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Metabolic effects of short-chain ceramide and glucosylceramide on sphingolipids and protein kinase C

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Recent studies have identified a potential role for glucosylceramide (GlcCer) in growth promotion and hormonal signalling. In an effort to demonstrate a growth-promoting activity of GlcCer, we prepared a GlcCer having a short-chain acid (octanoyl), in the belief that this glycolipid could be absorbed more readily and more uniformly by cultured cells. By using a mixture of two specific lecithins, dioleoylglycerophosphocholine and 1-stearoyl-2-palmitoylglycerophosphocholine, we were able to prepare dispersions containing a high molar proportion of the GlcCer and the related ceramide, octanoyl sphingosine. Unexpectedly, both sphingolipids inhibited protein and DNA synthesis in Madin-Darby canine kidney cells and produced large increases in the levels of the natural lipids, GlcCer, ceramide, free sphingosine, and an amine that may be glucosylsphingosine (GlcSph). Decreases were seen in the level of sphingomyelin and the proportion of protein kinase C in the cell membranes. The level of lactosylceramide was diminished by octanoyl GlcCer but elevated considerably by octanoyl sphingosine. Diacylglycerols were increased by the lecithins in the liposomes, but the exogenous sphingolipids had no effect. Octanoyl sphingosine labeled in the sphingoid base yielded labeled GlcCer and sphingomyelin labeled in both long-chain and very-long-chain fatty acid families, as well as the octanoyl version. The two families of ceramides, however, had relatively little radioactivity. Some of these changes are attributed to rapid hydrolysis of the added lipids with the formation, particularly from the ceramide, of sphingosine and its anabolic metabolite, GlcSph. Several observations support the idea that the octanoyl sphingosine inhibited the phosphocholinetransferase that synthesizes sphingomyelin while the octanoyl GlcCer inhibited GlcCer β-glucosidase and GlcCer galactosyltransferase. The use of unnatural short-chain lipids in the study of cell growth and other phenomena may result in unexpected changes in related metabolites and the findings from such experiments should therefore be interpreted cautiously.

Many reports have demonstrated that some kinds of glycosphingolipids play important roles in cell growth, proliferation, and development. Exogenous application of sphingolipids to cells provides one approach to the understanding of their function. Another approach involves depletion of cell glucosphingolipids (GSLs) by means of an inhibitor of glucosylceramide (GlcCer) synthase [1–4]. With the latter approach we have seen many biological changes but a question always arises: are the changes due to lack of GSLs or to a second, unknown effect of the inhibitor? This question can be answered to some extent by replacing the depleted lipids by specific exogenous GSLs, but a technical problem in lipid addition to intact cells has often been encountered.

The more polar glycolipids, such as gangliosides, are easily dispersed into culture media and incorporated into cells, but the less polar sphingolipids, such as GlcCer and ceramide (Cer), are very insoluble in water. Frequently used methods for adding these lipids to cell media, such as complexing with bovine serum albumin or cyclodextrin, or addition as solutions in ethanol or dimethylsulfoxide, have in our hands all yielded sedimentable suspensions that may adhere selectively only to some of the cells and regions of the culture dishes. Bovine serum albumin has the additional disadvantage of acting as a ‘lipid sponge,’ removing selected lipids from cell surfaces.

While we have used stable emulsions in a nonionic detergent for animal work [5], the detergent has been found to have some deleterious effect on cells, particularly at low cell densities [6]. We have also used liposomal mixtures of lecithin and sphingolipids, which seem to be stable, but relatively large amounts of lecithin were necessary and this lipid too acts as a lipid sponge and exerts its own biological effects. In previous studies short-chain sphingolipids were used as enzyme substrates [7, 8] and short-chain analogues were used as inhibitors of sphingolipid enzymes [9]. Many laboratories have taken advantage of the higher solubility and fluidity of dioctanoyl

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Abbreviations. Cer, natural ceramide; Dec, decanoyl; GlcCer, natural glucosylceramide; GlcSph, 1-*O*-glucosylsphingosine; GSL, glucosphingolipid; Lac, lactosyl; MDCK cells, Madin-Darby canine kidney cells; OctSph, *N*-octanoyl sphingosine; OctGlcSph, *N*-octanoyl glucosylsphingosine; PDMP, *threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol; PKC, protein kinase C; Sph, sphingosine; Ste, stearoyl; buffer A, 0.02% KCl, 0.8% NaCl, 0.02% KH_2PO_4, 0.115% Na_2HPO_4.

Enzymes. Ceramide glucosyltransferase (EC 2.4.1.80); β-glucosidase (EC 3.2.1.21).

glycerol, using it as a substitute for normal-chain diacylglycerols [10, 11]. *N*-Acetyl sphingosine has also been used as a type of Cer without full consideration of its possible secondary metabolic effects [12]. *N*-Octanoyl sphingosine produced a striking activation of a protein kinase in intact cells [13].

In this study, we have utilized the same approach, with liposomes consisting of a specific mixture of lecithins (controls) and lecithins with a short-chain sphingolipid [*N*-octanoyl glucosylsphingosine (OctGlcSph), *N*-decanoyl galactosylsphingosine (DecGalSph) or *N*-octanoyl sphingosine (OctSph)] were applied to cultures of Madin-Darby canine kidney (MDCK) cells, which incorporated the lipids and metabolized them. We found that OctGlcSph and OctSph, in particular OctSph, inhibited the growth of MDCK cells in low-density cultures. Marked increases in cell levels of normal fatty-acyl-chain Cer and GlcCer were also seen. The interrelationships and functions of sphingolipids, diacylglycerol and protein kinase C (PKC) in cell growth will be discussed.

