

Lung mast cells are a source of secreted phospholipases A₂

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Background: Secreted phospholipases A₂ (sPLA₂s) are released in plasma and other biologic fluids of patients with inflammatory, autoimmune, and allergic diseases.

Objective: We sought to evaluate sPLA₂ activity in the bronchoalveolar lavage fluid (BALF) of asthmatic patients and to examine the expression and release of sPLA₂s from primary human lung mast cells (HLMCs).

Methods: sPLA₂ activity was measured in BALF and supernatants of either unstimulated or anti-IgE-activated HLMCs as hydrolysis of oleic acid from radiolabeled *Escherichia coli* membranes. Expression of sPLA₂s was examined by using RT-PCR. The release of cysteinyl leukotriene (LT) C₄ was measured by means of enzyme immunoassay.

Results: Phospholipase A₂ (PLA₂) activity was higher in the BALF of asthmatic patients than in the control group. BALF PLA₂ activity was blocked by the sPLA₂ inhibitors dithiothreitol and Me-Indoxam but not by the cytosolic PLA₂ inhibitor AZ-1. HLMCs spontaneously released a PLA₂ activity that was increased on stimulation with anti-IgE. This PLA₂ activity was blocked by dithiothreitol and Me-Indoxam but not by AZ-1. HLMCs constitutively express mRNA for group IB, IIA, IID, IIE, IIF, III, V, X, XI_{IA}, and XI_{IB} sPLA₂s. Anti-IgE did not modify the expression of sPLA₂s. The cell-impermeable inhibitor Me-Indoxam significantly reduced (up to 40%) the production of LTC₄ from anti-IgE-stimulated HLMCs.

Conclusions: sPLA₂ activity is increased in the airways of asthmatic patients. HLMCs express multiple sPLA₂s and release 1 or more of them when activated by anti-IgE. The sPLA₂s released by mast cells contribute to LTC₄ production by acting in an autocrine fashion. Mast cells can be a source of

sPLA₂s in the airways of asthmatic patients. (*J Allergy Clin Immunol* ■■■■;■■■:■■■-■■■.)

Key words: Lung mast cells, secreted phospholipase A₂, leukotriene C₄, arachidonic acid

Phospholipases A₂ (PLA₂s) are key enzymes involved in the mobilization of arachidonic acid from membrane phospholipids.¹ This is the initial step in the metabolic cascade, leading to the synthesis of eicosanoids (prostaglandins, leukotrienes [LTs], and other). PLA₂s thought to play a role in arachidonic acid release are currently classified as high-molecular-weight cytosolic phospholipases A₂ (cPLA₂s) and low-molecular-weight secreted PLA₂s (sPLA₂s).¹

Ten isoforms of sPLA₂s have been identified in human cells and tissues.² sPLA₂s are released in extracellular fluids during local or systemic inflammation.³ In addition, it has been previously shown that sPLA₂ activity is detectable in the bronchoalveolar lavage fluid (BALF) of healthy individuals.⁴ This activity is increased in the airways of patients with inflammatory lung diseases (pneumonia, adult respiratory distress syndrome, and sarcoidosis).³ Moreover, sPLA₂ activity is also increased in the BALF of patients with bronchial asthma^{5,6} and in the nasal fluid of patients with allergic rhinitis after local allergen challenge.⁷ These observations indicate that sPLA₂ enzymes can be released during allergic reactions in both the upper and lower airways. However, these studies did not provide information on the cellular sources of these enzymes.

The role of sPLA₂ isoforms in airway inflammation has been investigated in rodents. For example, several sPLA₂s, such as GI_{IA}, GI_{ID}, GI_{IE}, GV, and GX, are overexpressed in lung biopsy specimens in experimentally induced pulmonary inflammation.^{8,9} In particular, GX, the isoform with the highest phospholipolytic activity in mammalian cells *in vitro*,^{10,11} is constitutively expressed in the lung.^{8,9} Interestingly, GX expression did not change on LPS- or carrageenin-induced lung inflammation,⁸ whereas it was significantly increased in the airways of mice with ovalbumin-induced asthma.⁹ Moreover, knocking out GX reduced all the histologic and functional features associated with the inflammatory response and airway remodeling in this model of asthma. These studies demonstrated that certain sPLA₂s play an important role in the pathogenesis of inflammatory and allergic diseases of the lung.

The expression of sPLA₂ isoforms in the upper and lower airways has been examined in patients with chronic rhinosinusitis or pneumonia.^{12,13} Immunohistochemistry revealed that low levels of human GI_{IA} (hGI_{IA}) were expressed in the nasal epithelium and submucosal glands of healthy donors, whereas the expression of this sPLA₂ was increased in patients with rhinosinusitis.¹² In the lung only human GX (hGX) was detected

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Abbreviations used

BALF:	Bronchoalveolar lavage fluid
BMMC:	Bone marrow–derived mast cell
cPLA ₂ :	Cytosolic phospholipase A ₂
Ct:	Cycle threshold
DTT:	Dithiothreitol
GAPDH:	Glyceraldehyde-3-phosphate dehydrogenase
hGIIA:	Human GIIA
hGX:	Human GX
HLMC:	Human lung mast cell
IC ₅₀ :	Inhibitory concentration of 50%
LTC ₄ :	Leukotriene C ₄
OA:	Oleic acid
PLA ₂ :	Phospholipase A ₂ *
PMSF:	Phenylmethylsulfonyl fluoride
qPCR:	Real-time quantitative PCR
sPLA ₂ :	Secreted phospholipase A ₂

in bronchial epithelial cells and subepithelial interstitium of both healthy donors and patients with pneumonia.¹³ In inflamed, but not normal, lung tissue hGIIA was found in vascular smooth muscle cells and bronchial chondrocytes, whereas human GIID, GV, and GX were found in epithelial cells and macrophages.¹³ Immunostaining analysis of cells from induced sputum demonstrated that hGX was expressed by bronchial epithelial cells and macrophages in healthy donors and patients with asthma.¹⁴ However, in asthmatic patients the expression of hGX was significantly higher than in healthy subjects and was further increased during exercise-induced bronchoconstriction. These observations indicate that sPLA₂ expression is upregulated in human airways during inflammatory and allergic disorders and suggest that cells resident in the lung might produce distinct sPLA₂s.

