Lipid metabolic changes caused by short-chain ceramides and the connection with apoptosis

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The effects of the short-chain ceramides D-*erythro*-*N*-acetylsphingosine (C_2-ceramide), 6-[*N*-(7-nitrobenz-2-oxa-1,3-diazole-4-yl)amino]hexanoyl-D-*erythro*-sphingosine (NBD-ceramide) and *N*-[4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-pentanoyl]-D-*erythro*-sphingosine (DMB-ceramide) on the incorporation of [^{14}C]acetate into baby-hamster kidney (BHK) fibroblasts have been examined. C_2-ceramide at concentrations up to 20 μM caused an inhibition of synthesis of phosphatidylcholine (PtdCho), sphingolipids and cholesterol within 2 h. Similar effects in BHK cells were seen using other radioactive tracers ([^{3}H]water, [^{3}H]palmitate and [^{3}H]choline) and using HL60 cells labelled with [^{14}C]acetate. The inhibition of PtdCho synthesis corresponded to an accumulation of label in diacylglycerol and triacylglycerol, probably as a consequence of cytidylyltransferase blockade. With [^{3}H]choline label, the decrease in sphingomyelin synthesis could be partly accounted for by accumulation of a slow-moving lipid, likely to be C_2-sphingomyelin. NBD-ceramide also reduced sphingomyelin and cholesterol biosynthesis, but had much less effect on PtdCho and acylglycerols. In contrast, the only apparent effect of DMB-ceramide was to inhibit synthesis of sphingomyelin, with a reciprocal increase in DMB-sphingomyelin synthesis. However, all of these short-chain ceramides caused massive apoptosis after 18 h, whereas addition of *N*-acetyldihydrosphingosine or elevation of natural ceramide by treatment of cells with sphingomyelinase had little effect on lipid synthesis or apoptosis. The present findings suggest that the apoptotic effect of short-chain ceramides is sometimes associated with inhibition of cytidylyltransferase, but is more closely correlated with a competitive inhibition of normal sphingomyelin biosynthesis.

Key words: BHK, cytidylyltransferase, diacylglycerol, sphingomyelin, triacylglycerol.

INTRODUCTION

There is considerable support for the idea that ceramides can influence signal-transduction pathways [1,2], particularly those controlling cell growth [3], differentiation [4], response to stress [5] and programmed cell death (apoptosis) [6–8]. An important part of the evidence for a signalling role for ceramides has depended on the use of short-chain ceramides such as *N*-acetylsphingosine (C_2-ceramide), which, unlike the normal long-chain ceramides, are able to rapidly enter cells when added exogenously. Although the use of these compounds has been questioned [9], it is notable that the biological effects of C_2-ceramide are not shown at all by the closely related compound *N*-acetyldihydrosphingosine (C_2-dihydroceramide), suggesting that the specific molecular characteristics of C_2-ceramide are crucial for its biological activities.

The mechanisms by which short-chain ceramides exert their effects on cells are unclear. It has been proposed that they act as lipid second messengers by altering the phosphorylation state and hence the activity of certain crucial enzymes [1]. However, there is good evidence that short-chain ceramides at similar concentrations to those which have physiological effects can directly alter lipid metabolism by substituting for endogenous ceramide in normal biosynthetic pathways. In particular it has been shown that these ceramides inhibit incorporation of radiolabelled serine into sphingolipids and can themselves be metabolized to the corresponding sphingomyelins and glucosylceramides [10–14]. These effects suggest that there is a competition between short-chain ceramide and endogenous ceramide for conversion into the more hydrophilic sphingolipids. The present study, using baby-hamster kidney (BHK) fibroblasts, shows that three different apoptotic short-chain ceramides have various metabolic effects, including inhibition of sphingolipid, phosphatidylcholine (PtdCho) and cholesterol biosynthesis. The only characteristic which they all share, however, is the ability to inhibit normal synthesis of sphingomyelin.

MATERIALS AND METHODS

D-*erythro*-*N*-Acetylsphingosine (C_2-ceramide) and *Bacillus cereus* sphingomyelinase were obtained from Sigma Chemical Co. D-*erythro*-*N*-acetyldihydrosphingosine was obtained from Calbiochem. 6-[*N*-(7-Nitrobenz-2-oxa-1,3-diazole-4-yl)amino]hexanoyl-D-*erythro*-sphingosine (NBD-ceramide) and *N*-[4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-pentanoyl]-D-*erythro*-sphingosine (DMB-ceramide) were products of Molecular Probes Inc. Eugene, OR, U.S.A. [2-^{14}C]Sodium acetate (50 mCi/mmol), [^{3}H]palmitate (54 Ci/mmol), [^{3}H]choline

Abbreviations: BHK, baby-hamster kidney. C_2-ceramide, D-*erythro*-*N*-acetylsphingosine; NBD-ceramide, 6-[*N*-(7-nitrobenz-2-oxa-1,3-diazole-4-yl) amino]hexanoyl-D-*erythro*-sphingosine; DMB-ceramide, *N*-[4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-pentanoyl]-D-*erythro*-sphingosine; PtdCho, phosphatidylcholine; PtdSer, phosphatidylserine; PtdEtn, phosphatidylethanolamine; SM, sphingomyelin; GluCer, glucosylceramide; DAG, diacylglycerol; TAG, triacylglycerol.

