Sphingolipid Metabolism Is a Crucial Determinant of Cellular Fate in Nonstimulated Proliferating Madin-Darby Canine Kidney (MDCK) Cells*

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The present report was addressed to study the influence of sphingolipid metabolism in determining cellular fate. In nonstimulated proliferating Madin-Darby canine kidney (MDCK) cells, sphingolipid de novo synthesis is branched mainly to a production of sphingomyelin and ceramide, with a minor production of sphingosylphosphocholine, ceramide 1-phosphate, and sphingosine 1-phosphate. Experiments with 32P as a radioactive precursor showed that sphingosine 1-phosphate is produced mainly by a de novo independent pathway. Enzymatic inhibition of the de novo pathway and ceramide synthesis affected cell number and viability only slightly, without changing sphingosine 1-phosphate production. By contrast, inhibition of sphingosine kinase-1 activity provoked a significant reduction in both cell number and viability in a dose-dependent manner. When sphingolipid metabolism was studied, an increase in de novo formed ceramide was found, which correlated with the concentration of enzyme inhibitor and the reduction in cell number and viability. Knockdown of sphingosine kinase-1 expression also induced an accumulation of de novo synthesized ceramide, provoking a slight reduction in cell number and viability similar to that induced by a low concentration of the sphingosine kinase inhibitor. Taken together, our results indicate that the level of de novo formed ceramide is controlled by the synthesis of sphingosine 1-phosphate, which appears to occur through a de novo synthesis-independent pathway, most probably the salvage pathway, that is responsible for the MDCK cell fate, suggesting that under proliferating conditions, a dynamic interplay exists between the de novo synthesis and the salvage pathway.

Sphingolipids are considered predominant building blocks of biological membranes. However, it is now accepted that they regulate several aspects of cell behavior (1). The de novo synthesis of sphingolipids begins with the condensation of serine and a fatty acyl-CoA by serine palmitoyltransferase (SPT)2 (2) to form 3-ketosphinganine, which is followed by its reduction to dihydroxyphosphoglycerate (DHS), which is further acylated to dihydroceramide (DH Cer), which is then desaturated to form ceramide (Cer). Cer is also produced by the salvage pathway, initiated by hydrolysis of sphingomyelin (SM) by the action of SMs. Ceramide is the central core lipid in the metabolism of sphingolipids; it can either be phosphorylated by ceramide kinases to ceramide 1-phosphate (Cer-1-P) or be utilized for the synthesis of SM or glycosphingolipids. Cer can also be broken down by ceramidases to sphingosine (Sph), which is in turn phosphorylated by sphingosine kinases (SKs) to form sphingosine 1-phosphate (Sph-1-P). Sph-1-P can be degraded by specific phosphatases to generate Sph or by a lyase that cleaves it irreversibly into ethanolamine 1-phosphate and palmitaldehyde, thus being the only pathway for sphingolipid degradation (3).

Many of the biosynthetic intermediates are formed during both the de novo synthesis and the salvage pathway: In addition, particularly Cer, Sph, and their phosphorylated products, Cer-1-P and Sph-1-P, are highly bioactive and can affect cell behavior. Cer is implicated in differentiation (4, 5), cell cycle arrest (6–8), apoptosis (9), and senescence (10), whereas Sph has been proposed to induce cell death by a Cer-independent mechanism (11). In opposition to Cer and Sph, Sph-1-P promotes proliferation, survival, and inhibition of apoptosis (12, 13). Cer-1-P has been shown to have mitogenic properties and has recently been described as a potent inhibitor of apoptosis and inducer of cell survival (14). DHS is considered an endogenous inhibitor of protein kinase C (15), and DH Cer has recently been reported as a mediator of autophagy in DU145 cells (16).

It is well documented that different types of cells respond to stress, cytokines, and growth factors by activating SMs and/or by increasing de novo synthesis (15–17). Both metabolic pathways increase the intracellular level of Cer, which in turn, depending on the expression and activity of Cer-metabolizing enzymes, can lead the metabolic pathway to the formation of pro-proliferating/survival or cell-arresting/death metabolites.

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§ The abbreviations used are: SPT, serine palmitoyltransferase; DHS, dihydroxyphosphoglycerate; TDS, 1,2-di-O-dihydroxyphosphoglycerate; Cer, ceramide; Cer-1-P, ceramide 1-phosphate; DH Cer, dihydroceramide; SM, sphingomyelin; Sph, sphingosine; SK, sphingosine kinase; Sph-1-P, sphingosine 1-phosphate; MDCK, Madin-Darby canine kidney; DMEM/F12, Dulbecco’s modified Eagle’s medium with nutrient mixture F-12; FBS, fetal bovine serum; SPC, sphingosylphosphocholine; L-CS, L-cycloserine; FB1, fumonisin B1; siRNA, small interfering RNA.

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In contrast to the well-documented importance of sphingolipid metabolism in determining cellular fate under stress conditions or under the effect of external stimuli, very little is known about its role in basal nonstimulated conditions. In a previous work, we showed differential branching of the sphingolipid metabolic pathways according to the developmental stage in rat renal papillae (18). We have shown that the developmental regulation of SK expression and activity leads sphingolipid metabolism to the formation of Sph-1-P in the neonatal period and of Cer in adult tissue. Considering the well-known biological properties of both metabolites, the results were consistent with the immature-proliferative and fully differentiated-quiescent stages of neonatal and adult tissues, respectively. Because we used ex vivo tissue from neonatal and adult rats, we were unable to demonstrate whether such different branching of the sphingolipid metabolism was either a cause or a consequence of the developmental stage.

