Elevation of Intracellular Glucosylceramide Levels Results in an Increase in Endoplasmic Reticulum Density and in Functional Calcium Stores in Cultured Neurons*

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Gaucher disease is a glycosphingolipid storage disease caused by defects in the activity of the lysosomal hydrolase, glucocerebrosidase (GlcCerase), resulting in accumulation of glucocerebroside (glucosylceramide, GlcCer) in lysosomes. The acute neuronopathic type of the disease is characterized by severe loss of neurons in the central nervous system, suggesting that a neurotoxic agent might be responsible for cellular disruption and neuronal death. We now demonstrate that upon incubation with a chemical inhibitor of GlcCerase, conduritol-B-epoxide (CBE), cultured hippocampal neurons accumulate GlcCer. Surprisingly, increased levels of tubular endoplasmic reticulum elements, an increase in [Ca\(^{2+}\)], response to glutamate, and a large increase in [Ca\(^{2+}\)], release from the endoplasmic reticulum in response to caffeine were detected in these cells. There was a direct relationship between these effects and GlcCer accumulation since co-incubation with CBE and an inhibitor of glycosphingolipid synthesis, fumonisin B\(_1\), completely antagonized the effects of CBE. Similar effects on endoplasmic reticulum morphology and [Ca\(^{2+}\)], stores were observed upon incubation with a short-acyl chain, nonhydrolyzable analogue of GlcCer, C\(_8\)-glucosylthiocteramide. Finally, neurons with elevated GlcCer levels were much more sensitive to the neurotoxic effects of high concentrations of glutamate than control cells; moreover, this enhanced toxicity was blocked by pre-incubation with ryanodine, suggesting that [Ca\(^{2+}\)], release from ryanodine-sensitive intracellular stores can induce neuronal cell death, at least in neurons with elevated GlcCer levels. These results may provide a molecular mechanism to explain neuronal dysfunction and cell death in neuronopathic forms of Gaucher disease.

Glucosylceramide (GlcCer),¹ a degradation product of complex glycosphingolipids (GSLs), is hydrolyzed in lysosomes by the acid hydrolase, glucocerebrosidase (β-glucosylcerebrosidase (GlcCerase). Mutations in the human GlcCerase gene cause a reduction in GlcCerase activity and accumulation of GlcCer, which results in Gaucher disease, the most common lysosomal storage disease (1). As for all lysosomal storage diseases, significant clinical heterogeneity is observed in Gaucher disease, with three main types known, varying from a chronic non-neuronopathic type (type 1) to infantile (type 2) and juvenile (type 3) neuronopathic types (2). The acute neuronopathic type of the disease is characterized by severe loss of neurons in the central nervous system and early onset of the disease (1). A molecular explanation for the neuronal dysfunction associated with neuronopathic forms of Gaucher disease is currently lacking, and no neurotoxic agent has been identified.

In the current study, we have analyzed the effects of treating cultured hippocampal neurons with an active site-directed inhibitor of GlcCerase, conduritol-B-epoxide (CBE) (3). We previously demonstrated that GlcCer accumulates in neurons upon CBE treatment, resulting in changes in axonal morphology, with an increase in the length of the axon plexus and in the number of axonal branch points per cell (4), although CBE had no effect on dendrite development (5). Co-incubation with CBE and inhibitors of sphingolipid synthesis (i.e. fumonisin B\(_1\) (FB\(_1\)), an inhibitor of acylation of sphingoid long-chain bases (6)), antagonizes the effects of CBE, and FB\(_1\) itself reduces the rate of axonal growth (4, 7, 8). We now demonstrate that in addition to these morphological changes, GlcCer accumulation causes changes in neuronal functionality, inasmuch as neurons show increased levels of tubular endoplasmic reticulum (ER) elements, a large increase in [Ca\(^{2+}\)], release from the ER in response to glutamate or caffeine stimulation, and are more sensitive to glutamate-induced neurotoxicity. This is the first time that changes in neuronal functionality have been reported in neurons with elevated GlcCer levels and may help unravel the mechanisms that lead to neuronopathic forms of Gaucher disease.

