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Blockade of Human Group X Secreted Phospholipase A_2 (GX-sPLA$_2$)-induced Airway Inflammation and Hyperresponsiveness in a Mouse Asthma Model by a Selective GX-sPLA$_2$ Inhibitor*[S]

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Group X (GX) phospholipase A_2, a member of a large group of secreted phospholipases A_2 (sPLA$_2$s), has recently been demonstrated to play an important *in vivo* role in the release of arachidonic acid and subsequent formation of eicosanoids. In a Th2 cytokine-driven mouse asthma model, deficiency of mouse GX (mGX)-sPLA$_2$ significantly impairs development of the asthma phenotype. In this study, we generated mGX-sPLA$_2^{-/-}$ mice with knock-in of human GX (hGX)-sPLA$_2$ (*i.e.* hGX-sPLA$_2^{+/+}$ knock-in mice) to understand more fully the role of GX-sPLA$_2$ in these allergic pulmonary responses and to assess the effect of pharmacological blockade of the GX-sPLA$_2$-mediated responses. Knock-in of hGX-sPLA$_2$ in mGX-sPLA$_2^{-/-}$ mice restored the allergen-induced airway infiltration by inflammatory cells, including eosinophils, goblet cell metaplasia, and hyperresponsiveness to methacholine in the mGX-sPLA$_2$-deficient mice. This knock-in mouse model enabled the use of a highly potent indole-based inhibitor of hGX-sPLA$_2$, RO061606 (which is ineffective against mGX-sPLA$_2$), to assess the potential utility of GX-sPLA$_2$ blockade as a therapeutic intervention in asthma. Delivery of RO061606 via mini-osmotic pumps enabled the maintenance *in vivo* in the mouse asthma model of plasma inhibitor concentrations near 10 μM, markedly higher than the IC$_{50}$ for inhibition of hGX-sPLA$_2$ *in vitro*. RO061606 significantly decreased allergen-induced airway inflammation, mucus hypersecretion, and hyperresponsiveness in the hGX-sPLA$_2^{+/+}$ knock-in mouse. Thus, development of specific hGX-sPLA$_2$ inhibitors may provide a new pharmacological opportunity for the treatment of patients with asthma.

The liberation of arachidonic acid from the *sn*-2 position of cell membrane phospholipids by phospholipases A_2 for the biosynthesis of eicosanoids (prostaglandins, leukotrienes, and others) is a well established process. Studies with genetically altered mice and small molecular weight inhibitors have established that cytosolic phospholipase $A_2\alpha$ (cPLA$_2\alpha$ [3]; group IVA PLA$_2$) can mediate arachidonate release, leading to eicosanoids in a variety of mammalian cells stimulated with a diverse set of agonists (1). There are five other isoforms of this enzyme, but the physiological functions of these isoforms have not been established. The mammalian genome also encodes a large number of secreted PLA$_2$s (sPLA$_2$s) (2). The functions of most of these enzymes are not yet known, but increasing evidence supports a role of the group X (GX) sPLA$_2$ in arachidonate release, leading to eicosanoids.

In a mouse model of asthma that is driven by Th2 cytokines, we found a major effect of mouse GX (mGX)-sPLA$_2$ deficiency (3). In this initial study of allergen (*i.e.* ovalbumin (OVA))-induced airway inflammation in the mGX-sPLA$_2$-deficient mouse, OVA-treated mGX-sPLA$_2^{-/-}$ mice compared with wild-type mice had a marked reduction in interstitial edema and the influx of eosinophils and other inflammatory cells, including CD4$^+$ and CD8$^+$ T cells, into the bronchoalveolar lavage (BAL) fluid and lung tissue. Whereas mGX-sPLA$_2^{+/+}$ mice had significant airway hyperresponsiveness to methacholine and remodeling, including goblet cell metaplasia and mucus hypersecretion after OVA challenge, these features of the asthma phenotype were not present in mGX-sPLA$_2^{-/-}$ mice (3). Th2 cytokine expression is a molecular hallmark of asthma. Levels of Th2 cytokines IL-4, IL-5, and IL-13 in the lungs were decreased in mGX-sPLA$_2^{-/-}$ mice compared with wild-type controls after OVA treatment. Furthermore, the cyclooxygenase products prostaglandin E_2 and prostaglandin D_2 and the 5-lipoxygenase products leukotriene B_4 and cysteinyl leukotrienes C_4, D_4, and E_4 of arachidonic acid metabolism were significantly reduced in mGX-sPLA$_2^{-/-}$ mice after OVA treatment compared with wild-type controls (3). These data indicated that mGX-sPLA$_2$ plays a key role in eicosanoid generation and that the decreased release of arachidonate metabolites secondary to mGX-sPLA$_2$ deficiency impairs the Th2 responses in this asthma model. Thus, development of a selec-

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[3] The abbreviations used are: cPLA$_2\alpha$, cytosolic phospholipase $A_2\alpha$; sPLA$_2$, secreted PLA$_2$; GX, group X; mGX, mouse GX; hGX, human GX; OVA, ovalbumin; BAL, bronchoalveolar lavage.

tive GX-sPLA$_2$ inhibitor may be a novel therapeutic intervention in asthma.

We have begun to study inflammatory cells in culture to better understand how GX-sPLA$_2$ is involved in eicosanoid biosynthesis, including an understanding of how it augments arachidonate release along with cPLA$_2\alpha$. Addition of human GX (hGX)-sPLA$_2$ exogenously to primary human eosinophils leads to cysteinyl leukotriene production in a process that involves an increase in intracellular calcium and activation of MAPK and cPLA$_2\alpha$ (4). The molecular mechanisms for this hGX-sPLA$_2$/cPLA$_2\alpha$ interaction remain to be elucidated, but these cellular studies support our mouse studies, which demonstrate a role of mGX-sPLA$_2$ in eicosanoid formation and airway inflammation in a mouse model of allergic asthma.

