Brefeldin A Promotes Hydrolysis of Sphingomyelin*

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Corinne M. Linardic, Supriya Jayadev, and Yusuf A. Hannun†

From the Departments of Medicine and Cell Biology, Duke University Medical Center, Durham, North Carolina 27710

The hydrolysis of sphingomyelin (SM) is a key reaction in the "sphingomyelin cycle," which plays a role in the regulation of cell proliferation and differentiation (Okazaki, T., Bell, R. M., and Hannun, Y. A. (1989) J. Biol. Chem. 264, 19076–19080). SM is produced from endoplasmic reticulum-derived ceramide and is delivered to organelle membranes in a regulated manner, presumably through the same endomembrane trafficking system used for sorting and delivery of proteins. Since brefeldin A (BFA) interferes with this endomembrane trafficking system and thus alters normal membrane and organelle distribution, we investigated the effect of BFA on SM levels in HL-60 leukemia cells. BFA caused a dose-dependent decrease of 20–25% in cellular SM levels, with effects observed at concentrations of BFA as low as 0.10 μg/ml. BFA effects on SM levels were noted as early as 5 min and were maximal by 20 min, with no further SM hydrolysis observed up to 60 min following treatment with BFA, suggesting the presence of a fixed SM-sensitive pool. BFA did not cause SM hydrolysis at 16 °C, a temperature that inhibits the effects of BFA on endomembrane mixing. The very early effects and temperature dependence of BFA-induced SM hydrolysis suggest that the mechanism of hydrolysis may be closely related to endomembrane mixing. These studies are beginning to define important interrelationships between membrane trafficking and topology, SM metabolism, and cell regulation.

Within the past few years, sphingolipids have been recognized as bioactive molecules in cell regulation and signal transduction (1–3). One of the simplest sphingolipids, sphingomyelin (SM), has been implicated as a key lipid in the signal transduction events that occur during the differentiation of the human HL-60 myeloid leukemia cell line. In this "sphingomyelin cycle," various inducers of HL-60 differentiation, including 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃), tumor necrosis factor-α and γ-interferon, cause hydrolysis of cellular SM in HL-60 cells (4, 5). SM is hydrolyzed by the induced activation of a neutral sphingomyelinase, yielding ceramide (the lipid moiety) and choline-phosphosphate (the water-soluble head group) (4). In separate studies, cell-permeable analogs of ceramide have been shown to mimic the effects of 1,25(OH)₂D₃ and tumor necrosis factor-α on cell proliferation and differentiation (5, 6). Thus, SM turnover through the "SM cycle" is emerging as a key metabolic pathway involved in the regulation of cell proliferation and differentiation.

Because we are studying the role of SM in signal transduction, we are also interested in the cellular machinery that modulates SM synthesis and intracellular distribution. The synthesis, trafficking, and delivery of SM to intracellular membranes is not well understood. Current literature suggests that SM is synthesized in the Golgi apparatus from endoplasmic reticulum (ER)-derived ceramide (7–9). Based on the premise that proper trafficking and sorting of lipids must occur to maintain proper cell membrane lipid distribution, we investigated whether the fungal metabolite brefeldin A (BFA) would affect SM levels in the cell.

BFA was initially identified as an antiviral and cytotoxic agent (10); later studies showed that it inhibited intracellular protein transport and protein secretion (11). Within 30 s of BFA application, a 110-kDa Golgi-associated protein (known as β-COP) and other peripherally bound proteins dissociate from the Golgi membrane and are dispersed within the cytoplasm (12–14). Within 15 min, the Golgi apparatus collapses into the ER, as monitored by fluorescence studies (15). Recently it has been suggested that BFA affects not only trafficking from ER to Golgi but trafficking between many endomembranes (16–18).

Reflecting the increasing interest in BFA biology, a number of recent studies have examined the effects of BFA on cellular lipids. BFA causes decreased synthesis of some lipids, whereas it causes increased synthesis of other lipids. Specifically, BFA has been shown to uncouple biosynthesis of certain glycosphingolipids (19, 20), whereas it slightly enhances the biosynthesis but does not affect the trafficking and delivery of phosphatidylethanolamine (21).

In the present study, BFA was found to induce hydrolysis of up to 20–25% of total cellular SM. The effect was time-, dose-, and temperature-dependent, with onset as early as 5 min and maximal hydrolysis occurring at 5 μg/ml. The implications of these studies on the regulation and topology of SM hydrolysis and the role of SM in cell regulation are discussed.

