Characterization of Ceramide Synthesis

A DIHYDROCERAMIDE DESATURASE INTRODUCES THE 4,5-TRANS-DOUBLE BOND OF SPHINGOSINE AT THE LEVEL OF DIHYDROCERAMIDE

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Ceramide (N-acylsphingosine) biosynthesis has been proposed to involve introduction of the 4,5-trans-double bond of sphingosine after synthesis of dihydroceramide (i.e. N-acylsphinganine). For the first time, the in vitro conversion of dihydroceramide to ceramide has been demonstrated using rat liver microsomes and N-[1-14C]octanoyl-δ-erythro-sphinganine (st-H₂Cer) and either NADH or NADPH as co-substrate; the apparent K_m values for st-H₂Cer and NADH were 340 and 120 μM, respectively. Molecular oxygen is required for enzymatic activity, and cyanide, divalent copper, as well as antibodies raised against cytochrome b₅ are inhibitory, which suggests that this enzyme should be named dihydroceramide desaturase based on these similarities with the mechanism of Δ⁵-desaturase (stearyl-CoA desaturase). Factors that influenced the activity of dihydroceramide desaturase include the alkyl chain length of the sphingoid base (in the order C₁₈ > C₁₂ > C₈) and fatty acid (C₈ > C₁₈); the stereochemistry of the sphingoid base (δ-erythro > l-threeo-dihydroceramides); the nature of the headgroup, with the highest activity with dihydroceramide, but some (~20%) activity with dihydrosphingomyelin (activity was not detected with dihydroglucosylceramide, however); and the ability to utilize alternative reductants (ascorbic acid could substitute for a reduced pyridine nucleotide, but was inhibitory at higher concentrations). Dihydroceramide desaturase was inhibited by dithiothreitol, which suggests that it might be possible to alter ceramide synthesis by varying the thiol status of hepatocytes. Consistent with this hypothesis, when rat hepatocytes were cultured in varying concentrations of N-acetylcysteine (5 and 10 mM), there was a decrease in the relative incorporation of [14C]serine into [14C]ceramide. These studies have conclusively established the pathway of ceramide synthesis via desaturation of dihydroceramide and have uncovered several properties of this reaction that warrant further consideration for their relevance to both sphingolipid metabolism and signaling.

Over the last few years, it has become clear that ceramide plays important roles in the metabolism of cells. It is involved as a second messenger in what has become known as the sphingomyelin cycle (reviewed in Ref. 1) and, to name but a few, serves as a mediator of cellular senescence (2), apoptosis, and differentiation in many cell types (3). It was also observed that not only ceramide derived from the sphingomyelin pool found in the plasma membrane but also that from de novo synthesis is involved in the cellular responses to inducers of apoptosis (4) and differentiation (5). These results suggest an important role of the anabolic pathway of ceramides in signal transduction. It is therefore very likely that sphingolipid biosynthesis is tightly regulated. Taking into consideration that dihydroceramide does not mimic the effects of ceramide (6, 7), one possible site of regulation might be dihydroceramide desaturase. In addition, ceramide is also involved in the transport of glycosylphosphatidylinositol-anchored proteins, for example in yeast (8).

The biosynthesis of the lipid anchor of all glycosphingolipids and sphingomyelin starts with the condensation of L-serine and palmitoyl-CoA catalyzed by the pyridoxal phosphate-dependent serine palmitoyltransferase (EC 2.3.1.50). Its product, 3-dehydro sphinganine, is immediately reduced by the NADPH-dependent 3-dehydro sphinganine reductase yielding δ-erythro sphinganine (see Ref. 9, and references therein). Whether this sphingoid base is first desaturated to form sphingosine and then acylated to yield ceramide (reviewed in Ref. 9) or first acylated and then desaturated as proposed by several authors (9–12) remained unclear for some time, although experimental results strongly favored the latter pathway. The discovery of funonisin B₁ as a potent inhibitor of the N-acylation of sphingoid bases (12, 13), helped us and others to demonstrate that, indeed, dihydroceramide is an intermediate in sphingolipid biosynthesis in many cell types (14, 15), whereas sphingosine is not. Thus, δ-erythro-sphinganine is first acylated and subsequent introduction of the 4,5-double bond by the dihydroceramide desaturase leads to the formation of ceramide.

The first three enzymes involved in sphingolipid biosynthesis are well characterized, and their subcellular localization and topology were found to be the cytosolic face of the endoplasmic reticulum (16, 17). We describe here for the first time an in vitro assay for dihydroceramide desaturase activity, which gave us the possibility to characterize this enzyme and study its substrate specificity in vitro.

