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Deficiency of Phospholipase A2 Receptor Exacerbates Ovalbumin-Induced Lung Inflammation

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Secretory phospholipase A2 (sPLA2) plays a critical role in the genesis of lung inflammation through proinflammatory eicosanoids. A previous in vitro experiment showed a possible role of cell surface receptor for sPLA2 (PLA2R) in the clearance of extracellular sPLA2. PLA2R and groups IB and X sPLA2 are expressed in the lung. This study examined a pathogenic role of PLA2R in airway inflammation using PLA2R-deficient (PLA2R−/−) mice. Airway inflammation was induced by immunosensitization with OVA. Compared with wild-type (PLA2R+/+) mice, PLA2R−/− mice had a significantly greater infiltration of inflammatory cells around the airways, higher levels of groups IB and X sPLA2, eicosanoids, and Th2 cytokines, and higher numbers of eosinophils and neutrophils in bronchoalveolar lavage fluid after OVA treatment. In PLA2R−/− mice, intratracheally instilled [125I]-labeled sPLA2-IB was cleared much more slowly from bronchoalveolar lavage fluid compared with PLA2R+/+ mice. The degradation of the instilled [125I]-labeled sPLA2-IB, as assessed by trichloroacetic acid-soluble radioactivity in bronchoalveolar lavage fluid after instillation, was lower in PLA2R−/− mice than in PLA2R+/+ mice. In conclusion, PLA2R deficiency increased sPLA2-IB and -X levels in the lung through their impaired clearance from the lung, leading to exaggeration of lung inflammation induced by OVA treatment in a murine model. The Journal of Immunology, 2013, 191: 1021–1028.

I t has been shown that eicosanoids, including leukotrienes (LT) and PGs, have crucial roles in the pathogenesis of airway inflammation (1–4). These eicosanoids are 5-lipoxygenase and cyclooxygenase metabolites of arachidonic acids, and the cellular availability of arachidonic acid is tightly controlled by hydrolysis of membrane phospholipids via the catalytic activity of phospholipases A2 (PLA2) (5–10). Previous animal and human studies showed that PLA2 is involved in the genesis of airway inflammation through proinflammatory eicosanoids (11–15).

A number of PLA2 subtypes have been identified and classified into different families based on their biochemical features and primary structures (5–10, 16). Mammalian PLA2 enzymes are classified into three main categories: intracellular cytosolic PLA2 (cPLA2), Ca2+-independent PLA2 (iPLA2), and secretory PLA2 (sPLA2), comprising 6 cPLA2, 9 iPLA2 and 11 sPLA2 (5–10, 16). sPLA2 possess several characteristic features, including a low molecular mass (typically, 13–18 kDa) and an absolute catalytic requirement for millimolar concentrations of Ca2+ (5–10, 16).

Two decades ago, a cell surface receptor for sPLA2-IB (phospholipase A2 receptor 1; PLA2R) was discovered (17). PLA2R, a so-called M-type PLA2 receptor, is a type I transmembrane glycoprotein with a molecular mass of 180 kDa and is composed of a large extracellular portion consisting of an N-terminal cysteine-rich region, a fibroconnectin-like type II domain, a tandem repeat of eight carbohydrate-recognition domains (CRDs) and, a short intracellular C-terminal region (10, 16, 18, 19). Three of the CRD-like domains (CRDs 3 to 5) are responsible for sPLA2 binding (10, 16, 18, 19). Among sPLA2 isozymes, mouse PLA2R has a high affinity to sPLA2-IB, -IIA, -IIE, -IIIF, and -X (18, 19). The intracellular C-terminal region contains a consensus sequence motif of coated pit-mediated-endocytosis that was originally identified in the low-density lipoprotein receptor (18–21). Previous in vitro studies have demonstrated that sPLA2-IB and S1A2-X are internalized and degraded via the lysosomal pathway after their binding to PLA2R, implicating a possible role of the receptor in the clearance of extracellular sPLA2 (18–22). sPLA2-IB and S1A2-X are expressed in the lung (11, 12, 23). The animal study using sPLA2-X–deficient mice showed that sPLA2-X produced proinflammatory eicosanoids and Th2 cytokines and played a critical role in genesis of allergen-induced airway inflammation (12). The human study indicated that sPLA2-X could have a role in the generation of proinflammatory eicosanoids in the airways, leading to the development of bronchial hyperresponsiveness (11). In addition, S1A2-IB was found to elicit the production of proinflammatory eicosanoids in the lung and the contraction of airway smooth muscles (24).
the pathogenic role of PLA2R in OVA-induced airway inflammation using PLA2R-deficient mice.

