Selective Functions in Human Eosinophils

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Secretory Phospholipases A₂ Activate Selective Functions in Human Eosinophils

Massimo Triggiani,² Francesco Paolo Granata,⁎ Barbara Balestrieri,⁎ Angelica Petrarelli,⁎ Giulia Scalia,† Luigi Del Vecchio,‡ and Gianni Marone*¹

Secretory phospholipases A₂ (sPLA₂)s are released in large amounts in the blood of patients with systemic inflammatory diseases and accumulate at sites of chronic inflammation, such as the airways of patients with bronchial asthma. Blood eosinophils or eosinophils recruited in inflammatory areas therefore can be exposed in vivo to high concentrations of sPLA₂. We have examined the effects of two structurally different sPLA₂ (group IA and group IIA) on several functions of eosinophils isolated from normal donors and patients with hypereosinophilia. Both group IA and IIA sPLA₂ induced a concentration-dependent release of β-glucuronidase, IL-6, and IL-8. Release of the two cytokines was associated with the accumulation of their specific mRNA. In addition, sPLA₂ induced the surface expression of CD44 and CD69, two major activation markers of eosinophils. Activated eosinophils release a variety of proinflammatory molecules, such as IL-5, leukotriene C₄ (LTC₄) and platelet-activating factor (PAF), and several proinflammatory molecules, including preformed mediators (e.g., cationic proteins and lytic enzymes), lipid mediators (e.g., leukotriene C₄ (LTC₄) and platelet-activating factor (PAF)), and several cytokines (e.g., IL-5 and IL-6) and chemokines (e.g., IL-8) (21). These molecules concur to promote further recruitment of inflammatory cells and to induce tissue injury.

In this study, we have examined the capability of two structurally different sPLA₂s (groups IA and IIA) to activate several immunological and biochemical functions of human eosinophils isolated from fluid of patients with rheumatoid arthritis (8–10), the bronchoalveolar lavage of patients with bronchial asthma (11, 12), and the nasal secretions of patients with allergic rhinitis (13, 14).

sPLA₂ released in plasma or within inflamed tissues are enzymatically active molecules, and they participate in systemic or local inflammatory reactions by releasing AA from outer cell membrane phospholipids (2, 15). However, increasing evidence suggests that at least some sPLA₂s (groups IA, IB, and IIA) also induce activation of cells by mechanisms unrelated to their enzymatic activity (16–20). For example, group IA and IIA sPLA₂ induce exocytosis and cytokine production from human lung macrophages by interacting with binding sites expressed on these cells (18). Group IB sPLA₂ induces bronchoconstriction and fibroblast proliferation via interaction with a 180-kDa receptor (M-type) (16, 17). In addition, group IB and IIA sPLA₂ activate mitogen-activated protein kinases and induce phosphorylation of cytosolic sPLA₂ in murine mast cells expressing the M-type receptor for sPLA₂ (19). These effects of sPLA₂s are reproduced by catalytically inactive sPLA₂s and, therefore, are thought to be independent from the enzymatic activity of these molecules.

Several groups have shown that sPLA₂s are released at sites of allergic reactions, such as the airways of patients with bronchial asthma and the nasal mucosa of patients with allergenic rhinitis (11–14). These tissues are the preferential areas of accumulation and activation of eosinophils. Activated eosinophils release a variety of proinflammatory molecules, including preformed mediators (e.g., cationic proteins and lytic enzymes), lipid mediators (e.g., leukotriene C₄ (LTC₄) and platelet-activating factor (PAF)), and several cytokines (e.g., IL-5 and IL-6) and chemokines (e.g., IL-8) (21). These molecules concur to promote further recruitment of inflammatory cells and to induce tissue injury.

In this study, we have examined the capability of two structurally different sPLA₂s (groups IA and IIA) to activate several immunological and biochemical functions of human eosinophils isolated from fluid.
normal donors and from patients with hypereosinophilia. Our results indicate that sPLA₂ induces the release of β-glucuronidase and the synthesis of IL-6 and IL-8. In addition, sPLA₂ promotes the expression of CD44 and CD69, two activation markers of eosinophils. Interestingly, the sPLA₂ examined in this study have no effect on IL-5, cysteinyl LTC₄, PAF, or superoxide anion production. Stimulation of selected functions in human eosinophils by sPLA₂ is mediated by mechanism(s) largely independent from the enzymatic activity and is associated with activation of extracellular signal-regulated kinases 1/2 (ERK1/2).