The present study was undertaken to elucidate whether intracellular sphingolipid metabolism can determine the cellular fate in nonstimulated conditions. Our most important findings were that in nonstimulated proliferating MDCK cells: 1) the level of de novo synthesized Cer is a determining factor to decide cellular fate; and 2) the de novo synthesis of Cer is under the control of the Sph-1-P, which appears to be formed through a de novo synthesis-independent pathway.

**EXPERIMENTAL PROCEDURES**

**Materials**—MDCK cells were from American Type Culture Collection. Carrier-free [32P]orthophosphate (specific activity 25 mCi/mmol) and [3H]serine (specific activity 34 Ci/mmol) were purchased from GE Healthcare, and [14C]palmitic acid (specific activity 55 mCi/mmol) was from PerkinElmer Life Sciences. X-ray film for autoradiography was from Eastman Kodak (specific activity 55 mCi/mmol) was from PerkinElmer Life Sciences. X-ray film for autoradiography was from Eastman Kodak Co. TLC silica gel plates were from Merck. Dulbecco’s modified Eagle’s medium with nutrient mixture F-12 (DMEM/F12; Invitrogen), fetal bovine serum (FBS; Invitrogen), penicillin-streptomycin (Invitrogen), Hoechst 33258 ( Molecular Probes), Lipofectamine 2000, and Stealth-siRNA were purchased from Invitrogen. Fumonisin B1, 1-cycloserine and threo-dihydrosphingosine, diacylglyceride kinase, alkaline phosphatase type VII, and recombinant sphingosine kinase-1 were from Sigma. Polyclonal anti-human sphingosine kinase-1 was from Oncogene. Monoclonal anti-Actin was from Chemicon. All other reagents and chemicals were of analytical grade and were from local commercial suppliers (Sigma, Merck, or Mallinkrodt).

**Cell Culture**—2 × 10^5 MDCK cells were plated in a 33-mm multiwell dish in 2 ml of DMEM/F12 containing 10% FBS, 100 IU/ml penicillin, and 100 µg/ml streptomycin and incubated for 24 h in a 5% CO2 incubator at 37°C. Then, the medium was changed for 2 ml of DMEM/F12 plus 0.5% FBS and antibiotics. After incubation overnight, the medium was changed again, and various inhibitors were added. Then cells were collected by trypsinization and counted, and cell viability was determined by the trypan blue exclusion procedure.

**Sphingolipid Metabolism Study**—Sphingolipid metabolism was evaluated using three different radioactive precursors. To study the de novo pathway, either 35 nCi/ml [14C]palmitic acid or 2 µCi/ml [3H]serine was added to each well 5 h with the inhibitors. In order to study phosphorylated sphingolipids, 1 µCi/ml [32P]orthophosphate was added 3 h before finishing inhibitors treatment.

**Sphingolipid Analysis**—After trypsinization, lipids were extracted by the method of Bligh and Dyer, and mild alkaline hydrolysis was performed for sphingolipid extraction (19). Sphingolipids were resolved by TLC using 1-butanol/acetic acid/water (3:1:1, v/v/v) as a solvent system and were visualized by autoradiography. The corresponding radioactive spots were scraped and counted in a scintillation counter. Sphingolipid species were identified by comparing with the respective retention factors of the standards. Data were normalized to the number of counts incorporated per cell.

The levels of endogenous Cer were measured by using the diacylglycerol kinase method as described by Perry et al. (20). The lipid substrate from 2.10^6 cells was solubilized by using mixed micelles containing nonionic detergent and phospholipids. The micelle-solubilized lipid extracts were subjected to a diacylglycerol kinase reaction wherein Cer was converted to a quantifiable product by the transfer of [32P]phosphate from [γ-32P]ATP to Cer. The enzymatic product was separated by TLC, identified by comigration with standard Cer-1-P, and quantified by using the slope of the curve obtained from standard Cer. Data were normalized to the amount of Cer/10^6 cells.

The levels of endogenous Sph-1-P were measured by the enzymatic method described by Edsall et al. (21). After trypsinization and washing, the cells were harvested in 1 ml of methanol containing 2.5 µl of HCl. Lipids were extracted by the addition of chloroform/1 M NaCl (1:1) plus 100 µl of 3 N NaOH. The basic aqueous phase was subjected to the action of alkaline phosphatase as described elsewhere (21). After acidifying, lipids were extracted twice with chloroform and evaporated under an N2 stream. The lipid extract was used as substrate for recombinant SK, and the assay was performed as described below. Data were normalized to the amount of Sph-1-P/10^6 cells.

**Hoechst 33258 Staining**—MDCK cells were plated on glass coverslips in a 33-mm multiwell dish and cultured as described above. After 6 h of treatment with the inhibitors, cells were washed three times with phosphate-buffered saline and then fixed with 4% paraformaldehyde and 0.12 M sucrose in phosphate-buffered saline for 30 min at room temperature. After fixation, buffer was washed, and cells were stained with Hoechst 33258 (5 µM final concentration) for 15 min in the dark at room temperature. The stained nuclei were examined by epifluorescence using an Olympus BX50 microscope, and photographs were taken with a CoolSnap digital camera with acquisition software Image Pro Plus (version 5.5).