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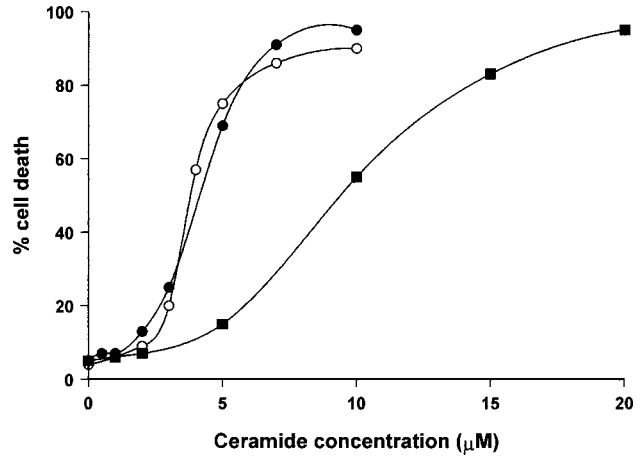


Figure 1 Dependence of BHK-cell apoptosis on the concentration of short-chain ceramides

Cells were incubated for at 37 °C in the presence of various concentrations of C_2-ceramide (squares), NBD-ceramide (closed circles) or DMB-ceramide (open circles) and the proportion of apoptotic cells was counted after 18 h (see the Materials and methods section). Values represent the means of duplicate determinations, which differed by no more than 10%. Cells were also treated with sphingomyelinase, but this treatment gave levels of apoptosis which could not be distinguished from those in untreated samples (results not shown). Similar results were obtained in two further experiments.

(15 Ci/mmol) and [^{3}H]water (250 mCi/ml) were from Amersham International.

Tissue culture

BHK fibroblasts were grown to half-confluence (about 5×10^5 cells/dish) on 3.5 cm-diameter plastic Petri dishes in RPMI-1640 medium buffered with Hepes at pH 7.4 and supplemented with 5% foetal-calf serum.

Incubation of cells

The growth medium was removed and replaced with 1 ml of RPMI-1640 containing from 0 to 20 μM C_2-ceramide or 0–10 μM NBD- or DMB-ceramide (added as a DMSO solution in no more than 4 μl) together with 2 μCi/ml [^{14}C]acetate or with either [^{3}H]choline (10 μCi/ml) or [^{3}H]water (1 mCi/ml) or [^{3}H]palmitate (5 μCi/ml). In some experiments, cells were grown in the continuous presence of [^{3}H]choline (1 μCi/ml) for 48 h before addition of various amounts of C_2-ceramide. In other experiments, larger samples of cells were grown to confluence in 75 cm^2 flasks, and triplicate flasks were incubated for 2 h with or without addition of 10 μM C_2-ceramide in the presence of [^{3}H]choline as described above. In all cases incubation was continued for 2 h at 37 °C before removing the medium and extracting the lipids from the cells.

In further experiments, cells were incubated for various times with 10 μM short-chain ceramides or with *B. cereus* sphingomyelinase (50 m-units/ml) for 2 h before extraction. In some experiments cell morphology was monitored and the proportion of cells which were spherical and possessed fragmented nuclei after 18 h incubation was measured using bisbenzamide staining (Hoechst 33258) [15].

Additional experiments were carried out in which HL-60 cells (a human leukocytic line) were incubated with [^{14}C]acetate and different concentrations of C_2-ceramide. The cells were incubated and lipids analysed as for BHK cells but with the difference that before lipid extraction the HL-60 cells were sedimented by centrifugation in polypropylene tubes.

Lipid analysis

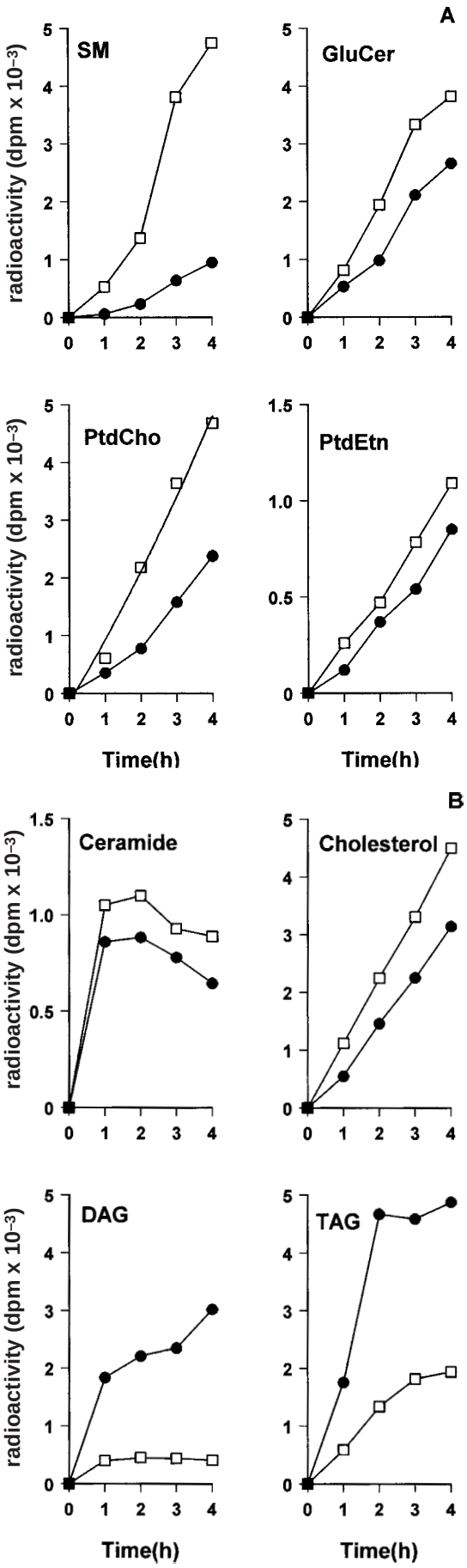
After incubation of BHK cells the medium was carefully removed and the lipids were extracted from the cells by the addition of 1.9ml of methanol/chloroform (2:1, v/v) to the dishes. Following transfer to polypropylene test tubes, the phases were split as described by Bligh and Dyer [16]. The chloroform phase was dried using a vacuum desiccator attached to a water pump and neutral lipids were separated in the solvent system benzene/diethyl ether/ethanol/acetic acid (500:400:20:1, by vol.). Spots were imaged on ^{3}H-sensitive screens and quantified on a Fuji PhosphorImager using imaging software supplied by Raytest Isotopenmessgerate G.m.b.H., Straubenhardt, Germany. The same plate was next run in either chloroform/methanol/acetic acid/water (75:45:12:2, by vol.) to separate phospholipids or in chloroform/methanol/water (70:30:5, by vol.) to separate phospholipids and glycolipids [17] and then was dried and phosphorimaged as before. In those experiments with [^{3}H]choline where it was necessary to resolve different molecular species of sphingomyelin, plates were run in chloroform/methanol/acetic acid water (75:45:12:4, by vol.). Standard amounts of radioactivity on the same plates were used to calibrate the spots in terms of d.p.m. When NBD-ceramide or DMB-ceramide were used, fluorescent spots were located and quantified by comparison with standard amounts of the same fluorescent ceramides using a Fuji CCD fluorescent imaging system (Raytest). In some experiments, the PtdCho and sphingomyelin spots were quantified by determination of total lipid phosphorus [18].

