The linoleic acid metabolite 13-HODE modulates degranulation of human polymorphonuclear leukocytes

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Abstract The effect of the linoleic acid metabolite 13-hydroxyoctadecadienoic acid (13-HODE) on degranulation of human polymorphonuclear leukocytes (PMNs) was investigated by measuring the expression of CD11b and CD67 on the plasma membrane. 13-HODE (5 μM) by itself induced degranulation of PMNs, but to a lesser extent as compared to PAF and fMLP. In addition, 13-HODE was found to inhibit the PAF-induced degranulation whereas an additive effect on the fMLP-induced PMNs degranulation was observed. These results indicate that 13-HODE can play a modulatory role in degranulation of PMNs.

Key words: 13-HODE; Polymorphonuclear leucocyte; Degranulation; CD11b; CD67

1. Introduction

Polymorphonuclear leukocytes (PMNs) are recruited to sites of inflammation in response to chemotactic stimuli, such as platelet-activating factor (PAF) and formyl-methionyl-leucyl-phenylalanine (fMLP). Subsequently, phagocytosis, production of reactive oxygen species and degranulation may occur. PMN degranulation is characterized by release of hydrolytic and proteolytic enzymes and by expression of receptors and proteins on the plasma membrane, originating from the membrane of granules and intracellular vesicles [1,2]. Several granule types exist in PMNs, which can be discriminated on the basis of their content and membrane markers. Degranulation of tertiary granules is characterized by release of gelatinase and upregulation of the heterodimeric glycoprotein CD11b/CD18 (CR3) on the plasma membrane [3,4]. The secretory vesicles contain cytochrome b_{558} and CD11b/CD18 [5]. Release of vitamin B_{12}-binding protein and lactoferrin from specific granules coincides with enhanced expression of the glycosyl phosphatidylinositol-linked membrane protein CD67 in addition to CD11b/CD18 on the plasma membrane [6–8]. The azurophilic granules are recognized by their content of myeloperoxidase, lysozyme and the membrane protein CD63 [6]. The different granule types exhibit different release kinetics, e.g. the secretory vesicles are readily mobilized compared to azurophilic granules [9,10].

Control of degranulation is important to assure that the PMN is activated only at the right place and time and to the proper extent. Arachidonic acid metabolites, which are produced by several cell types involved in the inflammatory process, e.g. PMNs and macrophages, modulate degranulation. The 5-lipoxygenase metabolites leukotriene B_4 (LTB_4) and 5-oxo-eicosatetraenoic acid (5-Oxo-ETE) induce PMN degranulation. 5-Oxo-ETE and 5-hydroxy-eicosatetraenoic acid (5-HETE) potentiate the PAF-induced degranulation [11,12]. In contrast, the 15-lipoxygenase metabolite of arachidonic acid, 15-hydroxy-eicosatetraenoic acid (15-HETE), has been demonstrated to inhibit the fMLP- and PAF-induced degranulation of PMNs [13].

Beside metabolism of arachidonic acid, inflammatory cells such as PMNs and macrophages convert linoleic acid, whereby 9- and 13-hydroxyoctadecadienoic acid (9- and 13-HODE) are produced [14–16]. 9- and 13-HODE have been shown to interfere with reactive oxygen species released by guinea pig pulmonary macrophages and posess chemotactic activity towards PMNs [17,18]. Chemoattractants, such as fMLP and PAF, also induce PMN degranulation. Because nothing is known about 13-HODE in this respect, we studied the effect of 13-HODE on degranulation of PMNs by measuring the expression of the granule membrane markers CD67 and CD11b on the plasma membrane.

2. Materials and methods

2.1. Materials

PAF, fMLP, linoleic acid, bovine serum albumin (BSA; fraction V) and lipoxidase I (type IV) were purchased from Sigma Chemical Co, St. Louis, USA. Percoll was obtained from Pharmacia Fine Chemicals AB, Uppsala, Sweden. Hespan (6% hydroxyethyl starch in 0.9% NaCl, sterile) was from Fresenius AG, Bad Homburg, Germany.

