A Truncated Epoxy-glucosylceramide Uncouples Glycosphingolipid Biosynthesis by Decreasing Lactosylceramide Synthase Activity*

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Introduction of an epoxide function at the C4 position of the glucose moiety of a truncated glucosylceramide (GlcCer) yields two stereoisomeric products namely a gluc- and a galacto-derivative.

The effects of both analogues on the [14C]serine incorporation into glycosphingolipids as well as on lactosylceramide (LacCer) synthase activity of primary cultured old chick embryos were investigated.

Whereas the galacto-derivative had no effect either on the pattern of labeled glycosphingolipids or on LacCer synthase activity, pretreatment (24 h) of neurons with the gluco-derivative caused a concentration dependent (IC50 = 8 μM) and irreversible decrease of the specific activity of LacCer synthase.

As a consequence the biosynthetic glycosphingolipid pattern of cultured neurons treated with the gluc-analogue changed as follows: the radioactive label of all glycosphingolipids biosynthetically derived from GlcCer was decreased while labeling of GlcCer increased significantly.

The inhibitory effect of the gluco-derivative on LacCer synthase activity was much less pronounced in vitro. Thus 250 μM analogue caused only about 30% inhibition of enzymatic activity.

Glycosphingolipids (GSL) are complex components of the outer leaflet of eucaryotic plasma membranes. They form cell-specific and species-specific patterns, which change during ontogenesis and cell transformation. Sialic acid-containing GSL, the so called gangliosides, are highly enriched in the nervous tissue (1–3).

Ceramide, the hydrophobic backbone of all sphingolipids, is formed from dihydroceramide by desaturation (4). Dihydroceramide is synthesized on the cytosolic face of the endoplasmic reticulum membrane (5). The stepwise addition of sugar moieties to ceramide is catalyzed by glycosyltransferases, which are localized in the Golgi membranes (6). On the cytosolic face of the Golgi membrane ceramide is glucosylated by UDP-glucose: ceramide glucosyltransferase (GlcCer synthase) (7–9). The resulting GlcCer is then converted into LacCer by UDP-galactose: glucosylceramide galactosyltransferase (LacCer-synthase or Gal-T 1). LacCer is the common precursor of the ganglio, globo, and neolacto series of GSL. Therefore the regulation of its biosynthesis should be of central importance for GSL metabolism (10). So far only a regulation of LacCer synthesis by low density lipoprotein has been described in normal and tumor cells (11, 12).

The topology of LacCer synthase in the cist Golgi membrane has not yet been unambiguously proven. The finding that mutant Chinese hamster ovary cells with an intact enzyme, but lacking the UDP-galactose translocase in the Golgi lumen, have greatly reduced levels of LacCer, suggests a luminal topology for LacCer synthase (13). Ghidoni and associates (14), however, reported a cytosolic orientation of the enzyme in rat liver Golgi.

The physiological importance of GSL is of central interest, yet not clear (15). One possibility to approach this problem is to specifically inhibit different steps of GSL biosynthesis and investigate the consequences of such inhibitions in different biological systems.

Compounds, isolated from different fungi, which specifically block enzymes leading to the formation of ceramide, were recently described (16, 17).

Three-1-phenyl-2-dodecanoylamino-3-morpholino-1-propanol (o-threo-PDMP) is the first synthetic inhibitor of GSL biosynthesis (18, 19). It is a ceramide analogue which inhibits GlcCer synthase activity. During the last years PDMP was used

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The abbreviations used are: GSL, glycosphingolipids; Cer, ceramide (N-acetylsphingosine); truncated Cer (12C12-Cer), (25R,4E)-2-(α-dodecanoylamino)-4-dodecen-3-ol; GlcCer, glucosylceramide, GlcCl = 1Cer; truncated GlcCer, (25R,4E)-1-(β-D-glucopyranosyl)oxoy)-2-(hex-4-enylamino)-4-octadecen-3-ol; epoxy-glycosylceramide, gluco-4-epoxy-4-C-methylhexylglycosyl ceramide or (25R,4E)-1-(1,4,7-anhydro-4-C-(hydroxymethyl)β-D-glucopyranosyl)oxoy)-2-(dodecanoylamino)-4-octadecen-3-ol; gangliosides, the outer leaflet of eucaryotic plasma membranes; they form cell-specific and species-specific patterns, which change during ontogenesis and cell transformation. Sialic acid-containing GSL, the so called gangliosides, are highly enriched in the nervous tissue (1–3).

