Ceramide metabolite, not intact ceramide molecule, may be responsible for cellular toxicity

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Ceramides, which are produced from the hydrolysis of sphingomyelin or synthesized from serine and palmitate in a *de novo* pathway, are regarded as important cellular signals for inducing apoptosis. However, controversy over this proposed role of ceramides exists. Using stable isotope labelling coupled with GC (gas chromatography)-MS and mass isotopomer distribution analysis, we have studied the metabolism of exogenous long-chain ceramides in HL60 cells. Our results do not support the concept of enhanced ceramide transport into cells induced by solvent mixtures of ethanol and hydrocarbons. In addition, cell toxicity does not correlate with the amount of intact ceramide in the cells. Our results are more consistent with a disturbance of sphingomyelin metabolism induced by the solvent mixture. The char-

acteristics of this disturbed sphingolipid disposition are the inhibition of dihydroceramide desaturation and an enhanced degradation of sphingomyelin. As a consequence, dihydroceramides accumulate and the cellular sphingomyelin content decreases. Inhibition of these pathways is most likely to be induced by the increased production of novel ceramide metabolites instead of by intact ceramides. Octadecane-1,2-diol is identified as a possible mediator. Treatments that divert ceramide degradation to the novel pathway are potential strategies in cancer therapy for inducing cell toxicity.

Key words: ceramide, mass isotopomer, octadecane-1,2-diol, sphingomyelin, sphingosine, stable isotope.

INTRODUCTION

Programmed cell death (apoptosis) is an important feature of many pathophysiological states [1]. Ceramides, which are produced from the hydrolysis of sphingomyelin or synthesized from serine and palmitate in a *de novo* pathway, are regarded as important cellular signals for inducing apoptosis [2]. However, controversy over this proposed role of ceramides exists [3–5]. Neither increase in ceramide levels nor correlation of ceramide levels with cellular effects is universally observed [6,7]. In addition, a definite signal target for ceramide has not been identified [8].

Mitochondrial involvement with the release of inter-membrane cytochrome *c* into the cytosol has emerged as a dominant mechanism for the initiation of apoptosis [9]. Ligation of receptors leads to the activation of death domains, cytochrome *c* release and caspase cascade. It has been proposed that ceramides are the endogenous signal for the execution of or amplification of these death cascades [7,10]. *In vitro*, ceramides enhance the release of mitochondrial cytochrome *c* [11], promote the mitochondrial permeability transition [12] and inhibit the mitochondrial electron-transport complex [13,14]. However, some reservations concerning the role of ceramide remain. One reason for this is the failure of natural long-chain ceramide to induce apoptosis in cultured cells. The proponents of the signalling role for ceramide feel that, in cell culture, the extracellular long-chain substrates fail to get inside the cell to the intracellular site of action [2,15]. However, Ji et al. [16] showed that natural ceramide could induce cellular apoptosis if the substrate is delivered into the cell in a mixture of ethanol and 2% (v/v) dodecane or decane. This has been cited as direct evidence for the role of natural ceramide in cellular apoptosis [2,17]. The mechanism of induction of activity by solvent mixtures was supposed to facilitate the delivery of exogenous lipids to the cells.

Using stable isotope labelling and analysis of the distribution of labelled molecules, we show that intact long-chain ceramide is not the species that initiates cell toxicity. Instead, the initiator responsible for the cellular effects could be a novel metabolite derived from ceramide degradation.

EXPERIMENTAL

Reagents

The regular serum medium for cell culture was RPMI 1640 (Sigma, St. Louis, MO, U.S.A.), supplemented with 10% (v/v) foetal bovine serum (Life Technologies, Grand Island, NY, U.S.A.) and antibiotics (400 units/l penicillin, 4 mg/l streptomycin and 10 µg/l amphotericin). Serum-free medium was prepared from commercially available RPMI 1640, supplemented with Hepes (10 mM), insulin (50 µg/l), transferrin (50 µg/l), sodium selenite (50 ng/l), penicillin (400 units), streptomycin (4 mg/l) and amphotericin (10 µg/l). [1,2,3,4-¹³C₄]Palmitate (percentage isotopic purity, 99+) was obtained from Isotec (Miamisburg, OH, U.S.A.). This labelled tracer was complexed with either serum or BSA to a final concentration of < 70 µM palmitate. Phospholipase C (*Bacillus cereus*) and sphingomyelinase (*B. cereus*) were purchased from Sigma. 1,3-Dipentadecanoylglycerol, heptadecanoic acid, pentadecanoic acid, 1,2-ditetradecanoylglycerophosphoethanolamine and 1,2-dipentadecanoylglycerophosphocholine were purchased from Sigma or Avanti Polar Lipids (Alabaster, AL, U.S.A.). 17-cer (*N*-heptadecanoylsphingosine) and 15-SM (*N*-pentadecanoylsphingophosphocholine), used as internal standards for ceramide and sphingomyelin respectively, were synthesized using the mixed anhydride method. The synthesis involves the reaction of acyl anhydride and either sphingosine or sphingophosphocholine

Abbreviations used: 16-cer, *N*-palmitoylsphingosine; 17-cer, *N*-heptadecanoylsphingosine; diH-16-cer, *N*-palmitoylsphinganine; diH-16-SM, *N*-palmitoyldihydrospingophosphocholine; GC, gas chromatography; NPE, *N*-palmitoylethanolamide; 15-SM, *N*-pentadecanoylsphingophosphocholine; 16-SM, *N*-palmitoylsphingophosphocholine; TMS, trimethylsilyl.