Mast cells play a primary role in the pathogenesis of bronchial asthma and rhinitis.¹⁵ These cells can be activated by IgE- and non-IgE-mediated stimuli to release a variety of preformed and *de novo* synthesized proinflammatory mediators.¹⁶ Mast cells are particularly abundant at the body's interface with the external environment, such as the mucosae of the respiratory and gastrointestinal tracts and the skin.¹⁷ This unique location justifies the important role of mast cells in allergic inflammation, as well as innate immunity and host defense against infections.¹⁶⁻¹⁸

Studies on the expression of sPLA₂s in mast cells have been primarily carried out in mice. Enomoto et al¹⁹ showed that bone marrow–derived mast cells (BMMCs) from BALB/cJ and C57BL/6J mice express all members of the group II subfamily of sPLA₂s, including GIIC, GIID, GIIE, GIIF, and GV. GIIA is expressed in BALB/cJ but not in C57BL/6J mast cells because the latter strain has a natural disruption of the gene encoding for GIIA. BMMCs from either strains do not express GIB and GX sPLA₂s.¹⁹ This and other studies²⁰ indicate that the expression pattern of sPLA₂ isoforms differs in mast cells with different phenotypes and from different animal species.

Marked biochemical and functional differences exist between murine and human mast cells, and in many cases information on cell activation and mediator production obtained in murine models was not confirmed in human mast cells.²¹ Data on

sPLA₂ expression in human mast cells are scarce because of the limited number of cells detectable in biopsy specimens or retrieved from biologic fluids. Immunohistochemistry studies demonstrated that human synovial²² and gut²³ mast cells express hGIIA. However, there are no data on the expression and function of sPLA₂s in mast cells purified *ex vivo* from human tissues. In this study we provide evidence that human lung mast cells (HLMCs) express mRNA for several sPLA₂s and release a PLA₂ activity with biochemical and pharmacologic characteristics similar to that of the PLA₂s secreted in the airways of patients with bronchial asthma.

METHODS**Reagents**

Percoll, dimethyl sulfoxide, L-glutamine, antibiotic-antimycotic solution (10,000 IU/mL penicillin, 10 mg/mL streptomycin, and 25 µg/mL amphotericin B), and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma (St Louis, Mo). Dithiothreitol (DTT) was from MP Biomedicals (Solon, Calif). Me-Indoxam and AZ-1 were prepared as previously described.^{11,24} Tritiated oleic acid (OA)-labeled *Escherichia coli* membranes were kindly provided by Dr Gianfrancesco Goracci (University of Perugia, Perugia, Italy). The rabbit anti-human Fce antibody was donated by Drs T. Ishizaka and K. Ishizaka (La Jolla Institute for Allergy and Immunology, La Jolla, Calif).

Study population

Bronchoalveolar lavage was performed in 14 patients with mild persistent asthma and 19 nonasthmatic subjects (see the **Methods** section in this article's Online Repository at www.jacionline.org). The study protocol was approved by the Ethical Committee of the University of Naples Federico II, and informed consent was obtained from each subject before bronchoscopy.

Bronchoalveolar lavage procedure

Bronchoscopy and bronchoalveolar lavage were performed according to a standardized protocol based on current National Heart, Lung, and Blood Institute guidelines (see also the **Methods** section in this article's Online Repository).²⁵

Cell isolation

The study protocol involving the use of human lung tissue was approved by the Ethical Committee of the University of Naples Federico II, and informed consent was obtained from patients undergoing thoracic surgery. Human mast cells were obtained from the lungs of patients undergoing thoracic surgery and were purified (>98%) by means of immunomagnetic selection, as previously described (see also the **Methods** section in this article's Online Repository).²⁶

Cell incubation

Mast cells suspended in PCG buffer²⁶ (10⁶/mL) were incubated (at 37°C for 15-120 minutes) with anti-IgE (0.03-1 µg/mL). For LTC₄ production, the cells (10⁵/mL) were preincubated (at 37°C for 15 minutes) with increasing concentrations (0.01-10 µmol/L) of Me-Indoxam or AZ-1 before stimulation (at 37°C for 30 minutes). The reactions were stopped by means of centrifugation (at 800g for 5 minutes at 4°C), and the cell-free supernatant was stored at -80°C for determination of PLA₂ activity, histamine release, LTC₄ production, or β-hexosaminidase release. The cell pellets were lysed with freeze-thaw cycles in distilled water, and aliquots were stored at -80°C for determination of total content of histamine or β-hexosaminidase.

PLA₂ assay

PLA₂ activity in BALF and HLMC supernatants was measured as previously described²⁷ by using tritiated OA-labeled *E coli* membranes. PLA₂ activity was determined in 50 mmol/L Tris HCl (pH 7.5) and 10 mmol/L

*The Roman numeral after the letter G indicates the group, and the uppercase letter after the numeral indicates the subgroup (eg, GIB indicates group IB PLA₂).

CaCl₂ in a total volume of 1.0 mL. The reaction was initiated by the addition of 0.1 μ Ci of tritiated OA-labeled *E coli* membranes. At the end of incubation (90 minutes at 37°C), the reaction was stopped by adding 2 mL of methanol, 1 mL of chloroform, and 50 μ L of 9% formic acid, and lipids were extracted and separated by means of thin-layer chromatography. Tritiated OA was measured by means of liquid scintillation counting (Tri-Carb 2800 TR; PerkinElmer, Waltham, Mass), and PLA₂ activity was expressed as picomoles of tritiated OA released per minute per milliliter of BALF or HLMC supernatants. Aliquots of BALF or HLMC supernatants were incubated (for 1 hour at 37°C) with 10 mmol/L DTT, 10 μ mol/L Me-Indoxam, 10 μ mol/L AZ-1, or 2 mmol/L PMSF before PLA₂ assay to examine the effect of various inhibitors on PLA₂ activity.

Mediator release assays

Histamine was measured in duplicate determinations by using a commercially available enzyme immunoassay (Immunotech, Praha, Czech Republic). β -Hexosaminidase was measured in duplicate determinations by using a colorimetric assay.²⁸ LTC₄ was measured in mast cell supernatants in duplicate determinations with a commercially available enzyme immunoassay (GE Healthcare, Fairfield, Conn). The linearity range of this assay was 15 to 1,000 pg/mL. Inhibition of LTC₄ production was expressed as a percentage of maximum response calculated as follows: $(R - R_b) / (R_{max} - R_b) \times 100$, where R is the release in samples treated with the inhibitor, R_b is the release in unstimulated samples, and R_{max} is the release in samples stimulated in the absence of the inhibitor.

