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5-HYDROPEROXYEICOSATETRAENOIC ACID (5-HPETE) ENHANCES THE SYNTHESIS OF 1-O-ALKYL-2-SN-ACETYL-GLYCERO-3-PHOSPHOCHOLINE (PAF) IN fMET-LEU-PHE-STIMULATED HL-60 CELLS: KEY ROLE OF DIACYLGLYCEROL (DAG) IN ACTIVATION OF PROTEIN KINASE C (PKC)

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We investigated the effect of 5-hydroperoxyeicosatetraenoic acid (5-HPETE) on the PAF formation in fMet-Leu-Phe-stimulated HL-60 cells. 5-HPETE was found to enhance the PAF synthesis in fmlp-stimulated cells without causing additional mobilization of intracellular calcium. However, a significant increase in diacylglycerol (DAG) levels due to 5-HPETE was observed, which in turn activated the protein kinase C (PKC). Obviously, PKC is responsible for the activation of phospholipase A_2 and the release of lyso-PAF and AA from complex lipid stores. Further, the dose-dependent increase in DAG production in absence of simultaneous increase in total inositol phosphates is indicative of an additional source for DAG besides PIP_2.

KEY WORDS: 5-HPETE, PAF, fMet-Leu-Phe, Diacylglycerol, Protein kinase C, HL-60 cells.

INTRODUCTION

The stimulation of human neutrophils with agonists like A23187 or fMet-Leu-Phe (fmlp) leads to a rapid release of arachidonic acid (AA) as well as 1-O-alkyl-2-lyso-glycero-3-phosphocholine (lyso-PAF).[1-5] However, the mechanisms for the action of these agonists are different. A23187 increases rapidly intracellular calcium, and thus activates phospholipase A_2 (PLA_2) and phospholipase C (PLC);[6-8] whereas fmlp activates the PLC in cells through a specific receptor via a G-protein, which then leads to breakdown of phosphatidylinositol-4,5-bisphosphate (PIP_2) into inositol-1,4,5-triphosphate (IP_3) and 1,2-diacylglycerol (DAG).[9-11] IP_3 and DAG serve as messengers for the mobilization of intracellular calcium and the activation of protein kinase C (PKC) respectively.[10,12] Activated PKC, together with intracellular calcium, has been reported to activate PLA_2.[13] Subsequently, released AA is metabolized mainly by 5-lipoxygenase to 5-hydroperoxytetraeicosaenoic acid (5-HPETE), which is further transformed to 5-hydroxyeicosatetraenoic acid (5-HETE) and leukotriene B_4

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(LTB4).[14–16] Lyso-PAF is acetylated by the enzyme acetyltransferase to 1-O-alkyl-2-sn-acetyl:glycero-3-phosphocholine (PAF).[3,17,18]

5-HETE has been shown to possess multiple roles in cellular metabolism. It has been demonstrated to mobilize intracellular calcium,[19,20] enhance the uptake of calcium by neutrophils,[21] augment the release of O_2^- by neutrophils,[22] increase the binding of (^{3}H)phorbol-12,13-dibutyrate to intact neutrophils,[22] and modulate potassium channels in neurons.[23] 5-HETE has also been shown directly or indirectly to stimulate PLA_2[24–26] and redistribute PKC in cytochalasin B-treated neutrophils.[19]

In the present paper, we will demonstrate that 5-HPETE can mobilize DAG, an activator of PKC, from different phospholipid pools of dimethyl-sulfoxide (DMSO)-differentiated HL-60 cells after stimulation with fmlp. Also, the activation of PKC is sufficient to mobilize AA and lyso-PAF from the complex lipid store without alteration in intracellular calcium.

EXPERIMENTAL PROCEDURES

HL-60 cells were supplied by Prof. G. Schultz, Institute of Pharmacology, Berlin and differentiated for 48 hours with 1.25% DMSO according to known procedures. The cells were harvested and labeled with 5.10^5 cpm (^{3}H)AA/10^7 cells (specific activity 80–135 Ci/mmol, Amersham, F.R.G.) by incubation at 37°C for 30 min. The cells were then washed twice with phosphate buffer saline (PBS) and resuspended to a concentration of 1.10^7 cells/ml for incubations. 60–65% of (^{3}H)AA was taken up by HL-60 cells. The viability of cells was examined by the trypan blue exclusion method.