In order to characterize the water-soluble components which were labelled with [^{3}H]choline, 0.1 ml of the aqueous layer from the phase partition of the large samples of cells was evaporated to dryness under vacuum and after dissolving in 10 μl of 30 mM EDTA, was chromatographed on Whatman no. 1 paper in ethanol/water (80:20, v/v) for 16 h, together with standards for choline ($R_F = 0.76$), phosphorylcholine ($R_F = 0.44$) and CDP-choline ($R_F = 0.17$). After drying, the papers were phosphorimaged and the spots corresponding to the standards were excised and eluted before quantification by liquid-scintillation counting.

RESULTS

Each of the three short-chain ceramides were effective in causing apoptosis in BHK cells after 18 h incubation. Cells were characteristically rounded and shrunken with blebbing surfaces and fragmented nuclei. The concentration which gave half-maximal apoptosis was about 10 μM for C_2-ceramide and about 4 μM for NBD-ceramide or DMB-ceramide (Figure 1). At the higher concentrations, some of the rounded cells were detached from the culture dish.

C_2-ceramide at 10 μM over incubation periods of up to 4 h had a dramatic effect on the distribution of labelling between different lipid classes of BHK cells (Figure 2). Incorporation of radioactivity into sphingomyelin (SM) was greatly reduced, and similar effects were seen on synthesis of glucosylceramide (GluCer) (Figure 2A), although there was little apparent effect on the labelling of ceramide (Figure 2B). Radioactivity in cholesterol was also decreased significantly (mean decrease in four experiments was 27 ± 10% at 4 h ($P \leqslant 0.05$). Labelling of PtdCho was markedly diminished in the presence of 10 μM C_2-ceramide, but labelling of phosphatidylethanolamine (PtdEtn) was decreased only by about 20% and phosphatidylserine (PtdSer)/PtdIns



labelling was largely unaffected (results not shown). The loss of label from PtdCho was almost entirely accounted for by an increase in labelling of diacylglycerol (DAG) and triacylglycerol (TAG), which rose 3–4 fold (Figure 2B).

Similar results were obtained using a 2 h incubation in the presence of different amounts of C_2-ceramide (Figure 3A) and, at the highest concentrations, labelling of PtdCho and SM was decreased by more than 70%. Comparable changes in the labelling pattern were obtained when cells were labelled with ^{3}H-labelled water or palmitate (Figure 4). For the experiment with [^{3}H]water, specific radioactivity was 0.4 mCi/ml (i.e. 0.4 mCi per 18 g of water, equivalent to 2 g of hydrogen), so that 1 g of hydrogen was equivalent to about 7×10^9 d.p.m. Since the total incorporation into PtdCho was 500 d..m. in 2 h, the rate of incorporation of hydrogen into PtdCho was $250/7 \times 10^9$ g-atoms/h, i.e. about 35 ng of atoms/h. Assuming that a PtdCho molecule contains about 60 hydrogen atoms which can exchange with [^{3}H]water [19], 35 ng atoms of hydrogen incorporation represents synthesis of about 0.6 nmol of PtdCho/h. Total PtdCho per dish was about 10 nmol, so that the rate of synthesis was about 6%/h, consistent with the observed cell doubling time of about 15 h.

Similar effects of C_2-ceramide on lipid labelling were seen with HL-60 cells incubated with [^{14}C]acetate. Again there was a decrease in labelling of PtdCho and an increase in acylglycerol labelling at higher concentrations of C_2-ceramide, although with these cells there was no apparent change in the proportion of radioactivity in cholesterol (Table 1). Also the relative decrease of label in PtdEtn was greater than in BHK cells and the proportion of label appearing in DAG was relatively less compared with TAG.

Labelling of PtdCho and SM in a 2 h incubation with [^{3}H]choline was similarly inhibited by C_2-ceramide, although no effect of C_2-ceramide was seen when cells had been labelled for 48 h with [^{3}H]choline or when measurements of lipid phosphorus were carried out (Figure 5). With the 2 h incubation with [^{3}H]choline a large proportion of the decrease in incorporation into normal SM could be accounted for by a rise in a labelled lipid running slightly faster than lysoPtdCho and which was visible as a weak band (X) when stained with iodine (Figure 6). This component is most likely to represent C_2-SM, since when cells were treated with sphingomyelinase this band decreased together with normal SM and a new radioactive spot, co-migrating with unlabelled C_2-ceramide, appeared when neutral lipids were separated in the benzene/diethyl ether/ethanol/acetic acid solvent system (results not shown). Consistent with this interpretation, about 80% of the decrease in SM labelling in cells treated with C_2-ceramide could be accounted for by a rise in labelling of the putative C_2-SM (the Table in the legend to Figure 6). In contrast, the labelling of lysoPtdCho did not change. Examination of the water-soluble components labelled by [^{3}H]choline showed that, whereas C_2-ceramide treatment caused little change in the radioactivity of phosphocholine, radioactivity in CDP-choline was significantly decreased (Table in the legend to Figure 6).