EXPERIMENTAL PROCEDURES

Materials

Dioleoylglycerophosphocholine, phosphatidylserine from bovine brain, cardiolipin from bovine heart, 1,2-dioleoyl-*sn*-glycerol, sphingomyelin from bovine brain, ceramide type III from bovine brain sphingomyelin, *n*-octyl β-D-glucoside, phenylmethylsulfonyl fluoride, histone III-S, L-cycloserine, sphingosine, phytosphingosine, and DEAE-cellulose were from Sigma Chemical. 1-Stearoyl-2-palmitoylglycerophosphocholine was from Avanti Chemical. Dithiothreitol was from Calbiochem. [γ-^{32}P]ATP (specific activity 25 Ci/mmol), [6-^{3}H]thymidine (specific activity 27 Ci/mmol) and Ecolume scintillation fluid were from ICN Radiochemicals. Dulbecco-modified Eagle's medium mixed 50/50 with Ham's F-12 was from Mediatech. [3-^{3}H]Sphingosine, from Du Pont NEN Products, was purified with a silica gel column and chloroform/methanol/2 M NH_4OH (80:20:2, by vol.). It was then acylated with octanoyl chloride and the resultant OctSph was purified with a silica gel column using chloroform, then chloroform/methanol (99:1).

Trypsin/EDTA (1 × solution) was from Irvine Scientific. Glucosylceramide was prepared from Gaucher spleen [14] and hydrolyzed to GlcSph [15]. Lactosylceramide (LacCer) was prepared from brain gangliosides by acid hydrolysis. OctSph, OctGlcSph and DecGalSph were prepared here previously. Cerebroside sulfate sodium salt ($GalCerSO_4$) was isolated from pig brain.

Preparation of liposomes

Liposomes were prepared by the method of Batzri and Korn [16], which involves rapid injection of an ethanolic solution of lipids into sterile phosphate-buffered saline (buffer A, 0.02% KCl, 0.8% NaCl, 0.02% KH_2PO_4, 0.115% Na_2HPO_4). In the preparation of lecithin liposomes, a solution containing 3.2 µmol dioleoylglycerophosphocholine (Ole_2Gro*P*Cho) and 3.2 µmol stearoylpalmitoylglycerophosphocholine (StePamGro*P*Cho) was evaporated to dryness with a stream of N_2 and redissolved in 210 µl ethanol. In the preparations of the other liposomes, the ethanolic lecithin solution also included 3.3 µmol OctSph, 3.2 µmol OctGlcSph, or 3.1 µmol DecGalSph. Each ethanolic solution (200 µl) was injected into 2.8 ml sterile buffer A. The liposomes were left for 1 h at room temperature before further dilution with medium.

Cell studies

Confluent Madin-Darby canine kidney (MDCK) cells were removed from the culture dishes with trypsin/EDTA solution and $5-10 \times 10^5$ cells (low density) or 5×10^6 cells (high density) were seeded into a round dish (80 × 20 mm, nominally 100 mm diameter) containing 8 ml serum-free medium and incubated at 37°C in the CO_2 incubator. The culture medium was replaced 24 h later by 7.75 ml of the same medium and 250 µl sterile buffer A containing 6.67% ethanol or by 250 µl liposomes. The concentrations of the sphingolipids in the medium were thus 33 µM OctSph, 32 µM OctGlcSph and 31 µM DecGalSph. The concentration of each lecithin was 32 µM.

In the study of thymidine incorporation, 10 µl [^{3}H]-thymidine (10 µCi) was added to the dish 20 h after adding the liposomes, and the cells were incubated for another 4 h. The cells were scraped into a glass tube with 2 ml 10% (mass/vol.) trichloroacetic acid and 1.5 ml 6.7% trichloroacetic acid. The cell pellets collected by centrifugation at $2500 \times g$ for 20 min were dissolved in 1 – 2 ml 0.6 M NaOH. A portion of the NaOH was mixed with Ecolume after neutralization with dilute HCl and counted by liquid scintillation. Another portion was used to determine the protein content and to extract sphingolipids. Protein was determined by the method of Lowry et al. [17] or Bensadoun and Weinstein [18].

Lipid studies

The lipids in the NaOH solution, neutralized with acetic acid, were extracted by mixing with chloroform and methanol so that the ratio of chloroform/methanol/water was 10:5:4. The mixture was centrifuged for 10 min at $1000 \times g$, the upper layer was discarded, and the lower layer (without the interfacial material) was transferred into a glass tube. The interfacial material was extracted with fresh chloroform/methanol/water in the same ratio and the second lower layer was combined with the first one. The GSLs, sphingomyelin, and Cer in the extract were separated by TLC on silica gel 60 plates (HPTLC 10 × 10 cm, Merck) and visualized by charring.

For the analysis of free sphingoid bases, the dried lipids were analyzed by a modification of the *o*-phthalaldehyde chromatography method of Merrill et al. [19]. Tridecylamine was used as the internal standard and the amines were purified with a cellulose phosphate column prior to derivatization.

The assay for diacylglycerol was carried out according to Preiss et al. [20], using cells scraped into a glass tube with two 2-ml portions of cold methanol. Known amounts of dioctadecenoylglycerol (diolein) and ceramide were used to obtain a standard curve. In this method, OctSph, diacylglycerol, and Cer were converted to OctSph 1-phosphate, phosphatidic acid, and ceramide 1-phosphate, respectively, by diacylglycerol kinase from *Escherichia coli*.

Protein kinase C (PKC) activity

All procedures were carried out at 0 – 4°C. Cells were washed three times with 8 ml cold buffer A and scraped with 1 ml, 1.5 ml and 1.5 ml buffer B (20 mM Tris/Cl pH 7.5 at room temperature, 0.5 mM EDTA, 0.5 mM EGTA, 10 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride and 25 µg/ml leupeptin) into a glass centrifuge tube. The cell suspension was sonicated in ice water for 12 5-s periods with a probe sonicator, then centrifuged for 1 h at $40000 \times g$. The

Table 1. Preparation of liposomes. Solutions of the various lipids were dried down under a stream of N_2 gas. The amount of OctGlcSph, 0.75 µmol, was kept constant in all preparations; 500 µl buffer A was added to the dried lipids and sonicated for 15 min at 60°C with a microtip probe.