Materials and Methods

Reagents and buffers

Group IA sPLA₂ (from *Naja mossambica mossambica* venom), fatty acid-free human serum albumin, BSA, Histoque-1077,PIPES, 1-g-glutamate, antibiotic-antimycotic solution (10,000 U/ml penicillin, 10 ml/g streptomycin, and 25 mg/ml amphotericin B), Triton X-100, phenolphalein glucuronide, lyso phosphatidylcholine (lyso-PC; 1-palmitoyl-sn-glycero-3-phosphocholine), lysophosphatic acid (lyso-PA; 1-oleoyl-sn-glycero-3-phosphate), cytochrome c (from horse heart type VI), and superoxide dismutase (from bovine erythrocytes) were purchased from Sigma-Aldrich (St. Louis, MO). RPMI 1640, FCS, and eosin B were purchased from ICN Pharmaceuticals, Inc. (Irvine, CA). The Ca²⁺ ionophore A23187 and FMLP were purchased from Calbiochem (La Jolla, CA). AA, LTC₄, and PIPES buffer were made of 25 mM PIPES, 110 mM NaCl, and 5 mM Na₂VO₄, 1% Nonidet P-40, and 5% glycerol.

Cell preparations

Eosinophils were isolated from peripheral blood of 19 healthy donors with normal eosinophil count (<300/mm³) and 12 patients with primary hem-1077.000o eosinophilic syndrome (eosinophil count ranging from 500 to 2000/mm³). At the time of blood withdrawal, none of the patients was on or had been previously on treatment with corticosteroids or other cytotoxic drugs. Informed consent was obtained from both normal donors and hypereosinophils patients. Granulocytes were isolated by centrifugation over Histopaque-1077 and eosinophils were purified by negative immunomagnetic selection (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer’s instructions. Briefly, granulocytes were resuspended (5 × 10⁸/50 μl) in PBS supplemented with 0.5% BSA and 2 mM EDTA and were incubated (30 min, 4°C) with magnetic beads coated with a mAb anti-CD16. At the end of incubation, the cells were washed and passed through a magnetic field that retained CD16⁺ cells. The free-flowing cell population consisted of eosinophils with a purity ranging from 95 to 98%, as assessed by eosin B staining. The cells were then resuspended (2 × 10⁶ cells/ml) in RPMI 1640 and incubated as described below.

The experiments on neutrophils were performed with cells isolated from peripheral blood of healthy donors by centrifugation over Histoque-1077. The preparations of neutrophils used in these experiments consisted of a percentage of eosinophils <2%.

Cell incubations

Eosinophils were incubated (37°C, 20 min to 18 h) in RPMI 1640 containing various concentrations of group IA (from *N. mossambica mossambica*) or IIA sPLA₂ (recombinant human synovial PL A₂). All sPLA₂ preparations were repurified by size exclusion chromatography (19) before use and were routinely checked for LPS contamination (*Limulus* Amebocyte Test; ICN Pharmaceuticals). Preparations were discarded if LPS concentration was above the detection limit of the assay (0.125 endotoxin U/ml).

In all experiments, eosinophils were incubated with increasing concentrations of AA, lysophospholipids, or BBP-inactivated sPLA₂. In another group of experiments, eosinophils were incubated with group IIA sPLA₂ (10 μg/ml) in the absence or in the presence of BSA (0.5–5 mg/ml). At the end of the incubation, the supernatants were removed, centrifuged twice (1000 × g, 4°C, 5 min), and stored for up to 72 h at ~80°C for the subsequent determination of β-glucuronidase, IL-5, IL-6, IL-8, and LTC₄ release. The cell pellets were lysed with 0.1% Triton X-100 to determine the total cellular content of β-glucuronidase.

β-Glucuronidase assay

β-Glucuronidase activity in supernatants and cell pellets was measured by a colorimetric assay (22). β-Glucuronidase release was expressed as the percentage of the total cellular content in cell aliquots lysed with 0.1% Triton X-100. All experiments were conducted in triplicate.

ELISA and RT-PCR for IL-5, IL-6, and IL-8

Cytokine release in the supernatant of eosinophils was measured in duplicate determinations using commercially available ELISA kits for IL-5 (R&D Systems, Minneapolis, MN), IL-6, and IL-8 (Euro Clone, Devon, U.K.), according to the manufacturer’s instructions. The linearity range of the assay was between 8 and 500 pg/ml (IL-5), 6 and 200 pg/ml (IL-6), and 52 and 2000 pg/ml (IL-8). The results were expressed as picograms of cytokine per 10⁶ cells.

In the experiments for RT-PCR, eosinophils (5 × 10⁶) were incubated in FCS-free medium alone or with group IIA sPLA₂ (1 μg/ml). At the end of the incubation, RNA was isolated by the TRIzol technique (Invitrogen, Milan, Italy), according to the manufacturer’s instructions. Di-ethylpyrocarbonate-treated water without SDS was used for the final resuspension step; RNA was stored at ~80°C. Reverse transcription was performed with 5 mM MgCl₂, oligo(dT)₁₆ primer, and murine leukemia virus reverse transcriptase according to the manufacturer’s instructions (Applied Biosystems, Norwalk, CT) on a thermocycler (GeneAmp PCR System 2400; Applied Biosystems). PCR was performed using Taq polymerase (1–2.5 U reaction) at the annealing temperature of 60°C, with target-specific primers for IL-5 (5'-TCACGGCAATCTTGTGTACAA-3' and 3'-GGCGTTTTTCTTCCTCAACCC-5'), IL-6 (5'-ATGAACCTTCTCTCAAGCGCC-3' and 3'-GTCAGGTCGGACTCCCGAGAGG-5'), and IL-8 (5'-GATCGTGGCAACC TACAACACCTTGATTCCCTCTCT-3' and 3'-CTTTTAAACCCACCTTTCCTC-5'). Cycle numbers were 30 for IL-6 and 35 for IL-5. IL-5 RNA was normalized by RT-PCR for the constitutive marker gene β-actin (30 cycles). All PCR products, together with a DNA ladder as a size standard, were separated on 2.5% agarose gel, stained with ethidium bromide, and photographed. A semiquantitative measurement of relative cytokine mRNA expression was obtained by digital scanning and densitometric analysis (Scion Image, Frederick, MD).