**Transfection with Small Interference RNA**—MDCK cells were plated in a 12-multilwell dish (5 × 10^4 cells/well) in 1 ml of DMEM/F12 plus 10% FBS without antibiotics. After 24 h, cells were transfected for 24 h with 200 nm double-stranded siRNAs using Lipofectamine 2000 according to the manufacturer’s instructions. The transfection reagents were washed out, and cells were incubated for another 48 h in DMEM/F12 with 0.5% FBS. The sequences of siRNAs for human SK1 were GGGCA-AGGCUCUGCAAGCUTdTd and GAGCUGCAAGCCUU
Endogenous Sphingolipid Metabolism in MDCK Cells

GCCCTdTd, and the sequences of scrambled siRNA were CAGUCGCGUUUGCGACUGG and CCAGUCGCAAA-CGGGACUG (22).

Enzyme Assays—SPT was assayed as described by Merrill et al. (23). Cell homogenates, 150 μg of protein, and 1 mM L-[3H]serine (specific radioactivity 13,333 cpm) were incubated in 100 mM Hepes, pH 8.3, 5 mM dithiothreitol, 2.5 mM EDTA, and 50 μg of protein. The reaction proceeded for 35 min and was terminated with the addition of 0.2 ml of 0.5 M NH4OH on ice. After the addition of 1.5 ml of chloroform/methanol (1:2, v/v), 2 ml of 0.5 M NH4OH and 50 μg of DHS were added. Then, the sample was centrifuged, the aqueous phase was removed, and the organic phase was washed twice with 2 ml of water. The extracted organic phase was dried under an N2 stream, and radioactivity was determined by scintillation counting. Data were normalized to the amount of Sph-1-P formed per mg of protein.

Sphingosine kinase activity was measured as described by Oliver et al. (26). Cells were harvested by trypsinization and resuspended in lysis buffer (20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.5 mM deoxyxypiridine, 15 mM NaF, β-mercaptoethanol, 1 mM Na3VO4, 40 mM β-glycerophosphate, 0.4 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 20% glycerol). Cells were lysed by repeated freezing and thawing. An aliquot of lysates was incubated with 50 μg of Sph (in 4 mg/ml fatty acid-free bovine serum albumin) and 1 mM [γ-32P]ATP (10 μCi dissolved in 200 mM MgCl2). The reaction was carried out for 30 min at 37 °C and stopped on ice by adding 20 μl of 1 N HCl and 800 μl of chloroform/methanol/HCl (100:200:1, v/v/v). After 10 min at room temperature, 240 μl of chloroform and 240 μl of 2 M KCl were added, and samples were centrifuged. The organic layer was extracted and dried under an N2 stream. Lipids were resolved by TLC using 1-butanol/ acetic acid/water (3:1:1, v/v/v) as the solvent system. Labeled Sph-1-P was visualized by autoradiography, and the corresponding radioactive spot was scraped and counted in a scintillation counter. Data were normalized to the amount of Sph-1-P formed per mg of protein.

Immunoblot Analysis—After treatment with trypsin-EDTA, cells were collected by centrifugation at 3000 × g for 5 min. They were lysed in lysis buffer containing 50%–diluted phosphate-buffered saline plus 0.1% Triton X-100 for 1 h on ice. The lysate was homogenized with a 21-gauge needle and centrifuged at 18,000 × g for 30 min. Protein concentration was determined in the supernatant using the Lowry assay. An equal amount of protein was resuspended in 4× Laemmli sample buffer, boiled for 5 min, and resolved in a 12% SDS-polyacrylamide gel. After transfer to polyvinyldene difluoride, the membranes were blocked for 1 h in 10% nonfat milk in Tris-buffered saline plus 0.1% Tween-20 (TBST) and probed with a primary antibody against human SK1 (goat polyclonal, Oncogene) overnight, washed three times with TBST, and probed with secondary antibodies. The membranes were then rinsed, and the signal was detected using enhanced chemiluminescence reagent (ECL, Amersham Biosciences). Charge was controlled by parallel detection of β-actin in the same polyvinylidene difluoride membrane. Immunoblot signals were quantified by optical density on a Gel-Pro Analyzer 3.1.

Statistical Analysis—The results are expressed as the mean ± S.E. Data from controls and different treatments were analyzed by analysis of variance, and significant differences were assessed by an a posteriori Scheffé test (p < 0.05).

RESULTS

Sphingolipid Metabolism in Nonstimulated Proliferating MDCK Cells—After a “lag” period (~24 h, Fig. 1A), MDCK cells still proliferated at the rate of approximately one cycle of division every 24 h, although they were submitted to a low concen-
Endogenous Sphingolipid Metabolism in MDCK Cells

FIGURE 2. Inhibition of sphingolipid de novo synthesis affects MDCK proliferation without affecting \[^{32}P\]\textsubscript{Sph-1-P} production. Subconfluent MDCK cells were incubated in low-serum medium for 24 h in the presence of 0.5 mM \(\text{\textit{L}-cycloserine}\), an inhibitor of SPT, in order to block sphingolipid de novo synthesis. A and B, \(\text{\textit{L}-cycloserine}\) treatment induced a moderate decrease in cell number without affecting cell viability. C, radio-labeling with \[^{14}C\]palmitic acid showed a reduction in radioactive incorporation (32% of control) without losing the incorporation profile. D, \(\text{\textit{L}-cycloserine}\) selectively affected \[^{32}P\]\textsubscript{orthophosphate} incorporation into sphingolipids, decreasing the radioactive incorporation into SM and SPC, whereas no changes were observed in Sph-1-P and Cer-1-P. Control cultures are shown in white and \(\text{\textit{L}-cycloserine}\)-treated cells in gray. The results represent mean ± S.E. from three independent experiments (*, \(p < 0.001\); §, \(p < 0.005\)).

tration of FBS. Because experiments were performed at 48 h (Fig. 1A, arrow), the results obtained correspond to non-stimulated proliferating cells, which presented ~100% of viability. Sphingolipid metabolism was evaluated by using three radioactive precursors. As seen in Fig. 1B, when \[^{14}C\]palmitic acid was used as a metabolic precursor, most of the radioactivity was found associated with SM (50% of total radioactivity), and 24% remained associated with Cer. Intermediate metabolites were not accumulated, and the end products, sphingosylphosphocholine (SPC), DHS-1-P, and Cer-1-P, accounted for only 8, 6, and 9%, of total radioactivity, respectively.

