Selective Functions in Human Eosinophils

Massimo Triggiani, Francescopaolo Granata, Barbara Balestrieri, Angelica Petraroli, Giulia Scalia, Luigi Del Vecchio and Gianni Marone

*J Immunol* 2003; 170:3279-3288; doi: 10.4049/jimmunol.170.6.3279

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*The Journal of Immunology* is published twice each month by
The American Association of Immunologists, Inc.,
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Secretory Phospholipases A₂ Activate Selective Functions in Human Eosinophils

Massimo Triggiani, Francesco Paolo Granata, Barbara Balestrieri, Angelica Petrari, Giulia Scalia, Luigi Del Vecchio, and Gianni Marone

Secretory phospholipases A₂ (sPLA₂s) 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. In contrast, none of the sPLA₂ examined induced the production of IL-5, the de novo synthesis of leukotriene C₄ and platelet-activating factor, or the generation of superoxide anion from human eosinophils. Incubation of eosinophils with the major enzymatic products of the sPLA₂ (arachidonic acid, lysophosphatidylcholine, or lysophosphatidic acid) did not reproduce any of the enzymes’ effects. In addition, inactivation of sPLA₂ enzymatic activity by bromophenacyl bromide did not influence the release of β-glucuronidase or of cytokines. Stimulation of eosinophils by sPLA₂ was associated with activation of extracellular signal-regulated kinases 1/2. These results indicate that sPLA₂ selectively activate certain proinflammatory and immunoregulatory functions of human eosinophils through mechanism(s) independent from enzymatic activity and from the generation of arachidonic acid. The Journal of Immunology, 2003, 170: 3279–3288.

Phospholipases A₂ (PLA₂s) are a family of enzymes that release fatty acids, including arachidonic acid (AA), from the sn-2-position of phospholipids (1, 2). Two major classes of PLA₂ have been identified and characterized: low-m.w. secretory PLA₂ (sPLA₂) and high-m.w. cytosolic PLA₂ (3). Within each class, there are several isoforms that have distinct primary structures, Ca²⁺ requirements, and cellular sources. sPLA₂ were initially found in reptile (group IA) and bee venoms (group III) and in mammalian pancreatic fluid (group IB). It was shown later that a variety of sPLA₂ can be expressed and released in human tissues (3). Increased levels of extracellular sPLA₂ have been detected in the plasma of patients affected by systemic inflammatory diseases such as acute pancreatitis (4), septic shock (5), extensive burns (6), and autoimmune diseases (7). These molecules also accumulate in inflammatory fluids such as the synovial 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 sPLA₂ (groups IA, IB, and IIA) also activate inflammatory 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 PLA₂ in murine mast cells expressing the M-type receptor for sPLA₂ (19). These effects of sPLA₂ are reproduced by catalytically inactive sPLA₂ and, therefore, are thought to be independent from the enzymatic activity of these molecules.

Several groups have shown that sPLA₂ are released at sites of allergic reactions, such as the airways of patients with bronchial asthma and the nasal mucosa of patients with allergic 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 sPLA₂ (groups IA and IIA) to activate several immunological and biochemical functions of human eosinophils isolated from...
normal donors and from patients with hypereosinophilia. Our results indicate that sPLA₂-S induce the release of β-glucuronidase and the synthesis of IL-6 and IL-8. In addition, sPLA₂-S promote the expression of CD44 and CD69, two activation markers of eosinophils. Interestingly, the sPLA₂-S examined in this study have no effect on IL-5, cyssteinyl LTCP, PAF, or superoxide anion production. Stimulation of selected functions in human eosinophils by sPLA₂-S 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, Histoaque-1077, PIPES, 1-glutamine, antibiotic-antimycotic solution (10,000 U ml−1 penicillin, 10 mg/ml streptomycin, and 25 mg/ml amphotericin B), Triton X-100, phenolphenalein glucuronide, lysophosphatidylcholine (lyso-PC; 1-palmitoyl-sn-glycerol-3-phosphocholine), lysophosphatidic acid (lyso-PA; 1-oleoyl-sn-glycerol-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 (Irvine, CA). The Ca²⁺ ionophore A23187 and FMLP were purchased from Calbiochem (La Jolla, CA). AA, LTC₄, and PAF were purchased from Biomol (Plymouth Meeting, PA). [⁹¹H]Acetic acid (Na salt; 1.9 Ci/ml) was purchased from DuPont NEN (Boston, MA). GM-CSF was purchased from PeproTech (Rocky Hill, NJ). FITC-conjugated anti-CD44 and anti-CD69 and PerCP-conjugated anti-CD45 mAbs were purchased from BD Biosciences (San Jose, CA). PE-conjugated anti-CD45R0 mAb was obtained from DAKO (Glostrup, Denmark).