In this study, we wanted to take a pharmacological approach to block the action of GX-sPLA$_2$ in a mouse asthma model. This requires an inhibitor that not only is selective among the full set of mammalian sPLA$_2$s but also has sufficiently good pharmacokinetic properties to be used over several days in the mouse asthma model. In our previous work, we synthesized a large number of analogs of the indole-based sPLA$_2$ inhibitors developed by workers at Eli Lilly and Co. (5). In that study, we discovered a highly potent inhibitor that is specific for hGX-sPLA$_2$. This compound unfortunately does not inhibit mGX-sPLA$_2$ for reasons that are apparent from the examination of the x-ray crystal structure of related inhibitors bound to hGX-sPLA$_2$ (5, 6). Thus, in this study, we generated a mouse that expresses hGX-sPLA$_2$ instead of mGX-sPLA$_2$ under the control of the mGX-sPLA$_2$ promoter. In a genetic knock-out, the level of GX-sPLA$_2$ is reduced to zero, and this may be an unrealistic achievement using a small molecular weight inhibitor of the enzyme. Thus, it is interesting to compare results obtained by pharmacological blockade with those obtained in the knock-out. In addition to allowing us to test our hGX-sPLA$_2$-selective inhibitor in a mouse model of allergic asthma, the hGX-sPLA$_2$ knock-in mouse would allow us to test if the airway inflammation that is lost in mGX-sPLA$_2{}^{-/-}$ mice is recovered after introduction of the human enzyme. Genetic knock-outs contain genome elements near the knock-out site from the mouse strain used to generate the gene disruption (129/SvEvBrd in this case), and it is always possible that the phenotype of the mGX-sPLA$_2{}^{-/-}$ mouse is controlled, at least in part, by genome differences between inbred strains of mouse around the GX-sPLA$_2$ locus.

In this study, we found that hGX-sPLA$_2$ can rescue the loss in airway inflammation seen in the mGX-sPLA$_2{}^{-/-}$ mouse. In addition, our highly potent inhibitor of hGX-sPLA$_2$, RO061606, significantly reduced allergen-induced airway inflammation and hyperresponsiveness in a mouse model of asthma. These results indicate that hGX-sPLA$_2$ is an important target amenable for the development of novel anti-asthma drugs.

EXPERIMENTAL PROCEDURES

Reagents—The hGX-specific sPLA$_2$ inhibitor RO061606 was prepared as described (compound 12b) by Oslund *et al.* (5). Recombinant sPLA$_2$s were prepared as described previously (5).

Pharmacokinetic Studies of RO061606—Pharmacokinetic studies of RO061606 were carried out following the procedure described by Kraus *et al.* (7). The compound was dissolved in 5% Me_2SO, 7% Tween 80, and 3% EtOH in sterile PBS at 5 mM. Wild-type mGX-sPLA$_2{}^{+/+}$ C57BL/6J mice (6–8 weeks old) were dosed intraperitoneally at 20 mg of drug/kg of mouse with a volume of 300 μl. At timed intervals of 30, 60, 120, and 360 min, 40 μl of tail blood was collected using a capillary tube. The plasma was isolated from the blood by centrifugation, collected, and stored at −80 °C until analysis by LC-MS as described below.

Drug Delivery Using Mini-osmotic Pumps—To verify that the minipump drug delivery method would provide suitable for drug plasma concentrations, RO061606 plasma concentrations in wild-type C57BL/6J mice dosed via ALZET® Model 2001 mini-osmotic pumps (DURECT Corp., Cupertino, CA) were assessed over a 116-h period. Drug-filled pumps (45 mM RO061606 in 1:1 Me_2SO/propylene glycol, with the compound dissolved first in Me_2SO, followed by addition of propylene glycol) were inserted subcutaneously in mice at two pumps per mouse, and mouse tail blood (~40 μl) was collected at timed intervals of 6, 24, 72, 96, and 116 h with a capillary tube. Plasma was isolated by centrifugation and stored at −80 °C until analysis.

Frozen plasma samples from drug-treated mice were thawed at room temperature and gently stirred on a Vortex mixer; 10 μl of plasma was removed and transferred to a 500-μl Eppendorf tube, followed by addition of 11 μl of acetonitrile containing 400 pmol of LY315920 (prepared as described previously (8)) as the internal standard. The mixture was briefly vortexed, followed by addition of 30 μl of acetonitrile to the sample. The sample was again vortexed to mix all of the components and then centrifuged at 13,500 rpm in a tabletop centrifuge for 10 min. The supernatant was removed and transferred to a new 500-μl Eppendorf tube and concentrated to dryness in a SpeedVac. The sample was then redissolved in 50 μl of 50% CH_3CN and 50% H_2O. The sample was vortexed and centrifuged at 13,500 rpm in a tabletop centrifuge for 5 min. Twenty-five microliters of the sample was transferred to an autosampler glass vial containing a 250-μl volume glass insert (Agilent 5183−2085), and 10 μl of this sample was analyzed by LC-MS. The LC-MS setup consisted of an Agilent HP 1100 chromatograph and a Bruker esquireLC electrospray ion trap mass spectrometer. Liquid chromatography was performed using an Agilent Zorbax SB-C18 column (3.5 μm, 2.1 × 100 mm) with a mobile phase consisting of water, 5% acetonitrile, and 1% acetic acid (solvent A) and acetonitrile and 1% acetic acid (solvent B). The mobile phase gradient consisted of 10% solvent B to 64% solvent B over 9 min at a flow rate of 0.2 ml/min; the flow rate was then increased to 0.35 ml/min with a gradient from 64 to 100% solvent B over 5 min. Drug concentrations were calculated by comparing the integrated peak area of RO061606 with that of the internal standard. The response factor was corrected for by comparing the integrated peak area of a known amount of RO061606 with the integrated peak area of the internal

standard. Integrations were performed using Bruker QUANT analysis software.

