Progressive Hypoxia Inhibits the de Novo Synthesis of Galactosylceramide in Cultured Oligodendrocytes*

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Myelination in the central nervous system is a perinatal event in which oligodendrocytes (OLG) elaborate membranous sheaths which envelope many axons (1). Myelination is vital for the normal function of many central nervous tracts. OLG are highly active during myelination, synthesizing three to four times their weight in myelin membranes/day (2). The metabolic requirements of these cells are thought to exceed those of other brain cell types at this time (3), making them likely to be highly susceptible to injury caused by nutrient deprivation (starvation) and energy impairment (hypoxia, carbon monoxide poisoning). Clinically, normal myelin formation has been shown to be highly sensitive to nutrient and energy deprivation insults (4). The glycosphingolipid galactosylceramide (GalCer) constitutes about 30% of the lipid of myelin and is rapidly synthesized during myelination (5). More than half of the GalCer in myelin exists as a 2-hydroxy fatty acid form (hydroxy fatty acid GalCer, HFA-GalCer), and this modification is unique to myelin. GalCer is also unique in that it contains unusually long fatty acid moieties (6). Long (18-carbon) and very long (l.v.) (24-carbon) fatty acid moieties are found in non-hydroxy GalCer, hence the terminology NFA-GalCer (C-18) and l.v. NFA-GalCer (C-24). HFA-GalCer contains predominantly (C-24). It has been suggested that GalCer (especially HFA-GalCer) can form tightly packed bilayers by virtue of extensive intermolecular hydrogen bonding, and that this property is responsible for the close and regular compaction which is essential for the normal functioning of the myelin sheath (7). The accepted biosynthetic pathway for GalCer (depicted in Fig. 1) involves fatty acylation of sphingosine to form ceramide. Acylation with a non-hydroxy fatty acid will define the ceramide as NFA-ceramide; acylation involving a 2-hydroxy fatty acid will result in HFA-ceramide. UDP-Gal:ceramide:galactosyltransferase (EC 2.4.1.47) is the enzyme activity which catalyzes the galactosylation of ceramide to form GalCer (8). Galactosylation of NFA-ceramide is considered rate-limiting in NFA-GalCer synthesis (9). However, it is 2-hydroxylation of free fatty acid, a reaction requiring molecular oxygen, that is thought to be the rate-limiting step in HFA-GalCer synthesis (10). In agreement with these theories of GalCer synthetic regulation is the observation that a large pool of NFA-ceramide is found in brain white matter, while HFA-ceramide is detectable in only trace amounts (11, 13). The locations in the cell of ceramide synthesis and galactosylation have been reported to be microsomal (12, 13). Studies of the cellular metabolism and trafficking of fluorescent derivatives of ceramide (14, 15) indicate that ceramide indeed exists as a precursor pool in a pre-Golgi area (possibly the endoplasmic reticulum), and that it is transported to the Golgi apparatus, where its conversion to more complex sphingolipids takes place (in cells other than OLG, ceramide is converted largely to glucosylceramide and to the phosphophospholipid sphingomyelin, SM). Continued transport through the Golgi results in vesicular budding at the trans-Golgi and translocation to

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The abbreviations used are: OLG, oligodendrocytes; HFA-GalCer, 2-hydroxy fatty acid form of galactosylceramide; NFA-GalCer, long chain non-hydroxy fatty acid form of GalCer; l.v. NFA-GalCer, very long chain non-hydroxy fatty acid form of GalCer; NFA-ceramide, non-hydroxy fatty acid form of ceramide; HFA-ceramide, 2-hydroxy fatty acid form of ceramide (HFA-ceramide); GI Cer, glucosylceramide; GMS, methylated glucocerebroside; GLC, gas liquid chromatography; TLC, thin layer chromatography.
Effects of Hypoxia on Galactosylceramide Synthesis in Oligodendrocytes

Pathway of Galactosylceramide Biosynthesis

- **Fatty-acyl CoA**
- **Sphingosine**
- **UDP-Galactose**

**Pathway of GalCer synthesis.** Condensation of non-hydroxy-fatty acyl-CoA (NFA) or 2-hydroxy-fatty acyl-CoA (HFA) with sphingosine yields NFA-ceramide or HFA-ceramide, respectively. Galactosylation of ceramide via a UDP Gal intermediate results in formation of GalCer (NFA or HFA species). Note that the rate-limiting step in HFA-GalCer synthesis is thought to be the hydroxylation of the free fatty acyl-CoA, while the galactosylation of NFA-ceramide is considered rate-limiting in NFA-GalCer synthesis (see text).

The age of the cultures was defined as the age of the pups at dissection, plus days in culture, and so OLG were used at approximately 25 days of age.

