of endotoxins in poultry PM was analyzed by utilizing the commercial LAL assay kit. Here, we also compared the levels of endotoxins present in the swine farm PM collected from a typical central Ohio swine confinement. As shown in Fig. 1B, both poultry and swine PM contained 0.64 and 0.63 EU/mg PM, respectively. This analysis confirmed that endotoxins were present in the poultry PM.

**Poultry PM induces release of AA by A549 lung epithelial cells**

PLA<sub>2</sub> upon activation catalyzes the release of AA from the membrane phospholipids that act as a precursor for the formation of eicosanoids (PGs and LTs) through the action of COXs and LOXs<sup>28</sup>. To investigate whether poultry PM could induce the activation of PLA<sub>2</sub> and cause the release of AA from the cell membrane, A549 cells prelabeled with [<sup>3</sup>H]AA were treated with different concentrations of the poultry PM for different lengths of time. As shown in Fig. 2, the poultry PM only at a dose of 1.0 mg/ml caused a significant increase in the release of [<sup>3</sup>H]AA at 1 h in A549 cells, as compared to the untreated control cells.

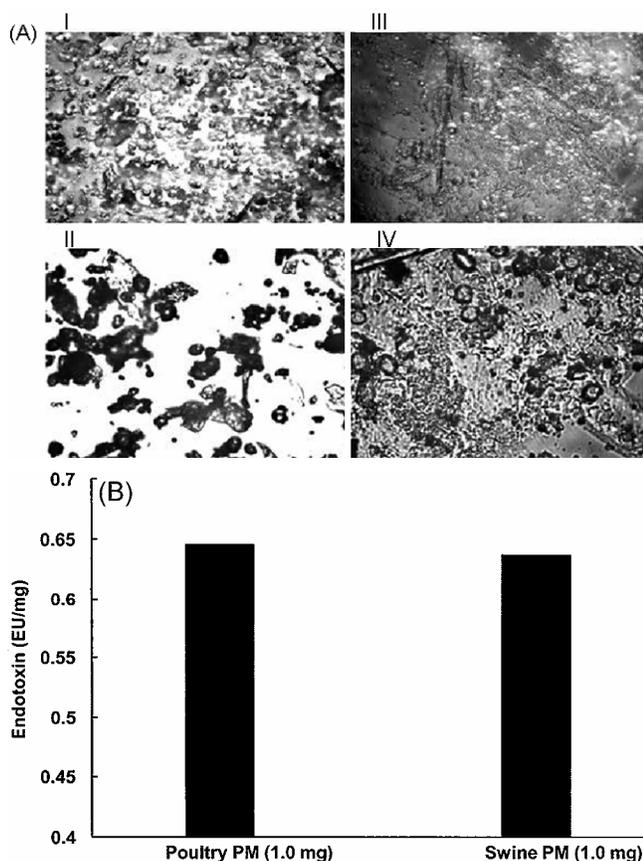


Fig. 1—(A): Microscopic nature of poultry PM [Freshly collected poultry PM was examined under light microscope (Olympus) as described under 'Materials and Methods'. Dry dust (I & III) was examined under 20X and 60X magnification. PM was suspended in PBS (50% wt/vol) and examined as aqueous suspension (II and IV) under 20X and 60X magnifications, respectively]; (B): Poultry PM contains endotoxin [Freshly collected poultry PM and swine PM suspended in PBS at a concentration of 1.0 mg/ml by continuous vortexing for 1 h. Levels of endotoxin in the aqueous suspensions of poultry and swine PM were determined by utilizing LAL assay as described in the 'Materials and Methods'. Data represent mean  $\pm$  SD of three independent determinations]

However, at 2 h of treatment of cells with the poultry PM at 0.1 and 1.0 mg/ml doses, the release of [ $^3$ H]AA from cells at the 0.1 mg/ml dose was slightly but not significantly elevated, whereas the same at 1.0 mg/ml dose was significantly greater, as compared to the same in the control untreated cells. At 3 h of treatment of cells with the poultry PM at 0.1 and 1.0 mg/ml doses, the release of [ $^3$ H]AA was significantly and slightly enhanced at the former dose, but was significantly elevated at the latter dose as compared to the same in the control untreated cells (Fig. 2). In comparison with the control untreated cells, poultry PM at a dose of 1.0 mg/ml induced a

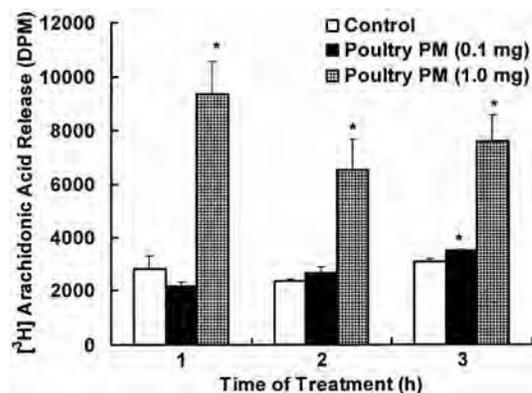


Fig. 2—Poultry PM induces release of AA by lung epithelial cells [A549 lung epithelial cells ( $5 \times 10^5$  cells/35-mm dish) were labeled with [ $^3$ H]AA (5  $\mu$ Ci) in complete RPMI medium for 12-18 h and then the cells were treated with basal RPMI medium or basal RPMI medium containing different concentrations of poultry PM (0.1 and 1.0 mg/ml) for 1, 2, and 3 h. At the end of incubation, [ $^3$ H]AA released into the medium was determined as described in 'Materials and Methods'. Data represent mean  $\pm$  SD of three independent experiments. \*Significantly different at  $P < 0.05$  as compared to control cells treated with basal RPMI medium alone]

significant and greater extent of release of [ $^3$ H]AA from A549 cells, as compared to the same at 2 and 3 h of treatment under identical conditions at 1, 2 and 3 h, respectively as compared to their respective control untreated cells.

Overall, this study revealed that the poultry PM at a dose of 1.0 mg/ml caused a significant enhancement of [ $^3$ H]AA release as an index of PLA<sub>2</sub> activation at 1 h of treatment of cells, which further was significantly higher as compared to the respective control untreated cells at 2 and 3 h of treatment under identical conditions, but was lower as compared to the same at 1 h of treatment of cells with 1.0 mg/ml dose. Thus, the study revealed that the poultry PM caused a significant dose- and time-dependent activation of PLA<sub>2</sub> in A549 lung epithelial cells, leading to the release of AA.

