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Inflammatory Activation of Arachidonic Acid Signaling in Murine P388D$_1$ Macrophages via Sphingomyelin Synthesis*

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Ceramide has emerged as an important lipid messenger for many cellular processes triggered via surface receptors. In the present study, inflammatory activation of P388D$_1$ macrophages with bacterial lipopolysaccharide (LPS) and platelet-activating factor (PAF) stimulated a transient accumulation of ceramide. Moreover, cell-permeable ceramide mimicked LPS/PAF in triggering arachidonate mobilization in these cells. LPS/PAF-induced ceramide synthesis did not result from sphingomyelinase activation but from increased *de novo* synthesis. Participation of this pathway in arachidonate signaling was detected since fumonisin B$_1$, an inhibitor of *de novo* ceramide synthesis, was able to inhibit the LPS/PAF-induced response. These studies have uncovered a new role for sphingolipid metabolism in cellular signaling and constitute evidence that products of the sphingomyelin biosynthetic pathway may serve a specific role in signal transduction by influencing the activity of the novel Group V secretory phospholipase A$_2$.

Phospholipase A$_2$ (PLA$_2$)[1] plays a key role in cellular signaling by generating a wide array of biologically active lipid mediators. PLA$_2$-mediated hydrolysis of glycerophospholipids results in the release of arachidonic acid (AA), which may either exert direct effects or serve as a substrate for the generation of other lipid messengers such as the prostaglandins and leukotrienes (1, 2). Two distinct PLA$_2$s have been shown to mediate the receptor-coupled AA release, namely the Group IV 85-kDa Ca^{2+}-dependent cytosolic PLA$_2$ (cPLA$_2$) and a 14-kDa Ca^{2+}-dependent secretory PLA$_2$ (sPLA$_2$) (3).

When triggered by immunoinflammatory stimuli, prostaglandin biosynthesis usually takes place in two phases, *i.e.* an immediate (minutes) and a delayed phase (hours) (4–6). Immediate prostaglandin production in macrophages and mast cells, two of the main eicosanoid-producing cells at the sites of inflammation, mostly arises from the AA liberated by the sPLA$_2$ (3, 5, 7). In P388D$_1$ macrophages (7) and mast cells as well (4, 5), this sPLA$_2$ is not the "classical" Group IIA enzyme that has been widely implicated in inflammatory reactions (8) and several other pathological conditions, including cancer (9), but the novel Group V sPLA$_2$ (7).

Following PAF receptor occupancy, Group V sPLA$_2$ is secreted from activated P388D$_1$ macrophages (7, 10) and rapidly associates with the outer membrane of the cells thereby, initiating AA release and prostaglandin production. The Group V enzyme is the only sPLA$_2$ these cells express (7). It is believed that the alterations in membrane lipid packing and asymmetry due to receptor occupancy cause activation of the sPLA$_2$ at the outer surface (11). Such a "membrane rearrangement" has been suggested to be mediated in part by receptor activation of sphingomyelin breakdown (12).

Most recently, a role for the cPLA$_2$ in this membrane rearrangement has been proposed for macrophages (3) and later confirmed to also occur in mast cells (6). cPLA$_2$ activation precedes and influences the subsequent activation of the sPLA$_2$ by generating a small transient burst of free AA that may contribute to destabilizing the membrane (3). The AA released by the cPLA$_2$ contributes little (3, 13) or nothing (5, 6) to immediate prostaglandin production, and most of it is retained intracellularly (3). Thus, quantitation of extracellular AA release in activated P388D$_1$ cells correlates best with sPLA$_2$ activation (3, 7, 10, 13).

In the present study we report a novel mechanism for regulating cellular AA signaling. Our data show that increased sphingomyelin biosynthesis during inflammatory signal transduction provides stimulatory signals allowing the sPLA$_2$ to act at the cellular surface.

