Phospholipase D-derived phosphatidic acid is involved in the activation of the CD11b/CD18 integrin in human eosinophils

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INTRODUCTION

Human eosinophils are potent effector cells in the host defence against invading (micro)-organisms, particularly in the killing of helminths [1,2]. It has also been demonstrated that eosinophils are cytotoxic for tumour cells [3]. The killing process is mediated by the release of cytotoxic proteins, such as major basic protein, eosinophil peroxidase, eosinophil cationic protein and eosinophil-derived neurotoxin (for review, see [4]), and by the production of toxic oxygen metabolites by a membrane-bound NADPH oxidase [5]. In vitro, these processes can be activated by serum-opsonized particles [serum-treated zymosan (STZ)], although this activation is highly dependent on priming, either by endogenous synthesis of platelet-activating factor (PAF) [6], or by in vitro priming with PAF [7,8]. Priming of the STZ-induced respiratory burst can also be achieved by treatment with the cytokines granulocyte-macrophage colony-stimulating factor, interleukin-3 or interleukin-5 [9]. Not much is known about the signals involved in eosinophil priming and activation. Priming of STZ responses consists, at least in part, of recruitment of cells to interact with and respond to STZ, because previous studies from our laboratory have demonstrated that PAF [8] and cytokines [9] enhance the number of eosinophils able to bind and respond to opsonized particles.

Binding of STZ to human eosinophils is inhibited by antibodies against the CD11b/CD18 integrin (complement receptor type 3) but not against complement receptor type 1 or FcyRII [9], and priming could be dissociated from CD11b/CD18 integrin upregulation [8]. This suggest that priming changes the affinity of the CD11b/CD18 integrin for its ligand iC3b, leading to an enhanced interaction between human eosinophils and STZ. The mechanism for this change in affinity has not yet been revealed. Van der Bruggen et al. [10] have also shown that CD11b/CD18 integrin is the important integrin for activation of human eosinophils by STZ.

The first human phospholipase D (PLD) enzyme was isolated from eosinophils [11]. Activation of PLD leads to the formation of phosphatidic acid (PA). Minnicozzi et al. [12] have demonstrated activation of PLD in human eosinophils upon stimulation with complement fragment C5a, the Ca2+ ionophore A23187 or with PMA. These authors did not investigate the role of PLD in the activation of eosinophil functions. Most studies on the role of PLD in the activation of granulocytes have been performed in neutrophils [13–23]. Many reports have shown that PLD is involved in respiratory burst activity in human neutrophils [18–22]. PLD activity has also been implicated in priming of human neutrophils [23].

The role of PLD in signal transduction can be investigated by...
performed experiments with ethanol (EtOH), because in the presence of EtOH the stable phospholipid phosphatidylethanol (PtdEt) is formed instead of PA [15]. In this study, we have investigated the role of PLD in priming and activation of human eosinophils, by determining the effect of EtOH on (1) priming by PAF and (2) activation by STZ. PLD-derived PA was found to be important, especially for the PAF-induced activation of the CD11b/CD18 integrin on eosinophils.

MATERIALS AND METHODS

Materials

STZ was prepared as described [24]. PAF (1-α-phosphocholine-β-acetyl-γ-O-hexadecylglycerol), α,β-propanolol, 1,2-DiC8-phosphatidylcholine (1,2-DiC8-PC), 1,2-DiC8-diacylglycerol (1,2-DiC8-DG), 1,3-DiC8-DG, 1-C8-monoacylglycerol (1,2-DiC8-MAG) and C18-lyso-PA were purchased from Sigma Chemicals (St. Louis, MO, U.S.A.). C8-ceramide was purchased from Calbiochem (La Jolla, CA, U.S.A.). 1,2-DiC8-PA was purchased from Avanti Polar Lipids (Alabaster, AL, U.S.A.).

Phosphatidylinositol (PI) was dissolved in DMSO at 500 times the final concentration and used at the final concentration for cell incubations. 1,2-DiC8-DG, 1,3-DiC8-DG and C8-ceramide were dissolved in DMSO at 2000 times the final concentration before use. 1,2-DiC8-PA, 1,2-DiC8-diacylglycerol and 1,2-DiC8-MAG were purchased from Avanti Polar Lipids (Alabaster, AL, U.S.A.).