2.2. Antibodies

The monoclonal antibody (MoAb) B13.9 (IgG_1) [7], directed to CD67, was purchased from the Central Laboratory of the Dutch Red Cross Blood Transfusion Service, Amsterdam, The Netherlands. Anti-CD11b MoAb (IgG_1) and the fluorescein isothiocyanate-labeled goat anti-mouse MoAb (GamFITC) were obtained from Becton Dickinson, San Jose, USA. The antibodies were diluted in phosphate-buffered saline (PBS) containing 2% (w/v) BSA and 0.02% (w/v) sodium azide.

2.3. Preparation of 13-HODE

13-HODE was biochemically prepared according to Garssen et al. [19]. Briefly, linoleic acid (600 μM) was incubated at 4°C with lipoxidase I (20,000 U/100 ml) in 0.1 M borate buffer, pH 9.0. 13-HPODE production was measured at intervals in a spectrophotometer (200–280 nm). The incubation was terminated by acidification to pH 4 with HCl. 13-HPODE was separated from non-metabolized linoleic acid by adsorption on a TLC-plate and subsequent development in hexane-diethylether-acetic acid (70:30:0.5). 13-HPODE was eluted from the silica with diethylether. Subsequently, 13-HPODE was reduced with sodium borohydrate. The purity of 13-HODE thus obtained was measured by reversed phase HPLC [20] and was found to be 95% (3% 9-HODE; 2% 13-HPODE). 13-HODE was stored in methanol at −80°C.

2.4. Isolation of human PMNs

Human PMNs were obtained from blood of normal healthy volunteers (Bloodbank, Red Cross Foundation, Utrecht, The Netherlands) anticoagulated by mixing with trisodium citrate-glucose (10:1; v/v). Erythrocytes were removed by addition of Hespan (11:3; v/v), mixing and subsequent centrifugation for 20 min at 100 × *g*. The resultant leuko-

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cyte-rich supernatant was removed and washed twice with PBS for 5 min at 400 × g. Then the cell suspension was layered over a discontinuous density gradient of Percoll. The required densities of Percoll (56% (v/v), 67% (v/v)) were obtained by mixing isoosmotic Percoll and 0.9% NaCl. Following centrifugation for 20 min at 1000 × g, the PMNs banded at the interface between the two densities of Percoll. The PMN band was carefully removed and washed with buffer containing 20 mM Hepes, 5 mM KCl, 145 mM naCl, 1.3 mM $CaCl_2$, 1 mM $MgSO_4$, 2.1 mM $NaHCO_3$, 5 mM glucose (pH 7.4). Finally, PMNs were resuspended in buffer to yield a concentration of 5×10^6 cells/ml. This PMN preparation contained >95% PMNs, with a viability of >99% (assessed by Trypan Blue exclusion).

2.5. Analysis of degranulation markers

Purified PMNs (5×10^5/ml) were prewarmed for 15 min in a shaking waterbath at 37°C and subsequently incubated with the compounds 13-HODE (5×10^{-6} M) for 10 min and PAF (100 nM) or fMLP (100 nM) for 5 min. The effect of 13-HODE on the PAF- and fMLP-induced degranulation was studied by preincubation with 13-HODE for 5 min and subsequent stimulation with PAF or fMLP for 5 min. The PMNs were centrifuged (1000 × g, 2 min, 4°C), washed with ice-cold PBS/2% (w/v) BSA/0.02% (w/v) azide (washing buffer), and incubated with the MoAb anti-CD67 or anti-CD11b for 30 min at 4°C. Thereafter, PMNs were washed three times with washing buffer and incubated with GamFITC for 30 min at 4°C. Subsequently, the PMNs were washed three times with washing buffer and analyzed by flow cytometry (FACScan: Becton Dickinson).