**Pathway of GalCer synthesis.** Condensation of non-hydroxy-fatty acyl-CoA (NFA) or 2-hydroxy-fatty acyl-CoA (HFA) with sphingosine yields NFA-ceramide or HFA-ceramide, respectively. Galactosylation of ceramide via a UDP Gal intermediate results in formation of GalCer (NFA or HFA species). Note that the rate-limiting step in HFA-GalCer synthesis is thought to be the hydroxylation of the free fatty acyl-CoA, while the galactosylation of NFA-ceramide is considered rate-limiting in NFA-GalCer synthesis (see text).

Materials and Methods

Primary Oligodendrocyte Culture—OLG were isolated from neonatal rat brain by a method similar to a modification (23) of the method of Cole et al. (27). OLG were homogenized by sonicating on ice with a Heat Systems/Ultrasonics sonifier (Plainview, NY). Sonicates (corresponding to 30 μg of protein) were extracted with 0.4 ml of 1 M borate buffer, pH 9.5, and washed with 1.3 ml of water and then theoretical upper phase distilled with 0.1 ml of each extract was allowed to reach room temperature for exactly 1 min in a glass scintillation vial, then was mixed with 0.1 ml of Luciferin/Luciferase assay mixture (Sigma, Kit FLAA, diluted 10-fold) and immediately counted for 10 s in a scintillation counter. The cpmol of duplicates were compared with an ATP standard curve for determination of ATP content, in picograms.

Labeling of Oligodendrocyte Lipids—OLG (one plate, corresponding to 0.05 μg of protein and approximately 0.3 million cells) were labeled with [9,10-3H]palmitic acid (60 Ci/mmol, Du Pont-New England Nuclear) in D- [6-3H]galactose (31.5 μCi/mmol, Amersham Corp), or [1-14C]lignoceric acid (a gift of Dr. Inderjit Singh) in Bottenstein (25) media, 3% CS or serum-free media. Typically cells were fed the media containing the radioactive media at the start of the experiment and labeled continually through the 6 h of hypoxic (or control) conditions. At the end of the labeling period, cells were harvested in buffered saline, sonicated as described above, and aliquots removed for ATP and protein (28) determinations. Lipids were then extracted from the sonicates in 3 ml of chloroform/methanol (C/M, 1:1, v/v) with nonradioactive GalCer (5 μg/sample, SUPELCO, Bellefonte, PA). The extract was then centrifuged for 10 min at 14,000 g at 4°C and washed with 1.3 ml of water and then theoretical upper phase (C/M/W, 3:4:7) as described by Folch, et al. (29). Part or all of the organic phase was then subjected to mild alkaline methanolysis (0.7 N NaOH in C/M at 37°C, at room temperature for 60 min) to destroy gycerolipids. Sphingolipids were chromatographed on 20 × 20-cm...
bore-impregnated silicic acid thin layer chromatography (TLC) plates (30) three consecutive times in the solvent system C/M/H,0
10% (30) GalCer and 24% in galactose. HFA-GalCer was
bands; and so the sphingosine could be easily quantitated. Results
labeled ceramides, GalCer, and sphingosine; thus, the fatty acid band
land Nuclear, 40 mCi/mmol), 15 pg of phosphatidylcholine (Sigma,
and Radin (33). The following were added to a 1.5 ml microcentrifuge
say-Activity was determined by a method similar to that of Brenkert
in the ceramide system above (11), the other on the GalCer system
proportions did not change in hypoxia. These results indicate that
galactosylation of either de novo synthesized ceramide or pre-existing
GalCer (5 pg) was added to samples spotted onto TLC plates and the
bands identified after development by orcinol staining (0.2% orcinol
in 2.0 M sulfuric acid, developed at 120 °C for 10 min). The bands
could then be scraped and counted as described. Orcinol staining
resulted in a loss of approximately 20% of the dpm present in a band
that was scraped. Sphingosine is known to have a relatively high
in an experience where this was treated the same, this did not result in any experimental artifacts. Labeled ceramides were separated by TLC in C/M/acetic acid (glacial)
(94:1:5) (11) and quantitated as described above. Ceramide
standards were obtained from Applied Science, Inc., PA. Glycero-
phospholipids were chromatographed in C/M/H2O (144:25:2.8), iden-
tified by their comigration with standards, visualized by iodine stain-
ing, and quantitated as above.