Materials and Methods

Materials

Rat monoclonal anti-PLA2R Ab was prepared as described in a previous report (25). Rabbit polyclonal anti-smooth muscle α-actin (αSMA) Ab and rabbit polyclonal anti-cytokeratin Ab were purchased from Abcam (Cambridge, U.K.). Recombinant mouse sPLA2-X was prepared as described previously (22). Recombinant human sPLA2-IB was obtained from R&D systems (Minneapolis, MN). Sodium [125I]-iodine (carrier-free, 3.7 GBq/ml) was purchased from Perkin Elmer (Boston, MA). ELISA kits for determination of IL-4 and IL-5 levels were obtained from R&D systems: the ELISA kit for IFN-γ was obtained from Raybiotech (Norcross, GA); the ELISA kits for LTB4, cysteinyl leukotrienes (CysLTs), PGD2, and PGE2 were obtained from Cayman Chemical (Ann Arbor, MI); the ELISA kit for 15-deoxy-Δ12,14-PGJ2 (15d-PGJ2) was obtained from Enzo Life Science (Farmingdale, NY); and ELISA kits for sPLA2-IB and sPLA2-X were obtained from Cusabio Biotech (Wuhan, China). Cell culture reagents were obtained from Sigma-Aldrich (Tokyo, Japan) and Invitrogen (Carlsbad, CA). Other chemicals were purchased from Sigma-Aldrich unless indicated otherwise.

Mice

The experimental protocol was approved by the University of Yamanashi Animal Care and Use Committee (approval reference no. 19-35), and procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (8th Edition, 2011). Details regarding the generation and characterization of PLA2R−/− mice (systemically deficient in PLA2R) were described previously (25, 26). PLA2R−/− female mice with a C57BL/6J background were randomly selected from five litters (two upper lobes and three lower lobes) per mouse and examined at a magnification of ×400. Each mouse had a score determined by the mean of the individual scores of 25 airs.

Immunostaining of lungs and cultured cells

The lung was fixed with 50% OCT (Tissue-Tek; Sakura Finetek, Tokyo, Japan) in PBS through the endotracheal tube. Thereafter, lungs were harvested, transferred, embedded in OCT, frozen, cut into 5-μm sections, and placed on slides. Histologic examination of the lung was determined using a dissecting microscope (23).

Lung histology

The trachea was exposed and catheterized as described before. Next, paraformaldehyde (4%) in PBS was instilled into the lung via the endotracheal tube with the use of a syringe at low pressure. Upper and lower lobes of the left lung were removed and fixed for 24 h in 4% paraformaldehyde. The tissues were embedded in paraffin, cut in 7-μm sections, and placed on slides. Histologic examination of the lung was determined by staining with H&E and periodic acid–Schiff (PAS). Peribronchial infiltration of inflammatory cells was graded on a semiquantitative scale, from 0 to 4; grade 0 was designated as no detectable inflammation; grade 1 was given when the bronchus was surrounded by a few inflammatory cells; grade 2 was assigned when the bronchus was surrounded by a layer one cell deep; grade 3 was given when the bronchus was surrounded by a layer two to four cells deep; grade 4 was assigned when the bronchus was surrounded by a layer more than four cells deep. The number of PAS-positive mucus-containing cells was determined as the percentage of total airway epithelial cells in each airway examined. For lung morphology, five mice per section were randomly selected from five sections (from upper two lobes and three lower lobes) per mouse and examined at a magnification of ×400. Each mouse had a score determined by the mean of the individual scores of 25 airs.

