Cell Biochem Biophys (2013) 67:415–429
DOI 10.1007/s12013-011-9329-7

ORIGINAL PAPER

Phospholipase A_2 Activation by Poultry Particulate Matter is Mediated Through Extracellular Signal-Regulated Kinase in Lung Epithelial Cells: Regulation of Interleukin-8 Release

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Published online: 20 December 2011

Abstract The mechanisms of poultry particulate matter (PM)-induced agricultural respiratory disorders are not thoroughly understood. Hence, it is hypothesized in this article that poultry PM induces the release of interleukin-8 (IL-8) by lung epithelial cells that is regulated upstream by the concerted action of cytosolic phospholipase A_2 ($cPLA_2$) and extracellular signal-regulated kinase (ERK). To test this hypothesis, the widely used cultured human lung epithelial cells (A549) were chosen as the model system. Poultry PM caused a significant activation of PLA_2 in A549 cells, which was attenuated by $AACOCF_3$ ($cPLA_2$ inhibitor) and PD98059 (ERK-1/2 upstream inhibitor). Poultry PM induced upstream ERK-1/2 phosphorylation and downstream $cPLA_2$ serine phosphorylation, in a concerted fashion, in cells with enhanced association of ERK-1/2 and $cPLA_2$. The poultry PM-induced $cPLA_2$ serine phosphorylation and IL-8 release were attenuated by $AACOCF_3$, PD98059, and by transfection with dominant-negative ERK-1/2 DNA in cells. The poultry PM-induced IL-8 release by the bone marrow-derived macrophages of $cPLA_2$ knockout mice was significantly lower. For the first time, this study demonstrated that the poultry PM-induced IL-8 secretion by human lung epithelial cells was regulated by $cPLA_2$ activation through ERK-mediated serine phosphorylation, suggesting a mechanism of airway inflammation among poultry farm workers.

Keywords Phospholipase A_2 · IL-8 · ERK · MAPK · Lung epithelial cell · Poultry dust · Agricultural PM · $cPLA_2$ serine phosphorylation · Inflammatory cytokines · Interleukin-8 · Respiratory epithelium · A549 cells

This work is dedicated to late Dr. Val Vallyathan of NIOSH who contributed significant findings to the Occupational Health Research.

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Introduction

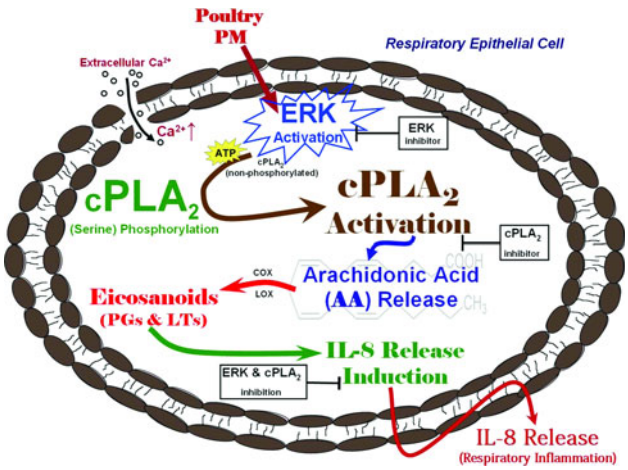
Airborne particulate matter (PM) has emerged as one of the noxious air pollutants in causing pulmonary and cardiopulmonary diseases in humans [1]. Agricultural PM such as poultry and swine dusts originate in the form of inorganic and organic dusts [2]. The poultry and swine farming, airborne PM often causes severe occupational respiratory and lung disorders and diseases [3–5].

Inflammation is a common symptom in all respiratory diseases. The prevalence of respiratory diseases in rural agricultural settings exceeds that of urban areas [6]. Agricultural PM contains inhalable bacterial toxins such as the endotoxins and, therefore, agriculture is considered as one of the most hazardous occupations [7, 8]. Increasing

incidences of respiratory disorders among the Central Ohio poultry farm workers have been reported [9]. Understanding the mechanism(s) of the organic agricultural PM-induced respiratory and pulmonary inflammatory responses can help prevent or alleviate the associated respiratory diseases.

Phospholipase A_2 (PLA_2), an enzyme belonging to the group of phospholipid hydrolases, plays a crucial role in the cascades of inflammation [10]. PLA_2, upon activation by an agonist, catalyzes the hydrolysis and release of the esterified unsaturated fatty acid (arachidonic acid, AA) at the sn-2 position of the membrane phospholipids [11]. Downstream conversion of the PLA_2-released AA by cyclooxygenases (COXs) and lipoxygenases (LOXs) leads to the formation of eicosanoids including prostaglandins (PGs) and leukotrienes (LTs), both of which act as important inflammatory mediators [12]. Agonists also induce the synthesis and secretion of inflammatory cytokines in certain cells. One such cytokine is the interleukin-8 (IL-8), which plays a pivotal role in recruiting the inflammatory cells like neutrophils and macrophages at the site of injury and inflammation [13–15]. Organic PM of agricultural origin tend to contain considerable amounts of endotoxins, which trigger IL-8 secretion by immunoactive cells [7].

Cytosolic PLA_2 ($cPLA_2$), a calcium-dependent member of the PLA_2 family of enzymes, is also known to be associated with membrane trafficking including endocytosis and secretion [10, 11]. Respiratory epithelium is the prime target of the inhaled noxious agents including the agricultural organic dusts such as the poultry PM. We have previously reported that poultry PM induces IL-8 release through $cPLA_2$, COX, and LOX in A549 lung epithelial cells [9]. Also, it is known that mitogen-activated protein kinases (MAPKs) regulate the activity of $cPLA_2$ [16]. Therefore, the regulation of the poultry-PM induced IL-8 release by respiratory epithelium through MAPK-mediated activation of $cPLA_2$ is conceivable. As the mechanism regarding the poultry PM-induced $cPLA_2$ activation and the subsequent release of IL-8 in respiratory epithelial cells has not been reported, in this article, we hypothesized that the poultry PM would induce the inflammatory cytokine release by lung epithelial cells through upstream serine phosphorylation of $cPLA_2$ mediated by extracellular signal-regulated kinase (ERK), a member of the MAPK family. Hence, we investigated the upstream activation of $cPLA_2$ through ERK-mediated serine phosphorylation and the downstream IL-8 secretion in human lung epithelial cells (A549) upon exposure to poultry PM collected from the Central Ohio poultry farms. The results of this study provided the first evidence that the poultry PM-induced IL-8 secretion by human lung epithelial cells was regulated by $cPLA_2$ activation through ERK-mediated serine phosphorylation (Scheme 1).



Scheme 1 Proposed mechanism of poultry PM-induced activation of $cPLA_2$ through ERK-1/2-mediated serine phosphorylation and regulation of IL-8 release in lung epithelial cells

Materials

Poultry PM (0.2–10 μm) were collected from the Wooster (Ohio) poultry and swine farm by the Agricultural Engineering Department of the Ohio State University. Human lung epithelial cells (A549) (passage 1–4) were purchased from Clonetics Corp. (San Diego, CA). RPMI 1640 medium, trypsin–EDTA, sodium pyruvate, fetal bovine serum (FBS), L-glutamine, antibiotic–antimycotic, and Dulbecco's phosphate buffer (PBS) were obtained from Gibco (Grand Island, NY). [^{3}H]AA acid was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Arachidonyl trifluoromethyl ketone ($AACOCF_3$) was obtained from Cayman Chemical (Ann Arbor, MI). PD98059 was obtained from Calbiochem (San Diego, CA). U0126 and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (St Louis, MO). Primary antibodies for $cPLA_2$, phosphoserine-$cPLA_2$, ERK-1, ERK-2, and phospho-ERK-1/2 raised in rabbit were obtained from Cell Signaling Technology, Inc (Danvers, MA). Secondary antibody (Anti-Rabbit IgG) and anti-β-actin antibody were obtained from Amersham Biosciences (Piscataway, NJ). Protein A/G plus Sepharose were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

Methods

Cell Culture

A549 cells were cultured in the RPMI 1640 medium supplemented with 10% FBS, antibiotics, pyruvate, and L-glutamine under a humidified environment of 95% air—5% CO_2 at 37°C in sterile T-75 cm^2 flasks. 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 a humidified environment of 95% air—5% CO_2 at 37°C for treatment with the desired pharmacological agent and/or poultry PM. A549 cells from passages 6–8 were used in the experiments.

[^{3}H]AA Acid Labeling and Assay of Phospholipase A_2 Activity

The activity of PLA_2 was assayed according to our previously published procedure [11]. A549 cells in 35-mm dishes (70% confluence) were labeled for 12 h with 1 ml of the medium containing 0.5 μCi/ml of [^{3}H]AA. Following treatment of cells, [^{3}H]AA released into the medium was measured in the Packard Tricarb 2900 TR liquid scintillation counter. PLA_2 activity was expressed as DPM of [^{3}H]AA released per dish.