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[18]. Using the same mixed anhydride procedure, we prepared N -[1,2,3,4- $^{13}\text{C}_4$]palmitoylsphingosine from [1,2,3,4- $^{13}\text{C}_4$]palmitate and sphingosine. We used this compound to study the uptake of natural ceramide by the cells.

Cell culture

HL60 human promyelocytic leukaemia cells were purchased from A.T.C.C. (Manassas, VA, U.S.A.). These cells were maintained in RPMI 1640 medium with 10% (v/v) foetal calf serum. Subculture was performed twice per week by diluting subconfluent cells in freshly prepared medium to a final concentration of 2×10^5 cells/ml. The cells were counted using a haemocytometer (Sigma). Cell viability was determined with Trypan Blue exclusion.

The cell treatment was performed in either 25 ml of regular 10% serum medium or serum-free medium in T-75 culture flasks at a cell density of 1×10^6 cells/ml. At the end of the experiments, the cells were collected by centrifugation. The collected cells were washed twice with PBS and brought up in 0.5 ml of Dulbecco's PBS (50 μl of this cell mixture was diluted with an equal volume of 1% deoxycholate for subsequent protein determination) and frozen for later lipid analysis. The protein content was determined using Lowry's reagent.

Lipid analysis

The procedure has been described in detail in our previous report [18]. In brief, the chloroform/methanol lipid extract from 3×10^7 cells was mixed with internal standards and then separated into several lipid classes through a silica-gel column by stepwise elution with solvents of increasing polarity. The final elution with methanol yielded two fractions. The earlier eluting fraction contained ceramide and phosphatidylethanolamines. Ceramide was extracted from this using iso-octane/ethyl acetate (3:1). The second methanol fraction, which contained phosphatidylcholines and sphingomyelins, was treated with sphingomyelinase. The liberated diacylglycerols and ceramides were then separated on another silica-gel column.

GC (gas chromatography) capillary analysis of ceramides

A Hewlett-Packard 5980 gas chromatograph was used for the analysis. The separation was achieved with a short column (5 m; 0.25 mm internal diameter; 0.25 μm coating) of methylsilicone (ZB-1 from Phenomenex, Torrance, CA, U.S.A.). The temperature zones for the instrument were set at 290 °C for both the injector and FID (flame-ionization detector). The initial oven temperature was 200 °C and was increased at 20 °C/min to 280 °C; this temperature was maintained for an additional 16 min. A splitless injection was used. Data analysis was performed with HP ChemStation software. Ceramides were analysed as TMS (trimethylsilyl, Me_3Si) derivatives after reaction with bis-trimethylsilyltrifluoroacetamide at room temperature for 10 min.

GC-MS measurement of ceramides

An Agilent Model 5973 Network Mass Selective Detector, coupled with a 6890 GC system, was used for the identification and determination of isotope enrichment. The inlet system was set at 270 °C, the MSD transfer line at 280 °C, MS source at 230 °C and MS quad at 150 °C. A column identical with that used for GC analysis was used. The oven temperature was set at 200 °C and was increased at 20 °C/min to 280 °C for 14 min.

The components of the sample were repetitively monitored from m/z 200 to 800 at a rate of 1.27 scans/s [19]. The use of rep-

etitive scan instead of selected ion monitoring of only a few selected ions of interest decreases the sensitivity of the analytic result. However, the scan mode has the advantage of allowing selection of ions of interest at a later date rather than having to choose all beforehand. In addition, compound identification is facilitated using this approach. The trade-off of loss of sensitivity is not a problem in the present study. Ceramide derived from the ceramide and sphingomyelin fractions is abundant enough to be analysed. Furthermore, a highly enriched tracer (> 99%) was used in the present study. The isotopic enrichments determined were typically in the range of 10–98%.

Mass isotopomer distribution analysis

Ceramide is synthesized from palmitate and sphingosine, both derived from labelled palmitate. Since the precursors are labelled as M4, the final ceramide product should contain isotopomers of M0, M4 and M8. If the fractional enrichment of the precursor palmitate pool is p and that of the sphingosine pool is s , then the mass isotopomer distribution of the ceramide would be $M0 = (1-p)(1-s)$; $M4 = (1-p)s + p(1-s)$; and $M8 = ps$, if both palmitate and sphingosine lead to a homogeneous single-ceramide pool serving as the precursor of sphingomyelin. The parameter p is calculated from the ratio of m/z 374 to (m/z 370 + m/z 374) of C_{16} ceramide derived from sphingomyelin. Similarly, s is calculated from the ratio of m/z 315 to (m/z 311 + m/z 315).