EXPERIMENTAL PROCEDURES

Materials—P388D$_1$ cells (TIB 63) were obtained from the American Type Culture Collection (Rockville, MD). Iscove's modified Dulbecco's medium (endotoxin, <0.05 ng/ml) was from BioWhittaker (Walkersville, MD). Fetal bovine serum was from Hyclone Laboratories (Logan, UT). Nonessential amino acids were from Irvine Scientific (Santa Ana, CA). [5,6,8,9,11,12,14,15-^{3}H]Arachidonic acid (specific activity, 100 Ci/mmol), [*methyl*-^{3}H]choline chloride (specific activity, 79 Ci/mmol), [U-^{14}C]serine (specific activity, 159 mCi/mmol), and [9,10-^{3}H]palmitic acid (specific activity, 54 Ci/mmol) were from NEN Life Science Products. *N*-[1-^{14}C]Hexadecanoylsphingosine (specific activity, 55 mCi/mmol) was from American Radiolabeled Chemicals (St. Louis, MO). LPS Re 595, 1,2-dioctanoyl-*sn*-glycerol, and fumonisin B$_1$ were from Sigma. *N*-Acetylsphingosine (C$_2$-ceramide) and D609 (tricyclodecan-9-yl xanthogenate) were from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA). PAF was from Calbiochem.

Cell Culture and Labeling Conditions—P388D$_1$ cells were maintained at 37 °C in a humidified atmosphere at 90% air and 10% CO_2 in Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and nonessential amino acids. Cells were plated at 10^6/well, allowed to adhere overnight, and used for experiments the following day. All experiments were conducted in serum-free Iscove's modified Dulbecco's medium.

Stimulation of P388D$_1$ Cells—The standard regimen for activating the P388D$_1$ cells with LPS and PAF has been described previously (14). Briefly, the cells were placed in serum-free medium for 30–60 min before the addition of LPS (200 ng/ml) for 1 h. After the LPS incubation, bovine serum albumin (0.1 mg/ml) was added, and the cells were then incubated with PAF, C$_2$-ceramide, DAG, or combinations of these stim-

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[1] The abbreviations used are: PLA$_2$, phospholipase A$_2$; cPLA$_2$, Group IV cytosolic Ca^{2+}-dependent phospholipase A$_2$; sPLA$_2$, secretory phospholipase A$_2$; AA, arachidonic acid; LPS, lipopolysaccharide; PAF, platelet-activating factor; DAG, diacylglycerol; PC, phosphatidylcholine; D609, tricyclodecan-9-yl xanthogenate.

uli for the time indicated. Concentrated solutions of C_2-ceramide and DAG were made up in dimethyl sulfoxide, and an aliquot was added to the cell cultures to achieve the indicated final concentrations. Appropriate controls were run to exclude any effects of the vehicle. When fumonisin B_1 was used, it was added 15 min before the cells were exposed to LPS. No intermediate washes were carried out along the procedure to prevent the burst of sphingomyelin synthesis that follows immediately after changing the medium (15).

Measurement of Extracellular AA Release and Cell-associated Free AA—Radiolabeling of the cells with [^{3}H]AA was achieved by including 0.5 μCi/ml [^{3}H]AA during the overnight adherence period (20 h). LPS-treated cells labeled with [^{3}H]AA were incubated with the different stimuli in the presence of 0.1 mg/ml bovine serum albumin for the time indicated. The supernatants were removed, cleared of detached cells by centrifugation, and assayed for radioactivity by liquid scintillation counting. More than 99% of the released radioactive material remains as unmetabolized AA under these experimental conditions (13).

For analysis of cell-associated free AA, the cell monolayers were scraped in 0.5 ml of 0.5% Triton X-100. Lipids were extracted according to Bligh and Dyer (16) and separated by thin-layer chromatography using *n*-hexane/diethyl ether/acetic acid (70:30:1, v/v/v) as a solvent system. Authentic AA was co-chromatographed and visualized by exposing the plates to iodine vapors. Areas containing AA were scraped into scintillation vials, and the amount of radioactivity was measured by liquid scintillation counting.

Measurement of Sphingomyelin Levels in [^{3}H]Choline-labeled Cells—For sphingomyelin determination, the cells were labeled with 0.5 μCi/ml [*methyl*-^{3}H]choline for 72 h. The cells were activated with LPS and PAF as described above. After the indicated times, supernatants were discarded, and the cell monolayers were scraped in 0.5 ml of 0.5% Triton X-100. Lipids were extracted according to Bligh and Dyer (16) and separated by thin-layer chromatography, using chloroform/methanol/acetic acid/water (50:30:8:5, v/v/v/v) as a solvent system (17). Spots corresponding to sphingomyelin were scraped into scintillation vials, and the amount of radioactivity was measured by liquid scintillation counting.

