Secretory group V phospholipase A2 regulates acute lung injury and neutrophilic inflammation caused by LPS in mice

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Secretory group V phospholipase A2 (sPLA2) is a family of lipolytic enzymes that catalyze the cleavage of fatty acids from the sn-2 position of phospholipids leading to the generation of free fatty acids and lysophospholipids (6, 9, 20, 27, 33, 36, 38). There are at least 12 isoforms of secreted PLA2 in mice (27, 23–27, 33); gVPLA2 binds to the outer cell membrane due to its high affinity to its interfacial binding site located at Trp-31 (13, 16, 17, 23–25). Mutation of Trp-31 to alanine reduces the hydrolytic activity of gVPLA2 (13, 16, 17, 23–25). Internalization of gVPLA2 by binding to heparan sulfate proteoglycan further hydrolyzes the nuclear membrane adding more arachidonic metabolites to the external membrane hydrolysis products, which have been taken up by the cell (16).

Deletion of the gene encoding gVPLA2 attenuates eosinophilic infiltration and airway hyperresponsiveness in allergic mice (24). However, there have been no studies on the effects of gVPLA2 in ALI and neutrophilic inflammation (NI). In this study, we investigated the effect of LPS-induced gVPLA2 on ALI and NI using pla2g5−/− KO and pla2g5+/+ littermate control mice. We found that gVPLA2 synthesis is induced by LPS administration and 2) an association between gVPLA2 activity and the severity of ALI as measured by change in pulmonary compliance, neutrophilic infiltration, and neutrophil activation. Our data are the first demonstration that 14-kDa secretory PLA2s, did not improve survival in patients with severe sepsis (41), even though LY-31592ONAS/S-5920 blocked oleic acid-induced acute lung injury (ALI) in rabbits (10). Previous investigations have reported that snake venom sPLA2 causes ALI in rats (31, 35) and that this enzyme hydrolyzes phospholipids in lung surfactant (5).

Secretory gVPLA2 is a 14-kDa protein and structurally similar to gIIaPLA2 (6, 20, 27, 33, 38). However, gVPLA2 has a 50-fold the hydrolytic capacity for outer cell surface phosphatidylcholine-rich plasma membrane compared with gIIaPLA2 in cell-free systems (13, 15). gVPLA2 is a proinflammatory enzyme that has diverse biological effects including airway inflammation (24), airway hyperresponsiveness (24), cell adhesion (25, 39), transcellular communication (25, 39), and generation of lipid mediators (4, 16, 17, 23, 25, 32, 39).

Secretory gVPLA2, which is not expressed in eosinophils (23, 25, 26, 39), is contained in neutrophils, epithelial cells, airway smooth muscle, T cells, and macrophages (7, 14, 20, 24–27, 33, 36). There is no known receptor for gVPLA2 (4, 20, 23–27, 33); gVPLA2 binds to the outer cell membrane due to its high affinity to its interfacial binding site located at Trp-31 (13, 16, 17, 23–25). Mutation of Trp-31 to alanine reduces the hydrolytic activity of gVPLA2 (13, 16, 17, 23–25). Internalization of gVPLA2 by binding to heparan sulfate proteoglycan further hydrolyzes the nuclear membrane adding more arachidonic metabolites to the external membrane hydrolysis products, which have been taken up by the cell (16).

Deletion of the gene encoding gVPLA2 attenuates eosinophilic infiltration and airway hyperresponsiveness in allergic mice (24). However, there have been no studies on the effects of gVPLA2 in ALI and neutrophilic inflammation (NI). In this study, we investigated the effect of LPS-induced gVPLA2 on ALI and NI using pla2g5−/− KO and pla2g5+/+ littermate control mice. We found that gVPLA2 synthesis is induced by LPS administration and 2) an association between gVPLA2 activity and the severity of ALI as measured by change in pulmonary compliance, neutrophilic infiltration, and neutrophil activation. Our data are the first demonstration that 14-kDa secretory gVPLA2 is an intracellular messenger protein in regulating LPS-induced vascular leak in mice in vivo.