Figure 2 Time course of incorporation of [^{14}C]acetate into BHK cells in the presence or absence of C_2-ceramide

Cells were labelled with [^{14}C]acetate in the absence (open squares) or presence (closed circles) of 10 μM C_2-ceramide. Incorporation of radioactivity into SM, GluCer, PtdCho, PtdEtn, ceramide, cholesterol, DAG and TAG was measured over a 4 h period as described in the Materials and methods section. Results are expressed as d.p.m. in each lipid class and represent the means of duplicate determinations, which varied from each other by no more than 5%. Similar results were obtained in three other experiments using different samples of cells.

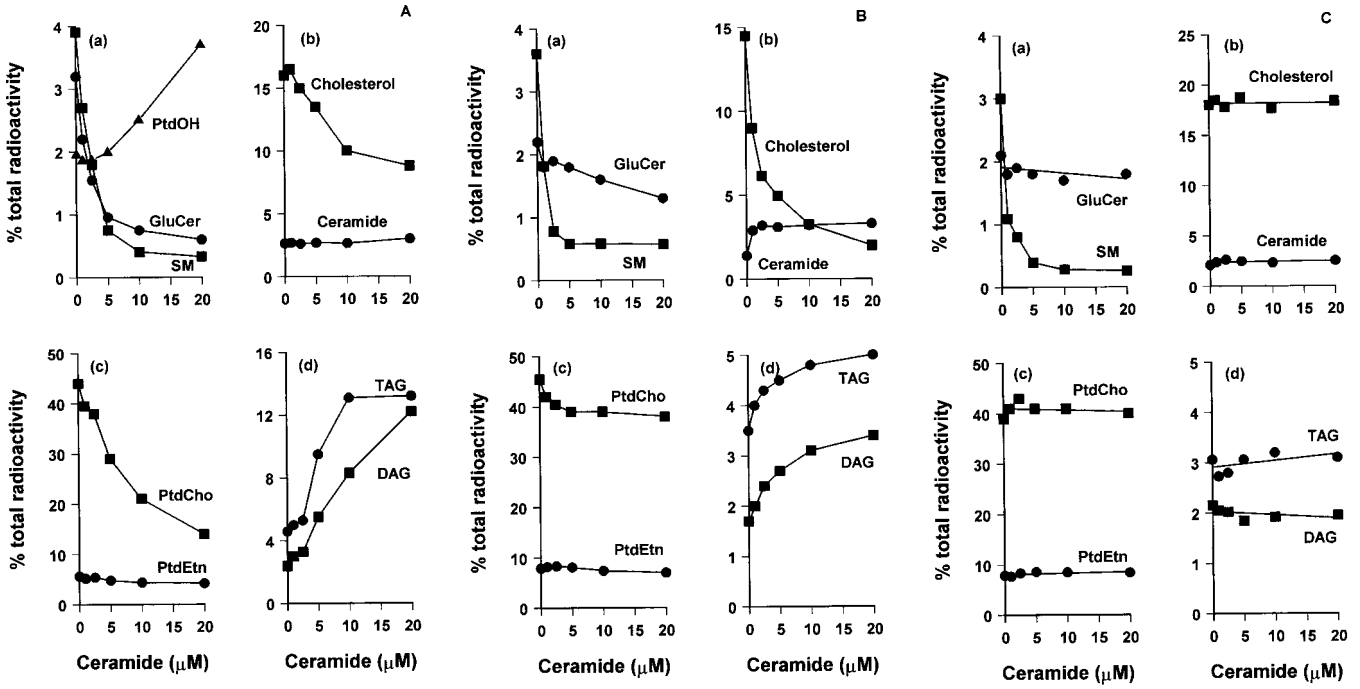


Figure 3 Dependence of incorporation of [^{14}C]acetate into BHK cell lipids on concentration of short-chain ceramides

BHK cells were incubated with [^{14}C]acetate, together with various concentrations of C_2-ceramide (**A**), NBD-ceramide (**B**) or DMB-ceramide (**C**) and the incorporation of radioactivity into the different lipid classes was measured over a 2 h period as described in the Materials and methods section. (a) SM (squares), GluCer (circles), PtdOH (triangles); (b) cholesterol (squares) and ceramide (circles); (c) PtdCho (squares) and PtdEtn (circles); (d) DAG (squares), TAG (circles). Phosphatidate (triangles) is included additionally in (**A**, part a). The results represent the means of duplicate determinations in one experiment which was repeated twice with essentially the same results. Total incorporation into lipids was 11684 ± 3994 d.p.m. (mean ± S.D. for three experiments).

With [^{14}C]acetate, the labelling of TAG did not increase at higher concentrations of C_2-ceramide, but the labelling of DAG continued to increase at concentrations up to 20 μM C_2-ceramide. A doubling of the radioactivity in a component having the same mobility as phosphatidic acid was also seen at higher concentrations of C_2-ceramide (Figure 3A). When plates were re-run in the second dimension in a solvent consisting of chloroform/methanol/7 M ammonium hydroxide (65:30:4, by vol.), this component scarcely moved from the origin, consistent with the behaviour of standard phosphatidic acid (results not shown). None of the above changes occurred in the presence of C_2-dihydroceramide at concentrations up to 50 μM (results not shown). However, similar decreases in labelling of SM and cholesterol were seen with NBD-ceramide (Figure 3B), although the effects on inhibition of PtdCho synthesis and the corresponding increase in acylglycerols was much less than with C_2-ceramide. On the other hand, DMB-ceramide had no effect at all on incorporation of label into lipids, apart from SM, whose radioactivity was decreased by up to 90% at the highest concentrations (Figure 3C). Both fluorescent ceramides were metabolized to the corresponding fluorescent SM, but it was notable that, whereas NBD-ceramide was converted into NBD-GluCer, no synthesis of the corresponding glycolipid was seen with DMB-ceramide (Figure 7).