Flow cytometry analysis of CD44 and CD69 expression

Eosinophils were suspended in RPMI 1640 supplemented with 2.5% FCS, 1% g-glutamate, and 1% antibiotic-antimycotic solution. Cells were cultured (10⁶/ml) for up to 48 h with and without group IIA sPLA₂ (0.1–10 μg/ml) or with 10 mg/ml GM-CSF used as positive control. The cultures were done in 12-well sterile, flat-bottom plates (BD Biosciences) previously coated with 1% BSA. At the end of incubation, the cells were harvested and washed twice with PBS. Expression of CD44 and CD69 was examined by direct immunofluorescence and flow cytometry (FACScalibur; BD Biosciences) according to the following protocol. Cells were suspended at a concentration of 2 × 10⁶/ml; 50 μl of cell suspension were incubated (4°C, 30 min) with saturating amounts of CD44-FITC, CD45RO-FITC, CD38-PE, and CD54-PerCP (1 μg). The pellets were then washed twice with PBS and were resuspended in 300 μl of PBS for analysis. CellQuest software (BD Biosciences) was used for acquisition and Paint-a-Gate software (BD Biosciences) was used for the analysis according to a three-parameter procedure. Although the samples contained almost exclusively eosinophils, these cells were identified according their light scatter properties and their typical expression of CD45 and CD45RO. The mean fluorescence intensity for the...
FIGURE 1. Effect of increasing concentrations of group IA and IIA sPLA₂ on the release of β-glucuronidase from eosinophils. The cells were incubated (37°C, 2 h) with the indicated concentrations of group IA and group IIA sPLA₂. At the end of the incubation, the supernatant was collected and centrifuged (1000 × g, 4°C, 5 min). β-Glucuronidase release was determined by a colorimetric technique (22). The values are expressed as the percentage of the total cellular content determined in cell aliquots lysed with 0.1% Triton X-100. The data are the mean ± SE of six experiments. *, p < 0.01 vs control.

Phosphorylation of ERKs

Purified eosinophils were suspended in PCG buffer. The cells (2.0 × 10⁶/sample) were incubated (37°C, 1–60 min) with 10 μg/ml group IA and IIA sPLA₂. At the end of incubation, the reactions were stopped by adding ice-cold PIPES buffer and then reblotted with anti-ERK1/2 Ab to verify equal protein content of each sample.

Statistical analysis

The data are expressed as the mean ± SE of the indicated number of experiments. The p values were determined with t test for unpaired samples (25).

Table I. sPLA₂-induced β-glucuronidase release from human eosinophils and neutrophils

<table>
<thead>
<tr>
<th></th>
<th>Group IA sPLA₂</th>
<th>Group IIA sPLA₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eosinophils</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(normal donors; n = 8)</td>
<td>6.7 ± 1.1</td>
<td>8.4 ± 1.2</td>
</tr>
<tr>
<td>(hypereosinophilic patients; n = 8)</td>
<td>10.4 ± 0.5**</td>
<td>14.3 ± 1.4***</td>
</tr>
<tr>
<td>(normal donors; n = 8)</td>
<td>10.6 ± 0.5</td>
<td>13.1 ± 0.7***</td>
</tr>
<tr>
<td>Neutrophils</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(normal donors; n = 8)</td>
<td>8.5 ± 0.6</td>
<td>9.6 ± 1.0</td>
</tr>
<tr>
<td>(normal donors; n = 8)</td>
<td>14.3 ± 1.4***</td>
<td>16.7 ± 1.8***</td>
</tr>
</tbody>
</table>

The cells were incubated (37°C, 2 h) with the indicated concentrations of group IA sPLA₂. At the end of the incubation, the supernatants were collected and centrifuged (1000 × g, 4°C, 5 min). β-Glucuronidase release was determined by a colorimetric technique (22). The values are expressed as the percentage of the total cellular content determined in cell aliquots lysed with 0.1% Triton X-100.

