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Deficiency of Phospholipase A2 Receptor Exacerbates Ovalbumin-Induced Lung Inflammation

Shun Tamaru,* Hitodo Mishina,* Yosuke Watanabe,* Kazuhiro Watanabe,* Daisuke Fujioka,* Soichiro Takahashi,* Koji Suzuki,* Takamitsu Nakamura,* Jun-ei Obata,* Kenichi Kawabata,* Yasunori Yokota,† Makoto Murakami,‡ Kohji Hanasaki,‡ and Kiyotaka Kugiyama*  

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.

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Abbreviations used in this article: ASM, airway smooth muscle; BALF, bronchoalveolar lavage fluid; BMMC, bone marrow–derived mast cell; cPLA2, intracellular cytosolic PLA2; CRD, carbohydrate-recognition domain; iPLA2, Ca2+-independent PLA2; LT, leukotriene; PAS, periodic acid–Schiff; PLZ, phospholipase A2; uSMA, smooth muscle α-actin; sPLA2, secretory PLA2.

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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 (α-SMA) Ab and rabbit polyclonal anti-cytoketatin Ab were purchased from Abcam (Cambridge, U.K.). Recombinant mouse sPLA₂-X were obtained as described previously (22). Recombinant human sPLA₂-IB was obtained from R&D systems (Minneapolis, MN). Sodium [125I] iodine (carrier-free, 3.7 GBq/mg) was purchased from Perkin Elmer (Boston, MA). ELISA kits for determination of IL-4 and IL-5 were obtained from R&D systems; the ELISA kit for IFN-γ was obtained from Raybiotech (Norcross, GA); the ELISA kits for LTβ, 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 similarly treated as described above. On day 24, the mice were euthanized by cutting the left renal artery after anesthesia with i.p. injection of 130 mg/kg ketamine and 8.8 mg/kg xylazine. Immediately after death, the lungs were lavaged for the experiments of bronchoalveolar lavage fluid (BALF). Thereafter, the lungs were removed for histological analysis. In another set of experiments, the lungs were removed for biochemical analysis. In some mice, crüttrated blood was obtained by intracardiac puncture under the anesthesia.

Bronchoalveolar lavage (BAL)

The trachea was exposed and catheterized with a 24-gauge polypropylene endotracheal tube. The lungs were lavaged 8 times with 0.5 ml of PBS to provide 4.0 ml of BALF (23). The BALF was collected by gentle aspiration. For each animal, 90% (3.6 ml) of the total injected volume was consistently recovered. The collected BALF was balanced and cooled to 4°C. The BALF was centrifuged at 300 × g for 10 min, and the nuclear cell count was determined using a hemocytometer. Differential cell counts were made after cytopsin centrifugation and staining with Diff-Quick. A total of 500 nuclear cells were counted under oil immersion microscopy using standard morphologic criteria (assessed by size, nucleus, and chromatin). Supernatants were stored at −80°C until assayed.

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 airways per section were randomly selected from five sections (five 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 airways. These histologic measurements were conducted by two investigators (H.M., K.W.) who were blinded to the study protocol for the mice. The interobserver and intraobserver variabilities for repeated measurements of 25 airways were 3.9 ± 0.5%, respectively, for the grading of peribronchial infiltration of inflammatory cells and 2.6 ± 0.3% and 3.2 ± 0.4%, respectively, for the percentage of PAS-positive mucus-containing cells, indicating high reproducibility in both measurements.

Immunostaining of lungs and cultured cells

The lung was instilled with 50% OCT (Tissue-Tek; Sakura Finetek, Tokyo, Japan) in PBS through the endotracheal tube. Thereafter, lungs were harvested, immediately embedded in OCT, frozen, cut into 5-μm sections and postfixed in acetone. For immunostaining, endogenous peroxidase was inactivated by incubating with 0.3% hydrogen peroxide for 30 min. After washing, the sections were blocked with 10% BSA, and then incubated with the primary Ab followed by a peroxidase-conjugated secondary Ab (Histofine Simple Stain; Nichirei Bioscience, Tokyo, Japan). To develop the brown coloration, the sections were treated with 3-diaminobenzidine tetrahydrochloride, a peroxidase substrate (Vector Laboratories, Burlingame, CA) and counterstained with hematoxylin. The primary Abs included a rat monoclonal anti-PLA₂R Ab (25), a rabbit polyclonal anti-α-SMA Ab, and a rabbit polyclonal anti-cytokeratin Ab. Some 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).