Measurement of Ceramide and Sphingomyelin Levels in [^{14}C]Serine-labeled Cells—For the simultaneous quantitation of sphingomyelin and ceramide, cells were incubated for 72 h with [U-^{14}C]serine. The cells were activated with LPS and PAF as described above. After the indicated times, supernatants were discarded, and the cell monolayers were scraped in 0.5 ml of 0.5% Triton X-100. Lipids were extracted according to Bligh and Dyer (16) and separated by thin-layer chromatography in the solvent system chloroform/benzene/ethanol (80:40:75, v/v/v) followed by another run in the mobile phase chloroform/methanol/28% ammonium hydroxide (65:25:5, v/v/v), as described (18, 19). Lipids were visualized by exposing the plates to iodine vapors. Fractions that co-migrated along with the unlabeled standards were scraped into scintillation vials, and the associated radioactivity was determined by liquid scintillation counting. Quantitation of sphingomyelin levels in [^{14}C]serine-labeled cells gave the same results as those found with [^{3}H]choline-labeled cells.

Determination of Diacylglycerol Production—Radiolabeling of the cells with [^{3}H]palmitic acid was carried out by including 0.5 μCi/ml [^{3}H]palmitic acid during the overnight adherence period (20 h). Lipids were extracted according to Bligh and Dyer (16) and separated by thin-layer chromatography using *n*-hexane/diethyl ether/acetic acid (70:30:1, v/v/v) as a solvent system. Authentic DAG was co-chromatographed and visualized by exposing the plates to iodine vapors. Areas containing DAG were scraped into scintillation vials, and the amount of radioactivity was measured by liquid scintillation counting.

Data Presentation—Assays were carried out in duplicate or triplicate. Each set of experiments was repeated at least three times with similar results. Unless otherwise indicated, the data presented are from representative experiments.

RESULTS

LPS/PAF Stimulation of P388D$_1$ Cells Elevates Intracellular Ceramide Levels—Stimulation of murine P388D$_1$ macrophages with nanomolar amounts of the inflammatory mediator PAF results in negligible cellular responses unless the cells are first treated with LPS. LPS acts just as a primer, *i.e* it does not stimulate the P388D$_1$ cells by itself but enables the cells to optimally respond to PAF (14, 20). Fig. 1*A* shows that treatment of [^{14}C]serine-labeled P388D$_1$ cells with LPS/PAF resulted in a sharp transient increase in ceramide levels. Generation of ceramide was detectable within 1 min after PAF addition, returning to baseline shortly thereafter. PAF stimulation of the LPS-primed cells also led to a rapid but sustained increase in [^{3}H]palmitate-labeled DAG levels (Fig. 1*B*), reaching a plateau after 5 min of PAF addition. In P388D$_1$ cells, phosphatidylcholine (PC) accounts for 60–70% of the palmitate incorporated into glycerophospholipids (13, 21). Thus, it is reasonable to assume that the bulk of ^{3}H-labeled DAG appearing after stimulation of [^{3}H]palmitate-labeled cells arises from PC. The LPS/PAF-induced DAG generation was not inhibited by D609 (1–25 μM), a purported PC-specific phospholipase C inhibitor (22).

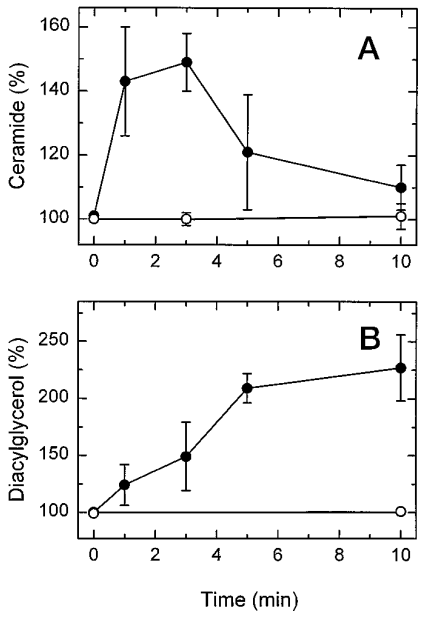

FIG. 1. **PAF-induced changes in ceramide (*A*) and DAG (*B*).** The LPS-treated cells, either labeled with [^{14}C]serine (*A*) or [^{3}H]palmitic acid (*B*), were incubated with (●) or without (○) 100 nM PAF for the times indicated. These data are given as mean values ± S.E. of three different experiments and are expressed as a percentage of the radioactivity in ceramide (*A*) or DAG (*B*) at time 0. The 100% value corresponds to 3,563 ± 2,023 cpm for *panel A* and 106,560 ± 37,790 cpm for *panel B*.