Treatment of BHK cells with exogenous sphingomyelinase drceased labelling of SM after 1 h, and there was a corresponding increase in ceramide radioactivity (Figure 8). There was also a decrease in labelling of cholesterol, but, in contrast with the effects of C_2-ceramide, there was a slight increase in labelling of glycolipids, little change in PtdCho and decreases in labelling of DAG and TAG (Figure 8). Even in the presence of sphingomyelinase for 18 h, with a consequent sustained 70% decrease in SM and a corresponding elevation of ceramide, the level of apoptotic cells was very similar to that in untreated samples (about 5%; results not shown).

DISCUSSION

Short-chain ceramides are potent apoptotic agents for a wide range of cells, and this is also true for BHK fibroblasts, where maximal effects of C_2-ceramide, NBD-ceramide and DMB-ceramide were seen in the micromolar range (Figure 1). However, these ceramides also have complex effects on biosynthesis of lipids measured as incorporation of [^{14}C]acetate (Figures 2 and 3) or as incorporation of ^{3}H-labelled water, palmitate (Figure 4) or choline (Figure 5). Inhibition of synthesis of SM is the most consistent and dramatic effect and is 90% complete at concentrations of ceramides around 10 μM (Figures 2–5).

The most plausible reason for this inhibition is that the artificial ceramide competes with natural (radioactive) ceramide for conversion into SM and glycolipids. Indeed there is previous evidence using fibroblasts that C_2-ceramide [12] or NBD-ceramide [20] are converted into the corrresponding SMs and GluCers. The amount of fluorescent SM synthesized from NBD-ceramide or DMB-ceramide was about 3 mmol/h per mol of cellular phospholipid and, given that endogenous SM represents about 75 mmol/mol of total phospholipid, the rate of normal SM synthesis should be about 5 mmol/h per mol of total phospholipid (based on a cell doubling time of 15 h), it can be seen that

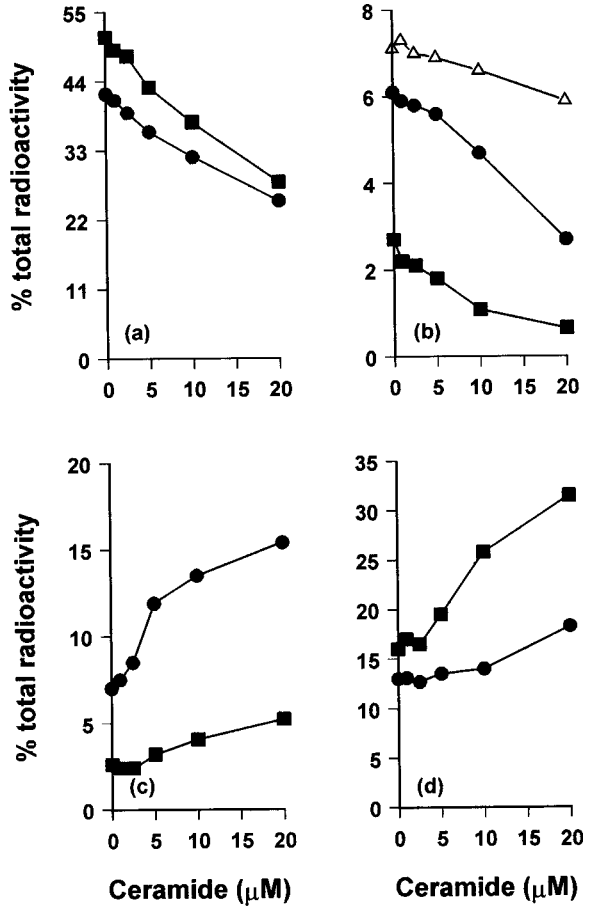


Figure 4 Effect of C_2-ceramide concentration on incorporation of radioactivity from [^{3}H]water or [^{3}H]palmitate into BHK cell lipids

BHK cells were incubated for 2 h in the presence of either [^{3}H]palmitate (5 μCi/ml) (filled squares) or [^{3}H]water (1 mCi/ml) (filled circles), together with various concentrations of C_2-ceramide. Lipids were extracted and analysed as as described in the Materials and methods section. Radioactivity in lipids is expressed as means of triplicate determinations in one experiment which was repeated with similar results. (**a**) PtdCho; (**b**) SM; (**c**) DAG; and (**d**) TAG. (**b**) Also contains data for cholesterol from cells labelled with [^{3}H]water (open triangles).Total radioactivity in the lipids of cells incubated with [^{3}H]palmitate was 255 000 d.p.m. and in cells incubated with [^{3}H]water was 1500 d.p.m. Similar results were obtained in one further experiment.

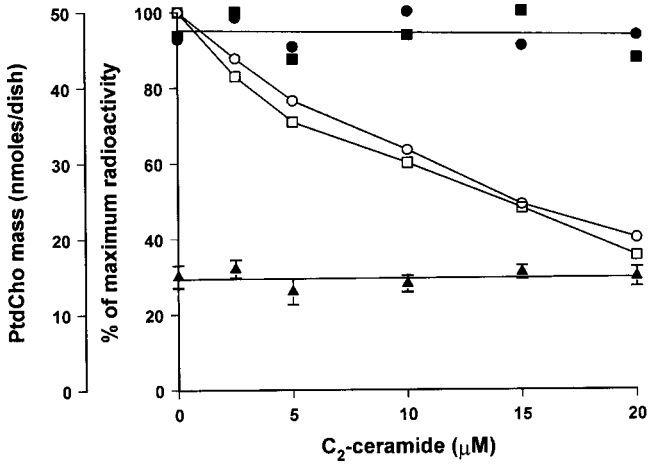


Figure 5 Effect of C_2-ceramide concentration on (a) incorporation of [^{3}H] choline into BHK cells and (b) lipid mass, measured as phosphate

