Communication

Stimulation of Platelet-activating Factor Synthesis in Human Endothelial Cells by Activation of the de Novo Pathway

PHORBOL 12-MYRISTATE 13-ACETATE ACTIVATES 1-ALKYL-2-LYSO-EN-GLYCERO-3-PHOSPHATE:ACETYL-CoA ACETYLTRANSFERASE AND DITHIOTHREITOL-INSENSITIVE 1-ALKYL-2-ACETYL-EN-GLYCERO-3-PHOSPHATE:ACETYL-CoA CHOLINEPHOSPHOTRANSFERASE*

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Human umbilical vein endothelial cells (HUVEC) produce platelet-activating factor (PAF) by a remodeling pathway involving a phospholipase A₂ followed by an acetyl-CoA-dependent acetyltransferase which acetylates a lyso-PAF intermediate to form PAF and is stimulated by a variety of agents that generate inflammatory and allergic responses. A second route for PAF synthesis in mammalian tissues is a de novo pathway, which requires the participation of three enzymes: 1-alkyl-2-lyso-sn-glycerol-3-phosphate (alkyllyso-GP): acetyl-CoA acetyltransferase, 1-alkyl-2-acetyl-sn-glycerol-phosphate phosphohydrolase, and dithiothreitol (DTT)-insensitive 1-alkyl-2-acetyl-sn-glycerol (alkylacetyl-G): CDP-choline cholinephosphotransferase. In the present study we show that protein kinase C activation by phorbol 12-myristate 13-acetate (PMA) induces PAF production in HUVEC by an increase of both alkyllyso-GP: acetyl-CoA acetyltransferase and DTT-insensitive alkylacetyl-G: CDP-choline cholinephosphotransferase. PAF synthesis, labeled precursors (\[^{1-3}\text{H}\]acetate and \[^{1-3}\text{H}\]choline) incorporation, and both enzyme activities of the de novo pathway increase concomitantly in response to different doses of PMA. PMA does not activate the enzymes of the remodeling pathway. We conclude that both remodeling and the de novo pathway for PAF synthesis are present in HUVEC and might be alternatively activated depending on the conditions of cell stimulation.

Platelet-activating factor (PAF) is a powerful mediator of cell-to-cell communication involved in many physiopathological conditions (for reviews see Refs. 1 and 2). In stimulated inflammatory cells (3-5) including endothelium (6, 7) PAF is produced by a remodeling pathway involving a phospholipase A₂ followed by an acetyl-CoA-dependent acetyltransferase which acetylates a lyso-PAF intermediate to form PAF (2). A second route for PAF synthesis in mammalian tissues is a de novo pathway, which begins with 1-alkyl-2-lyso-sn-glycerol-3-phosphate (alkyllyso-GP) as the precursor and requires the presence of three enzymes: alkyllyso-GP: acetyl-CoA acetyltransferase (8), 1-alkyl-2-acetyl-sn-glycerol-3-phosphate (alkylacetyl-GP) phosphohydrolase (9), DTT-insensitive 1-alkyl-2-acetyl-sn-glycerol (alkylacetyl-G): CDP-choline cholinephosphotransferase (10). The de novo pathway for PAF can be stimulated by neurotransmitters in chick retina (11, 12), by fatty acids in Ehrlich ascites cells (13), HL-60 cells (14), and rabbit platelets (15), and by phorbol 12-myristate 13-acetate (PMA) and 1-oleyl-2-acetylglycerol in human neutrophils (16, 17). DTT-insensitive cholinephosphotransferase activity is present in human umbilical vein endothelial cells (HUVEC) (18), but it is not activated by inflammatory stimuli, such as interleukin-1. The purpose of the present study has been to explore in HUVEC the behavior of the de novo enzymatic route in response to PMA stimulation. We show that PMA induces PAF synthesis by the de novo pathway regulating the activity of both alkyllyso-GP: acetyl-CoA acetyltransferase and DTT-insensitive alkylacetyl-G: CDP-choline cholinephosphotransferase.

