The role of de novo ceramide synthesis in the mechanism of action of the tricyclic xanthate D609

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Abstract  The cytotoxic effects of several chemotherapeutic drugs have been linked to elevated de novo ceramide biosynthesis. However, the relationship between the intracellular site(s) of ceramide accumulation and cytotoxicity is poorly understood. Here we examined the relationship between the site of ceramide deposition and inhibition of protein translation and induction of apoptosis by the anti-tumor/antiviral xanthate, D609. In Chinese hamster ovary (CHO)-K1, HEK-293, and NIH-3T3 cells, D609 caused rapid (1–5 min) and sustained eukaryotic initiation factor 2α (eIF2α) phosphorylation followed by apoptosis after 24 h. Concurrently, D609 stimulated de novo ceramide synthesis and increased ceramide mass 2-fold by 2 h in CHO-K1 cells. In D609-treated CHO-K1 cells, sphingomyelin synthesis was stimulated by brefeldin A, and C2-DMB-ceramide transport to the Golgi apparatus was blocked, indicating ceramide accumulation in the endoplasmic reticulum (ER). However, D609-mediated eIF2α phosphorylation, inhibition of protein synthesis, and apoptosis in CHO-K1 cells were not attenuated by fumonisin B1 or l-cycloserine. Interestingly, short-chain ceramide promoted eIF2α phosphorylation and inhibited protein synthesis in CHO-K1 cells, indicating that the effectiveness of endogenous ceramide could be limited by access to signaling pathways. Thus, expansion of the ER ceramide pool by D609 was not implicated in early (eIF2α phosphorylation) or late (apoptotic) cytotoxic events.—Perry, R. J., and N. D. Ridgway. The role of de novo ceramide synthesis in the mechanism of action of the tricyclic xanthate D609, J. Lipid Res. 2004. 45: 164–173.

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Ceramide is recognized as a key mediator of cell stress and apoptosis. Numerous studies have demonstrated that generation of ceramide, as either an early or a late response to various stimuli, is involved in subsequent downstream events that control cell survival [reviewed in refs. (1, 2)]. An emerging theme suggests that ceramide acts as an index or coordinator of cellular stress responses whereby elevation of ceramide beyond a certain threshold regulates protein phosphatases (3, 4) or kinases (5–7) involved in mitogenesis, apoptosis, or senescence.

By virtue of its central position in sphingolipid metabolism, ceramide is regulated by multiple synthetic and catabolic processes. Initially, ceramide generation in response to cytokine-activated neutral sphingomyelinases (SMases) (8–10) was identified as an important feature of signaling cascades. More recently, genotoxic drugs and environmental stress have been shown to increase ceramide by de novo biosynthesis (11). A variety of apoptotic and cytotoxic agents, such as etoposide (11), SDZ PSC 833 (12), daunorubicin (13), phorbol ester (14), and CPT-11 (15) have been shown to stimulate de novo ceramide synthesis by increasing serine palmitoyltransferase (SPT) or ceramide synthase activity. In etoposide-treated cells, ceramide accumulation occurred primarily by post-translational activation of SPT (11). Blockage of ceramide accumulation by fumonisin B1 treatment did not affect etoposide-mediated apoptosis, but appeared to be involved in a pathway that affected membrane permeability. Daunorubicin also elevated de novo ceramide synthesis and mass in leukemic cells, but in this case, by increasing the Vmax for ceramide synthase by 70% (13). Fumonisin B1 completely or partially inhibited DNA laddering and nuclear fragmentation in two leukemic cell lines treated with daunorubicin. Another study confirmed that daunorubicin elevated de novo ceramide synthesis, but concluded that ceramide was not involved in the activation of nuclear factor (NF)-κB by this drug (16). Thus, induction of cell stress and apoptosis by these chemotherapeutic agents involves activation of the biosynthetic pathway and

Abbreviations: BFA, brefeldin A; CHO, Chinese hamster ovary; DAG, diacylglycerol; eIF2α, eukaryotic initiation factor 2α; ER, endoplasmic reticulum; OSBP, oxysterol binding protein; PARP, poly(ADP-ribose) polymerase; PLC, phospholipase C; PKR, double-stranded RNA-dependent protein kinase; PtdCho, phosphatidylcholine; RAX, PKR activator X; SM, sphingomyelin; SPT, serine palmitoyltransferase.

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elevation of endogenous ceramide levels, which then engage downstream signaling targets.