Preparation of Cell Lysates, Immunoprecipitates, and Western Blotting

Preparation of cell lysates and immunoprecipitates, separation of proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and Western blotting were done according to our previously published procedures [17]. Cell lysates containing equal amounts of proteins were subjected to SDS-PAGE on 12% gels, transferred onto polyvinylidene difluoride (PVDF) membranes, and subjected to overnight immunoblotting with either anti-ERK-1/2 (1:2000 dilution) or anti-phospho-ERK-1/2 (1:1000 dilution) antibodies at 4°C. $cPLA_2$ and ERK-1/2 immunoprecipitates (IPs) from cells were prepared by treating the cell lysates containing equal amounts of protein (1 mg/ml) with 10 μl of rabbit polyclonal anti-$cPLA_2$ or anti-ERK-1/2 antibodies for 12 h at 4°C. IPs were captured on to the protein A/G agarose beads and then to SDS-PAGE on 8% gels. Proteins were then electrotransferred on to PVDF membranes, after which they were incubated in Tris-buffered saline containing 0.1% Tween-20 (TBST) containing 3% milk for 12 h at 4°C with rabbit primary anti-$cPLA_2$ or anti-ERK-1/2 antibodies (1:1000 dilution). The membranes were washed three times with TBST and incubated for 1–2 h at room temperature with horseradish peroxidase-conjugated goat anti-rabbit (1:2000 dilution) or goat anti-mouse secondary antibodies (1:5000 dilution). To verify equal protein loading on gels, β-actin in the cell lysates were probed as a marker with the aid of anti-β-actin antibody. The immunoblots were then developed on the film with the enhanced chemiluminescence (ECL) reagents according to the manufacturer's recommendation. The intensities of protein bands developed on film were quantified by digital densitometric analysis.

Transient Transfection of Cells

A549 cells were transiently transfected with wild-type (WT) or dominant-negative (DN) ERK-1 or ERK-2 cDNA according to our previously published procedure [17]. To obtain transfection efficiency following 18–24 h of transfection, the images of green fluorescent protein (GFP)-positive cells were captured using the Olympus fluorescent microscope at 50× magnification. The achieved transfection efficiency was in the range of 21–44% with an average of 32.6%.

Immunofluorescence Confocal Microscopy of $cPLA_2$ and Phosphoserine-$cPLA_2$

Translocation of $cPLA_2$ and appearance and localization of phosposerine-$cPLA_2$ in A549 cells were analyzed by immunofluorescence confocal microscopy by means of the rabbit anti-$cPLA_2$ and anti-phosphoserine-$cPLA_2$ antibodies (1:200 dilution) according to our previously published procedure [17]. The images were captured digitally on the Zeiss LSM 510 Confocal/Multiphoton Microscope at 488-nm excitation and 600-nm emission at 63× magnification.

Preparation of Bone Marrow-Derived Macrophages

$cPLA_2$ knockout (KO) mice were gifted by Dr. Joseph V. Bonventre of Harvard Medical School. Both the WT ($cPLA_2$ +/+) and $cPLA_2$ KO ($cPLA_2$ −/−) mice were bred on SV129/C57BL/6 background. They were bred, genotyped, and 1-month-old male $cPLA_2$ KO mice were used. To obtain differentiated bone marrow-derived macrophages (BMM), bone marrow progenitor cells were isolated from femur and tibia of mice and differentiated upon treatment with recombinant mouse M-CSF (R&D Systems (Minneapolis, MN)) as previously described [18, 19].

Assay for Interleukin-8 (IL-8)

Following the treatments of cells, 200 μl of the incubation medium was assayed for the IL-8 release utilizing the IL-8 ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's recommendations.

Aqueous Extraction

Appropriate volume of RPMI 1640 medium was added to a known amount of the poultry PM in a sterile, capped tube, and the mixture was vortexed at the high speed setting for

30 min at room temperature. The resulting poultry PM suspension was used for the treatment of cells.

Statistical Analysis

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

Results

Poultry PM Induces Arachidonic Acid Release by Lung Epithelial Cells in a Dose- and Time-Dependent Manner

To investigate whether poultry PM would induce the release of arachidonic acid (AA) from the epithelium, A549 lung epithelial cells pre-labeled with [^{3}H]AA were treated with varying concentrations (0.1, 1.0, and 10.0 mg/ml) of poultry PM for 1 and 2 h. Poultry PM, at a dose of 1.0 mg/ml, resulted in a significant increase in the release of [^{3}H]AA at 1 h (4-fold increase) as compared to the same in control untreated cells (Fig. 1a). Comparatively, at 2 h of treatment, the [^{3}H]AA released was lower than that was observed at 1 h of treatment. At 0.1 mg/ml dose of poultry PM, the release of [^{3}H]AA was slightly, but not significantly higher as in the case where the same at 1.0 mg/ml dose was significantly greater (3-fold increase) as compared to the same in control untreated cells. In shorter time-course study, the poultry PM-induced [^{3}H]AA release was found to be maximum at 60 min of treatment as compared to the same in cells exposed to poultry PM for 15, 30, and 45 min (Fig. 1b). Together these results revealed that poultry PM induced the release of AA by A549 cells in a dose- and time-dependent fashion suggesting the activation of PLA_2.

$cPLA_2$-Specific Inhibitor and ERK-1/2 Upstream Inhibitor Attenuate Poultry PM-Induced Arachidonic Acid Release by Lung Epithelial Cells

In order to confirm whether $cPLA_2$ would be responsible for the poultry PM-induced release of AA, A549 cells, pre-treated with $cPLA_2$-specific inhibitor ($AACOCF_3$, 0.1 μM) for 1 h, were challenged with poultry PM (1.0 mg/ml) for 1 h and the release of [^{3}H]AA by the cells was determined. $AACOCF_3$ treatment significantly attenuated the poultry PM-induced [^{3}H]AA release by cells as compared to the same in cells treated with poultry PM alone (Fig. 2a). These results confirmed that the poultry PM-induced AA release by lung epithelial cells was mediated by $cPLA_2$ activation.

The role of ERK in the poultry PM-induced release of AA was investigated in A549 cells after the pre-treatment of cells with the ERK-1/2 upstream inhibitor (PD98059, 20 μM) for 1 h and then the treatment of cells with poultry PM (1.0 mg/ml). PD98059 treatment significantly attenuated the poultry PM-induced [^{3}H]AA release by A549 cells as compared to the same in the cells treated with poultry PM alone (Fig. 2b). These results demonstrated that ERK-1/2-specific inhibitor attenuated the poultry PM-induced release of AA by A549 lung epithelial cells suggesting the

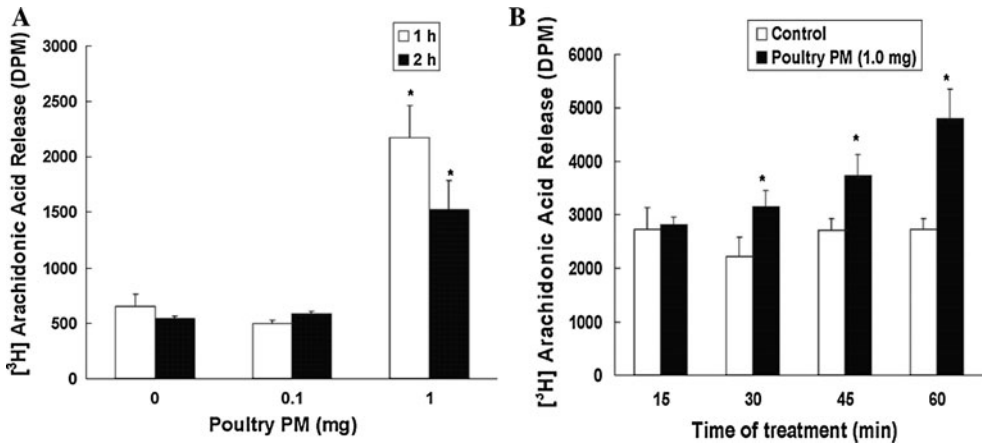


Fig. 1 Poultry PM induces arachidonic acid release by lung epithelial cells in a dose- and time-dependent manner. A549 cells, pre-labeled with [^{3}H]AA acid (0.5 μCi/ml) were treated with RPMI 1640 medium alone or RPMI 1640 medium containing poultry PM (0.1–1.0 mg/ml) for 1 and 2 h (**a**) and with RPMI 1640 medium alone or RPMI 1640 medium containing poultry PM (1.0 mg/ml) for 15–60 min (**b**). After the above treatments, release of [^{3}H]AA was determined by measuring the radioactivity in the medium as described in the "Materials" and "Methods" sections. Data represent ± SD of three independent experiments in triplicate. *Significantly different at $P < 0.05$ as compared with the control, untreated cells

