Blockade of Human Group X Secreted Phospholipase A2 (GX-sPLA2)-induced Airway Inflammation and Hyperresponsiveness in a Mouse Asthma Model by a Selective GX-sPLA2 Inhibitor*§

William R. Henderson, Jr.*1, Rob C. Oslund§, James G. Bollinger§, Xin Ye‡, Ying-tzang Tien§¶2, Jun Xue§, and Michael H. Gelb*§2

From the §Center for Allergy and Inflammation, Department of Medicine, and the Departments of ¶Chemistry and ¶¶Biochemistry, University of Washington, Seattle, Washington 98195-1700

Group X (GX) phospholipase A2, a member of a large group of secreted phospholipases A2 (sPLA2s), 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)-sPLA2 significantly impairs development of the asthma phenotype. In this study, we generated mGX-sPLA2−/− mice with knock-in of human GX (hGX)-sPLA2 (i.e. hGX-sPLA2+/+) knock-in mice) to understand more fully the role of GX-sPLA2 in these allergic pulmonary responses and to assess the effect of pharmacological blockade of the GX-sPLA2-mediated responses. Knock-in of hGX-sPLA2 in mGX-sPLA2−/− mice restored the allergen-induced airway inflammation by inflammatory cells, including eosinophils, goblet cell metaplasia, and hyperresponsiveness to methacholine in the mGX-sPLA2-deficient mice. This knock-in mouse model enabled the use of a highly potent indole-based inhibitor of hGX-sPLA2, RO061606 (which is ineffective against mGX-sPLA2), to assess the potential utility of GX-sPLA2 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 IC50 for inhibition of hGX-sPLA2 in vitro. RO061606 significantly decreased allergen-induced airway inflammation, mucus hypersecretion, and hyperresponsiveness in the hGX-sPLA2+/+ knock-in mouse. Thus, development of specific hGX-sPLA2 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 A2 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 A2α (cPLA2α; group IVA PLA2) 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 PLA2s (sPLA2s) (2). The functions of most of these enzymes are not yet known, but increasing evidence supports a role of the group X (GX) sPLA2 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)-sPLA2, deficiency (3). In this initial study of allergen (i.e. ovalbumin (OVA))-induced airway inflammation in the mGX-sPLA2-deficient mouse, OVA-treated mGX-sPLA2−/− 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-sPLA2+/+ 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-sPLA2−/− 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-sPLA2−/− mice compared with wild-type controls after OVA treatment. Furthermore, the cyclooxygenase products prostaglandin E2 and prostaglandin D2 and the 5-lipoxygenase products leukotriene B4 and cysteiny1 leukotrienes C4, D4, and E4 of arachidonic acid metabolism were significantly reduced in mGX-sPLA2−/− mice after OVA treatment compared with wild-type controls (3). These data indicated that mGX-sPLA2 plays a key role in eicosanoid generation and that the decreased release of arachidonate metabolites secondary to mGX-sPLA2 deficiency impairs the Th2 responses in this asthma model. Thus, development of a selective

* This work was supported, in whole or in part, by National Institutes of Health Grants R01 AI42989 (to W. R. H.) and R37 HL036235 (to M. H. G.).

§ The on-line version of this article (available at http://www.jbc.org) contains supplemental data and Figs. 1–3.

1 To whom correspondence may be addressed: Center for Allergy and Inflammation, UW Medicine at South Lake Union, University of Washington, 815 Mercer St., Seattle, WA 98109-4714. Tel.: 206-543-3780; Fax: 206-685-9318; E-mail: wrhchem@u.washington.edu.

2 To whom correspondence may be addressed: Depts. of Chemistry and Biochemistry, University of Washington, Campus Box 351700, Seattle, WA 98195-1700. Tel.: 206-543-7142; Fax: 206-685-8665; E-mail: gelb@chem.washington.edu.

3 The abbreviations used are: cPLA2α, cytosolic phospholipase A2α; sPLA2, secreted PLA2; GX, group X; mGX, mouse GX; hGX, human GX; OVA, ovalbumin; BAL, bronchoalveolar lavage.
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tive GX-sPLA₂ inhibitor may be a novel therapeutic intervention in asthma.