#### LPS fails to induce release of AA by A549 lung epithelial cells

[ $^3$ H]AA-labeled A549 cells were exposed to 1.0 mg/ml concentration of the poultry PM as well as 1 and 10  $\mu$ g concentrations of commercial LPS (*E. coli* 055:B5) for 1 and 2 h. The release of [ $^3$ H]AA by cells treated with the poultry PM was significantly higher (~2-fold), whereas LPS did not enhance the release of [ $^3$ H]AA from the cells under identical

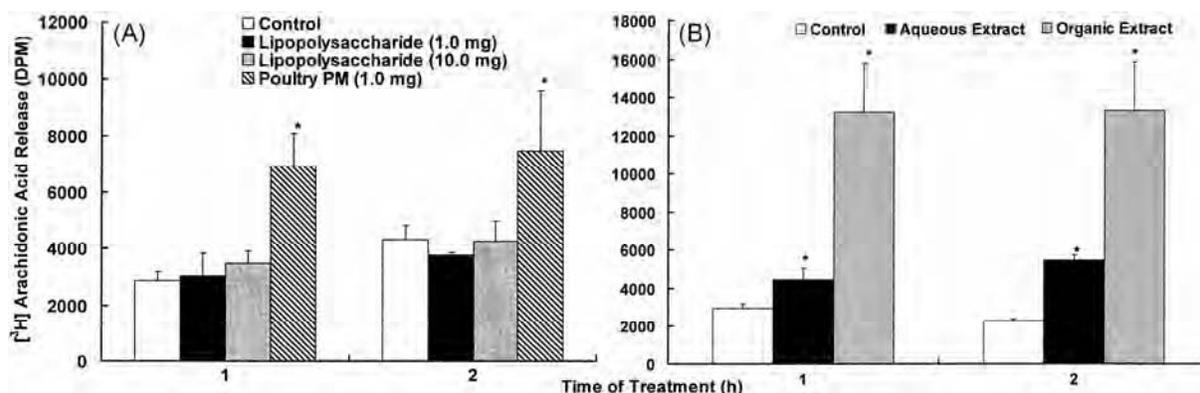


Fig. 3—(A): Lipopolysaccharide (LPS) fails to induce release of AA by lung epithelial cells [A549 lung epithelial cells ( $5 \times 10^5$  cells/35-mm dish) were labeled with [<sup>3</sup>H]AA ( $5 \mu$  Ci) in complete RPMI medium for 12–18 h and then the cells were treated with basal RPMI medium or basal RPMI medium containing *E. coli* LPS (1.0 and 10.0 mg/ml) and poultry PM (1.0 mg/ml) for 1 and 2 h at 37°C in a humidified environment of 5% CO<sub>2</sub>–95% air. At the end of incubation, [<sup>3</sup>H]AA released into the medium was determined as described in 'Materials and Methods'. Data represent mean  $\pm$  SD of three independent experiments. \*Significantly different at  $P < 0.05$  as compared to the respective control cells treated with basal RPMI medium alone]; (B): Organic solvent extract of poultry PM is a potent inducer of AA release by A549 lung epithelial cells [A549 lung epithelial cells ( $5 \times 10^5$  cells/35-mm dish) were labeled with [<sup>3</sup>H]AA ( $5 \mu$  Ci) in complete RPMI medium for 12–18 h and then the cells were treated with basal RPMI medium or basal RPMI medium containing poultry PM (1.0 mg/ml) or organic solvent (CHCl<sub>3</sub>: MeOH, 2:1, vol/vol) extract of poultry PM (1.0 mg) for 1 and 2 h at 37°C in a humidified environment of 5% CO<sub>2</sub>–95% air. At the end of incubation, [<sup>3</sup>H]AA released into the medium was determined as described in 'Materials and Methods'. Data represent mean  $\pm$  SD of three independent experiments. \*Significantly different at  $P < 0.05$  as compared to the respective control cells treated with basal RPMI medium alone.

conditions, as compared to the same in the control untreated cells (Fig. 3A). The study revealed that the commercial bacterial endotoxin (LPS) used in current study was not effective in inducing PLA<sub>2</sub> activation in the lung epithelial cells as opposed to the poultry PM which contained detectable amounts of endotoxin.

#### Organic solvent extract of poultry PM is a more potent inducer of AA release by A549 lung epithelial cells

Exposure of [<sup>3</sup>H]AA-labeled A549 cells to both aqueous and organic extracts of the poultry PM for 1 and 2 h showed that the organic extract induced significantly greater extents of [<sup>3</sup>H]AA release than the aqueous extract, as compared to the same in the control untreated cells (Fig. 3B).

#### Calcium chelators, cPLA<sub>2</sub>-specific inhibitor, and antioxidant (vitamin C) attenuate poultry PM-induced release of AA by A549 lung epithelial cells

To establish the role of calcium and oxidative stress in the poultry PM-induced activation of PLA<sub>2</sub> in the lung epithelial cells, the modulation of [<sup>3</sup>H]AA release by calcium chelators and vitamin C in the poultry PM-treated A549 cells was studied. A549 cells pre-labeled with [<sup>3</sup>H]AA were pre-treated with BAPTA, an intracellular calcium chelator for 1 h for effective internalization of the chelator, following

which, the cells were challenged with the poultry PM for 1 h. The cells treated with BAPTA showed a significant decrease in the poultry PM-induced [<sup>3</sup>H]AA release as compared to that observed in the cells treated with poultry PM alone (Fig. 4A). The cells treated with EGTA, an extracellular calcium chelator (5 mM) showed a significant and complete inhibition of the release of the poultry PM-induced [<sup>3</sup>H]AA release, as compared to that observed in cells treated with the poultry PM alone (Fig. 4A). These results indicated that both intracellular and extracellular calcium played a major role in the poultry PM-induced PLA<sub>2</sub> activation in the A549 cells.

As the calcium chelators inhibited the poultry PM-induced release of AA, the role of calcium-dependent PLA<sub>2</sub> (cPLA<sub>2</sub>) in the process was envisioned. In order to establish the role of cPLA<sub>2</sub> in the poultry PM-induced release of AA by A549 cells, cells were pre-treated with the cPLA<sub>2</sub>-specific inhibitor AACOCF<sub>3</sub> (0.1  $\mu$ M) for 1 h and then challenged with the poultry PM (1.0 mg/ml) for 1 h, following which the release of AA by A549 cells was determined. As shown in Fig 4B, AACOCF<sub>3</sub>, significantly inhibited the poultry PM-induced release of [<sup>3</sup>H]AA from A549 cells. These results revealed that cPLA<sub>2</sub> was responsible in part for the poultry PM-induced release of AA from A549 cells.