MATERIALS AND METHODS

Animals

Homozygous gVPLA2 KO (pla2g5−/−) and littermate control (pla2g5+/+) were derived from C57BL/6 mice as previously described (4, 32). C57BL/6 mice, aged 10–12 wk old, were maintained on standard laboratory Chow ad libitum. pla2g5−/− were bred in the University of Chicago Animal Facility. Briefly, mice heterozygous for disruption of the gene encoding group V sPLA2 (pla2g5) were crossed

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to a C57/BL6 background. Mating pairs of pla2g5^{−/−} and pla2g5^{+/+} mice were derived from the litters of C57/BL6 heterozygous breeding pairs. Experimental protocols conformed to the principles outlined by the Animal Welfare and the National Health services guidelines for the care and use of animal in biomedical research and were approved by the University of Chicago IACUC Review Board.

**RT-PCR**

An ~2-mm tail piece was cut from each weaning. DNA was isolated using TRIzol reagent (Life Technologies, Rockville, MD), cDNA was synthesized using 0.5 μg of total RNA in a 20-μl reverse transcription reaction using iScript cDNA synthesis kit (Bio-Rad Laboratories; Hercules, CA). The primers for gVPLA2 were sense 5'-GATGCAGCAGCGTTGATAG-3', reverse, 5'-TAATCTAATGGAAGAGCCTCAGGT-3'.

**Determination of gVPLA2 expression**

mRNA expression for gVPLA2. Total RNA was isolated using TRIzol reagent (Life Technologies, Rockville, MD). cDNA was synthesized using 0.5 μg of total RNA in a 20-μl reverse transcriptase reaction using iScript cDNA synthesis kit (Bio-Rad Laboratories; Hercules, CA). The primers for gVPLA2 were sense 5'-GATGCAGCAGCGTTGATAG-3', reverse, 5'-TAATCTAATGGAAGAGCCTCAGGT-3'.

**Immunohistochemistry.** Lungs were fixed by inflation with 10% paraformaldehyde solution at a pressure of 20 cmH2O and embedded in paraffin. Briefly, tissue sections were incubated for 1 h in primary antibody (MCL-3G1) or IgG1 antibody (negative control) diluted in normal saline solution. Antibody binding was prevented by incubating the tissue section for 15 min in 0.5% hydrogen peroxide solution, methanol (vol/vol), and normal goat serum (1/5 dilution). Slides containing lung tissues were incubated for 1 h in primary antibody (MCL-3G1) or IgG1 antibody (negative control) diluted in normal saline solution. Antibody binding was localized with a biotinylated secondary antibody, avidin-conjugated HRP, and diaminobenzidine chromogenic substrate (Vector Laboratories, Burlingame, CA). Stained airway microsections prepared from treated pla2g5^{+/+} or pla2g5^{−/−} mice were examined for the presence of gVPLA2 using light microscopy.

**Marine Model of ALI**

**Preparation of LPS.** Purified LPS from Escherichia coli (Siga-Aldrich, St. Louis, MO) was dissolved in 10 ml of endotoxin-free saline. The mixed solution was placed in a 37°C shaking water bath for 30 min and was subjected to sonication for 2 min in an ultrasonic water bath to obtain a homogenous solution. The LPS stock solution was aliquoted to the desired volume and stored in −20°C. The final dose of LPS used in this study was 10 mg/kg per intratracheal administration.

**Preparation of animals.** gVPLA2 wild-type littermate control (pla2g5^{+/+}) and KO (pla2g5^{−/−}) mice were anesthetized with ketamine-xylazine (100 mg/kg:20 mg/kg) and were instilled through a catheter inserted into the trachea with either sterile saline solution or 10 mg/kg LPS. The dose of LPS was chosen to be consistent with previous studies wherein the greatest neutrophil emigration in the lungs was achieved. Animals were studied 24 h after administration of LPS.