Mechanism of Ceramide Generation—Sphingomyelin hydrolysis by sphingomyelinase has been recognized as a major pathway for ceramide production in a wide variety of cell types (23–25). If sphingomyelinase activation is responsible for ceramide generation in a given cell system, increases in ceramide levels should parallel decreases in sphingomyelin levels (see for example Ref. 19). As shown in Fig. 2, this was clearly not the case in LPS/PAF-treated P388D$_1$ cells. Sphingomyelin levels rapidly increased immediately after PAF addition and remained elevated 10 min after cell challenge. It is interesting to note that ceramide elevation (Fig. 1*A*) seems to precede in time the appearance of sphingomyelin (Fig. 2). Thus these results suggest that ceramide elevation in LPS/PAF-treated cells is not due to sphingomyelinase-catalyzed breakdown of sphingomyelin. Instead, the data are compatible with the possibility that ceramide is the metabolic precursor of sphingomyelin.

Sphingomyelin synthesis is catalyzed by the enzyme sphingomyelin synthase, which transfers the phosphocholine moiety of PC to ceramide, giving rise to sphingomyelin and DAG (23–25). To evaluate the role of this enzyme during LPS/PAF activation, we designed a pulse-chase experiment (shown in Table I). The cells primed with LPS were exposed to labeled [^{14}C]ceramide (5 μCi/ml) for 1 min before addition of PAF, and after 10 min, radioactivity incorporated into sphingomyelin was measured. As shown in Table I, LPS/PAF-activated cells showed a greater conversion of ceramide to sphingomyelin than

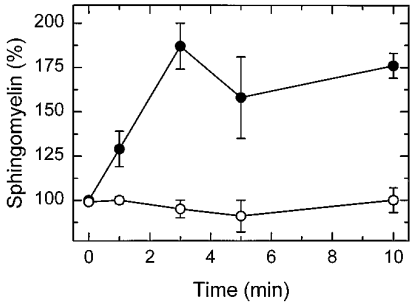

FIG. 2. **PAF-induced sphingomyelin production.** The LPS-treated cells, labeled with [^{3}H]choline, were incubated with (●) or without (○) 100 nM PAF for the times indicated. These data are given as mean values ± S.E. of three different experiments and are expressed as a percentage of the radioactivity in sphingomyelin at time 0. The 100% value corresponds to 7,271 ± 326 cpm.

TABLE I

Rapid conversion of ceramide to sphingomyelin in P388D$_1$ cells

Unlabeled LPS-treated cells were exposed to [^{14}C]ceramide (5 μCi/ml) for 1 min. Subsequently, 100 nM PAF, 10 μM ionophore A23187 or neither (control) was added, and the incubations proceeded for an additional 10-min period. Radioactivity in sphingomyelin was determined as described under "Experimental Procedures." The results are shown as means ± S.E. of three different experiments with duplicate determinations.

	Sphingomyelin synthesis	%
	cpm	
Control	15,850 ± 1,207	100
PAF	22,795 ± 1,439	144 ± 9
A23187	23,862 ± 2,392	151 ± 15

that observed in control cells. We used the Ca^{2+} ionophore A23187 as a positive control for this experiment, since this compound is known to rapidly activate sphingomyelin synthesis *de novo* in macrophages (26). As expected, the ionophore A23187 also accelerated the rate of conversion of ceramide to sphingomyelin (Table I). On the other hand, the rather high conversion of [^{14}C]ceramide into [^{14}C]sphingomyelin already observed in the controls supports the notion that sphingomyelin synthase rapidly attenuates cellular ceramide levels so that little free ceramide is formed as an intermediate of sphingolipid biosynthesis *de novo* (27). Hence, if ceramide arises *de novo* it will accumulate in a very transient fashion just as detected in LPS/PAF-treated cells (Fig. 1*A*).