A question that remains unanswered is the intracellular localization of the ceramide that is synthesized in response to these drugs. The active sites for the enzymes of ceramide synthesis are localized to the cytoplasmic surface of the endoplasmic reticulum (ER) (17). Ceramide is then transported to the Golgi apparatus by an ATP-dependent mechanism, where it is converted to sphingomyelin (SM) by SM synthase (18). In most cases, induction of ceramide synthesis is not accompanied by proportionate increases in SM or glucosylceramide synthesis (12, 19). This suggests that ceramide synthesis either exceeds its conversion to complex sphingolipids by subsequent steps in the pathway and accumulates in the ER, or is transported to sites where sphingolipid biosynthetic enzymes do not reside. This has important ramifications for ceramide-mediated signaling, as it appears that generation of ceramide in various organelles by targeted expression of bacterial SMase has differential effects on apoptosis (20).

D609 (tricyclodecan-9-yl xanthate) was recently shown to inhibit SM synthase, suggesting that it could be a useful agent to elevate ceramide levels and monitor subsequent effects on signaling pathways (21). D609 was originally identified as an antiviral/antitumor agent and inhibitor of bacterial phosphatidylcholine (PtdCho)-specific phospholipase C (PLC) (22). This observation has led to the widespread use of the drug to inhibit mammalian PtdCho-specific PLC, even though a mammalian form of this enzyme has not been conclusively identified or shown to be specifically inhibited by D609. D609 has been used extensively in cultured cell models to counteract the effects of other drugs or stimuli proposed to act via PtdCho-PLC (23–25), but in few instances have the primary targets of D609 been investigated. In this regard, it was recognized that xanthates are strong antioxidants (26, 27) and zinc chelators (28). Recently, a zinc-independent PtdCho-PLC was isolated from Pseudomonas aeruginosa that was insensitive to D609 (29), suggesting that the activity of D609 could be related to its chelation properties. Still others have suggested that D609 could have other primary targets involved in lipid signaling (21, 30, 31). Thus D609 may have mechanism(s) of action other than PtdCho-PLC inhibition that account for its antiviral/antitumor activity.

Here we report that D609 stimulates ceramide synthesis and induces a rapid phosphorylation of eukaryotic initiation factor 2α (eIF2α) within minutes, followed by apoptosis after 24 h. However, elevated endogenous ceramide synthesis and ceramide mass accumulation in the ER was not involved in eIF2α phosphorylation, inhibition of protein synthesis, or apoptosis. We conclude that the cytotoxic/apoptotic effects of D609 could be linked to stress-induced phosphorylation of eIF2α and inhibition of protein synthesis, while effects on sphingolipid metabolism are a more generalized response to drug-induced cell stress. This also demonstrates that the intracellular site of ceramide accumulation is an important determinant of its biological activity.

MATERIALS AND METHODS

Materials

The potassium salt of D609, C2-ceramide, and C2-dihydroceramide were purchased from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA). N-[5-(5,7-dimethyl boron dipropionethene difluoride)-1-pentanoyl]-α-erythrophosphinosine was from Molecular Probes, Inc. (Eugene, OR). Fumonisin B1 and 1-cycloserine were from Sigma Aldrich. Brefeldin A (BFA) and recombinant Escherichia coli sm PAL2-diaclyglycerol (DAG) kinase and z-VAD-fmk were purchased from Calbiochem (La Jolla, CA). Chelerythrine was purchased from LC Laboratories. [3H]serine and [γ-32P]ATP were from NEN/Mandel Life Science. 1-β[35S]methionine was from Amersham Biosciences. Tissue culture reagents and media were from Gibco-BRL. Silica gel 60 TLC plates were from BDH.

Cell culture

Chinese hamster ovary (CHO)-KI, HEK-293, and NIH-3T3 cells were grown in monolayers at 37°C in 5% CO2. CHO-KI and HEK-293 cells were cultured in DMEM containing 5% (v/v) FCS and 35 μg protein/ml (medium A). NIH-3T3 cells were cultured in DMEM containing 10% (v/v) FBS. Cells were subcultured in 60 mm dishes in 3 ml of medium. Cells received fresh medium on day 3, and experiments were started 18–24 h later. Stock solutions of reagents were prepared in the following manner: D609 (25 mg/ml stock or 93 mM) was freshly prepared in water and added directly to dishes at the indicated final concentrations; BFA (1 mg/ml stock) was dissolved in ethanol and added directly to dishes for a final concentration of 1 μg/ml; fumonisin B1 (10 mM stock) was dissolved in phosphate-buffered saline (PBS) and added directly to dishes at a final concentration of 50 μM or 100 μM; and 1-cycloserine (500 mM) was dissolved in water and added directly to dishes.

