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*J Immunol* 2007; 179:4800-4807; doi: 10.4049/jimmunol.179.7.4800

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Deletion of Secretory Group V Phospholipase A2 Attenuates Cell Migration and Airway Hyperresponsiveness in Immunosenstizied Mice

Nilda M. Muñoz,* Angelo Y. Meliton,* Jonathan P. Arm, ‡ Joseph V. Bonventre, ‡ Wonhwa Cho,§ and Alan R. Leff²∗∗†

We investigated the role of group V phospholipase A2 (gVPLA2) in OVA-induced inflammatory cell migration and airway hyperresponsiveness (AHR) in C57BL/6 mice. Repeated allergen challenge induced biosynthesis of gVPLA2 in airways. By aerosol, gVPLA2 caused dose-related increase in airway resistance in saline-treated mice; in allergic mice, gVPLA2 caused persistent airway narrowing. Neither group IIa phospholipase A2, a close homolog of gVPLA2, nor W31A, an inactive gVPLA2 mutant with reduced activity, caused airway narrowing in immune-sensitized mice. Pretreatment with MCL-3G1, a blocking Ab against gVPLA2, before OVA challenge blocked fully gVPLA2-induced cell migration and airway narrowing as marked by reduction of migrating leukocytes in bronchoalveolar lavage fluid and decreased airway resistance. We also assessed whether nonspecific AHR caused by methacholine challenge was elicited by gVPLA2 secreted from resident airway cells of immune-sensitized mice. MCL-3G1 also blocked methacholine-induced airway bronchoconstriction in allergic mice. Blockade of bronchoconstriction by MCL-3G1 was replicated in allergic pla2g5−/− mice, which lack the gene encoding gVPLA2. Bronchoconstriction caused by gVPLA2 in pla2g4−/− mice was comparable to that in pla2g4+/− mice. Our data demonstrate that gVPLA2 is a critical messenger enzyme in the development of AHR and regulation of cell migration during immunosenstization by a pathway that is independent of group IVa phospholipase A2.

Bronchial asthma is a chronic inflammatory disease of airways (1, 2) characterized by eosinophilic infiltrates (3, 4), mucus hypersecretion (5, 6), and hyperreactivity to inhaled allergen (7–9) and to nonspecific stimuli (10, 11). Phospholipases A2 (PLA2)³ are a superfamily of enzymes that hydrolyze the sn-2 acyl ester of phospholipids to generate fatty acids and lysophospholipids. Because the products of PLA2 hydrolysis, arachidonic acid in particular, serve as precursors for potent mediators of airway inflammation, leukotrienes, PLA2 has been implicated in the pathogenesis of asthma. Among multiple forms of mammalian PLA2, the involvement of two isoforms, 85-kDa cytosolic group IVa PLA2 (gIVaPLA2) and 14-kDa secretory type V PLA2 (gVPLA2), in inflammation has been shown by gene knockout studies.

GIVaPLA2 is the only PLA2 known to have genuine specificity for phospholipids containing sn-2 arachidonoyl group. Prior in vivo and in vitro studies have demonstrated that blockade of gIVaPLA2 inhibits 1) cellular adhesion/migration of eosinophils and lymphocytes (12–14), 2) synthesis of arachidonate metabolites (15–20), and 3) airway narrowing (21–23). Mammalian secretory 14-kDa PLA2-s, e.g., gVPLA2, gIIaPLA2, and gXPLA2, are inflammatory enzymes that are involved directly and indirectly in inducing inflammatory and allergic processes; however, the mode of action of these sPLA2 isoforms has not been fully established.