* p < 0.05 vs respective unstimulated.
** p < 0.01 vs respective unstimulated.
in β-glucuronidase release induced by group IA sPLA₂ between eosinophils from normal donors (blood eosinophils <300/mm³) and those from patients with hypereosinophilia (blood eosinophils >500/mm³). In addition, Table I shows that group IA sPLA₂ also induced the release of β-glucuronidase from human neutrophils (preparations containing <2% eosinophils) in a fashion quantitatively similar to that induced from eosinophils. These data indicate that sPLA₂s induce a noncytotoxic release of β-glucuronidase that is comparable in eosinophils (from normal donors and hypereosinophilic patients) and neutrophils.

**Effect of sPLA₂s on IL-5, IL-6, and IL-8 production from eosinophils**

In the next group of experiments, we determined whether, in addition to a preformed mediator such as β-glucuronidase, group IA and IIA sPLA₂s induced the production of IL-5, IL-6, and IL-8, three major cytokines produced by human eosinophils (26–28). In these experiments, the cells were incubated with increasing concentrations of sPLA₂s for 18 h (IL-5 and IL-6) or for 4 h (IL-8). Kinetic experiments indicated that these incubation times were optimal for sPLA₂-induced cytokine release (data not shown). Fig. 2 shows that both group IA (upper panel) and IIA (lower panel) sPLA₂s concentration dependently increase the basal secretion of IL-6 and IL-8 from eosinophils. In contrast, none of the sPLA₂s induced IL-5 release from cells incubated up to 24 h. The effect of sPLA₂s on the production of IL-6 and IL-8 was significant at 1 μg/ml and maximal at 10 μg/ml. There was no significant difference in sPLA₂-induced IL-6 and IL-8 production between eosinophils from normal donors and eosinophils from hypereosinophilic patients (data not shown).

We tested the hypothesis that sPLA₂s induce cytokine gene expression by evaluating IL-5, IL-6, and IL-8 mRNA expression by RT-PCR in eosinophils incubated with group IIA sPLA₂. Fig. 3 (upper panel) depicts the β-actin- and IL-6-specific RT-PCR amplification products from an experiment representative of three in which eosinophils were cultured for 12 or 18 h with medium alone or with group IIA sPLA₂. Fig. 3 (lower panel) depicts the β-actin- and IL-8-specific RT-PCR amplification products from an experiment representative of three in which eosinophils were cultured for 1 or 3 h with medium alone or with group IIA sPLA₂. We used different incubation times for IL-6 and IL-8 mRNA experiments because kinetics data showed that IL-6 release was delayed (16–24 h) compared with IL-8 release (2–6 h). Eosinophils constitutively express IL-6 and IL-8 mRNAs; incubation with group IIA sPLA₂ increased the amount of mRNA specific for both IL-6 and IL-8. There was no basal expression of IL-5 mRNA in eosinophil preparations and no sPLA₂-induced increase in our experimental conditions (data not shown). Densitometric analysis of the signal intensity of IL-6 and IL-8 mRNA amplification products, obtained in three experiments and expressed as the ratio with the corresponding signal intensity of β-actin, revealed that group IIA sPLA₂s induced a significant accumulation of IL-6- and IL-8 mRNA after 12 h and 3 h of incubation, respectively (Table II). These results indicate that sPLA₂-induced cytokine production is associated with the accumulation of their specific mRNA.

**FIGURE 2.** Effect of increasing concentrations of group IA and IIA sPLA₂s on the release of cytokines from eosinophils. The cells were incubated (37°C, 18 h for IL-5 and IL-6 and 4 h for IL-8) with the indicated concentrations of group IA (upper panel) and group IIA (lower panel) sPLA₂s. At the end of the incubations, the supernatant was collected and centrifuged (1000 × g, 4°C, 5 min). IL-5, IL-6, and IL-8 release were determined by ELISA. The data are the mean ± SE of six experiments. *, p < 0.05 vs control; **, p < 0.01 vs control.

**FIGURE 3.** RT-PCR of IL-6 and IL-8 in sPLA₂-stimulated eosinophils. Upper panel, β-Actin- (first row) and IL-6-specific RT-PCR amplification products from a representative experiment in which eosinophils were cultured for 12 and 18 h with RPMI 1640 alone (unstimulated) or group IIA sPLA₂ (1 μg/ml). The sizes for β-actin and IL-6 were 673 and 658 bp, respectively. A 100-bp DNA ladder was used as standard. Lower panel, β-Actin- (first row) and IL-8-specific RT-PCR amplification products from a representative experiment in which eosinophils were cultured for 1 and 3 h with RPMI 1640 alone (unstimulated) or group IIA sPLA₂ (1 μg/ml). The sizes for β-actin and IL-8 were 673 and 308, respectively. A 100-bp DNA ladder was used as standard.
Effect of sPLA₂ on CD44 and CD69 expression on human eosinophils