RT-PCR for sPLA₂s

Total RNA from HLMCs was extracted by using the SV total RNA isolation system (Promega, Madison, Wis), treated with RNase-free DNase I, and suspended in diethylpyrocarbonate-treated (DEPC) water. RNA concentration and quality were assessed by means of spectroscopy. One microgram of total RNA was reverse transcribed with 25 mmol/L MgCl₂, 50 μ mol/L oligo(dT), and 200 U of Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, Calif). Semiquantitative and real-time quantitative PCR (qPCR) were performed as previously described (see the Methods section and Table E1 in this article's Online Repository at www.jacionline.org).^{29,30}

Statistical analysis

Data are expressed as means $\pm$ SEs of the indicated number of experiments. *P* values were determined with the Student unpaired or paired *t* tests. Correlation was assessed by using the linear regression function of Microsoft (Redmond, Wash) Excel software.

RESULTS

Characterization of PLA₂ activity in BALF

Initial experiments were performed to verify the presence of PLA₂ activity in the airways of patients with bronchial asthma. PLA₂ enzymatic activity was determined in the cell-free unconcentrated BALF of 14 asthmatic patients and 19 control subjects. Fig 1, A, shows that low levels of PLA₂ activity were found in the BALF of control subjects (9.3 ± 0.9 pmol of tritiated OA/min/mL of BALF). PLA₂ activity was significantly increased in patients with asthma (28.3 ± 2.2 pmol of tritiated OA/min/mL of BALF, *P* < .01 vs control subjects). There was no significant correlation between the PLA₂ activity and the protein content of the BALF of asthmatic patients (see Fig E1, A, in this article's Online Repository at www.jacionline.org), whereas a positive correlation was found in the control group (see Fig E1, B, in this article's Online Repository).

To verify that the activity detected in the airways of asthmatic patients was due to the presence of sPLA₂s, we incubated the BALF with 2 different inhibitors of sPLA₂s (DTT and Me-Indoxam),¹¹ an inhibitor of cPLA₂s (referred to as AZ-1),³¹ or

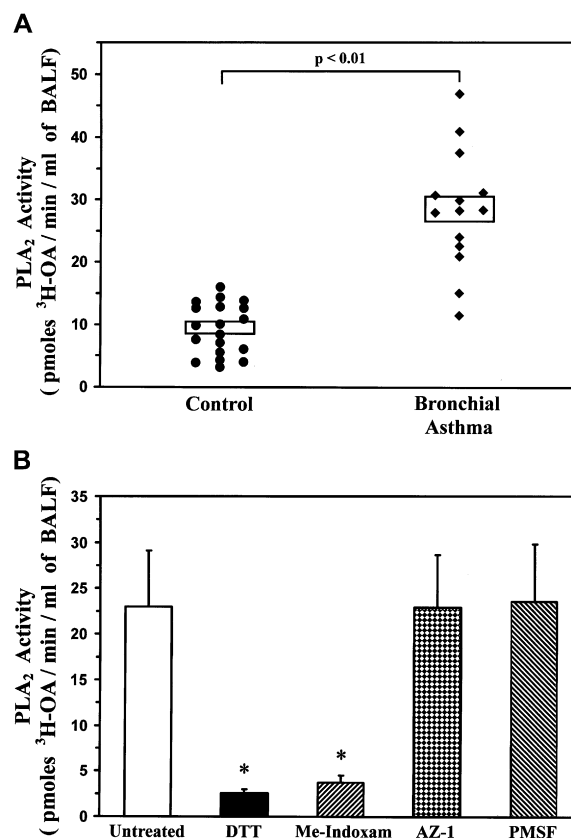


FIG 1. Characterization of PLA₂ activity in human BALF. **A**, BALF from control subjects (*n* = 19) and asthmatic patients (*n* = 14) was assayed for PLA₂ activity, as described in the Methods section. Boxes indicate means $\pm$ SEs. **B**, BALF was preincubated (for 1 hour at 37°C) with 10 mmol/L DTT, 10 μ mol/L Me-Indoxam, 10 μ mol/L AZ-1, or 2 mmol/L PMSF before the PLA₂ activity assay. Data are presented as means $\pm$ SEs of BALF from 4 asthmatic patients. **P* < .01 versus untreated.

an inhibitor of platelet-activating factor acetylhydrolase (PMSF).³² In particular, DTT is a reducing agent that alters the secondary structure of all sPLA₂s by reducing disulfide bonds, whereas Me-Indoxam is an active, site-directed, reversible, competitive inhibitor of sPLA₂ enzymatic activity.¹¹ The latter compound binds to sPLA₂ catalytic sites, thereby preventing the interaction of the enzyme with its substrate. Because the various sPLA₂ isoforms display structural differences in their catalytic sites,² the affinity and inhibitory effect of Me-Indoxam are different for the various human sPLA₂s. In particular, Me-Indoxam shows different potencies on the various sPLA₂ isoforms in *in vitro* assays with an inhibitory concentration of 50% (IC₅₀) of less than 100 nmol/L for hGIIA, hGIIIE, and hGV; an IC₅₀ of between 200 and 600 nmol/L for hGIB and hGX; and an IC₅₀ of greater than 2 μ mol/L for hGIID, hGIIF, hGIIL, and hGXIIA.¹¹ Fig 1, B, shows that the PLA₂ activity in the BALF of asthmatic patients was almost completely suppressed by means of incubation with either DTT or Me-Indoxam, whereas it was not affected by AZ-1 or PMSF. These results indicate that 1 or more sPLA₂s account for most of the enzymatic activity in the airways of asthmatic patients.

Release of sPLA₂s from HLMCs

The detection of sPLA₂ activity in the airways of patients with asthma led us to investigate the role of lung mast cells as a