Fumonisin B$_1$ Blocks Ceramide and Sphingomyelin Synthesis and Inhibits Extracellular AA Release in Activated P388D$_1$ Cells—To further evaluate the notion that the *de novo* biosynthetic pathway is responsible for the LPS/PAF-induced elevations in ceramide and sphingomyelin levels, we conducted experiments with the mycotoxin fumonisin B$_1$. Fumonisin B$_1$ behaves as a potent inhibitor of mammalian ceramide synthase (27). Ceramide synthase catalyzes the *N*-acylation of sphinganine or sphingosine with a long chain fatty acyl-CoA to form dihydroceramide or ceramide. Thus ceramide synthase is located one or two steps upstream of sphingomyelin synthase in the sphingolipid biosynthetic pathway (25, 27).

Fumonisin B$_1$ inhibited both ceramide (Fig. 3*A*) and sphingomyelin (Fig. 3*B*) elevations in activated P388D$_1$ cells in a dose-dependent manner. Almost complete inhibition of ceramide and sphingomyelin synthesis was found at a fumonisin B$_1$ dose as low as 1 μM. Of note, the IC$_{50}$ for inhibition of P388 ceramide synthase by fumonisin B$_1$ is 0.2 μM when assayed *in vitro* with 2 μM substrate (28).

Very importantly, 1 μM fumonisin B$_1$ partly suppressed the LPS/PAF-stimulated extracellular release of AA (30 ± 6% inhibition; Table II) while having no effect on the accumulation of cell-associated free AA (Table II). In P388D$_1$ macrophages,

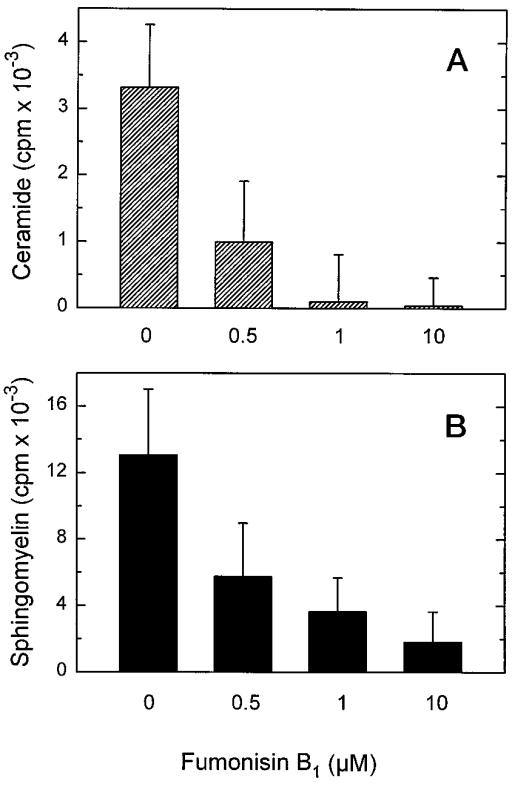

FIG. 3. **Dose response of the effect of fumonisin B$_1$ on ceramide (*A*) and sphingomyelin (*B*) production in activated P388D$_1$ cells.** For these experiments, [^{14}C]serine-labeled cells were used. The indicated concentrations of fumonisin B$_1$ were added to the cells 15 min before these cells were exposed to LPS. After the 1-h incubation with LPS, the cells were treated with 100 nM PAF for 3 min and the radioactivity in ceramide (*A*) and sphingomyelin (*B*) was determined. To highlight the effect of fumonisin B$_1$ on *stimulated* ceramide and sphingomyelin production, the radioactivity levels in unstimulated cells have been subtracted. These were 9,368 ± 1,362 for ceramide and 31,924 ± 1,375 for sphingomyelin. Fumonisin B$_1$ treatment did not significantly affect these basal values.

TABLE II

Effect of fumonisin B$_1$ on AA release

Fumonisin B$_1$ (1 μM) was added to the [^{3}H]AA-labeled cells 15 min before they were exposed to LPS. After the 1-h incubation with LPS, the cells were treated with 100 nM PAF for 10 min, and extracellular AA and cell-associated AA were determined as described under "Experimental Procedures." Control denotes the unstimulated cell response, *i.e.* that elicited in the absence of PAF.