In vivo clearance of sPLA2 from the lungs

Iodination of sPLA2-IB was performed with PD-10 columns (GE Healthcare, Amersham, U.K.) (25), yielding a specific radioactivity of 500 cpm/fmol. After anaesthesia by i.p. injection of 130 mg/kg ketamine and 8.8 mg/kg xylazine, the trachea was exposed and catherized with a polypropylene 24-gauge endotracheal tube. [125I]-Labeled sPLA2-IB (150 ng) was instilled into the lung through the endotracheal tube. After the tracheal instillation, the cervical incision was closed with a 5-0 silk suture, and the mice were returned to their cages. All treated mice recovered rapidly after surgery. After anaesthesia with ketamine and xylazine as described earlier, the lungs were lavaged to collect BALF 5, 30, or 60 min or 2 and 4 h after the instillation. BALF was spun at 300 × g for 10 min at −20°C. The collected BALF was placed on slides. Nuclei were stained with DAPI (Vector Laboratories). Images were acquired with an Olympus Fluoview 1000 confocal microscope (Milton Keynes, UK). The lung sections were incubated with normal rabbit, mouse, or goat IgG instead of primary Ab as negative controls. For double immunofluorescence staining of the lung, the sections were incubated with the indicated primary Abs followed by a secondary Ab with Alexa Fluor 488 or Alexa Fluor 647 (Invitrogen, Carlsbad, CA) (23).

In vitro clearance of sPLA2 from the lungs

Induction of allergic airway inflammation with OVA treatment

Mice were treated with i.p. injection of 10 μg of chicken egg OVA (Sigma-Aldrich) and 1.125 mg of adjuvant aluminum phosphate (Sigma-Aldrich) emulsified in 0.2 ml of sterile saline at days 0, 7, and 14. On days 21 to 23, mice were treated with intranasal administration of 50 μg of OVA in 0.05 ml saline after anaesthesia with i.p. injection of 130 mg/kg ketamine and 8.8 mg/kg xylazine. Control groups were treated with saline instead of OVA, otherwise similar to that described above. On day 24, the mice were euthanized by cutting the left renal artery after anesthesia with i.p. injection of 10% trichloroacetic acid (TCA), and the TCA-soluble radioactivity was measured.

Absys of cytokines, eicosanoids, and sPLA2

The concentrations of IL-4, IL-5, IFN-γ, LTB4, CysLTs, PGD2, sPLA2-IB, and sPLA2-X in BALF were measured using ELISA kits according to the manufacturer’s instructions. The plasma concentrations of sPLA2-IB and sPLA2-X were also measured. The enzymatic activity of sPLA2 in the culture medium was assayed using the sPLA2 Assay Kit (Cayman Chemical) exactly as recommended by the manufacturer. The eicosanoid levels in the culture medium of ASM cells and A549 cells (DS Pharma Biological, Osaka, Japan), a cell line of human alveolar basal epithelial cells, after incubation with or without sPLA2-IB were measured using ELISA kits. Expression levels of mRNA of cytokines in cultures of ASM cells and A549 cells were measured with real-time PCR.
Real-time PCR
Total RNA was extracted from the lungs or cultures of cells with a Qiagen RNaseq kit and DDNase I (Qiagen, Hilden, Germany). The mRNA expression levels were quantified with a two-step real-time PCR assay using SYBR Green I chemistry and a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA). The PCR primers are listed in Supplemental Table I. The GAPDH housekeeping gene was used to normalize gene expression.

Cultures of bone marrow-derived mast cells, Th2 cells, and ASM cells
Bone marrow–derived mast cells (BMMCs) were obtained by cultivation of bone marrow cells in RPMI 1640 medium containing IL-3 (5 ng/ml; Peprotech, Rocky Hill, NJ) and 10% FBS (27). After 3 wk, staining with May-Giemsa and toluidine blue showed that >95% of the cells were BMMCs. Th2 cells were generated from CD4+ T cells (3). CD4+ T cells were incubated with 5 nM [125I]-labeled sPLA2-I B for 2.5 h at 4˚C. [125I]-labeled sPLA2-I B cells were isolated using MACS (Miltex Biotech, Bergisch Gladbach, Germany) from spleen cells and activated by plating on T cell Activation Plates (BD Biosciences, San Jose, CA) in RPMI 1640 containing IL-2 (20 ng/ml), IL-4 (20 ng/ml; Peprotech), and anti–INF-γ Ab (10 µg/ml; Abcam). After 6 d of culture, Th2 cells were isolated by positive selection using a mouse IL-4 Secretion Assay (Miltex Biotech). ASM cells were cultured from explants of tracheas. The entire tracheas were split longitudinally and dissected into 2-3-mm squares. All segments were placed in a sterile Petri dish with DMEM containing 10% FBS and 100 µg/ml penicillin/streptomycin. The explants were incubated in a 5% CO2 chamber at 37˚C. Explanted tracheas were removed when the outgrowing cells became locally confluent. Once the dish became confluent, the cells were passaged. All examinations were performed on confluent cells at matched passage numbers (3 to 6). The immunohistochemical analysis showed that >95% of these cells were αSMA-positive cells.

