Biosynthesis of N-AcetylSphingosine by Platelet-activating Factor: Sphingosine CoA-independent Transacetylase in HL-60 Cells*

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Platelet-activating factor (PAF)1 is a potent lipid mediator involved in diverse pathophysiological processes, e.g. inflammation, allergic reactions, and many aspects of reproduction (see recent reviews by Hanahan (1992), Shukla (1992), Venable et al. (1993), and Snyder (1995a, 1995b)). The biosynthetic routes for PAF via either denovo (Lee et al., 1986, 1988; Renooij and Snyder, 1981; Woodard et al., 1987) or remodeling (Wykle et al., 1990; Lee, 1985) pathways are well established. In addition to the putative conversion of 1-alkyl-2-arachidonoyl-sn-glycero-3-phosphocholine (alkylarachidonoyl-GPC) to alkyl-lyso-GPC by phospholipase A2, in the remodeling pathway, alkyl-lyso-GPC can also be generated by a CoA-independent transacylase that transfers the long-chain acyl groups (primarily 20:4) from alkylacyl-GPC to other lysophospholipid acceptors containing either ethanolamine or choline, e.g. alk-1-ene-lyso-sn-glycero-3-phosphocholine (Lee et al., 1988, 1991; Nieto et al., 1991). The alkyl-lyso-GPC formed by the transacylase can subsequently be acetylated by the alkyllyso-GPC:acetyl-CoA acetyltransferase to produce PAF (Uemura et al., 1991; Nieto et al., 1991).

PAF is degraded by the acetylhydrolase to form acetate and alkyllyso-GPC (Blank et al., 1981, 1983b; Farr et al., 1980, 1983; Stafforini et al., 1987); the latter is rapidly transacylated to enter a membrane pool of alkylacyl-GPC that is highly enriched with arachidonic acid (MacDonald and Sprecher, 1991; and Snyder et al., 1992). Recently, we identified a unique membrane-associated CoA-independent transacylase that can transfer the acetate group from PAF to a wide variety of lysophospholipids (radyllyso-GPC, radyllyso-sn-glycero-3-phosphocholine, acyllyso-3-phosphoholamine, acyllyso-sn-glycero-3-phosphoserine, and acyllyso-sn-glycero-3-phosphoinositol), radyllyso-sn-glycero-3-phosphate, and long-chain fatty alcohols (Lee et al., 1992). This enzyme appears to be the preferential in vivo route for the biosynthesis of the ethanolamine plasmalogen and acyl analogs of PAF. It has been proposed (Lee et al., 1992) that the transacylase plays a role in the fine tuning of PAF biological responses and cross-talk between denovo and remodeling pathways of PAF biosynthesis via acetylation of intermediates involved in the generation of bioactive lipid molecules.

Sphingosine (see reviews by Hannun and Bell (1987, 1989), Merrill and Stevens (1989), Merrill (1991), Hannun and Lindic (1993), and Hannun and Bell (1993)) and its related metabolites such as ceramides (Kolesnick, 1991), sphingosine 1-phosphate (Zhang et al., 1991), and sphingosylphosphorylcholine (Desai et al., 1993) have also emerged as important signaling molecules involved in many cellular processes. The fact that sphingosine 1-phosphate and radyllyso-glycerophosphate, as well as sphingosylphosphorylcholine and radyllyso-GPC, are

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1The abbreviations used are: PAF, platelet-activating factor; GPC, sn-glycero-3-phosphocholine; HPLC, high performance liquid chromatography; C2-ceramide, N-acetylSphingosine; BSA, bovine serum albumin.
structurally similar to each other and acetylated sphingomyelin has been reported to possess PAF-like activity (Berdyshev and Getmanova, 1991) prompted us to investigate the possibility that the transacetylase may be able to donate the acetate group from PAF to sphingolipids, thus modifying the biological activities of both PAF and sphingolipid derivatives.