The profile of \[^{3}H\]serine incorporation showed that most of the radioactivity was found associated with Cer and SM, accounting for 37 and 24% of total radioactivity, respectively. SPC, Cer-1-P, and DHS-1-P appeared as minor end products with each one representing 10% of the total radioactivity.

The profile of \[^{32}P\]orthophosphate incorporation showed that Sph-1-P was the prominent phosphorylated sphingolipid metabolite formed, accounting for 45% of total radioactivity, followed by SPC (25%), SM (17%), and Cer-1-P (12.5%).

In the representative TLC plate colored by iodine vapors, it can be observed that SM is the major sphingolipid followed by SPC and Cer. The other sphingolipid were minor species and were almost undetectable. In the TLC plate colored by ninhydrin (a sensitive reagent to detect the free amine group), a single spot corresponding to the retention factor of Sph-1-P was observed.

Collectively, the results show that in nonstimulated proliferating MDCK cells, sphingolipid de novo synthesis leads to SM production efficiently, preserving a considerable level of de novo synthesized Cer. The level of \[^{14}C\]SM was higher than that of \[^{14}C\]Cer, but, when \[^{3}H\]serine was used as a metabolic precursor, the amount of radioactivity associated with Cer was higher than that of SM. This result is consistent with previous observations demonstrating that Cer containing C16 fatty acid is the species that is most involved in SM synthesis (22).

Effect of Serine Palmitoyltransferase Inhibition on Nonstimulated Proliferating MDCK Cells—The first step in sphingolipid de novo synthesis, which is catalyzed by the SPT, is rate-limiting for the entire pathway and, consequently, a major point of regulation. The treatment of subconfluent proliferating MDCK cells for 24 h with 0.5 mM \(\text{\textit{L}-cycloserine}\) (\(\text{\textit{L}-CS}\)), an inhibitor of SPT activity, induced a moderate decrease in cell number (79% of the cells present in the control cultures) without affecting cell viability (93%) (Fig. 2, A and B).

As a result of SPT inhibition, the overall incorporation of \[^{14}C\]palmitic acid to sphingolipids fell by about 68% (Fig. 2C). The \[^{14}C\] radioactivity associated with the various sphingolipids decreased without significant changes on the profile of distribution. Thus, the most important formed metabolites, SM and Cer, decreased by 74 and 79%, respectively, and the minor end products, Cer-1-P and SPC, decreased by 76 and 51%, respectively. No changes were observed in DHS and DHS-1-P (Fig. 2C).

As shown in Fig. 2D, the incorporation of \[^{32}P\] into the various sphingolipids was selectively affected by \(\text{\textit{L}-cycloserine}\). The treatment induced a 43 and 44% decrease in the radioactivity associated with SM and SPC, respectively, whereas no changes were observed in Sph-1-P and Cer-1-P. The fact that \[^{32}P\] incorporation into Sph-1-P was refractory to the inhibition of SPT indicates that inhibition of \textit{de novo} synthesis does not appear to inhibit \[^{32}P\]orthophosphate incorporation into Sph-1-P. On the other hand, \[^{32}P\]Cer-1-P was also resistant to SPT inhibition, thus suggesting that Cer-1-P can be synthesized from \textit{de novo} formed Cer (affected by SPT inhibition) and also from a preexisting pool of Cer (resistant to SPT inhibition).

Collectively, the results demonstrated that Sph-1-P appeared to be formed by a \textit{de novo} synthesis-independent pathway and that the inhibition of the committed step of the biosynthetic pathway for 24 h did not induce cell death and affected cell proliferation only slightly.

Effect of Fumonisin B1 on Nonstimulated Proliferating MDCK Cells—We were also interested in determining whether the inhibition of dihydroceramide synthase affects the proliferation and survival of subconfluent MDCK cells and the relationship between this effect and sphingolipid metabolism. As seen in Fig. 3A, after 24 h of treatment with fumonisin B1 (FB1),
The prominent effect of FB1 on the de novo pathway was the accumulation of $[^{14}C]$DHS (50-fold; Fig. 3C). Concomitantly, Cer de novo synthesis fell to 16% of the control, and $[^{14}C]$SM decreased to 8% of the control value. The total amount of $[^{14}C]$palmitate distributed into the various metabolites did not decrease, thus reflecting that the SPT activity was not affected.

As shown in Fig. 3D, $[^{32}P]$ radioactivity associated with Sph-1-P and Cer-1-P was not significantly affected by FB1. In contrast, the $[^{32}P]$ radioactivity associated with SPC and SM decreased to 30 and 34% of the control value, respectively. These results constitute a further demonstration that even though the formation of $[^{14}C]$- and $[^{32}P]$-labeled SM and SPC was coupled to Cer de novo synthesis, the formation of $[^{32}P]$Sph-1-P and $[^{32}P]$Cer-1-P appeared to be independent of Cer synthesis.