EXPERIMENTAL PROCEDURES

Reagents—Reagents for cell cultures were obtained from GIBCO (Paisley, Scotland); N-Corning (Corning, NY); PAF (1-O-acytetyl-2-lyso-sn-glycerol-3-phosphocholine) and lyso-PAF (1-O-acytetyl-2-lyso-sn-glycerol-3-phosphocholine) from Bachem Feinkemikalien (Bubendorf, Switzerland); \[^{1-3}\text{H}\]PAF (120 mCi/mmol), \[^{1-3}\text{H}\]acetate (1 Ci/mmol; the specific activity was adjusted by addition of unlabeled acetyl-CoA), \[^{1-3}\text{H}\]acetate (2 Ci/mmol), \[^{1-3}\text{H}\]choline chloride (59 mCi/mmol), CDP-\[^{1-3}\text{H}\]choline (59 mCi/mmol), and \[^{13}\text{C}\] arachidonic acid (60 mCi/mmol) from Amersham International (Bucks, United Kingdom); alkylacetyl-G (1-O-hexadecyl-2-acetyl-sn-glycerol) from Novabiochem AG (Lauffelfingen, Switzerland); all other products were from Sigma. Alkyllyso-GP and alkylacetyl-GP were prepared by phospholipase D treatment of lyso-PAF and PAF, respectively, according to the procedure described by Lee et al. (8) and purified (>90%) by preparative thin layer chromatography (TLC) as described (8).

PAF Production—PAF production was evaluated in HUVEC (35-mm diameter culture wells, \(5.0 \pm 0.4 \times 10^5\) cells, \(n = 22\)) grown and characterized as previously described (6), passed from 1 to 3 times. HUVEC were stimulated in 1 ml of M199 containing 0.25% bovine

\[^{1}\]The abbreviations used are: PAF, platelet-activating factor; alkyllyso-GP, 1-alkyl-2-lyso-sn-glycerol-3-phosphate; alkylacetyl-GP, 1-alkyl-2-acytetyl-sn-glycerol-3-phosphate; lyso-PAF, 1-alkyl-2-lyso-sn-glycerol-3-phosphocholine; alkylacetyl-G, 1-alkyl-2-acetyl-sn-glycerol; DTT, dithiothreitol; PMA, phorbol 12-myristate 13-acetate; AA, arachidonic acid; BSA, bovine serum albumin; EGTA, (ethylenebis(oxyethylenenitritio))tetraacetic acid; HUVEC, human umbilical vein endothelial cells.
serum albumin (M199-BSA). The reaction was blocked by adding 1 ml of acidified methanol to the incubation medium. Lipids were extracted according to a modification of Bligh and Dyer's procedure (19), with 50 mM acetic acid added to methanol to lower the pH of the aqueous phase to 3.0 (20). PAF associated to the cells and released into the medium was isolated, characterized, and measured as described (4). Results. For lipid extraction and purification procedures, the recovery of 10 nCi of [3H]PAF was 93–95%.

Incorporation of Radioactive Precursors into PAF—HUVEC (100-mm diameter culture wells, 2.1 ± 0.7 × 10^6 cells, n = 22) were labeled at 37°C with 80 nCi of [3H]acetate or with 20 µCi of [methyl-3H]choline in 4 ml of M199-BSA for 30 or 60 min, respectively, and then stimulated with PMA. The incubation was stopped as described, and the radioactivity incorporated into PAF fraction counted after separation by TLC. As for the PAF (see before), both radiolabeled lipids which co-migrated with a PAF standard on TLC were shown to be authentic PAF by the following evidence: 1) an identical elution volume on high performance liquid chromatography; 2) degradation of 98% of the radiolabeled products by phospholipase A^2 from Crotilus adamanteus, confirming that acetate had been incorporated at the sn-2 position (21); 3) degradation of 15% of the radiolabeled lipids by treatment with lipase A^2 from Rhizopus arrhizus, confirming that 85% of the products had an ether linkage at the sn-1 position (21); 4) a typical bioactivity causing aggregation of washed rabbit platelets (6), using a calibration curve with synthetic PAF for each series of assays. The complete characterization of PAF has been performed in three individual experiments out of four. The specificity of platelet aggregation was inferred from the inhibitory effect of 5 µM CV-3988 and 5 µM BN 52021 (11), two well known PAF antagonists. The biological activity was sensitive to previous treatment with phospholipase A^2 and resistant to the treatment with lipase A^2.

Enzymatic Analysis of HUVEC—HUVEC monolayers (100-mm diameter culture wells) were labeled for 24 h with 0.94 µCi of [3H]acetate in 5.5 ml of M199-BSA and then washed twice with M199-BSA and three times with M199 to remove unincorporated radioactivity (22). Total incorporation was 1,830,000 ± 240,000 cpm/dish. Experimental dishes were incubated with PMA or thrombin in 6 ml of M199-BSA. During 20-min incubation, aliquots of the medium were removed to determine the percentage of total cellular radioactivity released.