CD44 and CD69 are distinct cell surface activation markers for human eosinophils (29). CD44 is constitutively expressed, whereas little if any CD69 is expressed on quiescent eosinophils. However, both markers are significantly increased on eosinophils activated in vivo or in vitro (29). We analyzed the effects of group IIA sPLA₂ on the expression of these two eosinophil activation Ags. Fig. 4, A and B, shows that sPLA₂ enhanced the expression of CD44 in a concentration- and time-dependent fashion. In particular, the effect of sPLA₂ appeared after 24 h of incubation and reached maximal level after 48 h of incubation. As a control in these experiments, we used GM-CSF (10 ng/ml) that induces CD44 expression (29). Group IIA sPLA₂ also induced the expression of CD69 in a concentration- and time-dependent manner (Fig. 5, A and B). As previously shown (30), because CD69 is expressed

Table II. **Densitometric analysis of sPLA₂-induced mRNA expression of IL-6 and IL-8 in human eosinophils**

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>IL-6 (Ratio of IL-6:β-Actin)</th>
<th>IL-8 (Ratio of IL-8:β-Actin)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12 h</td>
<td>18 h</td>
</tr>
<tr>
<td>Unstimulated</td>
<td>0.18 ± 0.03</td>
<td>0.20 ± 0.04</td>
</tr>
<tr>
<td>Group IIA sPLA₂ (1 μg/ml)</td>
<td>0.48 ± 0.08*</td>
<td>0.28 ± 0.05</td>
</tr>
</tbody>
</table>

* Eosinophils were incubated for different times (1 or 3 h and 12 or 18 h for IL-8 and IL-6, respectively) with RPMI 1640 alone (unstimulated) or with group IIA sPLA₂. The results are expressed as the signal intensity ratio cytokine:β-actin. Data from three separate experiments are expressed as the mean ± SE. *p < 0.05 vs unstimulated.
at a low level in freshly isolated eosinophils, the effect of sPLA₂ is already near maximal after 24 h with only a small increase after 48 h of incubation. The maximal response induced by sPLA₂ (10 μg/ml) was comparable to that induced by 10 ng/ml of GM-CSF. These data confirm and extend previous observations (30) demonstrating that sPLA₂ induces the expression of two major activation markers on human eosinophils.

Effect of sPLA₂ on superoxide anion generation from human eosinophils

Eosinophils are a major source of oxygen radicals in hypereosinophilic diseases (31). In these experiments, we explored whether sPLA₂ induced superoxide anion generation from eosinophils. Fig. 6 shows that neither group IA nor group IIA sPLA₂ activated the production of superoxide anion from eosinophils isolated from normal donors or from patients with hypereosinophilia (data not shown). In the same experimental conditions, 5.9 ± 1.4 nmoles of superoxide anion/10⁶ cells were produced by eosinophils stimulated with the the Ca²⁺ ionophore A23187.

Effect of sPLA₂ on LTC₄ and PAF synthesis from human eosinophils

Extracellular sPLA₂s mobilize AA that is available for conversion to eicosanoids (15, 32). The major AA metabolite produced by human eosinophils is LTC₄, a potent bronchoconstrictor and proinflammatory mediator (33–35). In this group of experiments we evaluated the effect of sPLA₂ on the de novo synthesis of LTC₄. Group IA and group IIA sPLA₂s did not induce release of LTC₄ from eosinophils of either normal donors or hypereosinophilic patients (Fig. 7). Under the same experimental conditions, A23187 and FMLP, used as positive controls, induced the release of 21.3 ± 1.8 and 4.2 ± 0.5 ng of LTC₄/10⁶ cells, respectively (Fig. 7).

Eosinophils are a major source of PAF (36), a lipid mediator whose synthesis is biochemically related to AA metabolism (37). In three experiments, incubation of eosinophils with group IIA sPLA₂ up to 10 μg/ml did not induce the production of PAF as assessed by [³H]acetate incorporation (control, 22 ± 7; group IIA sPLA₂, 24 ± 8 cpm/10⁶ cells). In the same experiments, A23187

![FIGURE 6](image)

FIGURE 6. Effect of increasing concentrations of group IA and IIA sPLA₂ on superoxide anion generation from eosinophils. The cells were incubated (37°C, 30 min) with the indicated concentrations of group IA or IIA sPLA₂ or with A23187 (10⁻⁶ M), used as a positive control. At the end of the incubation, the supernatant was collected and centrifuged (1000 × g, 4°C, 5 min). Superoxide anion generation was determined by the superoxide dismutase-inhibitable reduction of cytochrome c. The data are the mean ± SE of four experiments. * p < 0.01 vs control.

![FIGURE 7](image)

FIGURE 7. Effect of increasing concentrations of group IA and IIA sPLA₂ on LTC₄ production from eosinophils. The cells were incubated (37°C, 1 h) with the indicated concentrations of group IA or IIA sPLA₂ or with A23187 (10⁻⁶ M) and FMLP (10⁻⁶ M), used as positive controls. At the end of the incubation, the supernatant was collected and centrifuged (1000 × g, 4°C, 5 min). LTC₄ production was determined by RIA. The data are the mean ± SE of four experiments. * p < 0.01 vs control.