Oxygen consumption was measured at 37 °C. Rates of oxygen consumption were calculated as the first derivative of the O2 concentration curves over a 60 s interval.

Measurement of PLD activity and PA synthesis

PLD activation was measured as described for granulocytes by Kessels et al. [13], with minor modifications. In short, eosinophils (5 x 10^6/μl) in incubation medium without HSA were incubated for 60 min at 37 °C with 5 μCi/ml of [3H]lyso-PAF. Thereafter, the eosinophils were washed, resuspended in incubation medium without HSA and allowed to recover from the labelling for 30 min at 37 °C. Subsequently, the eosinophils were centrifuged and resuspended in incubation medium. Eosinophils (10^6/ml, 0.6 ml) were activated in the presence of EtOH. For routine measurement of PLD activity, 0.5% (v/v) EtOH was used; for evaluating the effect of EtOH on PA synthesis, increasing concentrations of EtOH up to 1.5% (v/v) were used. The reaction was stopped by the addition of 3 ml of chloroform/methanol (1:2) plus 0.2 ml of 4 M NaCl. This Bligh and Dyer monophase was split with 1 ml of chloroform and 1 ml of 1 M NaCl by centrifugation (10 min, 1500 g). The lower chloroform phase containing the lipids was washed twice with 2 ml of 1 M NaCl and was dried under vacuum with centrifugation. The lipids were dissolved in 15 μl of methanol and were resolved by TLC on Silica gel 60, with chloroform/methanol/acetate acid (65:15:2) as the mobile phase. The amount of radioactivity comigrating with a PtdEt standard (Rf = 0.4) and with a PA standard (Rf = 0.2) was determined by scintillation counting after scraping the PtdEt spot or PA spot into a scintillation vial.

Measurement of STZ binding to human eosinophils

STZ binding was measured as described [8], with minor modifications. STZ particles were stained fluorescent green with streptavidin-FITC (0.2 μg per mg of STZ, 30 min at room temperature) after biotinylation of the particles (30 μg of biotin per mg of STZ, 2 h at room temperature). The fluorescent STZ particles retained the same ability to stimulate respiratory burst as did the original STZ particles. FITC-STZ (1 mg/ml) was added to the eosinophils (2 x 10^6/ml) and samples were taken at the indicated time points, fixed with ice-cold paraformaldehyde (1% w/v) in incubation medium and analysed in a FACScan flow cytometer (Becton and Dickinson, San José, CA, U.S.A.) using a ‘life-gate’ on the eosinophils to gate out the unbound STZ particles. Results were expressed as eosinophils that had bound at least one particle (i.e. detected as FL1-positive events) as a percentage of the total number of eosinophils (total events). This percentage is an underestimation of the amount of cells that actually have bound an STZ particle, because an aggregate of cells (bridged by STZ particles) is detected as a single FL1-positive event.

Measurement of CD11b expression on human eosinophils

The expression of surface CD11b was measured by indirect immunofluorescence and flow cytometry. After activation of the eosinophils, cells were washed and incubated with the primary antibody 44A (10 μg/ml) directed against CD11b for 45 min at 4 °C. Subsequently, the cells were washed and incubated with R-phycocerythrin-labelled goat (anti-mouse)-Ig (1:50; Dako A/S, Glostrup, Denmark) as the secondary antibody. After one wash, binding was quantified for 5000 cells with a FACScan flow cytometer (Becton and Dickinson) and expressed as mean fluorescence intensity.

Measurement of binding of human eosinophils to FN-coated wells

Plates (24-well) were coated with FN by incubation of 0.5 ml of FN (approx. 20 μg/ml) for 30 min at 37 °C, followed by washing of the wells with PBS. Five minutes after the addition of 0.5 ml of
eosinophils (2 × 10⁶/ml), the cells were stimulated by addition of an activating agent. Thereafter, supernatant (including the non-adherent cells) was harvested, and the wells were washed with PBS to remove loosely bound cells. Non-adherent and adherent cells were lysed in 1 % Triton X-100 and eosinophil adherence was quantified by measuring the lactate dehydrogenase (LDH) content in the lysates relative to the LDH content of the total number of cells added to the wells. LDH activity in the lysates was measured in a 50 mM KP¡ buffer (pH 7.4) in the presence of 0.15 mM pyruvate and 0.1 mM NADH at 25 °C. The conversion of NADH into NAD⁺ was determined spectrophotometrically as a decline in the absorbance at 340 nm.