Effect of SK Inhibition on Non-stimulated Proliferating MDCK Cells—As seen in Fig. 4, A and B, treatment for 24 h with an inhibitor of SK (D,L-threo-dihydrosphingosine (tDHS)) caused a clear deleterious effect on nonstimulated proliferating MDCK cells. Thus, the number of cells decreased (~60%), and viability was lost in 62% of the cells (Fig. 4, A and B). When sphingolipid metabolism was evaluated biochemically, we observed that the total amount of $[^{14}C]$palmitic acid radioactivity per cell increased by about 43% of the control value, explained mostly by a high accumulation of radioactive Cer, which presented an almost 300% increase (Fig. 4C). When $[^{3}H]$serine was used as a radioactive precursor, as shown in Fig. 3D, $[^{3}H]$Cer accumulated, reaching an almost 150% increase. Total $[^{3}H]$ radioactivity showed a 71% increase as compared with the control value. A significant accumulation of radioactive DHS was also observed with both radioactive precursors, whereas no significant changes were found in any other metabolite. When $[^{32}P]$ incorporation was evaluated, we observed that Sph-1-P was the only phosphorylated metabolite affected, showing a 50% decrease and thus reflecting the specific

![FIGURE 3. Effect of fumonisin B1 on proliferating MDCK cells. A and B, treatment of subconfluent MDCK cells with Fumonisin B1, an inhibitor of (dihydro)Cer synthesis, induces a slight decrease in both cell number and viability. C, $[^{14}C]$ palmitic acid incorporation assay revealed a reduction in the synthesis of all major sphingolipids accompanied by the accumulation of the most radioactive precursor in molecules of DHS. D, in contrast, the incorporation of $[^{32}P]$ was altered only in the cases of SPC and SM, and no changes were observed for Sph-1-P and Cer-1-P. Control cultures are shown in white and FB1-treated cells in gray. The results represent mean ± S.E. from three independent experiments (*, p < 0.001; §, p < 0.005).](https://example.com/figure3)

![FIGURE 4. Effect of SK inhibition on subconfluent MDCK cells. A and B, 24-h treatment with 25 μM tDHS, an inhibitor of SK, evoked a clearly deleterious effect on nonstimulated proliferating MDCK cells, causing an important reduction in cell number and viability. C and D, when the de novo synthesis was evaluated, a high accumulation of both $[^{14}C]$Cer (C) and $[^{3}H]$Cer (D) was observed. In both cases an increase in total radioactivity associated with the various sphingolipid metabolites was also observed. E, when $[^{32}P]$ incorporation was evaluated, only $[^{32}P]$Sph-1-P amount was decreased. Control cultures are shown in white and tDHS-treated cells in gray. The results represent mean ± S.E. from three independent experiments (*, p < 0.001; §, p < 0.005).](https://example.com/figure4)
inhibition of SK. Collectively, the results demonstrated that SK inhibition, besides inducing a decrease in the production of sphingosine, evoked an accumulation of de novo synthesized Cer and that such a metabolic condition was highly deleterious for proliferating MDCK cells.

Effect of Cer Synthesis Inhibition on tDHS-treated Cells—To determine whether SK inhibition had induced cell death because of the decrease in the production of sphingosine-1-phosphate or the accumulation of de novo synthesized Cer, cultured cells were treated with inhibitors of Cer synthesis over the inhibition of SK enzymatic activity. FB1 treatment partially reversed the decrease in cell number, whereas SPT inhibition evoked an almost complete recovery (Fig. 5A).

With respect to cell viability, the inhibition of the de novo synthesis pathway rescued the cells from death, with L-cycloserine being more efficient than FB1 (Fig. 5B). To visualize the effect of SK inhibition, we stained the cultured cells with Hoechst 33258.

Endogenous Sphingolipid Metabolism in MDCK Cells

To explore the enzymes involved in the accumulation of Cer induced by tDHS, we evaluated the activities of SPT and DH Cer/Cer synthases. tDHS produced an increase in SPT activity. FB1 treatment partially reversed the effect of the inhibition of SK (Fig. 5C, arrow heads), thus denoting an apoptotic process. When either FB1 or L-cycloserine was concomitantly added, the cell number was restored and the number of cells with apoptotic phenotype decreased significantly, thus reflecting protection of the cells from death.

Thereafter, we explored sphingolipid metabolism by using [14C]palmitic acid. As shown in Fig. 5C, the profile of [14C]palmitic acid distribution showed that FB1 over tDHS evoked a decrease in [14C]Cer, which fell to 50% of the value found in untreated cells, concomitantly with an accumulation of [14C]DHS and a decrease in [14C]SM and [14C]Cer-1-P. The analysis of the effect of L-cycloserine on tDHS revealed that the effect of the inhibition of SK was completely overcome by the inhibition of the SPT (Fig. 5D). The results obtained using [3H]serine were qualitatively the same (data not shown). Collectively, these results demonstrate that, despite SK inhibition, MDCK cell proliferation and viability were restored by avoiding the accumulation of de novo formed Cer, thus reflecting the prominent role of Cer in the determination of cell fate.