Analysis of labeled sphingolipids and phospholipids

To assess the effect of D609 on de novo sphingolipid and phospholipid synthesis, cells were preincubated for 4 h in serine-free medium A prior to pulse labeling with [3H]serine (5 μCi/ml) in the same medium. After incubation with [3H]serine and various pharmacological agents (refer to figure legends for times and concentrations), cells were rinsed twice with cold PBS and harvested in methanol-water (5:4; v/v). [3H]serine incorporation into sphingolipids and phospholipids was quantified after extraction of total lipid with chloroform-methanol (1:2;v/v) (32). [3H]serine incorporation into phospholipids was determined by TLC of total lipid extracts on silica gel 60 plates in a solvent system of chloroform-methanol-acetic acid-water (60:40:4:1; v/v/v/v). To measure [3H]serine incorporation into SM, ceramide, glucosylceramide, and sphinganine, total lipid extracts were subjected to hydrolysis in 0.1 N potassium hydroxide for 1 h at 37°C. Sphingolipids were extracted with chloroform-methanol (2:1;v/v) and separated by TLC in a solvent system of chloroform-methanol-acetic acid-water (60:40:4:1; v/v/v/v). To measure [3H]serine incorporation into phospholipids and sphingolipids were identified by fluorography and/or comigration with an authentic standard, scraped into vials, and quantified by scintillation counting

Quantification of cellular DAG and ceramide mass

DAG and ceramide mass from total lipid extracts were measured by the DAG kinase assay (34) and expressed relative to total cell protein (35).

Enzyme assays

Following D609 or mock treatments, cells were rinsed twice with cold PBS, scraped in the same buffer, and collected by cen-
trifugation at 2,000 g for 5 min. The cell pellets were homoge-
nized in 20 mM Tris-HCl (pH 7.7) and 10 mM EDTA (buffer A) by 10 passages through a 23-gauge needle, and total membrane
fraction was prepared by centrifugation of the homogenate at 400,000 g for 15 min at 4°C. The membrane fraction (pellet) was
resuspended in buffer A to a final concentration of ~1 mg/ml. SM
synthase and SPT activities were then measured as previously
described (36, 37).

C2-DMB-ceramide transport
The fluorescent ceramide analog C2-DMB-ceramide was used to
assess ceramide transport from the ER to the Golgi apparatus
by a modified protocol (38). Briefly, CHO-K1 cells were cultured
in medium A on glass coverslips. Fresh medium A was added to
coated glass slides and experiments were started 18–24 h later. Fol-

owing treatments (see legend of Fig. 7 for specific details), cells
were labeled with 1.25 mM C2-DMB-ceramide complexed with
1.25 mM fatty acid-free BSA in DMEM for 30 min at 4°C. Cells
were washed three times with DMEM at 4°C, followed by incubation
with DMEM containing 0.34 mg/ml fatty acid-free BSA for
0–30 min at 37°C. The experiment was terminated by rinsing
cells twice with cold PBS and fixing the cells in 3% (w/v) formal-
dehyde for 15 min. Finally, cells were washed twice with 5 mM
ammonium chloride, rinsed with distilled water, and mounted
on slides in 50 mM Tris-HCl (pH 9.0) containing 2.5% (w/v) 1,4-
diazabicyclo[2.2.2]octane and 90% glycerol. Cell images were
acquired using a Zeiss Axiovert 200 fluorescence microscope
equipped with an Axiocam digital camera and imported into Photoshop 5.0.

Immunoblotting and antibodies
Total and serine 51-phosphorylated eIF2α were detected by
immunoblotting with monoclonal antibodies (New England Bio-
labs). Endogenous oxysterol binding protein (OSBP) was de-
tected with a pan-OSBP antibody (39). Full-length and caspase-
proteolyzed poly(ADP-ribose) polymerase (PARP) were detected
by immunoblotting using a polyclonal antibody (Santa Cruz Bio-
technology, Inc.). For PARP detection, cells were extracted in 10
mM sodium phosphate (pH 7.4), 500 mM NaCl, 2 mM EDTA, 1
mM DTT, 100 μM PMSF, 1% (w/v) Triton X-100, and 1X pro-
tease cocktail (Pierce Biotecology), and a soluble fraction pre-
pared by centrifugation at 10,000 g for 10 min. Following trans-
fer of proteins from SDS-polyacrylamide gels to nitrocellulose,
filters were incubated with primary antibodies for 1 h in Tris (pH
7.4), 150 mM NaCl, 0.1% (v/v) Tween 20, and 5% (w/v) skim
milk powder. For total and phospho-eIF2α detection, filters were
incubated with primary antibodies in Tris-HCl (pH 7.4), 150 mM
NaCl, 0.04% (w/v) Tween 20, and 5% (w/v) BSA for 12 h at 4°C.
Filters were incubated with secondary goat anti-mouse or goat
anti-rabbit secondary antibodies coupled to horseradish peroxi-
dase, and proteins were detected by the chemiluminescence
method (Amersham Biosciences).