**EXPERIMENTAL PROCEDURES**

**Hippocampal Cultures**—Hippocampal neurons were cultured on poly-L-lysine-coated glass coverslips essentially as described (4, 9). Briefly, the dissected hippocampi of embryonic day 18 rats (Wistar), obtained from the Weizmann Institute Breeding Center, were dissociated by trypsinization (0.25% w/v, for 15 min at 37 °C). The tissue was washed in Mg\(^{2+}\)-/Ca\(^{2+}\)-free Hank’s balanced salt solution (Life Technologies, Inc.) and dissociated by repeated passage through a constricted Pasteur pipette. For biochemical analysis, cells were plated in minimal essential medium with 10% horse serum at a density of 240,000 cells/24-mm poly-L-lysine-coated glass coverslip. For morphological analysis, neurons were plated at a density of 6,000 cells/13-mm coverslip. After 3–4 h, coverslips were transferred into 100-mm Petri dishes or 24-well Multidishes that contained a monolayer of astroglia. Coverslips were

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4 The abbreviations used are: GlcCer, glucosylceramide; CBE, conduritol-B-epoxide; Dio, 3,3’-dihexyloxacarbocyanine iodide; ER, endoplasmic reticulum; FB\(_1\), fumonisin B\(_1\); GlcCerase, glucocerebrosidase; C\(_8\)-Glc-S-Cer, C\(_8\)-glucosylsphingosine; C\(_8\)-DMB-GlcCer, N-[5-(5, 7-dimethyl-bodipy)-1-pentanoyl]-n-erythro-glycosylphosphoginosine; GSL, glycosphingolipid; RR, ryanodine receptor.
placed with the neurons facing downwards and were separated from the glia by paraffin "feet." Cultures were maintained in serum-free minimal essential medium that included N2 supplements (9), ovalbumin (0.1%), glutamine, and 0.6% (w/v) glucose (19), and transferred to a new multi-well dish containing the same medium but not containing a glia monolayer. After 25 min at 37 °C, neurons were incubated with the C5-DMB-GlcCer on day 2 for 24 h. Top panels, phase-contrast micrographs; bottom panels, immunofluorescence. Bar = 10 μm.

**TABLE I**

| Lipid  | Control (day 4) | CBE-treated (day 4) | -Fold change (day 4) | Control (day 9) | CBE-treated (day 9) | -Fold change (day 9) |
|--------|----------------|---------------------|----------------------|----------------|---------------------|----------------------|
| GT1b   | 15878 ± 2233   | 13227 ± 1737        | 0.80                 | 18299 ± 4883   | 13718 ± 3017        | 0.76                 |
| GD1b   | 10576 ± 3006   | 10214 ± 3240        | 0.90                 | 11712 ± 3810   | 9933 ± 2902         | 0.86                 |
| GD1a   | 10988 ± 1020   | 10360 ± 1288        | 0.97                 | 13248 ± 4451   | 10498 ± 2862        | 0.81                 |
| GM1    | 11222 ± 2448   | 12902 ± 2585        | 1.17                 | 10884 ± 3824   | 12580 ± 5172        | 1.13                 |
| GM2    | 4014 ± 699     | 4271 ± 290          | 1.08                 | 5980 ± 2428    | 5702 ± 1870         | 0.99                 |
| GM3    | 1108 ± 180     | 1757 ± 131          | 1.53                 | 2557 ± 1298    | 2998 ± 1449         | 1.19                 |
| GM4    | 4914 ± 1249    | 6069 ± 1475         | 1.20                 | 5417 ± 2515    | 6350 ± 2246         | 1.25                 |
| SM     | 5748 ± 3441    | 5397 ± 3365         | 0.81                 | 4668 ± 2392    | 3364 ± 1788         | 0.71                 |
| LacCer | 2580 ± 968     | 2524 ± 854          | 0.96                 | 4168 ± 2312    | 5987 ± 845          | 1.09                 |
| GlcCer | 1412 ± 468     | 5616 ± 1670         | 4.84                 | 2212 ± 920     | 12555 ± 5937        | 5.51                 |
| Cer    | 2331 ± 731     | 2073 ± 952          | 0.95                 | 8195 ± 2832    | 7248 ± 2616         | 0.88                 |