Mixture	Molar ratio of			
	OctGlcSph	$Ole_2GroPCho$	StePamGro*P*Cho	$GalCerSO_4$
1	1	1	1	1
2	1		1	
3	1		10	
4	1	1	1	
5	1		1	1

resultant liquid and pellet were processed by the method of Kolesnick and Clegg [21] to purify and assay the PKC contents.

The activity of PKC was defined as the kinase activity in the presence of activator (diacylglycerol and phosphatidylserine) minus the activity in the absence of activator.

RESULTS

The dispersion of sphingolipids

Dispersions of GlcCer and Cer that included a relatively large proportion of egg lecithin were apparently stable but in several experiments the dispersions produced no effects that could not also be produced by lecithin alone. We found the same problem even with short-chain Cer and GlcCer. We also tried to incorporate OctGlcSph and OctSph into bovine serum albumin, in a 1:1 molar ratio, according to the procedure of Merrill et al. [22]. A solution of sphingolipid in absolute EtOH (0.5 µmol in 0.1 ml) was rapidly injected into a bovine serum albumin solution (0.5 µmol in 0.25 ml buffer A), then sonicated with a dipping probe for 30-s intervals in an ice bath. Aggregated lipid could be seen in the mixture. Similar results were obtained when the bovine serum albumin solution was added to the dry lipid and sonicated.

We then tested the possibility of using mixtures of pure, synthetic lecithins which might be more effective than a natural mixture. Various ratios of coevaporated lipids in buffer A were used with a probe-type sonicator, containing a relatively high amount of OctGlcSph in order to minimize any effects of the lecithin (Table 1). The results were evaluated visually under a bright light. Mixture 4 yielded apparently homogeneous suspensions that showed no aggregation after storage for 24 h at room temperature. Preparations 1 and 5, containing $GalCerSO_4$, were clear initially but formed gelatinous particles. Mixtures 2 and 3 eventually formed a sediment. This set of trials was done with water as the sole solvent and it was necessary to heat and sonicate in order to make liposomes. Fortunately the MDCK cells appeared to grow well in 0.2% EtOH and the high solubility of mixture 4 in absolute EtOH ($\approx$ 15 mM each lipid) made it possible to simplify the preparation of stable dispersions by rapidly injecting the ethanolic solution into sterile medium, without heating or sonicating. The remaining studies used mixtures of $Ole_2GroPCho$/StePamGro*P*Cho (1:1, used as a control), with an equimolar amount of OctSph, OctGlcSph, or DecGalSph (final concentration of each lipid $\approx$ 32 µM).

Table 2. Effect of octanoyl glucosylsphingosine and octanoyl sphingosine on the growth of MDCK cells. 5×10^5 MDCK cells were seeded into an 8-cm dish containing 8 ml Dulbecco's modified Eagle's medium without serum; 24 h after the incubation, the cells were treated with control or lipid-supplemented medium, as described in Materials and Methods. Four dishes were used in each condition. DecGalSph was used as an additional control for lecithin effects; it showed no evidence of metabolism to sphingomyelin, long-chain cerebroside, or natural ceramide (data not shown).

Condition	Protein	$10^{-6} \times$ [^{3}H]Thymidine incorporation
	µg/dish	cpm/mg protein
Control medium	507 ± 61	1.28 ± 0.092
Buffer A	457 ± 47	1.38 ± 0.064
Lecithins	429 ± 39	1.38 ± 0.085
32 µM OctGlcSph	330 ± 38[a]	0.88 ± 0.034[a]
31 µM DecGalSph	440 ± 63	1.37 ± 0.079
33 µM OctSph	230 ± 11[a]	0.91 ± 0.067[a]

[a] $P < 0.001$.

Effects of OctGlcSph and OctSph on the growth of MDCK cells

It has frequently been reported that specific glycosphingolipids affect cell growth, stimulating proliferation in some studies, stimulating differentiation in others. Recently, it has been shown that the depletion of GlcCer by PDMP treatment resulted in the inhibition of cell and organ growth [1, 5, 23]. The addition of OctGlcSph to the MDCK medium was expected to produce the opposite effect. To confirm this supposition, we measured [^{3}H]thymidine incorporation and protein content of the cells. [^{3}H]Thymidine incorporation into the cells at low density was significantly inhibited by treatment with OctGlcSph liposomes (63%) or OctSph liposomes (65%; Table 2). The protein content per incubation dish was also decreased by the treatments (Table 2). The values shown in the table were variable between experiments but OctGlcSph and (particularly) OctSph liposomes were always inhibitory to low-density culture cells. Permeability tests with trypan blue showed no evidence that these effects were due to a general type of toxicity.

There was no significant effect of 0.2% ethanol or lecithin liposomes. DecGalSph liposomes had no effect on cell proliferation, showing the importance of the glucose moiety. The cells cultured at high density with control liposomes or OctSph liposomes showed little difference in protein synthesis or conversion of thymidine to DNA (data not shown).

Incorporation of OctGlcSph and OctSph into MDCK cells and changes in sphingolipid composition

Marked accumulation of GlcCer, OctGlcSph, Cer, and OctSph could be seen by charring-TLC after incubation of low-density cultures with OctGlcSph liposomes (lane 3 in the illustrative plates of Figs 1 and 2). The short-chain sphingolipids separated distinctly from the normal twin bands (C_{24} and C_{18} families) of the naturally occurring lipids. LacCer, the galactose derivative of GlcCer, was decreased (Fig. 1, lane 3; the changes may not be visible in the photograph because of the relatively low concentrations). These lipids were identified not only by their excellent matches in R_F with standards but also by their stability in alkali.