EXPERIMENTAL PROCEDURES

Materials

Buffers and salts were purchased from Merck (Darmstadt, Germany). [14C]-Labeled fatty acids, CHAPS, NADH and other biochemicals were purchased from American Type Culture Collection.

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1 The abbreviations used are: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; Cer, ceramide; H₂Cer, dihydroceramide; st-Cer, semitaurated ceramide (N-[1-14C]octanoyl-δ-erythro-
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were supplied by Sigma (Munich, Germany). Pyrogallol was provided by Fluka (Buchs, Switzerland). X-Omat scientific imaging film was purchased from Eastman Kodak Co. Male Wistar rats were supplied by Charles River Wiga GmbH (Sulzfeld, Germany). b-erythro-C18-sphinganine and b-erythro-C14-sphingosine were kindly provided by Dr. Karl Clasen (Bio Diagnostic GmbH, H tiger Oldendorf, Germany); b-erythro-glucosylsphingosine by Gundo Wilkening (Kekule-Institut), and N-[6-3H]octanoyl-b-erythro-C14-sphingosine by Dr. Katussevani Bernardi (Institut für Immunologie, Kiel, Germany).

Chicken anti-rat cytochrome b5 antibodies and chicken control IgG were kindly provided by Dr. John B. Schenkman (Department of Pharmacology, University of Connecticut, Farmington, CT). The activity and specificity against rat liver microsomes were shown by Western blot analysis using horseradish peroxidase-coupled anti-chicken IgG as secondary antibody. The anti-cytochrome b5 antibodies bound exclusively to cytochrome b5, while treating the blot with the chicken control IgG showed no detectable staining.

Preparation of Rat Liver Microsomes

All solutions were prepared a day before use and stored at 0–4 °C. Male Wistar rats (50–55 days old, 200–250 g) were starved for 12 h and killed by decapitation. The livers were excised and washed twice in ice-cold sucrose/phosphate buffer (0.25 M sucrose, 100 mM NaH2PO4/Na2HPO4, pH 7.4). All subsequent procedures were carried out at 0–4 °C. Individual livers were weighed, minced with a scalpel, added to the same sucrose/phosphate buffer (0.5 g of liver/ml) and homogenized with 5 up-and-down strokes at 600 rpm in a Braun glass homogenizer with a loose-fitting Teflon pestle. The homogenate was centrifuged at 680 x g for 10 min. The supernant was saved, and the pellet was resuspended and centrifuged the same way as described above. The combined supernatants were centrifuged at 10,000 x g for 10 min and the resulting supernatant at 105,000 x g for 1 h. The pellet was resuspended in alkaline phosphate buffer (100 mM NaH2PO4/Na2HPO4, pH 8.0) (1 g of liver/ml). After centrifugation at 105,000 x g for 1 h, the pellet finally was resuspended in phosphate buffer (100 mM NaH2PO4/Na2HPO4, pH 7.4) (2.5 g of liver/ml), frozen in aliquots in liquid nitrogen, and stored at −80 °C.

Preparation of Silica Gel/Sodium Borate Thin Layer Chromatography Plates

Defatted plates were each coated with a mixture of 30 ml of double-distilled water, 0.8 g of Na2B4O7·10H2O, and 14 g of Silica gel G 60 and were allowed to dry for 2 days at room temperature (18).

Preparation of 14C-Labeled Substrates

The labeled substrates were synthesized by activation of 1-[14C]fatty acid with N-hydroxysuccinimide and dicyclohexylcarbodiimide for subsequent acylation of the respective sphingoid base as described previously (19). The crude product mixtures were evaporated under a stream of nitrogen. Purification first involved separation by TLC with CHCl3/CH3OH/2N ammonia (65:25:4, v/v) for Cer and CHCl3/CH3OH/2N ammonia (65:25:4, v/v) for st-GlcCer. The solution was sonicated with a stream of nitrogen in a 1.5-ml test tube. After dissolving in ethanol/dodecane (98:2, v/v), the solution was added to the reaction mixture and incubated for 15 min at 37 °C.

The reaction was terminated by addition of 200 μl of CHCl3/CH3OH (83:17, v/v) on ice. Lipid extraction was achieved by addition of 343 μl of CH3OH and 22 μl of CH3Cl, and vigorously mixing for 20 min. Phases were separated by centrifugation, and the lower phase was collected.