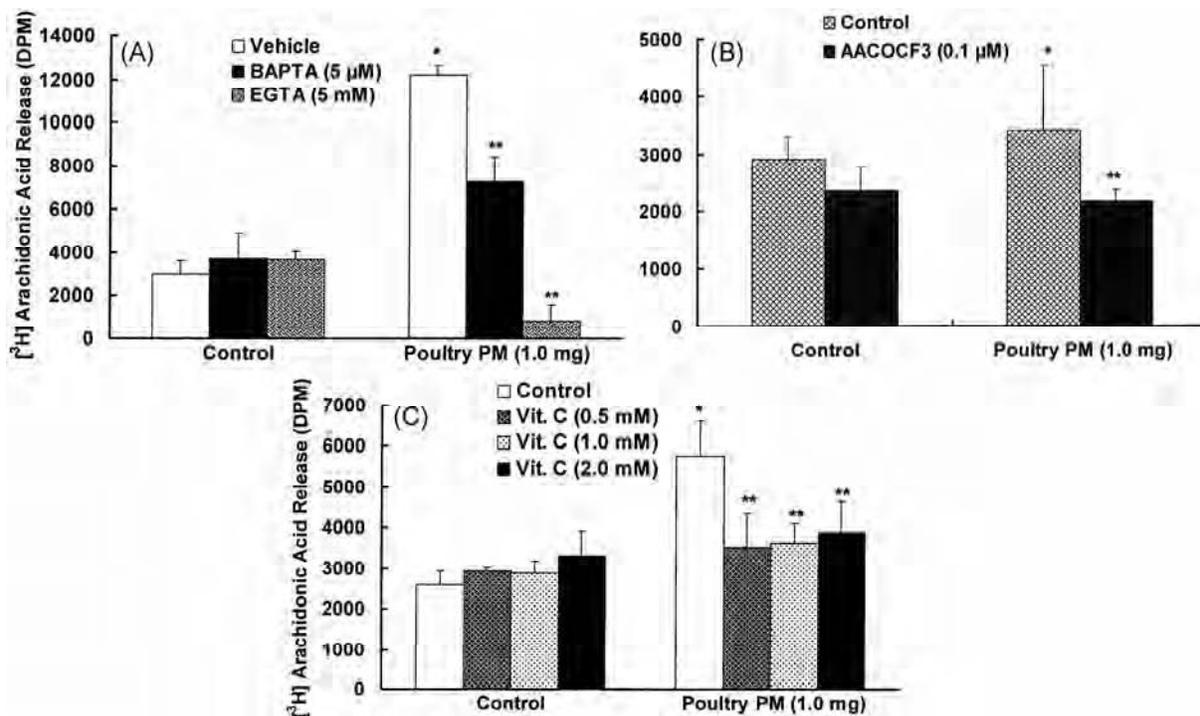


Fig. 4—Calcium chelators, cPLA<sub>2</sub>-specific inhibitor, and antioxidant (vitamin C) attenuate poultry PM-induced release of AA by A549 lung epithelial cells [A549 lung epithelial cells ( $5 \times 10^5$  cells/35-mm dish) were labeled with [<sup>3</sup>H]AA (5  $\mu$  Ci) in complete RPMI medium for 12-18 h. To study the role of intracellular calcium, cells were pretreated for 1 h with basal RPMI medium or basal RPMI medium containing BAPTA (5  $\mu$ M) [A] and then treated with basal RPMI medium or basal RPMI medium containing poultry PM (1.0 mg/ml) for 1 h at 37°C in a humidified environment of 5% CO<sub>2</sub>-95% air. To demonstrate cPLA<sub>2</sub> activation, cells were pretreated with basal RPMI medium or basal RPMI medium containing cPLA<sub>2</sub>-specific inhibitor, AACOCF<sub>3</sub> (0.1  $\mu$ M) for 1 h and then treated with basal RPMI medium or basal RPMI medium containing poultry PM (1.0 mg/ml) for 1 h at 37°C in a humidified environment of 5% CO<sub>2</sub>-95% air [B]. To study the role of extracellular calcium [A] and oxidative stress [C], cells labeled with [<sup>3</sup>H]AA were treated with basal RPMI medium or basal RPMI medium containing EGTA (5 mM) or vitamin C (0.5, 2.0 mM) or basal RPMI medium containing poultry PM (1.0 mg/ml) or basal RPMI medium containing poultry PM (1.0 mg/ml) + EGTA (5 mM) or poultry PM (1.0 mg/ml) + vitamin C (0.5-2.0 mM) for 1 h at 37°C in a humidified environment of 5% CO<sub>2</sub>-95% air. At the end of treatment, [<sup>3</sup>H]AA released into the medium was determined as described in 'Materials and Methods'. Data represent mean  $\pm$  SD of three independent experiments. \*Significantly different at P<0.05 as compared to the respective control cells treated with basal RPMI medium alone. \*\*Significantly different at P<0.05 as compared to the cells treated with basal RPMI medium containing poultry PM alone]

Reactive oxygen species (ROS) and oxidative stress have been shown to be associated with the activation of PLA<sub>2</sub> in several cellular systems<sup>23,24,29</sup>. Therefore, in order to determine the role of oxidative stress in poultryPM-induced PLA<sub>2</sub> activation, we investigated whether vitamin C, a well-established antioxidant, would attenuate the poultry PM-induced AA release by A549 cells. As shown in Fig. 4C, poultry PM (1.0 mg/ml) caused a significant increase in the release of [<sup>3</sup>H]AA from the cells after 1 h exposure of cells, as compared to the same in control untreated cells under identical conditions. The co-treatment with vitamin C (0.5-2.0 mM) significantly attenuated the poultry PM-induced [<sup>3</sup>H]AA release by A549 cells (Fig. 4C). Although vitamin C alone at any tested concentration did not

alter the extent of basal release of [<sup>3</sup>H]AA by the cells, even at a concentration of 0.5 mM, it caused a significant attenuation of the poultry PM-induced [<sup>3</sup>H]AA release by cells that remained unaltered even by increasing the concentration of vitamin C up to 2.0 mM. These results demonstrated that ROS and oxidative stress were involved in the poultry PM-induced PLA<sub>2</sub> activation and release of [<sup>3</sup>H]AA in A549 lung epithelial cells.

#### Poultry PM induces formation and release of cyclooxygenase (COX)-catalyzed AA metabolites (eicosanoids) by A549 lung epithelial cells

COX catalyzes the conversion of AA into eicosanoids, including the PGs and thromboxanes<sup>25</sup>. A549 cells treated with the poultry PM (1.0-10.0 mg/ml) for 1 and 2 h were assayed for the release of total

PGs, thromboxane A<sub>2</sub> and 8-isoprostane. A significant dose-dependent increase in the release of total PGs was observed in cells treated with the increased doses of the poultry PM, as compared to that released by the control untreated cells at both 1 and 2 h of incubation (Fig. 5A).

When assayed for release of thromboxane A<sub>2</sub> (measured as thromboxane B<sub>2</sub>, TXB<sub>2</sub>), cells showed elevated levels of release of TXB<sub>2</sub> even after 30 min of incubation with the poultry PM (1.0 mg/ml), as compared to the control untreated cells (Fig. 5B). An increase in the time of incubation from 30-120 min did not appear to enhance the extent of release of TXB<sub>2</sub> at 60, 90 and 120 min of incubation time with the poultry PM.

Release of 8-isoprostane, an eicosanoid marker for oxidative stress was significantly elevated in the cells treated with the poultry PM (1.0 mg/ml) for 30-120 min (Fig. 5C). Even at 30 min of treatment of cells with the poultry PM, a significant release of 8-isoprostane from the cells was observed. The increase of time of treatment of cells with the poultry PM up to 120 min did not enhance the same at any chosen time of treatment. Together, these results

indicated that the poultry PM induced the formation and release of COX-catalyzed PGs (eicosanoids) in the lung epithelial cells.