EXPERIMENTAL PROCEDURES

Cell Culture—HL-60 cells were purchased from the ATCC (Rockville, MD) and grown in RPMI 1640 media supplemented with 10% fetal calf serum at 37 °C and 5% CO₂. All media and fetal calf serum were purchased from Gibco-BRL. These stock cells were maintained at cell densities between 2 × 10⁶ and 1 × 10⁶ cells/ml. For all experiments, cells were grown in serum-free RPMI 1640 media supplemented with ITS (5 μg/ml insulin, 5 μg/ml transferrin, and 5 ng/ml sodium selenite; Sigma). Passages 20–30 were used for the experiments described.

Metabolic Labeling of HL-60 Cells with [³H]Choline—HL-60 cells were washed in Dulbecco's phosphate-buffered saline and resuspended in serum-free RPMI 1640 with ITS at a density of 2 × 10⁶ cells/ml [³H]Choline (Du Pont-New England Nuclear) was added at
In order to characterize the time course of the effects of treatment, cells were pelleted, and cellular lipid was extracted with \[^{3}H\] choline for an additional 30 min (beyond the described 2 h) in water baths set at 37 or 16°C. Extraction was continued by the addition of 0.8 ml of water to form a Bligh-Dyer monophase, which was then separated into a biphase by the addition of 1 ml of chloroform:methanol (1:2, v/v). Extraction was continued by the addition of 0.8 ml of water to form a Bligh-Dyer monophase, which was then separated into a biphase by the addition of 1 ml of chloroform and 1 ml of water (22). The resulting upper (aqueous) phase was aspirated and discarded, whereas the lower (organic) phase containing the labeled lipids was aliquotted and dried down under N\(_2\) gas. The resulting lipid film was resuspended in 80 \(\mu\)l of chloroform; 20 \(\mu\)l was spotted onto a thin layer chromatography plate lane (Whatman), and 20 \(\mu\)l (in duplicate) was analyzed for phospholipid phosphate. The TLC plate was developed in chloroform:methanol:acetic acid:water (50:30:8.5, v/v), dried, and exposed to film. Spots identified as SM and PC by the fluorogram were scraped into scintillation fluid and counted (RackBeta, LKB Wallac, Turku, Finland). SM and PC counts/min were normalized by phospholipid phosphate measurements (23).

RESULTS AND DISCUSSION

BFA Promotes Hydrolysis of SM but Not PC—Since BFA is known to interfere with endomembrane trafficking and thus disrupt the organization of intracellular membranes and organelles, we wondered whether BFA would have any effect on cellular SM levels. HL-60 cells were metabolically labeled with \[^{3}H\] choline for 3 days in order to label SM and PC. \[^{3}H\] Choline-labeled cells were then washed and resuspended in fresh media and treated with 5 \(\mu\)g/ml BFA for 20 min. After treatment, cells were pelleted, and cellular lipid was extracted and separated on TLC. Quantitation of the radioactively labeled lipids showed that approximately 15% of cellular SM was hydrolyzed, whereas PC remained constant (Fig. 1). This observation prompted us to investigate more closely the effects of BFA on SM hydrolysis in HL-60 cells.

Time and Dose Dependence of BFA Effects on SM Hydrolysis—In order to characterize the time course of the effects of BFA on SM hydrolysis, cells were treated at time 0 with 5 \(\mu\)g/ml BFA, and aliquots were removed for lipid analysis at intervals up to 60 min. Decreases in SM were observed at the earliest time points examined (10 min) and were maximal at 20 min with hydrolysis of up to 25% of SM (Fig. 2). In further experiments, significant decreases in SM were apparent as early as 5 min (see Fig. 2), inset. SM levels did not recover to base line after BFA treatment but remained at approximately 80% of control. This is in contrast to the previously observed effects on SM levels induced by tumor necrosis factor-\(\alpha\) and 1,25(OH)\(_{2}\)D\(_{3}\), where SM levels recover to base line after a few hours despite the continued presence of these inducers (4, 5). Therefore, BFA causes very early hydrolysis of SM that appears to persist in the continued presence of BFA.

Previous investigators have shown that the concentration of BFA necessary to inhibit trafficking and protein secretion varies with the cell system being employed. Therefore, we investigated the effect of BFA over a fairly wide range of concentrations, from 0.10 to 7 \(\mu\)g/ml. Dose-dependent hydrolysis was observed from 0.10 to 3 \(\mu\)g/ml (Fig. 3). BFA at 3 \(\mu\)g/ml produced a maximal response of 27% hydrolysis. Concentrations above 3 \(\mu\)g/ml caused minimal further decreases in SM levels.