Role of sPLA₂ enzymatic activity on the activation of human eosinophils

The major enzymatic products of sPLA₂s, AA and lysophospholipids, modulate several biochemical functions of inflammatory cells, including eosinophils (38). In particular, sPLA₂-induced expression of CD69 has been related to the generation of lysophospholipids (30). To understand whether the effects of sPLA₂ were due to the enzymatic activity, we designed two sets of experiments. We initially tested the hypothesis that enzymatic products of sPLA₂ could be involved in sPLA₂-induced activation of eosinophils by evaluating the effect of AA, lyso-PC, or lyso-PA on the release of β-glucuronidase and IL-8 from CD44 and CD69. Table III shows that AA, lyso-PC, and lyso-PA, at concentrations up to 10⁻⁵ M, did not affect any of the functions activated by group IIA sPLA₂. We also performed experiments to rule out that endogenous lipid mediators could have affected sPLA₂-induced activation of eosinophils. In these experiments, the cells were stimulated with group IIA sPLA₂ in the presence of BSA. At concentrations up to 5 mg/ml, BSA did not modify the sPLA₂-induced release of β-glucuronidase and IL-8 (data not shown). These results indicate that the major enzymatic products of sPLA₂ are not involved in sPLA₂-induced activation of eosinophils.

In another group of experiments, eosinophils were incubated with group IA or IIA sPLA₂, in which catalytic activity was inactivated by treatment with BPB for 4 h at 37°C (20). This procedure resulted in the irreversible inhibition of >98% of enzymatic activity (data not shown). Fig. 8 shows that both BPB-inactivated sPLA₂s (group IA and group II) retain their ability to induce the release of β-glucuronidase (upper panel) and IL-8 (lower panel) from eosinophils. However, although not statistically significant, the release of β-glucuronidase and IL-8 induced by enzymatically inactive sPLA₂s tended to be lower than that induced by the active molecule. Taken together, the results of these groups of experiments indicate that the enzymatic activity of sPLA₂ of both snake and human origin, is not primarily responsible for inducing degranulation and cytokine release.
Activation of ERK1/2 by sPLA₂ in human eosinophils

The aforementioned results indicated that the enzymatic activity of sPLA₂s was not mainly responsible for eosinophil activation and suggested that the effects of sPLA₂s could be mediated by alternative mechanisms. Binding of sPLA₂s to the M-type receptor expressed on mast cells and macrophages has been shown to activate intracellular signaling through the ERK1/2 pathway (19, 39). To verify this mechanism in human eosinophils, cells were stimulated with group IA sPLA₂ (10 μg/ml). The reaction was stopped at various time points and cytosolic extracts were subjected to Western blot with anti-phospho-ERK1/2 Ab (Fig. 9, upper gel). Phosphorylation became evident after 30 s and persisted up to 15 min. Reprobing the membrane with the anti-ERK1/2 Ab (nonphosphorylated forms) confirmed equal protein loading (Fig. 9, lower gel). Similar results were obtained when eosinophils were stimulated with group IIA sPLA₂ (data not shown). Phosphorylation of ERK1/2 was not detected under our experimental conditions when the cells were incubated with AA or lysophospholipids up to 10⁻⁵ M (data not shown).

Discussion

Extracellular PLA₂s are attracting increasing attention as mediators of local and systemic inflammation. Several studies have shown that sPLA₂s can activate inflammatory cells by various mechanisms either related or unrelated to their enzymatic activity.