Generation of mGX-sPLA$_2$-deficient Mice and hGX-sPLA$_2$ Knock-in (hGX-sPLA$_2^{+/+}$) Mice—Homozygous mGX-sPLA$_2$-deficient mice on a C57BL/6J background were obtained by breeding mGX-sPLA$_2$-deficient mice on a 129/SvEvBrd/C57BL/6J mixed background (9) for 12 generations with wild-type C57BL/6J mice, carrying the heterozygotes forward in the breeding cycle. Finally, heterozygous mice were bred to obtain homozygous mGX-sPLA$_2$-deficient mice.

A transgenic hGX-sPLA$_2^{+/+}$ mouse on a C57BL/6J background in which the cDNA coding for hGX-sPLA$_2$ is inserted into exon 1 of the mGX-sPLA$_2$ gene was generated. This hGX-sPLA$_2^{+/+}$ mouse expresses hGX-sPLA$_2$ under the control of the mGX-sPLA$_2$ promoter. The genomic DNA sequence including the regions surrounding the hGX-sPLA$_2$ cDNA is provided in the supplemental data. The primers used for genotyping are shown in the DNA sequence. The neomycin sequence (used for clonal selection) has been removed using Cre recombinase. After generation of the hGX-sPLA$_2^{+/+}$ knock-in mouse, it was bred for 10 generations with C57BL/6J mice.

To explore the expression of hGX-sPLA$_2$ in the knock-in mouse, we carried out time-resolved immunofluorescence for hGX-sPLA$_2$ protein using a previously reported procedure (10) and mouse testis extracts. The amount of hGX-sPLA$_2$ protein was measured to be 6.7 pg/mg of testis protein for the hGX-sPLA$_2^{+/+}$ mouse *versus* an assay signal corresponding to 0.9 pg/mg of testis protein in the mGX-sPLA$_2$-deficient mouse. (The signal is slightly higher in the wild-type mouse, corresponding to 1.6 pg/mg of testis protein, presumably due to a small amount of cross-reactivity of the hGX-sPLA$_2$-specific IgG with mGX-sPLA$_2$ protein.) These picogram values were obtained from the observed fluorescence signals based on a standard curve prepared with known amounts of recombinant hGX-sPLA$_2$.

Mouse Asthma Model Studies—Wild-type mGX-sPLA$_2^{+/+}$ C57BL/6J mice, mGX-sPLA$_2$-deficient C57BL/6J mice, and hGX-sPLA$_2^{+/+}$ mice also on a C57BL/6J background were immunized intraperitoneally with 100 μg of OVA (0.2 ml of 0.5 mg/ml) complexed with alum on days 0 and 14. Mice were anesthetized intraperitoneally with 0.2–0.3 ml of ketamine (6.5 mg/ml)/xylazine (0.44 mg/ml) in normal saline before receiving an intranasal dose of 100 μg of OVA (0.05 ml of 2 mg/ml) on day 14 and 50 μg of OVA (0.05 ml of 1 mg/ml) on days 26–28. Control groups received 0.2 ml of normal saline with alum intraperitoneally on days 0 and 14 and 0.05 ml of saline without alum intranasally on days 14 and 26–28.

To determine the effect of hGX inhibition in the OVA-treated hGX-sPLA$_2^{+/+}$ mice, RO061606 was dissolved at a concentration of 45 mM in 50% Me$_2$SO and 50% propylene glycol by first dissolving the compound in Me$_2$SO (ACS-grade, Fischer), followed by addition of propylene glycol (Sigma). The inhibitor solution was transferred into an ALZET® Model 2001 mini-osmotic pump following the manufacturer's instructions. The pumps were inserted subcutaneously at two pumps per hGX-sPLA$_2^{+/+}$ mouse on day 25 of the OVA challenge model described above.

On day 29, mice from all study groups underwent pulmonary function testing for assessment of airway hyperresponsiveness to aerosolized methacholine and collection of BAL fluid and lung tissue for determination of airway inflammation, edema, and mucus hypersecretion. To verify that drug was present during the final OVA challenge phase in the hGX-sPLA$_2^{+/+}$ mice administered RO061606, blood from these mice was also collected on day 29 and used for LC-MS analysis as described above.

Pulmonary Function Testing—On day 29 (24 h after the last intranasal challenge with OVA or saline), invasive pulmonary mechanics were measured in mice in response to methacholine as described previously (3). Mice received aerosolized solutions of methacholine (0, 0.78125, 1.5625, 3.125, 6.25, and 12.5 mg/ml in normal saline), and lung resistance was determined from measures of pressure and flow and expressed as centimeters of H_2O/ml/s using a commercial plethysmography system (resistance/compliance system with a Model PLY4111 plethysmograph and BioSystem XA software, Buxco Research Systems).