	Extracellular AA	Cell-associated AA
	cpm	*cpm*
Control	17,492 ± 1,468	2,787 ± 544
Control plus fumonisin B$_1$	18,538 ± 2,008	2,212 ± 900
PAF	51,865 ± 1,534	4,488 ± 650
PAF plus fumonisin B$_1$	39,861 ± 4,370	5,175 ± 100

accumulation of cell-associated free AA is a hallmark of cPLA$_2$ activation, whereas extracellular AA release is a hallmark of sPLA$_2$ activation (3). These differential effects on AA release at two distinct locations suggest that fumonisin B$_1$ is affecting AA mobilization in P388D$_1$ macrophages by selectively blunting the sPLA$_2$.

The Role of the Sphingomyelin Biosynthetic Pathway in AA Mobilization in P388D$_1$ Cells—Collectively, the above results indicate that sphingomyelin biosynthesis is increased in LPS/PAF-treated P388D$_1$ cells and that this pathway provides stimulatory signals leading to sPLA$_2$-mediated AA mobilization. An important feature of the sphingolipid biosynthetic route is that the last enzyme of the pathway, sphingomyelin synthase,

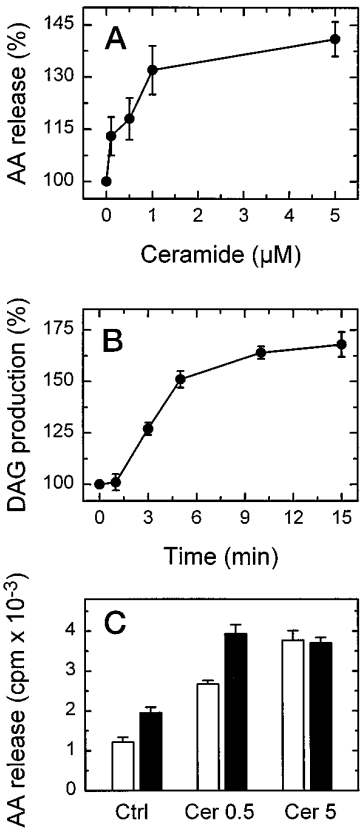

FIG. 4. **Exogenous ceramide and DAG stimulate AA mobilization in P388D$_1$ cells.** *A*, dose response of the effect of C_2-ceramide on AA release from [^{3}H]AA-labeled, LPS-treated cells (15-min incubation). These data are expressed as a percentage of the response observed in the absence of ceramide. The 100% value corresponds to 7655 ± 375 cpm. *B*, time course of the effect of 1 μM C_2-ceramide on DAG accumulation in [^{3}H]palmitate-labeled, LPS-treated cells. These data are expressed as a percentage of the radioactivity in DAG at time 0. The 100% value corresponds to 10,042 ± 360. The LPS-treated cells labeled with [^{3}H]AA were incubated with (■) or without (□) 5 μM DAG for 10 min (*C*). In addition, the incubations received 0.5 μM C_2-ceramide (*Cer 0.5*), 5 μM C_2-ceramide (*Cer 5*), or not either (*Ctrl*), as indicated.

serves not only to regulate ceramide and sphingomyelin levels but also to provide an alternative route for the synthesis of PC-derived DAG (23–25). Thus we sought to study the relationship between ceramide and DAG regarding AA release by using cell-permeable analogs of these lipids.

Fig. 4*A* shows that exogenous addition of C_2-ceramide to P388D$_1$ cells induced a concentration-dependent release of [^{3}H]AA to the extracellular medium. C_2-ceramide doses as low as 0.1 μM were sufficient to induce a significant response. C_2-ceramide also increased DAG levels in cells labeled with [^{3}H]palmitic acid (Fig. 4*B*). It is worth noting that the time course of ceramide-induced DAG closely parallels that of receptor-stimulated extracellular AA release, including a characteristic 1-min lag (*cf.* Fig. 4*B* and Fig. 2*A* in Ref. 12). Ceramide-induced DAG was not inhibited by D609 (1–25 μM).