#### Poultry PM induces formation and release of LOX-catalyzed AA metabolites (eicosanoids) and activation of arachidonate LOX in A549 lung epithelial cells

In mammalian cells, the arachidonate LOXs catalyze the conversion of AA of membrane phospholipids into AA metabolites, including the AA hydroperoxides and LTs<sup>30</sup>. The LOX-catalyzed AA metabolites also play a critical role in the inflammatory cascades<sup>30,31</sup>. As the organic agricultural dusts (PM) are known to cause inflammation in the respiratory tract<sup>32,33</sup>, here, we investigated whether the poultry PM would induce the formation of LOX-catalyzed AA metabolites (LTs) in the A549 lung epithelial cells. Following treatment of cells with the poultry PM (1.0 mg/ml) for 60-120 min, LTB<sub>4</sub> and LTC<sub>4</sub> released into the medium were determined. Even at 60 min of treatment, poultry PM induced a significant increase in the release of LTB<sub>4</sub>, as compared to the control untreated cells (Fig. 6A). However, upon increasing the time of treatment of cells to 90 and 120 min, the poultry PM although

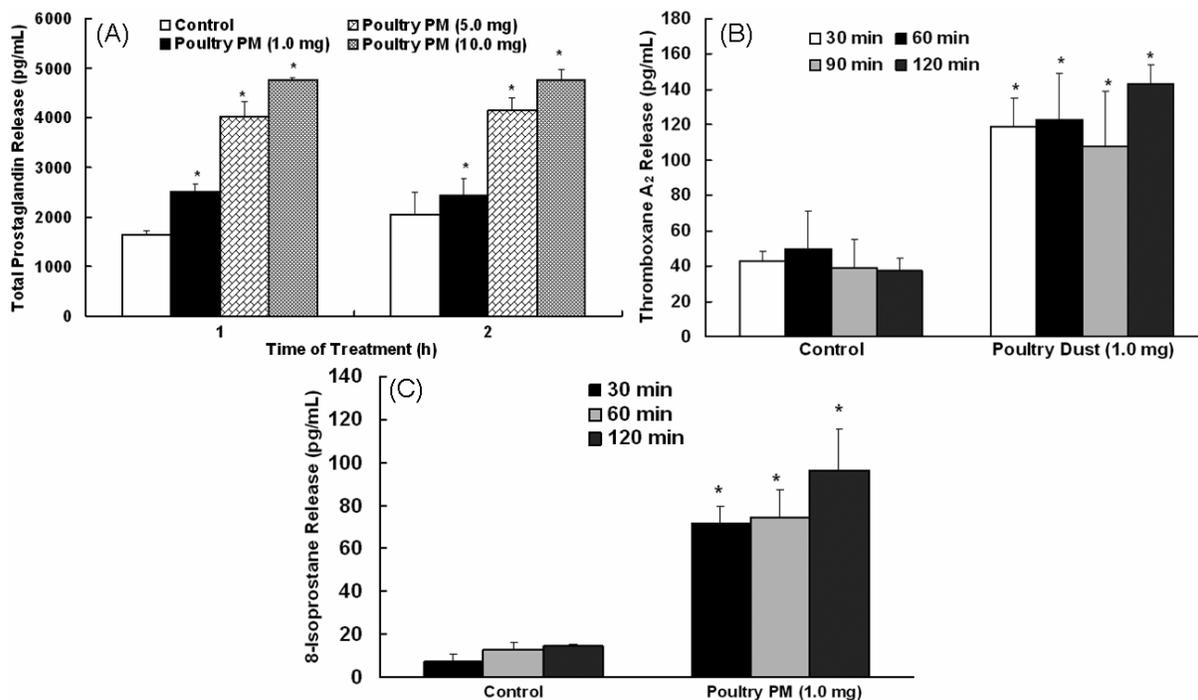


Fig. 5—Poultry PM induces formation and release of cyclooxygenase (COX)-catalyzed AA metabolites by A549 lung epithelial cells [A549 lung epithelial cells ( $5 \times 10^5$  cells/35-mm dish) were treated with basal RPMI medium or basal RPMI medium containing poultry PM (1.0-10.0 mg/ml) for 1 and 2 h at 37°C in a humidified environment of 5% CO<sub>2</sub>-95% air. Following treatment of cells, release of total prostaglandins [A], thromboxane A<sub>2</sub> as thromboxane B<sub>2</sub> [B] and 8-isoprostane [C] into the medium was determined as described in 'Materials and Methods'. Data represent mean  $\pm$  SD of three independent experiments. \*Significantly different at  $P < 0.05$  as compared to the respective control cells treated with basal RPMI medium alone]

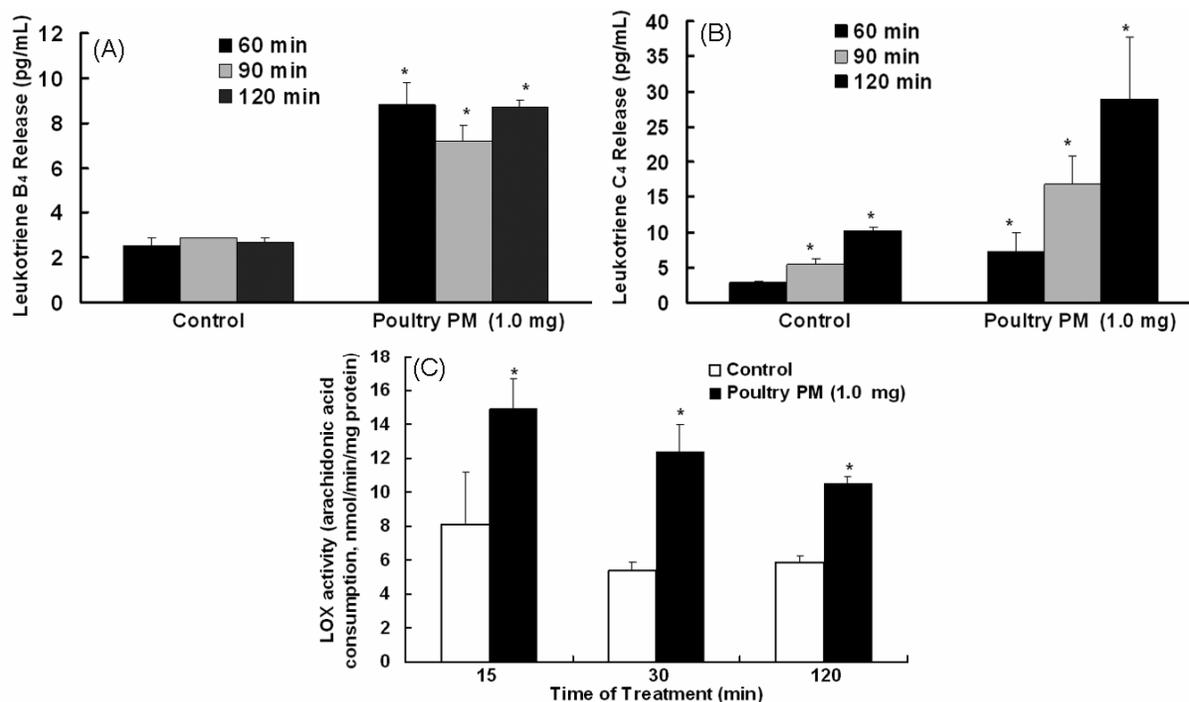


Fig. 6—Poultry PM induces formation and release of LOX-catalyzed AA metabolites and activation of arachidonate LOX in A549 lung epithelial cells [A549 lung epithelial cells ( $5 \times 10^5$  cells / 35-mm dish) were treated with basal RPMI medium or basal RPMI medium containing poultry dust (1.0 mg/ml) for 15–120 min at 37°C in a humidified environment of 5% CO<sub>2</sub>–95% air. At the end of treatment, release of total leukotriene B<sub>4</sub> [A], leukotriene C<sub>4</sub> [B] and activity of AA LOX [C] were assayed as described in ‘Materials and Methods’. Data represent mean  $\pm$  SD of three independent experiments. \*Significantly different at  $P < 0.05$  as compared to the respective control cells treated with basal RPMI medium alone]

induced a significant release of LTB<sub>4</sub> as compared to the same in the corresponding control untreated cells at those treatment times, the extent of release of LTB<sub>4</sub> in the poultry PM-treated cells at 90 and 120 min remained the same as seen in their counterparts treated for 60 min. This observation indicated that further prolongation of the time of treatment of cells with the poultry PM beyond 60 min did not alter the extent of LTB<sub>4</sub> release.