Assay of Enzymatic Activities—HUVEC (100-mm diameter culture wells) were stimulated with PMA at 37°C in 6 ml of M199-BSA. The plates were put on ice, the supernatants removed, and the cells detached with a rubber policeman in 2 ml of enzyme lysis buffer (10 mM Tris-HCl, pH 7.4, containing 0.25 mM succrose, 1 mM DTT to measure lyso-PAF:acetyl-CoA acetyltransferase; 20 mM Tris-HCl, pH 8.4, containing 0.5 mM NaF to measure alkyllyso-GP:acetyl-CoA acetyltransferase; 10 mM Tris-HCl, pH 7.4, containing 0.25 mM succrose, 1 mM DTT, 5 mM mercaptoethanol, 50 mM NaF to measure alkyllyso-GP:acetyl-CoA acetyltransferase; and 1 mM DTT to measure DTT-insensitive alkyllyso-GP:acetyl-CoA acetyltransferase). Cell lysates was obtained with three times freezing-thawing. Proteins were determined by the Bio-Rad assay kit.

Enzyme activities were measured using the assay conditions to measure lyso-PAF:acetyl-CoA acetyltransferase that have been described in detail (11, 18). Alkyllyso-GP:acetyl-CoA acetyltransferase was measured according to Lee et al. (8). 150 µg of lysate protein were incubated for 5 min at 37°C in 0.5 ml of 0.1 M Tris-HCl, pH 8.4, containing 50 mM NaF, 25 mM sodium vanadate, 25 µM alkyllyso-GP, 600 µM [3H]acetyl-CoA (2 µCi). Enzymatic activity was linear up to 200 µg of lysate protein. Products for each assay were analyzed by TLC using as solvent system: chloroform/methanol/concentrated NH₄OH, 65:35:8, v/v/v (R₀ of alkyllyso-GP = 0.11; Rₚ of alkyllyso-GP = 0.94) to identify alkyllyso-GP, and chloroform/methanol/acetic acid, 96:4:1, v/v/v (R₀ of alkyllyso-GP = 0.43) to quantify alkyllyso-GP which can be rapidly synthesized from the former product due to the high alkyllyso-GP phosphohydrolase activity (9). Alkyllyso-GP:acetyl-CoA acetyltransferase was expressed as nanomoles of [3H]acetate transferred to alkyllyso-GP including the radioactivity found in the product of hydrolysis of alkyllyso-GP. Alkyllyso-GP represented no more than 15% of the total products.

RESULTS

Biochemical Analysis of PAF—In the presence of PMA, HUVEC synthesized PAF in a clear-cut dose-dependent manner (Fig. 1, panel A). Maximal stimulation of PAF synthesis was observed at 250 nM PMA, and no further increase was observed at 1 µM PMA both after 2 and 20 min of incubation. The synthesis started shortly after the stimulus (2 min) and was maintained nearly constant over a 20-min period. H7 (1-(5-isouquinolinesulfonyl)-2-methylpyperazin), a known inhibitor of protein kinase C (24), elicited a dose-dependent block of PMA-induced PAF synthesis (not shown).

PAF-induced PAF synthesis was also evaluated by measuring the incorporation of [3H]acetate and of [methyl-3H]choline into the fraction co-migrating with synthetic PAF. Incubation of HUVEC in the presence of PMA enhanced the incorporation of both labels into the fraction co-migrating with synthetic PAF (Fig. 1, panel B) 1.4-fold (Fig. 1, panels B and C). The dose-response pattern of this incorporation showed that maximal response was reached at 250 nM PMA, both after 2 and 20 min of incubation. A time dependence of labels incorporation was also observed at each PMA concentration (Fig. 1, panels B and C). Less then 15% (range, 6–15%) of [3H]acetate-labeled lipid product was degraded by treatment with phospholipase A^2 suggesting that the majority of the compound produced by HUVEC activated by PMA and co-migrating in a TLC system with PAF is the 1-O-alkyl form rather than the 1-acyl form.

Thrombin (0.5 units/ml, 5 min), a well known activator of the remodeling pathway in HUVEC (6, 7), promoted the incorporation of [3H]acetate (8-fold) into PAF molecule as well, but through the Ca^2+-dependent lyso-PAF:acytyl-CoA acetyltransferase enhancement of [methyl-3H]choline incorporation into PAF was not observed after thrombin challenge (not shown).