Production of PGD2 and Th2 cytokines in cultures of BMMCs and Th2 cells
This study examined whether the constituent cells of the inflamed lung were capable of producing PGD2 and Th2 cytokines in response to sPLA2-IB. BMMCs were stimulated with sPLA2-IB. After 30 min, the levels of PGD2 in the culture medium were measured using ELISA kits (Cayman Chemical). Th2 cells were stimulated by various concentration of PGD2. After 4 h, the levels of IL-4 and IL-5 in the culture medium were measured using ELISA kits (R&D Systems).

Internalization and degradation of sPLA2-IB in cultured ASM cells
Cultures of ASM cells on 24-well culture plates were incubated for 2.5 h at 37˚C with 5 nM [125I]-labeled sPLA2-IB in binding medium (HBBS containing 0.1% BSA) in the absence or presence of a 50-fold excess of unlabeled sPLA2-IB (22). The supernatants were removed, and the cells were treated with an acidic buffer (50 mM glycine, 0.1 M NaCl, pH 3.0) for 10 min at 4˚C. After washing with PBS, the cells’ associated radioactivity was measured after solubilization of the cells with 1 N NaOH by a γ counter. For the assessment of sPLA2-IB degradation, the cells were incubated with 5 nM [125I]-labeled sPLA2-IB for 2.5 h at 4˚C, washed with PBS, resuspended in binding medium, and incubated for the indicated times at 37˚C (22). At the end of incubation, the supernatants were removed and precipitated with 10% TCA, and the TCA-soluble radioactivity was measured with a γ counter. To examine the effects of PLA2-R on the enzymatic activity of sPLA2-IB, added to the culture medium, ASM cells in 24-well culture plates were incubated with mouse sPLA2-X (3 µg/ml) (22) at 37˚C for the indicated times. After incubation, the supernatant was collected by centrifugation of the culture medium at 1000 × g for 5 min at 4˚C, and the enzymatic activity of sPLA2-X in the supernatant was measured. Among sPLA2-IB isozymes with a high affinity for PLA2-R, sPLA2-X was selected because of its potent enzymatic activity (6).

Statistical analysis
Unless otherwise stated, all data are expressed as means ± SE. Statistical comparisons of responses were performed using Student t test. When more than two groups were compared, a one-way ANOVA was performed followed by a Scheff test for post hoc comparison of group means. The serial changes were compared between two genotyped mice using two-way ANOVA for repeated measures followed by post hoc testing with a Scheff test. The results were accepted as significantly different when p < 0.05.

Results
Histologic analysis of OVA-induced airway inflammation
Compared with saline-treated mice, both PLA2R−/− and wild-type (PLA2R+/+) mice after OVA treatment showed an infiltration of inflammatory cells around the airways and blood vessels and hypersecretion of mucus in the airways (Fig. 1A). These histologic changes in the lung after OVA treatment were greater in PLA2R−/− mice than in PLA2R+/+ mice (Fig. 1B, 1C).

Expression of PLA2R in PLA2R+/+ lung
In PLA2R+/+ mice, the immunohistochemical analysis revealed strong immunoreactivity of PLA2R in the circumference of airway and faintly in the alveolar wall in the lung after OVA treatment (Fig. 2A–D). The negative control staining had no immunoreactivity to PLA2R (data not shown). Confocal immunofluorescence microscopy showed that PLA2R immunoreactivity was colocalized in airway smooth muscle expressing αSMA and in alveolar epithelial cells expressing cytokeratin in PLA2R+/+ lung (Fig. 2E–L).