BAL Fluid and Blood Collection—After completion of plethysmography on day 29, the left lung was tied off at the mainstem bronchus, and the right lung was lavaged three times with 0.5 ml of normal saline. After centrifugation at 250 $\times$ *g* for 5 min at 4 °C, total BAL fluid cells were counted with eosinophils stained with 0.05% eosin (11). In the OVA-treated hGX-sPLA$_2^{+/+}$ mouse group administered RO061606, blood was collected, and the plasma was isolated by centrifugation and stored at −80 °C until analysis of drug levels.

Lung Morphology—The upper and lower lobes of the left lung were fixed for 24 h in 10% neutral buffered formalin solution. The tissues were embedded in paraffin and cut into 5-μm sections. For lung morphometry, 10 airways (0.4–0.7 mm in diameter and surrounded by smooth muscle cells) per mouse were randomly selected and examined by the histopathologist (Y. T. T.) blinded to the protocol design (11). The sections were stained with hematoxylin and eosin to evaluate airway total inflammatory cell infiltration (11) on a semiquantitative scale ranging from 0 to 4+, and the number of neutrophils and eosinophils per unit of lung tissue area (*i.e.* 2200 μm^2 of tissue area) and edema were determined as described previously (12). The sections were stained with Alcian blue/periodic acid-Schiff reaction to identify airway goblet cells and mucus (11). The number of goblet cells was determined as the percent of total airway epithelial cells in each airway examined. Mucus occlusion of the airway diameter was assessed on a 0–4+ scale, with each airway section assigned a score for airway diameter occlusion by mucus as described previously (11).

RESULTS

Generation and Characterization of the hGX-sPLA$_2^{+/+}$ Knock-in Mouse—We generated a transgenic mouse in which the cDNA coding for hGX-sPLA$_2$ replaces exon 1 of the mGX-sPLA$_2$ gene. This mouse expresses hGX-sPLA$_2$ under the control of the mGX-sPLA$_2$ promoter. As described under "Experimental Procedures," the expression of hGX-sPLA$_2$ was verified in mouse testes by time-resolved

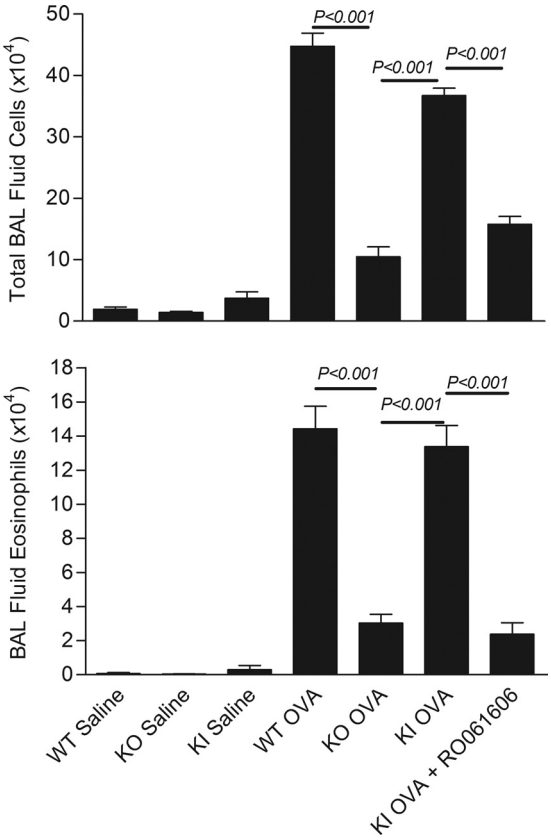

FIGURE 1. **Restoration of allergen-induced inflammatory cell infiltration of BAL fluid in mGX-sPLA$_2$-deficient mice by hGX-sPLA$_2$ knock-in and its blockade by RO061606.** BAL fluid was obtained on day 29 from saline- and OVA-treated GX-sPLA$_2^{+/+}$, mGX-sPLA$_2^{-/-}$, and hGX-sPLA$_2^{+/+}$ mice (in the absence or presence of RO061606 minipump administration), and the number of total inflammatory cells (*upper panel*) and eosinophils (*lower panel*) was determined. Data represent the mean ± S.E. (n = 5–8 per group). A one-way analysis of variance test was used. *KO*, knock-out; *KI*, knock-in.