RESULTS

D609 stimulates phosphorylation of eIF2α
Phosphorylation of eIF2α on serine 51 results in inhibition
ofprotein translation initiation via its sequestration of the guanine
nucleotide exchange factor eIF2B (40). Phosphorylation of eIF2α can be catalyzed by four kinases in response to a variety of stress signals including viral in-
fection, heat shock, serum withdrawal, heme deficiency, oxidative stress, and cytokines [as reviewed in ref. (41)].

Because D609 was reported to have antiviral and anti-
tumor activity, we tested whether the activity of this drug
could be mediated via phosphorylation of eIF2α and sub-
sequent suppression of protein translation. Preliminary
dose-response studies indicated that 375 μM D609 (100
μg/ml) had maximal effects on eIF2α phosphorylation
and sphingolipid metabolism (see later figures) and as
such, this concentrations was used in all experiments.
Three cell lines (CHO-K1, HEK-293, and NIH-3T3) were

Fig. 1. D609 stimulates rapid phosphorylation of eukaryotic initia-
tion factor 2α (eIF2α) in Chinese hamster ovary (CHO)-K1, HEK
293, and NIH 3T3 cells. A: Cells were treated with 375 μM D609 for
the indicated times, cell extracts were prepared, and proteins (10
μg) were separated on SDS-8% PAGE and immunoblotted for
phospho- or total eIF2α as described in Materials and Methods. As
a positive control, cells were treated with thapsigargin (20 μM) for
2 h. B: CHO-K1 cells were treated with 375 μM D609 (open circle)
or solvent control (closed circle) for up to 2 h and pulse labeled
with [35S]methionine for the last 30 min of each treatment time.
Total cell lysates were prepared and analyzed for [35S]methionine
incorporation into TCA-precipitated protein. Results are the mean
and SEM for three independent experiments. C: Treatments were
as described in A. Immunoblots were probed with an oxysterol
binding protein (OSBP)-specific antibody to detect the hyper- and
hypophosphorylated forms of OSBP.

166 Journal of Lipid Research Volume 45, 2004
regulating on the cell type, reached a maximum between 5 min and 30 min. The extent of eIF2α phosphorylation in response to thapsigargin, an inhibitor of calcium reuptake into ER stores and a known activator of eIF2α phosphorylation (42), after 2 h was similar to D609 treatment at 1–2 h. Stimulation of eIF2α phosphorylation by D609 was accompanied by a 50% decrease in protein synthesis at 30 min that was sustained for 2 h (Fig. 1B). To assess whether D609 induced the phosphorylation of other proteins in addition to eIF2α, we examined OSBP phosphorylation (Fig. 1C). Phosphorylation of serines 381, 384, and 387 in OSBP results in an apparent increase in molecular mass by SDS-PAGE, and is regulated by the cholesterol and sphingolipid content of the cell (39, 43) and by oxidative stress (Perry and Ridgway, unpublished observations). D609 increased phosphorylation of OSBP, albeit slightly delayed relative to eIF2α, as indicated by a shift to the higher molecular mass form (44). Thapsigargin treatment for 2 h did not affect OSBP phosphorylation. Thus an early event following D609 treatment is phosphorylation of eIF2α and inhibition of protein synthesis.