For a two-pool system with one unlabelled pool and one active labelled pool, the enrichment of precursor N -palmitate is unknown and assumed to be p' . A fraction f represents the fraction of the precursor ceramide pool that is actively converting into sphingomyelin. This f is equal to p/p' , since the apparent p is diluted from p' by the unlabelled pool. The true enrichment of the precursor sphingosine then becomes $s' = s/f$. The mass isotopomer distribution from this system would be $M0 = (1-f) + (1-p')(1-s')f$; $M4 = (1-p')s'f + s'(1-p')f$; and $M8 = p's'f$. A simple mathematical relationship of these parameters was formulated using Excel (Microsoft). The p' values were varied until the derived mass isotopomer distributions of M0, M4 and M8 are the closest fit to the experimentally derived distribution. In general, this close fit is within 0.01 of experimentally derived values.

Searching for a metabolite in the lipid extract

The first methanol fraction eluted from the silica-gel column was dried. Reduction was performed with sodium borohydride (1 mg) in methanol in an ice bath for 0.5 h. The reduced lipids were then extracted and derivatized with bis-trimethylsilyltrifluoroacetamide. This derivatized sample was analysed with repetitive scanning MS after GC capillary column separation using a longer column (30 m) than that described for the analysis of ceramide in the previous section. The initial temperature for this column was 80 °C and was increased at 30 °C/min to a final temperature of 280 °C; this temperature was maintained for another 20 min. Repetitive scanning at a rate of 8 scans/min from m/z 90 to 500 was used for the acquisition of mass spectra.

Synthesis of octadecane-1,2-diol

This compound was prepared by the reaction of 2-hydroxy-octadecanoic acid (1 μmol ; Matreya, Pleasant Gap, PA, U.S.A.) with lithium aluminium hydride (1 mg) in dry tetrahydrofuran for 10 min (see Figure 6). After decomposing the unchanged lithium aluminium hydride with excess ethyl acetate, the mixture

Figure 1 Mass isotopomer distribution analysis of the 16-SM enrichments from cells treated with 0.2% ethanol/decane (49:1, v/v) for 4 h

In this sample, the M4 enrichment of sphingosine (m/z 315) is 10.6% and that of *N*-palmitate (m/z 374) is 11.5%. The distribution of mass isotopomers (M0, M4 and M8) is derived from the mass fragments, m/z 666, 670 and 674. These mass fragments contain labels from both sphingosine and *N*-palmitate. The distributions that are actually determined are indicated by the open bars. The expected distributions from a single homogeneous pool system, calculated from the experimentally determined enrichments on sphingosine and *N*-palmitate, are indicated by the shaded bars. Apparently, a one-pool system is not consistent with the experimental results. Using a two-pool system model, the calculated distributions with a new SM pool of 13.7% are indicated by the black bars. In this model, 13.7% of the SM pool is derived from newly synthesized sphingomyelin with a precursor ceramide of 77% enrichment on sphingosine and 84% enrichment on *N*-palmitate. The other 86.3% of the SM pool is an old completely unlabelled pool. The enrichment of *N*-palmitate from the new pool happened to be the same as medium palmitate enrichment.

was dried. The residue was then extracted with ethyl acetate/isooctane. After conversion into its TMS derivative, the compound was analysed with GC-MS using the same conditions as described for other metabolites.

RESULTS AND DISCUSSION

Cellular concentration of ceramide

At high concentrations (20 μ M) of 16-cer (*N*-palmitoylsphingosine) in the medium, the solvent mixture of ethanol/dodecane (49:1) or ethanol/decane (49:1, v/v) did not enhance the uptake of long-chain ceramide. There is essentially no difference in these two solvent mixtures, except that ethanol/decane is slightly more potent than ethanol/dodecane in inducing cell toxicity. The basal 16-cer content was 0.17 ± 0.05 nmol/mg of protein ($n = 3$). When exogenous 16-cer was added in ethanol, the cellular 16-cer increased to 3.5 ± 1.5 nmol/mg of protein ($n = 8$). A significantly lower cellular content (1.78 ± 1.2 nmol/mg, $n = 11$) of 16-cer was found with ethanol/decane. Therefore the addition of hydrocarbon to ethanol did not enhance the transport of long-chain ceramide

into cells. These cells are presented with 250 nmol of 16-cer for every mg of cellular protein; thus the fraction of exogenous 16-cer that is in or around the cells is relatively small, e.g. < 1%. With the addition of lower concentrations of 16-cer in the solvent mixture, the cells died completely within 24 h. In contrast, the growth of control cells and those treated with 16-cer in ethanol was not affected. Clearly, cell toxicity requires the participation of 16-cer, but is not related to the cellular concentration of unmetabolized ceramide.

The solvent mixture affects basal sphingomyelin metabolism

Since the killing of cells is not related to exogenous 16-cer concentrations and the solvent mixture controls did not induce cell toxicity, we next studied whether the solvent mixture changed cellular metabolism to predispose the cells towards additional exogenous ceramide. As shown in Table 1, the solvent mixture did not affect the 'apparent' metabolism of endogenous ceramide. The enrichments of sphingosine and *N*-palmitate from 16-cer were not affected; neither were the concentration and ratios of enrichment. The amount of protein in the cells was also not significantly altered. However, the solvent mixture induced a significant change in sphingomyelin metabolism. The amount of sphingomyelin decreased, and the enrichments on both sphingosine and *N*-palmitate increased significantly. This increase in label occurred equally between sphingosine and *N*-palmitate, since the ratio of the labels in these two parts of the molecule was not altered. From isotopomer distribution analysis (Figure 1), the increase in labels is a result of increase in the percentage of newly synthesized sphingomyelin (Table 1, % new SM), which could be brought about by increased *de novo* synthesis from ceramide, increased catabolism of old unlabelled sphingomyelin or a combination of the two. Since the amount of sphingomyelin is decreased, the contribution from increased degradation of old sphingomyelin is the most probable mechanism.