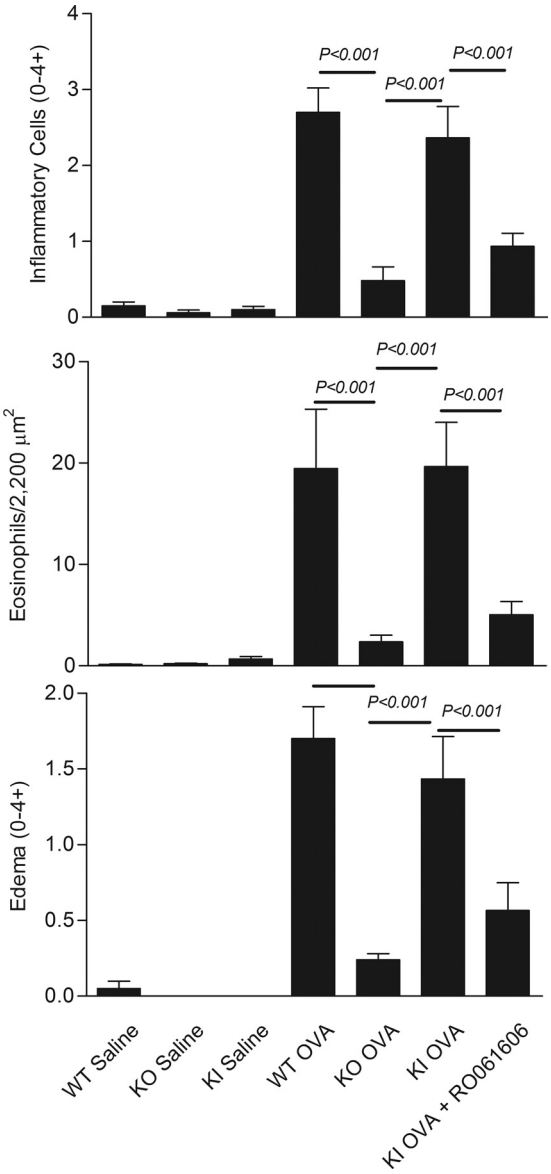

FIGURE 2. **Restoration of allergen-induced airway inflammation and edema in mGX-sPLA$_2$-deficient mice by hGX-sPLA$_2$ knock-in and its blockade by RO061606.** The intensity of the total inflammatory cell infiltrate (0–4+ scale), number of eosinophils per unit area (2200 μm^2), and airway edema (0–4+ scale) in the lungs on day 29 of the study groups described in the legend to Fig. 1 were determined by morphometric analysis. Data represent the mean ± S.E. (n = 5–8 per group). A one-way analysis of variance test was used. *KO*, knock-out; *KI*, knock-in.

immunofluorescence ELISA using an antiserum specific for this protein. The mRNA for hGX-sPLA$_2$ was also detected by RT-PCR analysis of mouse spleen, thymus, and embryonic stem cells obtained from the hGX-sPLA$_2^{+/+}$ knock-in mouse (data not shown).

Restoration of Allergen-induced Airway Inflammation and Hyperresponsiveness by Knock-in of hGX-sPLA$_2$ in the mGX-sPLA$_2$-deficient Mouse—On day 29, 24 h following the final intranasal OVA challenge in animals from each experimental group, the effect of hGX-sPLA$_2$ knock-in on allergen-induced airway inflammation and hyperreactivity in the mGX-sPLA$_2$-deficient mouse was determined. OVA-treated mGX-sPLA$_2^{+/+}$ mice had a large increase in total inflammatory cells recovered in BAL fluid compared with the saline control group (4.5×10^5/ml in OVA-treated mGX-sPLA$_2^{+/+}$ mice *versus* 0.2×10^5/ml in saline-treated controls; $p < 0.0001$) (Fig. 1, *upper panel*); ~27% of the BAL fluid cells were eosinophils in the OVA-treated mGX-sPLA$_2^{+/+}$ mice (Fig. 1, *lower panel*). As we have previously shown in mGX-sPLA$_2^{-/-}$ mice on a mixed 129/SvEvBrd/C57BL/6J background (9), the total number of inflammatory cells and eosinophils in the BAL fluid of OVA-treated mGX-sPLA$_2$-deficient mice on a pure C57BL/6J background was significantly reduced compared with that in wild-type controls (Fig. 1). Compared with saline-treated controls (Figs. 2 and 3 and supplemental Fig. 1, *upper panels*), a marked infiltration of inflammatory cells (eosinophils, neutrophils, and mononuclear cells) around the airways and blood vessels and interstitial edema (Fig. 2 and supplemental Fig. 1, *middle left panel*) with goblet cell metaplasia and mucus occlusion of the airways (Fig. 3 and supplemental Fig. 1, *middle right panel*) were observed in OVA-treated mGX-sPLA$_2^{+/+}$ mice. These features of allergen-driven airway inflammation and mucus hypersecretion were substantially decreased in OVA-treated mGX-sPLA$_2^{-/-}$ mice compared with wild-type mGX-sPLA$_2^{+/+}$ controls (Figs. 1–3 and supplemental Fig. 1, *lower panels*). Knock-in of hGX-sPLA$_2$ in the mGX-sPLA$_2^{-/-}$ mouse

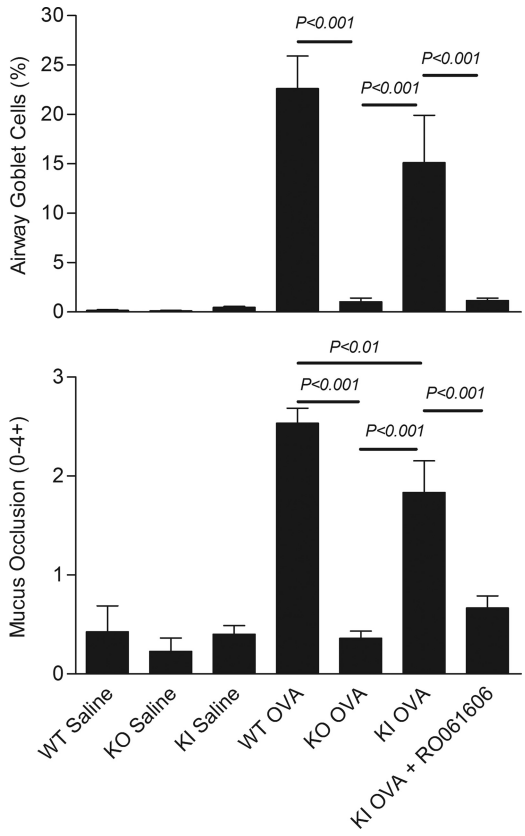

FIGURE 3. **Restoration of allergen-induced goblet cell metaplasia and mucus hypersecretion in mGX-sPLA$_2$-deficient mice by hGX-sPLA$_2$ knock-in and its blockade by RO061606.** The percentage of total airway epithelial cells positive for mucus glycoproteins and occlusion of airway diameter by mucus (0–4+ scale) in the lungs on day 29 of the study groups described in the legend to Fig. 1 were determined by morphometric analysis. Data represent the mean ± S.E. (n = 5–8 per group). A one-way analysis of variance test was used. *KO*, knock-out; *KI*, knock-in.