Table 1

| Treatment | Ceramide | Sph-1-P |
|-----------|----------|---------|
| Ceramide  | 45.8 ± 5.3 | 3.70 ± 0.61 |
| Ceramide  | 271.4 ± 23.5* | 0.85 ± 0.12* |
| Ceramide  | 61.2 ± 8.7 | 0.82 ± 0.15* |
| Ceramide  | 250.8 ± 31.5* | 0.13 ± 0.05* |

After 6 h of treatment with the SK inhibitor, a diminution in the average cell number present in a ×400 field was observed, and 7.5% of the cells presented chromatin condensation and nuclei fragmentation (versus 1.2% in control cultures; Fig. 5C, arrow heads), thus denoting an apoptotic process. When either FB1 or L-cycloserine was concomitantly added, the cell number was restored and the number of cells with apoptotic phenotype decreased significantly, thus reflecting protection of the cells from death.

TABLE 1

Ceramide and S1P content in control and treated proliferating MDCK cells

Proliferating MDCK cells were treated with tDHS, tDHS, and FB1 or L-CS and then subjected to lipid extraction. Ceramide and Sph-1-P levels were determined as described under "Experimental Procedures." Concentrations of ceramide and Sph-1-P are given as pmol/mg of protein. Data shown are mean ± S.D. of three independent experiments. * significantly different from the control (p < 0.05).

Effect of tDHS on SPT and DH Cer/Cer Synthase Activities—To explore the enzymes involved in the accumulation of Cer induced by tDHS, we evaluated the activities of SPT and DH Cer/Cer synthases. tDHS produced an increase in SPT activity.

Effect of tDHS on SPT and DH Cer/Cer Synthase Activities—To explore the enzymes involved in the accumulation of Cer induced by tDHS, we evaluated the activities of SPT and DH Cer/Cer synthases. tDHS produced an increase in SPT activity.

FIGURE 5. Inhibition of Cer synthesis reversed the decrease in cell number and viability due to SK inhibition. A and B, the combination of tDHS (black) with the inhibitors of Cer synthesis, FB1 (gray) and L-cycloserine (dark gray), caused a reduction in the effect in cell number and viability due to SK inhibition. The results represent mean ± S.E. from three independent experiments (§, p < 0.001; *, p < 0.005). C, this effect was clearly observed at 6 h of incubation when nuclei morphology was studied using Hoechst 33258 staining; an increase in the number of cells with apoptotic nuclei-phenotype was observed (pictures represent a ×400 field). D, results of control cultures are shown in white. The results represent mean ± S.E. from three independent experiments (**, p < 0.001; *, p < 0.005). D, Analysis of sphingolipid metabolism showed a reduction in [14C]Cer accumulation. Results of control cultures are shown in white. The results represent mean ± S.E. from four independent experiments (**, p < 0.05; §, p < 0.005).
activity reflected by a 7.5-fold rise in the amount of the enzymatic product (Table 2). DHcer/Cer synthase activity was also affected by TDHS, as a 4-fold increase in Cer was obtained after TDHS treatment of the cells. The addition of FB1 did not change SPT activity but almost abolished the activity of DHcer/Cer synthase. The addition of t-CS attenuated the increase in SPT activity induced by TDHS but did not affect the activity of DHcer/Cer synthase.

Down-regulation of SK1 Expression and Its Effect on Cellular Fate and Sphingolipid Metabolism—To specifically determine the contribution of basal endogenous SK1 to the proliferation and survival of MDCK cells as well as to the control of their sphingolipid metabolism, we used an SK1-specific siRNA. Because canine SK1 has not been cloned yet, we decided to use a human SK1-specific siRNA. Initially, we checked the expression and activity of the enzyme. Western blot analysis showed that protein expression was ~50% of that in the scrambled siRNA-treated cells (Fig. 6A), whereas the enzymatic activity was reduced to 60% of the control value.

To evaluate the biological effects of SK1 loss, we next examined cell number and viability (Fig. 6, B and C). SK1 knockdown provoked a decrease in cell number (50% of the cells present in the control) and also moderately affected their viability (20% of nonviable cells in siRNA-SK1-treated cells), thus confirming the contribution of endogenous SK1 in maintaining MDCK cell proliferation and viability.

We next evaluated the de novo synthesis pathway. SK1 knockdown evoked a 100% increase in 14C radioactivity-associated Cer. Besides Cer, 14C-SM and 14C-Cer-1-P were also increased (Fig. 6D).

Taken together, the results demonstrated that, in fact, Cer de novo synthesis is under the control of SK1, because the decrease in enzyme expression and activity induced 100% increase in de novo formed Cer. Under these conditions, although proliferation was significantly affected, cell viability was not massively impaired.

The Level of Cer Accumulation and MDCK Proliferation and Survival Correlates with the Degree of SK Inhibition—As shown above, treatment with 25 μM TDHS induced a 3-fold increase in 14C-Cer when compared with control cultures and caused a dramatic reduction in both cell number and viability. On the other hand, transfection of subconfluent MDCK cells with SK1 siRNA induced a smaller increase in 14C-Cer accumulation (as well as a significant accumulation of 14C-SM) and a lower reduction in cell number and survival. Therefore, to determine whether the reduction in cell number and viability correlated with the degree of 14C-Cer accumulation, we incubated subconfluent MDCK cells in the presence of increasing concentrations of TDHS. As shown in Fig. 7, A and B, at low concentrations of the SK inhibitor (2.5 and 5 μM) neither cell number nor viability was affected, but a 40 and a 62% decrease in cell num-

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**TABLE 2**

Serine palmitoyltransferase and ceramide synthase activities in control and treated proliferating MDCK cells

| Treatment       | SPT Activity | Cer Synthase Activity |
|-----------------|--------------|-----------------------|
| Control         | 0.32 ± 0.08  | 3.00 ± 0.91           |
| TDHS            | 2.41 ± 0.53* | 6.09 ± 0.95*          |
| TDHS + FB1      | 2.13 ± 0.43* | 0.09 ± 0.05*          |
| TDHS + L-CS     | 0.50 ± 0.07  | 4.41 ± 1.21           |

