Lysophosphatidic Acid Induces Chemotaxis, Oxygen Radical Production, CD11b Up-Regulation, Ca\(^{2+}\) Mobilization, and Actin Reorganization in Human Eosinophils via Pertussis Toxin-Sensitive G Proteins

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Lysophosphatidic acid (LPA) is a bioactive lipid mediator, which is generated by secretory type II phospholipase A\(_2\) and is thought to play a major role in the pathogenesis of atopic diseases. In this study, the biological activity of LPA on human eosinophils was characterized. We showed by reverse transcription and PCR that human eosinophils express the mRNA of the LPA receptors endothelial differentiation gene (EDG)-2 and EDG-7. Experiments revealed that LPA has chemotactic activity toward eosinophils, stimulates the production of reactive oxygen metabolites, and induces up-regulation of the integrin CD11b. Signal pathway measurements indicated Ca\(^{2+}\)-mobilization from intracellular stores and transient actin polymerization upon stimulation with LPA. Cell responses elicited by LPA were inhibited by pertussis toxin indicating that in eosinophils the LPA receptor(s), presumably EDG-2 and/or EDG-7, are coupled to G\(_{i/o}\) proteins. Moreover, LPA-induced activation of eosinophils could be completely blocked by the EDG-2/EDG-7 antagonist diacylglycerol pyrophosphate. In addition, at optimal doses the changes induced by LPA were comparable to those obtained by the other well-characterized chemotaxins. These results indicate that LPA is a strong chemotaxin and activator of eosinophils. These findings point to a novel role of LPA in the pathogenesis of diseases with eosinophilic inflammation such as atopic diseases as chemotaxin as well as activator of proinflammatory effector functions. The Journal of Immunology, 2004, 172: 4480 – 4485.

Eosinophils are considered as major effector cells in several inflammatory diseases such as parasitic infections, asthma bronchial, rhinitis allergica, bullous dermatoses, or vasculitis (1–6). Accumulation and stimulation of the proinflammatory activity of eosinophils at inflammatory sites are presumably caused by various chemotactic agents. Currently, the complement split product C5a, ATP, the phosphatidylycerine-derivate platelet-activating factor (PAF), and the CC chemokine ligands CCL5, CCL11, and CCL13 are well-characterized eosinophil chemotaxins (7–12). In addition to migration, these molecules stimulate eosinophil effector functions, such as the production of reactive oxygen metabolites and up-regulation of CD11b (11–13). Stimulation of leukocytes by chemotaxins requires binding to membrane-spanning ligand-specific receptors, which interact at the intracellular site of the plasma membrane with pertussis toxin-sensitive heterotrimeric guanine nucleotide-binding proteins (G proteins) (14, 15). Activated G proteins (GTP-form) dissociate into the GTP \(\alpha\)-subunit and free \(\beta\gamma\)-dimers. The latter activates phospholipase \(\mathrm{C}_{\beta}\), which cleaves phosphatidyl-inositol-4,5-bisphosphate into diacylglycerol and the Ca\(^{2+}\)-mobilizing inositol trisphosphate (16). In addition, G proteins in leukocytes control the reorganization of the actin cytoskeleton. The mechanism underlying actin reorganization presumably involves interaction of phosphoinositides with the actin-binding proteins and requires participation of small GTP-binding proteins of the rho family (17, 18). Intracellular Ca\(^{2+}\)-transients and actin reorganization in concert regulate cell response such as the migration, the production of reactive oxygen species, and up-regulation of integrins (13, 19).