The extraction procedure was repeated twice with 200 μl of CHCl3/CH3OH (83:17, v/v), each. The combined organic phases were evaporated under a stream of N2 and dissolved in 30 μl of CHCl3/CH3OH (1:1, v/v). The lipid mixture was separated by TLC using silica gel/sodium borate plates. The following were used as developing systems: CHCl3/CH3OH (9:1, v/v) for Cer/CH3Cer, CHCl3/CH3OH/2N ammonia (65:25:4, v/v) for st-GlcCer/GlcCer, and CHCl3/CH3OH/2N ammonia (60:35:8, v/v) for st-H2Cer/st-SSM. TLC was done twice to achieve a better separation of radiolabeled compounds.

The lipids were visualized by autoradiography, and the RF values for st-H2Cer and st-Cer were 0.7 ± 0.1 and 0.6 ± 0.1, respectively (depending on the homogeneity of the plates; however, a ΔRF of at least 0.1 was always observed). The lipids were recovered from the plate by scraping, and the radioactivities determined by liquid scintillation counting in a Packard 1900CA Tri-Carb analyzer using Ultima Gold (Packard, Frankfurt, Germany) as the scintillation liquid, after allowing the samples to stand at room temperature for 1 h. A background value of radioactivity, which was subtracted from each corresponding value, was obtained by scraping an area of about the same size in each lane of the plate containing no detectable radioactivity.

Sphingolipid Biosynthesis by Intact Hepatocytes

Rat hepatocytes were isolated and placed in primary culture, and sphingolipid biosynthesis from [14C]serine was analyzed as described previously (22, 23). Briefly, to each dish (approximately 1 mg of protein/dish) was added 1 ml of Dulbecco’s modified Eagle’s medium containing Ci of [14C]serine (for a total serine concentration of 1 mM) and 0, 5, 10, or 15 mM N-acetylcysteine (or sodium acetate). After incubation for 12 h, the lipids were extracted and acid-hydrolyzed to release the radiolabeled sphinganine and sphingosine backbones, and the radiolabeled sphingoid bases were quantified by thin layer chromatography and scintillation counting (22, 23).

Miscellaneous Procedures

Protein concentrations were measured by the method of Bradford (24) using bovine serum albumin as standard protein.

Presentation of Data

All data presented are means of at least duplicate determinations. Most single values are the mean of three to four separate determinations. All individual values are in the range of ±5% up to ±15% of the mean.

The results described in this report are for rat liver microsomes; however, activity was also detected in the homogenates of rat lung, rat brain, mouse liver, and mouse brain, as well as in cultured mouse cerebellar neurons and rat B104 neuroblastoma cells.
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RESULTS AND DISCUSSION

Conversion of Dihydroceramide to Ceramide by Rat Liver Microsomes—The in vitro assay described under “Experimental Procedures” allowed us to characterize the enzymatic reaction that is responsible for converting dihydroceramide to ceramide. Examination of the lipid extracts from these assays by TLC (Fig. 1, lane 1) revealed that [14C]st-Cer was produced from [14C]st-H2Cer, and that these compounds accounted for at least 90% of the radiolabel. The identity of these compounds was confirmed by fast atom bombardment mass spectroscopy of the radiolabel. The structures of st-H2Cer (substrate) and st-Cer (product) are shown on the right side. The [14C]-radiolabel is indicated by an asterisk.

Dihydroceramide desaturase was assayed as described under “Experimental Procedures” using st-H2Cer as the lipid substrate and intact rat liver microsomes (lane 1) or heat-inactivated microsomes (10 min, 95 °C, lane 2) as the enzyme source. The reaction was terminated by addition of CHCl3/CH3OH (83:17, v/v), and lipids were extracted and resolved by TLC using CHCl3/CH3OH (9:1, v/v) as the solvent system. Radioactive spots were visualized by autoradiography. The structures of st-H2Cer (substrate) and st-Cer (product) are shown on the right side. The [14C]-radiolabel is indicated by an asterisk.

The requirement for an electron donor (rather than acceptor) suggests that this reaction is catalyzed by a desaturase rather than a dehydrogenase. If so, molecular oxygen would be required as the electron acceptor in the desaturation process, and an oxygen scavenger, such as pyrogallol (1,2,3-trihydroxybenzene), should be inhibitory. As shown in Fig. 2, replacing the air in the assay tube with argon caused a small reduction in enzymatic activity (the lack of complete inhibition is not surprising because the solutions were not purged of residual oxygen); moreover, the conversion of st-H2Cer to st-Cer was strongly inhibited by adding increasing concentrations of pyrogallol, with >90% inhibition at 30 mM (no activity was detected at concentrations higher than 30 mM). The inhibition by pyrogallol was less potent if the assays were conducted under an oxygen atmosphere rather than argon; thus, it can be concluded that molecular oxygen is required for enzymatic introduction of the double bond into st-H2Cer.