Agonist-stimulated Release of [14C]Acidity in HUVEC—Table 1 shows the effect of 250 nM PMA on the release of [14C]acidity from [14C]AA-labeled cells. No release was observed over a 20-min incubation period, indicating that phospholipase A^2 did not seem to be influenced by PMA in that experimental condition. Thrombin (0.5 unit/ml), used as a positive control, elicited a significant release of [14C] radioactivity (Table 1).

Enzyme Activities of the de Novo and Remodeling Pathways in PMA-stimulated HUVEC—The activities of both alkyllyso-GP:acytyl-CoA acetyltransferase and DTT-insensitive alkyllyso-GP:CDP-choline cholinephosphotransferase resulted in an increase in HUVEC treated with PMA. The increase of both enzyme activities was evident as early as 2 min after PMA administration, was maintained over a 20-min period, and reached the maximum at 250 nM PMA concentration, both after 2 and 20 min of incubation (Fig. 2, panels A and B). So far, PAF synthesis, labeled precursor incorporation, and both enzyme activities of the de novo pathway increased concomitantly in response to different doses of PMA (Figs. 1 and 2).

No differences in lyso-PAF:acytyl-CoA acetyltransferase activity were observed between control and PMA-stimulated cells at all of the times and concentrations tested. Basal activity was 2.1 ± 0.66 nmol/min/mg of protein; after 2 min of incubation with 25, 250, and 1000 nM PMA, enzyme activity was 2.11 ± 0.29, 2.39 ± 0.43, and 2.63 ± 0.13, respectively, and similar values were obtained after 20 min of incubation, whereas thrombin (0.5 unit/ml) enhanced lyso-PAF:acytyl-CoA acetyltransferase activity about 7-fold within 5 min.
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**DISCUSSION**

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**FIG. 1.** Synthesis of PAF (A) and incorporation of [3H]acetate (B) and [methyl-3H]choline (C) into PAF molecule in HUVEC treated with PMA. Panel A, HUVEC (35-mm diameter culture wells) were stimulated in 1 ml of M199-BSA for 2 or 20 min at 37 °C with different concentrations of PMA (nm: 25, 250, 1000). After stimulation the amount of PAF generated was measured as described under "Experimental Procedures." Each point is the mean of three determinations in one typical experiment. Three experiments were done with similar results. Panel B, HUVEC (100-mm diameter culture wells) were prelabeled with [3H]acetate (20 μCi/ml, 30 min at 37 °C). Cells were stimulated for 2 (C) or 20 (A) min with different concentrations of PMA (nm: 25, 250, 1000), and incubation was stopped by lipid extraction. Lipids were fractionated by TLC as described under "Experimental Procedures." The radioactivity of lipids co-migrating with synthetic PAF was counted and expressed as percent increase of radiolabel incorporated into PAF molecule over basal value of non-treated cells (85,224 ± 17,045 cpm/dish). Values are given as the mean ± S.D. from four experiments each done in triplicate. Incorporation obtained after PMA treatment was compared versus control by Student's t test: *, p < 0.05; **, p < 0.02; ***, p < 0.005. Panel C, HUVEC (100-mm diameter culture wells) were prelabeled with [methyl-3H]choline (5 μCi/ml, 60 min at 37 °C). Cells were stimulated for 2 or 20 min with different concentrations of PMA (nm: 25, 250, 1000) and treated as described in panel B. Incorporation of radiolabel into PAF was expressed as percent increase of radiolabel incorporated into PAF molecule over basal value of non-treated cells (18,590 ± 3,118 cpm/dish). Values are expressed as the mean ± S.D. of four experiments, each done in triplicate. Incorporation obtained after PMA treatment was compared versus control by Student's t test: *, p < 0.05; **, p < 0.02; ***, p < 0.005.

**TABLE I**

| Radioactivity released | 1 min | 2 min | 5 min | 10 min | 20 min |
|------------------------|-------|-------|-------|--------|--------|
| Control                | 0.31  | 0.48  | 0.79  | 1.29   | 2.70   |
| PMA (250 nm)           | ±0.13 | ±0.17 | ±0.18 | ±0.29  | ±0.23  |
| Thrombin (0.5 unit/ml) | ±0.13 | ±0.16 | ±0.31 | ±0.18  | ±0.11  |

* Incubation time.