Lysophosphatidic acid (LPA) is a natural occurring water-soluble phospholipid that was originally identified as a key molecule in de novo lipid biosynthesis. Meanwhile it is also regarded as an important extracellular mediator regulating a broad range of biological functions (20, 21). At present, different pathways of LPA generation involving either specific oxidative degradation of low-density lipoproteins or cleavage of phosphatidic acid by secretory type II phospholipase A\(_2\) (sPLA\(_2\)) are known (22–25). Independently of the generation process, LPA is stored within the cell at concentrations up to 60 \(\mu\)M and can be released very fast into the extracellular space (26). Recently, several studies suggested a role for LPA as regulator of immunological functions. LPA regulates secretion of IL-2 and prevents apoptosis in T cells (27–29). It is a growth factor for B cells and it stimulates adhesion of monocytes and neutrophils to endothelial cells (30, 31). In addition, LPA regulates the trafficking, cytokine production, and T cell-activating...
functions of dendritic cells (32). Cellular recognition of LPA is mediated by G protein-coupled receptors of the EDG family such as EDG-2, EDG-4, and EDG-7 (33). The downstream biochemical events linking LPA to its pleomorphic activities are complex, because these receptors couple to different G proteins. Depending on cell type, G_3 as well as G_{q11/L2}, couple EDG-2, EDG-4, and EDG-7 to phospholipases, trigger tyrosine phosphorylation, and regulate rho-dependent actin reorganization (34–36).

Generation of LPA in acute lung injury is mediated by sPLA_2 and huge amounts of sPLA_2 activity could be detected in nasal and bronchoalveolar lavage fluids in allergic patients after Ag challenge (22, 37–39). Therefore, we characterized the biological activity of LPA in human eosinophils.

Materials and Methods

Materials

LPA, recombinant human C5a, PMA, lysophosphatidylcholine, Ficoll-Paque, pertussis toxin, and lucigenin were obtained from Sigma-Aldrich (Deisenhofen, Germany); diocetylphosphatidyl acid 8:0, and diacylglycerol pyrophosphate (DGPP) 8:0 purchased from Avanti Polar Lipids (Alabaster, AL); N-[7-nitrobenz-2-oxa-1,3-diazol-4-yl]-phallacidin (NBD-phallacidin) from BD Biosciences (Heidelberg, Germany); 1-[2-(5-carboxy-oxazol-2-yl)-6-aminoisobenzofuran-5-oxyl]-2-(2‘-amino-5‘-methylphenoxy)-ethane-N,N,N’,N’-tetraacetic acid, pentaoctoxymethyl ester (fura 2) from Calbiochem (La Jolla, CA); immunomagnetic beads (Dynabeads M-450) were purchased from Dynal (Denmark); PE-conjugated CD11b (LEU 15) was from BD Biosciences.

Isolation of eosinophils

Human eosinophilic granulocytes were isolated from heparin-anticoagulated (10 U/ml) blood of healthy nonatopic volunteers by Ficoll separation and negative selection with anti-CD16 Ab-coated Dynabeads (11). In brief, human granulocytes (10^7 cells) were incubated with 500 µl of 2 × 10^6 coated beads/ml for 1 h at 4°C. Bead-coupled polymorphonuclear cells were removed by repeated magnetic separation steps. Resulting eosinophils were resuspended. Pappenheim staining judged the purity of isolated eosinophils ≥96%. Contaminating cells represent neutrophils. Viability was checked routinely and was not influenced by LPA.

RT-PCR analysis

The mRNA of eosinophils was isolated with QIAshredder and RNeasy kits (Qiagen, Hilden, Germany). Messenger RNA, Moloney murine leukemia virus reverse transcriptase, and pd(N)_(1-8) primers (Life Technologies, Gaithersburg, MD) were used to obtain cDNA (32). All oligonucleotides used as primers in RT-PCR were designed to recognize sequences specific for each target cDNA. Primer sequences are as follows: EDG-2 receptor (464 bp product), sense 5’-CGG CAG GTA GTG GTG GTC-3’ and antisense 5’-CGC ACG TAG AAG A-3’; EDG-4 receptor (375 bp product), sense 5’-TCG CGG TAG GAG TAA ATG ATG-3’ and antisense 5’-TCG CGG CAG TGA TCA AAA ACA GA-3’; EDG-7 receptor (310 bp product), sense 5’-CAG ACA AGC AAA ATG AGC-3’ and antisense 5’-GC CAC ACT GAG CCG ACT-3’. The cDNA of the 2-microglobulin (259 bp product), sense 5’-GTT CAC TGA GGC TAT CCA GCG TA-3’ and antisense 5’-TCG CGG CAG GTA GTG GTG GTC-3’ was amplified using primers 5’-CCT TGA GGC TAT CCA GCG TA-3’ and antisense 5’-CAG ACA AGC AAA ATG AGC-3’.