Dose-response relationship

A dose-response relationship was investigated. The cell toxicity of 16-cer in ethanol/decane can be effected with an 'apparent' concentration of 40 nM in a medium containing 3×10^7 HL60 cells (approx. 2 mg of protein). The total amount of 16-cer added to the cells equals the endogenous 16-cer in these cells. As shown in Table 2, the cellular content of 16-cer is slightly increased at a 1 nmol dose. Increasing the amount of the exogenous dose by 100-fold did not change the amount of cellular 16-cer significantly. However, in the effective doses, the highly significant change is the accumulation of the precursor-saturated diH-16-cer (*N*-palmitoylsphinganine). In untreated cells, diH-16-cer exists only in trace amounts (Figure 2).

Table 1 Basal ceramide and sphingomyelin metabolism in HL60 cells 4 h after the addition of either ethanol or ethanol/decane

Ethanol (50 μ l) or 50 μ l of ethanol/decane (49:1) was added to HL60 cells (3×10^7 cells) in 25 ml of serum-free medium containing labelled palmitate. The cells were incubated at 37 °C for 4 h. The statistics were compared with paired *t* test.

	N	Cer Amount (nmol/mg)	E, % (sph)	E, % (pa)	Ratio	SM Amount (nmol/mg)	E, % (sph)	E, % (pa)	Ratio	% new SM	Protein (mg)
Ethanol	4	0.47 ± 0.14	43.43 ± 5.36	55.07 ± 1.00	0.789 ± 0.100	11.32 ± 1.03	8.13 ± 0.37	9.02 ± 0.77	0.904 ± 0.044	12.33 ± 2.45	1.75 ± 0.08
Ethanol/decane	4	0.60 ± 0.23	46.24 ± 9.15	56.01 ± 3.14	0.822 ± 0.130	10.07 ± 0.74	11.06 ± 0.79	12.32 ± 1.03	0.899 ± 0.040	17.5 ± 3.30	1.71 ± 0.10
<i>t</i> test		ns	ns	ns	ns	$P = 0.023$	$P = 0.001$	$P = 0.006$	ns	$P = 0.036$	ns

Abbreviations: Cer, amount of ceramide; SM, amount of sphingomyelin; E, % (sph), isotope enrichments on sphingosine (% of m/z 315); E, % (pa), isotope enrichments on *N*-palmitate (% of m/z 374); ratio, ratio of enrichments on sphingosine to enrichments on *N*-palmitate; % new SM, percentage of newly synthesized sphingomyelin, calculated based on the two-compartment model; ns, not significant.

Table 2 Dose-response relationship between exogenously added 16-cer and cellular protein, ceramide, sphingomyelin and cell growth

HL60 cells (3×10^7 cells) were suspended in 25 ml of serum-free RPMI 1640 medium containing 13 μ M labelled palmitate. The cells were collected after incubation at 37 °C for 4 h. Small aliquots of these cells were further incubated for 24 h and counted. The collected cells were analysed for ceramide and sphingomyelin with capillary GC and GC-MS as described in the Experimental section. The amount of protein was determined in samples incubated for 4 h.

16-cer,exo (nmol)	16-cer (nmol)	diH-16-cer (nmol)	16-SM (nmol)	Protein (mg)	Cell#, %
0	0.85 $\pm$ 0.25	0.04 $\pm$ 0.10	19.39 $\pm$ 3.88	1.70 $\pm$ 0.11	100
0.01	1.20	0.39	17.02	1.61	107
0.1	1.22	0.56	14.37	1.34	84
1	1.35 $\pm$ 0.07	1.92 $\pm$ 0.69*	9.18 $\pm$ 0.16*	0.65 $\pm$ 0.01*	0
10	1.40 $\pm$ 0.29*	2.26 $\pm$ 0.97*	9.62 $\pm$ 1.34*	0.58 $\pm$ 0.27*	0
100	1.40 $\pm$ 0.28*	1.72 $\pm$ 0.59*	8.27 $\pm$ 1.36*	0.94 $\pm$ 0.19*	0
100†	0.71 $\pm$ 0.11	0	16.56 $\pm$ 3.54	1.64 $\pm$ 0.09	100

Abbreviations: 16-cer,exo, exogenously added 16-ceramide in ethanol/decane(49:1); cell#, %, percentage of living cells (with ethanol/decane as 100 % control) when counted after 24 h.

* Significant difference from controls; there is no difference between parameters comparing doses from 1 to 100 nmol.

† 16-cer in ethanol only, with a final amount of 100 nmol.