OctGlcSph also produced a small reduction in sphingomyelin (Fig. 1, lane 3; the changes may not be visible in the

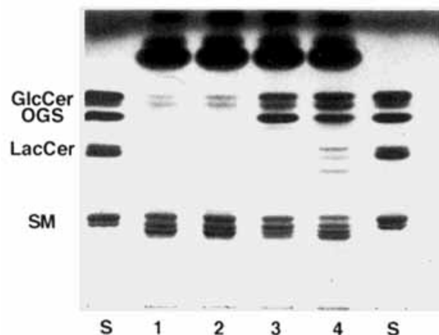

Fig. 1. Thin-layer chromatogram showing charred polar lipids from MDCK cells after a 24-h incubation with different sphingolipids. Lipids that were alkali-stable were extracted from the solution of cell pellet in 0.6 M NaOH and developed with chloroform/methanol/water (60:35:8). Aliquots containing an equal amount of protein (0.45 mg) were used for extraction. S, standard lipids [GlcCer doublet, OctGlcSph, LacCer doublet (natural LacCer), sphingomyelin doublet (SM)]. Lane 1, buffer A/ethanol control cells; lane 2, lecithin liposomes/ethanol control cells; lane 3, OctGlcSph (OGS) liposomes; lane 4, OctSph liposomes. Similar plates were obtained in two other experiments.

Table 3. Radioactive lipids formed by MDCK cells from [^{3}H]choline. MDCK cells (0.5×10^6) were incubated 24 h with 10 μCi [*methyl*-^{3}H]choline, then the medium was replaced with standard medium containing mixed lecithin liposomes or liposomes containing octanoyl sphingosine and incubation was continued for 24 h. The lipids were chromatographed with chloroform/methanol/acetic acid/water (100:60:16:8), the TLC plate was exposed to X-ray film, and the labeled regions were scraped off and counted. Data from a similar experiment showed an even greater OctSph-induced decrease in the amount of synthesized sphingomyelin. The unknown lipid migrated just below lecithin.

Lipid	Amount formed by	
	control cells	OctSph cells
	cpm	
Lecithin	15860	16320
Unknown	420	320
C_{24} sphingomyelins	2880	1860
C_{18} sphingomyelins	2760	1910
C_8 sphingomyelin	–	2540

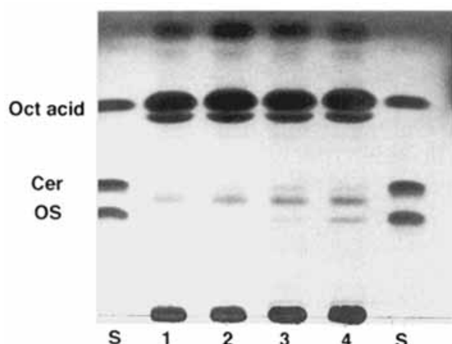

Fig. 2. Thin-layer chromatogram showing the charred less polar lipids from cells incubated with OctSph and OctGlcSph. TLC was done as in Fig. 1 but developed with chloroform/acetic acid (9:1). The top band in the standard is octanoic acid (Oct acid). Lane labels as in Fig. 1. Similar plates were obtained in two other experiments. The heavy band corresponding to octanoic acid contains the free fatty acids formed from the ester lipids by the reaction with alkali. The smaller band just below it is probably cholesterol and the band at the top of the plate may be cholesterol esters, which are more stable to alkali than the glyceryl esters.

photograph). It may be noted that the standard sphingomyelin used here, derived from brain, exhibited just the two C_{24} and C_{18} family bands. However, the sphingomyelin from control MDCK cells exhibited three major bands. The lowest band is probably due to hydroxylated sphingomyelins, which have been found in bovine kidney sphingomyelins [24]. The sphingomyelin from OctGlcSph-treated cells (lane 3) also contained an even slower band, evidently due to utilization of OctSph by sphingomyelin synthase.

In the OctSph-treated cells there were similar accumulations of the GSLs (lane 4, Figs 1 and 2). It should be noted that here too the increases in the natural lipids occurred in both acyl families. It is evident that some of the OctSph had been converted to OctGlcSph, OctLacCer, and short-chain sphingomyelin. A striking difference between OctSph and OctGlcSph is that the latter lowered the natural LacCer level while the former increased it. Also the naturally occurring sphingomyelin families decreased more than with OctGlcSph.

Confirmation of the identity of the octanoyl sphingomyelin was obtained by prelabeling cells with 10 μCi [*methyl*-^{3}H]choline for 24 h. The cells were then incubated another 24 h with either OctSph liposomes or lecithin alone. A TLC plate of the cell lipids yielded a radioautogram that showed the usual wide dark band for labeled lecithin and a pair of labeled sphingomyelins in both groups of cells. The OctSph-treated cells also showed a labeled octanoyl sphingomyelin band below the slower of the two sphingomyelin families. Measurement of the tritium in each band (Table 3) showed in addition that the OctSph had slowed the phosphocholine transfer to natural Cer, as indicated by the paler charred spots in Fig. 1.

An interesting discovery was made by comparing the radioautograph (not shown) and the charred plate (Fig. 1). The latter showed a distinct sphingomyelin band corresponding to hydroxylated sphingomyelin but the radioautogram did not show this family of lipids. Evidently the hydroxylation step occurs with older Cer or sphingomyelin molecules; the recently formed molecules (made during the 48-h incubation) were not appreciably oxidized.

Cells treated with lecithin liposomes alone showed no marked changes except for a small increase in GlcCer (Fig. 1, lane 2).

The figures show that both OctGlcSph and OctSph were taken up from liposomes and metabolized by MDCK cells, and induced marked changes in the sphingolipid composition of the cells. Similar changes in sphingolipid composition were also observed when high-density cultures were treated with lecithin or OctGlcSph liposomes (data not shown). The specificity of the effects was shown by the lack of distinct changes when the galactosyl analog (DecGalSph) was included in the cell medium even though the glycolipid was readily absorbed (data not shown).