restored the OVA-induced airway inflammatory responses. Compared with saline-treated controls (Fig. 4, *upper left panel*), the OVA-treated hGX-sPLA$_2^{+/+}$ mice had significantly increased total inflammatory cells and eosinophils in the BAL fluid (Fig. 1) and lung tissue (Figs. 2 and 4) and airway edema (Figs. 2 and 4). Airway goblet cell number and mucus release were significantly augmented in hGX-sPLA$_2^{+/+}$ mice after allergen treatment (Figs. 3 and 4, *middle right panel*) compared with saline controls (Figs. 3 and 4, *upper right panel*).

Whereas airway hyperreactivity to aerosolized methacholine (*i.e.* lung resistance assessed by invasive plethysmography) was significantly increased in the OVA-treated mGX-sPLA$_2^{+/+}$ mice compared with saline controls, lung resistance responses in the mGX-sPLA$_2^{-/-}$ mice were not significantly different between the OVA and saline treatment groups (Fig. 5). Airway hyperreactivity was restored in the hGX-sPLA$_2^{+/+}$ mice (Fig. 5).

Pharmacokinetic Studies of the hGX-sPLA$_2$-specific Inhibitor RO061606 in Mice—Prior to evaluation of our hGX-sPLA$_2$-specific inhibitor in the mouse model of allergic asthma, we needed to evaluate the pharmacokinetic profile of the compound in mice. As shown in Fig. 6, a single intraperitoneal dose of RO061606 in C57BL/6J mice led to a peak plasma concentration of ~40 μM, followed by a decline in inhibitor over several hours (inhibitor levels measured by LC-MS of plasma treated with acetonitrile to precipitate proteins). The mechanism of plasma clearance was not investigated. Because the allergic asthma model studied here requires multiday dosing with the allergen OVA and with the goal to maintain RO061606 in plasma at concentrations higher than ~1 μM (well above the IC$_{50}$ for inhibition of hGX-sPLA$_2$ *in vitro* (5)), we used implantable mini-osmotic pumps to provide continuous delivery of the inhibitor over several days. As shown in supplemental Fig. 2, when two C57BL/6J mice were dosed with RO061606 via mini-osmotic pumps, inhibitor concentrations near 10 μM were maintained in plasma for at least 5.5 days. This seems to be a reasonable dose for studies with OVA-induced airway inflammation. Approximately 90% of RO061606 was expected to be bound to serum albumin based on our observed shift in the *in vitro* IC$_{50}$ of RO061606 for hGX-sPLA$_2$ when albumin was present in the assay (data not shown). Thus, a dose of 10 μM represents ~10-fold the *in vitro* IC$_{50}$ of RO061606 for hGX-sPLA$_2$, which should ensure that most of the enzyme is inhibited. On the basis of these data, we pursued studies of OVA-dependent airway inflammation in mice dosed over extended days with RO061606 via osmotic minipumps.

Blockade of Allergen-induced Airway Inflammation and Hyperresponsiveness in the hGX-sPLA$_2^{+/+}$ Knock-in Mouse by RO061606—To determine the effect of the hGX-sPLA$_2$-specific inhibitor RO061606 on the asthma phenotype, RO061606 was administered to OVA-sensitized hGX-sPLA$_2^{+/+}$ mice beginning on day 25 prior to the intranasal OVA challenge period on days 26–28, with airway inflammation and pulmonary responsiveness assessed on day 29. The robust asthma phenotype seen in OVA-challenged hGX-sPLA$_2^{+/+}$ mice without RO061606 treatment as described above was markedly diminished by RO061606, with significant reductions observed in allergen-induced infiltration of the BAL fluid (Fig. 1) and lung tissue (Figs. 2 and 4, *lower left panel*), airway edema (Figs. 2 and 4, *lower left panel*), goblet cell metaplasia and mucus hypersecretion (Figs. 3 and 4, *lower right panel*), and airway hyperresponsiveness to methacholine (Fig. 5) by hGX-sPLA$_2^{+/+}$ inhibition. We also checked mice used in these experiments for the presence of RO061606 in plasma. As shown in supplemental Fig. 3, the hGX-sPLA$_2$ inhibitor was present at 7–20 μM in plasma on day 29 in five mice.

We did not test the hGX-sPLA$_2$ inhibitor in wild-type mice, which express mGX-sPLA$_2$. The inhibitor had some affinity for the mouse enzyme (IC$_{50}$ = 2 μM) (data not shown), and thus, the presence of the inhibitor at ~10 μM in plasma may result in some inhibition of the mouse enzyme. The use of the hGX-sPLA$_2^{+/+}$ mice ensured that most, if not all, of the human enzyme would be inhibited because the IC$_{50}$ for the human enzyme is 50 nM.