**Generation of Pressure-Volume Curves (Transtracheal Static Compliance)**

Mice weighing 25–32 g were anesthetized with intraperitoneal ketamine-xylazine (100 mg/kg:20 mg/kg). The animals were placed in a supine position, and a tracheostomy was performed using an 18-gauge needle secured with a 3–0 suture. The intubated animal was connected to a computer-controlled small animal ventilator (Flexivent; Scireq, Vancouver, Canada) that delivered a tidal volume of 6 ml/kg at a frequency of 120 breaths/min (24); the mice were allowed to breathe 100% oxygen for 5 min. The ventilator was stopped, and the tube connected to the trachea was clamped to ambient air. Lungs were inflated with ≥30 cmH2O transhastorial pressure, and volume was gradually decreased in 0.1-ml increments. The static lung compliance was determined by recording the lung pressure change associated with each volume change in the system when the trachea was occluded to ambient air (zero flow). Two or more full inflation-deflation cycles were determined for each mouse to achieve a volume history, and the pressure-volume characteristics of the lungs were generated.

**Determination of Wet: Dry Weight Ratio (Lung Fluid Accumulation)**

Animals treated as above were killed, and lungs were immediately excised, blotted dry, and weighed. Lung dry weights were recorded after treatment in an oven for 7 days at 65°C. The lung wet: dry weight ratio was determined by dividing the wet weight by the dry weight. The data were normalized to wild-type (WT) control where 1 = 0 represents no fluid accumulation in the lung.

**Total and Differential Inflammatory Cell Counts**

NI was assessed 24 h after LPS treatment in 1) pla2g5^{+/+} mice alone, no MCL-3G1, 2) pla2g5^{−/−} mice + MCL-3G1 MAb, or 3) pla2g5^{−/−} mice alone. The trachea was exposed and cannulated with an 18-gauge needle; bronchoalveolar lavage (BAL) was performed by injecting 0.8 ml of PBS into the lung and gently aspirating the fluid. The procedure was performed 3X to recover a total volume of 2–3 ml. The BAL fluid samples were centrifuged to obtain the cell pellet until analysis. Cytoslides were prepared and stained with Diff-Quick (Dade Diagnostics, Deerfield, IL), and the total and differential cells counts were determined using a hemocytometer (24).

**Data Analysis**

All values were expressed as means ± SE. The Student’s t-test was used for comparison between two groups. When more than two groups were compared, differences among the groups were determined by one-way ANOVA. The differences between groups were considered significant if P < 0.05.
RESULTS

Expression of Secretory gVPLA2

We first examined the expression of gVPLA2 in airway microsections obtained from gVPLA2 wild-type littermate control (pla2g5+/+) mice 24 h after LPS administration. Representative photomicrographs (n = 6) demonstrated that LPS caused upregulation of gVPLA2 expression as determined by immunohistochemical staining (Fig. 1). Intracellular gVPLA2 was detected in abundant quantities in microsections of pla2g5+/+ airways receiving LPS (Fig. 1A). gVPLA2 was weakly expressed in saline-treated pla2g5+/+ airways or parenchyma (Fig. 1B) compared with LPS-treated pla2g5+/+ mice. Expression of gVPLA2, as indicated by dark brown color, was evident in peripheral airways and lung parenchyma (Fig. 1A). IgG1, an isotype-matched, irrelevant antibody, was used as negative control (Fig. 1A and B).

To confirm further the specificity of MAb directed against gVPLA2, airway microsections from saline- and LPS-treated gVPLA2-KO mice (pla2g5−/−) were stained with MCL-3G1 MAb or IgG1 antibody. Expression of gVPLA2 was neither localized for pla2g5−/− mice receiving saline nor LPS (Fig. 1, C and D). Negative control (IgG1 stained) is shown in Fig. 1, C and D.

We next determined the mRNA expression for gVPLA2 in lung tissues obtained from these same animals by RT-PCR analysis (32). In pla2g5+/+ mice, mRNA expression for gVPLA2 was upregulated after LPS exposure compared with saline-exposed mice (Fig. 2). All data were normalized per 18S housekeeping gene and expressed as the ratio of gVPLA2 mRNA/18S. mRNA expression for gVPLA2 was 0.97 ± 0.36-fold/18S for pla2g5+/+ mice after LPS administration compared with baseline control expression of 0.48 ± 0.20-fold/18S for saline-treated pla2g5+/+ mice (P < 0.05). By contrast, mRNA expression for gVPLA2 was 0.006 ± 0.002-fold/18S for pla2g5−/− mice after LPS treatment (P < 0.001 vs. LPS-treated pla2g5+/+ mice) and 0.005 ± 0.002-fold/18S for pla2g5−/− mice having saline treatment (P < 0.001 vs. LPS-treated pla2g5+/+ mice).