The increased level of normal Cer, due to OctSph, could be the result of hydrolysis of the absorbed short-chain lipid to sphingosine and octanoic acid by ceramidase. The increased level of sphingol (sphingoid base) would then be expected to increase the rate of *N*-acylation by the various Cer synthases [25]. Sphingols, applied exogenously to cell cultures, have usually been found to inhibit growth by a variety of mecha-

Table 4. Sphingol contents of MDCK cells. Each dish was seeded with 10^6 cells and, after growth for 24 h in stock medium, buffer A or liposomes were added in fresh medium. The liposomes were prepared from lecithins alone, lecithins + OctGlcSph, or lecithins + OctSph. After 24 h of additional culture, the cells were washed, removed with methanol, and analyzed for sphingosine and a peak tentatively identified as GlcSph; n.d. = not detectable.

Condition	Sphingosine	GlcSph
	pmol/mg protein	
Buffer A controls	38 ± 2	n.d.
Lecithin controls	39 ± 9	n.d.
OctGlcSph + lecithin	452 ± 61	218 ± 7
OctSph + lecithin	466 ± 74	11 ± 11

nisms [26]; thus an increased sphingol level could conceivably account for the observed growth inhibition produced by OctSph. Accordingly we assayed the cells for sphingol content by fluorometric chromatography.

As shown in Table 4, treatment of MDCK cells at low culture density with OctGlcSph and OctSph caused a marked increase in Sph and a peak corresponding in elution time to GlcSph. The Sph content of the OctSph- and OctGlcSph-treated cells was about 12 times that of the control cells. In the control cells, treatment with lecithin liposomes caused no increase in Sph level. A very large increase in the level of GlcSph was seen in the cells incubated with OctGlcSph and a smaller increase with OctSph.

In the cells at high density culture (data not shown), lower levels of Sph were seen. Here too OctGlcSph liposomes caused an increase in Sph and GlcSph. The lower contents can be explained as being due to slower sphingolipid metabolism in cells near confluence. Lecithin liposomes had no effect. The results are consistent with the idea that the short-chain Cer and GlcCer, after uptake from the medium, were partially hydrolyzed to free bases which were then recycled with normal fatty acids to form normal Cer and GlcCer (and LacCer). The reacylation was not rapid enough to prevent accumulation of some free sphingol.

Further evidence came from cells incubated with liposome-containing sphingosine-labeled OctSph, specific activity 69000 cpm/μmol. The radioautogram from a TLC plate, prepared in the usual way [3], showed distinct bands corresponding to labeled GlcCer and sphingomyelin as well as OctGlcSph and octanoyl sphingomyelin (Fig. 3, lane 1). This confirms the identification of the slower bands seen in the charred TLC plates, the result of utilization of OctSph by the two synthases. Lipids that were not treated with alkali (lane 3) yielded the same results, showing there were no labeled ester lipids. Some OctLacCer could also be seen in the cell lipids.

Curiously, the Cer region of the plate, checked further with a less polar solvent, showed unexpectedly little radioactivity in the natural Cer bands. (The silica gel bands were counted directly to confirm this.) Yet radioactive mixed ceramides must have formed from the exogenous OctSph in order to yield the labeled GlcCer and sphingomyelin. This contradiction with the charred plate (Fig. 2) is discussed below.

An interesting side observation in the labeled OctSph experiment is that the medium contained not only unutilized OctSph but also labeled OctGlcSph and octanoyl sphingomyelin (Fig. 3, lanes 2 and 4). Perhaps these short-chain sphingolipids are not as tightly bound to membranes as the natural longer-chain lipids.

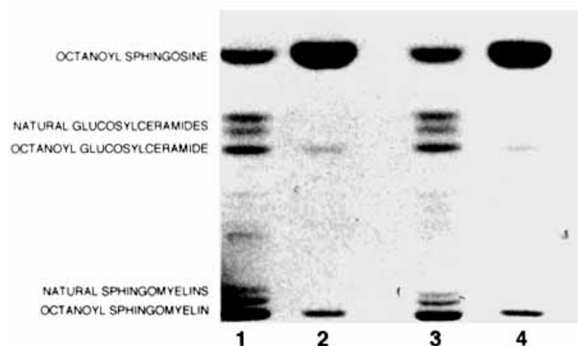

Fig. 3. Radioautogram from TLC plate prepared with the MDCK cell lipids after incubation for 24 h with octanoyl [^{3}H]sphingosine in lecithin liposomes. The cell lipids, in lane 1, were treated with alkali to destroy glyceryl esters and then chromatographed as in Fig. 1; the cell lipids in lane 3 were chromatographed directly. The culture medium was also extracted with chloroform/methanol and the lipids processed similarly (lanes 2 and 4). The fastest band is *N*-octanoyl sphingosine, the triplet bands below this are GlcCer (including OctGlcCer), and the triplet bands near the bottom are sphingomyelin (including octanoyl sphingomyelin).

The lipids shown in Figs 1 and 2 were chromatographed after treatment with alkali. When a different set of total cell lipids was chromatographed in the same way, without alkaline cleavage, a relatively dark band was seen migrating between Cer and GlcCer only in the cells incubated with OctSph and OctGlcSph. Judging by the position of the band, it could be a fatty acyl ester of GlcCer. Such esters are prominent in skin [27, 28] and have also been found in human Gaucher spleen [29].