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* The enzymes used include: UDP-glucose, ceramide β1–1 glucosyltransferase (EC 2.4.1.80) or GlcCer-synthase; UDP-galactose, galactosylceramide β1–1 galactosyltransferase (EC 2.4.1.1) or LacCer synthase or galactosyltransferase 1 or Gal-T 1; UDP-galactose, N-acetylglactosaminyl-α(N-acetylgalactosaminyl)-galactosyl-glucosylceramide β1–3 galactosyltransferase (EC 2.4.1.62) or GalNAc synthase or galactosyltransferase II or Gal-T II; CMP-N-acetylneuraminylceramide β1–2 sialyltransferase (EC 2.4.9.70) or GnT II synthase or sialyltransferase 1 or SAT 1; CMP-N-acetylneuraminyl, (N-acetylmuraminyl)-galactosyl-glucosylceramide β1–3 galactosyltransferase (EC 2.4.1.62) or GalNAc synthase or galactosyltransferase II or Gal-T II; CMP-N-acetylneuraminyl, galactosyl-glucosylceramide α2–3 sialyltransferase (EC 2.4.9.8) or GnT II synthase or sialyltransferase II or SAT II; CMP-N-acetylneuraminyl, monosialganglcoside (3Gn1) α2–3 sialyltransferase (EC 2.4.9.92) or GnT III synthase or sialyltransferase IV or SAT IV.
in a large number of studies, reporting a diversity of physiological effects caused by this compound via its depleting effect on GlcCer or higher anabolic products (20, 21).

Very recently the synthesis of epimeric glycosylceramide derivatives, obtained by introducing an epoxide function at the C4 position of the glucose moiety of GlcCer, was reported (22). These truncated derivatives were designed as potential inhibitors of LacCer synthase. We demonstrate in the present study that the gluco-analogue of these compounds exhibits an inhibitory effect on GSL biosynthesis as well as on LacCer synthase activity, whereas the epimeric galacto-derivative does not.

**EXPERIMENTAL PROCEDURES**

**Materials**

Eight-day-old chick embryos were obtained from Prof. Greuel, Institut für Veterinär- und Lebensmittelhygiene, University Bonn. L-[3-14C]Serine (2.13 GBq/mmol), CMP-[14C]NeuAc (10.5 GBq/mmol), UDP-[14C]glucose (10.5 GBq/mmol), and UDP-[14C]galactose (10.5 GBq/mmol) were purchased from Amer sham Corp. (Braunschweig, Federal Republic of Germany). Epoxy-derivatives were synthesized in the laboratory of Prof. Schmidt in Konstanz as previously described (22).

Truncated GlcCer and truncated Cer were synthesized in our laboratory (23, 24). Culture medium (Dulbecco's modified Eagle's medium), trypsin, deoxyribonuclease, bovine serum albumin, and fetal calf serum were purchased from Life Technologies Inc. Karlsruhe, FRG) and plastic Petri dishes were obtained from Falcon (Heidelberg, FRG). Thin-layer Silica Gel 60 plates and LiChroprep RP18 were supplied by Merck (Darmstadt, FRG). GlcCer (purified from human Gaucher's spleen), CMP-NeuAc, UDP-glucose, and UDP-galactose, β-galactosidase from bovine liver, MOPS, CHAPS, and Triton X-100 were obtained from Sigma (München, FRG). The scintillation mixture Pico Fluor 40 was from Packard (Frankfurt, FRG), Sephadex G-25 superfine was from Pharmacia (Freiburg, FRG), and cacodylate was from Fluka (Neu Ulm, FRG). All other reagents and solvents used were of analytical grade quality.

**Cell Culture**

 Cultures of pure neurons were prepared from the method described by Dreyfus et al. (25). Briefly, telencephalons of 8-day-old chick embryos were dissected and freed from their meninges. Cells were isolated by mild trypsinization (0.05%, mass/volume) followed by trituration of the suspension was then centrifuged and resuspended in Dulbecco's modified Eagle's medium containing 20% heat-inactivated fetal calf serum and plated onto poly(L-lysine)-coated 35-mm diameter Petri dishes (5 x 10⁶ cells/dish). At day 4 of culture, cells were incubated in medium containing 2% methionine, 20% fetal calf serum, and 10% horse serum in fresh medium. After the indicated times, cells were harvested and enzyme activities measured immediately in homogenates obtained by resuspending the cells in 0.3 M sucrose mechanically or by a 1-min sonication on an ice bath.

**Labeling and Isolation of Sphingolipids**

Sphingolipids were labeled by feeding cells with L-[3-14C]serine (2 µCi/ml). After the indicated times cells were harvested and lipids extracted from cell pellet with 5 ml of chloroform/methanol/water/pyridine (60:30:6:1, by volume) for 48 h at 48 °C. Phospholipids were degraded by mild alkaline hydrolysis with methanolic NaOH (50 µl) for 2 h at 37 °C. Then, the lipid extracts were desalted by reversed-phase chromatography on silica gel LiChroprep RP18, applied to TLC, and chromatographed with chloroform, methanol, 0.22% aqueous CaCl₂ (60:35:8, by volume); sphingolipids were visualized by fluorography.