Incubation Conditions

To a sample of the HL-60 cell suspension (0.5 ml; 5.10^6 cells) after equilibration at 37°C for 5 min were added either 0.4 ml PBS or 5-HPETE (final concentration 0–20 μM) simultaneously with 0.1 ml fmlp (final concentration, 1 μM) to start the reaction. After 3 min the incubation was stopped by the addition of 3.8 volume of methanol:chloroform:glacial acetic acid (2:1:0.04, v/v).

Determination of PAF

In this study we have determined only C_{16}-PAF released by HL-60 cells, which constitutes the major component of the several PAF species known.[27]

The samples were extracted according to Bligh and Dyer[28] and purified with the help of HPLC as described.[29] Fractions containing PAF underwent a second Bligh and Dyer extraction. The organic phase was dried under nitrogen and the residue was reconstituted in Tyrpde buffer. Radioactivity was counted in scintillation counter[30].

Assay of Diacylglycerol

5.10^6 cells/ml were equilibrated at 37°C for 5 min before starting the reaction by simultaneous addition of 0.4 ml of 5-HPETE (final concentration, 0–20 μM) and 0.1 ml of fmlp (final concentration, 1 μM). The reaction was stopped after 3 min and the mixture was extracted according to Bligh and Dyer.[28] Diacylglycerol was assayed as described.[31–33] For the time course study, incubation was carried out with simultaneous addition of 10 μM 5-HPETE and 1 μM fmlp for various times.

Assay of Intracellular Calcium

Intracellular calcium was determined by a modified method as described.[34] Briefly, 5.10^6 cells were treated with 5 μM FURA-2/AM (Serva, F.R.G.) at 37°C for 30 min. After washing with PBS, cells were resuspended in PBS and equilibrated for 5 min before challenging simultaneously with 5-HPETE (final concentration, 0–20 μM) and fmlp (final concentration, 1 μM). The fluorescence was measured using a Hitachi F 4000 fluorescence spectrophotometer. Intracellular calcium concentrations were quantified as described.[35,36]

Assay of Inositol Phosphates (Total IP)

HL-60 cells were incubated with 0.5 μCi of myo-(2-^{3}H) inositol (10–20 Ci/mmol, Amersham, F.R.G.) in EGTA containing buffer at 37°C for 3 hours. After washing twice with PBS, cells were resuspended in PBS without EGTA. Cell suspensions (0.5 ml; 5.10^6 cells) were incubated simultaneously with 0.4 ml 5-HPETE (final concentration, 0–20 μM) and 0.1 ml fmlp (final concentration, 1 μM) for 1 min. After stopping the reaction as described above, inositol phosphates were separated by anion-exchange chromatography as reported.[37] ^{3}H-inositol phosphates indicated the sum of inositol-1-phosphate (IP_1), inositol-1,4-biphosphate (IP_2) and inositol-1,4,5-triphosphate (IP_3).

RESULTS

We have used 5-HPETE in our study to reduce the inhibition of 5-lipoxygenase by 5-HETE.[38] Further, 5-HPETE has been suggested to autocatalyze the 5-lipoxygenase enzyme,[38] and is not rapidly transformed to 5-HETE in HL-60 cells as compared with human neutrophils (unpublished).

Table I shows that exposure of HL-60 cells to 1 μM fmlp for 3 min leads to an adequate formation of ^{3}H-PAF. Exogenously added 5-HPETE potentiated the formation of ^{3}H-PAF in a dose-dependent manner, and peaked at a concentration of 10 μM 5-HPETE. Further addition of 5-HPETE upto 20 μM did not alter the formation of PAF. Cell viability was not affected at the concentrations used. In the absence of fmlp, 5-HPETE was incapable of stimulating detectable PAF synthesis at all concentrations.