2.6. Data Analysis

Results are expressed as mean fluorescence intensity (MFI) ± S.E.M. The difference between two MFI values was expressed as delta MFI, i.e., MFI of stimulated cells minus MFI of cells under basal conditions. Paired Student's *t* test was used to evaluate statistical significance of the data; $P < 0.05$ was considered significant.

3. Results

When PMNs were incubated for 10 min with 5 μM 13-HODE a significant increase of the CD11b expression with 45 ± 12% was observed. In addition, the expression of CD67 was upregulated with the same extent by 13-HODE (Fig. 1, Table 1).

As shown in Table 1, stimulation of PMNs with PAF (100 nM) or fMLP (100 nM) also increased the expression of CD11b and CD67 on the plasma membrane. Both degranulation markers were upregulated to a greater extent after stimulation with PAF or fMLP than with 13-HODE.

To determine the effect of 13-HODE on the PAF- and fMLP-induced expression of CD11b and CD67, PMNs were preincubated for 5 min with 13-HODE whereafter PAF or fMLP was added. After pretreatment with 13-HODE, the PAF-induced upregulation of CD11b was significantly inhibited with 24 ± 7% (Fig. 2). 13-HODE also inhibited the PAF-induced increase of the expression of CD67 with 14 ± 8%. In contrast, preincubation of PMNs with 13-HODE enhanced the upregulation of CD11b induced by fMLP with 29 ± 8% (Fig. 2). The fMLP-induced expression of CD67 was increased with 69 ± 11% from 240 ± 26 MFI to 332 ± 42 MFI in PMNs preincubated with 13-HODE.

Table 1
Increase of expression of the degranulation markers CD11b and CD67 on the plasma membrane of PMNs

	CD11b	CD67
13-HODE	125 ± 29*	45 ± 5*
PAF	337 ± 34*	124 ± 23*
fMLP	281 ± 31*	135 ± 20*

PMNs (5×10^5/ml) were incubated with 13-HODE (5 μM), PAF (100 nM) or fMLP (100 nM). Results are expressed as ΔMFI ± S.E.M. of experiments performed with PMNs isolated from eight (CD11b) or five (CD67) different donors; *$P < 0.05$ compared to non-stimulated cells (MFI = 311 ± 36 MFI for CD11b and 105 ± 19 MFI for CD67 expression).