DISCUSSION

Higher sPLA$_2$ activity is present in the BAL fluid of asthmatics compared with controls (13), and sPLA$_2$ activity increases in the nasal lavage fluid (14) and BAL fluid (15, 16) of asthmatic patients after specific allergen challenge. In recent studies to

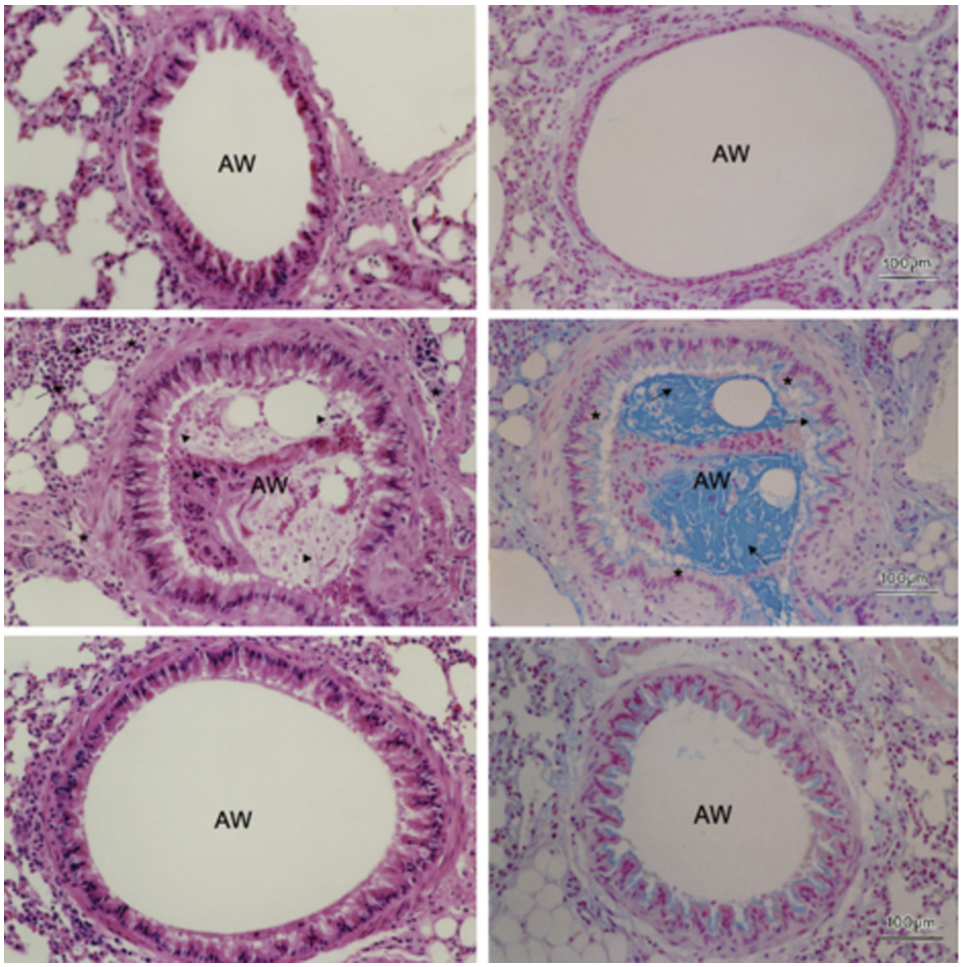

FIGURE 4. **Restoration of the asthma phenotype in mGX-sPLA$_2$-deficient mice by hGX-sPLA$_2$ knock-in and its blockade by RO061606.** Lung sections of hGX-sPLA$_2^{+/+}$ mice administered saline (*upper panels*) or OVA (*middle* and *lower panels*) in the absence (*middle panels*) or presence (*lower panels*) of RO061606 treatment were stained with hematoxylin and eosin (*left panels*) or Alcian blue/periodic acid-Schiff (*right panels*). The airways (*AW*) of saline-treated hGX-sPLA$_2^{+/+}$ mice were free of inflammatory cells (*upper left panel*) and mucus (*upper right panel*). In contrast, the airways of OVA-treated hGX-sPLA$_2^{+/+}$ mice were surrounded by eosinophils and other inflammatory cells (*arrows*), with edema (*asterisks*) in the lung interstitium and occlusion of the airway lumen by mucus (arrowheads) noted (*middle left panel*). The sPLA$_2^{+/+}$ mice after OVA treatment exhibited hyperplasia of airway goblet cells (*asterisks*) and mucus hypersecretion (*arrows*), as shown in the *middle right panel*. The inflammatory cell infiltration (*lower left panel*) and mucus release (*lower right panel*) were markedly reduced in the OVA-treated hGX-sPLA$_2^{+/+}$ mice after RO061606 treatment. *Scale bars* = 100 μm. Representative sections are shown from $n = 5–8$ per group.

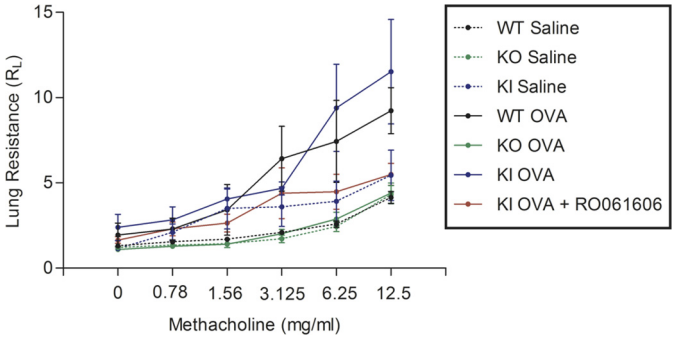

FIGURE 5. **Restoration of allergen-induced airway hyperresponsiveness in mGX-sPLA$_2$-deficient mice by hGX-sPLA$_2^{+/+}$ knock-in is blocked by RO061606.** Allergen-induced airway hyperresponsiveness was assessed by invasive plethysmography on day 29 in saline- and OVA-treated GX-sPLA$_2^{+/+}$, mGX-sPLA$_2^{-/-}$, and hGX-sPLA$_2$ knock-in mice (in the absence or presence of RO061606 treatment) as the degree of bronchoconstriction in response to aerosolized methacholine (0, 0.78125, 1.5625, 3.125, 6.25, and 12.5 mg/ml). Lung resistance was calculated as described under "Experimental Procedures" and is shown as the percentage of the base-line response to aerosolized normal saline. Data represent the mean ± S.E. ($n = 5–8$ per group). *KO*, knock-out; *KI*, knock-in.