gIIaPLA2 is a close homolog of gVPLA2; however, unlike gVPLA2, gIIaPLA2 has low affinity for zwitterion phosphatidylincholine-rich outer plasma membranes and little ability to generate free fatty acids and lysophospholipids (24–27). Although the relative contributions of cytosolic gIVaPLA2 and secretory PLA2 differ between cells and tissues, different PLA2-s may act synergistically in producing proinflammatory lipid mediators (28–30). Among the secretory PLA2-s, gVPLA2 is a unique enzyme because it: 1) possesses interfacial binding properties and high affinity/ activity and hydrolyzes for phosphatidylcholine-rich outer cell plasma membrane to see generate lysophospholipids and free fatty acids (24, 31–33); 2) can enter mammalian cells in a heparan sulfate-dependent manner and acts intracellularly by transmembrane transport caused by binding to surface heparan sulfate proteoglycan (25, 33, 34); 3) acts on the perinuclear membrane to generate bioactive lipid mediators through both gIVaPLA2-dependent (32, 33) and -independent mechanisms (34, 35); and 4) up-regulates eosinophil surface CD11b/CD18 expression (35) causing adhesion to the endothelial counterligand, ICAM-1 (35, 36).

We have shown previously that blockade of eosinophil migration caused by inhibition of gIVaPLA2 corresponded to attenuation

³ Abbreviations used in this paper: PLA2, phospholipase A2; GVPLA2, group V phospholipase A2; GIVaPLA2, cytosolic group IVa PLA2; AHR, airway hyperresponsiveness; pla2g5−/−, gVPLA2-null mice; WT, wild type; pla2g5+/−, gVPLA2 WT mice; pla2g4−/−, cytosolic gIVaPLA2-null mice; pla2g4+/−, cytosolic gIVaPLA2 WT mice; Rrs, airway lung resistance; MCh, methacholine; BALF, bronchoalveolar lavage fluid.

Received for publication May 15, 2007. Accepted for publication July 17, 2007.

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of airway hyperresponsiveness (AHR) in allergic guinea pigs (21). Other studies have demonstrated substantially greater AHR in immunosensitized gIVaPLA2 wild-type (WT) mice than in gIVaPLA2 knockout mice (23, 34, 37). Although much attention has been focused on gIVaPLA2 as the critical regulating enzyme in integrin adhesion (12–14) and eicosanoid generation (15–20), the signal initiating stimulus that triggers the activation of inflammatory cells to cause cell migration and AHR in chronic asthma has not been defined. We have demonstrated that gIVaPLA2 can trigger the biosynthesis and release of LTB4 from human neutrophils (32, 33) in a gIVaPLA2-dependent manner and LTC4 from human eosinophils in a gIVaPLA2-independent manner (34, 35). We and others have also reported recently that epithelial cells are a natural source of gIVaPLA2 (35, 36) and that in vitro transcellular migration of gIVaPLA2 secreted from activated epithelial cells causes secretion of LTC4 from adherent eosinophils by a pathway that is independent of gIVaPLA2 (35, 36).

In this study, we hypothesized that gVPLA2 is an intercellular messenger protein for induction of airway inflammation in vivo and subsequent AHR. To test this hypothesis, we measured the effect of MCL-3G1, a blocking mAb against gVPLA2, (38, 39), in cell migration and AHR in immunosensitized airways. We assessed whether nonspecific AHR elicited by methacholine (MCh) challenge was caused by endogenous gVPLA2, secreted from airways of immunosensitized mice. In additional studies, we used gVPLA2null mice to confirm the contributory role of gVPLA2 in cell migration and AHR. Finally, we determined whether gVPLA2-induced AHR and airway inflammation were independent of gIVaPLA2 activation. Our data are the first demonstration that 1) gVPLA2 regulates AHR in immunosensitized mice and 2) induction of AHR by gVPLA2 is independent of cytosolic gIVaPLA2.

Materials and Methods

Isolation of secretory PLA2s

The mutagenesis of gVPLA2 to the W31A mutant was performed as described previously (32–35). gIIaPLA2, gIVaPLA2, and W31A were expressed in Escherichia coli and refolded as described previously (32–35). Mouse mAb directed against human gVPLA2, MCL-3G1, was produced from the Hybridoma Facility of the University of Chicago (Chicago, IL) (38–39). We have shown previously that MCL-3G1 is a functional blocking Ab (35) that prevents the effects of secreted gVPLA2 from cultured epithelial cells causes secretion of LTC4 from adherent eosinophils by a pathway that is independent of gIVaPLA2 (35, 36).