cultures of airway smooth muscle (ASM) cells (see below) in eight-well chamber slides were washed with PBS and fixed with methanol for 10 min at −20°C. Next, the cultured cells were stained as described above. Nuclei were stained with DAPI (Vector Laboratories). Images were acquired with an Olympus Fluoview 1000 confocal microscope (Olympus, Tokyo, Japan) and processed using FV10-ASW software version 1.0 (Olympus).

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 anesthesia by i.p. injection of 130 mg/kg ketamine and 8.8 mg/kg xylazine, the trachea was exposed and catheterized 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 anesthesia 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 to pellet cells, and the radioactivity in the cell-free supernatant of the BALF was measured with a γ counter (AccuFLEX γ7000; Aloka, Tokyo, Japan). The cell-free supernatant of the BALF harvested 4 h after instillation was precipitated with 10% trichloroacetic acid (TCA), and the TCA-soluble radioactivity was measured.

Assays of cytokines, eicosanoids, and sPLA₂

The concentrations of IL-4, IL-5, IFN-γ, LTβ, 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 Biotechnical, 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 RNeasy kit and DNase 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-IIB for 2.5 h at 4˚C. After washing with PBS, the cells’ associated radioactivity was measured. For the assessment of sPLA2-IIB degradation, the cells were washed with PBS, resuspended in binding medium, and incubated 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 β counter. For the assessment of sPLA2-IIB degradation, the cells were incubated with 5 nM [125I]-labeled sPLA2-IIB 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 β counter. To examine the effects of PLA2R on the enzymatic activity of sPLA2, 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 isoforms 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 Scheffe test for post hoc comparison of group means. The serial changes were compared between two genotype pairs using two-way ANOVA for repeated measures followed by post hoc testing with a Scheffe 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 PLA2-R 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 PLA2-R (data not shown). Confocal immunofluorescence microscopy showed that PLA2-R 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-IIB, -IIE, and -X in lung tissue after OVA treatment between the two genotyped mice (Fig. 3B–D). sPLA2-IIF 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-IIB and -X, eicosanoids (LTB4, CysLTs, and PGD2), Th2 cytokines (IL-4 and IL-5) in both PLA2R+/+ and -/− mice (Fig. 3C–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+/+ and PLA2R+/− mice after treatment with saline or OVA (Fig. 3O). There was no detectable plasma levels of sPLA2-IIB and -X in both PLA2R+/+ and -/− mice after treatment with either saline or OVA (data not shown).

In vivo clearance of sPLA2-IIB instilled into the lungs

To determine the in vivo kinetics of PLA2-IIB clearance from the lungs, [125I]-labeled sPLA2-IIB was instilled intratracheally into the lung. In PLA2-R+/− mice, the instilled [125I]-labeled sPLA2-IIB was cleared much more slowly from the BALF, as 50% of [125I]-labeled sPLA2-IIB remained in the BALF after 120 min (Fig. 4A). In contrast, in PLA2R+/+ mice, only 10% of [125I]-labeled sPLA2-IIB remained after 120 min. Four hours after instillation, degradation of the instilled [125I]-labeled sPLA2-IIB, as assessed by TCA-soluble radioactivity in BALF, was inhibited in PLA2R+/− mice compared with PLA2R+/+ mice (Fig. 4B).

In vitro clearance of sPLA2-IIB in cultures of ASM cells

Immunohistochemical analysis showed that PLA2-R protein was expressed in cultured ASM cells obtained from PLA2R+/+ mice but not in PLA2R+/− mice (data not shown). The levels of cell-associated [125I]-labeled sPLA2-IIB 2.5 h after the incubation at 37˚C was significantly lower in cultures of ASM cells from PLA2R+/− lung than those from PLA2R+/+ lung (Fig. 4C). The TCA-soluble radioactivity in the culture medium 90 and 120 min after the...
incubation at 37°C was significantly lower in cultures of ASM cells from PLA2R
+/− 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−/− lung (Fig. 4E).