In contrast to LTB<sub>4</sub> release, LTC<sub>4</sub> release increased significantly with time of treatment of A549 cells with the poultry PM (1.0 mg/ml) from 60–120 min, as compared to the control untreated cells (Fig. 6B). On the other hand, in the control untreated cells also, the extent of release of LTC<sub>4</sub> was significantly increased in a time-dependent manner from 60–120 min, suggesting that these cells exhibited a time-dependent increase in the formation and release of basal levels of LTC<sub>4</sub>, which was further enhanced by the treatment of cells with the poultry PM along with the increase in time of treatment of cells. Overall, these studies revealed that the poultry PM induced the release of LOX-catalyzed eicosanoid metabolites, such as LTB<sub>4</sub> and LTC<sub>4</sub> in A549 lung epithelial cells.

As this study revealed that the poultry PM induced the formation of arachidonate LOX-catalyzed eicosanoids, such as LTB<sub>4</sub> and LTC<sub>4</sub> in A549 cells, here we investigated whether the poultry PM would activate the arachidonate LOX activity in A549 cells *in vitro*. Following the treatment of cells with basal RPMI medium or RPMI medium containing the poultry PM (1.0 mg/ml) for 15–120 min, LOX activity in the cell lysates was determined by measuring the formation of conjugated dienes in exogenously added AA as the LOX substrate. Control untreated cells exhibited noticeable activation of LOX even at 15 min of incubation and slightly but not significantly decreased at 30 and 120 min of incubation of cells at 37°C under identical conditions (Fig. 6C). On the other hand, cells treated with the poultry PM for 15 min exhibited significant LOX activation, as compared with the same in the control untreated cells (Fig. 6C). The poultry-PM induced activation of LOX although was significantly greater than that observed in the corresponding control untreated cells at 30 and 120 min of incubation, the same at those time points in the poultry PM-treated cells was slightly lower as compared to that observed at 15 min

of treatment. Thus, this study established that the poultry PM induced the activation of arachidonate LOX in A549 lung epithelial cells.

#### Poultry PM induces interleukin-8 (IL-8) release by A549 lung epithelial cells in a dose- and time-dependent manner

Organic agricultural dusts (PM) have been shown to induce the release of inflammatory cytokines including IL-8<sup>15,36</sup>. Therefore, here, we examined whether the poultry PM would induce the release of IL-8 in A549 cells. Although the poultry PM (0.1 and 1.0 mg/ml) induced a slight but significant increase of IL-8 release at 4 h of treatment as compared to the same in the control untreated cells at 8 h of treatment of cells, the IL-8 release in A549 cells was significantly higher as compared to the same in the corresponding control untreated cells and in the poultry PM-treated cells at 4 h (Fig. 7A). Comparison of the extent of IL-8 release in the cells treated with different doses of the poultry PM (0.1, 1.0 and 10.0 mg/ml) for 6 and 8 h, it was observed that there was a significant dose-dependent increase in the extent of IL-8 release in the poultry PM-treated cells at 6 h, but the same at 8 h was significantly and markedly higher in a dose-dependent manner (Fig. 7B). At 8 h of treatment of cells with the poultry PM (0.1, 1.0 and 10.0 mg/ml), although a significant dose-dependent increase in the extent of IL-8 release was observed up to 1.0 mg/ml, at 10.0 mg/ml dose, the extent of IL-8 release was significantly higher as compared to the same in control untreated cells, but was lower than that seen in cells treated with 1.0 mg/ml dose under identical conditions (Fig. 7B). Thus, it was evident from these results that the poultry PM induced the release of the inflammatory cytokine IL-8 in a

time- and dose-dependent manner in A549 lung epithelial cells.

#### Calcium chelators (EGTA and BAPTA) and antioxidants (vitamin C and NAC) attenuate poultry PM-induced IL-8 release by A549 lung epithelial cells

Earlier experiments of the current study demonstrated that the calcium chelators and antioxidant (vitamin C) significantly attenuated the poultry PM-induced AA release from A549 cells. Therefore, it was reasonable to envision that calcium and ROS (oxidative stress) could play an upstream role in regulating the poultry PM-induced IL-8 release by A549 cells. Here, we investigated whether the calcium chelators (EGTA, the extracellular calcium chelator and BAPTA, the intracellular calcium chelator) and antioxidants (vitamin C and NAC) would modulate the poultry PM-induced IL-8 release by A549 cells.

As shown in Fig. 8A, the poultry PM (1.0 mg/ml) significantly induced the IL-8 release in A549 cells at 8 h of treatment, as compared to the same in the corresponding control untreated cells. Both BAPTA (5  $\mu$ M) and EGTA (5 mM) significantly attenuated the poultry PM-induced IL-8 release from cells, wherein EGTA exhibited greater inhibitory effect, suggesting that extracellular pools of calcium apparently play a major regulatory role in the poultry PM-induced IL-8 release by A549 cells. Antioxidants — vitamin C (1 mM) and NAC (0.1 mM) significantly attenuated the poultry PM-induced IL-8 release at 8 h of treatment of cells with the poultry PM, suggesting that ROS (oxidative stress) appeared to regulate the poultry PM-induced IL-8 release by A549 cells (Figs 8B and C).

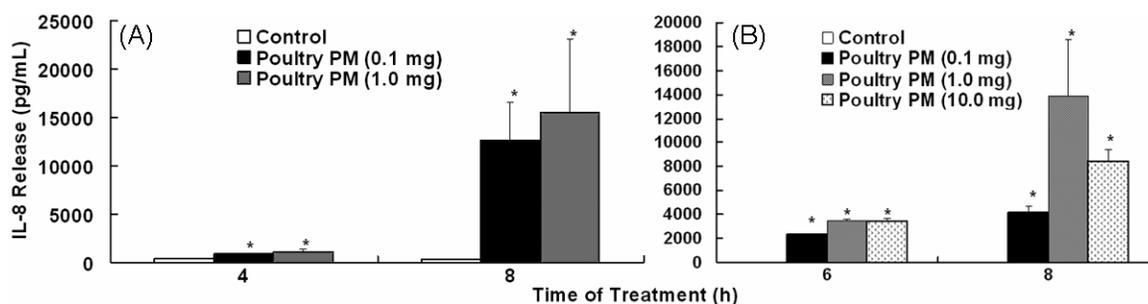


Fig. 7—Poultry PM induces IL-8 release by lung epithelial cells in a dose- and time-dependent manner [A549 lung epithelial cells ( $5 \times 10^5$  cells/35-mm dish) were treated with basal RPMI medium or basal RPMI medium containing poultry PM (0.1 and 1.0 mg/ml) for 4 and 8 h [A] or poultry PM (0.1, 1.0 and 10.0 mg/ml) for 6 and 8 h [B] at 37°C in a humidified environment of 5% CO<sub>2</sub>–95% air. At the end of treatment, release of IL-8 into the medium was assayed as described in 'Materials and Methods'. Data represent mean  $\pm$  SD of three independent experiments. \*Significantly different at  $P < 0.05$  as compared to the respective control cells treated with basal RPMI medium alone]