BHK cells were incubated in the presence of [^{3}H]choline for either 48 h (1 μCi/ml, 20 Ci/mol choline (closed symbols) or for 2 h (10 μCi/ml, 200 Ci/mol of choline) (open symbols), together with various amounts of C_2-ceramide (present for 2 h in both cases). Radioactive PtdCho and SM were extracted and analysed as described in the Materials and methods section. Additional unlabelled replicates were taken for measurement of lipid phosphorus in PtdCho. This experiment was one of two which gave closely similar results. Maximum incorporation of radioactivity into PtdCho in this experiment was 300 459 d.p.m. after 2 h and 486 542 d.p.m. after 48 h. Total incorporation of radioactivity into SM in this experiment was 8113 d.p.m. after 2 h and 85 183 d.p.m. after 48 h. Circles, PtdCho radioactivity; squares, SM radioactivity; triangles, PtdCho phosphorus.

the synthesis of fluorescent SM represents a considerable proportion of the amounts synthesized from endogenous ceramide in untreated cells. A calculation based on radioactivity incorporated into lipids from [^{3}H]water leads to a similar conclusion (see the Results section). Thus it is not surprising that the amount of radioactivity incorporated into sphingolipids is decreased so much by the fluorescent ceramides. Indeed, the fact that, unlike NBD-ceramide, DMB-ceramide is not converted into glycolipids and also has no effect on glycolipid labelling (Figure 7) supports the idea that inhibition of sphingolipid labelling is due to competition with endogenous ceramide. A similar metabolic competition seems to be the best explanation

Table 1 Changes in distribution of lipid radioactivity in HL-60 cells incubated with various concentrations of C_2-ceramide

HL-60 cells (about 1×10^6 cells/ml) were incubated with various amounts of C_2-ceramide in the presence of [^{14}C]acetate, and lipids were extracted and analysed after 2 h as described in the Materials and methods section. The results shown represent the percentage of total lipid radioactivity in each lipid class (means of triplicates $\pm$ S.D.) from a single experiment which was repeated once more with substantially the same outcome. Total incorporation of radioactivity into lipid in this experiment was 80 338 $\pm$ 4772 d.p.m.

	Percentage of total lipid radioactivity in each lipid class						
[C_2-ceramide] (μM)	SM	PtdCho	PtdSer/PtdIns	PtdEtn	Cholesterol	DAG	TAG
0	1.18 $\pm$ 0.08	33.2 $\pm$ 0.8	8.2 $\pm$ 0.4	10.8 $\pm$ 0.3	4.4 $\pm$ 0.2	4.6 $\pm$ 0.1	8.7 $\pm$ 0.2
1.25	1.07 $\pm$ 0.05	31.8 $\pm$ 0.6	9.1 $\pm$ 0.8	11.6 $\pm$ 0.9	4.9 $\pm$ 0.3	4.5 $\pm$ 0.5	8.7 $\pm$ 0.9
2.5	0.74 $\pm$ 0.08	30.9 $\pm$ 0.9	7.9 $\pm$ 0.5	10.7 $\pm$ 0.6	4.2 $\pm$ 0.3	4.1 $\pm$ 0.6	9.3 $\pm$ 0.6
5	0.57 $\pm$ 0.06	28.6 $\pm$ 0.5	7.6 $\pm$ 0.6	6.6 $\pm$ 0.8	4.8 $\pm$ 0.4	4.8 $\pm$ 0.2	12.9 $\pm$ 1.1
10	0.43 $\pm$ 0.04	24.6 $\pm$ 0.8	8.4 $\pm$ 0.8	5.7 $\pm$ 0.9	5.1 $\pm$ 0.5	5.2 $\pm$ 0.4	14.9 $\pm$ 1.4
20	0.35 $\pm$ 0.05	22.5 $\pm$ 0.9	7.7 $\pm$ 0.7	4.8 $\pm$ 0.5	5.3 $\pm$ 0.4	6.9 $\pm$ 0.9	21.0 $\pm$ 2.1

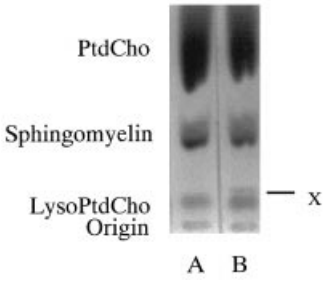


Figure 6 Synthesis of C_2-SM by BHK cells treated with C_2-ceramide

BHK cells were grown to confluence in 75 cm^2 flasks and triplicate samples were either incubated with [^{3}H]choline alone (50 μCi in 5 ml) or with [^{3}H]choline and 10 μM C_2-ceramide for 2 h. Lipids were extracted and analysed as described in the Materials and methods section and the TLC plate was phosphorimaged before staining with iodine. Spots were photographed and identified by comparison with lipid standards before being excised and counted for radioactivity by liquid scintillation. A, untreated cells; B, cells incubated in the presence of 10 μM C_2-ceramide. Putative C_2-SM is labelled X. The asterisk indicates significant difference from untreated control ($P < 0.01$). The aqueous layer from the phase partition was also analysed for radioactivity in phosphocholine and CDP-choline (see the Materials and methods section). The Table below represents the distribution of radioactivity in the labelled spots; values are given as mean d.p.m. $\pm$ S.D. for triplicate flasks of cells. This experiment was repeated once again with essentially the same result, namely a greater than 10-fold rise in radioactivity in the putative C_2-SM, accompanied by a greater than 50% decrease in the labelling of PtdCho, SM and CDP-choline.