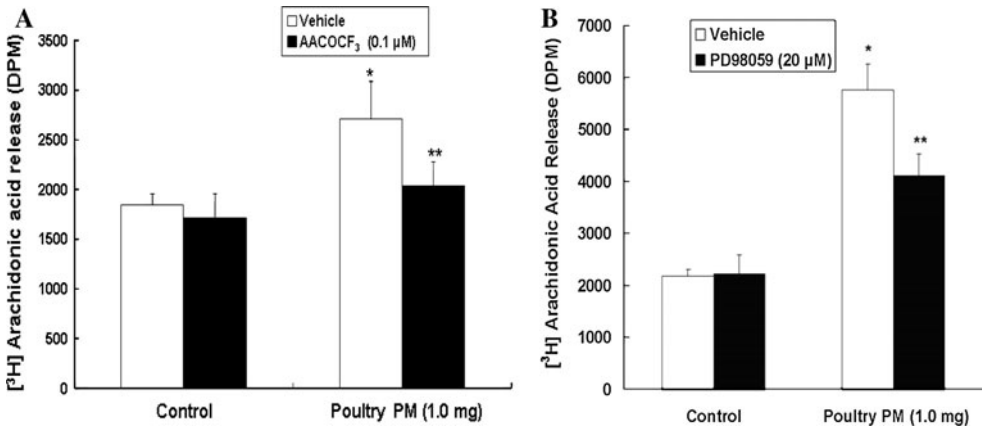


Fig. 2 $cPLA_2$-specific inhibitor ERK-1/2 upstream inhibitor attenuate poultry PM-induced arachidonic acid release by lung epithelial cells. A549 cells, pre-labeled with [^{3}H]AA acid (0.5 μCi/ml) were pre-treated for 1 h with RPMI 1640 medium alone or RPMI 1640 medium containing $cPLA_2$-specific inhibitor ($AACOCF_3$, 0.1 μM) (**a**) or ERK-1/2 upstream inhibitor (PD98059, 20 μM) (**b**) and then treated with RPMI 1640 medium alone or RPMI 1640 medium containing poultry PM (1.0 mg/ml) for 1 h. Following treatments, release of [^{3}H]AA acid was determined by measuring the radioactivity in the medium as described in the "Materials" and "Methods" sections. Data represent ± SD of three independent experiments in triplicate. *Significantly different at $P < 0.05$ as compared with the control untreated cells. **Significantly different at $P < 0.05$ as compared with the poultry PM-treated cells

role of ERK-1/2 in the poultry PM-induced activation of $cPLA_2$.

Poultry PM Induces Serine Phosphorylation of $cPLA_2$ in Lung Epithelial Cells in a Dose- and Time-Dependent Manner

As the previous experiment has demonstrated the involvement of ERK in the poultry PM-induced activation of $cPLA_2$ in A549 cells, in this study, we investigated whether the enzyme activation would be associated with the serine phosphorylation of $cPLA_2$. SDS-PAGE and Western blotting analyses of proteins revealed that poultry PM (0.1–1.0 mg/ml) induced serine phosphorylation in a time-dependent manner (0–60 min) in A549 cells. Poultry PM-induced serine phosphorylation of $cPLA_2$ was observed to be the highest at 1.0 mg/ml (Fig. 3a, b). The serine phosphorylation of $cPLA_2$ gradually increased from 0 to 30 min, reached a maximum at 30 min, and then declined from 30 to 60 min (Fig. 3c, d) in A549 cells as compared to the same in the control untreated cells. From these results, it was evident that poultry PM induced serine phosphorylation of $cPLA_2$ in lung epithelial cells in a time- and dose-dependent fashion.

Poultry PM Induces Serine Phosphorylation of $cPLA_2$ and Phosphorylation of ERK-1/2 in Lung Epithelial Cells in a Concerted Fashion

To further demonstrate that the serine phosphorylation of $cPLA_2$ and ERK-1/2 activation (phosphorylation) would be associated in a concerted fashion, in this article, we investigated whether poultry PM would induce ERK-1/2 phosphorylation and serine phosphorylation of $cPLA_2$ in a temporal fashion with the aid of SDS-PAGE and Western blotting analysis of proteins. From the results, it was evident that poultry PM (1.0 mg/ml) induced serine phosphorylation of $cPLA_2$ in a time-dependent manner from 0 to 30 min (Fig. 4a). Serine phosphorylation of $cPLA_2$ peaked at 30 min of treatment of cells with poultry PM, while the extent of $cPLA_2$ phosphorylation at 60 min under identical conditions declined compared to the same in the control untreated cells (Fig. 4b).

On the other hand, poultry PM (1.0 mg/ml) induced ERK-1/2 phosphorylation in A549 cells in a time-dependent fashion (0–15 min) with a maximum extent at 30 min of treatment (Fig. 3c, d). At 30 and 60 min of exposure, although the extent of ERK-1/2 phosphorylation was significantly higher in cells treated with poultry PM, it declined as compared to the same at 15 min of treatment (Fig. 4c, d). These results demonstrated that poultry PM induced ERK-1/2 phosphorylation before inducing the serine phosphorylation of $cPLA_2$ in A549 cells. Furthermore, these results revealed that the poultry PM-induced ERK-1/2 phosphorylation declined gradually after 15 min of treatment while the serine phosphorylation of $cPLA_2$ gradually increased up to 30 min of treatment. Overall, these results demonstrated that poultry PM induced ERK-1/2 phosphorylation (activation) upstream of serine phosphorylation of $cPLA_2$ in A549 cells in a concerted fashion.

Poultry PM Enhances Association of $cPLA_2$ with ERK-1/2 in Lung Epithelial Cells

The previous experiments of this study have revealed that the poultry PM-induced $cPLA_2$ activation was regulated by ERK-1/2 and associated with the upstream

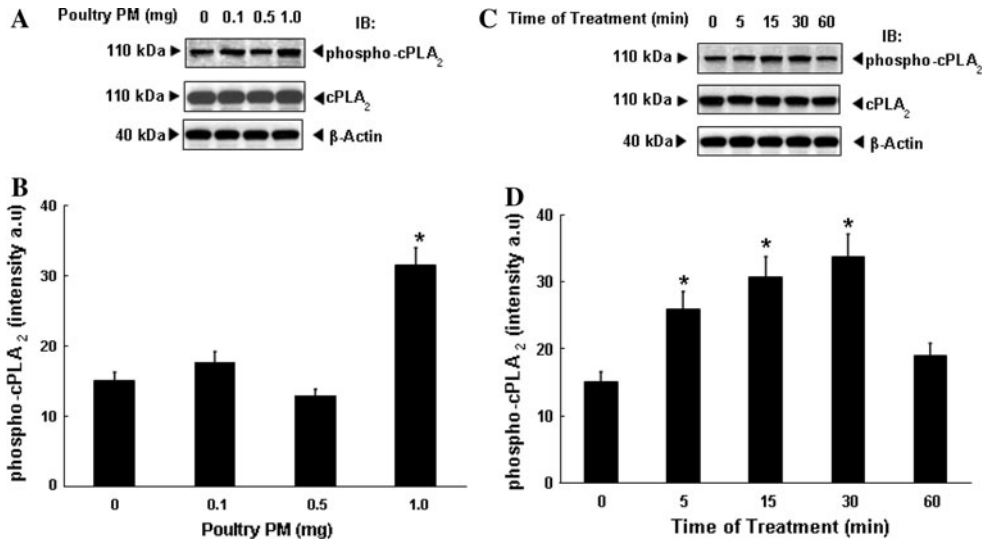


Fig. 3 Poultry PM induces serine phosphorylation of $cPLA_2$ in lung epithelial cells in a dose- and time-dependent manner. A549 cells were treated with RPMI 1640 medium alone or RPMI 1640 medium containing poultry PM (0.1–1.0 mg/ml) for 1 h (**a**, **b**), and with RPMI 1640 medium containing poultry PM (1.0 mg/ml) for 0–60 min (**c**, **d**). After the above treatments, proteins in cell lysates were subjected to SDS-PAGE and Western blotting analysis with $cPLA_2$-specific and phosphoserine-$cPLA_2$-specific antibodies, and the intensities of phosphoserine-$cPLA_2$ protein bands were digitally determined as described in the "Materials" and "Methods" sections. Each Western blot is a representative of three independent experiments conducted under identical conditions. Data represent ± SD of three independent experiments in triplicate. *Significantly different at $P < 0.05$ as compared with the control, untreated cells (**b**) and the cells treated with poultry PM at 0 min (**d**)