Comparison of mRNA expression levels of PLA2-R and sPLA2 in the lungs and the number of inflammatory cells and levels of sPLA2, eicosanoids, and Th2 cytokines in BALF after treatment with saline or OVA between PLA2R+/+ and PLA2R−/− mice
In PLA2R−/− mice, mRNA levels of PLA2-R in lung tissue were not changed significantly after OVA treatment compared with saline treatment (Fig. 3A). There were no significant differences in the mRNA expression levels of sPLA2-IB, −IIIB, and −X in lung tissue after OVA treatment between the two genotyped mice (Fig. 3B–D). sPLA2-IIIB expression was not detectable in OVA-treated lung in the two genotyped mice (data not shown). Compared with saline treatment, BALF after OVA treatment showed an increase in the number of eosinophils, neutrophils, and lymphocytes, the levels of sPLA2-IB and −X, eicosanoids (LTB4, CysLTs, and PGD2), Th2 cytokines (IL-4 and IL-5) in both PLA2R−/− and PLA2R+/+ mice (Fig. 3E–N). These increased levels in BALF after OVA treatment were greater in PLA2R−/− lungs than in PLA2R+/+ lungs (Fig. 3E–N). IFN-γ levels in BALF were not changed significantly after OVA treatment, and the IFN-γ levels were similar between PLA2R−/− mice and PLA2R+/+ mice after treatment with saline or OVA (Fig. 3O). There was no detectable plasma levels of sPLA2-IB and −X in both PLA2R−/− and PLA2R+/+ mice after treatment with either saline or OVA (data not shown).

In vivo clearance of sPLA2-IB instilled into the lungs
To determine the in vivo kinetics of sPLA2 clearance from the lungs, [125I]-labeled sPLA2-IB was instilled intratracheally into the lung. In PLA2R−/− mice, the instilled [125I]-labeled sPLA2-IB was cleared much more slowly from the BALF, as 50% of [125I]-labeled sPLA2-IB remained after 120 min. Four hours after instillation, degradation of sPLA2-IB in cultures of ASM cells
Cultures of ASM cells on 24-well culture plates were incubated for 2.5 h at 37˚C with 5 nM [125I]-labeled sPLA2-IB in binding medium (HBBS containing 0.1% BSA) in the absence or presence of a 50-fold excess of unlabeled sPLA2-IB (22). The supernatants were removed, and the cells were treated with an acidic buffer (50 mM glycine, 0.1 M NaCl, pH 3.0) for 10 min at 4˚C. After washing with PBS, the cells’ associated radioactivity was measured after solubilization of the cells with 1 N NaOH by a γ counter. For the assessment of sPLA2-IB degradation, the cells were incubated with 5 nM [125I]-labeled sPLA2-IB for 2.5 h at 4˚C, washed with PBS, resuspended in binding medium, and incubated for the indicated times at 37˚C (22). At the end of incubation, the supernatants were removed and precipitated with 10% TCA, and the TCA-soluble radioactivity was measured with a γ counter. To examine the effects of PLA2-R on the enzymatic activity of sPLA2-IB, added to the culture medium, ASM cells in 24-well culture plates were incubated with mouse sPLA2-X (3 µg/ml) (22) at 37˚C for the indicated times. After incubation, the supernatant was collected by centrifugation of the culture medium at 1000 × g for 5 min at 4˚C, and the enzymatic activity of sPLA2-X in the supernatant was measured. Among sPLA2-IB isozymes with a high affinity for PLA2-R, sPLA2-X was selected because of its potent enzymatic activity (6).

Statistical analysis
Unless otherwise stated, all data are expressed as means ± SE. Statistical comparisons of responses were performed using Student t test. When more than two groups were compared, a one-way ANOVA was performed followed by a Scheff test for post hoc comparison of group means. The serial changes were compared between two genotyped mice using two-way ANOVA for repeated measures followed by post hoc testing with a Scheff test. The results were accepted as significantly different when p < 0.05.
incubation at 37˚C was significantly lower in cultures of ASM cells from PLA2R^2/2 lung compared with those from PLA2R^+/+ lung (Fig. 4D). The residual enzymatic activity of sPLA2-X exogenously added to the culture medium gradually decreased in cultures of ASM cells from PLA2R^+/+ lung, whereas the sPLA2-X activity was barely changed in those from PLA2R^2/2 lung (Fig. 4E).