EXPERIMENTAL PROCEDURES

Materials—1-Hexadecyl-2-[3H]acetyl-GPC (10 Ci/mmol), [2-palmitoyl-9,10-3H] dipalmitoyl-GPC (42.0 Ci/mmol), hexadecyl-[3H]arachidonoyl-GPC (146 Ci/mmol), and [3H]-acetyl-CoA (3.9 Ci/mmol) were purchased from DuPont NEN. Sodium [3H]acetate was from the Amersham Corp. Phospholipase D from Streptomyces chromofuscus, hexadecyl-arachidonoyl-GPC, phosphatidylethanolamine from bovine brain (containing 60% plasmalogen), α-erythro-dihydroxyphosphoglycerine (phinganine), ceramide, sphingomyelin, stearylamine, acetyl-CoA, and CoA were from Sigma, whereas α-erythro-phosphoglycerine, sphingosylphosphorylcholine, and C2-ceramide were obtained from Matreya, Inc. α-erythro-sphingosine, β-threo-sphingosine, and γ-threo-sphingosine were generous gifts from Dr. A. H. Merrill (Emory University, Atlanta, GA).

Preparation of Various Lipid Analogs—1-Alk-1-enyl-2-lyso-sn-glycerol-3-phosphoethanolamine (lysoplasmalogen) was generated from plasmalogen-containing ethanolamine glycerolipids by treatment with monomethylamine to cleave the sn-1 and sn-2 acyl linkages (Clark and Dawson, 1981). N-Acetylsphingosine (C2-ceramide) was prepared from sphingosine by the method of Gaver and Sweeley (1966). N,O2-triacetylsphingosine and O2-diacetylcysteine were made by heating sphingosine and ceramide with 1.5 ml of acetic anhydride and 0.3 ml of pyridine at 100 °C for 45 min, respectively. O-Acetylsphingomyelin was synthesized as described by Berdyshev and Getmanova (1991). O-Acetylcysteine was obtained from O-acetylsphingomyelin via a phospholipase C reaction (Mavis et al., 1972). Sphingosine 1-phosphate was prepared by reacting sphingosylphosphorylcholine with phospholipase D from S. chromofuscus (Wolf and Gross, 1985).

Cell Culture and Membrane Isolation—Methods used to culture undifferentiated and differentiated HL-60 cells in serum-free medium, supplementation with arachidonate (10 μM, 24 h), and the isolation of membrane fractions (100,000 g × 60 min pellet) were the same as described previously (Uemura et al., 1991).

Enzyme Assays—Standard incubations for measuring the PAF: sphingosine CoA-independent transacylase reaction were similar to that reported for PAF:lysophospholipid CoA-independent transacylase (Lee et al., 1992) except the concentrations of [3H]PAF were varied and 50 μM sphingosine was added as an equimolar mixture with fatty acid-free bovine serum albumin (BSA). Ceramide, sphingomyelin, stearylamine, sphinganine, sphingosine 1-phosphate, and sphingosine 1-phosphate associated with the membrane fraction (100,000 × g 60 min pellet) of undifferentiated HL-60 cells. Hexadecyl-[3H]acetyl-GPC (1,000 Ci/mmol, 0.3 μCi in 50 μl 0.1% BSA-saline) was incubated with 50 μM sphingosine (in 50 μl of 0.1% BSA-saline), 1 mM sodium acetate, 5 mM EGTA, and 100 mM Tris-HCl (pH 7.4), with/without or boiled (100 °C for 10 min) membrane proteins (100 μg) in a final volume of 0.5 ml for 60 min at 37 °C. Reaction products were analyzed after lipid extraction by TLC and liquid scintillation counting as described under “Experimental Procedures.” Authentic standards, PAF, N-acetylsphingosine (N-Ac-Sph), and N,O2-triacetylsphingosine (N,O2-Ac-Sph) migrated to the areas indicated by the brackets below the fraction numbers.
Transacetylase and Biosynthesis of C2-Ceramide

Characterization of the Reaction Kinetics—Investigation of the time-dependent generation of the C2-ceramide by the CoA-independent transacetylase revealed that the reaction rate was not linear and exhibited a lag period (Fig. 2). In addition, a small but significant amount of C2-ceramide was formed in the absence of an enzyme source (Fig. 2). This nonenzymatic formation of C2-ceramide increased substantially with increasing concentration of PAF and pH (data not shown). However, at fixed concentrations of PAF and sphingosine, the amounts of N-acetylsphingosine produced by both enzymatic (Fig. 3A) and nonenzymatic (Fig. 3B) reactions were inversely related to the molar ratio of BSA to sphingosine, i.e., the reaction rate was much higher at lower molar ratios of BSA to sphingosine (e.g., 1:27). On the other hand, the enzymatic reaction rate tended to be more linear at higher molar ratios of BSA to sphingosine (e.g., 1:1) than at lower molar ratios of BSA to sphingosine (Fig. 3C).