**Measurement of cytosolic free calcium concentration**

Cytosolic free Ca²⁺ concentration was measured with Indo-1-loaded eosinophils exactly as described previously [7].

**Measurement of eosinophil viability**

Eosinophils were resuspended in incubation medium to a concentration of 10⁷ cells/ml and were incubated with 1.5 % (v/v) EtOH. A 20 μl volume of this suspension was mixed with 20 μl of fluorescein diacetate (FDA; 250 ng/ml in PBS) or with 20 μl of ethidium bromide (EB; 5 μg/ml in PBS) and were incubated for 10 min at room temperature. Cells were analysed in a FACSscan flowcytometer (Becton and Dickinson). Viable cells take up and hydrolyse FDA, resulting in a green fluorescence signal, and dead cells are stained red with EB.

**RESULTS**

**Activation of PLD by opsonized particles**

In our initial experiments, we investigated the activation of PLD in human eosinophils activated by serum-opsonized particles (STZ). However, in contrast to human neutrophils, eosinophils require priming for optimal interaction with STZ [7,8]. Without priming, human eosinophils hardly bind STZ particles, and under these conditions synthesis of PtdEt (as a measure of PLD activity) was found to be low (Figure 1). We therefore also performed these experiments with STZ addition after pre-treatment of the cells with PAF (1 μM) for 2 min at 37 °C. As shown in Figure 1, addition of STZ to PAF-primed eosinophils leads to the formation of PtdEt, after a lag time of about 30 s. The PAF treatment itself (1 μM of PAF for 2 min at 37 °C) resulted in a 2-fold increase in PtdEt formation [control medium, 461 ± 51 d.p.m./10⁶ eosinophils (mean ± S.E.M., n = 6); after treatment with 1 μM PAF for 2 min at 37 °C, 1041 ± 230 d.p.m./10⁶ eosinophils (mean ± S.E.M., n = 6), P < 0.05 Student’s t-test], indicating that PAF activates PLD.

**Role of PLD in PAF priming and STZ stimulation of respiratory burst**

Next, we investigated the relevance of PLD for PAF-primed and STZ-activated respiratory burst in human eosinophils by performing experiments with EtOH. In the presence of EtOH, production of PA after PLD activation can be expected to be lower, because PtdEt is formed instead of PA. For routine measurement of PLD activity, 0.5 % (v/v) EtOH is used [15]. However, in the presence of this concentration of EtOH, STZ-induced PA accumulation was inhibited by only 52 ± 8 % (mean ± S.E.M., n = 3). Table 1 shows that in the presence of 1.5 % EtOH a strong inhibition of PA accumulation is observed. This high concentration of EtOH did not inhibit PLD activity itself, because more than 80 % of the counts present in PA after stimulation with PAF-STZ in the absence of EtOH were recovered in the sum of PA and PtdEt in the presence of 1.5 % EtOH (Table 1).

<table>
<thead>
<tr>
<th>Preincubation</th>
<th>Stimulation</th>
<th>Respiratory burst</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>STZ</td>
<td>2.3 ± 0.5</td>
</tr>
<tr>
<td>PAF</td>
<td>STZ</td>
<td>12.4 ± 0.4</td>
</tr>
<tr>
<td>PAF → EtOH</td>
<td>STZ</td>
<td>10.5 ± 0.7</td>
</tr>
<tr>
<td>EtOH → PAF</td>
<td>STZ</td>
<td>2.1 ± 0.7</td>
</tr>
</tbody>
</table>

**Table 1 Effect of EtOH on PAF-STZ-induced synthesis of phosphatidic acid**

Eosinophils were loaded with [³H]lyso-PAF. After preincubation for 5 min at 37 °C, the cells (10⁷/ml) were incubated with 1 μM PAF for 2 min and with EtOH (1.5 % v/v) for 2 min in the order indicated. Subsequently, the cells were activated with STZ (1 mg/ml) for 5 min. Results are expressed as means ± S.E.M. of three different experiments.