**Identification and Quantitative Evaluation of Glycosphingolipids**

All glycosphingolipids were identified from their Rf values (3, 25). Radioactive bands were scraped from the TLC plate and measured by liquid scintillation counting.

**Incorporation of [14C]Galaactose into Trichloroacetic Acid-precipitable Cell Material**

Cells were labeled for 24 h with [14C]galactose (1 µCi/ml). After the indicated times cells were washed five times with ice-cold phosphate-buffered saline. A solution of trichloroacetic acid (10%) was added and cell proteins were precipitated at 10 min at 4 °C. Then cells were washed five times with ice-cold phosphate-buffered saline. After the indicated times cells were harvested and enzyme activities measured immediately in homogenates obtained by resuspending the cells in 0.3 M sucrose mechanically or by a 1-min sonication on an ice bath.

**Presentation of Data**

Results are depicted as means of at least three different experiments. All enzyme reactions were assayed in duplicate and S.D. values never exceeded 15%.

**RESULTS**

The Effect of Epimeric Epoxy-glycosylceramide Derivatives on the GSL Pattern of Cultured Neurons—The structures of the two epimeric 4-epoxy-4-C-methylene-glycosylceramides used in the present study as potential inhibitors of LacCer formation are presented in Fig. 1. Addition of these derivatives to the culture medium of embryonic chick neurons caused different...
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Labeling with [14C]-serine

controls galacto-analogue [25μM]  
gluco-analogue [25μM]

changes of the labeling of cellular GSL pattern after feeding [14C]serine (Fig. 2).

In the presence of 25 μM gluco-derivative (Fig. 1) the incorporation of [14C]serine into LacCer and higher GSL was reduced by about 45%, while the labeling of GlcCer increased 3.6-fold compared to control cells as assayed by determination of incorporated radioactivity (Fig. 2).

When the cells were treated in a similar way with 25 μM galacto-derivative (Fig. 1) for 24 h, the GSL pattern obtained after metabolic labeling with [14C]serine remained unchanged (Fig. 2).

Thus, only the gluco-derivative of glycosylceramide seems to exhibit an effect on LacCer synthesis, while the epimeric galacto-derivative does not influence this biosynthetic step. Under the described conditions neither the cells showed any morphological changes compared to control cells nor was the protein content/dish influenced by the derivatives, suggesting that neither of the components had an effect on cell viability. Higher concentrations of the analogues (50 μM, 24 h) were, however, cytotoxic.

Similar results were obtained in primary cultured neurons from cerebella of 5-day-old mice isolated as described before (3), where the incorporation of [14C]galactose into the GSL fraction starting with LacCer was reduced up to 50% by the gluco-derivative compared to control cells (data not shown). LacCer synthase activity was, however, much better measurable in the homogenate of the embryonic neurons, which were still dividing during the first days in culture (25). Therefore all enzyme measurements were done in chick embryonic neurons.

The Effect of Epimeric Epoxy-glucosylceramide Derivatives on Enzyme Activities of Cultured Neurons—The reduced incorporation of labeled serine into LacCer and the ganglioside fraction in the presence of gluco-epoxy-glucosylceramide could be due to the inhibition of LacCer synthase and/or another enzyme activity involved in GSL biosynthesis. To verify this assumption we have performed parallel experiments in which we have measured LacCer synthase activity in cell homogenate after pretreatment of cells with the gluco- or galacto-derivative instead of feeding [14C]serine.

The results are shown in Fig. 3. LacCer synthase activity was decreased by 70% in cells pretreated with the gluco-derivative compared to untreated control cells. No effect on LacCer synthase activity was noticed in homogenate derived from cells pretreated with the galacto-derivative. In this case the specific activity was comparable to that measured in the homogenate of control cells.

No other enzymes of ganglioside biosynthesis tested were affected by pretreatment of cells with the gluco-derivative. Thus the specific activities of GlcCer, G₃₇₃, G₄₃, G₆₃, and G₃₃ synthases remained unchanged compared to control cells (data not shown).

In another set of experiments we measured under similar conditions incorporation of [14C]galactose into trichloroacetic acid-precipitable cell material. No changes of incorporated radioactivity could be measured between control and pretreated...
cells (data not shown), suggesting that galactosylation of proteins is also not affected by this compound.

Thus epoxy-GlcCer seems to specifically affect LacCer synthase activity. This effect was concentration dependent (Fig. 4), half-maximal inhibition being obtained at a concentration of 8 μM derivative.

To clarify if the described effect of epoxy-GlcCer on LacCer synthesis is reversible, cells were treated with the gluco-derivative, then culture medium containing the inhibitor was changed, cells were washed, and then the culture was continued in an inhibitor-free medium. After 24 h culturing in the absence of epoxy-GlcCer, LacCer synthase activity decreased even more, reaching a level comparable to that of cells cultured for 48 h in the presence of inhibitor (Fig. 5). This result strongly suggests an irreversible, most probably covalent, binding of the gluco-derivative to the enzyme.