**FIGURE 1.** Comparison of histological changes in the lung after OVA-treatment between PLA2R^2/2 and PLA2R^+/+ mice. (A) Representative pictures of H&E (upper panels) or PAS (lower panels) staining of lungs after OVA treatment. Scale bars in H&E staining, 200 μm; scale bars in PAS staining, 50 μm. Sections are representative of five mice. (B) Semiquantitative analysis of the severity of peribronchial inflammation. Peribronchial inflammation was scored on a scale of 0–4; grade 0 was designated as no detectable inflammation, grade 1 was given when the bronchus was surrounded by a few inflammatory cells; grade 2 was assigned when the bronchus was surrounded by a layer one cell deep; grade 3 was given when the bronchus was surrounded by a layer 2-4 cells deep; grade 4 was assigned when the bronchus was surrounded by a layer more than four cells deep. (C) The number of PAS-positive, mucus-containing cells, expressed as a percentage of total airway epithelial cells in each airway. In each mouse, 25 airways per mouse were randomly selected and examined at a magnification of ×400. n = 7 mice in each experiment. *p < 0.05, †p < 0.05 compared with the respective genotype after saline treatment.+/+ denotes PLA2R^+/+ mice and ^2/2 denotes PLA2R^2/2 mice.

**FIGURE 2.** Expression of PLA2-R in the lungs after OVA-treatment. (A–D) Representative immunostaining images of lungs from PLA2R^+/+ mice after treatment with saline (A) or OVA (C) and lung from PLA2R^2/2 mice after saline (B) or OVA treatment (D) using an Ab against PLA2-R (brown). (F–H and J–L) Representative immunofluorescence images of lung from PLA2R^+/+ mice after OVA treatment using Abs against PLA2-R (F and J, green), αSMA (G, red), PLA2-R and αSMA (H), cytokeratin (K, red) or PLA2-R and cytokeratin (L). (E and I) H&E staining of lung (blue rectangular area) corresponding to that in images of F–H and J–L, respectively. Scale bars in panels (A)–(D), (E), (F)–(H), (I), and (J)–(L), were 50, 200, 20, 200, and 20 μm, respectively. Sections are representative of five mice.
Production of PGD$_2$ and Th2 cytokines in BMMCs and Th2 cells

This study examined whether the constituent cells of the inflamed lung were capable of producing PGD$_2$ and Th2 cytokines in response to sPLA$_2$-IB. The addition of sPLA$_2$-IB significantly increased PGD$_2$ levels in the culture medium of BMMCs (Fig. 5A). The addition of PGD$_2$ to Th2 cells significantly increased levels of Th2 cytokines IL-4 and IL-5 levels in the culture medium (Fig. 5B).

Production of eicosanoids and cytokines in cultures of ASM cells and A549 cells in response to sPLA$_2$-IB

This study examined whether there is a difference in production of anti-inflammatory or proinflammatory eicosanoids and cytokines in response to sPLA$_2$-IB in ASM cells through PLA$_2$R-mediated mechanisms. Among eicosanoids and cytokines that we examined (28), there was not significant increase in their levels in ASM cells from either PLA$_2$R$^{+/+}$ mice or PLA$_2$R$^{-/-}$ mice (Supplemental Figs. 1A, 2A), except that levels of PGD$_2$ and PGE$_2$ increased at 100 nM of human sPLA$_2$-IB in culture of ASM cells from PLA$_2$R$^{+/+}$ mice (Supplemental Fig. 1A). The levels of PGD$_2$ and PGE$_2$ in the culture medium of ASM cells from PLA$_2$R$^{-/-}$ mice did not change in response to the same dose of catalytically inactive human sPLA$_2$-IB pretreated for 4 h with bromophenylacylbromide (1 mM; Sigma-Aldrich), a blocker of sPLA$_2$-IB activity (Supplemental Fig. 1B). Similarly, sPLA$_2$-IB did not induce either eicosanoid or cytokine levels in cultures of A549 cells, a cell line of human alveolar basal epithelial cells that express PLA$_2$R (Supplemental Fig. 1C and Fig. 2B).