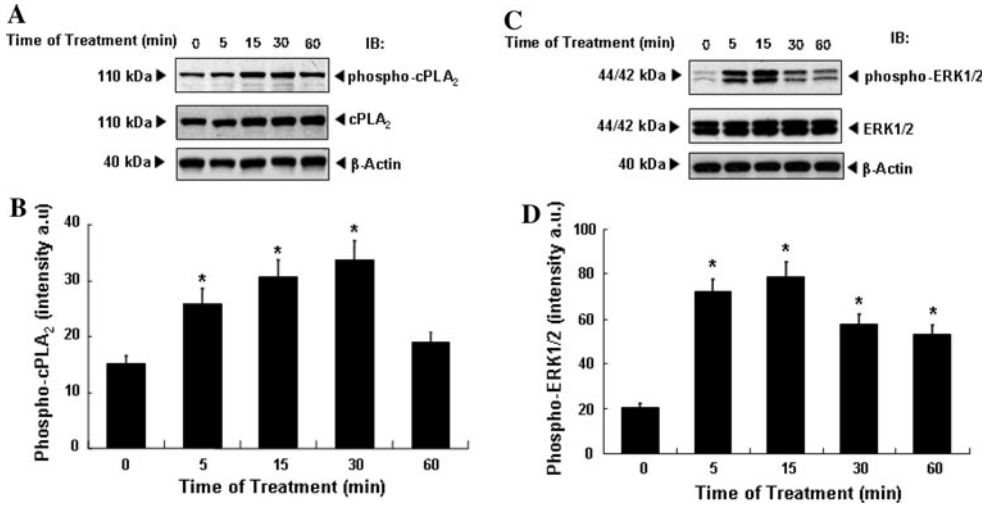


Fig. 4 Poultry PM induces serine phosphorylation of $cPLA_2$ and phosphorylation of ERK-1/2 in lung epithelial cells in a concerted fashion. A549 cells were treated with RPMI 1640 medium containing poultry PM (1.0 mg/ml) for 0–60 min. After the above treatment, proteins in cell lysates were subjected to SDS-PAGE and Western blotting with $cPLA_2$-specific and phosphoserine-$cPLA_2$-specific antibodies (**a**, **b**) and ERK-1/2-specific and phospho-ERK-1/2-specific antibodies (**c**, **d**), after which intensities of phosphoserine-$cPLA_2$ and phospho-ERK-1/2 protein bands were digitally determined as described in the "Materials" and "Methods" sections. Each Western blot is a representative of three independent experiments conducted under identical conditions. Data represent ± SD of three independent experiments in triplicate. *Significantly different at $P < 0.05$ as compared with the cells treated with poultry PM at 0 min

phosphorylation of ERK-1/2 and downstream serine phosphorylation of $cPLA_2$ in A549 cells. Therefore, here, we investigated whether poultry PM would affect the association of ERK-1/2 and $cPLA_2$ in A549 cells. SDS-PAGE and Western blotting analysis of the IPs of $cPLA_2$ from cells treated with poultry PM (1.0 mg/ml for 30 min) showed significantly elevated phosphoserine-$cPLA_2$ formation and enhanced association and

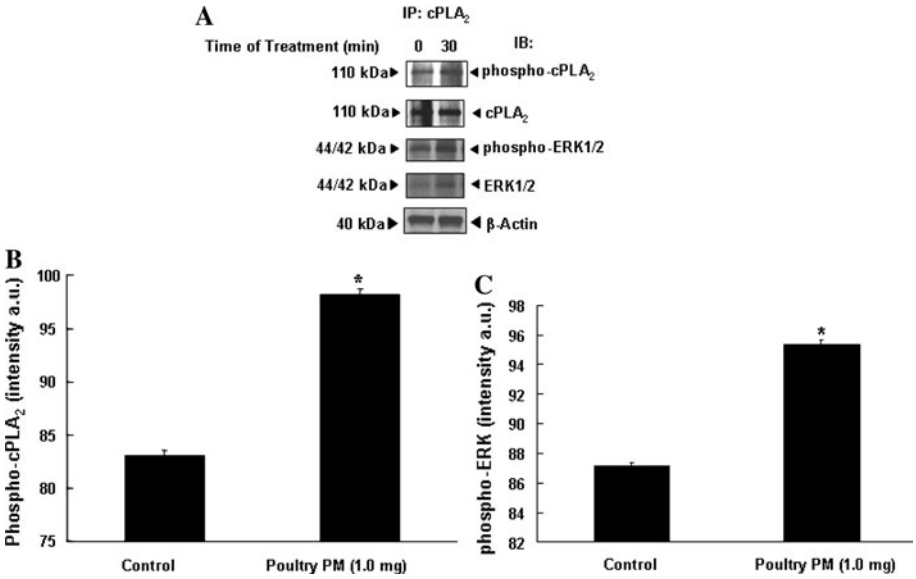


Fig. 5 Poultry PM enhances association of $cPLA_2$ with ERK-1/2 in lung epithelial cells. A549 cells were treated with RPMI 1640 medium containing poultry PM (1.0 mg/ml) for 0 and 30 min. After the above treatments, immunoprecipitates (IPs) of $cPLA_2$ from cell lysates were subjected to SDS-PAGE and Western blotting with $cPLA_2$-specific, phosphoserine-$cPLA_2$-specific, ERK-1/2-specific, and phospho-ERK-1/2-specific antibodies (**a**), and the intensities of phosphoserine-$cPLA_2$ (**b**) and phospho-ERK-1/2 (**c**) protein bands were digitally determined as described in the "Materials" and "Methods" sections. Each Western blot is a representative of three independent experiments conducted under identical conditions. Data represent ± SD of three independent experiments in triplicate. *Significantly different at $P < 0.05$ as compared with the cells treated with poultry PM at 0 min

phosphorylation of ERK-1/2 as compared to the same in the control cells treated with poultry PM (1.0 mg/ml for 0 min) (Fig. 5a, c). These results demonstrated that poultry PM enhanced the association of $cPLA_2$ with ERK-1/2 in lung epithelial cells.

Poultry PM Induces Translocation and Serine Phosphorylation of $cPLA_2$ in Lung Epithelial Cells

At this juncture, this study demonstrated the poultry PM-induced activation and serine phosphorylation of $cPLA_2$ in A549 cells as revealed by the [^{3}H]AA release assay and the SDS-PAGE and Western blotting analysis of proteins. Using the confocal immunofluorescence microscopy, here, we conducted studies to confirm in situ translocation and serine phosphorylation of $cPLA_2$ in the poultry PM-treated A549 cells. Poultry PM (1.0 mg/ml) induced translocation of $cPLA_2$ in A549 cells in a time-dependent fashion (0–60 min) (Fig. 6a). At 15 min of treatment with poultry PM, an intense translocation and visualization of $cPLA_2$ in cells in situ were observed which were increasingly robust at 30 and 60 min of treatment as compared to the same in the cells at 0 min of treatment or in the control untreated cells at 60 min of incubation. Furthermore, poultry PM induced in situ serine phosphorylation of $cPLA_2$ in a dose-dependent manner (0–1.0 mg/ml) in A549 cells at 1 h of treatment (Fig. 6b). These results confirmed that poultry PM induced in situ translocation and serine phosphorylation of $cPLA_2$ in A549 cells.

ERK-1/2 Inhibition Attenuates Poultry PM-Induced Serine Phosphorylation of $cPLA_2$ in Lung Epithelial Cells

Previous experiments have revealed that the upstream ERK-specific inhibitor (PD98059) attenuated the poultry PM-induced $cPLA_2$ activation and the poultry PM-induced upstream ERK-1/2 phosphorylation and downstream $cPLA_2$ serine phosphorylation. Therefore, here, we investigated whether the serine phosphorylation of $cPLA_2$ induced by poultry PM in A549 cells would be mediated by ERK-1/2 by means of the SDS-PAGE and Western blotting analysis of proteins and confocal immunofluorescence microscopy. As shown in Fig. 7a, b, PD98059 (10 μM) significantly attenuated the poultry PM-induced (1.0 mg/ml for 1 h) serine phosphorylation of $cPLA_2$ in A549 cells as revealed by the SDS-PAGE and Western blotting analysis of proteins. Complementary to this observation, PD98059 also attenuated the poultry PM-induced in situ serine phosphorylation of $cPLA_2$ in A549 cells under identical conditions (Fig. 7c). These results demonstrated that the poultry PM-induced $cPLA_2$ serine phosphorylation was mediated by ERK-1/2 in A549 cells.