When the cells were exposed to exogenous DAG (1,2-dioctanoyl-*sn*-glycerol, 5 μM) a small but significant release of AA to the extracellular medium was also detected, which suggests that DAG is also able to activate the sPLA$_2$ (Fig. 4*C*). When DAG was applied together with a submaximal dose of ceramide (0.5 μM), the effect observed was equal to the sum of the effects of the two compounds when added separately (Fig. 4*C*). Interestingly, DAG had no effect if added along with a maximal ceramide dose (5 μM). Collectively these data suggest that both C_2-ceramide and DAG stimulate the sPLA$_2$-mediated extracellular AA release by identical mechanisms. These results, together with the notion that ceramide increases DAG levels (Fig. 4*B*), suggest that perhaps at least part of the effect of ceramide on AA release is mediated by the DAG that ceramide itself generates via sphingomyelin synthase.

DISCUSSION

We herein demonstrate that engagement of PAF receptors in LPS-treated P388D$_1$ cells triggers the activation of a novel pathway for phospholipid metabolism that provides stimulatory signals for sPLA$_2$ activation and subsequent AA release. Thus, activation of the cells results in increased ceramide and sphingomyelin biosynthesis, which leads to parallel DAG production via the PC:ceramide exchange reaction catalyzed by sphingomyelin synthase. Activation of the *de novo* pathway for sphingolipid biosynthesis was first reported by Bose *et al.* (28) in P388 and U937 cells during daunorubicin-induced apoptosis. The regulatory checkpoint of this pathway was found to be the ceramide synthase, *i.e.* the enzyme situated one or two steps upstream of sphingomyelin synthase in the sphingolipid biosynthetic pathway, depending on whether ceramide arises from sphingosine or sphinganine. In these studies, the biologically active species generated was ceramide, as expected (28). However, the unanticipated finding in our study is that in addition to any effect of ceramide *per se*, the DAG generated by the sphingomyelin biosynthetic pathway also appears to play a role in AA mobilization. This is supported by the results presented in Fig. 4*C*, showing an additive response to a combination of suboptimal doses of ceramide and DAG in inducing extracellular AA release but a lack of effect of DAG if added along with a maximal ceramide dose. The finding that ceramide is rapidly converted to sphingomyelin by the cells (Table I) also supports this contention.

In addition to the one provided by sphingomyelin synthase, there are at least four other cellular pathways that can eventually lead to generation of DAG during cellular activation conditions. Thus, DAG activation of sPLA$_2$ could theoretically be mediated through multiple phospholipid metabolic pathways. These are (i) the hydrolysis of inositol lipids by phosphoinositide-specific phospholipase C, (ii) phospholipase C cleavage of other phospholipids, (iii) phospholipase D/phosphatidate phosphohydrolase, and (iv) the glycerolipid biosynthetic pathway. Of these, phospholipase C cleavage of phospholipids appears not to operate in LPS/PAF-stimulated P388D$_1$ cells, as manifested by the lack of effect of the specific inhibitor D609 (22) on the response. However, we have recently found that phospholipase D activation is unrelated to AA mobilization in LPS/PAF-stimulated P388D$_1$ macrophages, arguing against a role for the DAG generated through this pathway in sPLA$_2$ activation (20). Similarly, we have found that bromoenol lactone, a compound that blocks *de novo* DAG biosynthesis (21), is without effect on AA mobilization (3), arguing against a role for that pathway in sPLA$_2$ activation. Finally, we have detected that polyphosphoinositide-derived DAG production has ceased within the first 2 min of activation,[2] *i.e.* well before the sPLA$_2$ begins to act (3). This lack of a temporal relationship, along with the notion that AA release in LPS/PAF-activated P388D$_1$ macrophages is not modulated by protein kinase C (20), makes it unlikely that the DAG produced by phospholipase C cleavage of inositol lipids has a significant role in sPLA$_2$ activation. In addition, polyphosphoinositide-derived DAG, highly enriched in AA, does not contribute to overall AA release by serving itself as a substrate for the DAG lipase pathway, as judged from studies using the specific DAG lipase inhibitor RHC 80267.[2]

Collectively, the above data indicate that sphingomyelin

[2] J. Balsinde and E. A. Dennis, unpublished data.

synthesis constitutes the only DAG-generating system that is specifically linked to $sPLA_2$ activation. This observation should lend more importance to the molecular mechanism underlying $sPLA_2$ activation by products of the sphingomyelin biosynthetic pathway.