The effect of [3H]PAF concentrations on the formation of N-acetylsphingosine was determined at a molar ratio of BSA: sphingosine of 1 (Fig. 4). Maximal enzyme activity occurred between 10 and 20 μM of [3H]PAF, with an apparent Km of 5.4 μM for PAF. The effect of sphingosine concentrations on the generation of N-acetylsphingosine by the PAF:sphingosine CoA-independent transacylase can be seen in Fig. 5. A Lineeweaver-Burk plot gave a curved reciprocal line with an upward divergence (Fig. 5B). This type of kinetic behavior makes the determination of the Michaelis constant difficult and also suggests that sphingosine not only serves as a substrate but may act as an activator (Dixon and Webb, 1964) for the transacetylase.

Substrate Stereochemical Configuration Requirements—Sphingosine can exist as four stereoisomers but only the d-erythroisomer of sphingosine is synthesized in biological systems (Fujino and Zabin, 1962; Karlsson, 1970). Similarly, we found that the d-erythrospingosine can act as an acceptor for [3H]acetate from [3H]PAF. l-Threo-, l-erythro-, and d-threosphingosine had just 9.2, 6.7, and 3.0%, respectively, of

![Figure 2](http://www.jbc.org/)

**Fig. 2. Time course for the formation of N-acetylated sphingosine (N-Ac-Sph) by the PAF:sphingosine CoA-independent transacetylase associated with the membrane fraction (100,000 g 60 min pellet) of undifferentiated HL-60 cells.** The assay system was the same as described in the legend of Fig. 1 except the incubation times were varied as indicated. Results, the average of duplicate determinations with variations < 10%, are representative of five similar experiments.
the activity exhibited by D-erythrospingosine.

Substrate Specificity and Competition—Sphinganine, stearylamine, ceramide, sphingomyelin, sphingosine 1-phosphate, and sphingosylphosphorylcholine were also tested to determine whether they could serve as acceptors for the transfer of [3H]acetate from [3H]PAF. The products were identified by their identical co-migrations on TLC plates with authentic standards. Only sphinganine exhibited some capability to accept the acetate group from PAF. Ceramide, sphingosylphosphorylcholine, stearylamine, sphingomyelin, and sphingosine 1-phosphate were inactive as acetate acceptors for the transacylase.

The possibility of sphingosine accepting a long-chain acyl group, instead of the short-chain acetyl grouping, was examined by incubating 1 μM (0.3 μCi) [3H]-dipalmitoyl-GPC or hexadecyl[3H]arachidonoyl-GPC instead of [3H]PAF with 50 μM sphingosine (complex with equal molar of BSA) using the same conditions under which PAF:sphingosine CoA-independent transacylase was assayed. Under these conditions, no measurable amount of ceramide could be detected. Furthermore, when sphingosine, ceramide, or alkyllyso-GPC was incubated with [3H]acetyl-CoA in the presence of membrane proteins from undifferentiated HL-60 cells, only alkyllyso-GPC (75 pmol/min-mg protein) but neither sphingosine nor ceramide was acetylated by the lyso-PAF:acetyl-CoA acetyltransferase. These results are consistent with our finding that the amounts...
of acetate transferred from PAF to sphingosine remained the same when [3H]PAF and sphingosine were incubated either in the presence or absence of sodium acetate (1 mM) with membrane proteins of HL-60 cells (data not shown). Moreover, no acetate was incorporated into sphingosine when 1 mM sodium [3H]acetate (0.3 μCi) was incubated under the same conditions with 50 μM sphingosine and membrane proteins of undifferentiated HL-60 cells.