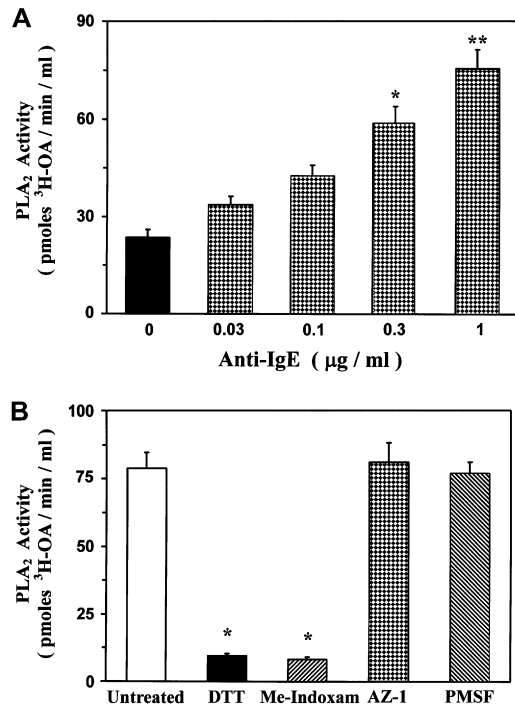


FIG 2. Characterization of PLA₂ activity in supernatants of HLMCs. **A**, HLMCs were incubated (for 30 minutes at 37°C) with anti-IgE. PLA₂ activity was determined in supernatants, as described in the Methods section. Data are presented as means ± SEs of 4 experiments. **P* < .05 versus unstimulated; ***P* < .01 versus unstimulated. **B**, Supernatants of anti-IgE-stimulated HLMCs were preincubated (for 1 hour at 37°C) with 10 mmol/L DTT, 10 μmol/L Me-Indoxam, 10 μmol/L AZ-1, or 2 mmol/L PMSF before the PLA₂ activity assay. Data are presented as means ± SEs of 4 experiments. **P* < .01 versus untreated.

potential source of these enzymes. In this group of experiments, we explored the capacity of primary human mast cells purified from the lung parenchyma (HLMCs) to release sPLA₂ activity on immunologic activation. Fig 2, A, shows that HLMCs spontaneously released PLA₂ activity (23.6 ± 2.4 pmol of tritiated OA/min/mL of HLMC supernatant). Stimulation of HLMCs with anti-IgE (0.03-1 μg/mL) concentration-dependently increased the release of PLA₂ activity, with a maximum at 1 μg/mL (75.6 ± 5.7 pmol of tritiated OA/min/mL of HLMC supernatant). To understand whether the PLA₂ activity released by lung mast cells was from an sPLA₂ and had the same biochemical properties as those present in the BALF of asthmatic patients, supernatants of anti-IgE-activated HLMCs were preincubated with the PLA₂ inhibitors described in the previous section. Fig 2, B, shows that the PLA₂ activity released by HLMCs was completely blocked by the reducing agent DTT and by the site-directed sPLA₂ inhibitor Me-Indoxam at concentrations known to inhibit most of the human sPLA₂s *in vitro*.¹¹ In contrast, AZ-1 and PMSF had no effect on PLA₂ activity released from mast cells. These data indicate that immunologically activated HLMCs released a PLA₂ activity with biochemical and pharmacologic properties of an sPLA₂.

In the next group of experiments, we examined the kinetics of release of sPLA₂ activity and histamine from anti-IgE-stimulated HLMCs. In 3 different preparations of HLMCs, the release of the sPLA₂ activity was detectable already after 15 minutes of stimulation and peaked at 30 minutes (Fig 3, A). The kinetics of sPLA₂ release were similar to those of histamine (half-time of sPLA₂ release, 15.3 ± 3.2 minutes; half-time of histamine release, 12.8 ±

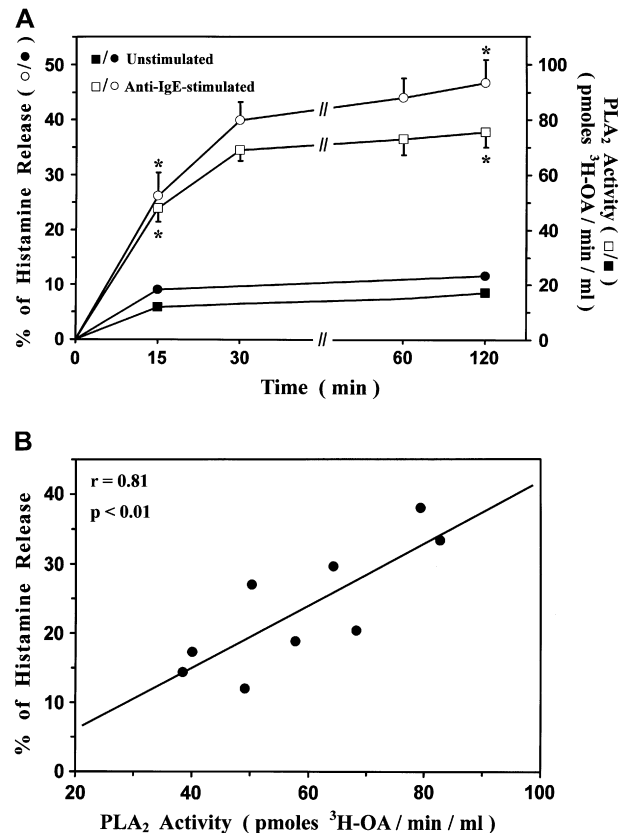


FIG 3. Release of sPLA₂s and histamine from anti-IgE-stimulated HLMCs. **A**, HLMCs were incubated with or without anti-IgE (1 μg/mL). Histamine release and PLA₂ activity were determined in supernatants, as described in the Methods section. Data are presented as means ± SEs of 4 experiments. **P* < .01 versus unstimulated. **B**, HLMCs were incubated (for 30 minutes at 37°C) with anti-IgE (1 μg/mL). Histamine release and PLA₂ activity were determined as described above. Correlation between histamine release and sPLA₂ activity was assessed by using the linear regression function of Microsoft Excel.

2.6 minutes). Data obtained with HLMCs from 9 different donors indicated that there was a significant correlation between maximal release of sPLA₂s (expressed as biologic activity) and of histamine (expressed as the percentage of the total cellular content) when mast cells were stimulated with an optimal concentration of anti-IgE (1 μg/mL; Fig 3, B). These data suggest that the sPLA₂ is stored as a preformed mediator within mast cells and is rapidly released on immunologic activation.