Table III. Effect of sPLA₂ enzymatic products on human eosinophils

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>β-Glucuronidase (%) of total cellular content</th>
<th>IL-8 (pg/10⁶ cells)</th>
<th>CD44 (MFI)</th>
<th>CD69 (MFI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>6.32 ± 0.68</td>
<td>340 ± 57</td>
<td>70.3 ± 6.4</td>
<td>24.7 ± 3.1</td>
</tr>
<tr>
<td>AA, 10⁻⁷ M</td>
<td>5.93 ± 0.35</td>
<td>313 ± 71</td>
<td>69.0 ± 4.4</td>
<td>24.3 ± 2.6</td>
</tr>
<tr>
<td>AA, 10⁻⁶ M</td>
<td>5.66 ± 0.41</td>
<td>308 ± 72</td>
<td>71.7 ± 5.1</td>
<td>26.7 ± 4.0</td>
</tr>
<tr>
<td>AA, 10⁻⁵ M</td>
<td>6.03 ± 0.37</td>
<td>325 ± 67</td>
<td>67.0 ± 6.5</td>
<td>26.3 ± 3.3</td>
</tr>
<tr>
<td>Lyso-PC, 10⁻⁷ M</td>
<td>6.57 ± 0.73</td>
<td>332 ± 50</td>
<td>69.7 ± 5.2</td>
<td>24.3 ± 3.9</td>
</tr>
<tr>
<td>Lyso-PC, 10⁻⁶ M</td>
<td>6.92 ± 0.75</td>
<td>350 ± 47</td>
<td>68.7 ± 3.9</td>
<td>23.0 ± 3.7</td>
</tr>
<tr>
<td>Lyso-PC, 10⁻⁵ M</td>
<td>6.96 ± 0.58</td>
<td>352 ± 63</td>
<td>71.3 ± 4.9</td>
<td>25.3 ± 2.9</td>
</tr>
<tr>
<td>Lyso-PA, 10⁻⁷ M</td>
<td>6.01 ± 0.69</td>
<td>362 ± 61</td>
<td>73.3 ± 8.4</td>
<td>25.7 ± 3.5</td>
</tr>
<tr>
<td>Lyso-PA, 10⁻⁵ M</td>
<td>6.10 ± 0.57</td>
<td>328 ± 58</td>
<td>69.0 ± 7.5</td>
<td>26.0 ± 2.8</td>
</tr>
<tr>
<td>Lyso-PA, 10⁻³ M</td>
<td>6.20 ± 0.74</td>
<td>344 ± 64</td>
<td>69.3 ± 4.3</td>
<td>23.3 ± 4.3</td>
</tr>
<tr>
<td>Group IIA sPLA₂, 10 μg/ml</td>
<td>11.06 ± 0.95***</td>
<td>713 ± 97**</td>
<td>109.3 ± 10*</td>
<td>55.3 ± 6.3**</td>
</tr>
</tbody>
</table>

* p < 0.05 vs respective controls (none).
** p < 0.01 vs respective controls (none).

The cells were incubated (37°C, 2 h for β-glucuronidase release, 4 h for IL-8 release, 48 h for CD44 and CD69 expression) with the indicated concentrations of AA, lyso-PC, lyso-PA, and group IIA sPLA₂. At the end of the incubation, the supernatant was collected and centrifuged (1000 × g, 4°C, 5 min). β-Glucuronidase release was determined by a colorimetric technique (22). IL-8 release was measured by ELISA. CD44 and CD69 expression were analyzed as described in Materials and Methods and expressed as the MFI. The values of β-glucuronidase are expressed as the percentage of the total cellular content determined in cell aliquots lysed with 0.1% Triton X-100. The values represents the mean ± SE of three experiments.
(17–20, 39–41). We have examined the effects of two structurally
different sPLA2s on several functions of human eosinophils. Both
group IA and II A sPLA2s induce the release of the specific granule
enzyme β-glucuronidase, the production of IL-6 and IL-8, and the
expression of activation markers (CD44 and CD69) on human eo-
sinophils. In contrast, sPLA2s have no effect on IL-5, LTC4, PAF,
and superoxide anion production.

Large quantities of sPLA2 are released in the blood of patients
with systemic inflammatory diseases. For example, concentrations
up to 4 μg/ml sPLA2 have been detected in the plasma of patients
with septic shock (5). In addition, calculation of the specific ac-
tivity of human group II A sPLA2 (42) and dilution of alveolar fluid
due to the bronchoalveolar lavage (43) indicate local concentra-
tions of sPLA2 in the airways of patients with bronchial asthma
ranging between 2 and 10 μg/ml (12). Therefore, eosinophils may
be exposed in vivo to concentrations of sPLA2 that efficiently activate
selected biochemical and immunological functions in these cells. Furthermore, eosinophils are a major cellular source of
group II A sPLA2 (44). This observation suggests that sPLA2 may
function as an autocrine stimulus for eosinophil activation.

Interestingly, both group IA and II A sPLA2s are more potent stimuli for IL-8 production than are other eosinophil agonists like
PAF, RANTES, and PMA, and they do not require cell priming
(28, 45). These data support the concept that sPLA2s may be rel-
levant stimuli for systemic and/or local activation of eosinophils in
generic or hypereosinophilic disorders usually associated with
high circulating levels of IL-6 and IL-8 (28, 46–48).

Surprisingly, sPLA2 do not induce, at least in vitro, IL-5 pro-
duction. Previous data suggest that the production of cytokines is
differentially regulated in normal human eosinophils. For example,
secretory IgA induces the release of IL-8 but not of IL-5 (45). In
addition, the production of IL-8, but not of other cytokines, in
human eosinophils is closely associated with degranulation (45).
Our data support the hypothesis of differential regulation of cyto-
kine transcription in eosinophils by showing that sPLA2 selectively
activate intracellular signals leading to IL-8 and IL-6, but not IL-5, expression. Furthermore, sPLA2s induce the release of a
prefomed mediator (β-glucuronidase), but not of de novo synthe-
sized mediators (LTC4, PAF, and superoxide anion). These obser-
vations suggest that activation by sPLA2 is restricted to selected
signaling pathways within human eosinophils. Our results indicate
that incubation of eosinophils with sPLA2s induces a rapid acti-
vation of ERK1/2. This signaling pathway is crucial for degrau-
dation and cytokine production, but not for other functions in hu-
mans eosinophils (49). Although these data clearly indicate that
sPLA2s generate intracellular signals in eosinophils, additional ex-
nperiments are needed to understand the mechanism(s) underlying the selectivity of effect of sPLA2s.