*a* Gangliosides are named according to Svennerholm (46). SM, sphingomyelin; LacCer, lactosylceramide; Cer, ceramide.
prior to addition of 1 mCi $^{45}$Ca$^{2+}$ (18.8 mCi/mg, Amersham Pharmacia Biotech, UK) for various times at 37 °C (22). The reaction was terminated by removing coverslips from the wells, washed by dipping five times in medium, and then adding 0.65 ml of NaOH (0.5M) for 3 h. $^{45}$Ca$^{2+}$ was quantitatively extracted by adding NaOH for 16 h and then for another 2 h. NaOH extracts were pooled and $^{45}$Ca$^{2+}$ were determined by liquid scintillation counting.

RESULTS

Accumulation and Localization of GlcCer—Incubation with CBE results in accumulation of GlcCer in cultured hippocampal neurons (4) and in neuroblastoma cells (23, 24). In hippocampal neurons, a correlation was observed between the extent of inhibition of GlcCer degradation after a 3-day incubation with CBE and the changes in axonal branching patterns (4). To quantify GlcCer accumulation after longer times of incubation with CBE, hippocampal neurons were metabolically labeled with $[^3H]$dihydrosphingosine (14), a precursor of GSLs (15). After 4 and 9 days, there were 4.8- and 5.5-fold increases, respectively, in $[^3H]$GlcCer levels in CBE-treated neurons compared with control cells (Table I) and a 7-fold increase after a 14-day incubation (Fig. 1a). However, there were no significant changes in levels of other GSLs (15) or of lactosylceramide, ceramide, or sphingomyelin (Table I).

To determine the intracellular site of GlcCer accumulation after CBE-treatment, neurons were incubated with a short acyl chain fluorescent derivative of GlcCer, C$_5$-DMB-GlcCer (12). In CBE-treated neurons, C$_5$-DMB-GlcCer accumulated mainly in lysosomes, which appear as discrete puncta located in the perikarya and dendrites (Fig. 1b), as observed in previous studies in hippocampal neurons using short acyl chain fluores-
Effects of GlcCer Accumulation on ER Morphology—We next examined the intracellular morphology of neurons treated with CBE for various times in culture. Upon incubation with DiO, a generic marker of the ER (16, 17), CBE-treated neurons showed significantly more tubular ER elements (Fig. 2, a and b). No nuclear labeling was observed, but the density of labeling of ER elements above the nucleus was significantly higher in CBE-treated cells (Fig. 2b), resulting in a marked (~3-fold) increase in ER density compared with untreated cells (Fig. 3a). No effects on ER morphology were observed upon incubation with FB1, but the increased levels of DiO-labeling of the ER induced by CBE was blocked by co-incubation with CBE and FB1 (Fig. 3a).

The increase in ER density was confirmed using an antibody to the RR, a calcium channel located in the ER of hippocampal neurons (27). Incubation with either CBE or with a nonhydrolyzable analogue of GlcCer (C₈-Glc-S-Cer) (11, 13), resulted in an increase in RR labeling in a perinuclear region of the neurons, corresponding to the ER (Fig. 2, c and d). Quantification of labeling intensity demonstrated an ~2-fold increase in RR density on the ER after incubation with either CBE or C₈-Glc-S-Cer (Fig. 3b). Moreover, preliminary analysis by electron microscopy revealed an increase in the surface density of the ER in CBE-treated neurons after a 5-day incubation (3.29 ± 0.98 ER intersections per μm test line in control cells compared with 4.79 ± 1.29 ER intersections per μm test line in CBE-treated neurons).