Expression of mRNA for sPLA₂s in HLMCs

The results obtained thus far have indicated that human mast cells release 1 or more isoforms of sPLA₂s. We therefore examined constitutive gene expression of the known human isoforms of sPLA₂s in resting HLMCs by means of RT-PCR by using target-specific primers for the various sPLA₂s (see Table E1 in this article's Online Repository). Fig 4 shows the PCR amplification signals of 2 experiments representative of 4 different preparations of HLMCs. PCR fragments of the expected size encoding for hGIB (341 bp), hGIIA (434 bp), hGIID (294 bp), hGIII (120 bp), hGIIF (211 bp), hGIII (500 bp), hGV (358 bp), hGX (370 bp), hGXIIA (105 bp), and hGXIIIB (141 bp) were amplified in all HLMC preparations at subsaturating cycle numbers (35

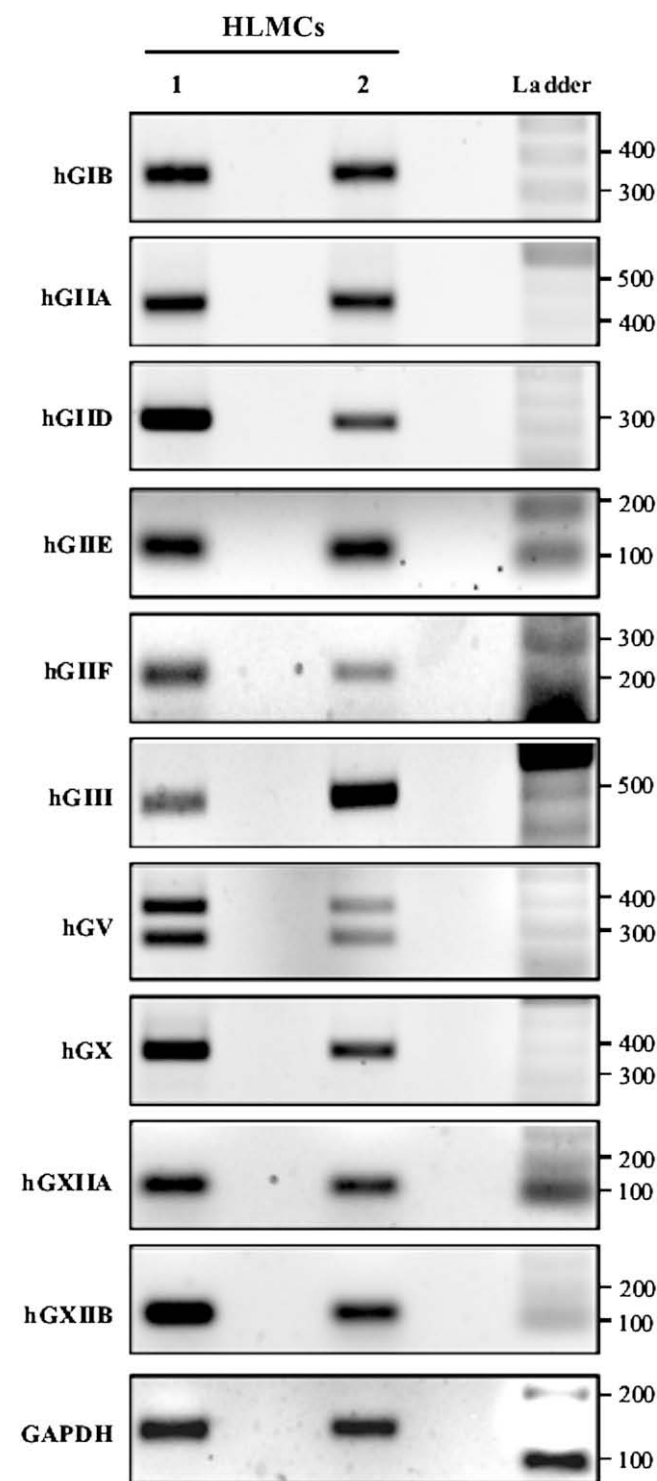


FIG 4. Expression of sPLA₂s in HLMCs. HLMCs from 2 different lung preparations were lysed, and total RNA was extracted. Expression of sPLA₂s was evaluated by means of semiquantitative PCR, as described in the Methods section. RT-PCR amplification products were separated on 2% agarose gel, stained with ethidium bromide, and photographed.

cycles). As previously reported in human neutrophils, the primers used to evaluate hGV mRNA expression in HLMCs also generated a 251-bp PCR product that was identical to the hGV mRNA from nucleotides 24 to 381 but lacked the untranslated

TABLE I. Expression of sPLA₂s in human lung mast cells

sPLA ₂ s	mRNA expression (Δ Ct)*		P value
	Unstimulated†	Anti-IgE†	
hGIIA	15.73 $\pm$ 1.11	16.34 $\pm$ 1.17	.179
hGIID	9.36 $\pm$ 1.03	9.90 $\pm$ 0.47	.225
hGIII	12.06 $\pm$ 0.94	13.46 $\pm$ 0.64	.155
hGV	9.09 $\pm$ 0.96	9.36 $\pm$ 0.54	.322
hGX	4.84 $\pm$ 0.38	5.29 $\pm$ 0.29	.222
hGXIIA	6.81 $\pm$ 0.78	7.54 $\pm$ 0.99	.211
hGXIIIB	7.43 $\pm$ 1.03	7.99 $\pm$ 1.28	.119

*mRNA expression is based on qPCR, and data are expressed as Δ Ct (see the Methods section in this article's Online Repository). A Δ Ct of less than 10 means high to medium expression, a Δ Ct of 10 to 15 means medium to low expression, and a Δ Ct of greater than 15 means low expression. Data are presented as the means $\pm$ SEs of 3 different donors. P values were determined by using the Student paired *t* test.

†The cells were incubated (at 37°C for 3 hours) in the absence (unstimulated) or presence of anti-IgE (1 μ g/mL). At the end of incubation, mRNA expression of sPLA₂s was evaluated as described above.

region corresponding to exon 4.²⁴ These data indicate that primary HLMCs constitutively express mRNA for most human sPLA₂s.

To investigate whether anti-IgE challenge of HLMCs modifies the expression of sPLA₂s, we next examined mRNA for the major sPLA₂ isoforms (hGIIA, hGIID, hGIII, hGV, and hGX) in both resting and anti-IgE-activated HLMCs. To this end, we carried out qPCR in 3 different preparations of HLMCs incubated (at 37°C for 3 hours) in the absence (unstimulated) or presence of anti-IgE (1 μ g/mL). These experiments allowed an accurate quantification of the sPLA₂s constitutively expressed by HLMCs. Table I shows that human mast cells express high levels of hGIII, hGV, and hGX; intermediate levels of hGIID and hGIIA; and low levels of hGIII and hGIIA. Stimulation with anti-IgE did not enhance the expression of any sPLA₂s examined. We rather observed a tendency toward a reduction in the expression of all sPLA₂s, but these results did not reach statistical significance.