<table>
<thead>
<tr>
<th>Simulation</th>
<th>PA spot (d.p.m.)</th>
<th>PtdEt spot (d.p.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1278 ± 576</td>
<td>354 ± 126</td>
</tr>
<tr>
<td>PAF-STZ</td>
<td>12225 ± 693</td>
<td>561 ± 165</td>
</tr>
<tr>
<td>PAF-1.5% EtOH-STZ</td>
<td>3018 ± 780</td>
<td>6945 ± 378</td>
</tr>
</tbody>
</table>

**Figure 1 Time course of PtdEt formation induced by STZ in human eosinophils**

Eosinophils were loaded with [³H]lyso-PAF. After preincubation for 5 min at 37 °C in the presence of 0.5 % EtOH, the cells (10⁷/ml) were incubated in the absence (○) or presence (●) of 1 μM PAF (2 min) and were activated with STZ (1 mg/ml). Results are expressed as means ± S.E.M. of four different experiments.

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shows that addition of EtOH after treatment with PAF results in only a minor inhibition of STZ-induced respiratory burst, suggesting that PLD activity is not rate-limiting for respiratory burst. However, when EtOH was added 2 min before treatment with PAF, 1.5%. EtOH caused almost complete inhibition of STZ-induced respiratory burst. This was not caused by a different time of incubation with EtOH before the addition of STZ, because incubation with EtOH (after PAF priming) for 4 min before STZ addition did not result in a stronger inhibition (results not shown). Taken together, these results indicate that EtOH has a strong inhibitory effect on PAF priming and only a minor effect on STZ stimulation, suggesting that PLD-derived products are involved in PAF priming.

To rule out the possibility that the high concentration of EtOH interferes with processes other than PA accumulation, we determined its effect on the PAF-induced increase in cytosolic free Ca^{2+}. EtOH did not affect this response (results not shown), indicating that PAF in the presence of 1.5% EtOH still binds to the PAF receptor and can induce signal transduction via phospholipase C. Cell viability was also not influenced by the high concentration of EtOH, because FDA staining resulted in 94%±2% (mean±S.D., n=3) and 93%±3% (mean±S.D., n=3) positive cells in the absence and presence of 1.5% EtOH respectively. Staining with EB revealed 5%±2% (mean±S.D., n=3) dead cells, both in the absence and in the presence of 1.5% EtOH.

Effect of EtOH on STZ binding to human eosinophils
We next measured the effect of EtOH on the binding of STZ to human eosinophils. In previous studies we have shown that one of the effects of priming is the enhancement of STZ binding [8]. Figure 2 clearly shows that it is only when EtOH is added before treatment with PAF that a strong inhibition of STZ binding is observed. Although 1.5% EtOH is required for complete inhibition of STZ binding, significant inhibition was already observed at lower concentrations of EtOH. PAF-induced binding of STZ particles to human eosinophils was completely inhibited by 10 μg/ml of the MoAb 44A directed against CD11b (results not shown).

Table 3  Effect of propranolol on the binding of FITC-labelled STZ to human eosinophils

<table>
<thead>
<tr>
<th>Incubation</th>
<th>2 min</th>
<th>5 min</th>
<th>10 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solvent-STZ</td>
<td>2±1</td>
<td>5±2</td>
<td>9±4</td>
</tr>
<tr>
<td>PRO-STZ</td>
<td>4±1</td>
<td>5±1</td>
<td>7±1</td>
</tr>
<tr>
<td>Solvent-3 nM PAF-STZ</td>
<td>5±2</td>
<td>10±3</td>
<td>15±5</td>
</tr>
<tr>
<td>PRO-3 nM PAF-STZ</td>
<td>28±4*</td>
<td>39±4*</td>
<td>42±3**</td>
</tr>
</tbody>
</table>