Effect of inhibitors of sphingolipid metabolism

One possibility is that OctGlcSph and OctSph produced an elevation in sphingol levels by stimulating their synthesis *de novo*. L-Cycloserine, an inhibitor of 3-keto-sphinganine biosynthesis [30], was applied for 24 h to MDCK cells at low-density culture with or without lecithin and OctSph liposomes. The Cer and diacylglycerol in the cells were phosphorylated with diacylglycerol kinase and [^{32}P]ATP and chromatographed. The TLC radioautogram shows that the formation of Cer in control cultures was markedly blocked by incubation with 2 mM cycloserine (Fig. 4, lanes 2 and 4 vs 1 and 3). Evidently the Cer normally formed by hydrolysis of GlcCer and sphingomyelin was so rapidly hydrolyzed or reutilized for sphingolipid synthesis that it could not maintain the normal level during the 24-h exposure to cycloserine. In contrast, the cells incubated with OctSph liposomes (lanes 5 vs 6) maintained a high level of total ceramide (normal Cer plus OctSph) whether or not cycloserine was present. Evidently the rapid uptake and hydrolysis of OctSph maintained Sph at so high a level that resynthesis of Cer was not appreciably affected by cycloserine's blockage of Sph biosynthesis. Quantitative data are presented in the next section.

In all cases, treatment of the cells with cycloserine resulted in a small decrease in diacylglycerol content of the cells. The marked accumulation of GlcCer and OctGlcSph induced by OctSph was unaffected by the presence of cycloserine (data not shown). However, in cells treated with normal medium or with lecithin liposomes, cycloserine produced a marked depletion of GlcCer. We have recently shown the same loss of GlcCer in mice treated with cycloserine [5]. The results suggest

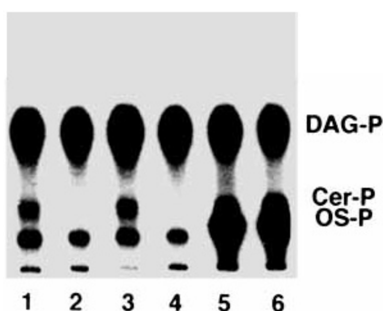

Fig. 4. Effect of 2 mM D-cycloserine on MDCK cells incubated 24 h with buffer A/ethanol alone, PtdCho liposomes, or OctSph/PtdCho liposomes. After treatment of the lipids with [^{32}P]ATP and diacyl glycerol kinase, they were chromatographed on a TLC plate with chloroform/methanol/acetic acid (65:15:5) and exposed to Kodak X-Omat film. DAG-P, diacylglycerol phosphate formed from diacylglycerol; Cer-P, ceramide phosphate formed from Cer; OS-P, phosphate formed from OctSph. Lanes 1 and 2 are derived from buffer A control cells; lane 2 included cycloserine. The radioactive spot below ceramide phosphate appeared in control runs with diacylglycerol alone; it may be lysophosphatidic acid formed from the diacylglycerol phosphate by a trace of phospholipase contaminating the commercial kinase. Lanes 3 and 4 are from two other groups of controls, treated with mixed lecithins alone; lane 4 included cycloserine. Lanes 2 and 4 show the virtual absence of Cer due to blockage of sphingol synthesis by cycloserine. Lane 5, from cells grown with OctSph, shows a large spot for OctSph-*P*, obscuring the intense Cer-P spot. It overlaps the lower spot, the putative lysophosphatidic acid seen in all the lanes. Lane 6 shows the lipids from cells grown with OctSph + cycloserine; comparison with lane 5 shows that the cycloserine did not cause a decrease in OctSph or Cer. (The film was slightly overexposed to show the Cer-P spots more clearly.)

that the rate of GlcCer formation is distinctly reduced by a lack of sphingol unless exogenous OctSph or OctGlcSph is available as a source of sphingosine or Cer.

A similar test using conduritol B epoxide for 24 h showed that GlcCer accumulated markedly in the cells (although not as much as with OctSph or OctGlcSph). This inhibitor blocks β-glucosidase so it causes a model form of Gaucher disease, with GlcCer appearing in part because of normal synthesis and in part because of hydrolysis of LacCer and the higher glycolipids. When cycloserine was also included in the incubation medium, the level of GlcCer was normalized, indicating that most of the conduritol-epoxide-induced accumulation was the result of blocking GlcCer hydrolysis, rather than coming from hydrolysis of the higher GSLs. GlcCer is the major glycolipid in MDCK cells [31] so this finding is not surprising.

Effect of OctGlcSph and OctSph on diacylglycerol and ceramide levels

The level of diacylglycerol, which is well known as an activator of PKC, was determined (together with Cer) by using [^{32}P]ATP and diacylglycerol kinase from *E. coli*. Incubation of lecithin, OctGlcSph, or OctSph liposomes with cells at low density all resulted in a 50% increase in diacylglycerol in the cells (Table 5). Evidently all three types of liposomes generated diacylglycerol from the lecithins in the liposomes.

In this assay system, the conversion efficiency of OctSph to OctSph-*P* by diacylglycerol kinase was about 70% of that of Cer. Unfortunately the spots for Cer-*P* and OctSph-*P* were too close to permit separate scraping and counting, so the

Table 5. Diacylglycerol and ceramide content of MDCK cells. 5×10^5 MDCK cells were seeded into an 8-cm dish. Cells were incubated 24 h with or without liposomes, then the diacylglycerol, Cer, and OctSph were converted to phosphatidic acid, ceramide phosphate, and octanoylsphingosine phosphate, respectively, by diacylglycerol kinase. Some phosphatidic acid was produced by blank incubations, possibly derived from the cardiolipin in the incubation medium, due to a phospholipase contaminant in the kinase; this value was subtracted from the sample values. The value of Cer for OctGlcSph and OctSph liposomes studies includes both normal-chain Cer and OctSph. Each value shown is an average ± SD from three dishes.