Animals

Homozygous gVPLA2-deficient mice (pla2g5+/−) and littermate control (pla2g5−/−) mice were derived as described (40–41). Heterozygous mice were bred to a C57BL/6 background and were bred to derive N11 C57BL/6 pla2g5−/− and WT control pla2g5−/+ mice. Homozygous BALB/c gIVaPLA2null mice (pla2g4−/−) and littermate controls (pla2g4+/−) were derived as described (20, 29, 37). Control C57BL/6 mice, 6–8 wk old, were purchased from The Jackson Laboratory. These mice were housed in specific pathogen-free conditions at the University of Chicago animal facility. Experimental protocols conform to the principles outlines by the Animal Welfare and the National Health services guidelines for the care and use of animals in biomedical research.

Immunosensitization

Animals were sensitized by i.p. injection of 10 μg of chicken egg OVA (Sigma-Aldrich) and 1.125 mg of alum in 0.2 ml of sterile saline at days 0, 7, and 14. On days 21–23, animals were placed in an incubation chamber and were challenged with 1% OVA for 40 min using a DeVelbess nebulizer (39). Similar procedures were performed in pla2g5−/−, pla2g4−/−, and littermate control mice.

Secretory gVPLA2 expression

Immunohistochemistry. Tissue sections were examined for the presence of immunohistochemical staining in mice using MCL-3G1 mAb (38, 39). Frozen mouse tissues (~5 to ~6-μm-thick sections were subsequently cut and fixed in 10% formalin solution. Endogenous peroxidase activity and nonspecific Ab binding were prevented by incubating the sections for 15 min in 0.5% H2O2, methanol (vol/vol) and normal goat serum (1/5 dilution). Sections were incubated for 1 h in primary Ab (MCL-3G1) diluted in normal saline solution. Ab binding was localized with a biotinylated secondary Ab, avidin-conjugated HRP, and diaminobenzidine chromogenic substrate (Vector Laboratories).

Immunoblotting analysis. Lung protein extracts (40 μg/lane) were run on SDS-PAGE and transferred to nitrocellulose membrane. gVPLA2 expression was probed with MCL-3G1, a mAb directed against gVPLA2, and secondary goat anti-mouse IgG1 (HRP conjugated) as previously described (38–39).

PCR. An ~2-mm tail was cut from each weaning. DNA was isolated, and genotyping was performed using PCR. Mice with the required genotype, pla2g3−/−, pla2g5−/−, pla2g4−/−, and pla2g4+/− were kept until immunologically mature. Primers used for gVPLA2 are: forward, 5′-GATGCA CGACCCTTGTTATG-3′; reverse, 5′-TAATCTCAATTGGAAGACCCCT AGGT-3′. Primers for NEO are: forward, 5′-GCTTGTCGGACGTTGTC ACTC-3′. Primers used for gIVaPLA2 genotyping are: number 1, 5′-CG ACTCTACAGTGCCCCATC; number 2, 5′-GGGAATCTTCTGTGCTG TTTCC; number 3, 5′-TTGTTCTACTCATTTGTTGTTTCA.

Preparation of animals

Mice were anesthetized with 30 mg/ml xylazine plus 80 mg/ml ketamine i.p. and were ventilated quasisinusoidally at a frequency of 150 breaths/min (tidal volume, 10 ml/kg) as described previously (42). Increasing concentrations of sPLA2 and MCh were delivered by Aeroneb, an ultrasonic nebulizer device attached to the ventilator. All outcome parameters of the flexiVent (Sartorius) were calculated using well-validated mathematical models with a sound physical basis (42). The change in airway resistance (Rrs) was expressed as centimeters of H2O per milliliter per second.

Measurement of AHR

Concentration-response curve. Rrs as a function of airway narrowing was measured using a computer-controlled small-animal ventilator as described previously (42). MCh (0–10 mg/ml) or 14–1400 ng/ml gVPLA2, gIIaPLA2, or W31A were given via Aeroneb to mice. Rrs was measured during continuous nebulization (15 s) of MCh, gVPLA2, gIIaPLA2, or W31A. MCh concentration-response curves were generated in treated pla2g5+/− and pla2g5−/− mice, and similar measurements were performed. Nebulization of gVPLA2 was performed in allergic pla2g4+/− mice, which lack genes encoding gIVaPLA2. Littermate control mice.