Figure 2 GC analysis of 16-cer (peak 1) and diH-16-cer (peak 2) with 17-cer as an internal standard (peak 3) from 3×10^7 HL60 cells after lipid extraction, silica-gel column chromatographic separation and analysis as TMS derivatives

The identities of the peaks were confirmed with GC-MS analysis. A1, control cells, after 4 h incubation, in serum-free RPMI 1640 medium; A2, with the addition of 100 nmol of 16-cer in ethanol/decane (49:1, v/v). The final concentration of ethanol was 0.2 % and that of decane was 0.004 %. B1, with the addition of 100 nmol of sphingosine in ethanol; B2, with the addition of 100 nmol of NPE to ethanol.

The accumulation of diH-16-cer is due to the inhibition of desaturation instead of increased hydrolysis from dihydrosphingomyelin. This mechanism is supported by the following observations. First, diH-16-SM (*N*-palmitoyldihydrosphingophosphocholine) is only a small percentage (1.86 ± 0.26 %, $n = 7$) compared with the more abundant 16-SM. After treatment in the presence of 16-cer (4 μ M or 100 nmol/flask) and ethanol/decane for 4 h, the

diH-16-SM to 16-SM ratios were slightly, but significantly ($P = 0.04$, t test), increased to 2.21 ± 0.30 %. Furthermore, the isotopic enrichments (% of m/z 315) on the dihydrosphingosine backbone of diH-16-cer (81.8 ± 2.8 %, $n = 6$) were significantly higher than those from diH-16-SM (17.5 ± 3.9 %, $P < 0.0001$, t test). This relationship indicates that diH-16-cer is the precursor, whereas diH-16-SM is the product in a typical precursor-product relationship.

Associated with the increase in diH-16-cer are the highly significant decreases in cellular 16-SM and protein in treated cells. Within 24 h, all cells with decreased sphingomyelin and protein contents died. In contrast, when 16-cer was added in ethanol, the cellular parameters were not affected. During the same incubation period, the cellular content of phosphatidylcholines (199 ± 13 nmol, controls versus 206 ± 13 nmol treated; $n = 3$) and their steady-state enrichments (67 ± 4 % controls versus 65 ± 6 % treated) were not affected by either treatment.

Mass isotopomer distribution analysis

To probe the mechanism of altered ceramide and sphingomyelin metabolism, the cells were grown in stable isotope-labelled [$1,2,3,4\text{-}^{13}\text{C}_4$]palmitate-enriched medium. As shown in Figure 1, from the incorporation of label into sphingosine and *N*-palmitate of ceramide and sphingomyelin, the metabolic fluxes through the biosynthetic sequence can be determined [19,20]. The M4 versus M0 enrichments on sphingosine (m/z 315 versus m/z 311) and *N*-palmitate (m/z 374 versus m/z 370) from cellular 16-cer and 16-SM are shown in Table 3. After a 4 h incubation, the ceramide enrichments reach a steady state. The enrichments on the sphingosine portion are consistently lower than that on *N*-palmitate, with a ratio of approx. 0.8. The lower sphingosine enrichment compared with that of *N*-palmitate results from the recycling of unlabelled sphingosine. This steady-state pattern is a consequence of two pools of ceramide (Figure 3). The synthetic pool is labelled, whereas the degradation pool is unlabelled. As shown in Table 3, the sphingosine enrichments from 16-cer in ethanol are slightly, but significantly, decreased from the controls. With an unchanged *N*-palmitate enrichment, this pattern results from increased recycling of unlabelled sphingosine, most probably derived from the hydrolysis of exogenous ceramide. In contrast, after the inclusion of decane, the sphingosine enrichments are slightly, but significantly, increased. Therefore decane has the effect of inhibiting the production and recycling of sphingosine. In both treatments, the added ceramide does not contribute significantly to cellular ceramide pools. Otherwise, the enrichments of both sphingosine and *N*-palmitate should be diluted equally by the entry of unlabelled 16-cer. These results rule out enhanced transport from the inclusion of decane. Instead, the results are more consistent with a mechanism of altered metabolism.

Table 3 Isotope enrichments on sphingosine (m/z 315 versus m/z 311) and *N*-palmitate (m/z 374 versus m/z 370) of cellular 16-cer and 16-SM after 4 h incubation

C₁₆ ceramide (100 nmol) in 50 μ l of ethanol or ethanol/decane (49:1).

	16-Cer			16-SM		
	m/z 315, %	m/z 374, %	Ratio	m/z 315, %	m/z 374, %	% new SM
Controls	48.15 $\pm$ 2.69	56.61 $\pm$ 2.48	0.85 $\pm$ 0.03	11.42 $\pm$ 1.06	12.55 $\pm$ 1.02	17.5 $\pm$ 3.3
In ethanol/decane	51.50 $\pm$ 2.54*	55.77 $\pm$ 1.22	0.92 $\pm$ 0.03*	2.51 $\pm$ 0.57*	2.80 $\pm$ 0.51*	2.9 $\pm$ 1.2*
In ethanol	43.05 $\pm$ 3.03*	58.53 $\pm$ 2.52	0.74 $\pm$ 0.02*			

* Significant difference obtained from paired t test.