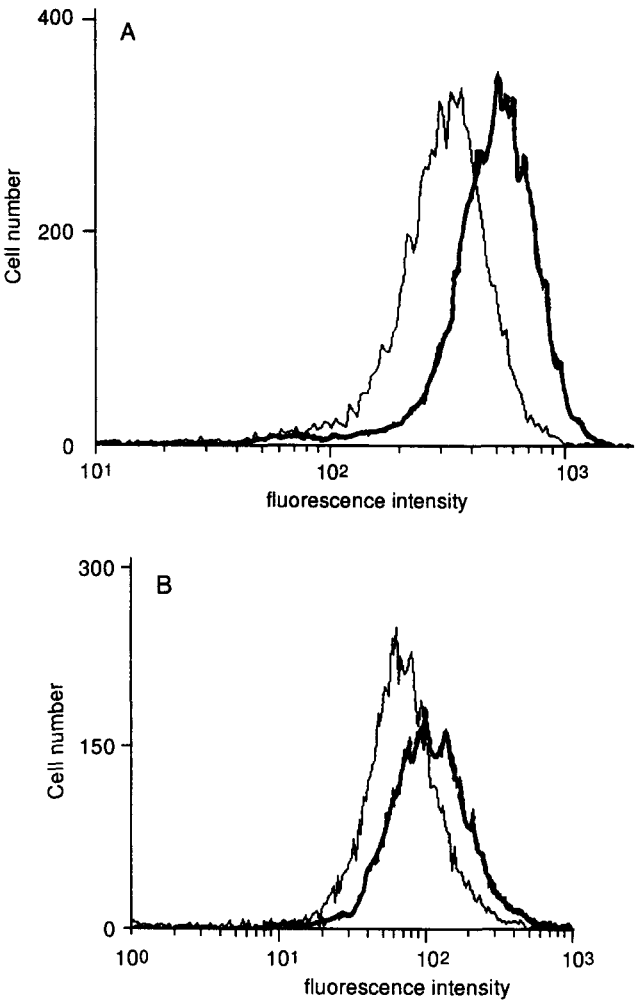

Fig. 1. Flow cytometry analysis of the basal and 13-HODE (5 μM) induced expression of CD11b (A) and CD67 (B) on the plasma membrane of PMNs (5×10^5/ml). Representative histograms of eight (A) or five (B) experiments are shown, indicating basal expression (thin line) and 13-HODE-induced expression (thick line).

4. Discussion

In the present study it is demonstrated that the linoleic acid metabolite 13-HODE induced degranulation of human PMNs. This degranulation was detected by measuring the expression of CD11b, which is a marker for degranulation of secretory vesicles, tertiary and specific granules and CD67, which is a marker for specific granules, on the plasma membrane. 13-HODE (5 μM) upregulated both degranulation markers to the same extent. It is highly unlikely that small contaminations of 9-HODE or 13-HPODE in our 13-HODE preparation were responsible for the observed effects, since 9-HODE nor 13-HPODE have been reported to be more potent than 13-HODE on different leukocyte functions [17,18]. Similar to 13-HODE, the arachidonic acid metabolites 5-HETE, 5-OxoETE and LTB_4 have been reported to stimulate PMN degranulation [11].

Regarding the concentration in which 13-HODE was used, 5-HETE induced PMN degranulation at similar concentrations, i.e. 1–10 μM [21,22]. In addition, 13-HODE can be produced in concentrations of up to 1 μM under non-stimulated conditions [20]. These data indicate that our present effects of 13-HODE, observed at a 5-μM concentration, may be of physiological significance.

The precise mechanism by which PMN degranulation is accomplished is not completely understood. An elevation of the intracellular calcium concentration ($[Ca^{2+}]_i$) is generally considered to be important for degranulation [23]. However, 13-HODE induced PMN degranulation without a detectabe rise of $[Ca^{2+}]_i$ [24]. Other investigators also observed PMN degranulation without an increase of $[Ca^{2+}]_i$ [9]. Activation of protein kinase C (PKC) with the phorbol ester PMA induces PMN degranulation without a change in cytosolic Ca^{2+} [25]. Unsaturated fatty acids, including arachidonic and linoleic acid, can stimulate PKC and induce PMN degranulation [26,27]. It is conceivable that also 13-HODE induces degranulation of PMNs by activation of PKC. Several other studies suggest a modulatory role for $[Ca^{2+}]_i$ and the involvement of additional signalling pathways in the degranulatory process. Thus, Nube et al. reported the involvement of GTP-binding proteins in addition to a pronounced effect of Ca^{2+} on the dynamics of degranulation [28]. In permeabilized PMN preparations, GTPγS induced degranulation of azurophilic granules. Subsequent addition of Ca^{2+} augmented the GTPγS-induced degranulation [29]. Fatty acids, such as arachidonic acid and its metabolite 15-HETE, have been shown to interact with GTP-binding proteins [13,30]. We cannot exclude that 13-HODE exerts its degranulatory response by interaction with GTP-binding proteins.

In addition to its direct degranulatory activity a modulatory effect of 13-HODE on the PAF- and fMLP-induced degranulation was observed. 13-HODE attenuated the degranulatory response of PMNs to PAF. Previously, we observed a strong inhibitory effect of 13-HODE on the PAF-induced $[Ca^{2+}]_i$-increase [24]. Since $[Ca^{2+}]_i$ can modulate PMN degranulation, the decrease of PAF-induced degranulation caused by 13-HODE may result from the inhibitory effect of 13-HODE on the PAF-induced $[Ca^{2+}]_i$. This explanation is supported by the observation that the inhibitory effect of 15-HETE on the PAF-induced degranulation of PMNs coincides with a decreased rise of $[Ca^{2+}]_i$ [13]. The degranulatory response of PMNs to fMLP was additive on the 13-HODE-induced degranulation. In accordance, 13-HODE does not affect the fMLP-induced $[Ca^{2+}]_i$-increase [24].