Blockade of gVPLA2 or MCh-induced bronchoconstriction by MCL-3G1. Treatment 1 was an i.p. injection of 20 μg of MCL-3G1 daily (days 21, 22, and 23) and 60 min of OVA challenge by aerosol (after phase II; see Fig. 3A).

Treatment 2 was an i.p. injection of 20 μg of MCL-3G1 24 h after the last OVA challenge: 60 min later, gVPLA2, or MCh inhalation was performed (after phase II; see Fig. 3A).

Increase in Rrs was measured for all treated mice. Either saline or isotype-matched control (IgG1) was used as negative control.

Inflammatory cell count

The cell pellet was collected from bronchoalveolar lavage fluid (BALF), and differential cell counts were performed with Wright-Giemsa method.

Lung histology

Treated lungs were inflated to 20–25 cm of H2O before infusion of 10% formalin solution. Endogenous peroxidase activity and nonspecific Ab binding were prevented by incubating the sections for 15 min in 0.5% H2O2, methanol (vol/vol) and normal goat serum (1/5 dilution). Sections were incubated for 1 h in primary Ab (MCL-3G1) diluted in normal saline solution. Ab binding was localized with a biotinylated secondary Ab, avidin-conjugated HRP, and diaminobenzidine chromogenic substrate (Vector Laboratories).

Statistical analysis

Data are presented as mean ± SEM. Variation between three or more groups was analyzed using ANOVA followed by Fisher’s protected least significant difference. Variation between two groups was tested using a two-tailed Student t test. Statistical significance was claimed whenever p was <0.05.
specimens. The way section can be obtained only from pneumonectomy or autopsy histological staining (data not shown). However, full thickness airways demonstrated bronchoconstriction in allergic mice but not phocytes. The H&E-stained transverse cross-sections of peripheral filtrated with inflammatory cells, particularly, eosinophils and lymphocytes. In OVA-sensitized mice acutely challenged with OVA (allergic mice), the lungs were heavily infiltrated with eosinophils (34, 35, 38). Preliminary studies revealed expression of gVPLA2 in abundant quantities in microsections of allergic mice (Fig. 2D, inset) compared with nonallergic mice (Fig. 2C, E, inset). Secretory gVPLA2 expression (dark brown stain) was evident in epithelial cells, airway smooth muscle, and inflammatory cells, mostly macrophages (see red arrow), surrounding the bronchial airways (Fig. 2F). Immunoblotting analysis showed that gVPLA2 was highly expressed in lungs of allergic mice (Fig. 2G); modest expression of gVPLA2 was observed in lungs of nonallergic mice. Excised heart from allergic mice was used as positive control because gVPLA2 is highly expressed in this organ (43, 44). These data demonstrate that gVPLA2 is inducible in murine airways by Ag-induced immune sensitization.

Secretory gVPLA2-induced bronchoconstriction in nonallergic and allergic mice

We next examined the effect of gVPLA2 in causing airway narrowing in nonallergic and allergic mice. Inhalation of gVPLA2 caused concentration-related increase in airflow lung resistance (Rrs; cm H2O/ml/s) in nonallergic mice (Fig. 3A). By contrast, maximal bronchoconstriction was elicited at 14 ng/ml gVPLA2 and was sustained thereafter in allergic mice. Baseline Rrs was 0.48 ± 0.08 cm H2O/ml/s and increased to 1.44 ± 0.30 cm H2O/ml/s in nonallergic mice (p < 0.05); in allergic mice, Rrs increased from 1.27 ± 0.0968 cm H2O/ml/s at baseline to 2.4 ± 0.188 cm H2O/ml/s after 14 ng/ml gVPLA2 (p < 0.05). Greater concentrations of gVPLA2 had no further effect on Rrs in allergic mice.