	Radioactivity (d.p.m.)	
Lipid	(A) Untreated	(B) ($+C_2$-ceramide)
PtdCho	2039107 $\pm$ 124091	1057632 $\pm$ 50763*
SM	18270 $\pm$ 994	6316 $\pm$ 663*
LysoPtdCho	3514 $\pm$ 674	2993 $\pm$ 569
X (C_2-SM)	861 $\pm$ 49	10133 $\pm$ 403*
Phosphocholine	1089980 $\pm$ 103057	1221101 $\pm$ 137338
CDP-choline	246733 $\pm$ 39644	118217 $\pm$ 15742*

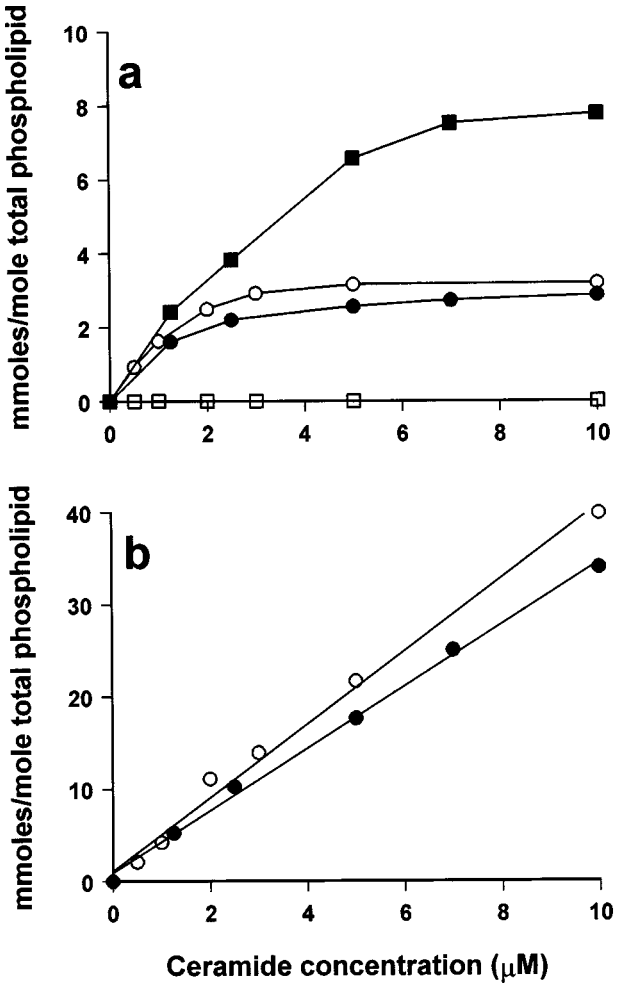


Figure 7 Metabolism of fluorescent ceramides to SMs and GluCers by BHK cells

BHK cells were incubated with 0–10 μM NBD-ceramide or DMB-ceramide for 1 h and then the lipids were extracted and analysed for fluorescent metabolites and for total lipid phosphorus. NBD-lipids are shown as closed symbols, and DMB-lipids are shown as open symbols. (**a**) GluCers (squares) and SMs (circles); (**b**) ceramides bound to cells. Results represent the means of duplicate determinations from one experiment which was repeated on two further occasions with essentially the same results.

for the inhibition of sphingolipid labelling observed with C_2-ceramide, particularly because the fall in labelling of normal SM is accompanied by a reciprocal rise in the labelling of a slow-moving lipid, which is probably C_2-SM (Figure 6).

Both with NBD-ceramide and C_2-ceramide at higher concentrations (but not with DMB-ceramide) there was a significant decrease in cholesterol synthesis (Figures 2 and 3), although the effect was greater with NBD-ceramide. There is evidence that a decrease in plasma membrane SM may cause a decrease in cholesterol synthesis [21,22] and this is confirmed by the results of treating BHK cells with sphingomyelinase (Figure 8). However, no decrease in cholesterol synthesis was seen with DMB-ceramide, despite the fact that it greatly decreased incorporation of radioactivity into SM (Figure 3C). Of course it should be noted that the total synthesis of SM (normal plus DMB-SM) was little changed, so it may be that DMB-SM (unlike C_2-SM or NBD-SM) can substitute for normal SM so far as cholesterol homoeostasis is concerned.

Quantitatively the largest effect of C_2-ceramide on BHK cells was the decrease in synthesis of PtdCho (Figures 2A, 3A, 4 and 5). Since there was little change in total lipid synthesis, it appeared that essentially all of the decrease in PtdCho labelling could be accounted for by an increase in label in DAG and TAG (Figures 2B and 3A). The DAG was not generated by lipase action on PtdCho, since in cells labelled to equilibrium with [^{3}H]choline, there was no apparent loss of label from PtdCho in the presence of C_2-ceramide and neither was there any change in the mass of PtdCho as measured by phosphate determination (Figure 5). The simplest way of interpreting this data is that one of the steps of PtdCho synthesis is inhibited so that DAG is unable to react with CDP-choline and, consequently, either accumulates or is acylated to form TAG (although a small amount may be phosphorylated to form phosphatidate; Figure 3A). Indeed, the further observation that radioactivity in CDP-choline is less in cells exposed to C_2-ceramide (Table in the legend to Figure 6) indicates that C_2-ceramide decreases the level of this crucial metabolite and it is this factor which limits the synthesis of PtdCho.