Time after STZ addition

The results presented above suggest that PLD is involved in PAF priming. Nevertheless, the disadvantage of the use of EtOH is that the production of both second messengers derived from PLD activity [i.e. PA and diradylglycerides (DRG)] is inhibited. We therefore measured the sensitivity of PAF-induced STZ binding to inhibition by propranolol. Propranolol at relatively high concentrations acts as an inhibitor of PA-phosphatase activity [27] and thereby diminishes the formation of DRG from PA. Propranolol appeared to have no effect on the PAF-induced STZ binding; preincubation of human eosinophils with 200 μM propranolol for 5 min and subsequent priming with PAF (1 μM) and activation with STZ resulted in 38±7% (mean±S.E.M., n=4) of eosinophils binding STZ particles, compared with 62±4% (mean±S.E.M., n=4) in the absence of propranolol. This implies that PA, and not DRG, is the important PLD-derived molecule for priming.

Induction of STZ binding to human eosinophils by propranolol or endogenous PA

In the next set of experiments, we tried to induce STZ binding to human eosinophils by an increase in PA levels, either by inhibition of the breakdown of PA by adding propranolol or by addition of cell-permeable DiC8-PA. Obviously, the effect of propranolol depends on a small rate of PA formation and breakdown. Table 3 shows that propranolol alone does not induce binding of STZ particles to human eosinophils. However, in the presence of suboptimal concentrations of PAF (3 nM), propranolol and PAF appeared to have a synergistic effect on STZ binding.

To further strengthen the hypothesis that elevation of PA levels results in enhanced STZ binding, we have performed experiments with addition of cell-permeable DiC8-PA to human eosinophils. Figure 3 shows that preincubation with DiC8-PA enhanced STZ binding in a dose- and time-dependent fashion. Although optimal binding was observed with 200 μM DiC8-PA for 30 min at 37°C, suboptimal concentrations of PA and shorter incubation time also resulted in detectable STZ binding.
Table 4 shows that the DiC8-PA-induced STZ binding to human eosinophils was diminished in the presence of 10 μg/ml of the MoAb 44A directed against CD11b, indicating that DiC8-PA-induced STZ binding to human eosinophils is mediated by the CD11b/CD18 integrin.

We next investigated whether other cell-permeable lipids also could induce binding of STZ particles to human eosinophils. As shown in Table 4, preincubation with 1,2-DiC8-PC, 1-C8-MAG, C18-lyso-PA or C8-ceramide did not result in binding of FITC-STZ to human eosinophils, indicating that the effect of DiC8-PA is not mediated by the (Di)-C8-fatty acid moiety. On the other hand, activation of human eosinophils with PMA, 1,2-DiC8-DG or 1,3-DiC8-DG did result in enhanced CD11b expression.

Adherence of human eosinophils to FN-coated wells

We next investigated the effect of PAF and DiC8-PA on the adherence of human eosinophils to FN-coated wells, because previous work had demonstrated that, under our experimental conditions, binding of human eosinophils to the wells is mediated mainly via the CD11b/CD18 integrin. Figure 5 shows that addition of PAF or DiC8-PA to human eosinophils resulted in adherence to FN-coated wells. These results indicate
that PAF and DiC8-PA induce the CD11b/CD18-dependent adherence of human eosinophils.

DISCUSSION

In this study we have found indications that PLD-derived PA plays an important role in the activation of the CD11b/CD18 integrin in human eosinophils. We have shown previously [8,9] that binding of STZ to human eosinophils is mediated by the CD11b/CD18 integrin. Although IgG is present on the STZ particles, IgG molecules do not contribute significantly to the binding of STZ to human eosinophils [8–10]. Because, under the experimental conditions used (1 μM PAF for 2 min), PAF does not induce upregulation of the CD11b/CD18 integrin (see Figure 4 and [8]), the enhanced binding of STZ after PAF treatment is presumably caused by activation of CD11b/CD18 integrin through a change in the affinity of the CD11b/CD18 integrin for its ligand iC3b. The finding that the effect of PAF on STZ binding is strongly diminished in the presence of 1.5 % EtOH and enhanced by propranolol (in the presence of suboptimal concentrations of PAF) indicates that PLD-derived PA is an important molecule for the PAF-induced activation of the CD11b/CD18 integrin.