**FIG. 2.** Effect of PMA on HUVEC activities of alkyllyso-GP:acetyl-CoA acetyltransferase (A) and of DTT-insensitive alkyllyso-GP:CDP-choline phosphotransferase (B). HUVEC (100-mm diameter culture wells) were exposed to different concentrations of PMA for 2 or 20 min at 37 °C in 6 ml of M199-BSA (control). Incubation was stopped by removing the medium and scraping the cells on ice in ice-cold extraction buffer as detailed under "Experimental Procedures." Panel A, alkyllyso-GP:acetyl-CoA acetyltransferase activity was expressed as nanomoles of [3H]acetate transferred to alkyllyso-GP and the hydrolysis product alkyllyso-G per min and per mg of lysate protein. Panel B, alkyllyso-GP:CDP-choline phosphotransferase activity was expressed as nanomoles of [methyl-3H]choline transferred to alkyllyso-G per min and per mg of lysate protein. Values are given as the mean ± S.D. from five experiments each done in triplicate. Enzyme activity after PMA treatment was compared versus control by Student's t test: *, p < 0.05; **, p < 0.02; ***, p < 0.005.

**PAF Stimulates the de Novo Synthetic Pathway for PAF in HUVEC**
acetyl-G-CDP-choline cholinephosphotransferase, whereas it does not activate the enzymes of the remodeling pathway. This conclusion is supported by the evidence that PMA stimulates, in a dose-dependent manner, (a) the generation of PAF, (b) the incorporation of $[^3H]$acetate and [methyl-$^3$H] choline into PAF, and (c) both alkyllyso-GP:acytoll-CoA acetyltransferase and DTT-insensitive cholinephosphotransferase (21, 22). PMA promotes the incorporation of labeled acetate into 1-O-alkyl-2-lyso-glycerol-3-phosphocholine forming 1-O-alkyl-2-acytoll-sn-glycerol-3-phosphocholine, which was first identified and termed PAF. Our results suggest that, in HUVEC, both alkyllyso-GP:acytoll-CoA acetyltransferase (8) and DTT-insensitive alkylacytoll-G:CDP-choline cholinephosphotransferase (23) are absent in PMA-stimulated cells. These data agree with previous ones showing the lack of PMA effect on the remodeling pathway in rat alveolar macrophages (4) and in human vascular endothelial cells (30).

As to DTT-insensitive alkylacytoll-G:CDP-choline cholinephosphotransferase, our findings are in keeping with previous studies on neutrophils, which showed 1-oleyl-2-acytoll-glycerol-dependent cholinephosphotransferase activation and PAF synthesis (17), and the requirement of well determined concentrations of alkylacytoll-G for optimal expression of the PMA-elicited PAF synthesis (16). In addition, we report the novel finding that PMA exerts a regulatory role on the activity of alkyllyso-GP:acytoll-CoA acetyltransferase. The detailed biochemical characterization of both alkyllyso-GP:acytoll-CoA acetyltransferase and DTT-insensitive alkylacytoll-G:CDP-choline cholinephosphotransferase has been reported (8, 10). In most tissues, the specific activity of alkyllyso-GP:acytoll-CoA acetyltransferase is the lowest among the three enzymes of the de novo pathway (8-10); therefore, it appears that this enzyme would be the rate-limiting step in the production of PAF. However, it has been shown that only the decrease of the DTT-insensitive cholinephosphotransferase (and not of the acetyltransferase) accounts for the decreased rate of PAF synthesis during the development of renal necrosis (31). This observation, together with the finding that PAF generated by chick retinas upon stimulation with neurotransmitters is due to an increase in the DTT-insensitive alkylacytoll-G:CDP-choline cholinephosphotransferase (11, 12), points to an important regulatory role of this enzyme in the de novo synthesis of PAF. Our results suggest that, in HUVEC, both alkyllyso-GP:acytoll-CoA acetyltransferase and DTT-insensitive alkylacytoll-G:CDP-choline cholinephosphotransferase (whose specific activities are very similar) are regulatory enzymes and that changes in their activity may be due to PKC-induced enzyme phosphorylation mediated by PMA. While inflammatory stimuli (i.e. thrombin)-induced increase of intracellular Ca$^{2+}$ results in activation of the remodeling pathway (25), it most probably inhibits the synthesis of PAF through the de novo pathway, since Ca$^{2+}$ is an inhibitor of both alkyllyso-GP:acytoll-CoA acetyltransferase (8) and DTT-insensitive alkylacytoll-G:CDP-choline cholinephosphotransferase (10).

PKC-induced activation of PAF synthesis through the de novo pathway may take place when HUVEC are stimulated by agents which can activate PKC in the absence of any Ca$^{2+}$ transient, i.e. cis-fatty acids (32), elevated glucose concentration (33), and selective oxidative modification of the enzyme regulatory domain (34).

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