Condition	Diacylglycerol	Ceramide
	nmol/mg protein	
Buffer A control medium	8.25 ± 0.028	1.12 ± 0.16
PtdCho liposomes	12.0 ± 0.14	1.37 ± 0.053
OctGlcSph liposomes	11.9 ± 0.35	4.02 ± 0.021
OctSph liposomes	12.7 ± 1.41	11.7 ± 3.32

reported values refer to the sum of both types and are therefore a little low. As observed semiquantitatively in Fig. 2, lecithin liposomes did not influence the Cer level of the cells while OctGlcSph and OctSph liposomes caused a 3.6-fold and 10-fold increase, respectively, in the total ceramide (Cer plus OctSph). From Fig. 2, it is evident that a significant portion of the measured increase in Cer produced by OctSph was due simply to uptake of OctSph.

DecGalSph (not shown in the table) also caused a 50% increase in diacylglycerol, due to the presence of lecithins in the liposomes. The ceramide level was unchanged but a radioactive band, possibly formed from DecSph by hydrolysis of the galactolipid (0.72 nmol/mg protein) could be seen.

Effect of OctGlcSph and OctSph on protein kinase C in MDCK cells

Because of the known involvement between diacylglycerol and PKC, we assayed the soluble and particulate PKC activity in MDCK cells seeded at low density (Table 6). On the one hand, in both the no-liposome control and the lecithin-treated cells, around 75% of the total activity of PKC was found in the soluble fraction. (A similar percentage was found in MDCK cells by Godson et al. [32].) On the other hand, in the OctGlcSph-treated and OctSph-treated cells, the PKC activity in the soluble fraction reached about 90% of the total activity. These results may signify that elevated levels of Cer or GlcCer reduce the binding between plasma membranes and PKC.

β-Glucosidase as a factor in the accumulation of GlcCer

OctGlcSph was clearly converted to OctSph to some extent by the action of β-glucosidase (Fig. 2). This OctSph evidently gave rise to the formation of much normal-chain Cer and the greater availability of the latter evidently led to greater production of normal-chain GlcCer (Fig. 1). However, another factor in GlcCer elevation should be considered. It may be supposed that the OctGlcSph competed with normal GlcCer for access to glucosidase, giving rise to accumulation of normal GlcCer. Evidence that this inhibition was actually operative came from the observation (Table 4) that GlcSph accumulated in the OctGlcSph-treated cells, but not in OctSph-treated cells. This lysolipid is known to be synthesized from sphingosine by a glucosyltransferase [33] and is known

Table 6. Protein kinase C activity of MDCK cells exposed to short-chain sphingolipids. 5×10^5 MDCK cells were seeded into an 8-cm dish; 24 h after the incubation, the cells were treated with or without liposomes for 24 h in the CO_2 incubator. Four dishes were used in each condition and pooled with buffer as described in the text and the cells were sonicated at 0°C. A unit of protein kinase activity is the amount catalyzing the incorporation into substrate of 1 pmol phosphate/min. The specific activities are calculated from the total protein in the cells. Incubation conditions as in the other tables.

Added lipid	Protein kinase C activity of				
	soluble fraction		particulate fraction		Proportion in particulate ($100 \times C/[A + C]$)
	activity (A)	specific activity (B)	activity (C)	specific activity (D)	
	units	units/mg	units	units/mg	%
Buffer A control	44.4	71.8	13.4	21.7	23.2
Lecithin control	44.3	66.0	15.3	22.8	25.7
OctGlcSph	41.6	64.6	5.81	9.02	12.3
OctSph	7.56	83.1	0.90	9.89	10.6

to accumulate in Gaucher patients, who lack the glucosidase acting on both GlcSph and GlcCer [34]. A further factor is the ability of Sph [35] and GlcSph [36] to inhibit β-glucosidase. OctGlcSph itself is hydrolyzed to OctSph and probably competes with other glucosides, including GlcSph, for the enzyme's reaction site. AcGlcSph was found to be both a good substrate and a strong inhibitor of β-glucosidase [8].

DISCUSSION

The present study has shown that MDCK cells in low-density cultures undergo characteristic changes in growth and sphingolipid composition when exposed for 24 h to approximately 32 μM OctGlcSph and OctSph. Though MDCK cells at high-density culture incorporated OctGlcSph as well as the cells at low density, there was no change in DNA synthesis or protein content of the cells. The following discussion is focused on the cells at low-density culture.

We first studied the problem of making stable dispersions of the sphingolipids and found that a specific mixture of two pure lecithins produced stable or relatively stable dispersions containing a high proportion of sphingolipid. It is often observed that a mixture of two kinds of detergents produces superior dispersions; lecithins can be considered to be zwitterionic detergents. When these dispersions were tested with MDCK cells, we made the unexpected discovery that they inhibited cell growth, despite the ample previous evidence that GlcCer could stimulate cell growth [4]. Analysis of the cells showed that both short-chain lipids were readily taken up by the cells and produced high levels of normal-chain GlcCer, which made the growth inhibition findings even more perplexing. Further chemical and isotopic analysis yielded clues which may explain the inhibitory action.

First, there is the possibility that the octanoyl lipids and their anabolic products (OctLacCer, octanoyl gangliosides, etc.) inhibited growth. They would certainly tend to produce membranes with abnormal properties. Moreover, since marked changes in the lipid composition of MDCK cells were produced by the octanoyl lipids, we can assume that the properties of the cell membranes were considerably affected. Other researchers using short-chain lipids seem to have assumed that the lipids had no deleterious effects of this type.

Second, OctSph and OctGlcSph produced high levels of normal Cer, which may inhibit growth by direct action of the sphingolipid on a newly discovered protein kinase. This kinase is activated very quickly by even very low Cer levels [13]. It phosphorylates the EGF receptor (and other proteins), a process that might block the growth-promoting action of tyrosine kinase activation. Inhibition of GlcCer synthase with PDMP [2–4] also caused accumulation of Cer and also inhibited cell growth and proliferation. OctSph, which induced a higher level of Cer than OctGlcSph (Table 5), was a more effective inhibitor of protein synthesis in MDCK cells (Table 2). Thus these observations implicate an inhibitory role for Cer. However, patients with the genetic disorder, ceramidosis (Farber's disease), accumulate large amounts of Cer [38] yet are not notable for retardation of growth.