This interpretation is not convincing by itself, but previous work [23] has shown that C_2-SM (the SM corresponding to C_2-ceramide) has a powerful inhibitory effect on cytidylyltransferase,

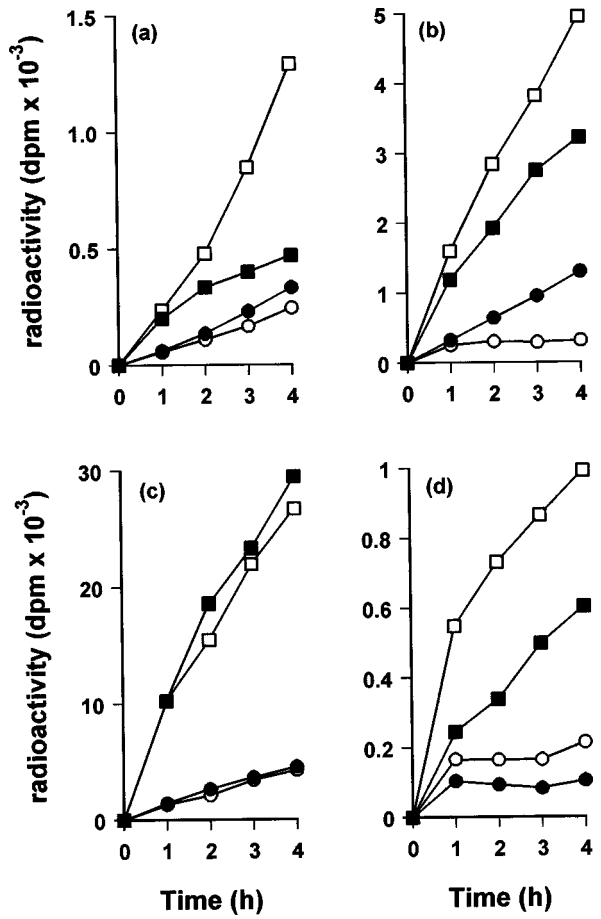


Figure 8 Time course of labelling of lipids in BHK cells incubated with [^{14}C]acetate in the absence or presence of sphingomyelinase

BHK cells were incubated for up to 4 h with [^{14}C]acetate in the absence (open symbols) or presence (closed symbols) of 50 m-units of *B. cereus* sphingomyelinase. Lipids were extracted and analysed as described in the Materials and methods section. Results represent the means of triplicate determinations in one experiment which was repeated on two further occasions with essentially the same results. (**a**), SM (squares) and ganglioside G_{M3} (circles); (**b**) cholesterol (squares) and ceramide (circles); (**c**), PtdCho (squares) and PtdEtn (circles); (**d**), TAG (squares) and DAG (circles).

the enzyme which catalyses the synthesis of CDP-choline (the rate-limiting step in PtdCho synthesis) [24]. Since C_2-ceramide is converted into C_2-SM by fibroblasts (Figure 6; [12]), it seems plausible that the inhibition of PtdCho synthesis in BHK cells is caused by C_2-SM synthesized from the added C_2-ceramide. Such an interpretation is supported by the evidence that other artificial choline lipids such as hexadecylphosphocholine and 1-*O*-octadecyl-2-*O*-methylglycero-3-phosphocholine ('ET-18-OCH3') have an inhibitory effect on PtdCho synthesis through their action on cytidylyltransferase [25–27]. No increase in production of acylglycerols or phosphatidate has previously been reported using these inhibitors of cytidylyltransferase, but such accumulations would be logical consequences of this metabolic blockade.

The large inhibition of lipid synthesis induced by C_2-ceramide could be the cause of its marked apoptotic effects (Figure 1). Indeed, recent work shows that defective synthesis of PtdCho is by itself sufficient to cause apoptosis; thus mutant Chinese-hamster ovary ('CHO') cells possessing a temperature-sensitive cytidylyltransferase undergo apoptosis when exposed to temperatures which inactivate this enzyme [28], and HeLa cells or HL60 cells are rescued from apoptosis caused by inhibitors of PtdCho synthesis if cytidylyltransferase is up-regulated [26] or if lyso-PtdCho is added as an alternative source of PtdCho [27]. There is even a possibility that C_2-ceramide as a natural metabolite could affect normal lipid synthesis, since at least in HL60 cells, endogenous C_2-ceramide can be formed from platelet-activating factor [29].

However, with BHK cells, NBD-ceramide had a much smaller inhibitory effect on PtdCho synthesis than did C_2-ceramide, and DMB-ceramide had no effect at all, in spite of the fact that these fluorescent ceramides were apoptotic at lower concentrations than C_2-ceramide (Figure 1). Indeed, DMB-ceramide caused no changes in labelling of any lipid apart from SM, so comparing the different short-chain ceramides it appears that only the ability to inhibit synthesis of normal SM consistently correlates with apoptosis.

Neither breakdown of surface SM by exogenous sphingomyelinase nor the consequent accumulation of ceramide of natural fatty acid composition caused apoptotic changes in BHK or HT29 cells [14]. This was consistent with the observations of Zhang et al. [30], who showed that exogenous sphingomyelinase was not apoptotic in tumour cells, even though it converted large amounts of cell-surface SM into ceramide, whereas expression of cloned bacterial sphingomyelinase which broke down only an internal pool of SM did cause apoptosis. Exogenous sphingomyelinase had no significant effect on PtdCho synthesis in BHK cells, although it did decrease labelling of cholesterol and acylglycerols (Figure 8). There was no evidence that synthesis of SM was decreased by this treatment, since total radioactivity in SM plus ceramide was similar in treated and untreated cells, so this again accords with the notion that the ability of ceramides to cause apoptosis correlates with their capacity to competitively inhibit synthesis of normal SM.

A significant conclusion from the present study is that although C_2-ceramide may still be a useful tool for the investigation of apoptotic mechanisms, it needs to be used with some caution, because in addition to any effects it may possess as a cell-surface messenger molecule it has multiple effects on internal lipid metabolism, some of which (e.g. inhibition of cytidylyltransferase) could well be important in the control of apoptosis. Nevertheless, it seems clear that inhibition of cytidylytransferase is not the only 'metabolic' effect of short-chain ceramides which could be involved in apoptosis, since DMB-ceramide causes apoptosis without any apparent effect on PtdCho metabolism, although, significantly, it does inhibit synthesis of normal SM, as do the other apoptotic short-chain ceramides. At this stage it is not clear whether inhibition of SM synthesis could be a generally important process in the control of apoptosis, although it would not be surprising if interference with the molecular structure of this major lipid component of the cell surface had important morphological and functional consequences.

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