The characteristics of this activity (presence in microsomes, utilization of a reduced pyridine nucleotide, and requirement for oxygen) are similar to the microsomal stearoyl-coenzyme A desaturase (Δ⁹ desaturase, reviewed in Ref. 25). This enzyme is part of a multi-enzyme complex, which consists of NADH-cytochrome b₅ reductase, cytochrome b₅, and Δ⁹ desaturase. The knowledge about this system and its inhibitors gave us the opportunity to study further the likelihood that conversion of st-H2Cer to st-Cer is catalyzed by an analogous desaturase. First, cyanide is known to inhibit the Δ⁹ desaturase activity, because it is bound more tightly than O₂, and we found that NaCN also inhibits the in vitro conversion of st-H2Cer to st-Cer with an IC₅₀ of 100 µM. Second, divalent copper blocks electron transport to molecular oxygen in Δ⁹ desaturase (by inhibiting the formation of intermediate superoxide anions from O₂⁻) (26) and was inhibitory for dihydroceramide desaturation as well (with an IC₅₀ of 31 µM). Third, antibodies raised against cytochrome b₅ have been found to inhibit plasmalogens desaturase utilizing the cytochrome b₅ electron transport system (27). Likewise, when rat liver microsomes were incubated for 30 min with different amounts of anti-cytochrome b₅ antibodies prior to the desaturase assay, formation of ceramide was inhibited by 48% up to 82% compared with controls when varying the dilution of the antibody serum from 1:1000 to undiluted, respectively (four different dilutions; data not shown).

Despite these similarities, an iron(II)-chelator (bathophenanthroline sulfonate) that inhibits Δ⁹ desaturase by removing non-heme bound and catalytically important iron(II)
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DE-18/8-H₂Cer

LT-18/8-H₂Cer

DE-18/12-H₂Cer

DE-18/18-H₂Cer

DE-12/8-H₂Cer

LT-12/8-H₂Cer

DE-8/8-H₂Cer

DE-18/8-H₂SM

DE-18/8-GlcH₂Cer

DE-18-sphinganine

Relative Enzymatic Activity

0.0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0

Variation of Type of Sphingolipid

Variation of Sphingoid Base Chain Length

Variation of Acyl Chain Length

Reference

Fig. 3. Substrate specificity of the dihydroceramide desaturase in vitro. Dihydroceramide desaturase was assayed under “Experimental Procedures” using the lipid substrates indicated in this figure. Method C was used for solubilization. Results are expressed relative to the enzymatic activity of dihydroceramide desaturase using st-H₂Cer as substrate. A relative enzymatic activity of 1.0 equals 17.8 nmol of st-Cer formed/h/mg of protein. DE, d-erythro; LT, l-threo; numbers refer to the number of carbon atoms in the moieties of the molecules and are stated in the order: sphingoid base/fatty acid.

(28) had no effect on dihydroceramide desaturation. Thus, dihydroceramide desaturase and Δ⁶ desaturase are likely to have similar electron transport system(s), but are clearly distinct enzymes.

Utilization of Different Dihydroceramides by This Desaturase—A Lineweaver-Burk plot of the activity with varying concentrations of N-octanoyl-d-erythro-C₁₈₈-sphinganine (st-H₂Cer) (and 20 mM NADH) produced an apparent Kₘ for this substrate of 340 μM. Other dihydroceramide analogs that differed in chain length, stereochemistry, and type of headgroup were also analyzed (Fig. 3).³ The in vitro activity decreased as the chain length of the amide-linked fatty acid of the H₂Cer was increased (i.e. 18/8 > 18/12 > 18/18), presumably due to the greater insolubility of the longer chain homologs. No desaturation of the unmodified sphingoid base (sphinganine) was detected.

The activity was lower with a truncated sphinganine analog than with the natural, 18-carbon sphingoid base (cf. 8/8 and 18/8-H₂Cer) (Fig. 3). The stereochemistry of the sphingoid base had a large effect on activity; the activity with N-octanoyl-o-erythro-C₁₈₈-sphinganine (DE-18/8-H₂Cer) was approximately 10-fold higher than with the unnatural l-threo-isomer (LT-18/8-H₂Cer); a 2-fold difference was seen with DE-12/8-H₂Cer versus LT-12/8-H₂Cer.