FIGURE 1. Comparison of histological changes in the lung after OVA-treatment between PLA2R−/− 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) Semi-quantitative 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 −/− denotes PLA2R−/− mice.

FIGURE 2. Expression of PLA2R 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−/− mice after saline (B) or OVA treatment (D) using an Ab against PLA2R (brown). (E−H and I−L) Representative immunofluorescence images of lung from PLA2R+/+ mice after OVA treatment using Abs against PLA2R (F and J, green), αSMA (G, red), PLA2R and αSMA (H), cytokeratin (K, red) or PLA2R 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 PGD₂ and Th2 cytokines in 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. The addition of sPLA₂-IB significantly increased PGD₂ levels in the culture medium of BMMCs (Fig. 5A). The addition of PGD₂ 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 sPLA₂-IB

This study examined whether there is a difference in production of anti-inflammatory or proinflammatory eicosanoids and cytokines in response to sPLA₂-IB through PLA₂R-mediated mechanisms. Among eicosanoids and cytokines that we examined (28), there was not significant increase in their levels in ASM cells from either PLA₂R⁻⁻ mice or PLA₂R⁺⁺ mice (Supplemental Figs. 1A, 2A), except that levels of PGD₂ and PGE₂ increased at 100 nM of human sPLA₂-IB in culture of ASM cells from PLA₂R⁻⁻ mice (Supplemental Fig. 1A). The levels of PGD₂ and PGE₂ in the culture medium of ASM cells from PLA₂R⁻⁻ mice did not change in response to the same dose of catalytically inactive human sPLA₂-IB pretreated for 4 h with bromophenylacylbromide (1 mM; Sigma-Aldrich), a blocker of sPLA₂-IB activity (Supplemental Fig. 1B). Similarly, sPLA₂-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 PLA₂R (Supplemental Fig. 1C and Fig. 2B).

Discussion

This study demonstrated that the OVA-induced airway inflammation, characterized by more eosinophils and neutrophils in the lung, the number of inflammatory cells and the levels of sPLA₂-IB and -X, eicosanoids, IL-4, IL-5, and IFN-γ in BALF after treatment with saline or OVA between PLA₂R⁺⁺ and PLA₂R⁻⁻ mice. (A–D) mRNA expression levels of lung PLA₂R (A), sPLA₂-IB (B), -IE (C), and -X (D) after treatment with saline or OVA in PLA₂R⁺⁺ and PLA₂R⁻⁻ mice. The expression levels were normalized to GAPDH mRNA expression. (E–O) Measurements in BALF. (E) Number of eosinophils. (F) Number of neutrophils. (G) Number of lymphocytes. (H) sPLA₂-IB levels. (I) sPLA₂-X levels. (J) LTB₄ levels. (K) CysLTs levels. (L) PGD₂ levels. (M) IL-4 levels. (N) IL-5 levels. (O) IFN-γ levels in BALF. n = 6–8 mice in each experiment. *p < 0.05, †p < 0.05 compared with the respective genotypes after saline treatment. ⁺⁺ denotes PLA₂R⁺⁺ mice, and ⁺⁻ denotes PLA₂R⁻⁻ mice. n.d., Not detected.
BAM 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, LTβs, 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−/− lungs than PLA2R+/+ lungs. 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, -IFF, 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