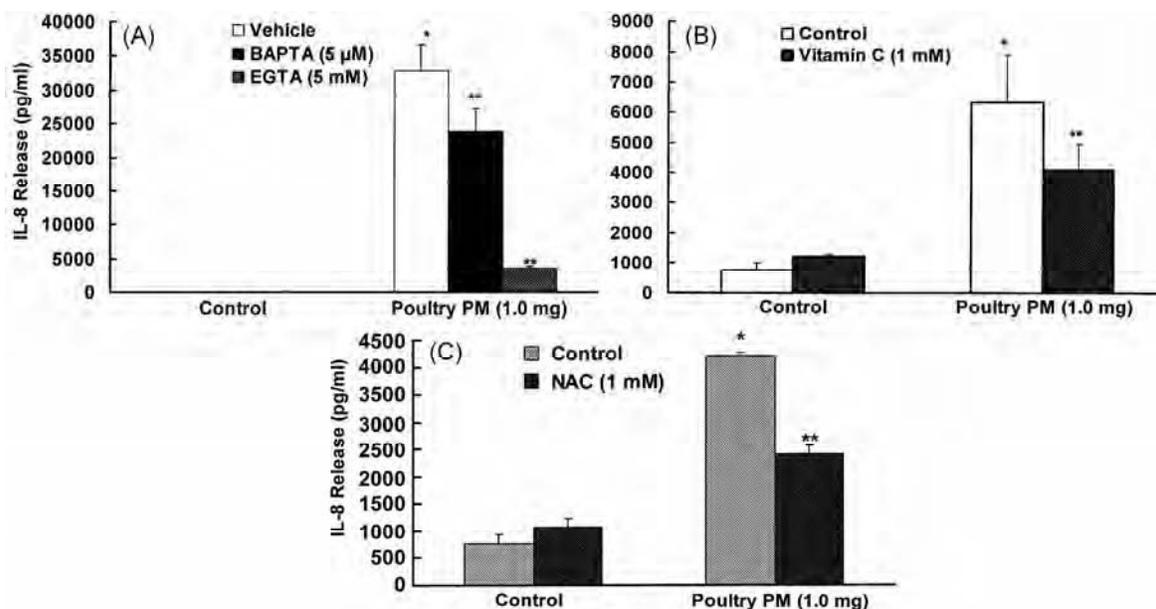


Fig. 8—Calcium chelators and antioxidant (vitamin C) attenuate poultry PM-induced IL-8 release [To test the role of calcium A549 lung epithelial cells, A549 lung epithelial cells ( $5 \times 10^5$  cells/35-mm dish) were treated with basal RPMI medium or basal RPMI medium containing BAPTA (5  $\mu$ M) alone or EGTA (5 mM) alone or basal RPMI medium containing poultry PM (1.0 mg/ml) alone or poultry PM (1.0 mg/ml) + BAPTA (5  $\mu$ M) or poultry PM (1.0 mg/ml) + EGTA (5 mM) for 8 h [A] at 37°C in a humidified environment of 5% CO<sub>2</sub>–95% air. To test the role of oxidative stress, A549 lung epithelial cells were treated with basal RPMI medium alone or basal RPMI medium containing vitamin C (1 mM) or poultry PM (1.0 mg/ml) or poultry PM (1.0 mg/ml) + vitamin C (1 mM) [B] and basal RPMI medium containing NAC (1 mM) or basal RPMI medium containing poultry PM (1.0 mg/ml) + NAC (1 mM) [C] at 37°C in a humidified environment of 5% CO<sub>2</sub>–95% air. At the end of treatment, release of IL-8 into the medium was assayed as described in ‘Materials and Methods’. Data represent mean  $\pm$  SD of three independent experiments. \*Significantly different at  $P < 0.05$  as compared to the respective control cells treated with basal RPMI medium alone. \*\*Significantly different at  $P < 0.05$  as compared to the cells treated with poultry dust alone]

#### COX- and LOX-specific inhibitors attenuate poultry PM-induced IL-8 release by A549 lung epithelial cells

Our earlier experiments of the current study revealed that the poultry PM induced the cPLA<sub>2</sub>-catalyzed release of AA and the formation and release of COX- and LOX-catalyzed AA metabolites (eicosanoids) during the initial stages of treatment of A549 cells upstream of the poultry PM-induced release of IL-8 from the cells. Also, it has been established that AA released from the membrane phospholipids acts as the substrate for COXs and LOXs, leading to the formation of eicosanoids<sup>28</sup>. Therefore, the upstream regulatory role of COX- and LOX-generated AA metabolites (PGs and LTs) in the poultry PM-induced IL-8 release by A549 cells was investigated with the aid of COX- and LOX-specific inhibitors. As shown in Fig. 9A, the poultry PM (1.0 mg/ml) significantly induced the IL-8 release at 8 h of treatment in A549 cells, as compared with the same in the control untreated cells. The poultry

PM-induced IL-8 release in cells was significantly attenuated by the two tested COX-specific inhibitors—ibuprofen (500  $\mu$ M) and aspirin (500  $\mu$ M), wherein the latter was more effective (Fig. 9A). These results demonstrated that the COX-generated AA metabolites appeared to regulate the poultry PM-induced downstream release of IL-8 release by the A549 cells.

As shown in Fig. 9B, the poultry PM (1.0 mg/ml) significantly induced the release of IL-8 from A549 cells at 8 h of exposure, as compared to the same in control untreated cells. NDGA (50 and 100 M), the general LOX-specific inhibitor caused a significant attenuation of the poultry PM-induced IL-8 release by the A549 cells, wherein 100  $\mu$ M dose of NDGA caused a greater and significant attenuation of the poultry PM-induced IL-8 release (Fig. 9B). These results revealed that the upstream generation of LOX-generated AA metabolites regulated the downstream IL-8 release by the A549 cells exposed to the poultry PM.