Role of endogenous sPLA₂s in the generation of LTC₄ from HLMCs

sPLA₂s contribute to the generation of eicosanoids in murine mast cells^{19,27,33,34} and macrophages.^{10,35} However, it is still debated whether this contribution is due to intracellular or extracellular actions of sPLA₂s. Our experiments demonstrated that human mast cells secrete 1 or more sPLA₂s based on results obtained with Me-Indoxam. It is worth noting that, being cell impermeable, Me-Indoxam is able to inhibit the activity of sPLA₂s only when they are secreted in the extracellular space.³⁶ Thus we were able to evaluate the role of endogenous PLA₂s on LTC₄ production once these enzymes have been released by immunologically activated mast cells. In these experiments mast cells were stimulated with anti-IgE in the presence of increasing concentrations (0.1–10 μ M) of Me-Indoxam or AZ-1, a potent and cell-permeable inhibitor of GIV-cPLA₂. At the end of incubation, LTC₄ production was determined in the supernatants. Fig 5 shows that the GIV inhibitor AZ-1 caused a complete suppression of LTC₄ synthesis (IC₅₀, 40.3 $\pm$ 7.9 nmol/L). However, Me-Indoxam also inhibited, in a concentration-dependent fashion, up to 40% of anti-IgE-induced LTC₄ release. Neither Me-Indoxam

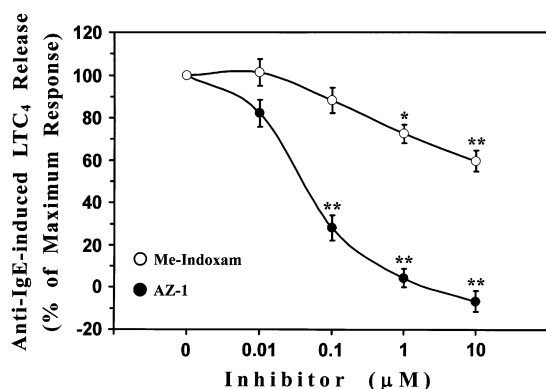


FIG 5. Effect of sPLA₂ and cPLA₂ inhibitors on LTC₄ production from anti-IgE-stimulated HLMCs. The cells were preincubated (for 15 minutes at 37°C) with increasing concentrations of Me-Indoxam or AZ-1 before stimulation (for 30 minutes at 37°C) with anti-IgE (1 μg/mL). LTC₄ production was determined in supernatants by using an enzyme immunoassay. Inhibition of LTC₄ production was expressed as a percentage of maximum response. Data are presented as means ± SEs of 4 experiments. **P* < .05 versus anti-IgE; ***P* < .01 versus anti-IgE.

nor AZ-1 significantly influenced anti-IgE-induced degranulation of mast cells, as assessed by means of β-hexosaminidase release (data not shown). These results indicate that LTC₄ synthesis in mast cells is primarily due to the GIV-cPLA₂. However, sPLA₂s, once secreted, significantly contribute to this process by augmenting IgE-mediated cysteinyl LT production.

DISCUSSION

Patients with mild persistent asthma have higher levels of sPLA₂ activity in BALF than nonasthmatic control subjects. Primary lung mast cells constitutively express mRNA for several sPLA₂s and release, on immunologic activation, sPLA₂ activity with biochemical characteristics similar to those of the sPLA₂s detected in the BALF of asthmatic patients. Endogenous sPLA₂s released by mast cells significantly contribute to IgE-mediated production of cysteinyl LTs.

Our results indicate that low levels of sPLA₂s are detectable in the airways of control subjects and that this activity is increased in patients with mild asthma. A significant correlation between sPLA₂ activity and protein content exists in the BALF of control subjects. This correlation is lost in asthmatic patients, suggesting that sPLA₂ enzymes are selectively released in the airways of these patients. These results are in line with previous studies showing that bronchial antigen challenge in asthmatic patients increases sPLA₂ activity in the BALF 3- to 5-fold during the late-phase reaction (ie, 4-20 hours after challenge).^{5,6}

Several cells involved in the pathogenesis of asthma, including eosinophils, basophils, TH₂ cells, epithelial cells, macrophages, and fibroblasts, express sPLA₂s.²⁰ Our work provides the first characterization of sPLA₂s in human mast cells and indicates that they might be a source of these proinflammatory molecules in patients with asthma. An interesting observation of this study is that, in contrast to other inflammatory cells involved in asthma, mast cells not only express sPLA₂s but also release 1 or more of these enzymes on stimulation. Epithelial cells, macrophages, and eosinophils in induced sputum of asthmatic patients express various sPLA₂s¹⁴; the ability of these cells to release sPLA₂s was not investigated. This is a relevant issue because eosinophils,³⁷

basophils,³⁸ and macrophages²⁹ synthesize 1 or more sPLA₂s but fail to release them, at least *in vitro*. Thus mast cells are rather unique among effector cells in bronchial asthma because they secrete sPLA₂s when activated by IgE.

The release of sPLA₂s from stimulated mast cells is rapid and coincident with that of histamine. However, stimulation with anti-IgE does not modify the mRNA expression of any sPLA₂s. These findings suggest that sPLA₂s are synthesized and stored as preformed mediators. The correlation between the release of sPLA₂ activity and histamine by activated mast cells supports the hypothesis that these mediators are stored together within secretory granules. Early studies demonstrated that sPLA₂s are contained within granules of rodent mast cells.^{19,39} Moreover, unpublished data from our laboratory also indicate that sPLA₂s of the group II subfamily, identified by means of immunohistochemistry, colocalize with tryptase in skin mast cells from patients with mastocytosis.

Another unique feature of human mast cells is the expression of mRNA for a number of sPLA₂s. This is at variance with most human inflammatory cells, which express a restricted profile of sPLA₂ isoforms.²⁰ The amount of mRNA for the various sPLA₂s is rather different because HLMCs constitutively express high levels of hGIII, hGV, and hGX; medium levels of hGIID and hGIIF; and low levels of hGIIA and hGIIE. These results suggest that these cells might synthesize different quantities of the various sPLA₂s. The detection of sPLA₂ proteins in mast cells by using conventional techniques (eg, Western blotting) is limited by the low number of cells retrieved from specimens of lung tissue. Thus although our data indicate that mast cells produce messages for all sPLA₂s, they do not define which isoforms are translated into proteins, secreted, or both. However, information on the sPLA₂s secreted by stimulated mast cells can be inferred from the data obtained with Me-Indoxam. This compound inhibits hGIB, hGIIA, hGIIE, hGV, and hGX with an IC₅₀ of less than 600 nmol/L and hGIID, hGIIF, hGIII, hGXIIA, and hGXIIIB with an IC₅₀ of greater than 2 μmol/L. Thus it is conceivable that HLMC supernatants contain those sPLA₂s that can be blocked by Me-Indoxam (hGIB, hGIIA, hGIIE, hGV, and hGX) rather than those sPLA₂s that are poorly sensitive or nonsensitive to the inhibitory effect of this compound (hGIID, hGIIF, hGIII, hGXIIA, and hGXIIIB). Further studies with more sensitive and specific techniques for sPLA₂ detection will define which sPLA₂ proteins are synthesized and released by human mast cells.