To test the specificity of airway responses elicited by gVPLA2, we next examined the effect of gIIaPLA2, a close homolog of gVPLA2 (25, 31–35), and W31A, which has a point mutation at the putative interfacial binding site of gVPLA2 and much reduced hydrolytic activity (32–35), in causing bronchoconstriction in allergic mice (Fig. 3B). Neither gIIaPLA2 nor W31A was effective in causing an increase in Rrs at any concentration tested indicating the specificity of gVPLA2 and the requirement for interfacial unique membrane binding property in eliciting airway narrowing in immune-sensitized mice.

MCL-3G1, a gVPLA2 blocking Ab, attenuated gVPLA2-induced bronchoconstriction and cell migration in allergic mice

To determine whether inhibition of endogenous gVPLA2 by MCL-3G1 would block AHR caused by immunosensitization, 1) isotype matched-control (IgG1) or 2) 20 µg of MCL-3G1 (after phase I) was administered to mice for 3 consecutive days before OVA challenge (Fig. 4A, treatment 1). The change in Rrs caused by gVPLA2 was measured 24 h after the last OVA challenge (after phase II). Maximal airway narrowing was 2.9 ± 0.011 cm H2O/ml/s in response to 14 ng/ml gVPLA2 for control mice receiving isotype-matched control Ab, IgG1. Higher concentrations of gVPLA2 had no further effect on Rrs (Fig. 4B, □). The contractile response to increasing concentrations of gVPLA2 was attenuated by MCL-3G1 to baseline level (Fig. 4B, ■). In separate studies, MCL-3G1 was injected 24 h after phase II (Fig. 4A, treatment 2), and the mice were challenged with gVPLA2 (Fig. 4B, ■). Bronchoconstriction elicited by gVPLA2 also was blocked fully in those mice receiving MCL-3G1 24 h after the last OVA challenge and gVPLA2 inhalation.

The total BALF cell count was 1240 ± 98.6 × 103 cells in mice receiving 20 µg of MCL-3G1 after phase II compared with 153 ± 14.6 × 103 cells for mice receiving 20 µg of MCL-3G1 after phase I and before each of three inhalation challenges with OVA in phase II.
II (Fig. 4 C, p < 0.001). The total inflammatory cell count decreased from 1100 ± 87 × 10³ cells to 120 ± 635 × 10³ cells for eosinophils (p < 0.001), from 160 ± 52.5 × 10³ cells to 23 ± 13.5 × 10³ cells for macrophages (p < 0.05) and from 115 ± 29 × 10³ cells to 10.3 ± 3.5 × 10³ cells for lymphocytes (p < 0.05). These data demonstrate that blockade of endogenous gVPLA₂ is sufficient to block both AHR (Fig. 4 B) and cell migration before phase II (Fig. 4 C) caused by immunosensitization. By contrast, after full sensitization with OVA (after phase II), MCL-3G1 did not affect the migration of inflammatory cells; however, the airway narrowing still was blocked significantly (Fig. 4 A).

**FIGURE 2.** Expression of gVPLA₂ in saline-treated (nonallergic) and OVA-sensitized/-challenged airway (allergic). Values are representative of five mice per group. A, nonallergic airway microsection stained with isotype-matched control, IgG1; B, allergic airway microsection stained with isotype-matched control, IgG1; C, nonallergic airway microsection stained with MCL-3G1 mAb; D, allergic airway microsection stained with MCL-3G1 mAb; E, inset, nonallergic airway stained with MCL-3G1, IgG1; F, inset, allergic airway stained with MCL-3G1 mAb, red arrows indicate the expression of gVPLA₂ in the airway epithelium (EPI), airway smooth muscle (ASM) and macrophages (mΦ); G, lung and heart homogenates from nonallergic and allergic mice probed with MCL-3G1 mAb and analyzed by Western blot. The expression of gVPLA₂ in airway microsection was visualized by light microscopy.