It has been reported previously that the activation of PKC is required for the synthesis of PAF and LTB_4.[13] but PKC activation alone (e.g. with phorbol ester) is not sufficient for the synthesis of PAF and LTB_4, the synthesis requires mobilization of intracellular calcium as well.[13] In order to investigate if the potentiation of PAF formation by 5-HPETE is related to an additional increase in intracellular calcium, we measured the levels of intracellular calcium by adding various concentrations of 5-HPETE with fmlp to HL-60 cells (Table II). Fmlp (1 μM) caused a rapid increase in intracellular calcium level from 212 $\pm$ 37 nM (mean $\pm$ SEM) basal value to 538 $\pm$ 22 mM peak value. Surprisingly, no additional increase of intracellular calcium level was observed with any concentration of 5-HPETE. However, in the absence of fmlp, the mobilization of intracellular calcium by 5-HPETE concentrations was of the same magnitude as with fmlp alone. The maximum intracellular calcium level was achieved with 5 μM 5-HPETE. It is conceivable that the application of 1 μM fmlp caused maximum mobilization of intracellular calcium, so that 5-

TABLE 1
Effect of 5-HPETE on the synthesis of PAF (C_{16} species) by differentiated HL-60 cells in presence and absence of 1 μM fmlp

	Experiment	PAF (dpm)
1.	cells alone	36 ± 17
2.	cell + 1 μM fmlp	507 ± 37
3.	cells + 1 μM fmlp + 1 μM 5-HPETE	612 ± 52
4.	cells + 1 μM fmlp + 2.5 μM 5-HPETE	1194 ± 78
5.	cells + 1 μM fmlp + 5 μM 5-HPETE	2112 ± 44
5.	cells + 1 μM fmlp + 10 μM 5-HPETE	2474 ± 81
6.	cells + 1 μM fmlp + 20 μM 5-HPETE	2568 ± 91
7.	cells + 1 μM 5-HPETE	48 ± 21
8.	cells + 2.5 μM 5-HPETE	50 ± 12
9.	cells + 5 μM 5-HPETE	98 ± 22
10.	cells + 10 μM 5-HPETE	107 ± 43
11.	cells + 20 μM 5-HPETE	94 ± 36
12.	vehicle	41 ± 19

5.10^6 labeled HL-60 cells were equilibrated for 5 min before starting the reaction by addition of various concentrations of 5-HPETE and 1 μM fmlp. For experimental details see *Experimental Procedures*. Values represent the mean ± SEM of 3 separate experiments

HPETE was unable to mobilize intracellular calcium from empty stores. We examined, therefore, the effect of various concentrations of 5-HPETE together with suboptimal concentrations of fmlp (10–100 nM) and found similar results as shown in Table II. This suggests that 5-HPETE may bind to same receptors as fmlp. Treatment of HL-60 cells with pertussis toxin caused significant inhibition of intracellular calcium release by 5-HPETE (manuscript in preparation). The presence of receptors for 5-HETE on human neutrophils has been speculated upon in a previous report.[19]

TABLE 2
Effect of 5-HPETE on the release of intracellular calcium by differentiated HL-60 cells in presence and absence of 1 μM fmlp. Values represent the mean ± SEM of 3 separate experiments. For experimental details see *Experimental Procedures*.

	Experiment	Concentration of intracellular calcium (nM)
1.	cells alone	212 ± 27
2.	cell + 1 μM fmlp	538 ± 22
3.	cells + 1 μM fmlp + 1 μM 5-HPETE	528 ± 39
4.	cells + 1 μM fmlp + 2.5 μM 5-HPETE	544 ± 12
5.	cells + 1 μM fmlp + 5 μM 5-HPETE	551 ± 18
5.	cells + 1 μM fmlp + 10 μM 5-HPETE	577 ± 33
6.	cells + 1 μM fmlp + 20 μM 5-HPETE	603 ± 42
7.	cells + 1 μM 5-HPETE	410 ± 34
8.	cells + 2.5 μM 5-HPETE	524 ± 46
9.	cells + 5 μM 5-HPETE	561 ± 17
10.	cells + 10 μM 5-HPETE	596 ± 24
11.	cells + 20 μM 5-HPETE	610 ± 73
12.	vehicle	206 ± 29

5.10^6 labeled HL-60 cells were equilibrated for 5 min before starting the reaction by addition of various concentrations of 5-HPETE and 1 μM fmlp. For experimental details see *Experimental Procedures*. Values represent the mean ± SEM of 3 separate experiments

TABLE 3
Effect of 5-HPETE on total inostol phosphates (total IP) and DAG of differentiated HL-60 cells after stimulation with 1 μM fmlp. Values represent the % increase of control and are mean of three separate experiments.