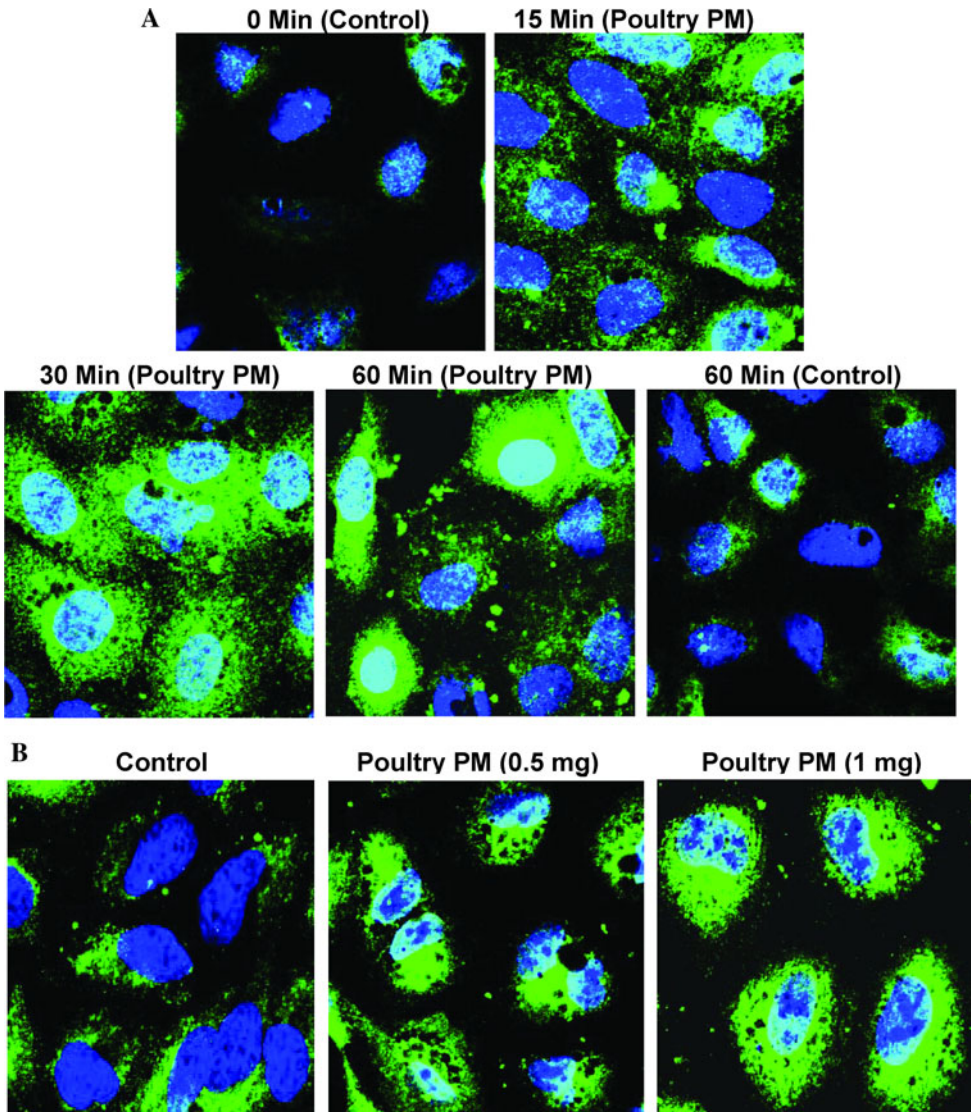


Fig. 6 Poultry PM induces translocation and serine phosphorylation of $cPLA_2$ in lung epithelial cells. A549 cells grown on glass coverslips were treated with RPMI 1640 medium alone for 60 min or RPMI 1640 medium containing poultry PM (1.0 mg/ml) for 0–60 min (**a**) or with RPMI 1640 medium alone or RPMI 1640 medium containing poultry PM (0.5 and 1.0 mg/ml) for 1 h (**b**), following which $cPLA_2$ and phosphoserine-$cPLA_2$ were localized respectively, by immunofluorescence confocal microscopy at a magnification of 63× aided by immunostaining with $cPLA_2$-specific and phosphoserine-$cPLA_2$-specific antibodies as described in the "Materials" and "Methods" sections. Each micrograph is a representative of three independent experimental observations conducted under identical conditions

DN ERK-1/2 Transient Transfection Inhibits Serine Phosphorylation of $cPLA_2$ in Lung Epithelial Cells

Using the pharmacological approach, in the previous experiments, it was demonstrated that the ERK-1/2 upstream inhibitor (PD98059) attenuated the poultry PM-induced serine phosphorylation of $cPLA_2$ in A549 cells, suggesting the ERK-mediated $cPLA_2$ serine phosphorylation. In order to further confirm the role of ERK-1/2 in mediating the poultry PM-induced serine phosphorylation of $cPLA_2$ in A549 cells, in this study, we adopted the molecular biological strategy involving the transient transfection of cells with cDNA of WT and DN ERK-1 and ERK-2 after which we investigated the role of ERK-1 and ERK-2 in the poultry PM-induced $cPLA_2$ serine phosphorylation in those cells (Fig. 8a) with the aid of SDS-PAGE and Western blotting analysis. Poultry PM (1.0 mg/ml for 1 h) significantly induced the serine phosphorylation of $cPLA_2$ in the vector-transfected control cells and WT ERK-1- and WT ERK-2-transfected cells as compared to the same in the untreated control, WT ERK-1-, and WT ERK-2-transfected A549 cells (Fig. 8b, c). Both the DN ERK-1 and ERK-2 transfections significantly attenuated the poultry PM-induced serine phosphorylation of $cPLA_2$ in A549 cells as compared to the same in the poultry PM-treated vector-, WT ERK-1-, and WT ERK-2-transfected cells under identical conditions (Fig. 8c). This molecular biological approach involving the WT and DN ERK-1 and ERK-2 transfection strategies confirmed that the poultry PM-induced $cPLA_2$ serine phosphorylation in A549 cells was mediated by both ERK-1 and ERK-2.

Poultry PM Induces ERK-1/2 Phosphorylation in Bone Marrow-Derived Macrophages of $cPLA_2$ Knockout Mice

In order to confirm that $cPLA_2$ would act as a substrate for ERK-1/2-mediated serine phosphorylation in cells, in this