As one means of addressing the question as to whether a single enzyme or two different enzymes catalyze the acetylation of lysophospholipids and sphingosine by PAF, mixed substrate experiments were conducted. Results indicate that sphingosine, acyllyso-GPC, and sphingosylphosphorylcholine at equal molar concentration with that of lysoplasma1ogen (50 μM) inhibited the production of acetylated plas1ogen by 20, 55, and 27%, respectively. On the other hand, lysoplasma1ogen, acyllyso-GPC, and sphingosylphosphorylcholine at 50 μM had no effect on the acetylation of 50 μM sphingosine. When the concentration of sphingosine was reduced to 25 μM, 50 μM lysoplasma1ogen (molar ratio of sphingosine to lysoplasma1ogen, 1:2) was still ineffective in inhibiting N-acetylsphingosine formation. With 10 μM sphingosine, a mere 13% decrease in N-acetylsphingosine generation was caused by 50 μM lysoplasma1ogen. In addition, sphingosine 1-phosphate, not a substrate for the transacetylase, exerts no influence on the transacetylation of either lysoplasma1ogen or sphingosine. Thus, either the CoA-independent transacetylase has a higher substrate affinity for sphingosine than any of these other substrate analogs or the possibility of two isoforms of the transacetylase might be involved in the transfer of the acetate from PAF to sphingosine and lysophospholipids.

Tissue Distribution—Distribution of PAF:sphingosine transacetylase activity in various tissues and undifferentiated HL-60 cells was studied and compared with that of PAF:lysoplasma1ogen transacetylase (Fig. 7, A and B). Kidney has the highest PAF:sphingosine transacetylase activity, whereas both kidney and lung have the highest PAF:lysoplasma1ogen transacetylase activities. In general, the patterns of tissue distribution for both transacetylases are similar with the exception of rat lung and the undifferentiated HL-60 cells. PAF: sphingosine transacetylase activity is much higher than that of PAF:lysoplasma1ogen transacetylase in undifferentiated HL-60 cells, and the reverse applied to the two transacetylase activities in the lung.
Inhibitor Studies—In order to determine the amino acid residues that may participate in the transacetylase reactions, the effects of diethyl pyrocarbonate (modifies histidine residues) and N-ethylmaleimide (reacts with the —SH group of cysteine) on the transacetylases were studied (Table I). Diethyl pyrocarbonate and N-ethylmaleimide inhibit both PAF:lysoplasmalogen and PAF:sphingosine transacetylase activities, but to a different degree. Diethyl pyrocarbonate is more effective as an inhibitor for PAF:lysoplasmalogen transacetylase than that of PAF:sphingosine transacetylase, whereas the re-
verse is true for N-ethylmaleimide.

Formation of N-acetylsphingosine (C2-Ceramide) by Intact HL-60 Cells—Both differentiated and undifferentiated HL-60 cells can synthesize N-acetylsphingosine when [3H]PAF (1 μM) and sphingosine (50 μM) are supplemented in the incubation medium for 1 h; the estimated amounts of N-acetylsphingosine formed are in the range of 0.6–0.8 μM based on the assumption that 1 cell = 6.3 × 10^{-13} liter (Ladinsky and Westring, 1967). The amounts of C2-ceramide produced depend on the concentration of [3H]PAF and sphingosine, as well as the length of incubation time (data not shown). Similar amounts of N-acetylsphingosine are also formed when excess sodium acetate (5 mM) was included in the incubation medium to exclude CoA-mediated acetylation. These results suggest that PAF:sphingosine transacylase is responsible for the production of C2-ceramide in intact HL-60 cells. In addition, using the methodologies described under “Experimental Procedures,” the endogenous cellular levels of C2-ceramide were determined to be 19.0 ± 0.6, and 28.2 ± 6.3 (n = 3) pmol/10^7 cells or 3.0 and 4.5 μM, in differentiated and undifferentiated HL-60 cells, respectively.