For the inhibitory effect of EtOH, relatively high concentrations of EtOH (1.5 %, v/v) are required. However, such a high concentration of EtOH does not seem to interfere with other processes, because: (1) addition of EtOH after PAF priming only moderately affected STZ binding and respiratory burst activation; (2) eosinophil viability in the presence of 1.5 % EtOH was 95 %; (3) 1.5 % EtOH did not affect the PAF-induced increase in the intracellular Ca²⁺ concentration in human eosinophils, indicating that PAF in the presence of EtOH is still able to bind to its receptor and induce activation of phospholipase C; and (4) most of the PA synthesized in the absence of EtOH was, in the presence of 1.5 %, EtOH, recovered in PLD-derived products, indicating that 1.5 % EtOH does not strongly affect PLD activity per se.

The second indication for a role of PA in the activation of the CD11b/CD18 integrin in human eosinophils is the finding that elevation of PA, through inhibition of PA breakdown by propranolol in the presence of suboptimal concentrations of PAF, results in enhanced binding of STZ particles to human eosinophils. Moreover, addition of cell-permeable PA (DiC8-PA) to human eosinophils results in CD11b/CD18-dependent binding to STZ particles or FN-coated wells. Taken together, we have found that elevation of intracellular PA leads to enhanced adhesiveness of eosinophils via the CD11b/CD18 integrin.

Several other lipids were tested for their ability to induce binding of FITC-STZ to human eosinophils, but only 1,2-DiC8-DG and 1,3-DiC8-DG were effective. 1,2-DiC8-DG, but not 1,3-DiC8-DG, is a potent activator of protein kinase C (PKC) [28]. Activation of PKC by PMA also results in STZ binding to human eosinophils. However, Ganong et al. [28] have shown that because of facile acyl group migration between the 2 and 3 positions, preparations of 1,3-DiC8-DG of greater than 99 % purity could show considerable PKC activation (up to 70 % of that of 1,2-DiC8-DG), unless the 1,3-DiC8-DG was subjected to HPLC purification immediately before use. Because we did not perform this HPLC purification, we cannot exclude that the observed effects of 1,3-DiC8-DG are mediated by contamination with 1,2-DiC8-DG. We have observed that PMA, 1,2-DiC8-DG and 1,3-DiC8-DG, in contrast to PAF and DiC8-PA, induce upregulation of the CD11b/CD18 integrin. This suggests that different mechanisms are responsible for the enhanced STZ binding. PA-induced STZ binding is mediated by activation of already expressed CD11b, whereas upregulation of CD11b might support enhanced STZ binding after PKC activation.

Our study does not reveal the mechanism by which PLD-derived PA affects the CD11b/CD18 integrin. We cannot exclude the possibility that not PA itself but a metabolite of PA (different from DRG) mediates the binding of STZ to human eosinophils. Three reports describe the regulation of β₃-integrins by lipids derived from activated granulocytes [29–31]. However, none of these has succeeded in characterizing the lipid. Our study confirms the finding that β₃-integrins are regulated by lipids. In addition, we describe indications that the lipid is derived from PLD activity.

Alternatively, the action of PA may be mediated by the activation of one or more kinases. Phosphorylation events have been suggested to be involved in the activation of CD11b/CD18 integrin. The α subunit (CD11b) is constitutively phosphorylated, but the β subunit (CD18) becomes phosphorylated after activation [32,33]. Threonine phosphorylation in particular is recognized as being important for the regulation of CD11/CD18 avidity [34,35]. PA-dependent protein phosphorylation has been described by several investigators [36–38]; thus, activation of CD11b/CD18 integrin by PA might be mediated by one or more kinases.

In conclusion, in this study we made three observations, indicating that PLD-derived PA is involved in the PAF-induced CD11b/CD18-dependent adhesion of human eosinophils, without upregulation of CD11b: (1) inhibition of PLD-derived PA formation by EtOH results in diminished STZ binding; (2) elevation of PLD-derived PA levels by propranolol in the presence of suboptimal concentrations of PAF results in enhanced STZ binding; and (3) addition of cell-permeable DiC8-PA results in enhanced binding to STZ particles or FN-coated wells. In contrast, enhanced STZ binding to human eosinophils by activation of PKC is associated with upregulation of CD11b.

REFERENCES


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