Isotypic control Abs, FITC-conjugated mouse IgG1, and PE-conjugated IgG2 were purchased from Immunotech (Marseille, France) and Caltag (Burlingame, CA), respectively. Rabbit anti-phospho-ERK1/2 Ab and rabbit anti-ERK1/2 Ab were purchased from New England Biolabs (Beverly, MA). FITC-conjugated anti-CD16 was purchased from Amersham Pharmacia Biotech (Buckinghamshire, U.K.).

Group IIA (recombinant human synovial) sPLA₂ was a generous gift from J. Winkler (Smith Kline and Beecham, King of Prussia, PA). Bromophenacyl bromide (BPB)-inactivated group I A sPLA₂, prepared as previously described (20), was kindly provided by A. Fonteh (Wake Forest University, Winston-Salem, NC). BPB-inactivated group IIA sPLA₂ was prepared with the same protocol (20). IL-5, IL-6, IL-8, and -β-actin primers were designed by D. Essayan (Johns Hopkins University, Baltimore, MD) and were produced and purified by the Johns Hopkins DNA Core Facility. All other reagents were from Carlo Erba (Milan, Italy).

PIPES buffer was made of 25 mM PIPES, 110 mM NaCl, and 5 mM KCl. PCG buffer (was made of PIPES buffer containing 1 mM CaCl₂ and 1 mM glucose (pH 7.4)). Lysis buffer for Western blot of ERK1/2 was made by the Johns Hopkins DNA Core Facility. Lysis buffer for Western blot of ERK1/2 was made by the Johns Hopkins DNA Core Facility.

Cell preparation

Eosinophils were isolated from peripheral blood of 19 healthy donors with normal eosinophil count (<300/mm³) and 12 patients with primary hypereosinophilic 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 hypereosinophilic 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 × 10⁷/50 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 passaged 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 Histopaque-1077. The preparations of neutrophils used in these experiments contained a percentage of eosinophils <2%.

Flow cytometry analysis of CD44 and CD69 expression

Eosinophils were suspended in RPMI 1640 supplemented with 2.5% FCS, 1% L-glutamine, and 1% antibiotic-antimycotic solution. Cells were cultured (10⁴/ml) for up to 48 h with and without group IIA sPLA₂ (1 μg/ml) or with 10 ng/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-PE, and CD16-PerCP in a 15 ml Tubes. 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
Phosphorylation of ERKs

FIGURE 1. Effect of increasing concentrations of group IA and IIA sPLA2s 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 sPLA2s. 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 × 10^6/sample) were incubated (37°C, 1–60 min) with 10 μg/ml group IA and IIA sPLA2s. At the end of incubation, the reactions were stopped by adding ice-cold PIPES buffer, and the samples were microfuged for 15 s. Cell pellets were immediately lysed in lysis buffer. Cell lysates were kept on ice for 20 min and then microfuged for 10 min at 4°C. Supernatant was collected as a protein extract containing lysed cell components without nuclei pellets were immediately lysed in lysis buffer. Cell lysates were kept on ice though comparisons were made on the basis of an equal number of cells, equal protein content of each sample.

Results

Effect of sPLA2s on β-glucuronidase release from human eosinophils

Initial experiments were performed to determine whether group IA (from cobra venom) and IIA (recombinant human synovial) sPLA2 induced the release of the lysosomal enzyme β-glucuronidase from human eosinophils isolated from normal donors. The cells were incubated (37°C, 2 h) with various concentrations of group IA or IIA sPLA2. Fig. 1 shows that group IA and IIA sPLA2s induce the release of β-glucuronidase from purified human eosinophils in a concentration-dependent fashion. The effect of sPLA2s was significant at a concentration of 1 μg/ml, whereas concentrations >10 μg/ml did not induce further release of β-glucuronidase. None of the sPLA2s used in these experiments significantly influenced cell viability, as assessed by trypan blue exclusion (data not shown). In addition, the release of LDH, used as an indicator of cytotoxicity, in sPLA2-treated eosinophil supernatants was always <4%.

Additional experiments were performed to compare sPLA2-induced β-glucuronidase release from eosinophils isolated from hyper eosinophilic patients with eosinophils or neutrophils isolated from normal donors (Table I). There was no significant difference and membrane-bound anti-rabbit Ig Ab was visualized with the ECL Western blotting detection reagent (Amersham Pharmacia Biotech) and HyperECL luminescence detection film (Amerham Pharmacia Biotech). Although comparisons were made on the basis of an equal number of cells, membranes were stripped with stripping buffer (7 M guanidine hydrochloride in distilled water) and then rebotted with anti-ERK1/2 Ab to verify equal protein content of each sample.