DISCUSSION

We have demonstrated that an enzyme from the membrane fraction of undifferentiated HL-60 cells can transfer the acetate group of PAF to sphingosine with the formation of C2-ceramide as depicted by Reaction 1. The product was identified as N-acetylsphingosine by its co-migration with an authentic standard on thin-layer plates, its sensitivity to acid hydrolysis, its resistance to alkaline treatment, which indicated the presence of an amide linkage, and its ability to form dibenzoate derivatives. Several other lines of evidence also support the notion
that this reaction is catalyzed by a CoA-independent transacylase. The reactions were normally carried out in the presence of excess unlabeled sodium acetate (1 mM) in order to prevent or minimize any radioactive acetate released from the PAF being directly incorporated into sphingosine. In addition, the presence of CoA up to 0.5 mM in the incubation mixture had no effect on the generation of N-acetylsphingosine (data not shown). Under the assay conditions where lysoplasmalogens are acetylated by the acetyl-CoA acetyltransferase, sphingosine does not serve as a substrate. Furthermore, transacylation of the amino group on sphingosine apparently is highly specific for short-chain acyl groups (i.e., acetate), because dipalmitoyl-GPC and hexadecylarachidonoyl-GPC cannot transfer either palmitate or arachidonate, respectively, to sphingosine under similar experimental conditions. However, the possibility could also exist that dipalmitoyl-GPC and hexadecylarachidonoyl-GPC can not serve as a substrate due to their inability to insert into membrane. A small but significant amount of N-acetylsphingosine is produced from [3H]PAF and sphingosine nonenzymatically (chemical) (Fig. 2 and 3B), and the reaction rate is not linear with incubation time (Fig. 2). However, both of these problems can be circumvented by complexing sphingosine with equal molar amounts of BSA (Fig. 3). Reasons for why complexing the sphingosine with BSA would reduce the nonenzymatic formation of N-acetylsphingosine between sphingosine and PAF and improve the linearity of the reaction are not clear at present. It is possible that sphingosine may exist in both monomeric and aggregate forms in the absence of BSA, which could favor the chemical formation of N-acetylsphingosine. Furthermore, Merrill (1991) has reported the formation of 1:1 molar complex of sphingosine with BSA stabilizes the amphotheric sphingosine molecule. Nevertheless, even though the sphingosine-BSA complex reduces the nonenzymatic formation of N-acetylsphingosine and provides satisfactory linear kinetics for the reaction, it also diminishes the reaction rate significantly (Fig. 3). When kinetic parameters were measured using the sphingosine-BSA complex as the substrate, the calculated apparent $K_m$ for [3H]PAF (5.4 $\mu M$, Fig. 4) of the PAF:sphingosine transacylase is in the same range as we previously reported for the PAF:lysoplasmalogen transacylase (12.0 $\mu M$) (Lee et al., 1992). The apparent $K_m$ for lysoplasmalogen was 106.4 $\mu M$ (Lee et al., 1992), whereas the apparent $K_m$ for sphingosine in this study could not be assessed due to its kinetic behavior (Fig. 5).

Among the variety of sphingosine-related compounds (i.e., stearylamine, sphinganine, sphingosine-1-phosphate, sphingosylphosphorylcholine, ceramide, and sphingomyelin) tested, only sphinganine, sphingosylphosphorylcholine, and ceramide showed limited ability to accept the acetate group from PAF (Fig. 6). These results indicate the transacylase has a strict substrate structural requirement for sphingolipids in that it prefers to transfer the acetate group to the $\text{-NH}_2$ instead of $\text{-OH}$ group; moreover, the presence of $\text{-OH}$ and trans double bond within the structure appears to potentiate the transacylase activity. For example, stearylamine is inactive, whereas sphinganine (a metabolite of the de novo biosynthetic pathway (Merrill, 1991)) is less active than sphingosine (a turnover product of sphingolipids) as a substrate for the transacylase. In addition, neither lysoplasmalogen and related substrate analogs (i.e., acyllyso-GPC) nor sphingosine analogs (i.e., sphingosylphosphorylcholine and sphingosine-1-phosphate) affected the transfer of acetate from PAF to sphingosine, whereas the transfer of acetate from PAF to lysoplasmalogen was easily inhibited by the presence of these substrate analogs (with the exception of sphingosine-1-phosphate).

These findings suggest that the transacylase has a different affinity for sphingosine and lysoplasmalogens. Consistent with these findings are the observations that concentration-dependent responses to the modification of cysteine and histidine residues of the transacylases by the inhibitors are different for the acetylation of sphingosine and lysoplasmalogens (Table 1).