	Experiment	% Increase of control	
		Total IP	DAG
1.	cells alone	0	0
2.	cells + 1 μM fmlp	247	298
3.	cells + 1 μM fmlp + 1 μM 5-HPETE	282	333
4.	cells + 1 μM fmlp + 2.5 μM 5-HPETE	321	356
5.	cells + 1 μM fmlp + 5 μM 5-HPETE	324	403
6.	cells + 1 μM fmlp + 20 μM 5-HPETE	296	521
7.	vehicle	7	11

Besides intracellular calcium, DAG is another potent activator of PKC.[12] It seemed pertinent, therefore, to determine the breakdown products of PIP_2 inositol phosphates as well as DAG after stimulation of HL-60 cells with various concentrations of 5-HPETE in the presence and absence of 1 μM fmlp. As shown in Table III, the stimulation of HL-60 cells with 1 μM fmlp alone caused an increase of total IP level by 250% as compared with the basal value. Addition of 5-HPETE and fmlp together resulted in an additional dose-dependent enhancement of total IP levels reaching a peak value at a concentration of 2.5 μM; further increases of the 5-HPETE concentration did not alter the total IP level. On the other hand, DAG levels were found to increase in a dose-dependent manner with added 5-HPETE up to a maximum con-

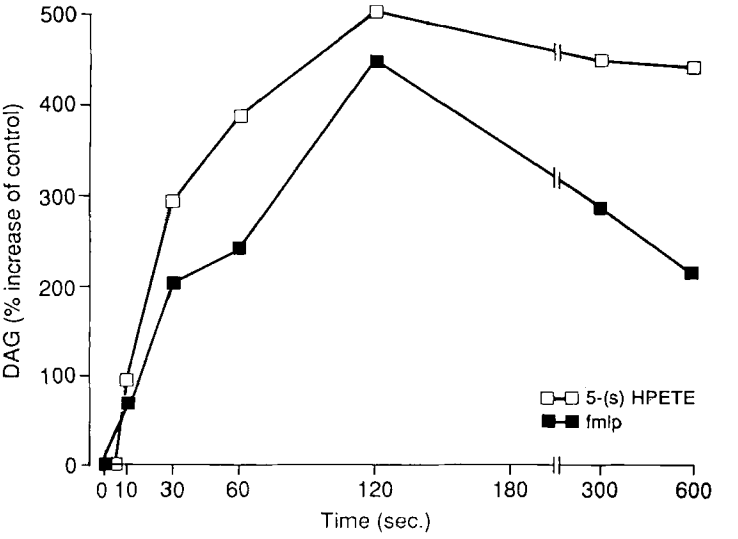

FIGURE 1 Effect of 5-HPETE on DAG production
HL-60 cells were stimulated with 1 μM fmlp in absence and presence of 10 μM 5-HPETE. For experimental details see *Experimental Procedures*. Values represent the mean of three separate experiments.

centration of 20 μM 5-HPETE. This dissociation in total IP levels and DAG levels after treatment with 5-HPETE of fmlp-stimulated HL-60 cells is indicative of an additional source for DAG other than PIP_2.

The above observation was further supported by a time course study of DAG production in HL-60 cells treated with 1 μM fmlp and 10 μM 5-HPETE (Figure 1). It can be seen that maximum production of DAG is achieved with and without 5-HPETE within 120 seconds. Moreover, 5-HPETE caused an additional release of about 50% DAG in comparison to fmlp alone. A significant increase is observed even as soon as 10 seconds after the stimulation of cells. This observation is in contradiction to a previous report where no DAG could be detected within the first 30 seconds.[36] Another striking feature shown in Figure 1 is the sustaining level of DAG during the period of observation (10 min); DAG levels achieved with fmlp alone decreased significantly during this period.