Regulation of ceramide synthesis by D609

A recent report showed that eIF2α phosphorylation was stimulated by short-chain ceramide via phosphorylation of PKR activator X (RAX), an activator of the double-stranded RNA-dependent protein kinase (PKR) (4). Because exogenous ceramides can stimulate stress kinases (5, 7, 38), it was proposed that this pathway could be responsible for activation of RAX/PKR, phosphorylation of eIF2α, and inhibition of translation. This, coupled with reports that various chemotherapeutic drugs activate ceramide synthesis, suggested that D609 could stimulate eIF2α phosphorylation by increasing endogenous ceramide levels. Initial studies in CHO-K1 cells confirmed that D609 (180–375 μM) caused a partial delay in SM resynthesis after bacterial SMase treatment (results not shown), consistent with inhibition of SM synthase activity involved in a salvage pathway for SM resynthesis (21). However, the effects of D609 on de novo sphingolipid synthesis have not been reported, raising the possibility that activation of ceramide synthesis or inhibition of SM synthase could contribute to the observed changes in sphingolipid metabolism in response to this drug. To test this possibility, ceramide and sphingolipid synthesis was measured in CHO-K1 cells treated with D609 for up to 2 h by pulse-labeling with [3H]serine (Fig. 2A). D609 caused a rapid enhancement of [3H]serine incorporation into ceramide that reached a maximum of 10-fold by 1 h. Interestingly, D609 increased [3H]serine incorporation into SM by 2- to 3-fold. D609 also caused a dramatic inhibition of phosphatidylserine synthesis but did not affect [3H]serine incorporation into phosphatidylethanolamine. The effect of D609 on SM and ceramide metabolism appeared to be a common response in cultured cells, because a similar increase in [3H]serine incorporation into SM and ceramide was observed in HEK-293 and, to a lesser extent, NIH-3T3 cells (Fig. 2B).

To determine whether increased de novo ceramide synthesis resulted in increased ceramide mass, CHO-K1 cells were treated with D609 for up to 2 h and ceramide and DAG mass in lipid extracts was analyzed by the DAG kinase method (Fig. 3A). D609 caused a significant increase in ceramide mass by 1 h and a continued increase to 2-fold by 2 h. The increase in ceramide mass in response to D609 was completely blocked by fumonisin B1, an inhibitor of ceramide synthase, confirming that it was derived solely from de novo synthesis. D609 also significantly decreased DAG levels to a maximum of 50% by 30 min. The decrease in DAG mass was not affected by fumonisin B1, suggesting it occurs independently of D609 effects on sphingolipid metabolism.

To establish which enzyme(s) in ceramide biosynthesis were affected by D609, CHO-K1 cells were treated with D609 or D609 plus fumonisin B1, and [3H]serine incorpora-
ration into ceramide and sphinganine was measured (Fig. 4). As expected, fumonisin B1 inhibited ceramide synthesis and elevated \[^{3}H\]sphinganine production, consistent with inhibition of ceramide synthase. Pretreatment of cells with fumonisin B1 completely blocked the increase in \[^{3}H\]serine incorporation into ceramide caused by D609. Moreover, the decrease in ceramide synthesis following D609 and fumonisin B1 treatment was accompanied by a reciprocal 10-fold increase in \[^{3}H\]sphinganine levels, suggesting that biosynthetic steps prior to ceramide synthase were stimulated by D609.

The activity of SPT, which catalyzes the initial step in sphingolipid synthesis, is stimulated by genotoxic drugs by unknown mechanisms (11). To determine whether D609 had a similar effect, membranes isolated from CHO-K1 cells treated with D609 were assayed for SPT activity. SPT activity in membranes from CHO-K1 cells treated with D609 was increased by 25–30% between 30 min and 120 min, but was only significant at the 30 min time point. (Fig. 5A). SPT activity returned to control values by 4 h. Addition of D609 (375 μM) to untreated CHO-K1 cell membranes did not affect SPT activity, thus ruling out a direct effect of the drug (result not shown). The in vitro activity of SM synthase in membranes from D609-treated CHO-K1 cells was unchanged over the first 2 h and was slightly inhibited by 4 h (Fig. 5B). We confirmed the results of Luberto et al. (21) that D609 (375 μM) inhibited SM synthase activity by >80% when added directly to the in vitro assay (results not shown). Collectively, the results in Figs. 4 and 5 show that D609 stimulates sphingolipid synthesis and ceramide mass accumulation primarily via increased SPT activity.

**ER deposition of ceramide in D609-treated cells**

The biological activity of endogenous ceramide is probably dependent on transport to other locations in the cell where it can affect various signaling processes or be further metabolized. It is presently unknown whether ceramide accumulation following stimulation of de novo synthesis is retained in the ER or transported to other sites. If ceramide accumulation in D609-treated cells was restricted to the ER, then BFA could be used to merge the ER and Golgi compartments and make ER-associated ceramide accessible to SM synthase (45). Consistent with previous reports in CHO-K1 cells (46, 47), BFA increased SM synthesis but had no effect on \[^{3}H\]serine incorporation into sphinganine (Spa) and ceramide (Cer) was measured as described in Materials and Methods. The results are mean and SEM for triplicate determinations from a representative experiment that was repeated twice with similar results.
tively minor compared with the reciprocal changes in labeled ceramide and SM. Addition of D609 or BFA also promoted a 3-fold increase in glucosylceramide synthesis, but the effect of the two agents was not additive.