Effects of GlcCer Accumulation on [Ca²⁺]ᵢ Release from the ER—We next examined the functionality of increased levels of ER elements and of the RR. Initially, the responses of control and CBE-treated cells to glutamate were examined. Following a pulsed application of glutamate, there was a typical increase in fluo-3 AM fluorescence that peaked after about 2–3 s and decayed back to control levels within 15–20 s (Fig. 4), as previously observed (16). In CBE-treated cells, the peak response to glutamate was 63% larger than that of control cells (Fig. 4). To examine if this larger response to glutamate is related to changes in calcium stores, we used caffeine, a more selective releaser of calcium from stores (27). Caffeine caused a transient increase in free [Ca²⁺]ᵢ (Fig. 5, a and b), which recovered back to baseline levels within 10–20 s in control cells (Fig. 6). A 3-fold increase in the magnitude of the [Ca²⁺]ᵢ response to caffeine was recorded in cells incubated with CBE for 4 days (Figs. 5, c and d, and Fig. 6) or with C₈-Glc-S-Cer for 4 days (Fig. 6), with a marked increase in both the peak and duration of the response; similar data were obtained using a ratiometric dye, Fura-2 (not shown). The response was totally blocked upon co-incubation with CBE and FB1, which reduced [Ca²⁺]ᵢ changes to below levels of the control response (Fig. 6). As previously observed in control cells (16, 27), the response of CBE-treated cells to caffeine was blocked in the presence of ryanodine (not shown). No effects were observed on caffeine-stimulated [Ca²⁺]ᵢ release after short-term incubation (12 h) with either CBE or C₈-Glc-S-Cer.

To examine the functional consequences of the increase in Ca²⁺-sensitive calcium stores and the increased response to glutamate, the sensitivity of neurons to glutamate toxicity (10 mM, 1 h) was examined. Neurons incubated with either CBE or C₈-Glc-S-Cer were much more sensitive than control cells to glutamate-induced neuronal cell death, and this effect was abolished by co-incubation with CBE and FB1 (Fig. 7a). Remarkably, pre-incubation with ryanodine for 1 h completely blocked the neurotoxic effects of glutamate in a dose-dependent manner (Fig. 7b), demonstrating that the release of Ca²⁺ from the ER is responsible for glutamate-induced neuronal cell death, at least in neurons with elevated GlcCer levels.
There were no changes in basal fluorescence levels before caffeine change in fluorescence ($F$) control (10 mM, 1 h) is shown for untreated cells and for cells treated for 4 days

Results are means ± S.E. for 2–21 coverslips per treatment. Note that the rate of increase of free [Ca$^{2+}$]$_i$ is proportional to the rate of decrease of free [Ca$^{2+}$]$_i$, in all experimental conditions: CBE-treated neurons ($\Delta F/F$ per s = 0.24 for increase, and 0.49 for decrease, ratio = 0.49); Glc-S-Cer-treated neurons ($\Delta F/F$ per s = 0.27 for increase and 0.68 for decrease, ratio = 0.40); control ($\Delta F/F$ per s = 0.10 and 0.19, ratio = 0.53); FB1-treated ($\Delta F/F$ per s = 0.05 and 0.15, ratio 0.33); CBE + FB1-treated neurons ($\Delta F/F$ per s = 0.02 and 0.05, ratio = 0.40).