FIGURE 1. Human eosinophils express the mRNA for EDG-2 and EDG-7 receptors. RT-PCR analysis was performed on mRNA isolated from human eosinophils. Experiments were repeated four times with identical results.

FIGURE 2. LPA elicits chemotaxis of human eosinophils. Eosinophils were exposed to the indicated concentrations of LPA for 90 min at 37°C in Boyden chambers. Data are expressed as mean ± SEM (n = 7). Global differences between groups: p ≤ 0.0001 (ANOVA); ***, p ≤ 0.001 compared with untreated cells (Tukey multiple comparison test); **, p ≤ 0.01 compared with untreated cells (Tukey multiple comparison test); *, p ≤ 0.05 compared with untreated cells (Tukey multiple comparison test).

FIGURE 3. Dose dependency of the LPA-induced CD11b expression in eosinophils. A. Eosinophils were activated with the indicated concentrations of LPA for 30 min at 37°C. Expression of CD11b was analyzed by flow cytometry with PE-conjugated anti-CD11b Abs. Data are means ± SEM (n = 7). Global differences between groups: p ≤ 0.0001 (ANOVA); ***, p ≤ 0.001 compared with untreated cells (Tukey multiple comparison test); **, p ≤ 0.01 compared with untreated cells (Tukey multiple comparison test); *, p ≤ 0.05 compared with untreated cells (Tukey multiple comparison test). B. Representative histogram of the anti-CD11b-stained eosinophils stimulated with 50 µM LPA (3) compared with control buffer (2). Histogram (1) represents the isotype control. Representative data of one experiment are shown. The experiment was repeated seven times with identical results.
FIGURE 4. LPA activates the respiratory burst in eosinophils. The time courses of the lucigenin-dependent chemiluminescence response in eosinophils upon stimulation with the indicated concentrations of LPA are shown. Representative data of one experiment are shown. The experiment was repeated five times with identical results.

ACG GCA GGC ATA CT-3'. Thirty PCR cycles were run at 94°C (denaturation, 1 min), 62°C (annealing, 1 min), and 72°C (extension, 1 min). The generated products were subjected to electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining. The identity of the PCR products was confirmed by sequencing after cloning using pCRII vectors. Controls run without reverse transcriptase yield no PCR products.

Migration assay
Chemotactic activity of eosinophils was assessed using the modified Boyden chamber technique (11). Stimuli were placed into bottom wells, which were covered by a 3-μm pore polycarbonate membrane. Eosinophils (5 × 10⁵ eosinophils/well) were added to the upper compartment and incubated at 37°C in a humidified atmosphere. After 1 h incubation the migrated cells at 37°C were counted. Eosinophils (5 × 10⁵ eosinophils/ml) were added to the upper compartment and incubated at 37°C in a humidified atmosphere. After 1 h incubation the migrated cells in the lower part of the Boyden chamber were lysed by adding 0.1% Triton X-100, and the β-glucuronidase activity of the lysates were determined photometrically. The chemotactic index was calculated as the ratio between stimulated cells and cells in the control medium. Each experiment was performed in triplicate.