DISCUSSION

In the present study we have investigated the effect of 5-HPETE on PAF formation by fmlp-stimulated differentiated HL-60 cells. Although 5-HPETE at all concentrations used in our study was incapable alone of stimulating a detectable PAF synthesis, it potentiated PAF synthesis in a dose-dependent manner in concert with 1 μM fmlp (Table I). In previous studies 5-HPETE has been reported to modulate A23187-induced PAF formation in human neutrophils by direct activation of PLA_2.[39] Those observations were primarily based on the inhibition of PAF formation by nordihydroguaretic acid (NDGA).[39] But, the stimulation of HL-60 cells with fmlp leads to a receptor-G protein coupled activation of PLC with subsequent generation of IP_3 and DAG as intracellular messengers.[9] IP_3 triggers the rapid elevation of intracellular calcium, which in turn activates PKC and also PLA_2.[39]

Although 5-HPETE in the absence of fmlp does not synthesize detectable amounts of PAF (Table I), it mobilizes intracellular calcium by approx. 300 nM depending upon the concentration of 5-HPETE applied (Table II). However, this level of intracellular calcium is incapable alone of stimulating PLA_2.

The synthesis of PAF by HL-60 cells stimulated with fmlp alone (Table I) in our studies, and a report which showed complete inhibition of PAF and LTB_4 synthesis by long chain amines like sphingosine (15 μM), stearylamine (25 μM) and palmitoylcarnitine (25 μM) in A23187-stimulated human neutrophils,[13] are indicative of a PKC-mediated mechanism for the activation of PLA_2. Further, it has been reported that PKC requires an elevated intracellular calcium to trigger the PLA_2 activity.[13]

However, our observations do not show any additional increase in the intracellular calcium level due to 5-HPETE in fmlp-stimulated cells. But a significant and rapid increase in DAG level, which was sustained for 10 min, was caused by 10 μM of 5-HPETE (Figure 1). The results obtained with higher levels of DAG with 5-HPETE are interesting because fmlp-stimulated HL-60 phagocytes have recently been shown to demonstrate a 10-fold, calcium-mediated, increase in the affinity for DAG site on PKC.[33] This may explain the enhancement of PAF formation by 5-HPETE in HL-60 cells stimulated with fmlp.

Table III shows another striking feature of our observations. Whereas (^{3}H) inositol phosphates showed maximum PAF formation with 2.5 μM of 5-HPETE from fmlp-stimulated cells, the formation of DAG continued to increase in a dose-dependent

manner up to 20 μM of 5-HPETE. This is further supported by Figure 1 which shows the maximum accumulation of DAG at 2 min. This level is sustained for another 10 min suggesting that either DAG is accumulating after the PIP_2 hydrolysis, or it is generated from some membrane pools other than PIP_2. The latter possibility can also be derived from the fact that the hydrolysis of PIP_2 to IP_3 is completed within 30 seconds,[40] whereas DAG production increased steadily for 2 min. Phosphatidylcholine, and also inositol-1-phosphate, have been suggested as the major precursor of DAG production in different cell systems as substrates for PLC.[33] However, inositol-1-phosphate does not serve in absence of calcium as a substrate for PLC,[33] so it is likely that 5-HPETE or its transformed product 5-HETE, which have been described as modulators of intracellular calcium,[22] act as cofactors for the metabolism of inositol-1-phosphate.

The dose-dependent increase in the synthesis of phosphatidic acid by 5-HPETE (1—20 μM) in fmlp-stimulated HL-60 cells (data not shown) ruled out any possible accumulation of DAG.

We conclude that the enhancement of fmlp-stimulated PAF formation by 5-HPETE in differentiated HL-60 cells is primarily a result of increased DAG production from different phospholipid pools. As already reported in literature,[33] the increased sensitivity of PKC for DAG sites enables it to activate PLA_2 and thus mobilize lyso-PAF and AA from complex lipid stores.

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