Our results extend the previous observation that sPLA2 induces
the expression of surface activation markers on eosinophils (30).
CD44 and CD69, although not specific of eosinophils, are over-
expressed on cells activated in vivo, such as the eosinophils re-
trieved from the bronchoalveolar lavage, or in vitro by incubation
with cytokines (29, 50). The function of these markers in asthma
and in hypereosinophilic disease is unknown. Interestingly, how-
ever, CD44 is highly expressed on leukocytes from patients with
rheumatoid arthritis (51), a disease in which blood levels of sPLA2
are significantly increased. This observation supports the hypoth-

osis that sPLA2 activate a broad spectrum of inflammatory cells.
In line with this hypothesis is our observation that sPLA2 induced
degranulation of neutrophils in a fashion comparable to that of
eosinophils.

The activation of inflammatory cells induced by sPLA2 could
occur through at least three mechanisms: 1) generation of AA and
lyso phospholipids by direct enzymatic activity on membrane phos-
pholipids with the concurrent generation of AA metabolites, 2)
interaction of sPLA2s with membrane peptidoglycans through
their heparin-binding sites, and 3) activation of specific receptors,
i.e., the M-type or N-type.

The enzymatic activity of sPLA2s generates AA and lysophospho-
 lipids, two intracellular messengers capable of inducing such
cellular responses as exocytosis and cytokine expression (38, 52).
In addition, LTC4, the main AA metabolite synthesized by eosino-
phils, may function as an autocrine stimulus for these cells (53,
54). Therefore, the enzymatic activity may be, at least in part,
responsible for the eosinophil activation induced by sPLA2s. How-

ever, a number of observations exclude that catalytic activity plays
a primary role in the induction of degranulation and IL production.
First, sPLA2-induced activation of eosinophils is not associated
with significant LTC4 generation. Second, incubation of eosino-
phils with the major enzymatic products of sPLA2, AA, lyso-PC,
or lyso-PA, did not affect β-glucuronidase or IL-8 secretion. Fi-
nally, inactivation of sPLA2 enzymatic activity by PPB did not
abolish its capacity to induce eosinophil activation. These data
indicate that, in other inflammatory cells (18–20), the catalytic
activity of sPLA2s is not mainly responsible for their ability to
induce the release of lysosomal enzymes and the de novo synthesis
cytokines and chemokines. However, inactivation of PL2 activity
tends to reduce the effect of sPLA2 on β-glucuronidase and IL-8 production, suggesting that one or more of the enzymatic
products of sPLA2s may enhance the effects exerted by sPLA2s on
eosinophils.

Several sPLA2 bind to peptidoglycans on cell membranes
through their heparin-binding site (55, 56). This interaction results
in the internalization of sPLA2, which is then capable of generating
intracellular signals leading to gene expression (57). Both group
IA and II A sPLA2 possess a heparin-binding site (58). Therefore,
a potential mechanism by which these molecules may activate eo-
sinophils is through interaction with membrane peptidoglycans.
Moreover, it is conceivable that sPLA2, at least at high concen-
trations, could induce membrane perturbations, which in turn may
trigger eosinophil activation.

Finally, sPLA2s may activate eosinophils by interacting with
one or more specific receptors, i.e., the M-type or the N-type (59).
These receptors are expressed in a variety of tissues and inflam-
matory cells, including macrophages, monocytes, and mast cells,
and they are functionally coupled to different cellular responses
(17–19, 42, 59, 60). The lack of specific Abs or ligands for these
receptors in human cells currently precludes their characterization
on eosinophils. However, we provide evidence that both group IA
and II A sPLA2s induce activation of ERK1/2, a signaling pathway
that has been coupled to activation of the M-type receptor in other
cell types (19, 39). These data support the hypothesis that sPLA2
may activate human eosinophils by interacting with the M-type
receptor. However, our data do not exclude the possibility that
activation of N-type receptor or of other as yet uncharacterized
receptors may be involved in sPLA2-induced activation of
eosinophils.

We were unable to confirm the observation by Urasaki et al. (30)
that enzymatically generated lysophospholipids are responsible for
the expression of CD69 on eosinophils. This discrepancy may be
explained by differences in the procedure for purification of eo-
sinophils, i.e., density gradients vs immunomagnetic selection.
In any event, our results do not exclude the possibility that more
than one mechanism underlies the effect of sPLA2s on eosinophils.

In conclusion, our results indicate that sPLA2s selectively acti-
uate certain proinflammatory and immunomodulatory functions in
eosinophils with mechanism(s) that appear mainly independent
from their enzymatic activity. Activation of human eosinophils by sPLA₃s may concur with the eicosanoid-forming capacity of these molecules to induce systemic and/or local activation of these cells in inflammatory and allergic disorders.

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References