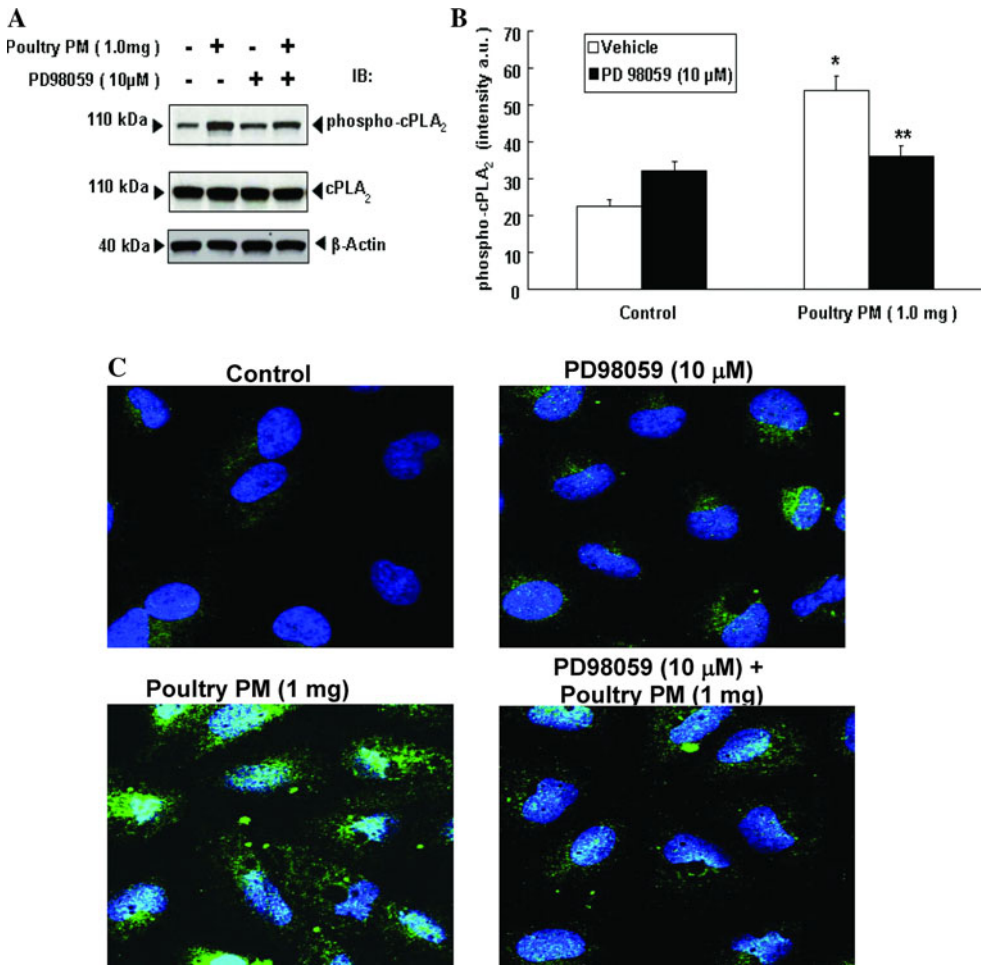


Fig. 7 ERK-1/2 inhibition attenuates poultry PM-induced serine phosphorylation of $cPLA_2$ in lung epithelial cells. A549 cells, pre-treated with RPMI 1640 medium alone or RPMI 1640 medium containing ERK-1/2 upstream inhibitor (PD98059, 10 μM) for 1 h, were treated with RPMI 1640 medium alone or RPMI 1640 medium containing poultry PM (1.0 mg/ml) for 1 h. Following treatment, proteins in cell lysates were subjected to SDS-PAGE and Western blotting with $cPLA_2$-specific and phosphoserine-$cPLA_2$-specific antibodies (**a**) and the intensities of phosphoserine-$cPLA_2$ protein bands were digitally determined as described in the "Materials" and "Methods" sections (**b**). Data represent ± SD of three independent experiments in triplicate. *Significantly different at $P < 0.05$ as compared with the control untreated cells. **Significantly different at $P < 0.05$ as compared with the poultry PM-treated cells. **c** A549 cells grown on glass coverslips were pre-treated with RPMI 1640 medium alone or RPMI 1640 medium containing ERK-1/2 upstream inhibitor (PD98059, 10 μM) for 1 h and then were treated with RPMI 1640 medium alone or RPMI 1640 medium containing poultry PM (1.0 mg/ml) for 1 h. At the end of treatments, cells were examined for in situ serine phosphorylation of $cPLA_2$ by confocal immunofluorescence microscopy at a magnification of 63× aided by immunostaining with $cPLA_2$-specific and phosphoserine-$cPLA_2$-specific antibodies as described in the "Materials" and "Methods" sections. Each micrograph is a representative of three independent experimental observations conducted under identical conditions

study, we chose the bone marrow-derived macrophages isolated from WT and $cPLA_2$ KO mice. Macrophages are also known to release IL-8 upon agonist challenge [20], and we have previously established the procedure for the isolation and culture of bone marrow-derived macrophages [18]. Therefore, in this study, we used the bone marrow-derived macrophages isolated from WT and $cPLA_2$ KO mice, exposed them to poultry PM (1 mg/ml for 30 and 60 min), and then examined the ERK-1/2 phosphorylation and $cPLA_2$ serine phosphorylation in cellular proteins using SDS-PAGE and Western blotting analysis. The results revealed that poultry PM induced significantly robust serine phosphorylation in WT cells which had constitutively expressed $cPLA_2$ (Fig. 9a, b). On the other hand, bone marrow-derived macrophages isolated from $cPLA_2$ KO mice lacked constitutively expressing $cPLA_2$ and did neither exhibit the presence of $cPLA_2$ nor the serine phosphorylation of $cPLA_2$, and the poultry PM-induced $cPLA_2$ serine phosphorylation was not detected because of the absence of substrate ($cPLA_2$) for ERK-1/2 in these cells. However, poultry PM, under the identical conditions, induced significant and robust ERK

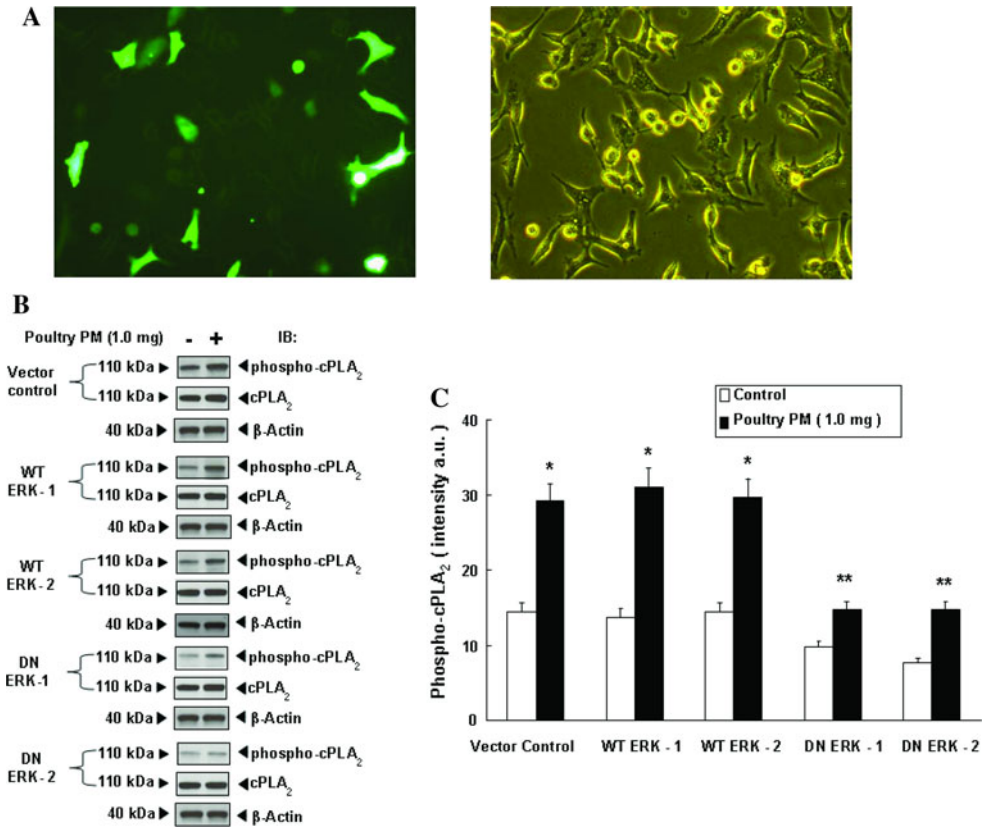


Fig. 8 DN ERK-1/2 transient transfection inhibits serine phosphorylation of $cPLA_2$ in lung epithelial cells. A549 cells, transiently transfected with Vector, WT ERK-1, WT ERK-2, DN ERK-1, and DN ERK-2 DNA (as shown by GFP fluorescence in **a** with 30–40% transfection efficiency) were treated with RPMI 1640 medium alone or RPMI 1640 medium containing poultry PM (1.0 mg/ml) for 1 h. Following treatment, proteins in cell lysates were subjected to SDS-PAGE and Western blotting with $cPLA_2$-specific and phosphoserine-$cPLA_2$-specific antibodies (**b**) and the intensities of phosphoserine-$cPLA_2$ protein bands were digitally determined (**c**) as described in the "Materials" and "Methods" sections. Each Western blot is a representative of three independent experiments conducted under identical conditions. Data represent ± SD of three independent experiments in triplicate. *Significantly different at $P < 0.05$ as compared with the control untreated cells. **Significantly different at $P < 0.05$ as compared with the poultry PM-treated cells transfected with Vector, WT ERK-1, and WT ERK-2 DNA

phosphorylation in both WT and $cPLA_2$ KO bone marrow-derived macrophages which underscored that in the absence of constitutively expressed $cPLA_2$ in the $cPLA_2$ KO bone marrow-derived macrophages, ERK-1/2 was still active and, as $cPLA_2$ (the substrate for ERK-1/2) was absent, the serine phosphorylation of $cPLA_2$ in cells exposed to poultry PM was not evident.

$cPLA_2$-Specific Inhibitor and ERK-1/2 Upstream-Specific Inhibitors Attenuate Poultry PM-Induced IL-8 Release by Lung Epithelial Cells

Previously, it was demonstrated that ERK-1/2 was involved in the poultry PM-induced activation and phosphorylation of $cPLA_2$ in A549 cells. Therefore, in this study, we investigated whether $cPLA_2$ and ERK-1/2 would be involved in the poultry PM-induced inflammatory cytokine, IL-8 release by A549 cells. We pretreated A549 cells for 1 h with the $cPLA_2$-specific inhibitor ($AACOCF_3$, 10 μM) and ERK-1/2-upstream-specific inhibitors (PD98059, 20 μM and U0126, 20 μM), then treated them with poultry PM (1.0 mg/ml for 12 h) after which IL-8 released into the medium was assayed. Poultry PM significantly induced IL-8 release by A549 cells. Both the $cPLA_2$-specific and ERK-1/2 upstream-specific inhibitors significantly attenuated the poultry PM-induced IL-8 release by A549 cells (Fig. 10a, b). These results demonstrated that both $cPLA_2$ and ERK-1/2 were involved in the poultry PM-induced IL-8 release by A549 cells.