The role of sPLA₂s in asthma is still under investigation. Some of these molecules, such as hGX,^{9,14} can participate in airway inflammation and remodeling through at least 3 mechanisms.

First, sPLA₂s can contribute to prostaglandin and LT biosynthesis by potentiating the effect of cPLA₂s. The results shown in Fig 5 indicate that LTC₄ production in stimulated HLMCs is primarily dependent on GIV-cPLA₂. Nevertheless, the observation that the cell-impermeable sPLA₂ inhibitor Me-Indoxam reduces LTC₄ production by 40% indicates that the sPLA₂s released by HLMCs contribute to LT production by cross-talking with GIV-cPLA₂. These data are reminiscent of those obtained with murine mast cells and macrophages showing that sPLA₂s alone do not initiate LTC₄ production but potentiate the eicosanoid-forming capacity of GIV-cPLA₂.^{27,33,35} The mechanisms of the cross-talk between cPLA₂s and sPLA₂s are still unclear, but it is currently believed that sPLA₂-induced intracellular signals might increase the activation of the GIV-cPLA₂.³⁴ Several isoforms of sPLA₂s (GIB, GIIA, GV, and GX) bind to a specific

M-type receptor, which generates intracellular signals leading to proinflammatory responses in target cells.⁴⁰ Me-Indoxam blocks not only the catalytic activity of sPLA₂s¹¹ but also the receptor-mediated activation of inflammatory cells^{30,40} by preventing the binding of sPLA₂s to the M-type receptor.⁴¹ Of relevance to the present study, GIB, GIIA, and GV activate the M-type receptor expressed on murine BMMCs, thereby activating GIV-cPLA₂ and promoting arachidonate mobilization and eicosanoid production.⁴² Therefore the enhancement of LT production by sPLA₂s in HLMCs might be due to an autocrine effect on the M-type receptor expressed on these cells.

A second mechanism by which sPLA₂s might promote inflammation in asthma is through their nonenzymatic, receptor-mediated activation of inflammatory cells.⁴⁰ We and others demonstrated that several sPLA₂s (GIB, GIIA, and GX) activate cytokine and chemokine production^{30,43-45} by interacting with the M-type or other receptors expressed on human inflammatory cells.

Finally, sPLA₂s might contribute to the pathogenesis of asthma *in vivo* through the degradation of surfactant phospholipids. Alterations of the physicochemical properties of surfactant occur in asthma and are associated with airway obstruction and hyperreactivity.⁴⁶ sPLA₂s hydrolyze surfactant phospholipids, generating lysophospholipids that, in turn, alter surfactant properties and induce proinflammatory effects.⁴⁷ Together, these observations help explain why knocking out just 1 sPLA₂ isoform (GX) dramatically reduces allergic inflammation.⁹

The capacity of mast cells to secrete sPLA₂s might also be relevant to the role of these cells in innate immunity.¹⁶⁻¹⁸ Several sPLA₂s have potent bactericidal activity.^{48,49} In addition, GIII sPLA₂ inhibits HIV replication by blocking viral entry into the cells.⁵⁰ Our results raise the interesting hypothesis that sPLA₂s are mediators supporting the role of mast cells in innate immunity.

In conclusion, sPLA₂s released by immunologically activated mast cells have biochemical properties similar to those of the enzymes secreted in the airways of asthmatic patients, indicating that mast cells might be a major source of sPLA₂s in asthma. The demonstration that sPLA₂s are released by mast cells further reinforces the concept that these molecules have an important role in inflammation and tissue remodeling in asthma.

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Clinical implications: HLMCs can be a source of sPLA₂s in the airways of asthmatic patients. PLA₂s secreted by mast cells are implicated in LT synthesis and might provide a novel therapeutic target in asthma.

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METHODS

Study population

Fourteen patients (8 male and 6 female patients; age range, 20–54 years) were classified as having mild persistent asthma on the basis of clinical history and pulmonary function studies.^{E1} All patients were atopic as documented by a positive skin test result for at least 1 aeroallergen. Inhaled corticosteroids were discontinued at least 4 weeks before bronchoscopy, and only short-acting β_2 -agonists on demand were allowed. As a control group, we enrolled patients (14 male and 5 female patients; age range, 18–60 years) with a single lung lesion in whom the BALF used for the experiments was obtained from the contralateral side. They had no history of atopic diseases and at the time of bronchoscopy were free of respiratory symptoms and had forced vital capacity and FEV₁ values of greater than 90% of the predicted value.

Bronchoalveolar lavage procedure

A flexible fiberoptic bronchoscope (Olympus BF type P20; Olympus, Center Valley, Pa) was wedged into a segmental or subsegmental bronchus, and 3 fractions (50 mL each) of saline preheated at 37°C were introduced. Recovered fluid was pooled and filtered through 2 layers of sterile gauze. The fluid was then centrifuged twice at 800g for 10 minutes at 4°C and stored at –80°C for determination of PLA₂ activity and total protein content by a Bradford-based assay (Bio-Rad, Hercules, Calif).

Purification of human lung mast cells

Lung fragments chopped with scissors were dispersed into their cellular elements by means of enzymatic digestion with pronase (Calbiochem, San Diego, Calif), chymopapain (Sigma), collagenase, and elastase (Calbiochem). The mast cell suspension was enriched (>80%) by means of flotation over Percoll (Sigma) density gradients and then purified by using positive immunomagnetic selection with the CD117 Microbead Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Mast cell numbers and purity were determined

by means of toluidine blue staining. The purity of mast cells used in the experiments was always greater than 98%.

RT-PCR

For semiquantitative PCR, equivalent templates of cDNAs were amplified by using target-specific primers for hGIB, hGIIA, hGIID, hGIIIE, hGIIF, hGIIL, hGV, hGX, hGXIIA, hGXIIIB, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; see Table E1). The PCR products were separated on 2% agarose gel, stained with ethidium bromide, and visualized by using the image analysis system ChemidocXRS (Bio-Rad).