The accumulation of ceramide in the ER of D609-treated cells (Fig. 6) and the relatively poor conversion to SM (Figs. 1, 6) implies that ceramide export from the ER is inhibited or saturated. To distinguish between these two possibilities, the fluorescent ceramide analog C5-DMB-ceramide was used as a marker of ceramide transport to the Golgi apparatus (48). CHO-K1 cells were treated with or without D609 for 2 h prior to loading with C5-DMB-ceramide for 30 min at 4°C. Subsequent transport of the fluorescent analog to the Golgi apparatus was then monitored after incubation at 37°C for 30 min. (Fig. 7). In control cells, C5-DMB-ceramide exited the ER network and appeared in compact perinuclear regions corresponding to the Golgi apparatus after 30 min at 37°C. In D609-treated cells, C5-DMB-ceramide was initially found in a diffuse perinuclear region and reticular network. There was no evidence of compact Golgi-perinuclear staining after 30 min at 37°C, suggesting that transport to the Golgi apparatus was inhibited. Finally, to determine whether inhibition of C5-DMB-ceramide export was the result of dilution in a large ER pool of endogenous ceramide in D609-treated cells, CHO-K1 cells were pretreated with fumonisin B1 to prevent endogenous ceramide accumulation. However, this explanation seems unlikely, because the C5-DMB-ceramide staining pattern in D609/fumonisin B1-treated cells was similar to that of D609-treated cells at 0 min and 30 min. D609 and fumonisin B1 had a minimal effect on the structure of the Golgi apparatus, as determined by immunofluorescence microscopy (Fig. 5).
munostaining for giantin (Fig. 7, right panels). Giantin localization was similar to that of C_2-DMB-ceramide in control cells at 30 min, but not to the diffuse perinuclear staining observed in D609- or D609/fumonisin B1-treated cells at either 0 min or 30 min.

**Elevated endogenous ceramide is not linked to D609-mediated phosphorylation of eIF2α or apoptosis**

Having established that D609 elevates endogenous ceramide levels in the ER primarily by stimulation of de novo synthesis, we next assessed whether this was linked to the phosphorylation of eIF2α shown in Fig. 1. In these experiments, eIF2α phosphorylation on serine 51 was measured in D609-treated CHO-K1 cells that were pretreated with fumonisin B1 or the SPT inhibitor l-cycloserine in order to block the increase in ceramide and sphinganine (Fig. 8A). As expected, D609 treatment increased phosphorylation of eIF2α after 30 min. However, neither l-cycloserine nor fumonisin B1 had any effect on D609 activation of eIF2α phosphorylation. CHO-K1 cells also displayed a significant increase in eIF2α phosphorylation when exposed to exogenous C_2-Ceramide, but not the dihydro analog, indicating that signaling pathways that respond to exogenous ceramide were intact in these cells. Changes in phosphorylation status of eIF2α shown in Fig. 8A were correlated with protein translation as measured by [³⁵S]methionine incorporation (Fig. 8A). Fumonisin or l-cycloserine did not block the inhibition of protein translation by D609; and C_2-ceramide, but not the dihydro analog, inhibited translation. The exception was bacterial SMase treatment, which did not affect eIF2α phosphorylation (Fig. 8A) but partially inhibited protein synthesis (Fig. 8B).

Elevation of ceramide levels by increased de novo synthesis or SM hydrolysis is often associated with or required for apoptosis. Thus it was feasible that ceramide production in response to D609, while not affecting protein translation, could trigger apoptosis. Detection of caspase proteolysis of 116 kDa PARP into the characteristic 85 kDa fragment revealed that D609 treatment for 24 h caused limited induction of apoptosis in the three cell lines compared with a 2 h treatment with the protein kinase inhibitor chelerytherine (Fig. 9). Treatment of cells with fumonisin B1 or l-cycloserine did not prevent PARP cleavage in D609-treated CHO-K1 or NIH 3T3 cells. In HEK 293 cells, the small amount of PARP cleavage induced by D609 was inhibited by fumonisin B1 but not by l-cycloserine. Cleavage of PARP in D609- and chelerytherine-treated cells was blocked by the broad-spectrum caspase inhibitor z-VAD-fmk. Thus, caspase activation and apoptosis in response to D609 is not dependent on de novo ceramide generation.