**FIGURE 3.** Nonresponsiveness to gIIaPLA₂ and the inactive W31A gVPLA₂ mutant. A, Increase in airway resistance (Rrs, cm H₂O/ml/s) elicited by gVPLA₂ in nonallergic mice (□, n = 5 mice) and allergic mice (■, n = 5 mice); B, absence of change in airway lung resistance (Rls, cm H₂O/ml/s) elicited by gIIaPLA₂, a close homolog of gVPLA₂ (□, n = 4 mice) and W31A, an inactive mutant of gVPLA₂ (■, n = 4 mice) in allergic mice. *p < 0.05 and †, p < 0.01 vs control value of nonallergic mice for each group compared with allergic mice. Data are expressed as mean ± SEM.

Initial studies established the increase in AHR measured as change in Rrs in nonallergic and allergic mice (Fig. 5 A). Baseline Rrs in allergic mice was 1.36 ± 0.02 cm H₂O/ml/s and increased progressively to 5.55 ± 0.43 cm H₂O/ml/s with inhalation of 10 mg/ml MCh (p < 0.01 vs baseline value). There was no significant increase in Rrs at any concentration of MCh ≤ 10 mg/ml in the nonallergic group. Pretreatment with i.p. MCL-3G1 after phase I and before each challenge with OVA blocked substantially the increase in Rrs caused by MCh-inhalation (see Fig. 4 A, treatment I). Similarly, i.p. MCL-3G1 administered after phase II still...
FIGURE 4. Effect of MCL-3G1, a gVPLA2 blocking Ab, on gVPLA2-induced bronchoconstriction and cell migration before and after OVA challenge. A Protocol design: administration of MCL-3G1 before gVPLA2 or MCh inhalation. B Change in airway resistance (Rrs; cm H2O/ml/s) caused by gVPLA2, Isotype-matched control (IgG1; □, no MCL-3G1; n = 5 mice). Blockade of AHR by 20 μg of MCL-3G1 alone administered (after phase I) daily before OVA challenge performed on days 21, 22, and 23 (■, treatment 1; n = 5 mice) and 20 μg of MCL-3G1 administered (after phase II) 24 h after the last OVA challenge (■, treatment 2; n = 5 mice). C The total BALF and differential cell count. *, p < 0.001 and †, p < 0.01 vs MCL-3G1 before OVA challenge (after phase I). Data are expressed as mean ± SEM. IgG1 isotype-matched control was used as negative control in allergic mice (n = 5 mice per group).

FIGURE 5. Cumulative concentration-response curve to methacholine (MCh) in the presence or absence of MCL-3G1 in nonallergic and allergic mice. Change in airway resistance (Rrs; cm H2O/ml/s) caused by MCh. A. □, nonallergic, no MCL-3G1 (n = 7 mice); ■, allergic mice, no MCL-3G1 (n = 7 mice); □, MCL-3G1 administered (after phase I) before OVA challenge (n = 5 mice); ■, MCL-3G1 administered 24 h after the last OVA challenge (n = 5 mice). B–E, Representative airway microsections stained with H&E from the same experimental mice in A. B, Nonallergic airway, not receiving MCL-3G1; C, allergic airway, not receiving MCL-3G1; D, MCL-3G1 administered after OVA-sensitization (phase I) and daily before OVA challenge (phase II); E, MCL-3G1 administered 24 h after the last OVA challenge (after phase II). F–H, Histological identification of gVPLA2 expression in airway of immune-sensitized mice; F, allergic airway, stained with isotype-matched control. IgG1; G, allergic mice stained with MCL-3G1; H, MCL-3G1 administered after OVA sensitization (phase I) and daily before OVA challenge (before phase II), stained with MCL-3G1. *, p < 0.05; †, p < 0.01, and ‡, p < 0.001 compared with nonallergic mice, to MCL-3G1 treated mice before OVA challenge, and to 24 h after the last OVA challenge.