We have begun to study inflammatory cells in culture to better understand how GX-sPLA₂ is involved in eicosanoid biosynthesis, including an understanding of how it augments arachidonate release along with cPLA₂. Addition of human GX (hGX)-sPLA₂ exogenously to primary human eosinophils leads to cysteinyll leukotriene production in a process that involves an increase in intracellular calcium and activation of MAPK and cPLA₂ that involves an increase in intracellular calcium and activation of MAPK and cPLA₂ (4). The molecular mechanisms for this hGX-sPLA₂/cPLA₂ interaction remain to be elucidated, but these cellular studies support our mouse studies, which demonstrate a role of mGX-sPLA₂ 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-sPLA₂ in a mouse asthma model. This compound unfortunately does not inhibit mGX-sPLA₂ for reasons that are apparent from the examination of the x-ray crystal structure of related inhibitors bound to hGX-sPLA₂ (5, 6). Thus, in this study, we generated a mouse that expresses hGX-sPLA₂ instead of mGX-sPLA₂ under the control of the mGX-sPLA₂ promoter. In a genetic knock-out, the level of GX-sPLA₂ 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-sPLA₂-selective inhibitor in a mouse model of allergic asthma, the hGX-sPLA₂ knock-in mouse would allow us to test if the airway inflammation that is lost in mGX-sPLA₂ 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-sPLA₂⁻/⁻ mouse is controlled, at least in part, by genome differences between inbred strains of mouse around the GX-sPLA₂ locus.

In this study, we found that hGX-sPLA₂ can rescue the loss in airway inflammation seen in the mGX-sPLA₂⁻/⁻ mouse. In addition, our highly potent inhibitor of hGX-sPLA₂, RO061606, significantly reduced allergen-induced airway inflammation and hyperresponsiveness in a mouse model of asthma. These results indicate that hGX-sPLA₂ is an important target amenable for the development of novel anti-asthma drugs.

EXPERIMENTAL PROCEDURES

Reagents—The hGX-specific sPLA₂ inhibitor RO061606 was prepared as described (compound 12b) by Oslund et al. (5). Recombinant sPLA₂s 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% Me₂SO, 7% Tween 80, and 3% EtOH in sterile PBS at 5 mM. Wild-type mGX-sPLA₂⁻/⁻ 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 Me₂SO/proplyene glycol, with the compound dissolved first in Me₂SO, 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% CH₃CN and 50% H₂O. 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.
standard. Integrations were performed using Bruker QUANT analysis software.

**Generation of mGX-sPLA2-deficient Mice and hGX-sPLA2 Knock-in (hGX-sPLA2+/−) Mice**—Homozygous mGX-sPLA2-deficient mice on a C57BL/6j background were obtained by breeding mGX-sPLA2-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-sPLA2-deficient mice.

A transgenic hGX-sPLA2+/− mouse on a C57BL/6j background in which the cDNA coding for hGX-sPLA2 is inserted into exon 1 of the mGX-sPLA2 gene was generated. This hGX-sPLA2+/− mouse expresses hGX-sPLA2 under the control of the mGX-sPLA2 promoter. The genomic DNA sequence including the regions surrounding the hGX-sPLA2 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 "typing are shown in the DNA sequence. The neomycin

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-sPLA2+/− 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 H2O/ml/s using a commercial plethysmograph 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 × 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-sPLA2+/− 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 μm² 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-sPLA2+/− Knock-in Mouse**—We generated a transgenic mouse in which the cDNA coding for hGX-sPLA2 replaces exon 1 of the mGX-sPLA2 gene. This mouse expresses hGX-sPLA2 under the control of the mGX-sPLA2 promoter. As described under “Experimental Procedures,” the expression of hGX-sPLA2 was verified in mouse testes by time-resolved
immunofluorescence ELISA using an antiserum specific for this protein. The mRNA for hGX-sPLA2 was also detected by RT-PCR analysis of mouse spleen, thymus, and embryonic stem cells obtained from the hGX-sPLA2+/−/− knock-in mouse (data not shown).