Transthoracic Static Compliance: Pressure-Volume Curves

We next measured the transthoracic static compliance, an index of lung edema caused by ALI. Pressure-volume (P-V) curves were generated in 1) saline-treated pla2g5+/+ mice (n = 7 mice), 2) LPS-treated pla2g5+/+ mice (n = 8 mice), 3) LPS-treated pla2g5−/− mice (n = 6 mice), and 4) MCL-3G1 plus LPS-treated pla2g5+/+ mice (n = 5 mice) in vivo. Lung volume in experimental animals was measured begin-

![Fig. 1. Localization of group V phospholipase A2 (gVPLA2) expression caused by intratracheal administration of LPS or sterile saline in murine airways. gVPLA2 littermate control (pla2g5+/+) or gVPLA2 knockout (pla2g5−/−) mice were treated with LPS or saline control for 24 h, and gVPLA2 expression was determined by immunohistochemical analysis. Microsections of airways and lung parenchyma were stained with either MAb directed against gVPLA2 (A–D, left) or isotype-match control, IgG1 (A–D, right). Representative photomicrographs from 6 airway microsections of pla2g5+/+ mice were shown that gave similar results. Original magnification, ×100 pixel. Ab, antibody.](http://ajplung.physiology.org/)}
Fig. 2. Effect of LPS on mRNA expression for gVPLA2. mRNA expression for gVPLA2 caused by LPS in wild-type (WT) littermate control (pla2g5+/+) or gVPLA2 knockout (pla2g5−/−) mice was measured in lung homogenates by RT-PCR as described in MATERIALS AND METHODS. The upregulation of mRNA was normalized as the ratio of mRNA/18S compared with vehicle-stimulated control airways. RT-PCRs were performed in triplicate. The data represent the means ± SE of 6 samples; *P < 0.05 and **P < 0.001 by Student’s t-test.

LPS-Induced Neutrophilic Inflammation: Histological Examination

Histological examination revealed increased cellular infiltrates in lungs of mice receiving intratracheal LPS (Fig. 5A); these changes were much less pronounced in saline-treated pla2g5+/+ mice (Fig. 5B). Airway neutrophilic inflammation caused by LPS activation was substantially attenuated in gVPLA2 KO mice (pla2g5−/−; Fig. 5C) and in pla2g5+/+ mice treated with MCL-3G1, a MAb directed against gVPLA2 (Fig. 5D) before LPS treatment.

Cell Infiltration in BAL Fluid

Intratracheal instillation of LPS caused pulmonary inflammation characterized by cellular infiltrates into the alveolar space, as reflected by the increased number of neutrophils recovered in the BAL fluid (Fig. 6). NI was attenuated in gVPLA2 KO mice and MCL-3G1-treated mice; NI was not observed in saline-treated pla2g5+/+ mice. Quantification of the number of cellular inflammatory cells in BAL was assessed by differential cell counting after Diff-Quick staining (Fig. 6). However, total BAL fluid cell number was greater in LPS-treated pla2g5+/+ littermate controls than LPS-pretreated mice even after instillation with the same concentration of LPS (Fig. 6A). Baseline (pla2g5+/+ + saline) total cell count was 0.20 × 10⁶ ± 0.05 cells and increased to 5.33 × 10⁶ ± 1.27 cells after LPS administration (P < 0.01). Total cell number caused by LPS in gVPLA2 KO (pla2g5−/−) mice decreased to 2.73 × 10⁶ ± 0.87 cells (P < 0.05 vs. LPS-treated pla2g5+/+ mice) and 3.17 × 10⁶ ± 0.87 cells for wild-type pla2g5+/+ mice receiving MCL-3G1 before LPS exposure (P < 0.05 vs. LPS-treated pla2g5+/+ mice).