While some of the increase in normal Cer in OctSph-treated cells may have been due to rapid hydrolysis and reacylation of its sphingol, part of the increase could be due to simple reversal of the normal action of acid ceramidase. This enzyme is able to carry out the synthesis of Cer from free sphingol and fatty acid and one assay method even takes advantage of the reaction [39]. Moreover, studies with doubly labeled Cer have furnished strong evidence for some recycling by this enzyme, which can introduce new fatty acids into the lipid without releasing the sphingol for other reactions [40].

A second source of the increased level of Cer, particularly in cells exposed to OctSph, can be attributed to the direct inhibition of ceramide:lecithin phosphorylcholinetransferase (sphingomyelin synthase) by OctSph. The OctSph may act as a competitive substrate, blocking the utilization of normal Cer for sphingomyelin formation, causing loss of the phosphosphingolipid (Fig. 1) and accumulation of natural Cer. Sphingomyelin may itself play a significant role in cell growth [41, 42], possibly through its enzymatic hydrolysis to Cer or reversal of sphingomyelin synthase. This phosphorylcholinetransferase catalyzes what is obviously a low-energy reaction and the equilibrium point may well depend on the concentrations of the four reactants. The inhibitory effect of OctSph on the synthase was seen in Fig. 1 and Table 3; OctGlcSph produced a smaller effect, due to its incomplete conversion to OctSph by glucosidase action. When Cer accumulation was induced by PDMP [4], there was a concomitant increase in diacylglycerol, due to the stimulation of sphingomyelin synthase by the high level of Cer, but no such effect was seen here, evidently because of the inhibitory effect of OctSph on the synthase.

Explanation of the accumulation of natural Cer in the OctSph-treated cells must take into consideration the fact that sphingosine-labeled OctSph gave rise to little labeled natural

Cer (Fig. 3) yet it did yield GlcCer and sphingomyelin in which the Cer moieties were labeled. This supports the idea that sphingomyelin synthase catalyzes the reverse reaction in addition to the synthetic reaction. In other words, the exogenous OctSph reacted with lecithin to form octanoyl sphingomyelin and diacylglycerol and this increased amount of diacylglycerol reacted with endogenous sphingomyelin (relatively low in radioactivity) to form lecithin and mixed ceramides of low specific activity. Additional evidence for the reversibility of the enzyme reaction comes from the finding that dioctanoyl glycerol stimulated sphingomyelin breakdown and increased the level of Cer [43]. In addition, cell incubations with doubly labeled sphingomyelin have yielded the conclusion that 10–15% of the degraded phosphosphingolipid was utilized to form Cer and lecithin [44]. The labeled GlcCer that did form must have come from a specific Cer pool formed from the sphingosine moiety of the OctSph. This pool of molecules (in the Golgi membranes or lysosomes?) did not enter the major pool, seen on the charred TLC plate. Instead it was directly converted to GlcCer and sphingomyelin.

Third, we found that OctSph, OctGlcSph, and PDMP [4] all produced increased levels of Sph, a metabolite that, when added exogenously, has usually been found to result in growth inhibition. This inhibition has generally been attributed to inhibition of protein kinase C by the sphingol, although there is evidence that sphingosine actually produces growth inhibition by forming Cer [45]. The sphingol effect is consistent with our finding that OctSph and OctGlcSph lowered the specific activity of PKC in the cell membranes (Table 6). Cells that were treated with PDMP, the glucosyltransferase inhibitor, accumulated not only Cer and Sph, but also *N,N*-dimethylsphingosine [2]. The latter sphingol, which is derived from sphingosine by methylation [46], was also found to inhibit PKC; presumably its level increased in our cells treated with octanoyl lipids. The same kind of growth inhibition may have been caused by the accumulation of what seemed to be GlcSph (Table 5). This lysolipid, like other lysolipids, appears to be toxic to cells.

A fourth factor that may influence growth was the increased formation of LacCer produced by OctSph (Fig. 1). This GSL has been found to stimulate proliferation in smooth muscle cells from aorta but it inhibited growth of other cell types [47]. Unlike OctSph, OctGlcSph inhibited the formation of LacCer (the decrease is not readily seen in the printed photograph). This effect can be explained as due to inhibition of GlcCer galactosyltransferase by OctGlcSph, which may act as a competitive substrate. Testing of this synthase with homogenates of human kidney proximal tubule cells has shown that OctGlcSph is indeed a good inhibitor (S. Chatterjee, personal communication). The finding that OctSph and OctGlcSph differed somewhat in their effects on LacCer suggests that the OctSph and OctGlcSph were taken into different sites in the cells. The differing concentrations of OctGlcSph in the cells treated with OctGlcSph and OctSph may also affect the inhibitory action.

A paper that appeared just as we finished this study showed that HL-60 cells, like our MDCK cells, formed much Cer in response to OctSph addition to the medium [48]. The increase was much greater, and there was also an accumulation of diacylglycerol. The latter effect was attributed to inhibition of diacylglycerol kinase by Cer, an inhibition that could be shown directly with purified kinase. Our cells did not show this change in diacylglycerol levels, possibly because our incubation time was somewhat longer or because our OctSph was added as part of a lecithin liposome. HL-60 cells that were treated with tumor necrosis factor also showed an elevation in Cer but not in diacylglycerol [49].

In summary, the growth inhibition produced by OctSph and OctGlcSph can be attributed to several enzyme reactions and their products. Our analytical findings demonstrate the importance for other investigators of carrying out similar searches for metabolic changes when abnormal lipids are added to cells.

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