It is difficult at present to assess whether two different enzyme activities carry out the transfer of the acetate from PAF to lysophospholipids and sphingosine. Even though PAF:lysoplasmalogen transacylase differs from PAF:sphingosine transacylase in its substrate specificities, tissue distribution (Fig. 7), and enzyme inhibitor responses (Table 1), both enzymatic activities respond to temperature inactivation in a similar manner when either preincubating the membrane fractions at 60°C for various times or when assayed at different incubation temperatures (data not shown). We are currently attempting to purify the transacylase protein(s) and clone the cDNA(s) for the transacylases in order to address this important issue.

Recently, Liu and Subbaiah (1994) reported that purified lecithin-cholesterol acytransferase from human plasma can catalyze the transfer of the acetyl group from PAF to lysophosphatidylcholine (acyllyso-GPC) forming acyl analog of PAF. Lecithin-cholesterol acytransferase with a molecular mass of 65,000 - 69,000 Da (Marcel, 1982; Chung et al., 1979) is synthesized in the liver and secreted into plasma where it associates with high density lipoproteins (Marcel et al., 1980; Glomset, 1972). It requires serine, histidine, and cysteine for catalytic activity (Jauhiainen and Dolphin, 1986). On the other hand, the PAF-dependent transacylase we investigated is a membrane-associated enzyme. In addition, the PAF:lysophospholipid transacylase (solubilized from rat kidney membranes with 0.02% Tween 20) has an estimated molecular mass of 47 kDa (determined by Sephacyr S-200 column),2 and both histidine and cysteine (Table 1), but not serine (not inhibited by phenylmethylsulfonyl fluoride) (Lee et al., 1992) are required for expression of its activity. Furthermore, the PAF-dependent transacylases do not transfer long-chain acyl groups to acceptor molecules. These properties indicate that lecithin-cholesterol acytransferase and transacylase are two distinct enzymes.

C$_2$-ceramide, which has been extensively used by many investigators as an unnatural, cell-permeable analog of long-chain acyl-ceramides, possesses many of the biological activities associated with the naturally occurring ceramides containing long-chain acyl moieties. At micromolar concentrations, it stimulates the activities of a mitogen-activated protein kinase (Raines et al., 1993), a cytosolic protein phosphatase (Dobrowsky and Hannun, 1992), and protein phosphatase 2A (Dobrowsky et al., 1993) and induces programmed cell death (Obeid et al., 1993), cell cycle arrest (Iaydev et al., 1995), cellular differentiation, and c-myc down-regulation (Kim et al., 1991). In addition, C$_2$-ceramide inhibits the stimulation of DNA synthesis and sphingolipid $D$ activity by phosphatidic acid and lysophosphatidic acid (Gomez-Mun˜oz et al., 1994). It

\[ \text{REACTION 1.} \]

\[
\begin{align*}
\text{[acyl]} + \text{PC} &\rightarrow \text{[acyl]PC} \\
\text{[acyl]PC} &\rightarrow \text{[acyl]PC} + \text{BSA}
\end{align*}
\]

2 T.-c. Lee and M.-c. Ou, unpublished data.
also inhibits fMet-Leu-Phe- and phorbol 12-myristate 13-acetate-induced superoxide formation in neutrophils (Wong et al., 1995). However, unlike sphingosine and sphingosylphosphorylcholine, the C2-ceramide does not inhibit protein kinase C (Hannun and Bell, 1987) or cause Ca^{2+} release (Ghosh et al., 1990). Furthermore, C2-dihydroceramide (N-acetylsphinganine) acts instead as an inhibitor of protein phosphatase 2A (Dobrowsky et al., 1993) and fails to induce apoptosis (Obied et al., 1993) or block fMet-Leu-Phe-activated superoxide generation (Wong et al., 1995). Our results on the identification and characterization of PAF:sphingosine transacytase, the demonstration of the formation of C2-ceramide by this enzyme in intact HL-60 cells, and that this enzyme only uses the naturally occurring stereoisomer, d-erythro-sphingosine, as a substrate, suggest that one function of the PAF:sphingosine transacytase is to modulate the biological responses of PAF and sphingosine by producing a different signaling molecule, C2-ceramide. The detection of physiological levels of C2-ceramide in HL-60 cells further supports the notion that C2-ceramide is a naturally occurring lipid mediator.

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