Real-time quantitative PCR (qPCR) was performed on the iCycler (Bio-Rad) with the Platinum SYBR Green qPCR kit (Invitrogen). Target-specific primers for hGIIA, hGIID, hGIIIE, hGIIF, hGIIL, hGV, hGX, and GAPDH suitable for qPCR were designed by using the Beacon Designer 3.0 (Bio-Rad; see Table E1). After an initial denaturation step at 94°C for 30 seconds, amplification was performed with 40 cycles of denaturation (94°C) for 30 seconds, annealing (55°C) for 30 seconds, and primer extension (72°C). The data were analyzed with iCycler iQ analysis software (Bio-Rad), and the mRNA signals in each sample were normalized to that of the GAPDH mRNA. The quantification of the mRNA target was obtained by calculating the relative expression of the reference gene compared with GAPDH. Data were expressed as Δ cycle threshold (Ct), which was calculated as the number of PCR cycles for liftoff for the target mRNA of interest minus the number of PCR cycles for liftoff for GAPDH mRNA: $\Delta Ct = Ct(\text{gene of interest}) - Ct(\text{GAPDH})$. The Ct values for GAPDH were typically around 23. A ΔCt of less than 10 means high to medium expression, a ΔCt of 10 to 15 means medium to low expression, and a ΔCt of greater than 15 means low expression.

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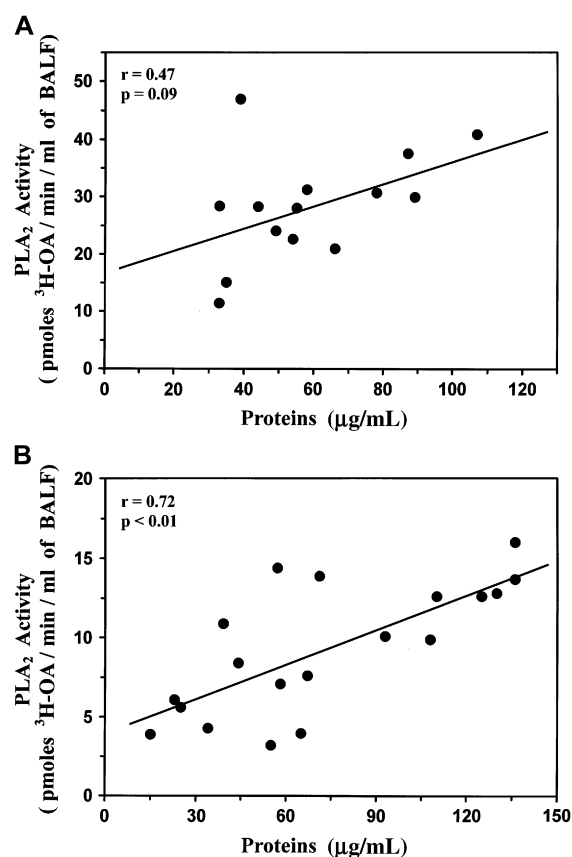


FIG E1. Correlation between PLA₂ activity and protein content in the BALF. Cell-free BALF from patients with bronchial asthma (**A**; $n = 14$) and control subjects (**B**; $n = 19$) was assayed for PLA₂ activity by using tritiated OA-labeled *E coli* membranes, as described in the Methods section, and for total protein content by using a Bradford-based assay. Data are plotted as a function of PLA₂ activity versus protein content. Correlation was assessed by using the linear regression function of Microsoft Excel software.

TABLE E1. Primer sequences and conditions for RT-PCR and qPCR

Target*	Product length (bp)	Ta	Primer (5'-3')	GenBank accession no. or reference
hGIB (RT-PCR)	341	60°C	Forward: TCCTTGTGCTAGCTGTGCTG Reverse: TGAAGGCCTCACACTCTTTG	Degousee et al ²⁴
hGIIA (RT-PCR)	434	57°C	Forward: ATGAAGACCCTCCTACTGTT Reverse: TCAGCAACGAGGGGTGCTCC	Degousee et al ²⁴
hGIIA (qPCR)	117	55°C	Forward: ACCCTCCCTCCCTACCCTAAC Reverse: AATCTGCTGGATGTCTCATTCTGG	NM_000300
hGIID (RT-PCR)	294	60°C	Forward: GAAAATGCCCATCCTCTCCT Reverse: AAACGCAGTCGCTTCTGGTA	Degousee et al ²⁴
hGIID (qPCR)	85	55°C	Forward: CGGACTAGGTGGCAGAGG Reverse: GGTCTTCAGGTGGTCATAGC	NM_012400
hGIIIE (qPCR)	120	53°C	Forward: GGTGTTCTTTGCCTCCTG Reverse: CCGCAGTAACAGCCATAGTC	NM_014589
hGIIIF (qPCR)	211	55°C	Forward: GACCCACACCCTCTCTCC Reverse: GATGCTCTCCCTGCTTACG	NM_0221819
hGIII (RT-PCR)	500	60°C	Forward: TGCCTACAGAATCAGCACGA Reverse: TTGAGCAGCTGGAACCTCGAT	Degousee et al ²⁴
hGIII (qPCR)	100	55°C	Forward: GCGAGCAGCAGGGCAGAG Reverse: AGAATCTCCAACCTCCACACCACAG	NM_015715
hGV (RT-PCR)	358	60°C	Forward: TTGGTTCTGGCTTGTAGTGTG Reverse: TGGGTTGTAGCTCCGTAGGTTT	Degousee et al ²⁴
hGV (qPCR)	100	55°C	Forward: GCAACATTGCGACACAGTCC Reverse: CGGTCACAGGCACAGAGG	NM_000929
hGX (RT-PCR)	370	60°C	Forward: GATCCTGGAACCTGGCAGGAA Reverse: TCAGTCACACTTGGGCGAGT	Degousee et al ²⁴
hGX (qPCR)	94	55°	Forward: GCTGCTGCTGCTTCTACC Reverse: CACAGTTCCTGCCAGTTCC	NM_003561
hGXIIA (RT-PCR)	105	55°C	Forward: TGTTGTGTCAGGTGCCAGGAG Reverse: AAGGCGGCGTTCAGGTAC	BC_017218
hGXIIIB (RT-PCR)	115	55°C	Forward: GCCTGTGATTCCCTGGTTGAC Reverse: ACTCTTCCTTCTCCTCCTCTGC	NM_032562
GAPDH (qPCR)	141	55°C	Forward: GTCCACTGGCGTCTTCAC Reverse: CTTGAGGCTGTTGTCATACTTC	Granata et al ³⁰

*For PLA₂s, *h* stands for human, the Roman number after the letter G indicates the group, and the letter in caps after the number indicates the subgroup (eg, hGIB indicates human group IB PLA₂s).

Ta, Annealing temperature.