Production of LTC4 and PAF

LTC4 release was measured by RIA, as previously described (23). The linearity range of the assay was between 80 and 5000 pg/ml. The results were expressed as nanograms of LTC4 per 10^6 cells. The production of PAF was determined in both supernatants and cell pellets as the incorporation of [3H]acetate (22). The results were expressed as cpm of [3H]acetate incorporated into PAF/10^6 cells.

Generation of superoxide anion

Superoxide anion production was measured as superoxide dismutase-inhibitable reduction of cytochrome c according to the method of Pick and Mize (24). The results were expressed as nanomoles of superoxide anion per 10^6 cells.

Lactate dehydrogenase (LDH) assay

LDH release at the end of the incubation was determined as an index of cytotoxicity. LDH was measured in cell-free supernatants using a commercially available kit (Sigma-Aldrich).

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>Group IA sPLA2</th>
<th>Eosinophils (normal donors; n = 8)</th>
<th>Eosinophils (hypereosinophilic patients; n = 8)</th>
<th>Neutrophils (normal donors; n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unstimulated</td>
<td>6.7 ± 1.1</td>
<td>8.4 ± 1.2</td>
<td>10.6 ± 0.5</td>
</tr>
<tr>
<td>0.1 μg/ml</td>
<td>8.5 ± 0.6</td>
<td>9.6 ± 1.0</td>
<td>12.4 ± 1.0</td>
</tr>
<tr>
<td>1 μg/ml</td>
<td>10.4 ± 0.5</td>
<td>14.3 ± 1.4**</td>
<td>13.1 ± 0.7**</td>
</tr>
<tr>
<td>10 μg/ml</td>
<td>16.3 ± 1.3**</td>
<td>17.0 ± 0.9**</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 sPLA₂ induce a nontoxic release of β-glucuronidase that is comparable in eosinophils (from normal donors and hypereosinophilic patients) and neutrophils.

**Effect of sPLA₂ 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 sPLA₂ 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 sPLA₂ 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) sPLA₂ concentrations dependently increase the basal secretion of IL-6 and IL-8 from eosinophils. In contrast, none of the sPLA₂ induced IL-5 release from cells incubated up to 24 h. The effect of sPLA₂ 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. 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 sPLA₂ 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 sPLA₂ 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 IIA (lower panel) sPLA₂. 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.

FIGURE 4. FACS analysis of CD44 expression on human eosinophils. A, Eosinophils were cultured (37°C, 48 h) with medium alone (unstimulated; dotted line) or with group IIA sPLA₂ (1 μg/ml; continuous line). At the end of the incubation, the cells were analyzed as described in Materials and Methods. The figure displays the results of one representative experiment of three. B, Eosinophils were incubated (37°C, 24–48 h) with medium alone (unstimulated), with increasing concentrations (0.1–10 μg/ml) of group IIA sPLA₂, and with GM-CSF (10 ng/ml) used as a positive control. At the end of the incubation, the cells were analyzed as described in Materials and Methods. The figure shows the mean ± SE of three experiments. *, p < 0.05 vs unstimulated.

FIGURE 5. FACS analysis of CD69 expression on human eosinophils. A, Eosinophils were cultured (37°C, 24 h) with medium alone (unstimulated; dotted line) or with group IIA sPLA₂ (1 μg/ml; continuous line). At the end of the incubation, the cells were analyzed as described in Materials and Methods. The figure displays the results of one representative experiment of three. B, Eosinophils were incubated (37°C, 24–48 h) with medium alone (unstimulated), with increasing concentrations (0.1–10 μg/ml) of group IIA sPLA₂, and with GM-CSF (10 ng/ml) used as a positive control. At the end of the incubation, the cells were analyzed as described in Materials and Methods. The figure shows the mean ± SE of three experiments. *, p < 0.05 vs unstimulated.
at a low level in freshly isolated eosinophils, the effect of sPLA_2 is already near maximal after 24 h with only a small increase after 48 h of incubation. The maximal response induced by sPLA_2 (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 sPLA_2s induce the expression of two major activation markers on human eosinophils.

**Effect of sPLA_2s 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 sPLA_2s induced superoxide anion generation from eosinophils. Fig. 6 shows that neither group IA nor group IIA sPLA_2 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/10^6 cells were produced by eosinophils stimulated with the Ca^{2+} ionophore A23187.

**Effect of sPLA_2s on LTC_4 and PAF synthesis from human eosinophils**

Extracellular sPLA_2s mobilize AA that is available for conversion to eicosanoids (15, 32). The major AA metabolite produced by human eosinophils is LTC_4, a potent bronchoconstrictor and proinflammatory mediator (33–35). In this group of experiments we evaluated the effect of sPLA_2s on the de novo synthesis of LTC_4. Group IA and group IIA sPLA_2s did not induce release of LTC_4 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 LTC_4/10^6 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 sPLA_2 up to 10 μg/ml did not induce the production of PAF as assessed by [3H]acetate incorporation (control, 22 ± 7; group IIA sPLA_2, 24 ± 8 cpm/10^6 cells). In the same experiments, A23187 (10^-6 M) induced the incorporation of 842 ± 67 cpm of [3H]acetate into PAF/10^6 cells.