Figure 3 A two-compartment model for the metabolism of cellular ceramide and sphingomyelin as determined from mass isotopomer distribution analysis

Ceramide is divided into two pools: the synthetic (S) pool and the D pool. The S pool is derived from the *de novo* pathway from serine (Ser) and palmitate (Pal). This pool is labelled when the cells are grown in labelled palmitate. Ceramide in this pool is used for the synthesis of sphingomyelin. The newly synthesized sphingomyelin is labelled. With the expansion of the new pool, the old unlabelled sphingomyelin pool is degraded to contribute ceramide to the D pool. This degradative D ceramide pool is essentially unlabelled within 4 h incubation. The ceramide in S and D pools is not directly exchangeable. There is only a small percentage of sphingosine, derived from hydrolysis of ceramide in the D pool, recycling to the S pool for the resynthesis of ceramide and sphingomyelin. The major portion of D pool ceramide is further metabolized and is not available for the resynthesis of sphingomyelin. Exogenous ceramide is delivered to the D pool, since it is not converted into sphingomyelin.

The increase in cellular ceramide after treatment in ethanol/decane is essentially endogenous in origin. This increase is a result of inhibited conversion into sphingomyelin. Not only is the cellular content of sphingomyelin decreased to only 50% of the controls (Table 2), the isotope enrichments on sphingosine and *N*-palmitate in sphingomyelin are also decreased (Table 3). Using a two-pool model of isotopomer distribution analysis of the enrichments of ions *m/z* 666 (M0), 670 (M4) and 674 (M8), the fractions of the newly synthesized sphingomyelin pools are derived. For control cells, 17.5% of the sphingomyelin pool is newly synthesized, whereas this fraction is only 2.9% in the treated cells. For a pool size of 19 nmol, the control cells synthesize 3.3 nmol of 16-SM in 4 h by the *de novo* pathway. In treated cells, the corresponding *de novo* synthesis rate is only 0.26 nmol, which means > 90% inhibition. However, the inhibition of sphingomyelin synthesis alone is not sufficient to explain the > 50% decrease in the total cellular pool of sphingomyelin. An additional contribution from enhanced degradation of sphingomyelin must also occur after treatment. On the basis of mass balance, 30% of the more than 10 nmol decrease in the sphingomyelin pool is derived from inhibited *de novo* synthesis (3 nmol) and 70% from enhanced degradation (7 nmol). Clearly, any exogenous 16-cer is immediately degraded inside the cells and does not contribute to the overall cellular ceramide pool. The inclusion of decane perturbs the degradation of ceramide.

Sphingosine does not affect sphingomyelin synthesis

The pathway for ceramide degradation is through hydrolysis to sphingosine [21]. We studied the possibility that accumulation of sphingosine from ceramide degradation causes cell death. As shown in Figure 2(B), the addition of sphingosine to cultured cells significantly increased cellular 16-cer (27 ± 13 -fold increase, $n = 7$), but the conversion into sphingomyelin was not

affected ($102 \pm 21\%$ of controls). Furthermore, there was no accumulation of diH-16-cer and no inhibition of cell growth (Figure 2).

Dihydrosphingosine also failed to inhibit sphingomyelin synthesis and cell growth

Biological activities of ceramides are supposed to be structure-specific. An unsaturation between carbons 4 and 5 of the sphingosine backbone is essential for this activity [22]. The saturated analogues, namely dihydrosphingosine and the corresponding dihydroceramides, are inert in terms of cellular apoptotic activity [22]. Dihydrosphingosine is readily taken up by the cells when added to the medium. In the presence of palmitate, dihydrosphingosine is converted into diH-16-cer (2.3 ± 0.3 ratio to 16-cer, $n = 3$) and 16-cer (5.9 ± 2.8 -fold increase). Despite the accumulation of intracellular long-chain 16-cer, the growth of cells is not affected. This result is consistent with the notion that dihydrosphingosine is an inert control for cellular apoptosis. It further indicates that the accumulation of dihydroceramide is not the cause of the cellular effects observed in the administration of 16-cer in the presence of solvent mixtures. Despite the increase in dihydroceramide and ceramide, the production of sphingomyelin is not affected ($104 \pm 4\%$ of controls, $n = 3$). This further supports the conclusion described in the previous section that the inhibition of sphingomyelin synthesis is the mechanism that is responsible for cellular apoptosis.

Effects of *N*-acylethanolamide on ceramide and sphingomyelin metabolism

To manipulate cellular sphingosine content, we resorted to the use of a ceramidase inhibitor, *N*-oleoylethanolamide, and its inactive homologue, NPE (*N*-palmitoylethanolamide) [23,24]. To our surprise, these compounds produced the same pattern of diH-16-cer accumulation and inhibition of sphingomyelin ($66 \pm 7\%$ of controls, $n = 4$) synthesis. In addition, *N*-acylethanolamides also inhibited the desaturation process of conversion of dihydroceramide into ceramide. When cells were grown in the presence of *N*-acylethanolamide and dihydrosphingosine, the ratios of cellular diH-16-cer to 16-cer are increased significantly ($197 \pm 72\%$ of controls, $n = 4$). Therefore it appears that *N*-acylethanolamide treatment has the same effect as that observed when the cells are treated with long-chain ceramide in a solvent mixture (Figure 2).