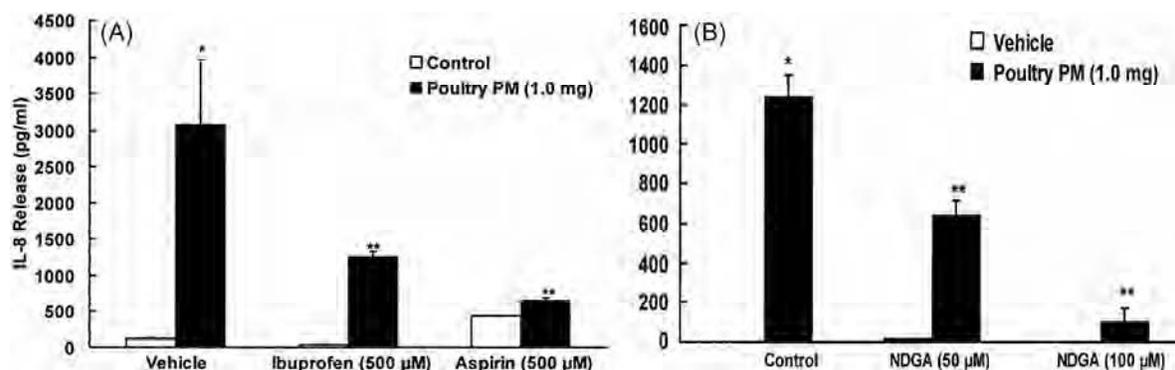


Fig. 9—COX- and LOX-specific inhibitors attenuate poultry PM-induced IL-8 release by A549 lung epithelial cells [To test the role of COX [A] and LOX [B], A549 lung epithelial cells ( $5 \times 10^5$  cells/35-mm dish) were treated with basal RPMI medium or basal RPMI medium containing COX-specific inhibitors (500  $\mu$ M ibuprofen or aspirin) or LOX-specific inhibitor (50 and 100  $\mu$ M NDGA) alone or poultry PM (1.0 mg/ml) alone or poultry PM (1.0 mg/ml) + COX-specific inhibitors or LOX-specific inhibitors for 8 h at 37°C in a humidified environment of 5% CO<sub>2</sub>–95% air. At the end of treatment, release of IL-8 into the medium was determined as described in 'Materials and Methods'. Data represent mean  $\pm$  SD of three independent experiments. \*Significantly different at  $P < 0.05$  as compared to the respective control cells treated with basal RPMI medium alone. \*\*Significantly different at  $P < 0.05$  as compared to the cells treated with poultry PM alone.

## Discussion

The current study revealed that the airborne poultry PM induced the release of AA by the activation of cPLA<sub>2</sub> in the A549 lung epithelial cells. The poultry PM was also found to induce the secretion of inflammatory cytokine IL-8 by the lung epithelial cells which was significantly attenuated by the COX- and LOX-specific inhibitors. The results of the current study demonstrated for the first time the upstream role of cPLA<sub>2</sub> and COX- and LOX-derived eicosanoids in the regulation of the poultry PM-induced IL-8 release by the human lung epithelial cells. Overall, the current study established a direct link between the poultry PM-induced inflammatory cytokine release through the upstream activation of cPLA<sub>2</sub>- and eicosanoid-mediated signaling.

Agricultural organic PM has been implicated as the cause of airway inflammation and respiratory irritation among farm workers<sup>34</sup>. Epidemiological studies indicate high morbidity and mortality arising from certain respiratory diseases among agricultural workers<sup>35</sup>. Owing to the non-specific nature of symptoms and the overlapping pattern of agricultural respiratory conditions, they are often mistaken for bacterial or viral infections<sup>34</sup>. Studies of allergen-induced airway responses show that cPLA<sub>2</sub> is an important effector of airway hyperreactivity in mouse. The effect of cPLA<sub>2</sub> on airway response is likely to be due to alterations in the downstream products of phospholipid metabolism<sup>28</sup>.

PLA<sub>2</sub> plays an upstream regulatory role in the release of AA for the formation of eicosanoids by specific hydrolysis of *sn*-2 ester linkage of the membrane phospholipids during activation by a wide variety of stimuli<sup>19</sup>. Thus, the physiological roles of PLA<sub>2</sub> are to maintain the membrane phospholipid turnover and repair by catalyzing the deacylation (hydrolysis) of unsaturated fatty acid in the *sn*-2 position of the membrane phospholipids and facilitate the reacylation (insertion of new unsaturated fatty acid) process. Under pathophysiological scenario, PLA<sub>2</sub> is activated by stresses and catalyzes the release of AA from the membrane phospholipids, thus acting as a rate-limiting enzyme to provide the substrate for the eicosanoid-synthesizing enzymes.

Inflammation is known as a major symptom of the organic dust-induced acute and chronic respiratory diseases, including asthma, organic toxic dust syndrome, hypersensitivity and pneumonitis<sup>34</sup>. Airway inflammation may be caused by immediate or delayed hypersensitivity. Other mechanisms, such as the activation of inflammatory cells by toxic agents or exposure to high levels of toxicants may be of considerable importance in causing airway and lung diseases<sup>32</sup>. IL-8, a chosen biomarker of inflammation is determined to assess the potency of organic dusts capable of inducing respiratory inflammation<sup>33,36</sup>.

Studies have shown that swine dust is a potent stimulus for IL-8 release in lung epithelial cells and alveolar macrophages<sup>37</sup>. Therefore, the present study

focused on the upstream regulatory role of cPLA<sub>2</sub> in secretion of the poultry PM-induced inflammatory cytokine IL-8, both being potent endogenous mediators of the inflammatory pathway. Moreover, the connection between the important inflammatory pathways, such as the eicosanoid formation and IL-8 release in the respiratory epithelium under the agonist challenge has not been reported so far. Hence, the current study attempted to demonstrate the upstream activation of cPLA<sub>2</sub> and the formation of COX- and LOX-derived bioactive eicosanoids and their involvement in the downstream regulation of inflammatory cytokine release in the well established human lung epithelial (A549) cell model exposed to the airborne agricultural (poultry) PM.

The results of the present study showed that poultry PM induced the release of AA upon activation of cPLA<sub>2</sub> in a dose-dependent manner. PLA<sub>2</sub> inhibition is of clinical significance in the pathway as it can block the synthesis of three important lipid mediators of inflammation (PGs, LTs and platelet aggregating factor), making PLA<sub>2</sub> an ideal target for anti-inflammatory drugs. However, mammalian cells express three different types of PLA<sub>2</sub> which are regulated by the complex signaling pathways<sup>23,24</sup>, making it all the more important to understand the definitive role of cPLA<sub>2</sub> and the events occurring at the molecular level, so that cPLA<sub>2</sub> can be selectively blocked. cPLA<sub>2</sub> is found in the cells in the absence of Ca<sup>2+</sup> and translocates to the particulate fraction in the presence of submicromolar amounts of Ca<sup>2+</sup>(ref 28).

Our results showed that cPLA<sub>2</sub> activation in human lung epithelial cells induced by the poultry PM was attenuated by the calcium chelators, suggesting that increased intracellular calcium led to the activation of Ca<sup>2+</sup>-dependent cPLA<sub>2</sub> and release of AA from the membrane phospholipids, which was subsequently metabolized into eicosanoids, including PGs and LTs. One of the striking observations of the current study was that the extracellular Ca<sup>2+</sup> chelator EGTA offered effective and significant attenuation of the poultry PM-induced AA release (cPLA<sub>2</sub> activation) and IL-8 release by the A549 lung epithelial cells. This observation led to the credence that the poultry PM also induced the uptake of extracellular Ca<sup>2+</sup>, probably by activating the Ca<sup>2+</sup> transport or channels in the lung epithelial cells, which warrants further investigation.

Calcium also seems to promote binding of cPLA<sub>2</sub> to phospholipid vesicles<sup>38</sup>. The role of calcium in

cPLA<sub>2</sub> activation in the lung epithelial cells exposed to the poultry PM was established by the use of intracellular (BAPTA/AM) and extracellular calcium chelators (EGTA) in the current study. Both the extracellular and intracellular calcium chelators were found to attenuate the poultry PM-induced activation of Ca<sup>2+</sup>-dependent cPLA<sub>2</sub>, as observed from the release of AA from the human lung epithelial cells. The residual oil fly ash (ROFA), an airborne PM composed of high levels of heavy metals that has been shown to induce the release of inflammatory cytokines in respiratory cells<sup>39,40</sup>, was used as a positive control. ROFA, which was used as a surrogate particle in this study, caused PLA<sub>2</sub> activation similar to the poultry PM in the A549 lung epithelial cells (data not shown).