Actin polymerization
The filamentous (F)-actin content was analyzed by flow cytometry with NBD-phallacidin staining (12). Briefly, aliquots of cell suspension (5 × 10⁶ eosinophils/ml) were withdrawn at the indicated time intervals from a stirred and 37°C tempered sample compartment. Equal volumes of cells (100 μl) were fixed in a 7.4% formaldehyde buffer and mixed with the staining mixture containing 7.4% formaldehyde, 0.33 μM NBD-phallacidin and 1 mg/ml lysophosphatidylcholine. The fluorescence intensity was measured by flow cytometry.

Intracellular Ca²⁺-measurements
Intracellular-free Ca²⁺ was measured in fura 2 labeled cells using the digital fluorescence microscope unit Attofluor (Zeiss, Oberkochen, Germany) (32).

Lucigenin-dependent chemiluminescence
Eosinophils were resuspended to a density of 5 × 10⁶ cells/ml containing 200 μM lucigenin. Measurements were performed in triplicate at 37°C (11). The reaction after addition of stimuli to the cells was followed and expressed as intensity integral counts.

CD11b expression
The integrin CD11b was analyzed by flow cytometry with PE-conjugated anti-CD11b Abs. Eosinophils were activated with the indicated stimuli for 30 min at 37°C. The reaction was stopped by diluting the sample with 100-fold ice-cold buffer. Samples were incubated on ice for 40 min with PE-conjugated anti-CD11b Abs or respective unconjugated control Abs. The specific fluorescence index F(x) was calculated by subtraction of the autofluorescence in the presence of control Abs F(control) from the fluorescence of PE-conjugated Abs F(total) using the equation: F(x) = F(total) − F(control) (11).

Results
Eosinophils express the mRNA of the LPA receptors EDG-2 and EDG-7
Expression of mRNA for the different LPA receptors subtypes and β-microglobulin was analyzed in eosinophils by RT-PCR. Fig. 1 shows that eosinophils express the mRNA of EDG-2 and EDG-7. In contrast, we could not reveal the mRNA of EDG-4 in eosinophils, despite that this product could be detected by control experiments with isolated mRNA of dendritic cells (data not shown). No products were obtained after omitting reverse transcription in the reaction (data not shown).

LPA-induced chemotaxis, stimulated CD11b up-regulation and activated the respiratory burst
To prove functional expression of LPA receptors in eosinophils, chemotaxis experiments with Boyden chambers were performed. These experiments revealed that LPA has chemotactic activity for eosinophils (Fig. 2). Half-maximal and maximal reactions were observed at 10⁻⁶ M and 10⁻⁵ M LPA, respectively. Up-regulation of integrin molecules like CD11b is a feature during the migration response of eosinophils (12). Therefore, we analyzed the influence of LPA on the expression of CD11b by flow cytometry. Again, LPA induced a concentration-dependent response. Half-maximal and maximal effects were observed by these cell response at 5 × 10⁻⁶ M and 5 × 10⁻⁵ M LPA, respectively (Fig. 3). However, LPA had no influence on the activation markers CD44 and CD69 (M. Idzko and J. Norgauer, data not shown). Eosinophils can mediate their proinflammatory activity in tissue through generation of reactive oxygen metabolites. The activation of the respiratory burst...
by LPA was studied by lucigenin-dependent chemiluminescence (Fig. 4). These experiments revealed production of reactive oxygen species in a concentration-dependent manner. At all concentrations tested the continuous measurements indicated a rapid induction of the response with maximum values after 5 min.

LPA-induced actin polymerization and mobilized Ca\textsuperscript{2+} from intracellular stores

The influence of LPA on the actin network in eosinophils was analyzed by flow cytometry. This phospholipid caused a rapid and transient polymerization of actin molecules (Fig. 5). There was a transient increase of the F-actin content of \( \approx 50\% \) within 30 s. Half-maximal and maximal effects were observed at \( 5 \times 10^{-5} \) M and \( 5 \times 10^{-5} \) M LPA, respectively. Intracellular Ca\textsuperscript{2+} transients were followed in fura 2-labeled eosinophils by digital fluorescence microscopy. LPA induced a rapid and concentration-dependent intracellular Ca\textsuperscript{2+} response (Fig. 6). Because the Ca\textsuperscript{2+}-chelator EGTA had no effect on the magnitude or time course of induced Ca\textsuperscript{2+} transients, one can conclude that LPA stimulated mobilization of Ca\textsuperscript{2+} from intracellular stores (data not shown).