Exogenous 16-cer does not survive as intact molecule inside the cells

To study the uptake of exogenous long-chain ceramide by the cells, we synthesized 16-cer labelled only at the *N*-palmitoyl group. This labelled tracer contains M4 label only at the *N*-palmitate (M4 > 99%) group and no label on sphingosine. After incubating at an initial medium concentration of 0.2–0.4 μM for 4 h, we analysed the isotopic enrichments on the *N*-palmitate and sphingosine parts of cellular 16-cer. To our surprise, the M4 label is not confined to the *N*-palmitate group alone as would be predicted from the uptake of 16-cer. Instead, a labelling pattern ($n = 3$) of $5.58 \pm 1.66\%$ on sphingosine and $5.92 \pm 2.88\%$ on *N*-palmitate was found. This pattern indicates that exogenous ceramide taken up by the cells does not mix with the endogenous pool before being metabolized. It is more probable that the exogenous molecules are degraded to palmitate and then incorporated into the endogenous ceramide pool through *de novo* synthesis.

Figure 4 NPE is an integral part of the 16-cer structure

The cleavage between carbons 2 and 3 would yield NPE. However, it is not known whether a lyase exists to cleave this bond.

The fact that ^{13}C -palmitate-labelled exogenous 16-cer is totally degraded to palmitate before reaching the endogenous pool supports the proposal that degraded products, instead of intact ceramide, are responsible for cell killing. Although the sphingosine pathway produces palmitate as one of the end-products, our experimental evidence has essentially ruled out sphingosine as an intermediate in the degradation pathway. Not only did exogenous sphingosine fail to produce the same characteristic pattern, but also the amounts of cellular sphingosine and sphingosine phosphate were not altered (K.-Y. Tserng and R. L. Griffin, unpublished work).

Searching for a novel metabolite

From our results, it is apparent that intact long-chain ceramide is not responsible for the effect of cell killing. Furthermore, sphingosine, the only known obligatory metabolite in the further degradation of ceramide, does not appear to be the cause of the cellular effect either. In contrast, NPE is the only exogenous agent tested that imitates the effect that results in dihydroceramide accumulation preceding cell killing. As shown in Figure 4, NPE is an integral part of the molecular structure of 16-cer. It is not known whether 16-cer can be metabolized to NPE, which then triggers the effect. Such a conversion would require a lyase to cause a cleavage between carbons 2 and 3 of the ceramide molecule (Figure 4, broken line).

In our silica-gel column separation procedure of the lipid classes, both sphingosine and NPE are eluted in the same fraction as ceramide. We investigated this fraction to find an NPE-like molecule. There was no detectable increase in NPE in these cells. Since an aldehyde metabolite could be produced from the degradation pathway of ceramide, we also searched for a metabolite that could be an aldehyde or ketone in origin. Lipids in extracts were reduced with sodium borohydride and then analysed. As shown in Figure 5, compound M1 is increased in treated cells. The TMS derivative of this compound has a base ion at m/z 327 (see the inset of Figure 5A) and the highest ion occurs at m/z 415. Since the M-15 ion is the most common highest-mass ion in TMS derivatives in electron impact (70 eV)-induced fragmentation, the molecular mass of this derivatized M1 is expected to be 430. This fragmentation pattern is similar to 1-palmitoylglycerol and 1-stearoylglycerol (Figure 5). The corresponding mass fragments of 1-palmitoylglycerol bis-TMS occur at m/z 371 and 459, whereas those of 1-stearoylglycerol bis-TMS occur at m/z 487 and 399 respectively. In these vicinal diol TMS derivatives, the base peaks are derived from the removal of the 88 (trimethylsilanol-2H) neutral mass fragment from the M-15 ions. For the M1 metabolite, the M-15-88 mass fragment is found at m/z 327. This relationship indicates that compound M1 probably has a terminal

Figure 5 Increase in compound M1 in reduced lipids extracted from cells treated with 16-cer ($1\ \mu\text{M}$) in ethanol containing 2% decane (A)

For comparison, the corresponding chromatogram from control cell lipids is shown in (B). Samples were analysed as TMS derivatives. The 70 eV electron impact (EI) mass spectrum of M1 is shown in the inset of (A). This fragmentation pattern is similar to those derived from 1-palmitoylglycerol (peak near 8 min) and 1-stearoylglycerol (peak near 9 min). Compound M1 is confirmed to be octadecane-1,2-diol. C6-ceramide (2 nmol; C6-cer) was added as internal standard for the analysis. The amount of lipids was derived from 3×10^7 cells.

Figure 6 Synthesis of octadecane-1,2-diol (B) from reduction of 2-hydroxyoctadecanoic acid (A) by lithium aluminium hydride (LiAlH_4)

vicinal diol group and has 44 mass units less compared with 1-palmitoylglycerol. The potential metabolite that fits these criteria and that has the observed retention time would be an octadecanediol.

Dihydrosphingosine is known to undergo deamination induced by nitrite to a mixture of ketones, aldehydes and alcohols with a C_{18} -hydrocarbon chain [25]. Octadecane-1,3-diol is one of these products. We performed the degradation of dihydrosphingosine essentially as described in [25]. A product with retention time slightly higher than M1 (<0.1 min) is observed. This compound has mass fragments at m/z 325 and 415, consistent with a 1,3-diol structure on an octadecane hydrocarbon backbone. As expected, the M-15 fragment (m/z 415) is the same as the M1 metabolite. However, for 1,3-diol, the basal peak changes to m/z 325. This fragment is derived from the loss of 90 mass units (trimethylsilanol group) from the M-15 ion. The difference in retention time by GC from octadecane-1,3-diol and the mass fragmentation analogous to 1-acylglycerols make octadecane-1,2-diol probably identical with compound M1. The definite identification of M1 as

octadecane-1,2-diol is confirmed by comparison with a synthetic authentic sample derived from the reduction of 2-hydroxyoctadecanoic acid by lithium aluminium hydride (Figure 6).