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**Fig. 8.** Increased endogenous ceramide synthesis in D609-treated cells is not required for eIF2α phosphorylation. A: CHO-K1 cells were treated with 375 μM D609, D609 (375 μM) plus fumonisin B1 (100 μM), D609 plus l-cycloserine (2 mM), C_2-ceramide (100 μM), C_2-dihydroceramide (100 μM), bacterial sphingomyelinase (SMase) (50 mU), thapsigargin (20 μM), or appropriate solvent controls for 2 h. Cell extracts were prepared and total or serine 51-phosphorylated eIF2α was quantitated by immunoblotting, followed by densitometry. The ratio of phospho-to-total eIF2α was quantitated by immunoblotting, followed by densitometry. The ratio of phospho-to-total eIF2α was expressed as fold-increase relative to the water control. B: Cells were treated as described in A for 1 h. Protein synthesis (expressed relative to water-treated control) was determined by pulse labeling with [³⁵S]methionine for the final 30 min. Results are the mean and SEM for three independent experiments. FB1, fumonisin B1; l-CS, l-cycloserine; C_2-Cer, C_2-ceramide; C_2-dihydro, C_2-dihydroceramide; bSMase, bacterial SMase; thaps, thapsigargin.

**Fig. 9.** Endogenous ceramide synthesis is not linked to D609-induced caspase cleavage of poly(ADP-ribose) polymerase (PARP). CHO-K1 cells were treated with D609 (375 μM), fumonisin B1 (50 μM), l-cycloserine (2 mM), D609 plus fumonisin B1, D609 plus l-cycloserine, z-VAD-fmk (20 μM), D609 plus z-VAD-fmk, or no addition for 24 h. As a control for PARP cleavage, cells were treated with chelerytherine (20 μM), or chelerytherine plus z-VAD-fmk for 2 h. Cell extracts were prepared and immunoblotted for PARP using a polyclonal antibody as described in Materials and Methods.
DISCUSSION

We have investigated the relationship between activation of ceramide synthesis in the ER and its subsequent transport to the Golgi apparatus, and phosphorylation of eIF2α and inhibition of protein translation by the xanthate D609. Results show that although D609 strongly stimulated flux through the sphingolipid biosynthetic pathway, leading to elevated ceramide mass, this was not required for stimulation of eIF2α phosphorylation and apoptosis. Indeed, ceramide mass accumulation in the presence of D609 was restricted primarily to the ER, and D609 elicited a ceramide-independent stress response that resulted in rapid inhibition of translation and later induction of apoptosis. Inhibition of protein synthesis and induction of apoptosis could be related to the reported antiviral and antitumor activity of D609.

D609 was originally identified as a putative inhibitor of mammalian PtdCho-specific PLC based on inhibition of the Bacillus cereus enzyme. Although widely used as a pharmacological inhibitor of this enzyme, mammalian PtdCho-PLC has not been isolated or shown to be specifically inhibited by D609, except indirectly by changes in cellular phosphocholine and DAG levels. Recent evidence indicated that inhibition by D609 of an SM synthase activity involved in a salvage (SM resynthesis) pathway could potentially elevate ceramide and reduce DAG levels (21, 49). Our examination of the effects of D609 on de novo sphingolipid synthesis indicated that the drug caused rapid [3H]serine incorporation into ceramide, accompanied by a modest increase in [3H]sphinganine levels. Although de novo ceramide synthesis was stimulated by D609, the drug did not inhibit the SM synthase activity involved in de novo synthesis. This conclusion is based primarily on the observations that D609 stimulated SM synthesis (Figs. 2, 6), albeit much less than the total increase in ceramide, and SM synthesis was stimulated 10-fold by D609 in the presence of BFA (Fig. 6). Moreover, total SM synthase activity in membranes from D609-treated cells was either not affected or weakly inhibited at late time points. This is contrary to the reported inhibition of SM synthase by D609 in transformed lung fibroblasts (21). However, we confirmed that D609 inhibited SM synthase activity in CHO-K1 cell membranes in vitro, and in CHO-K1 cells, D609 caused a 20–40% inhibition of SM resynthesis following hydrolysis of [3H]serine-labeled SM by bacterial SMase (results not shown). A plausible explanation for these discrepancies is that different SM synthase enzymes are involved in salvage and de novo synthetic pathways, or excess de novo ceramide generation in response to D609 interfered with the measurement of SM resynthesis following bacterial SMase treatment.