**The Effect of Epoxy-glycosylceramide on LacCer Synthase Activity in Vitro**—To get more information on the interaction between gluco-epoxy-glycosylceramide and LacCer synthase, we tested its effect on enzymatic activity in vitro.

The assay was carried out under optimal conditions (see "Experimental Procedures"). The apparent $K_m$ for GlcCer was 25 μM and $V_{max} = 0.8$ nmol/h·mg.

When instead of GlcCer the two epoxy-glycosylceramides were added to the GalT-I assay as substrates, no formation of the respective LacCer derivatives was observed (data not shown).

To test the effect of epoxy-GlcCer in vitro, increasing amounts of this derivative were added to the assay mixture. Only a slight inhibition (30%) of LacCer synthase was measured in the presence of 250 μM gluco-derivative (Fig. 6).

**DISCUSSION**

The present study has shown that gluco-4-epoxy-4-C-methylglucosylceramide uncouples GSL biosynthesis in cultured neurons beyond GlcCer by inhibiting LacCer synthase activity and thus leading to an accumulation of GlcCer and to a decreased formation of complex glycosphingolipids.

This effect was observed only when a truncated derivative was used. Similar observations were made in biological tests where sphingosines or ceramides were employed (27-29). The 4-epoxy-GlcCer with C18-sphingosine and C18-fatty acid in its ceramide backbone showed none of the biological effects observed with its truncated analogue when applied to cultured neurons. Differences in cellular uptake of the two analogues may explain these results. Primary cultured embryonic chick neurons were chosen as the biological system because they exhibit a complex ganglioside pattern. Moreover, all enzymes involved in GSL biosynthesis show good specific activities in cell homogenate.

We have measured different glycosyltransferases involved in GSL biosynthesis after pretreatment of cells with epoxy-GlcCer. LacCer synthase activity was reduced in a concentration dependent manner while all the other measured enzyme activities were not affected by this compound.

The fact that even GalT-II (G, synthase) activity was not reduced by 4-epoxy-GlcCer suggests a high specificity of this substance for LacCer synthase. Both these galactosyltransferases catalyze the transfer of 1 galactose residue from UDP-galactose. In the case of LacCer synthase (GalT-I), galactose is
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Recently stimulation of smooth muscle cell proliferation by LacCer has been described (30). 4-Epoxy-GlcCer may be a useful tool to investigate the biological function of endogenous LacCer and higher GSL.

REFERENCES


linked to the glucose residue of GlcCer while in the case of G\textsubscript{M\textsubscript{1}}, synthase (Gal-T\textsubscript{1}), it is linked to the N-acetylglactosamine residue of G\textsubscript{M\textsubscript{1}}.

It has to be pointed out that the biological effect of 4-epoxy-glycosylceramide is highly stereospecific. While the gluco-isomer was strongly effective on LacCer synthase activity, the galacto-isomer was completely inactive. The inefficiency of the galacto-derivative also rules out an unspecific inhibition of LacCer synthase activity, e.g. by the amphiphilic nature of the gluco-isomer.

GlcCer with the same number of carbon atoms in its molecule as the truncated derivatives had also no effect on LacCer synthase activity. This suggests the importance of the epoxy group in the 4 position of the glucose moiety of GlcCer for this inhibitory effect.

Concerning the mechanism of inhibition one would assume an opening of the epoxide ring and subsequent covalent binding to the enzyme. The irreversibility of the inhibitory effect argues in favor of this hypothesis.

The inhibition of GaIT-I activity could be demonstrated in vitro only at much higher concentrations of the gluco-derivative. Pretreatment of intact cells with epoxy-GlcCer was essential for its inhibitory property at low concentrations. This leads to the conclusion that the correct membrane topology is important for the covalent binding of the inhibitor. We cannot, however, exclude the possibility that in intact cells the gluco-derivative accumulates in the Golgi membrane, where LacCer synthesis occurs.

On the other hand, decrease of LacCer synthase activity after pretreatment of cells with 4-epoxy-GlcCer could involve the covalent binding of the derivative to a protein either involved in the transcription of GaIT-I mRNA or in its translation. In addition, we cannot exclude at the present time the binding of the gluco-derivative to a part of LacCer synthase responsible for the transport of GlcCer from the cytosolic to the luminal surface of Golgi membranes (flip-flop protein).

The mechanism of PDMP, described as an inhibitor of GlcCer synthase, has also not yet been clarified. However, the inhibitory effect was reversible upon removal of PDMP from the culture medium and not dependent on a pretreatment of cultured cells.

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