Differential cell count demonstrated that neutrophils were the predominant cell type recovered from BAL fluid after LPS (Fig. 6B). Macrophages increased to a lesser extent, but this difference was not statistically significant (Fig. 6C). The number of neutrophils in the BAL fluid increased from 0.04 × 10⁶ ± 0.03 cells (saline-treated) to 5.07 × 10⁶ ± 1.25 cells after LPS administration (P < 0.01). A ±50% reduction in neutrophil migration from basal count was observed in LPS-treated KO (pla2g5−/−) mice (2.43 × 10⁶ ± 0.77 cells; P < 0.05 vs. LPS-treated pla2g5+/+ mice) and 2.95 × 10⁶ ± 0.74 cells in pla2g5−/− treated with MCL-3G1 + LPS (P < 0.05 vs. LPS-treated pla2g5+/+ mice).

LPS-Increased MPO Activity

MPO content, which is linearly proportional to its activity (see MATERIALS AND METHODS), was substantially increased by LPS in pla2g5+/+ mice and attenuated by specific blockade of gVPLA2 and in pla2g5−/− mice (Fig. 7A). Lung MPO concentration, expressed as optical density per 150 μg/ml protein (OD), increased to 1.17 ± 0.02 OD after LPS administration compared with 0.15 ± 0.02 OD for saline-treated pla2g5+/+ mice (Fig. 7A; P < 0.01). The concentration of MPO in pla2g5−/− mice treated with LPS was 0.52 ± 0.09 OD (P < 0.05 vs. LPS-treated pla2g5+/+ mice). Pretreatment of pla2g5+/+ (wild-type) mice with MCL-3G1 caused compara-
ble decrease in MPO concentration after LPS as for \(pla2g5^{-/-}\) mice.

Neutrophils in BAL fluid were identified as likely the source of MPO as determined by flow cytometric analysis (Fig. 7, B–D). Cells contained within the box are granulocytes from BAL fluid after saline or LPS treatment as determined by Gr1, a MAb used to detect the granulocytes including neutrophils. Cells outside the box are other cells in the BAL fluid aside from granulocytes. Granulocytes constituted 88.1% of all cells in the BAL fluid after treatment with LPS. Total cell population in BAL fluid of saline-treated \(pla2g5^{-/-}\) mice showed insignificant numbers of granulocytes (2.04%) as determined by Gr1 MAb staining (Fig. 7C). The isotype-matched IgG (LPS-treated \(pla2g5^{+/+}\) + PE-IgG antibody) served as control for Gr1 MAb alone and also showed no granulocyte infiltrates in BAL fluid (Fig. 7D). Histological examination of cytoslides showed that PMNs were the predominant granulocytes increasing in number in \(pla2g5^{+/+}\) mice.

**DISCUSSION**

The objective of this investigation was to determine the role of the highly hydrolytic phospholipase, gVPLA2, in mediating ALI induced by LPS. Studies were performed to assess whether LPS causes upregulation of gVPLA2 in murine airways. Further studies were performed to determine if this upregulation of gVPLA2 was responsible for decreased trans-thoracic static compliance, increased W/D weight ratio, NI,
and subsequent neutrophil activation caused by LPS. These physiological and inflammatory effects are commonly used to monitor ALI in animal models of ARDS (11, 21).

We and others (25, 39) previously have shown that gVPLA2 is secreted from epithelial cells to cause eosinophil activation and surface integrin adhesion of eosinophils to endothelial counterligands in vitro. Immune sensitization also was shown to upregulate gVPLA2 expression (24). Blockade of gVPLA2 by specific MAb attenuated substantially this effect in vivo and further blocked airway hyperresponsiveness to methacholine (24). In preliminary studies with neutrophils, we have demonstrated that gVPLA2 also is capable of activating neutrophil adhesion (16, 17). Thus, it is likely that activation of neutrophils caused by this protein results from transport of gVPLA2 from activated endothelium by LPS. However, neutrophils, epithelium, macrophages, T cells, or airway smooth muscle also contain gVPLA2 (7, 14, 16, 17, 19, 20, 24).

Secretory gVPLA2 has not been shown to be chemotactic, so the in vivo process for initial activation remains undefined. Because of the very small blood volume of mice, it has not been possible to measure directly the concentration of gVPLA2 after endothelial or epithelial activation in vivo or to determine which tissues participate in gVPLA2 secretion that can be demonstrated in vitro.