Conclusion

The ISI citation index [26] lists more than 100 citations of the findings of Ji et al. [16]. Some investigators used their work as supportive evidence that natural long-chain ceramides are as much capable of inducing cellular apoptosis as short-chain compounds if penetration into cells is enhanced [2,17]. Some more investigations have used this method for demonstrating the effects of natural ceramides on biological systems [8,27–29]. Our results have shown that ethanol with a trace of hydrocarbon does not enhance the transport of long-chain ceramide into cells. Despite the fact that cell toxicity occurs, this activity is independent of the cellular concentration of intact natural ceramide. Therefore the biological properties of exogenous long-chain ceramides documented in these investigations should be interpreted with caution.

It appears that, in the presence of an ethanol/hydrocarbon mixture, the cells are in an environment of enhanced sphingomyelin catabolism. We could not find in the literature any study on the effect of hydrocarbons on sphingomyelin degradation. However, there are reports documenting enhanced sphingomyelin hydrolysis after treatment of cells with volatile anaesthetics, such as halothane [30]. It has been proposed that this enhanced degradation of sphingomyelin is due to the activation of membrane-bound neutral sphingomyelinase, possibly from increased membrane fluidity [30].

The major pathway for sphingomyelin catabolism is hydrolysis to ceramide mediated by sphingomyelinase. Whether an enhanced sphingomyelin catabolism precipitated by the inclusion of ethanol/hydrocarbon is through the activation of sphingomyelinase requires further investigation. However, enhanced sphingomyelinase activity is observed in many treatments that result in cell toxicity [2]. Since the concentration of and enrichments on ceramide are not changed significantly under conditions of enhanced sphingomyelin catabolism, we propose that increased ceramide is removed by degradation to other metabolites. The pathway for degradation of ceramide is conversion into sphingosine through the action of ceramidase [21]. The quantitative contribution of this degradation pathway is not known, but it is generally assumed to be the major, if not the only one. To study this pathway, we inhibited ceramidase in the cell with the well-known specific inhibitor, *N*-oleoylethanolamide, and its inactive analogue, NPE. We found that the same pattern of increased dihydroceramide inhibited sphingomyelin synthesis, and cell toxicity exists here also. This result strongly suggests that NPE or its metabolite shares the same action site as ceramide metabolites in inducing cell toxicity.

Our observations explain the inconsistency concerning ceramide increase in cellular apoptosis, namely the fact that the intact ceramide is not the causative agent. It is the result of altered ceramide and sphingomyelin degradation. In the presence of trace hydrocarbon, the amount of ceramide needed to induce cell toxicity is only twice the endogenous level. We propose that ceramide can be degraded, at least in the presence of hydrocarbons, by an alternative and unknown pathway, to produce a toxic metabolite. Enhanced degradation of endogenous ceramide and the delivery of exogenous ceramide lead to the production of the novel metabolite and cellular toxicity.

In an attempt to identify metabolites that might be elevated from a degradation pathway of 16-cer other than the known sphingosine pathway, we observe an increase in octadecane-1,2-diol

in treated cells. Without an available 16-cer tracer labelled on the sphingosine backbone of the molecule, we do not know whether this octadecanediol is derived from the degradation of 16-cer or from the perturbation of other metabolic pathways. Since octadecanediol is observed only after sodium borohydride reduction, the original molecule must be an aldehyde or ketone. Hydroxy unsaturated aldehydes, such as 4-hydroxynonenal, are potent cytotoxic agents [31]. In addition, octadecane-1,2-diol is a known potent anti-tumour compound [32]. Furthermore, this type of long-chain diol or ketone-alcohol has the potential of being the substrate for diacylglycerol kinase. This enzyme has been used almost exclusively in the determination of the cellular content of ceramide and is instrumental in establishing the concept of ceramide signalling [2]. However, diacylglycerol kinase is known to have very broad substrate specificity; monoacylglycerols are among its known substrates [33]. The increase in cellular ceramide has not been reproduced in some of the more specific MS methods [6]. More work is needed to explore the implications of this observation.

Perturbation of sphingomyelin metabolism induced by trace hydrocarbons could be explored further as a model for the underlying mechanism responsible for the cell toxicity of ceramides. In addition, results from studies on cultured HL60 cells indicate that ceramide could be a potent anti-cancer agent, since the concentration of ceramide needed for tumour cell toxicity is already present in cells *in vivo*. Also important is the effort to determine the conditions which will induce the metabolic changes in sphingomyelin. Over the past several years, there have been a few proposals for testing the potential of ceramide as a chemotherapeutic agent [22]. Apparently, the variation in biological results, the insolubility of the testing substances and the high cost of the synthesis must have hindered progress in this area. Shifting the metabolic pathway of ceramide without any exogenous administration of relatively insoluble substances could be an attractive alternative to conventional treatment.

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