Somewhat surprisingly, dihydrosphingomyelin (DE-18/8-H₂SM) was a relatively good substrate for the desaturase, yielding 20% of the activity with DE-18/8-H₂Cer. In contrast, st-GlcH₂Cer was not desaturated at a detectable rate in vitro, although there was considerable hydrolysis of GlcH₂Cer by the microsomes (50–60% of the total radioactive substrate added) and Cer was produced, presumably due to desaturation of the st-H₂Cer. To exclude the possibility that GlcCer was formed from GlcH₂Cer, but was not detected because it was hydrolyzed, the hydrolysis was inhibited with conduritol β-epoxide (a potent β-glucosidase inhibitor that reduced the hydrolysis to <10% of the total radioactivity), and no desaturation of st-GlcH₂Cer was detected.

The conclusion that H₂SM is a substrate for the desaturase is more definitive than the lack of activity with st-GlcH₂Cer, because the latter might be affected by the physical state of the substrate or another artifact of the in vitro assay conditions. Nonetheless, these observations are consistent with studies that have been conducted in intact cells. Smith and Merrill (29) have reported that de novo sphingolipid biosynthesis from [¹⁴C]serine by J774 cells initially produces large amounts of complex sphingolipids with a [¹⁴C]sphinganine backbone, and which are converted to the more typical sphingosine-containing species over time. Another study of the metabolism of NBD-labeled sphingolipids by HT-29 cells⁴ has found that NBD-H₂Cer is initially incorporated into Glc-NBD-H₂Cer, but direct desaturation of Glc-NBD-H₂Cer was not observed. Thus, as was seen on our study of the desaturase activity in vitro, desaturation of GlcH₂Cer actually reflected hydrolysis of the glycoconjugate to the (NBD-)H₂Cer, which was oxidized and (in the case of the studies of the NBD-sphingolipids in intact cells) the glycosyl headgroup was added.

The addition of the product of the desaturase reaction, st-Cer, to the in vitro assay mixture (with DE-18/8-H₂Cer as the substrate) was found to inhibit the activity of the desaturase with an IC₅₀ of 140 μM. Future studies should determine whether this product inhibition is competitive or represents allosteric regulation of the desaturase.

Other Characteristics of Dihydroceramide Desaturase Activity in Vitro—The activity of dihydroceramide desaturase is relatively stable: approximately 50% of the activity was lost after 1 year of storage at −80 °C, or 7 days at 4 °C, or 3 h at 37 °C. When the assays were conducted at varying temperature, activity could be detected at 5 °C (about 8% of maximal activity) and steadily increased with the incubation temperature until a maximum of 43 °C (the half-maximal enzymatic activities were at 27 °C and 47 °C); above 60 °C, no activity was detected (data not shown).

Optimal activity was obtained over a fairly wide pH range (pH 6.5–9) (data not shown). A much lower activity was obtained at pH < 6.5; however, this may reflect a buffer effect (at least in part) because there was a large drop in activity of about 80% at pH 6 when the assay was conducted with citrate buffer instead of phosphate buffer. Nevertheless, further decrease of citrate buffer pH value (down to pH 4) resulted in further reduction of activity. When the assays were conducted at neutral pH, the same activities were found when HEPES/NaOH, MOPS/KOH, NaH₂PO₄/Na₂HPO₄, or glycine/NaOH were used over a range of concentrations (50–300 mM); however, the activity using Tris/HCl was reduced by approximately 30%.

Addition of various cations (CaCl₂ at 0.1–100 μM, MgCl₂ at 1 μM to 1 mM, MnCl₂ at 1 μM to 1 mM, FeCl₃ at 1–100 μM, KCl at 10–150 mM, NaCl at 10–100 mM, Na₂SO₄ at 10–100 mM, and EDTA at 10–100 mM) had no effect on the activity, whereas ZnCl₂ (at 1 mM) decreased the activity by 25% (data not shown). Thus, the enzyme is tolerant to many inorganic cations.

In contrast, the activity was inhibited 81% by addition of 1 mM dithiothreitol (DTT), a common reagent used to protect protein thiol groups from oxidation. This may explain why this reaction has not been observed in previous studies since 10 mM

³ The results shown in Fig. 3 were obtained using method C to solubilize the substrates, but similar results were found using the other two methods described under “Experimental Procedures.”