Expression of CD11b on the cell surface is not only indicative for PMN degranulation, but also subserves PMN adhesion to other cells. CD11b belongs to the integrin family of adhesion molecules and is involved in the adherence of PMNs to activated endothelial and epithelial cells by interaction with its counterstructure ICAM-1 present on these cells [31,32]. Following adhesion, PMNs transmigrate between the cells and migrate along a gradient of chemoattractant, such as PAF and fMLP, to the inflammatory region. We previously observed chemotactic activity of 13-HODE towards PMNs in vitro [18]. The effects of 13-HODE on the upregulation of CD11b also suggest an important role for 13-HODE in the recruitment of PMNs from the blood stream into the inflamed tissue. The possible role for 13-HODE in cell-cell interaction is supported

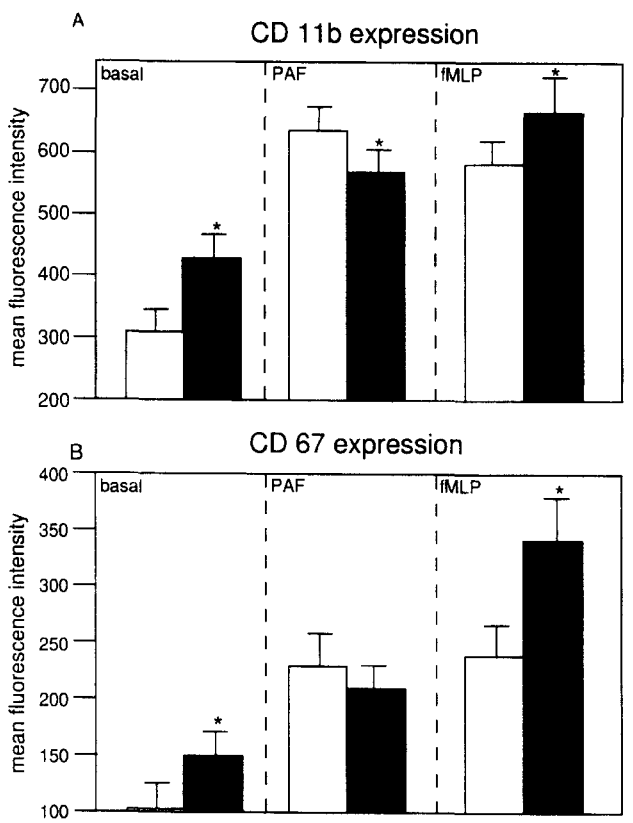

Fig. 2. Effect of 13-HODE (5 μM; black bars) on the basal, PAF (100 nM) and fMLP (100 nM) induced expression of CD11b (A) and CD67 (B) on the plasma membrane of PMNs. Results are expressed as MFI ± S.E.M. of experiments performed with PMNs isolated from eight (A) or five (B) different donors; $^*P < 0.05$.

by a number of other studies. Buchanan et al. observed a decreased adhesion between PMNs and endothelial cells after inhibition of 13-HODE synthesis in PMNs and endothelial cells, indicating a proadhesive role for 13-HODE towards PMNs [33]. In contrast, 13-HODE inhibited PMA-induced expression of the adhesion molecule GpIIb/IIIa on tumor cells [34]. Furthermore, it has been found that a decrease of the intracellular 13-HODE levels in endothelial cells corresponds with an enhanced expression of the vitronectin receptor and an increase of endothelial cell adhesivity [35].

In conclusion, results of the present study indicate that the linoleic acid metabolite 13-HODE, produced and released by several inflammatory cells including PMNs, induces and modulates PMN degranulation. Moreover, the effect of 13-HODE on the expression of the adhesion molecule CD11b, suggests a modulatory role for 13-HODE in the interaction of PMNs to other cell types.

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