Deficiency of Phospholipase A2 Receptor Exacerbates Ovalbumin-Induced Lung Inflammation

Shun Tamaru, Hideto 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

J Immunol 2013; 191:1021-1028; Prepublished online 1 July 2013; doi: 10.4049/jimmunol.1300738
http://www.jimmunol.org/content/191/3/1021

Supplementary Material http://www.jimmunol.org/content/suppl/2013/06/28/jimmunol.1300738.DC1

References This article cites 37 articles, 15 of which you can access for free at: http://www.jimmunol.org/content/191/3/1021.full#ref-list-1

Subscription Information about subscribing to The Journal of Immunology is online at: http://jimmunol.org/subscription

Permissions Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Deficiency of Phospholipase A2 Receptor Exacerbates Ovalbumin-Induced Lung Inflammation

Shun Tamaru,* Hitoko 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*

Sectional 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 pathogenetic role of PLA2R in airway inflammation using PLA2R-deficient (PLA2R−/−) mice. Airway inflammation was induced by immunoglobulin sensitization 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.

It 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-lipoxgenase 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 have shown 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 fibronectin-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 isoforms, mouse PLA2R has a high affinity to sPLA2-IB, -IIA, -IIE, -IIF, and -X (18, 19). The intracellular C-terminal region contains a consensus sequence motif of coated pit-mediated-endoctyosis that was originally identified in the low-density lipoprotein receptor (18–21). Previous in vitro studies have demonstrated that sPLA2-IB and sPLA2-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 sPLA2-X are expressed in the lung (11, 12, 23). The animal study using sPLA2–deficient mice showed that sPLA2-IB produced proinflammatory eicosanoids and Th2 cytokines and played a critical role in gene expression of allergen-induced airway inflammation (12). The human study indicated that sPLA2-X2 could have a role in the generation of proinflammatory eicosanoids in the airways, leading to the development of bronchial hyperresponsiveness (11). In addition, sPLA2-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-cytokinin 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] iodide (carrier-free, 3.7 GBq/μl) 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 LTβα, cysteinyl leukotrienes (CysLTs), PGD2, and PGE2 were obtained from Cayman Chemical (Ann Arbor, MI); the ELISA kit for 15-deoxy-Delta 12,14-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 obtained from Jackson Laboratory. Additional mice were obtained from Male C57BL/6J (Jackson Laboratory, Bar Harbor, ME) and from the laboratory colony maintained in the University of Yamanashi. Mice were housed in a temperature-controlled, 12-h light/dark cycle, and an autoclaved, sterile environment.

Bronchoalveolar lavage fluid (BALF)

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 cytoplasm 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 bronchi were 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 (from 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 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 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, harvested and fixed in 0.05 ml saline after anesthesia with i.p. injection of 130 mg/kg ketamine and 8.8 mg/kg xylazine. Immediately after washing, the sections were blocked 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).

antigen retrieval with 0.5% trypsin and 0.1% HCl for 10 min was performed. After blocking, the sections were incubated with a primary Ab (anti-PLA2R, anti-αSMA, and anti-cytokeratin Ab). Endogenous peroxidase was blocked with H2O2, and then sections were incubated with the primary Ab followed by a peroxidase-conjugated secondary Ab (Histone 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. As positive controls for immunostaining with primary Abs, 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 Fiji/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 at 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 cytoplasm 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.
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 isolated using MACS (Miltenyi Biotec, 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 (Miltenyi Biotec). ASM cells were cultured from explants of tracheae. The entire tracheae were removed, and the tracheal segments 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 tracheae 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 concentrations 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 (HBSS 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 incubated 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 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, isozymes with a high affinity for PLA2R, 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 genotyped mice 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 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 PLA2R and sPLA2 in the lungs and the number of inflammatory cells and levels of eicosanoids, and Th2 cytokines in BALF after treatment with saline or OVA between PLA2R+/+ and PLA2R−/− mice

In PLA2R−/− mice, mRNA levels of PLA2R 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, -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-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 in the BALF after 120 min (Fig. 4A). In contrast, in PLA2R+/+ mice, only 10% of [125I]-labeled sPLA2-IB remained after 120 min. Four hours after instillation, degradation of the instilled [125I]-labeled sPLA2-IB, as assessed by TCA-soluble radioactivity in BALF, was inhibited in PLA2R−/− mice compared with PLA2R+/+ mice (Fig. 4B).

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

Immunohistochemical analysis showed that PLA2R 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-IB 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 PLA2R2/2 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 PLA2R2/2 lung (Fig. 4E).

FIGURE 1. Comparison of histological changes in the lung after OVA-treatment between PLA2R2/2 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 PLA2R2/2 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 PLA2R2/2 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 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...
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, LTβ, 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 from 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 PLA2-R played a role in the clearance of sPLA2 from the lung and that a deficiency of PLA2-R 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 PLA2-R in a PLA2R−/− lung.

Mouse PLA2-R 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-IIIE 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 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 PLAR R internalizes and degrades sPLA₂ (22), a previous in vivo experiment failed to show the clearance effect of PLAR R on sPLA₂-IB in systemic circulation (26). In this regard, the current study clearly showed that PLAR R acted as a sink for sPLA₂ in the lung and that PLAR R might protect against sPLA₂-mediated airway inflammation through clearance of sPLA₂ in vivo. Moreover, it is known that PLAR R is expressed in a variety of cells and organs, including the kidney (10, 18, 25, 31). It remains to be determined whether sPLA₂ also expressed in a variety of cells and organs, including the kidney and other tissues. Further studies are needed to clarify whether sPLA₂-IB and sPLA₂-X in BALF were likely to contribute to the difference in the lung inflammation between the two genotypes.

Previous reports raised the possibility that the cytoplasmic tail of PLAR 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 cPLA₂ to induce eicosanoid generation (35, 36), and sPLA₂-R-mediated intracellular transduction signals can participate in the cross talk between sPLA₂-IB and cPLA₂ (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 PLAR-R-mediated mechanisms. We cannot rule out the possible effects of a putative signal transduction system on the present findings in PLAR R−/− mice; however, the cytoplasmic tail of PLAR 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 PLAR inhibited lung inflammation are still unclear. It remains undefined whether PLAR acts solely as a sink of sPLA₂ and whether PLAR 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, 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.

Acknowledgments

We thank A. Watanabe for technical assistance.

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.