⁴ Kok, J. W., Nicolova-Karakashian, M., Klappe, K., Alexander, C., and Merrill, A. H., Jr. (1997) J. Biol. Chem. 272, 21128–21136.
H2Cer, but reduced the amount of label in sphingosine-containing sphingolipids by rat hepatocytes.

As shown in Fig. 4, deacetylated to increase the levels of intracellular thiols (glutathione) (30–33). As shown in Fig. 4, deacetylated to increase the levels of intracellular thiols (glutathione) (30–33). As shown in Fig. 4, deacetylated to increase the levels of intracellular thiols (glutathione) (30–33). As shown in Fig. 4, deacetylated to increase the levels of intracellular thiols (glutathione) (30–33). As shown in Fig. 4, deacetylated to increase the levels of intracellular thiols (glutathione) (30–33). As shown in Fig. 4, deacetylated to increase the levels of intracellular thiols (glutathione) (30–33).

Dihydroceramide desaturase may have one (or more) disulfide bonds that is (are) required for protein stability and/or catalytic activity.

Conversion of Dihydroceramide to Ceramide by Intact Rat Hepatocytes—Previous studies have shown that radiolabeled serine is incorporated into Cer and SM by intact rat hepatocytes with little accumulation of H2Cer (22, 23); therefore, the desaturase reaction is not rate-limiting in these cells (at least, under the conditions used). Since dihydroceramide desaturase is inhibited by DTT, we examined whether elevation of cellular thiols would suppress the formation of ceramide(s) by cultured rat hepatocytes. These experiments were conducted by incubating the hepatocytes with increasing concentrations of N-acetyl-l-cysteine, which is taken up by hepatocytes and deacetylated to increase the levels of intracellular thiols (glutathione and protein thiols) (30–33). As shown in Fig. 4, N-acetyl-l-cysteine did not cause the accumulation of label in H2Cer, but reduced the amount of label in sphinganine-containing sphingolipids by approximately half. Since dihydroceramides have been found to accumulate in several other cell systems (9, 29), it would be interesting to know if this is due to a difference in the levels of dihydroceramide desaturase per se or its modulation by factors such as the cellular thiol status.

Conclusion—For the first time, it has become possible to demonstrate the in vitro conversion of dihydroceramide to ceramide, to assign the name dihydroceramide desaturase to the enzyme that catalyzes this reaction, and to delineate the pathway for introduction of the 4,5-trans-double bond of sphingosine into dihydroceramide (or dihydrosphingomyelin) is catalyzed by dihydroceramide desaturase, as shown. The diagram also illustrates how altering the cellular thiol state might affect signaling pathways that involve ceramide. It is by no means clear whether the step marked with an asterisk also occurs in vivo, because the subcellular site and topology of dihydroceramide desaturase is not known.

**Fig. 4. Effect of N-acetyl-l-cysteine on incorporation of [14C]serine into sphinganine- and sphingosine-containing sphingolipids by rat hepatocytes.** Primary cultures of rat hepatocytes were incubated with [14C]serine and the shown concentrations of N-acetyl-l-cysteine for 12 h; the amount of label in sphinganine- and sphingosine-containing sphingolipids was then determined as described under “Experimental Procedures.” The results are given as the mean ± S.D. (n = 3) for two separate hepatocyte preparations; the results designated by * and ** are significantly different from the control (no N-acetyl-l-cysteine) at p < 0.05 and p < 0.02, respectively.

Dihydroceramide desaturase activity.

**Fig. 5. The pathway for de novo sphingolipid biosynthesis.** Sphingolipid biosynthesis begins with the condensation of serine and palmitoyl-CoA followed by reduction of 3-ketosphinganine, and acylation of sphinganine to dihydroceramide. Introduction of the 4,5-trans-double bond of sphingosine into dihydroceramide (or dihydrosphingomyelin) is catalyzed by dihydroceramide desaturase, as shown. The existence of this pathway in vivo also depends on the topology of the enzymes involved. Sphingomyelin formation has been assigned to the luminal side of the Golgi membranes (35), whereas desaturation of dihydroceramide presumably occurs at ER membranes with so far unknown topology.

The sensitivity of dihydroceramide desaturase to thiols is also intriguing because thiols protect against cell injury by many agents (30–33), whereas depletion of glutathione can lead to cell death. The assay system described in this paper will allow further characterization of this important enzyme and elucidation of its role in cell regulation.

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