Effect of Temperature on BFA-promoted SM Hydrolysis—In order to investigate the mechanism of BFA-induced SM hydrolysis, we tested the ability of BFA to cause hydrolysis at lowered temperatures. It has been shown previously that temperatures less than 20°C inhibit the organelle mixing caused by BFA (24). Therefore, we hypothesized that if BFA-induced SM hydrolysis was also inhibited by reduced temperatures, the mechanism of hydrolysis might involve BFA-induced membrane redistribution. At 16°C, BFA failed to induce SM hydrolysis (Table I), suggesting that SM hydrolysis may indeed be related to membrane redistribution and organelle mixing.

We have observed a specific and early effect of BFA on SM levels in HL-60 cells. Resting cells treated with BFA lose up to 20–25% of total cellular SM in a time-, dose-, and temperature-dependent fashion. SM levels do not recover to base
that during its characteristic redistribution of membranes and SM in contact with a sphingomyelinase. In this case, the experimental Procedures." Cells were aliquotted into individual flasks or ethanol vehicle for 30 min. Cells were harvested, and the lipids were quantitated. These results are averages of duplicates and are expressed as percent of control.

| Temperature | Remaining sphingomyelin |
|-------------|-------------------------|
| °C          | Vehicle     | BFA        |
| 37          | 100.0 ± 1.9 | 88.0 ± 3.9 |
| 15          | 100.0 ± 1.0 | 101.6 ± 0.0 |

Effect of temperature on BFA-induced sphingomyelin hydrolysis

The effect of BFA on lipid hydrolysis is specific to SM and does not affect PC in the conditions outlined.

There are three possible explanations for the decrease in SM caused by BFA. First, BFA may stimulate a sphingomyelinase and in this way cause SM hydrolysis and loss. Preliminary studies from our laboratory suggest that BFA is unable to directly activate a neutral sphingomyelinase from HL-60 cells in an in vitro assay. It is possible, however, that BFA may cause indirect activation of a cellular sphingomyelinase in vivo. The second explanation is that BFA interferes with normal SM recycling by preferentially inhibiting the resynthesis of SM while normal SM breakdown continues, yielding a net loss of SM. Resynthesis of SM might be disrupted because of the block in trafficking of needed ceramide from the ER to Golgi. However, the components necessary for SM resynthesis (ceramide, ceramide-phosphate donor, and SM synthetase enzymes) are still present, albeit in a mixed ER-Golgi "organelle." Thus, one would expect instead an increase in SM levels, not a decrease. Indeed, this has been observed in a recent study by Brünning et al. (25), where BFA at a concentration of 5 μg/ml causes increased synthesis of SM from a radioactively labeled truncated ceramide. On the other hand, van Echten et al. (20) have shown that BFA inhibits resynthesis of SM and glycosphingolipids in murine cerebellar cells. The reasons for these discrepancies are unknown but may be a result of different methodological approaches or due to intrinsic differences in cell lines. The third explanation is that during its characteristic redistribution of membranes and organelles, BFA may act to bring previously sequestered pools of SM in contact with a sphingomyelinase. In this case, the intrinsic activity of sphingomyelinase(s) need not change. We favor this third possibility, as it is supported by previously published morphological evidence that demonstrates significant mixing of organellar membranes after BFA treatment (15). The experiment investigating the effect of temperature on BFA-induced hydrolysis also suggests that organellar mixing may be involved in the observed SM hydrolysis, since inhibiting organellar mixing, SM hydrolysis was also inhibited.

The significance of BFA-induced hydrolysis in HL-60 leukemic cells is 2-fold. First, we are interested in the possibility that BFA may cause the hydrolysis of a specific pool of SM and therefore define a "BFA-sensitive" pool. The identity of this pool is unknown but will be the object of further study in our laboratory. Second, a most intriguing question that is generated from these studies is the relationship of BFA-induced SM hydrolysis to the SM cycle. Although the effects of BFA on cell biology are rooted mostly in its effects on protein secretion, it is possible that BFA may act in the HL-60 system to activate the "SM cycle" and all of the downstream events turned on by the control. Based on the proposed role of the SM cycle in differentiation, we have predicted that BFA may have the capacity to cause differentiation of HL-60 cells if it hydrolyzes the same SM pool that is hydrolyzed in response to inducers of HL-60 differentiation (i.e., 1,25(OH)2D3, tumor necrosis factor-α, and γ-interferon). Ongoing experiments investigating the identity of the BFA-sensitive pool and the ability of BFA to induce differentiation of HL-60 cells support this hypothesis. Thus, these studies are beginning to define important interrelationships between membrane trafficking and topology, SM metabolism, and cell regulation.

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