Several lines of evidence lead us to conclude that D609 stimulated de novo ceramide synthesis at the SPT catalyzed step. First, treatment with the ceramide synthase inhibitor fumonisin B1 completely blocked elevated ceramide synthesis (by [3H]serine labeling) and mass in response to D609. Second, the addition of fumonisin B1 to D609-treated cells resulted in a reciprocal rise in [3H]sphinganine levels, indicating that a step prior to ceramide synthase was stimulated. And finally, in vitro assays of membranes from D609-treated cells revealed a modest but significant increase in SPT activity similar to that reported for other apoptotic agents (11, 50). Collectively, these results show that D609 activates the initial and rate-limiting step in sphingolipid synthesis, leading to a global increase in sphingolipid synthesis and elevated ceramide mass. Although the mechanism of SPT activation is unknown, the rapidity of activation in response to diverse pharmacological agents suggests a post-transcriptional mechanism involving a common apoptotic or stress-induced pathway.

The disparity between [3H]serine incorporation into ceramide versus that of SM and glucosylceramide indicated that ceramide export from the ER was inhibited by D609. This conclusion is supported by experiments with BFA, which enhanced SM synthesis in D609-treated cells by merging the ER and Golgi apparatus and effectively bringing expanded ceramide pools into contact with SM synthase. BFA was effective in reducing labeled ceramide levels by 60%. The incomplete conversion to SM indicates either that SM synthase was saturated with substrate, that not all ER ceramide was equally available to the enzyme, or that ceramide had been transported from the ER and was not affected by BFA. Regardless of the explanation, these results show that upregulation of de novo ceramide synthesis by D609 results in a substantial portion remaining in the ER. Ceramide accumulation in the ER could have resulted from either inhibition of export, transbilayer movement, or saturation of the export pathway (18). Like endogenous ceramide, transport of the fluorescent ceramide analog C2-DMB-ceramide from the ER to the Golgi was inhibited in the presence of D609 and not restored by reducing ceramide levels with fumonisin B1 (Fig. 7). This supports the idea that D609 blocks transport of ceramide from the ER or a preGolgi compartment to the Golgi apparatus. D609 may have other general effects on lipid and protein trafficking, because it has also been reported to inhibit prosaposin transport to the lysosomes in CHO-K1 cells (31).

Treatment with exogenous C2-ceramide stimulated phosphorylation of eIF2α by an unknown stress-activated kinase (4). The proposed mechanism involves phosphorylation of RAX(FACCT), which enhances its binding to and activation of the double-stranded RNA-dependent kinase PKR, one of several eIF2α kinases (42, 51). Contrary to results with short-chain ceramide analogs, which in CHO-K1 cells stimulated phosphorylation of eIF2α and inhibited protein translation (Fig. 9), endogenous generation of long-chain ceramides in response to D609 was not linked to eIF2α phosphorylation. This conclusion is based on the lack of effect of fumonisin B1 and t-cycloserine on D609-mediated eIF2α phosphorylation and inhibition of protein translation, and a delay between significant ceramide accumulation (30–60 min) and eIF2α phosphorylation (1–5 min). In addition, we show that prolonged treatment with D609 (24 h) induced apoptosis, as indicated by partial processing of the caspase 3 substrate, PARP. Although
D609 appeared to weakly induce apoptosis, decreasing ceramide synthesis with fumonisin B1 or t-cycloserine did not attenuate PARP cleavage. This apparent dichotomy between in vivo effects with endogenous ceramide versus exogenous analogs could be explained by differential access to signaling pathways. Exogenous short-chain ceramide can diffuse freely into cells and rapidly access cellular compartments and signaling pathways. In contrast, our results show that endogenous long-chain ceramides made via the de novo pathway can be restricted in their movement from the ER and are not involved in subsequent signaling events. This lack of effect of ER-localized ceramide is supported by a recently reported study examining ceramide generation by targeted expression of bacterial SMase in different organelles (20). In that study, ER expression of SMase in MCF7 cells increased ceramide levels significantly but did not induce apoptosis.

The results of the current study show that the site of generation and movement of endogenous ceramide are important considerations when examining its biological activity. A functional dichotomy probably exists between the effects of exogenous ceramide analogs or ceramide generated at the PM by SM hydrolysis and ceramide made in the ER. In the case of D609, stimulation of ceramide production generates a relatively static pool of ER ceramide that has restricted access to other compartments of the cell and is not involved in stress or apoptotic responses.

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