Inhibition of LPA-induced activation of eosinophils with the EDG-2/EDG-7 antagonist DGPP

Previously, it has been described that DGPP is an antagonist for EDG-2 and EDG-7 (40, 41). To show that LPA activation in eosinophils is receptor-mediated, experiments with this compound were performed. Pretreatment of eosinophils with DGPP completely inhibited the LPA-induced chemiluminescence response (Fig. 7A), Ca\textsuperscript{2+} mobilization (Fig. 7B), and the transient increase of actin polymerization (data not shown). To prove the metabolic activity of eosinophils after DGPP treatment, the chemiluminescence and calcium response with C5a was followed. DGPP treatment did not influence the C5a response (data not shown).

Pertussis toxin inhibited the LPA-induced cell responses

Pertussis toxin blocks cell activation induced by G\textsubscript{i} protein-coupled receptors (12). Pretreatment of eosinophils with pertussis toxin completely inhibited the LPA-induced chemiluminescence response (Fig. 8), the transient increase of actin polymerization and Ca\textsuperscript{2+} mobilization (data not shown). To prove the metabolic activity of eosinophils after pertussis toxin treatment, the chemiluminescence response with PMA was followed. Toxin treatment did not influence the phorbol ester-triggered response (data not shown).

Comparison of the activation profiles of different chemotaxins of eosinophils

The activation profile of LPA was compared with the responses provoked by other well-defined eosinophil activators such as C5a, PAF, CCL5, CCL11, and CCL13. Similar to LPA, the other eosinophil activators induced actin reorganization and triggered the respiratory burst in a concentration-dependent manner (data not shown).
sphingomyelinase conditioning of plasma membrane vesicles and phospholipase C and by phospholipase D or diacylglycerol kinase activation (22–25). Independently of the synthesis pathway up to 60 μM LPA can be stored within the cells and accumulation of LPA in this concentration range in extracellular fluids, including serum and inflammatory exudate, can be expected (26). Therefore, the cell response reported in the present study is likely to be of physiological relevance.

Intracellular Ca2+-transients and actin reorganization have been implicated in the intracellular signaling activation cascade of migration, CD11b up-regulation, and the NADPH oxidase (11, 12). As could have been expected for a chemotactic agent we have shown in this study that LPA induced a transient reorganization of the actin network. The precise regulation mechanisms for this actin response is not fully understood, however, it is believed to involve interaction of phospholipids with actin-binding proteins (17, 18). In addition, we demonstrated mobilization of Ca2+- from intracellular stores by LPA and that all cell responses induced by LPA were inhibited by pertussis toxin, which inactivates heterotrimeric G\textsubscript{i}-proteins by ADP-ribosylation (12). Beside coupling to G\textsubscript{i} protein, EDG are able to interfere with G\textsubscript{s} protein in certain cells (32).

However, we have found no evidence for alterations of the cAMP level in eosinophils by LPA (M. Idzko and J. Norgauer, unpublished observations). Therefore, these findings suggest that LPA in eosinophils specifically activates G\textsubscript{i} protein-coupled EDG-2 or/and EDG-7, and initiate similar signaling pathways than other chemoattractants such as the C5a, ATP, PAF, and the chemokines CCL5, CCL11, and CCL13 (11–14). Moreover, the lack of influence of LPA on certain activation markers such as CD44 and CD69 (Ref. 42 and M. Idzko and J. Norgauer, unpublished observations) suggests that LPA activates only a very specific set of cell responses associated with chemotaxis and oxygen radical production. It is very interesting to mention that CD44 and CD69 are directly influenced by sPLA\textsubscript{2} (42). Therefore one can assume that the effects after activation of sPLA\textsubscript{2} in vivo are more amplified in comparison to in vitro data with purified compounds and isolated eosinophils. In addition, this study performed direct quantitative comparison of LPA to these activators, which revealed not only an identical stimulation pattern, but also a similar magnitude of the provoked cell responses and intracellular signaling events. Therefore, one can assume that in vivo LPA might contribute to recruitment and proinflammatory activity of eosinophils to a similar extent as the other well-characterized agents.