Discussion

This study demonstrated that the OVA-induced airway inflammation, characterized by more eosinophils and neutrophils in...
BALF and inflammatory histologic changes in the airway, was stronger in PLA2R−/− mice than in PLA2R+/+ mice. PLA2R−/− mice had higher levels of sPLA2-IB and sPLA2-X in BALF after OVA treatment than did PLA2R+/+ mice. These higher levels were associated with greater levels of eicosanoids and Th2 cytokines in BALF from PLA2R−/− mice than from PLA2R+/+ mice. Previous animal and human studies showed that sPLA2 have an important role in the genesis of airway inflammation through the production of proinflammatory eicosanoids in a variety of inflammatory cells (11–15). Eicosanoids including CysLTs, LTβ2, and PGD2 cause lung inflammation directly or indirectly through induction of Th2 cytokines (1–4). In line with these previous reports, the present in vitro study showed that sPLA2-IB increased PGD2 in mast cells and that PGD2 induced Th2 cytokines (IL-4 and IL-5) in Th2 cells. Together, the results suggest that the greater increase in sPLA2-IB and sPLA2-X levels can result in higher levels of proinflammatory eicosanoids and Th2 cytokines, leading to enhanced inflammation in the airway after OVA treatment in PLA2R−/− mice compared with PLA2R+/+ mice.

In agreement with previous reports (10, 18, 19, 21, 22), the current study showed that cultures of ASM cells, expressing PLA2R, internalized and degraded sPLA2-IB. After the exogenous addition of sPLA2-X, the residual enzymatic activity of sPLA2-X decreased more slowly in the culture medium of ASM cells from PLA2R−/− mice than PLA2R+/+ mice. In line with these in vitro studies, the present in vivo experiments showed that intratracheally instilled sPLA2-IB decreased much more slowly in BALF of PLA2R−/− mice than that of PLA2R+/+ mice. In addition, after the instillation of [125I]-labeled sPLA2-IB, TCA-soluble radioactivity in BALF was lower in PLA2R−/− lungs than PLA2R+/+ lungs, indicating lower rates of degradation of sPLA2-IB in PLA2R−/− lung than PLA2R+/+ lung. These data suggested that PLA2R played a role in the clearance of sPLA2 from the lung and that a deficiency of PLA2R resulted in the increase in sPLA2 levels through impaired clearance of sPLA2. That result in turn appears to have led to exacerbation of sPLA2-mediated inflammation of the airway in PLA2R−/− mice. This scenario needs to be confirmed by additional experiments examining whether the increase in the OVA-induced inflammation of lung can be reversed by re-expression of PLA2R in a PLA2R−/− lung.

Mouse PLA2R has a high affinity for sPLA2-IB, -IIA, -IIE, -IIF, and -X (18, 19, 22). A previous report showed that sPLA2-IB and sPLA2-X are expressed in the bronchial epithelium and alveolar epithelial cells or infiltrated inflammatory cells (23). The increase in lung expression of sPLA2-IB and sPLA2-X mRNAs after OVA treatment was similar in PLA2R+/+ and PLA2R−/− mice. Thus, in...
the two genotypes, there might not be a difference in the production of these sPLA2 in OVA-treated lung. The low clearance of sPLA2 (rather than an increase in their production) could partly account for relatively higher levels of these sPLA2 in BALF of PLAR-?/? mice compared with those of PLAR-+/+ mice. It was unlikely that levels of sPLA2-IB and sPLA2-X in BALF were derived from plasma because their levels were not detectable in plasma in both genotyped mice after treatment with either saline or OVA. sPLA2-IIIE is expressed in lung alveolar cells as reported previously (29). Although lung expression of sPLA2-IIIE was weak in the current study, we cannot rule out the possibility that sPLA2-IIIE has a role in the increase in OVA-mediated inflammation in the lungs of PLAR-?/? mice. sPLA2-IIIF was not detectable in OVA-treated lungs. sPLA2-IIIA is naturally disrupted by a frameshift mutation in C57BL/6J background mice (30). Thus, sPLA2-IIIA and IIIF were unlikely to contribute to the difference in the lung inflammation between the two genotypes.