Restoration of Allergen-induced Airway Inflammation and Hyperresponsiveness by Knock-in of hGX-sPLA2 in the mGX-sPLA2-deficient Mouse—On day 29, 24 h following the final intranasal OVA challenge in animals from each experimental group, the effect of hGX-sPLA2 knock-in on allergen-induced airway inflammation and hyperreactivity in the mGX-sPLA2−/−/− mouse was determined. OVA-treated mGX-sPLA2−/−/− mice had a large increase in total inflammatory cells recovered in BAL fluid compared with the saline control group (4.5 × 10⁵/ml in OVA-treated mGX-sPLA2−/−/− mice versus 0.2 × 10⁵/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-sPLA2−/−/− mice (Fig. 1, lower panel). As we have previously shown in mGX-sPLA2−/−/− 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-sPLA2−/−/− 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-sPLA2−/−/− mice. These features of allergen-driven airway inflammation and mucus hypersecretion were substantially decreased in OVA-treated mGX-sPLA2−/−/− mice compared with wild-type mGX-sPLA2−/−/− controls (Figs. 1–3 and supplemental Fig. 1, lower panels). Knock-in of hGX-sPLA2 in the mGX-sPLA2−/−/− mouse
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restored the OVA-induced airway inflammatory responses. Compared with saline-treated controls (Fig. 4, upper left panel), the OVA-treated hGX-sPLA2+/+ 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-sPLA2+/+ 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-sPLA2−/− mice with saline controls, lung resistance responses in the mGX-sPLA2−/− mice were not significantly different between the OVA and saline treatment groups (Fig. 5). Airway hyperreactivity was restored in the hGX-sPLA2−/− mice (Fig. 5).

Pharmacokinetic Studies of the hGX-sPLA2-specific Inhibitor RO061606 in Mice—Prior to evaluation of our hGX-sPLA2-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/6 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 IC50 for inhibition of hGX-sPLA2 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 IC50 of RO061606 for hGX-sPLA2 when albumin was present in the assay (data not shown). Thus, a dose of 10 μM represents ~10-fold the in vitro IC50 of RO061606 for hGX-sPLA2, 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-sPLA2+/+ Knock-in Mouse by RO061606—To determine the effect of the hGX-sPLA2-specific inhibitor RO061606 on the asthma phenotype, RO061606 was administered to OVA-sensitized hGX-sPLA2+/+ 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-sPLA2+/+ 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-sPLA2+/+ inhibition. We also checked mice used in these experiments for the presence of RO061606 in plasma. As shown in supplemental Fig. 3, the hGX-sPLA2 inhibitor was present at 7–20 μM in plasma on day 29 in five mice.

We did not test the hGX-sPLA2 inhibitor in wild-type mice, which express mGX-sPLA2. The inhibitor had some affinity for the mouse enzyme (IC50 = 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-sPLA2+/+ mice ensured that most, if not all, of the human enzyme would be inhibited because the IC50 for the human enzyme is 50 nM.

DISCUSSION

Higher sPLA2 activity is present in the BAL fluid of asthmatics compared with controls (13), and sPLA2 activity increases in the nasal lavage fluid (14) and BAL fluid (15, 16) of asthmatic patients after specific allergen challenge. In recent studies to
identify the sPLA2s associated with asthma, we found that the sPLA2 activity in the BAL fluid of asthmatics was primarily either GX-sPLA2 or GIIA-sPLA2 (17). The levels of GX-sPLA2, but not GIIA-sPLA2, correlated with asthma severity, airway inflammation (sputum neutrophils), and eicosanoid release (BAL fluid prostaglandin E2); greater expression of GX-sPLA2 compared with other sPLA2s (GIIA-sPLA2 and GV-sPLA2) was also detected in the airway epithelia of the asthmatics (17). We previously found that GX-sPLA2 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 sPLA2s, with further increase in the airways of GX-sPLA2 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) was the gene most differentially expressed at
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-sPLA2 (18) and that recombinant hGX-sPLA2 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-sPLA2 inhibitor ROC-0929 (4). These studies suggest an important role for GX-sPLA2 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-sPLA2 and asthma was demonstrated in our previous study of allergen-induced airway inflammation in mice genetically deficient in mGX-sPLA2 (3). The results of this previous study are made stronger by the present results showing that expression of hGX-sPLA2 under the control of the mGX-sPLA2 promoter restores airway inflammation (mucus production, infiltration of inflammatory cells, and airway hyperresponsiveness) in mice lacking mGX-sPLA2. Furthermore, we have shown that this hGX-sPLA2-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-sPLA2 inhibitor, which in turn suggests that inhibitors of this sPLA2 may offer therapeutic benefit to humans suffering from asthma. Studies are under way to better understand the cellular basis of hGX-sPLA2 involvement in eicosanoid production and airway inflammation, which seems warranted given the dramatic phenotypes seen in mGX-sPLA2-deficient mice and the pharmacological results obtained in this study.

REFERENCES

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).
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doi: 10.1074/jbc.M111.235812 originally published online June 7, 2011

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