Deletion of MCL-3G1 gene inhibits AHR and cell migration in allergic mice: role of gIVaPLA2

We further assessed the specificity of our findings by examining the effect of gVPLA2 deletion on AHR and cell migration in mice genetically deficient in gVPLA2 (pla2g5−−/−) and WT littermate controls (pla2g5+−/+). The immunosensitized littermate controls pla2g5+−/− had greater airway responsiveness to MCh (Fig. 6A) than did the allergic pla2g5−−/− mice even after exposure to the same concentration of OVA. In allergic pla2g5+−/− littermate control mice, airway narrowing (absolute number minus baseline
Photomicrographs of representative lung tissues obtained from C57BL/6 mice. There was no difference in change in pla2g5 after 1.25 mg/ml MCh, 2) 0.5 and value) progressively increased by 1) 0.385 ± 0.05 cm H2O/ml/s after 1.25 mg/ml MCh, 2) 0.5 ± 0.02 cm H2O/ml/s after 2.5 mg/ml MCh, 3) 0.98 ± 0.11 cm H2O/ml/s after 5 mg/ml MCh, and 4) 1.79 ± 0.41 cm H2O/ml/s after 10 mg/ml MCh. An increase in Rrs caused by MCh also was blocked substantially in gVPLA2-deficient mice. There was no difference in change in Rrs for pla2g5+/− and pla2g5−/− saline-treated mice (p = NS).

Histological examination of airways revealed the absence of inflammatory cells and typical appearance of normal bronchial airways in nonallergic pla2g5−/− (Fig. 6B, upper left), nonallergic pla2g5−/− mice (Fig. 6B, lower left) as well as in allergic pla2g5−/− mice (Fig. 6C, lower right). By contrast, airways from allergic pla2g5−/− mice had extensive eosinophilic infiltrates within the submucosa that extended through the epithelial lining of the bronchial airways (Fig. 6C, upper right).

The total number of BALF cells increased significantly from 76.6 ± 18.4 × 10^3 cells to 918 ± 168 × 10^3 cells for pla2g5−/− mice after OVA challenge (Fig. 6D; p < 0.01), which was attenuated by >45% in allergic pla2g5−/− mice (481 ± 154 × 10^3 cells; p < 0.05 vs allergic pla2g5+/− mice) and to 0.58 cm H2O/ml/s after 0.11 cm H2O/ml/s after 5 mg/ml MCh, and 4) 0.98 cm H2O/ml/s after 14 ng/ml gVPLA2 (p < 0.002 vs control) and 1.67 ± 0.58 cm H2O/ml/s in response to 1400 ng/ml gVPLA2 (p < 0.01 vs 14 ng/ml gVPLA2; p < 0.002 vs control). These data are identical with those from allergic pla2g4−/− mice (p = NS for all comparisons) and indicate that gVPLA2-induced airway narrowing is effected by mechanisms distinct from gIVAPlA2 activation.

Discussion

The objective of this study was to characterize the role of gVPLA2 in cytopathological and morphological alterations that accompany the induction of AHR in allergic lungs of C57BL/6 mice. Our data show that immunosensitization caused 1) airway inflammation and remodeling (Fig. 1), 2) biosynthesis of gVPLA2 in the airways (Fig. 2), and 3) AHR to gVPLA2 (Fig. 3A) and MCh (Fig. 5A) compared with sham-sensitized mice. Injection i.p. of MCL-3G1, a gVPLA2 blocking Ab, before OVA challenge (after phase I) blocked cell migration and AHR (Figs. 4 and 5). An important finding in this study is that MCL-3G1 still blocked the bronchoconstriction caused by MCh in highly inflamed airways (Fig. 5A).

We also found that neither gIIaPLA2, a close homolog of gVPLA2, nor W31A, a mutant of gVPLA2, that does not bind to the outer plasma membrane to release arachidonic acid (32–34) caused development of AHR in allergic mice (Fig. 3B). Hence, these effects are specific to gVPLA2 and do not result from activation of the cytosolic gIVAPlA2 enzyme (Fig. 7). Blockade of cell migration...
and AHR was replicated by deletion of gVPLA2 in allergic pla2g5
data. Our study is the first to describe the involvement of gVPLA2 in AHR and airway inflammation in a murine model of allergic pulmonary inflammation. In this study, naive C57BL/6 mice were utilized as a proper control for pla2g5
data. Both strains were responsive to Ag challenge, and there was a clear and significant increase in airway lung resistance.