**FIGURE 4.** Clearance of instilled sPLA2-IB from the lung in vivo and internalization and degradation of sPLA2-IB in cultured ASM cells. (A and B) [125I]-Labeled sPLA2-IB (150 ng) was instilled into the lung. (A) Radioactivity in BALF 5, 30, 60, and 120 min after instillation of [125I]-labeled sPLA2-IB. The data at each point are expressed as a percentage of that observed 5 min (100%) after the instillation. *p < 0.05 compared with PLA2R+/+ mice at the corresponding time point. n = 6 mice in each experiment. (B) TCA-soluble radioactivity in BALF 4 h after the instillation of [125I]-labeled sPLA2-IB. n = 6 mice in each experiment. *p < 0.05. (C and D) Internalization and degradation of sPLA2-IB in cultured ASM cells. (C) Cell-associated radioactivity after incubation of cultures of ASM cells with 5 nM [125I]-labeled sPLA2-IB for 2.5 h at 37˚C in the absence or presence of a 50-fold excess of unlabeled sPLA2-IB. The specific cell-associated radioactivity is shown after correcting for nonspecific association. *p < 0.05. (D) Cultures of ASM cells were incubated with 5 nM [125I]-labeled sPLA2-IB for 2.5 h at 4˚C in the absence or presence of a 50-fold excess of unlabeled sPLA2-IB, washed with PBS, and incubated for 15, 30, 60, 90, and 120 min at 37˚C. The TCA-soluble radioactivity in the culture medium is shown after correcting for nonspecific degradation. *p < 0.05 compared with PLA2R−/− ASM cells at the corresponding time point. (E) Serial changes in residual enzymatic activities of sPLA2-X in the culture medium of ASM cells were measured after its addition to the culture medium. *p < 0.05 compared with PLA2R+/+ ASM cells at the corresponding time point. n = 6 mice in each experiment. +/− denotes PLA2R+/+ mice and −/− denotes PLA2R−/− mice.

**FIGURE 5.** PGD2, IL-4, and IL-5 production in BMMCs and Th2 cells. (A) PGD2 levels in culture media of BMMCs 30 min after stimulation with sPLA2-IB. (B) IL-4 and IL-5 levels in culture media of Th2 cells 4 h after stimulation with PGD2. n = 6 in each experiment. *p < 0.05 versus control.
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 PLA2R−/− mice compared with those of PLA2R+/+. 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 PLA2R−/− 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-IIA and IIF 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 PLA2R internalizes and degrades sPLA2 (22), a previous in vivo experiment failed to show the clearance effect of PLA2R on sPLA2-IB in systemic circulation (26). In this regard, the current study clearly showed that PLA2R acted as a sink for sPLA2 in the lung and that PLA2R might protect against sPLA2-mediated airway inflammation through clearance of sPLA2 in vivo. Moreover, it is known that PLA2R is expressed in a variety of cells and organs, including the kidney (10, 18, 25, 31). It remains to be determined whether PLA2R also functions as a clearance receptor for sPLA2 and blocks the biologic effects in these tissues. We previously discovered a soluble form of PLA2-R that is derived from membrane-bound PLA2-R by protease cleavage (18, 32). Soluble PLA2-R retains all the extra-cellular domains of the membrane-bound receptor and blocks the biologic functions of sPLA2-IB and -X after binding (18, 32). Therefore, it is possible that soluble PLA2-R has an additional role in the attenuation of airway inflammation in PLA2R−/− mice, although the current study did not elucidate its possible effects in lung tissue.

Previous reports raised the possibility that the cytoplasmic tail of PLA2-R is directly linked to a signal transduction system that induces cell proliferation, eicosanoid production, and inflammation independent of intrinsic enzymatic activities of sPLA2 (15, 33, 34). We and others showed that some sPLA2 act in concert with cPLA2α to induce eicosanoid generation (35, 36), and PLA2-R-mediated intracellular transduction signals can participate in the cross talk between sPLA2-IB and cPLA2α (37). However, the current study did not find any evidence that eicosanoids and cytokines were produced in response to sPLA2-IB in ASM cells through PLA2-R-mediated mechanisms. We cannot rule out the possible effects of a putative signal transduction system on the present findings in PLA2R−/− mice; however, the cytoplasmic tail of PLA2-R does not seem to transduce any specific signals other than internalization signals on the basis of its sequence (18–21). The precise mechanisms by which PLA2-R inhibited lung inflammation are still unclear. It remains undefined whether PLA2-R acts solely as a sink of sPLA2 and whether PLA2-R can directly transduce specific intracellular signals that lead to production of anti-inflammatory molecules or suppression of the production of proinflammatory molecules.

In conclusion, in a mouse model of OVA-mediated lung inflammation, PLA2-R deficiency increased sPLA2-IB and sPLA2-X levels in the lung through their impaired clearance from the lung, leading to exaggeration of lung inflammation. PLA2-R might play a protective role in the pathogenesis of lung inflammation.


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