**DISCUSSION**

In the current study, we demonstrate that elevation of intracellular GlcCer levels causes changes in the morphology and functionality of the ER in cultured hippocampal neurons. Although we have not unambiguously demonstrated that these effects are caused by accumulation of GlcCer in lysosomes (rather than accumulation in other intracellular compartments), our observations are of relevance for understanding the neuronal dysfunction and cell death that are observed in neuropathic forms of Gaucher disease, in which massive lysoosomal accumulation of GlcCer occurs (1). Only limited studies on the neuropathology of Gaucher patients exist, but changes in neuronal morphology have been observed, including dilated and distended smooth and rough ER in brains from neuropathic forms of Gaucher disease (28, 29), and marked dilations of the rough ER in corneal keratinocytes from the neuropathic form of Gaucher disease (30). Similar morphological findings were observed in the brains of mice fed CBE (31). Even less is known about the relationship between neuronal cell death and accumulation of GlcCer. We now demonstrate, for the first time, a direct relationship between GlcCer accumulation, Ca$^{2+}$ release from intracellular stores, and neuronal cell death. This finding may have implications not only for understanding neuronal cell death in Gaucher disease, but also for the neurotoxic roles of GlcCer in other neurodegenerative conditions.

Only limited data is available about the levels of GlcCer accumulation in Gaucher patients, and there are no systematic studies comparing levels of accumulation in various brain regions at various stages of progression of the disease. In one study, GlcCer levels were 20–80 times higher in the cerebral
cortex from a type 2 Gaucher patient and 5–40 times higher in the cerebellar cortex (32). In another study (33), GlcCer levels were elevated in both type 2 and 3 patients, but neuropathological findings were detected only in the type 2 brain. At this stage, we are unable to correlate levels of GlcCer accumulation in cultured neurons with the extent of neuronal dysfunction, but analysis of GlcCer accumulation (or the extent GlcCerase inhibition (34)) and changes in ER function, including [Ca\(^{2+}\)], release from the ER, are currently underway.

We have repeated most of the findings obtained using CBE with a nonhydrolyzable analogue of GlcCer, C\(_8\)-Glc-S-Cer, to confirm that the effects of CBE are indeed because of GlcCer accumulation and not because of a nonselective, pharmacological action of CBE. This is further confirmed by studies in which accumulation of GlcCer was prevented by co-incubation with FB\(_1\) and CBE. Using a similar approach, the accumulation of ganglioside GM\(_2\) was blocked in Tay-Sachs mice treated with an inhibitor of GlcCer synthesis (35). Thus, treatment of patients suffering from a sphingolipid storage disorder with inhibitors of sphingolipid synthesis may yet prove to be a viable therapeutic option.

There are two possibilities as to how intracellular accumulation of GlcCer could affect ER morphology and Ca\(^{2+}\) stores. First, GlcCer could directly affect the ER. In this scenario, GlcCer would need to be transported from its site of accumulation (in lysosomes and perhaps other organelles) to the ER. Both short acyl-chain analogues of GlcCer (11, 12), and also metabolically labeled GlcCer (36) can be transported out of endosomes and lysosomes and accumulate in the intracellular compartments (the ER and Golgi apparatus) where they are metabolized to higher order sphingolipids (11, 37). In hippocampal neurons, a fluorescent derivative of C\(_8\)-Glc-S-Cer, C\(_8\)-NBD-Glc-S-Cer, is internalized to various intracellular compartments, including lysosomes, but also the ER (data not shown). Whether GlcCer accumulation in the ER can alter the activity of ER Ca\(^{2+}\) channels is unknown, but modulatory effects of lyso-sphingolipids (38, 39), sphingoid long-chain bases (40), metabolites of sphingoid bases (41), and cerebroside (42) on Ca\(^{2+}\) mobilization have been observed. Alternatively, GlcCer could act indirectly on the ER, for instance, by altering levels of an intracellular signaling molecule. Incubation of Madin-Darby Canine Kidney cells with CBE results in decreased bradykinin-stimulated formation of inositol trisphosphate, whereas the opposite effect is observed upon inhibition of GlcCer synthesis (43, 44). Thus, when GlcCer accumulates, inositol trisphosphate levels may be chronically depleted, resulting in up-regulation of the RR in the ER that may in turn be responsible for increased Ca\(^{2+}\) release from the ER. Irrespective of the mechanism of action of GlcCer, this is the first time that changes in the functionality of neurons with elevated GlcCer levels have been observed and will provide the experimental tools for analyzing the relationship between GlcCer accumulation and neuronal functionality and development.

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