Free radicals (ROS) are produced in response to stimulus by endotoxins. Antioxidants, like ascorbic acid (vitamin C) are able to react with these free radicals and quench the chain reactions. NAC has also been shown to reduce oxidative stress both *in vivo* and *in vitro*<sup>41</sup>. Our results showed that both vitamin C and NAC attenuated the poultry PM induced cPLA<sub>2</sub> activation and IL-8 release, indicating the role of oxidative stress and ROS in the poultry PM-induced inflammatory cascade involving the IL-8 release through the upstream regulation of cPLA<sub>2</sub> activation.

Endotoxins of bacterial origin have been shown to be present in organic particulate matter and are potential cause for respiratory diseases<sup>42</sup>. Our results also showed the presence of high levels of endotoxin in the poultry PM. Bacteria/bacterial products in swine dust have also been shown to induce cytokine IL-8 release by the human alveolar macrophages and epithelial cells<sup>26,37</sup>. Earlier studies have also demonstrated that inhalation of corn dust extract results in an acute inflammatory response, elevated cellularity, such as neutrophils and high levels of IL-8 along with other cytokines in bronchoalveolar lavage (BAL)<sup>27</sup>. Lipopolysaccharides (LPS) have been well documented to trigger the IL-8 secretion<sup>43</sup>.

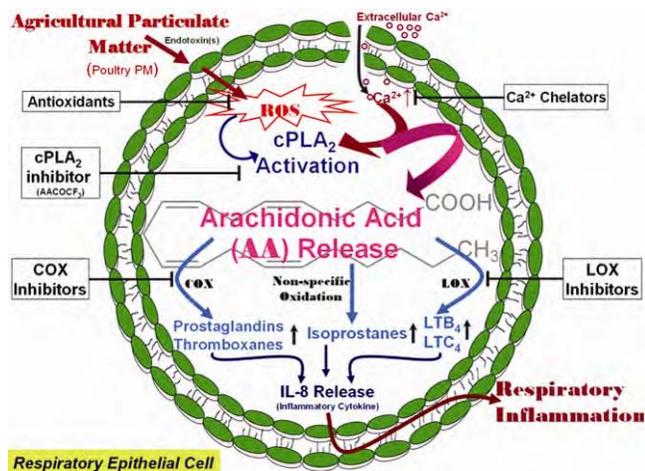
Organic dusts have also been shown to elicit similar IL-8 response as exhibited by LPS<sup>13</sup>. However, in the current study, *E. coli* LPS did not appear to induce the activation of cPLA<sub>2</sub> (release of AA) in the A549 lung epithelial cells, as opposed to the endotoxin-containing poultry PM which was effective to induce the activation of cPLA<sub>2</sub>. This observation suggested the important features: (i) *E. coli* LPS that was obtained commercially was

not effective in inducing cPLA<sub>2</sub> activation in the A549 cells and/or (ii) there could be a different type of endotoxin present in the Central Ohio poultry PM which was more effective in causing the activation of cPLA<sub>2</sub> in A549 cells. Nevertheless, the nature of the endotoxin present in the Central Ohio poultry PM needs thorough characterization.

The effect of organic extract of the poultry PM was examined because of the high levels of endotoxin found in the PM. Aqueous and organic extracts of the poultry PM were found to significantly increase AA release by the A549 cells as observed in the current study. Both epithelial cells and alveolar macrophages have been shown to contribute to the release of proinflammatory cytokines upon exposure to the swine dust<sup>44</sup>. Our results indicated that the organic extract of the poultry PM caused a greater release of AA by the cells, probably caused by the efficient extraction and greater cellular accessibility of the hydrophobic LPS/toxins in to the organic phase. This also suggested that another type of toxin (e.g. fungal toxin) might be present in the poultry PM or the type of endotoxins present in the agricultural particulate matter might be different from the commercially available bacterial endotoxins. It has been previously observed in several studies that when inflammatory agents, such as endotoxins are inhaled, proinflammatory cytokine release is induced<sup>13</sup>. IL-8, one among several inflammatory cytokines, is released in the process. Our results were consistent with the other reported findings showing that exposure of lung epithelial cells to the poultry PM caused a dramatic increase in IL-8 secretion<sup>12-16</sup>.

Earlier studies have also shown that COX- and LOX-derived eicosanoids are critical players in the lung pathophysiology<sup>20</sup>. PLA<sub>2</sub> has been shown to regulate the upstream production of PGs and LTs through COX and LOX pathways. The current study also revealed attenuation of the poultry PM-induced IL-8 release by COX- and LOX-specific inhibitors, suggesting a probable link between the upstream activation of cPLA<sub>2</sub> and formation of eicosanoids and the downstream release of IL-8 in the lung epithelial cell exposed to the poultry PM.

Since both cPLA<sub>2</sub> and IL-8 play a role in the inflammation process, it is crucial to understand how these two molecules interact, especially in the respiratory tract epithelium during exposure to the environmental toxins such as the airborne PM. IL-8 secretion could be exacerbated or inhibited,



**Schema**—Proposed mechanism of poultry PM-induced IL-8 release by A-549 lung epithelial cells through the activation of cPLA<sub>2</sub>, arachidonic acid (AA) release and formation of COX- and LOX-catalyzed AA metabolites (Eicosanoids)

depending on the activation of cPLA<sub>2</sub>. The results of the current study for the first time revealed the direct association of cPLA<sub>2</sub> activation and eicosanoid formation with IL-8 secretion in the respiratory epithelial cells (**Schema**).

However, the poultry PM-induced upstream activation of cPLA<sub>2</sub>, formation of the COX- and LOX-derived eicosanoids and the downstream release of IL-8 could very well be regulated by several protein kinases. It remains to be established whether other pathways, such as those involving the mitogen-activated protein kinases (MAPK) are involved in the activation of cPLA<sub>2</sub>, which could affect the release of poultry PM-induced IL-8 release. Therefore, a thorough understanding of the mechanisms of the poultry PM-induced release of pro-inflammatory cytokines (e.g. IL-8) by the respiratory epithelium regulated through the cPLA<sub>2</sub> and eicosanoid actions will unravel the mechanisms of pathogenesis of agricultural respiratory and lung diseases. IL-8 plays a major role in the recruitment of neutrophils to the site of inflammation prior to initiation of respiratory burst. The activation of neutrophils, such as the respiratory burst is crucial in the host-defense against the invading microorganisms<sup>45</sup>. However, these activities have also been implicated in the damage that is associated with inflammation regulated through the release of toxic ROS and eicosanoids. Overall, the direct link between the poultry PM-induced release of the inflammatory cytokine (IL-8) and the upstream activation of cPLA<sub>2</sub> and generation of eicosanoids

as demonstrated in the current study would likely offer mechanistic insights into the agricultural PM- and other airborne PM-induced respiratory tract inflammatory disorders for proper therapeutic intervention (**Schema**).

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