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**FIGURE 7.** Effect of SK inhibition degree on MDCK proliferation and survival. A and B, a dose-response experiment showed that the action of TDHS in cell number (A) and viability (B) depends on the inhibitor concentration. C, radiolabeled assays showed that both [14C]Cer accumulation and [32P]Sph-1-P reduction correlated perfectly with the increase in TDHS concentration. [14C]SM levels increased at low concentrations of the inhibitor until 10 μM TDHS. No further increase in [14C]SM was observed at higher TDHS concentrations. The results represent mean ± S.E. from three independent experiments.

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Fate and Sphingolipid Metabolism

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Endogenous Sphingolipid Metabolism in MDCK Cells
number was obtained with 10 and 25 μM inhibitor, respectively. Cell viability was also affected at the higher concentrations, showing 25 and 70% of loss of viability, respectively. The metabolic experiments using [14C]palmitic acid and 32P as radioactive precursors showed that after 5 μM of tDHS, a concentration-dependent decrease of [32P]Sph-1-P correlated with the accumulation of [14C]Cer. With respect to SM synthesis, from 5 to 10 μM tDHS, an increased level of [14C]SM, which did not parallel that [14C]Cer, was observed. After 10 μM tDHS no further increase in [14C]SM was observed, despite the concomitant accumulation of [14C]Cer. These data are consistent with those obtained in the siRNA-treated cells, where SK1 activity was only partially affected and where [14C]Cer increased up to 100% of the dosage of endogenous Sph-1-P. In fact, such an accumulation of endogenous Sph-1-P mass. Although we have no explanation for such a result, it is evident that the decrease in endogenous Sph-1-P without the increase in de novo formed Cer did not prevent the rescue of cells from death. However, further studies are necessary to confirm this finding.

Serine palmitoyltransferase has been indicated as the limiting enzyme in the de novo pathway of Cer synthesis and is also considered the major target for the regulation of the pathway. The determination of enzyme activities revealed that, in fact, the inhibition of SK produced an increase in SPT activity. However, besides increasing the SPT activity, the treatment with tDHS also induced a rise in DHSCer/Cer synthase activity. This result is consistent with a recent report by Laviad et al. (30), who characterized a ceramide synthase type 2, which is inhibited by Sph-1-P. It is possible that, under our experimental conditions, Sph-1-P exerts another point of Cer synthesis control by negatively modulating ceramide synthase 2 activity.

In this study, we examined sphingolipid metabolism in non-stimulated proliferating MDCK cells and its influence on the maintenance of the proliferation and survival of cells. The data presented here reveal that the level of de novo formed Cer is controlled by Sph-1-P synthesis, which seems to occur by a de novo synthesis-independent pathway, most probably the salvage pathway. Such a Sph-1-P-controlled level of Cer synthesis seems to be crucial for the maintenance of cell proliferation and survival.

The de novo pathway of sphingolipid biosynthesis includes the formation of highly biologically active metabolites, and the interruption of the pathway at any step is considered potentially deleterious to the cell because of both the accumulation of intermediates and the decrease in the metabolic product (27). However, neither the accumulation of DHS nor the dramatic decrease in Cer and SM synthesis affected the proliferation and survival of MDCK cells to any great extent. By contrast, the specific decrease in Sph-1-P formation evoked an important deleterious effect, reinforcing the notion of Sph-1-P as a survival molecule.

The key role of the de novo formed Cer in promoting cell death was further supported by the measurement of the endogenous content of Cer and Sph-1-P. At first, FB1 over tDHS produced a specific decrease in Cer content without affecting endogenous Sph-1-P, demonstrating that although the level of Sph-1-P remained low, cells were still recovered from death. Secondly, L-cycloserine, which produced a decrease in de novo formed Cer ([14C]Cer) failed to affect its endogenous mass, even though the cells were rescued from death, thus demonstrating the existence of an SPT-independent pathway of Cer formation that is not involved in cell death. It is possible that in proliferating MDCK cells, Cer is actively formed as an intermediate metabolite in a pathway initiated by the hydrolysis of complex sphingolipids and or SM and ending with the efficient formation of Sph-1-P. When Sph-1-P formation was inhibited by tDHS, Cer accumulated forming a pool of Cer (detected by the determination of endogenous mass) that was not involved in cell death. In turn, such a pathway could constitute a major source of Sph-1-P formation. To our knowledge, this pathway of Cer formation, which is sensitive to FB1 but not to L-cycloserine, is the one recently redefined by Kitatani et al. (29) as the salvage pathway. Another piece of evidence to support the notion that the accumulation of the de novo formed Cer is responsible for the cell death observed under our experimental conditions comes from the dosage of endogenous Sph-1-P. In fact, L-cycloserine treatment over tDHS produced an unexpected decrease in endogenous Sph-1-P mass.

DISCUSSION

In this study, we examined sphingolipid metabolism in non-stimulated proliferating MDCK cells and its influence on the maintenance of the proliferation and survival of cells. The data presented here reveal that the level of de novo formed Cer is controlled by Sph-1-P synthesis, which seems to occur by a de novo synthesis-independent pathway, most probably the salvage pathway. Such a Sph-1-P-controlled level of Cer synthesis seems to be crucial for the maintenance of cell proliferation and survival.