**Role of sPLA_2 enzymatic activity on the activation of human eosinophils**

The major enzymatic products of sPLA_2s, AA and lysophospholipids, modulate several biochemical functions of inflammatory cells, including eosinophils (38). In particular, sPLA_2-induced expression of CD69 has been related to the generation of lysophospholipids (30). To understand whether the effects of sPLA_2s were due to the enzymatic activity, we designed two sets of experiments. We initially tested the hypothesis that enzymatic products of sPLA_2s could be involved in sPLA_2-induced activation of eosinophils by evaluating the effect of AA, lyso-PC, or lyso-PA on the release of β-glucuronidase and IL-8 (data not shown). These results indicate that the major enzymatic products of sPLA_2s are not involved in sPLA_2-induced activation of eosinophils.

In another group of experiments, eosinophils were incubated with group IA or IIA sPLA_2, 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 sPLA_2s (group IA and group IIA) 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 sPLA_2s 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 sPLA_2s, 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 sPLA₂ 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. Group IA sPLA₂ induced phosphorylation of both ERK1 and ERK2 (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 (non-phosphorylated 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.
(17–20, 39–41). We have examined the effects of two structurally different sPLA₂ on several functions of human eosinophils. Both group IA and IIA sPLA₂ 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 eosinophils. In contrast, sPLA₂ 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 activity of human group IIA sPLA₂ (42) and dilution of alveolar fluid due to the bronchoalveolar lavage (43) indicate local concentrations 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 IIA sPLA₂ (44). This observation suggests that sPLA₂ may function as an autocrine stimulus for eosinophil activation.

Interestingly, both group IA and IIA 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 sPLA₂ may be relevant stimuli for systemic and/or local activation of eosinophils in allergic or hyper eosinophilic 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 production. 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 cytokine transcription in eosinophils by showing that sPLA₂ selectively activate intracellular signals leading to IL-8 and IL-6, but not IL-5, expression. Furthermore, sPLA₂ induce the release of a preformed mediator (β-glucuronidase), but not of de novo synthesized mediators (LTC₄, PAF, and superoxide anion). These observations suggest that activation by sPLA₂ is restricted to selected signaling pathways within human eosinophils. Our results indicate that incubation of eosinophils with sPLA₂ induces a rapid activation of ERK1/2. This signaling pathway is crucial for degranulation and cytokine production, but not for other functions in human eosinophils (49). Although these data clearly indicate that sPLA₂ generate intracellular signals in eosinophils, additional experiments are needed to understand the mechanism(s) underlying the selectivity of effect of sPLA₂.

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 overexpressed on cells activated in vivo, such as the eosinophils retrieved 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, however, 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 hypothesis 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 lysophospholipids by direct enzymatic activity on membrane phospholipids with the concurrent generation of AA metabolites, 2) interaction of sPLA₂ 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 sPLA₂ generates AA and lysophospholipids, 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 eosinophils, 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 sPLA₂. However, 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 eosinophils with the major enzymatic products of sPLA₂, AA, lyso-PC, or lyso-PA, did not affect β-glucuronidase or IL-8 secretion. Finally, inactivation of sPLA₂ enzymatic activity by BBP did not abolish its capacity to induce eosinophil activation. These data indicate that, as in other inflammatory cells (18–20), the catalytic activity of sPLA₂ is not mainly responsible for their ability to induce the release of lysosomal enzymes and the de novo synthesis of 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 sPLA₂ may enhance the effects exerted by sPLA₂ 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 IIA sPLA₂ possess a heparin-binding site (58). Therefore, a potential mechanism by which these molecules may activate eosinophils is through interaction with membrane peptidoglycans. Moreover, it is conceivable that sPLA₂, at least at high concentrations, could induce membrane perturbations, which in turn may trigger eosinophil activation.

Finally, sPLA₂ 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 inflammatory 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 IIA sPLA₂ 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 eosinophils, 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 sPLA₂ on eosinophils.

In conclusion, our results indicate that sPLA₂ selectively activate certain proinflammatory and immunomodulatory functions in eosinophils with mechanism(s) that appear mainly independent...
from their enzymatic activity. Activation of human eosinophils by sPLA₂-s 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.

Acknowledgments

We are indebted to Dr. Giuseppe Spadaro (Division of Clinical Immunology and Allergy, University of Naples Federico II, Naples, Italy) and Dr. Gennaro Liccardi (Division of Pneumology and Allergy, A. Cardarelli Hospital, Naples, Italy) for providing access to patients with hypereosinophilia. We are grateful to Jean Ann Gilder for text editing.

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