Although the present and previous in vitro experiments using cultured cells indicated that PLAR internalizes and degrades sPLA2 (22), a previous in vivo experiment failed to show the clearance effect of PLAR on sPLA2-IB in systemic circulation (26). In this regard, the current study clearly showed that PLAR acted as a sink for sPLA2 in the lung and that PLAR might protect against sPLA2-mediated airway inflammation through clearance of sPLA2 in vivo. Moreover, it is known that PLAR is expressed in a variety of cells and organs, including the kidney (10, 18, 25, 31). It remains to be determined whether PLAR also expresses in a variety of cells and organs, including the kidney, cytokines were produced in response to sPLA2-IB in ASM cells lungs of PLAR+/+ mice. sPLA2-IIF was not detectable in OVA-

33, 34). We and others showed that some sPLA2 act in concert of PLA2R is directly linked to a signal transduction system that PLA2R-mediated intracellular transduction signals can participate in the cross talk between sPLA2-IB and cPLA2 (16). Valentin, E., and G. Lambeau. 2000. Increasing molecular diversity of secreted PLA2 (rather than an increase in their production) could partly account for relatively higher levels of these sPLA2 in BALF of PLAR-?/? mice compared with those of PLAR-+/+. It was unlikely that levels of sPLA2-IB and sPLA2-X in BALF were derived from plasma because their levels were not detectable in plasma in both genotyped mice after treatment with either saline or OVA. sPLA2-IIIE is expressed in lung alveolar cells as reported previously (29). Although lung expression of sPLA2-IIIE was weak in the current study, we cannot rule out the possibility that sPLA2-IIIE has a role in the increase in OVA-mediated inflammation in the lungs of PLAR-?/? mice. sPLA2-IIIF was not detectable in OVA-

In conclusion, in a mouse model of OVA-mediated lung inflammation, PLAR deficiency increased sPLA2-IB and sPLA2-X levels in the lung through their impaired clearance from the lung, leading to exaggeration of lung inflammation. PLAR might play a protective role in the pathogenesis of lung inflammation.

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Disclosures
Y.Y. and K.H. are employees of Shionogi Research Laboratories, Shionogi and Co., Ltd. (Osaka, Japan). They contributed to the development of PLAR mice and an mAb against PLAR, but they did not contribute to the study design and the data analysis. The remaining authors have no financial conflicts of interest.

References


35. Han, W. K., A. Sapirstein, C. C. Hung, A. Alessandrini, and J. V. Bonventre. 2003. Cross-talk between cytosolic phospholipase A2 alpha (cPLA2 alpha) and secretory phospholipase A2 (sPLA2) in hydrogen peroxide-induced arachidonic acid release in murine mesangial cells: sPLA2 regulates cPLA2 alpha activity that is responsible for arachidonic acid release. J. Biol. Chem. 278: 24153–24163.


Supplemental Table 1. Sequences of PCR primers

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<th>Forward primers</th>
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The eicosanoids levels in the culture medium of cells after incubation with sPLA₂-IB.

(A), The eicosanoids levels in the culture medium of airway smooth muscle (ASM) cells after incubation for 1 h with or without 10 nM or 100 nM of human sPLA₂-IB.

+/+ denotes ASM cells from PLA₂R+/+ mice and -/- denotes ASM cells from PLA₂R−/− mice. n = 6-8 mice in each experiment.

* p < 0.05; † p < 0.05 compared with control in ASM cells from PLA₂R−/− mice.

(B), ASM cells were incubated with same dose of human sPLA₂-IB pre-treated for 4 hr with bromophenylacylbromide (1 mM), a blocker of sPLA₂-IB activity.

(C), The eicosanoids levels in the culture medium of A549 cells, a cell line of human alveolar basal epithelial cells after incubation with sPLA₂-IB.
The mRNA levels of cytokines in ASM cells and A549 cells after incubation with sPLA₂-IB.

(A), The cytokine mRNA levels in ASM cells after incubation for 6 hr with or without 10 nM or 100 nM of human sPLA₂-IB. +/+ denotes ASM cells from PLA₂R⁺/⁺ mice and −/− denotes ASM cells from PLA₂R⁻/⁻ mice. n = 6 mice in each experiment. n.d. = not detected. The mRNA levels were normalized to GAPDH mRNA expression and were expressed relative to the control of ASM cells from PLA₂R⁺/⁺ mice (= 1).

(B), The cytokine mRNA levels in A549 cells after incubation 6 hr with or without 10 nM or 100 nM of human sPLA₂-IB. n = 6-8 in each experiment. The data were expressed relative to the control (= 1).