DN ERK-1 Transient Transfection Inhibits Poultry PM-Induced IL-8 Release by Lung Epithelial Cells

Previous experiments have revealed that the ERK-specific inhibitors significantly attenuated the poultry PM-induced IL-8 release by A549 cells. In order to further confirm the role of ERK-1/2 in mediating the poultry PM-induced IL-8 release by A549 cells, in this study, we adopted the molecular

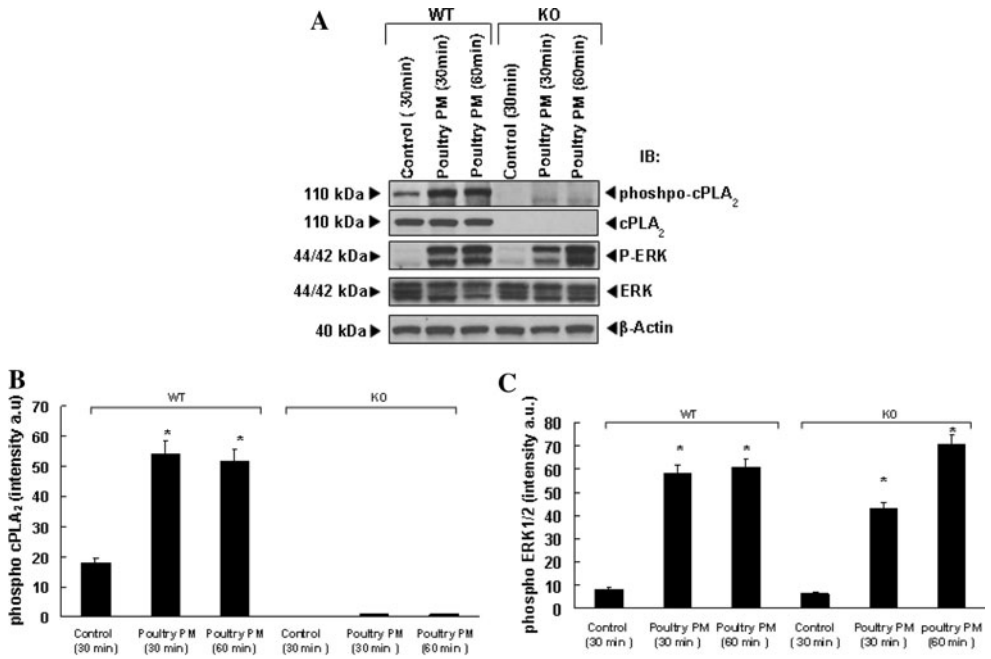


Fig. 9 Absence of $cPLA_2$ and serine phosphorylation of $cPLA_2$ in poultry PM-treated bone marrow-derived macrophages of $cPLA_2$ knockout mice exhibiting ERK-1/2 phosphorylation. Bone-derived macrophages, obtained from WT and $cPLA_2$ knockout (KO) animals, were treated with RPMI 1640 medium alone or RPMI 1640 medium containing poultry PM (1.0 mg/ml) for 30 and 60 min. Following treatment, proteins in cell lysates were subjected to SDS-PAGE and Western blotting with $cPLA_2$-specific, phosphoserine-$cPLA_2$-specific antibodies, and phospho-ERK-1/2 antibodies (**a**) and the intensities of phosphoserine-$cPLA_2$ (**b**) and phospho-ERK-1/2 (**c**) protein bands were digitally determined as described in the "Materials" and "Methods" sections. Each Western blot is a representative of three independent experiments conducted under identical conditions. Data represent ± SD of three independent experiments in triplicate. *Significantly different at $P < 0.05$ as compared with the control untreated cells

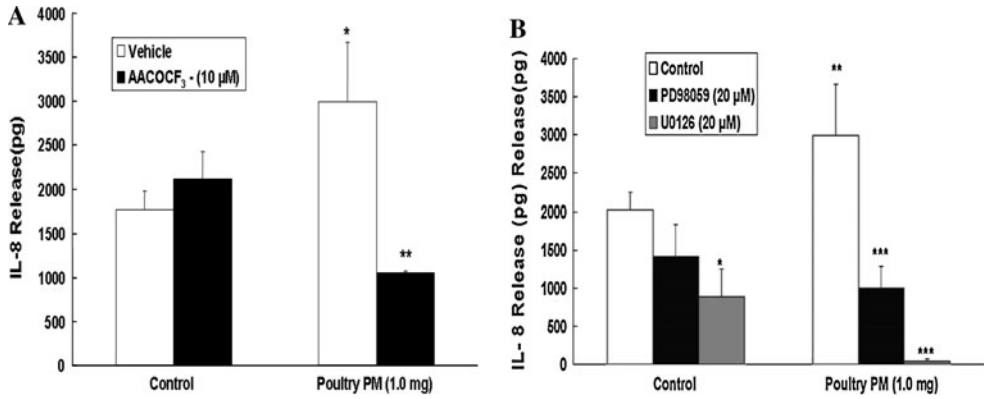


Fig. 10 $cPLA_2$-specific inhibitor and ERK-1/2 upstream-specific inhibitors attenuate poultry PM-induced IL-8 release by lung epithelial cells. A549 cells, in absence or presence of $cPLA_2$-specific inhibitor ($AACOCF_3$, 0.1 μM) (**a**) or ERK-1/2 upstream-specific inhibitors (PD90859 or U0126, 20 μM) (**b**) were treated with RPMI 1640 medium alone or RPMI 1640 medium containing poultry PM (1.0 mg/ml) for 24 h. At the end of treatment, IL-8 released into the medium was determined by ELISA as described in the "Materials" and "Methods" sections. Data represent ± SD of three independent experiments in triplicate. *Significantly different at $P < 0.05$ as compared with the control untreated cells. **Significantly different at $P < 0.05$ as compared with the control untreated cells. ***Significantly different at $P < 0.05$ as compared with the poultry PM-treated cells

biological approach involving the transient transfection of cells with cDNA of WT and DN ERK-1 and ERK-2, after which we studied the roles of ERK-1 and ERK-2 in the poultry PM-induced IL-8 release in those cells (Fig. 8a). Poultry PM (1.0 mg/ml for 12 h) significantly induced more IL-8 release in the WT ERK-1-transfected cells as compared to the same in the vector-transfected control cells, whereas, in the WT ERK-2-transfected cells, IL-8 release was higher as compared to the same in the vector-transfected control cells; in comparison to the WT ERK-1-transfected cells, the

extent of IL-8 release was lower (Fig. 11). On the other hand, the poultry PM-induced IL-8 release by the DN ERK-1-transfected cells was significantly lower as compared to the same in both the vector- and WT ERK-1-transfected cells. DN ERK-2 transfection did not appear to alter the poultry PM-induced IL-8 release by A549 cells as compared to the same in the vector-transfected control cells (Fig. 11). In contrast, the poultry PM-induced IL-8 release by DN ERK-2-transfected cells did not significantly change as compared with the same in WT ERK-2-transfected cells. These results demonstrated that ERK-1 was involved in the poultry PM-induced IL-8 release by A549 cells.

Absence of $cPLA_2$ Attenuates Poultry PM-Induced IL-8 Release by Bone Marrow-Derived Macrophages from $cPLA_2$ Knockout Mice

So far, this study has revealed that poultry PM induced $cPLA_2$ activation through upstream regulation by ERK-mediated serine phosphorylation of $cPLA_2$ which led to downstream IL-8 release by A549 cells. Previously, it was shown that poultry PM failed to induce $cPLA_2$ serine phosphorylation in the bone marrow-derived macrophages which were lacking constitutively expressed $cPLA_2$ isolated from the $cPLA_2$ KO mice. Using the bone marrow-derived macrophages from the $cPLA_2$ KO mice which lacked constitutively expressed $cPLA_2$, in this study, we investigated the role of $cPLA_2$ in the poultry PM-induced IL-8 release by the immunoactive cells. Poultry PM (0.1 mg/ml for 24 h) caused a significant and robust induction of IL-8 by the WT bone marrow-derived macrophages as compared to the same in the control, untreated cells. On the other hand, the poultry PM-induced IL-8 release by the $cPLA_2$ KO bone marrow-derived macrophages was significantly attenuated as compared to the same in the WT cells under identical conditions. These results confirmed the role of $cPLA_2$ in the poultry PM-induced IL-8 release by the bone marrow-derived macrophages.

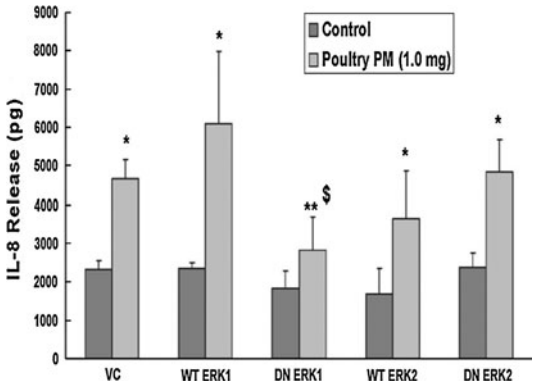


Fig. 11 DN ERK-1 transient transfection inhibits poultry PM-induced IL-8 release by lung epithelial cells. A549 cells, transiently transfected with Vector, WT ERK-1, WT ERK-2, DN ERK-1, and DN ERK-2 DNA (30–40% transfection efficiency) were treated with RPMI 1640 medium alone or RPMI 1640 medium containing poultry PM (1.0 mg/ml) for 24 h. At the end of treatments, IL-8 released into the medium was determined by ELISA as described in the "Materials" and "Methods" sections. Data represent ± SD of three independent experiments in triplicate. *Significantly different at $P < 0.05$ as compared with the control untreated cells. **Significantly different at $P < 0.05$ as compared with the poultry PM-treated cells

Discussion

As the mechanisms of the poultry PM-induced respiratory inflammation are not known, this study focused on the regulation of $cPLA_2$ activation and its role in the release of inflammatory cytokine, IL-8, by the human lung epithelial cells in culture. The results of this study demonstrated that poultry PM induced the upstream $cPLA_2$ activation through ERK-mediated serine phosphorylation of the enzyme which subsequently regulated downstream release of IL-8 by A549 cells.