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The involvement of Sph-1-P in regulating the de novo biosynthesis of Cer was first reported by van Echten-Deckert et al. (31). More recently, Taha et al. (22) have reported that in breast cancer cells, basal SK1 regulates ceramide synthesis and have proposed the de novo synthesis as the most important mechanism for SK1-mediated Cer accumulation. In addition, Le Stunff et al. (32) have reported that overexpression of Sph-1-phosphatase induces dephosphorylation of Sph-1-P and enhances Cer synthesis, thus suggesting that the SK1/Sph-1-P pathway, besides modulating Cer clearance, may reduce Cer synthesis. The data in the present study support the role of basal SK1 in regulating Cer de novo synthesis and are consistent with the notion that SK1/Sph-1-P down-regulates such a metabolic pathway. However, none of the above reports examined the metabolic pathway involved in the formation of Sph-1-P. Here, by using three different radioactive precursors and selective inhibitors, we were able to demonstrate that in non-stimulated proliferating MDCK cells, endogenous SK1 did not clear de novo formed Cer because neither [14C]Sph-1-P nor [3H]Sph-1-P was detected, and Sph-1-P appeared as the major 32P-labeled sphingolipid metabolite. This fact, together with the lack of
Endogenous Sphingolipid Metabolism in MDCK Cells

FIGURE 8. Sphingolipid metabolism in nonstimulated proliferating MDCK cells. Active coexistence of the de novo and salvage pathway determined the proliferating stage of MDCK cells. Sph-1-P most probably formed by the salvage pathway controls the de novo formed Cer by exerting a negative modulation of SPT and DHCer/Cer syntheses.

inhibitors of the de novo synthesis pathway to affect the formation of [32P]Sph-1-P, leads us to suggest that the modulation of Cer de novo synthesis occurs by endogenous SK1 activity, which most probably clears Cer coming from the salvage pathway. In other words, we propose that SK1 operating on the salvage pathway of sphingolipid metabolism exerts a control on de novo formed Cer. Our results reinforce the so-called sphingolipid rheostat concept (28), in the sense that the relative level of Cer and Sph-1-P determines cell fate for death, survival, or proliferation. In this context, SK1 has been proposed to play a role as an interconverting enzyme that simultaneously removes a proapoptotic signal (Cer) and creates a survival signal (Sph-1-P). However, our results reveal that in nonstimulated proliferating MDCK cells, it appears that there is not a channelling process but a process involving the cross-talking of separate pathways, where the proapoptotic signal (Cer) comes from the de novo synthesis and where the survival signal (Sph-1-P) appears to be formed by a different pathway, probably the salvage pathway (Fig. 8).

Under our experimental conditions, it appears that de novo formed Cer was preferentially drained to the formation of SM, thus constituting a major pathway to clear de novo synthesized Cer. In fact, under inhibition of Cer biosynthesis, the decrease in [14C]Cer was accompanied by a parallel diminution in [14C]SM, thus reflecting that both metabolites stem from the de novo pathway. It is interesting to note that the increase in de novo formed Cer was only partially accompanied by an increase in SM synthesis but that after a certain level of accumulated Cer no further increase in SM de novo synthesis was observed. It is evident that SM synthesis served to clear the basal level of de novo formed Cer but was unable to further metabolize the excess of accumulated Cer when its synthesis was uncontrolled. Because in our system SM appeared as an important end product of the metabolic pathway, the saturability of SM synthesis could be a major cause of Cer accumulation. Whether the limiting factor was the availability of the ceramide-transporting protein (CERT) or the SM synthase activity needs further demonstration. In any case it appears that the metabolic transformation of Cer to SM may constitute a regulatory point in sphingolipid metabolism, as suggested in Molt-4 cells treated with the death-inducing agent etoposide (33).

As a pivotal molecule, Cer can be phosphorylated to Cer-1-P, thus constituting another branch to clear Cer. By using the different radioactive precursors and the selective inhibition of the de novo pathway, we were able to demonstrate the existence of two separate pools of Cer-1-P: one dependent on and the other independent of de novo formed Cer. The first one was detected by the presence of [14C]Cer-1-P sensitive to the inhibition of Cer de novo synthesis, which has a limited capacity to clear de novo synthesized Cer, whereas the second one was denoted by the resistance of [32P]Cer-1-P to inhibition of the de novo synthetic pathway. In any case, Cer-1-P synthesis was unable to metabolize the increase in the level of [14C]Cer induced by the inhibition of Sph-1-P synthesis.

It is interesting to note that the high synchronism among the enzymes involved in sphingolipid metabolism allows an adequate branching of the pathway, thus avoiding the accumulation of intermediate metabolites that could affect cellular fate. It is also noticeable that the cell possesses an extremely low capacity to adapt to changes in sphingolipid metabolism, as only a minor rise in the level of de novo formed Cer can be cleared. This fact could be the reason that sphingolipid metabolism is so highly implicated in determining cell fate and could also explain the efficiency of its pharmacological manipulation to induce cell death. We suggest that under physiological proliferating conditions there exists a dynamic interplay between the de novo synthesis and the salvage pathway, where the Sph-1-P formed by the salvage pathway controls the de novo synthesis of Cer, thus maintaining an adequate level compatible with cellular physiology (Fig. 8).

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Endogenous Sphingolipid Metabolism in MDCK Cells

SEPTEMBER 12, 2008•VOLUME 283•NUMBER 37
JOURNAL OF BIOLOGICAL CHEMISTRY 25691

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