The phospholipids in biological membranes are distributed asymmetrically, *i.e.* choline phospholipids are located in the outer leaflet and amino phospholipids are located in the inner leaflet. Such a distribution may be responsible for the tight packing that prevents the $sPLA_2$ from penetrating the outer layer and hydrolyzing the phospholipids (11). However, the asymmetrical distribution of phospholipids in resting cells is lost in activated cells due to the increased appearance of amino phospholipids such as phosphatidylethanolamine and phosphatidylserine at the cell surface (29). Disruption of lipid asymmetry leads to reduced lipid packing, which in turn allows the $sPLA_2$ to attack its substrates at the outer leaflet (11). Consistent with this model, we have found that the bulk of AA released by the $sPLA_2$ on the outer surface of activated $P388D_1$ cells arises from phosphatidylethanolamine (3). It follows from these observations that any circumstance that promotes lipid packing rearrangements or reduction should increase $sPLA_2$ activity. DAG and possibly ceramide as well cause major perturbations in membrane bilayers (30) that increase PLA_2 activity *in vitro* (31–33) and *in vivo* (33–35). As a matter of fact, we have found that the stimulatory effect of DAG on extracellular AA release in $P388D_1$ cells (Fig. 4) lacks specificity, *i.e.* both 1,2-DAG and 1,3-DAG are capable of exerting the same effect, which indicates that the effect is not enzyme-mediated.[2]

Taken together, these results suggest a model for Group V $sPLA_2$ activation in stimulated $P388D_1$ cells whereby LPS/PAF induce rapid sphingomyelin synthesis, which leads to accumulation of ceramide and/or DAG at discrete sites on the membrane. These local accumulations destabilize the bilayer and render it susceptible to attack by the Group V $sPLA_2$. The current model predicts a close proximity between sphingomyelin synthase and the $sPLA_2$ at the cellular surface, which is consistent with the fact that sphingomyelin synthase has been found in the plasma membrane (36). Moreover, such a compartmentalization allows us to explain why no other DAG-generating mechanism is involved in $sPLA_2$ activation and attendant AA release.

From the data presented here and from previous studies, it is apparent that inflammatory activation of AA signaling involves activation of at least four enzymes that generate interacting lipid mediators. In the first place, a phosphoinositide-specific phospholipase C generates a Ca^{2+} transient (37) that is required for activation of the $cPLA_2$ in an intracellular compartment (13), probably the nuclear membrane and/or endoplasmic reticulum (38, 39). The $cPLA_2$ generates a burst of intracellular free AA that provides stimulatory signals for activation of the $sPLA_2$ at the cellular surface (3). $sPLA_2$ activation is also influenced by those products of the sphingomyelin biosynthetic pathway that accumulate in its vicinity, *i.e.* ceramide and/or the DAG produced by sphingomyelin synthase. Once adequately positioned, the $sPLA_2$ begins to hydrolyze phospholipids (particularly phosphatidylethanolamine (3)) and release AA to the extracellular medium. Part of this fatty acid will be recaptured by the cells (40, 41) and/or converted to prostaglandins (10).

Two important features of the above model should be stressed further. First, none of the bioactive products generated by the sphingomyelin biosynthetic pathway, *i.e.* ceramide and DAG, are required for $cPLA_2$ activation, as manifested by the lack of effect of fumonisin B_1 on $cPLA_2$-mediated intracellular AA release (Table II). Second, no source of DAG other than that provided by sphingomyelin synthase is utilized for AA signaling in LPS/PAF-stimulated $P388D_1$ macrophages. These two aspects highlight the exquisite compartmentalization mechanism that regulates AA mobilization in these cells. Mammalian cells contain two prostaglandin synthase isoforms (42, 43). It is likely that each of these is linked to different AA pools and hence to distinct PLA_2s (4–6). Thus, two PLA_2s, acting on different cellular AA pools at different locations and being regulated by separate but interacting mechanisms, confer on the system a great versatility to efficiently ensure both immediate and delayed generation of AA-derived mediators during the inflammatory challenge.

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