Agricultural PM has been implicated in pulmonary disorders and several occupational respiratory diseases [2]. Advances in the understanding of pathophysiology of airway diseases offer mechanisms of acute and chronic inflammation as related to the diseases [21]. Inflammation of airways is an initial response in organic PM-induced lung diseases [2], characterized by the release of eicosanoids and cytokines. An overproduction of the lipid mediators has been shown in inflammation and tissue disorders [22]. Inflammatory cytokines such as IL-8 are important players in mediating inflammation, and respiratory epithelial cells respond to different types of agonists including the agricultural PM [9, 20, 23]. There are three broad classes of PLA_2s in mammalian cells: secretory ($sPLA_2$), $cPLA_2$, and calcium-independent PLA_2 ($iPLA_2$) [24]. $cPLA_2$ has been implicated in lung pathophysiology and is a key regulatory enzyme in the eicosanoid synthesis [11, 25, 26].

Previously, we have shown that the poultry PM-induced IL-8 release is mediated by upstream activation of $cPLA_2$ and COX- and LOX-generated eicosanoids in A549 cells. In this study, we further showed the regulation of poultry PM-induced $cPLA_2$ activation in A549 cells through the upstream activation of ERK1/2- and the ERK1/2-mediated serine phosphorylation of $cPLA_2$. Studies suggest that $cPLA_2$ is a receptor-regulated enzyme involved in the inflammatory response [27]. $cPLA_2$ activation is regulated by multiple mechanisms including phosphorylation of serine residues and Ca^{2+} concentration [28]. Calmodulin-dependent protein kinase (CaM kinase) is thought to regulate the phosphorylation of serine in type IIα $cPLA_2$ and the subsequent AA

release [29]. MAP kinases are serine–threonine-specific protein kinases that include ERK1/2, JUNk, and p38MAPK [30]. $cPLA_2$ is a known cytoplasmic substrate for ERK1/2, and this study revealed that poultry PM caused ERK1/2 phosphorylation (activation) which in turn resulted in $cPLA_2$ serine phosphorylation. ERK1/2 has been shown to regulate $cPLA_2$ activation through serine phosphorylation involving the translocation of $cPLA_2$ to the membrane [28]. $cPLA_2$ is phosphorylated at multiple serine residues [22, 31] of which both S505 and S515 are important for AA release by vascular smooth muscle cells [32]. The results of this study were also in agreement with the previous reports made on the ERK-mediated serine phosphorylation associated with the regulation of $cPLA_2$ and suggested that $cPLA_2$ acted as a substrate for ERK1/2 in the poultry PM-exposed A549 cells.

Allergen-induced airway responses show that $cPLA_2$ is an important effector of airway hypereactivity in mouse which may be due to alterations in the downstream products of phospholipid metabolism [25]. Coal dust induces IL-6 release from mouse epidermal cells, JB6 cells, and A549 cells which may involve ERK and p38 MAPK pathways [33]. Endotoxin in organic dusts stimulates the phosphorylation of ERK1/2, p38MAPK, and JNK in airway epithelial cells and thereby leads to the downstream release of cytokines [34]. The results of this study concurred with the reported findings that poultry PM-induced IL-8 release in A549 cells was regulated upstream by ERK1/2.

Environmental agents, including the agricultural PM, have been shown to induce IL-8 secretion by different cellular models. IL-8, a common indicator for pulmonary inflammation, is used in bioassays to assess the potency of organic dust-induced respiratory inflammation [35, 36]. Swine dust is a potent stimulus for IL-8 release in lung epithelial cells and alveolar macrophages [37]. However, the role of $cPLA_2$ in the poultry PM-induced secretion of IL-8 by A549 cells has not been reported so far. Using the pharmacological inhibitors and the molecular-biological approaches, this study offered evidence showing that $cPLA_2$ activation through upstream activation of ERK1/2, in a concerted fashion, played an important role in the downstream release of IL-8 by lung epithelial cells. Although the bone marrow-derived macrophages from $cPLA_2$ KO mice used in this study were different from A549 cells, they served as an appropriate alternative model to confirm the role of $cPLA_2$ in the regulation of the poultry PM-induced IL-8 release in immunoactive cells such as lung epithelial cells. Although ERK1/2-mediated phosphorylation of $cPLA_2$ appeared to play a regulatory role in the poultry PM-induced IL-8 release in A549 cells, ERK1/2 independently could also regulate the release of IL-8. This was substantiated by our current findings that ERK1/2-specific inhibitors (PD98059 and U0126) were more effective in attenuating the poultry PM-induced IL-8 release in A549 cells as compared to the same caused by the $cPLA_2$-specific inhibitor ($AACOCF_3$) (Figs. 10, 11). Furthermore, this study also revealed that the poultry PM-induced IL-8 release by the bone marrow-derived macrophages from the $cPLA_2$ KO mice was significantly attenuated up to 40% as compared to the same in their counterparts obtained from the WT animals (Fig. 12). This study also suggested the concerted signaling actions of $cPLA_2$ and ERK in the regulation of the poultry PM-induced IL-8 release in A549 cells. In addition, ERK1/2 and $cPLA_2$ independently could have contributed to the poultry PM-induced IL-8 release by A549 cells which is the subject of further study in detail.

$cPLA_2$ is also known to be involved in transport and secretion [10]. Hence, $cPLA_2$ could regulate the secretion of IL-8 very well through either protein–protein interactions or free AA-mediated membrane phenomenon. Previously, we have shown that COX- and LOX-specific inhibitors effectively attenuate the poultry PM-induced IL-8 release in A549 cells [9]. Therefore, it is reasonable to surmise that the COX- and LOX-generated eicosanoids from $cPLA_2$-released AA from membrane phospholipids could also be among the potential players in causing the poultry PM-mediated IL-8 release by A549 cells. $cPLA_2$ has also been localized in mammalian cell nucleus [38, 39] including the A549 cells. This signifies the transcriptional regulatory activity of $cPLA_2$ [40]. Hence, $cPLA_2$ could also have played a role in

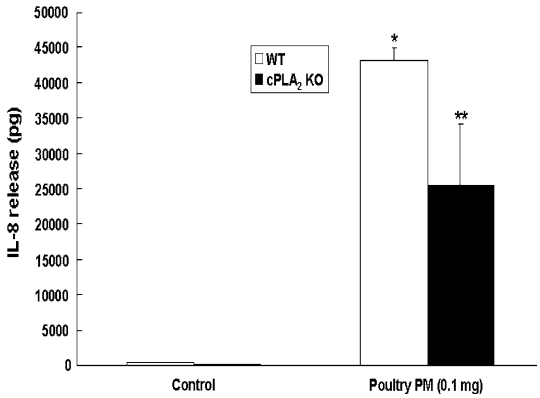


Fig. 12 Absence of $cPLA_2$ attenuates poultry PM-induced IL-8 release by bone marrow-derived macrophages from $cPLA_2$ knockout mice. Bone marrow-derived macrophages, obtained from WT and $cPLA_2$ knockout (KO) animals, were treated with RPMI 1640 medium alone or RPMI 1640 medium containing poultry PM (0.1 mg/ml) for 24 h. At the end of treatment, IL-8 released into the medium was determined by ELISA as described in the "Materials" and "Methods" sections. Data represent ± SD of three independent experiments in triplicate. *Significantly different at $P < 0.05$ as compared with the control untreated cells. **Significantly different at $P < 0.05$ as compared with the poultry PM-treated cells

the poultry PM-induced IL-8 release by the A549 cells through transcriptional regulation.

For the first time, this study demonstrated the concerted signaling actions of ERK1/2 and $cPLA_2$ in the regulation of poultry PM-induced IL-8 release by human lung epithelial cells through the serine phosphorylation of the enzyme. Second, this study also suggested the interdependent regulation of two major pools of inflammatory mediators such as eicosanoids and IL-8. Overall, the mechanism of regulation of $cPLA_2$-controlled eicosanoid generation by COX/LOX and IL-8 release could offer insights into effective therapeutic interventions of agricultural PM-induced respiratory and lung disorders.

Acknowledgments This study was supported by funds from the NIOSH (ROI OH008539), the NIH (HL40871), the Division of Pulmonary, Allergy, Critical Care, and Sleep Medicine, and Dorothy M. Davis Heart and Lung Research Institute, The Ohio State University College of Medicine.

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