These results indicate that LPA stimulates chemotaxis, respiratory burst, up-regulation of CD11b, actin polymerization, and intracellular Ca2+-mobilization via pertussis toxin-sensitive G\textsubscript{i} proteins. These findings point to a novel role of LPA in the pathogenesis of diseases with eosinophilic inflammation such as asthma bronchiale and rhinitis allergica as chemotaxin and activator of proinflammatory effector functions.

### Discussion

Recently, involvement of LPA-generating inflammation-associated sPLA\textsubscript{2} in the pathogenesis of atopic diseases such as asthma and rhinitis allergica has been suggested (22, 37–39). Eosinophils are thought to be major effector cells in these diseases (1–7). To elucidate the relation of sPLA\textsubscript{2} and these effector cells of allergic reactions, we characterized the biological activity of LPA on eosinophils. In this study, we have shown that human eosinophils express mRNA of the LPA receptors EDG-2 and EDG-7. Functional expression of the LPA receptors in eosinophils was proven in this study with cell studies analyzing chemotaxis, CD11b expression and production of reactive oxygen metabolites. Moreover experiments with the EDG-2/EDG-7 antagonist DGPP and pertussis toxin strongly indicate that the effect of LPA is mediated via specific receptors. Half-maximal and maximal migration responses toward LPA were observed at 10\textsuperscript{–6} M and 10\textsuperscript{–5} M LPA, respectively. Slightly higher concentrations were required to provoke CD11b up-regulation and production of reactive oxygen metabolites. Under pathological conditions LPA can be generated through cleavage of phosphatidic acid, which is produced either through

### Table 1. Influence of different activators of eosinophils on actin response and lucigenin-dependent chemiluminescence

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>F-actin</th>
<th>Chemiluminescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.00 (±0.00)**</td>
<td>0450 (±112)</td>
</tr>
<tr>
<td>LPA</td>
<td>1.43 (±0.04)***</td>
<td>8473 (±873)***</td>
</tr>
<tr>
<td>CCL5</td>
<td>1.51 (±0.03)***</td>
<td>7907 (±654)***</td>
</tr>
<tr>
<td>CCL11</td>
<td>1.48 (±0.05)***</td>
<td>8231 (±546)***</td>
</tr>
<tr>
<td>CCL13</td>
<td>1.53 (±0.04)***</td>
<td>7832 (±671)***</td>
</tr>
<tr>
<td>CSA</td>
<td>1.51 (±0.06)***</td>
<td>8867 (±841)***</td>
</tr>
<tr>
<td>PAF</td>
<td>1.45 (±0.03)***</td>
<td>7643 (±762)***</td>
</tr>
</tbody>
</table>

* Eosinophils were stimulated without or with 10\textsuperscript{–6} M LPA, 100 ng/ml CCL5, 100 ng/ml CCL11, 100 ng/ml CCL13, 10\textsuperscript{–7} M CSA, and 10\textsuperscript{–7} M PAF. The relative F-actin content was analyzed after 10 s stimulation with the indicated agents. Chemiluminescence response is given as integral (counts × 10\textsuperscript{3}) after 60 min. Data are means ± SEM (n = 5). Global differences between groups: p ≤ 0.0001 (ANOVA); p ≤ 0.001 (***). Compared with untreated cells (Tukey multiple comparison test).

### Acknowledgments

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### References