From these studies and others demonstrating that neutrophil cytokines and leukotriene B4 activate neutrophil adhesion (16, 17, 22) and subsequent migration, we postulated that upregulation and activation of gVPLA2 by LPS would cause vascular leak and contribute substantially to lung edema in mice. In a first series of studies, we demonstrated that gVPLA2 content of the lung parenchyma was upregulated substantially within 24 h of exposure to LPS (Fig. 1). No upregulation of gVPLA2 content was demonstrated in gVPLA2 KO mice or in isotype-matched controls. The baseline concentration of mRNA in lungs of littermate controls also was substantially upregulated 24 h after tracheal instillation of LPS (Fig. 2). These data indicate that LPS causes synthesis and upregulation of gVPLA2 content within 24 h.

Upregulation of gVPLA2 by LPS also corresponded to decreased transthoracic static compliance (Fig. 3), which corresponded to an increased W/D weight ratio (Fig. 4) in control mice. The decrease in transthoracic static compliance and increase W/D weight ratio was blocked almost completely by deletion of the gene encoding gVPLA2 (gVPLA2 KO mice) and by specific MAb directed against gVPLA2 (MCL-3G1).
These data suggest a substantial role for gVPLA2 in mediation of the response leading to ALI in this murine model. We also found that cell infiltrates (predominantly neutrophils) into the small peripheral airways and alveolar spaces caused by LPS was blocked substantially by MAb directed against gVPLA2 and were substantially attenuated in gVPLA2 KO mice (Fig. 5). gVPLA2 may serve to amplify neutrophil chemotaxis indirectly, possible by activation of smaller numbers of neutrophils elicited by LPS treatment alone (Fig. 6). A model for this process has been suggested from prior in vitro studies of neutrophil activation by gVPLA2 (16, 17). The precise sequence of events in vivo, however, has not yet been elucidated. Nonetheless, blockade of gVPLA2 by either gVPLA2 KO or neutralizing MAb against gVPLA2 substantially attenuated NI caused by LPS. Although substantial, NI was not completely blocked by these processes (Figs. 6 and 7). The absence of complete blockade of either MPO concentration or all cell migration, even in gVPLA2 KO mice, suggests that the pathogenesis of neutrophil activation is not linked solely to gVPLA2, but may also, to a lesser extent, be caused by LPS alone. Nagase and coworkers (28, 29) demonstrated increased lung elastance in mice treated with hydrochloric acid that was blocked by genetic deletion of the intracellular phospholipase gIVaPLA2, which is activated downstream by gVPLA2 in neutrophils (16, 17). In these studies, some neutrophil migration was blocked by gIVaPLA2 KO mice, suggesting that gIVaPLA2 may also play a role in neutrophil migration in vivo. The role of gVPLA2 and gIVaPLA2 in neutrophil migration in vivo is not yet known.
phils still were present in gIVaPLA2 KO mice, even though the indirect measurements of lung compliance were blocked fully and histological sections of lung were similar to those seen in gVPLA2 KO mice (Fig. 5). In these studies, we did not establish the downstream mechanism of gVPLA2 blockade of lung edema caused by LPS. However, it appears that substantial reduction in neutrophil secretion and NI, which is not absolute, may still be sufficient to block the formation of lung edema in ALI.

It is important to consider some further limitations of our findings. These studies are performed in a pathogenetic mouse model, and data cannot be extrapolated to therapeutics in the human situation. We also administered blocking antibody in each study 30 min before LPS administration. It is not clear whether post hoc administration of gVPLA2 MAb would block edema formation. The purpose of this paper was to determine the possible pathogenetic role of gVPLA2 as an effector of edema formation. The purpose of this paper was to determine the possible pathogenetic role of gVPLA2 as an effector of edema formation.

Further studies are also required to establish the downstream mechanism of gVPLA2 blockade of lung edema caused by LPS. However, it appears that substantial lung edema caused by LPS. Blockade of this enzyme or genetic deletion (KO) preventing synthesis of gVPLA2 blocks substantially the NI and increased lung elastance caused by LPS. The potential role of gVPLA2 in the prevention or treatment of ALI and NI in animal models and in humans remains to be assessed.

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