Asthma is a chronic disease characterized by AHR, cellular infiltrates, and reversible narrowing of airways (1–6). The airway inflammation is associated with infiltration of leukocytes, including T lymphocytes, macrophages, and eosinophils (1–6). However, the primary signals for the infiltration of the lung tissue with leukocytes have yet to be identified. Although cytokines enhance the differentiation, migration, and pathobiological efficiency of eosinophils, neither GM-CSF, nor IL-5, nor IL-3 activates eosinophils to cause bronchoactive mediator release (46). Prior studies have reported that cells that normally reside in bronchial airways can generate bronchoactive mediators, which can directly activate the neighboring inflammatory cells (35–36); yet the endogenous trigger for activation of inflammatory cell functions inside the lung has not been identified.

In this study, we found gVLA2 to be extremely potent and efficacious in causing bronchoconstriction (Fig. 3). Maximal bronchoconstriction was elicited in allergic mice with 14 ng/ml gVPLA2 (≈1 × 10−9 M; Fig. 3A); comparable bronchoconstriction required 2.5 mg/ml MCh (≈1 × 10−3 M; see Fig. 5A) (47). Because secreted gVPLA2 binds rapidly to the phosphatidylcholine-rich cell membrane of circulating inflammatory cells, the quantitation of this enzyme in BALF is not possible. Administration of MCL-3G1 attenuated both eosinophilic inflammation and AHR in allergic mice. Our data also demonstrate that blockade of gVPLA2 alone is sufficient to inhibit the AHR and eosinophilic migration elicited by MCh or gVPLA2 inhalation of immunosensitized mice.

Specificity of gVPLA2 in the induction of cell migration and AHR was confirmed by replicating our findings in gVPLA2-deficient mice. We found that in allergic pla2g5−/− mice, airway inflammation (Fig. 6C) and AHR to MCh challenge (Fig. 6A) were substantially reduced compared with immunosensitized pla2g5+/− mice. These data are identical with those from allergic mice treated with MCL-3G1 before OVA challenge (Fig. 5A). By contrast, there are no statistical differences in airway narrowing caused by gVPLA2 inhalation in allergic pla2g4+/+ and pla2g4−/− mice (Fig. 7). These data suggest 1) a functional role for gVPLA2 in induction of bronchoconstriction that does not utilize gIVaPLA2 activation (Fig. 7) and 2) a potential role of anti-gVPLA2 mAb in the future prevention or management of allergic inflammation and AHR.

It is important to note some limitations of our findings. Although our results define a unique mechanism by which AHR and cell migration into the airway lumen requires endogenously secreted gVPLA2, our data are based on studies in immunosensitized mice. The morphological changes of the airway in this process resemble those of human asthma; however, our data cannot necessarily be extrapolated to the human state given that mice do not spontaneously develop asthma (42). Nonetheless, our data suggest a potential role of human gVPLA2 as a messenger protein in allergic pulmonary inflammation and thus suggest the potential value of evaluating the efficacy of gVPLA2 inhibitors in humans.

In summary, we demonstrate that gVPLA2 inhibition by MCL-3G1, a specific blocking Ab against gVPLA2, causes blockade of inflammatory cell trafficking into the airway lumen and blocks bronchoconstriction to allergen challenge and MCh, a generalized measure of AHR, in immunosensitized airways. Furthermore, MCL-3G1 blocked AHR to both gVPLA2 and MCh when administered after OVA challenge of sensitized mice even in the presence of OVA-induced inflammation. Our data also show that gVPLA2 induces AHR by a mechanism that does not utilize a gIVaPLA2 pathway. Deletion of the gVPLA2 gene reduced allergic pulmonary inflammation and completely blocks the AHR to MCh challenge even in the immunosensitized state. Accordingly, blockade of endogenous secreted gVPLA2 could provide a potential new therapeutic approach for treating diverse phenotypes of human asthma.

Acknowledgment

We are grateful to the Hybridoma Facility, University of Chicago, for the production of MCL-3G1, a mAb directed against human gVPLA2.

Disclosures

The authors have no financial conflict of interest.

References