identify the sPLA$_2$s associated with asthma, we found that the sPLA$_2$ activity in the BAL fluid of asthmatics was primarily either GX-sPLA$_2$ or GIIA-sPLA$_2$ (17). The levels of GX-sPLA$_2$, but not GIIA-sPLA$_2$, correlated with asthma severity, airway inflammation (sputum neutrophils), and eicosanoid release (BAL fluid prostaglandin E_2); greater expression of GX-sPLA$_2$ compared with other sPLA$_2$s (GIIA-sPLA$_2$ and GV-sPLA$_2$) was also detected in the airway epithelia of the asthmatics (17). We previously found that GX-sPLA$_2$ was differentially overexpressed in the airway cells (airway epithelial cells and bronchial macrophages) of subjects with asthma and exercise-induced bronchoconstriction compared with other human sPLA$_2$s, with further increase in the airways of GX-sPLA$_2$ in these subjects after exercise challenge (9). Genome-wide expression profiling of this group of asthmatics with exercise-induced bronchoconstriction compared with asthmatic individuals without exercise-induced bronchoconstriction demonstrated that *TGM2* (transglutaminase 2) was the gene most differentially expressed at

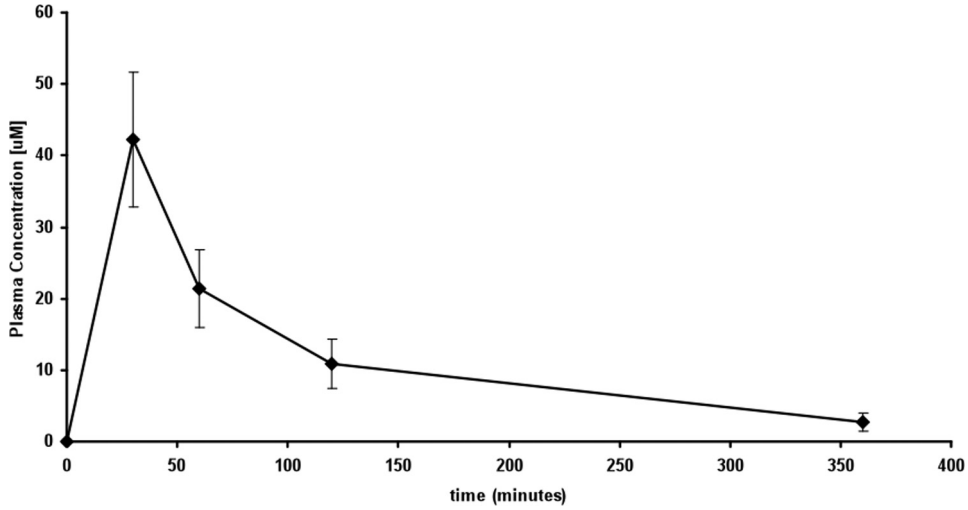

FIGURE 6. **Pharmacokinetics of RO061606 in mice.** Mice were dosed intraperitoneally at 20 mg/kg, and plasma levels were monitored for RO061606 at the indicated time points. Data represent the mean ± S.D. ($n = 3$).

base line between these two groups of asthmatics, with increased expression of *TGM2* found in airway epithelial brushing from the asthmatics with exercise-induced bronchoconstriction compared with non-asthmatic control subjects (18). Recent *in vitro* studies have demonstrated that recombinant human *TGM2* significantly augments the enzyme activity of GX-sPLA$_2$ (18) and that recombinant hGX-sPLA$_2$ induces the release of arachidonic acid and rapid synthesis of cysteinyl leukotrienes by human eosinophils, a p38- and JNK MAPK-mediated process that is blocked by the selective GX-sPLA$_2$ inhibitor ROC-0929 (4). These studies suggest an important role for GX-sPLA$_2$ in asthma pathogenesis by augmenting eicosanoid release, in particular, the proinflammatory cysteinyl leukotrienes involved in airway inflammation, remodeling, and hyperresponsiveness in this pulmonary disorder.

The functional association of GX-sPLA$_2$ and asthma was demonstrated in our previous study of allergen-induced airway inflammation in mice genetically deficient in mGX-sPLA$_2$ (3). The results of this previous study are made stronger by the present results showing that expression of hGX-sPLA$_2$ under the control of the mGX-sPLA$_2$ promoter restores airway inflammation (mucus production, infiltration of inflammatory cells, and airway hyperresponsiveness) in mice lacking mGX-sPLA$_2$. Furthermore, we have shown that this hGX-sPLA$_2$-augmented airway inflammation can be blocked by a specific inhibitor of this enzyme when delivered continuously into the blood over the allergen challenge period. This result shows that it is possible to reduce asthma-like symptoms in a mouse model of asthma with a specific hGX-sPLA$_2$ inhibitor, which in turn suggests that inhibitors of this sPLA$_2$ may offer therapeutic benefit to humans suffering from asthma. Studies are under way to better understand the cellular basis of hGX-sPLA$_2$ involvement in eicosanoid production and airway inflammation, which seems warranted given the dramatic phenotypes seen in mGX-sPLA$_2$-deficient mice and the pharmacological results obtained in this study.

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