Incorporation of [3H]Palmitic Acid into Different Moieties of
GalCer—Palmitic acid, a 16-carbon saturated fatty acid, is incorpo-
rated into newly synthesized sphingosine in cells, and is elongated to form
longer fatty acids utilizing glycosphingolipid synthase (see Fig. 1). To confirm that the [3H]palmitic acid used to label OLG is really incorporated into these moieties and so actually reflects de
ovo synthesis of ceramides and GalCer the following procedure (based on (32)) was performed: [3H]palmitic acid-labeled GalCer species
were chromatographed as described above, visualized with
iodine, scraped, and eluted from the silica gel with 70:20:12 (v/v/v) Individual GalCer species were transferred to a glass tube, dried, and
resuspended in 0.6 ml of 3 N HCl in water. The tubes were heated at
110 °C for 3 h to allow GalCer acid-hydrolysis to proceed. This
procedure liberates free fatty acid, sphingosine, and galactose from
glycosphingolipids. After hydrolysis, the reaction mixture was ex-
tracted with 3 ml of C/M (2:1) (29). The upper phase containing
galactosylceramide was recovered and quantitated by liquid scintillation
counting. The lower phase was divided into halves, half was chromatographed in the ceramide system above (11), the other on the GalCer system
above (31). The ceramide system separated free fatty acid from any
labeled ceramides, GalCer, and sphingosine; thus, the fatty acid band
was conveniently scraped and quantitated. The GalCer system sepa-
rated sphingosine from any labeled GalCer, fatty acid, or ceramide
bands; and so the sphingosine could be easily quantitated. Results
indicated that NFA-GalCer species were labeled 35% in the fatty acid
moiety, 41% in sphingosine, and 24% in galactose. HFA-GalCer was
labeled 94% fatty acid, 6% sphingosine, and 99% galactose. These
proportions did not change in hypoxia. These results indicate that
indeed labeled palmitate is incorporated mostly into the backbone of
the GalCer moiety, and so reflects de novo synthesis of GalCer. Only
a small amount of palmitate is recycled into galactose and used to
galactosylate ceramide. [3H]Galactose incorporation into GalCer species
occurs exclusively via galactosylation of ceramide, however, as
no [3H]galactose was incorporated to a detectable extent into
ceramide. Thus, GalCer species labeled with [3H]galactose may reflect
galactosylation of either de novo synthesized ceramide or pre-existing
pools of ceramide in the cell.

UDP-Galactose:ceramide:Galactosyltransferase (EC 2.4.1.47) Ass-
ay—Activity was determined by a method similar to that of Brenkert
and Berlin (33). The following were added to a 1.5 ml microcentrifuge
tube and dried under nitrogen. 0.23 nmol UDP-Gal (Sigma, in ethanol,
250,000 dpm of UDP-[1-3H]galactose (Du Pont-New England
Nuclear, 40 C/mmol), 15 μg of phosphatidylcholine (Sigma, in C/M),
6 μg each of HFA- and NFA-ceramides. The final com-
position of the incubation mixture (0.05 ml) was 100 mM Tris-HCl, pH
7.4, 1.0 mM MgCl2, 10 mM EDTA, and 20 μg of OLG protein or water
(for controls). Incubation was performed in a water bath for 5
half-second bursts (setting 3-4), and vortexed carefully. The mixture
was incubated at 37 °C for 1 h, and then was extracted as described
above, chromatographed on silicic acid plates, and the species of
GalCer quantitated by scraping and counting. Controls were always
done to verify that no GalCer synthesis occurred in the absence of
cell sonicate. UDP-Galactose:ceramide:galactosyltransferase activity to-
ward NFA-ceramide was always approximately 20% of activity toward
HFA-ceramide. Low activity toward NFA-ceramide has been ob-
served by a number of authors (33,36), and the reasons for this are not
known.

Incorporation of [3H]Galactose into OLG Glycoproteins—OLG were
labeled with 2 μCi/ml of [3H]galactose in Bottenstein's media, 3% CS as
described above, under hypoxic or control conditions. The
following procedure is based on Ref. 37: 50 μg of OLG protein,
determined by the method of Lowry (28), was precipitated with ice-
cold trichloroacetic acid (10%), along with 200 μg of carrier albumin.
After 2 h at 4 °C, the precipitates were filtered through Whatman GF/C glass fiber filters that had previously been washed with 1 ml
galactose in 10% trichloroacetic acid (to block nonspecific binding of
the label). The filters were washed extensively with cold trichloroac-
cetic acid, cold absolute ethanol, and cold diethyl ether (to remove
lipids). This process leaves only proteins on the filters, which were
then dried in air. The filters were then extracted in 1 N NaOH
overnight, neutralized, and quantitated by liquid scintillation coun-
ting. Results were expressed in dpm/50 μg protein, after subtraction
of blank (filter w/o protein) values.

RESULTS

Time Course of Oligodendrocyte Hypoxia—An important
principle that has emerged from the study of molecular mech-
anism of cell injury is that injury is a gradual process,
Incorporation of [3H]Palmitic Acid into Different Moieties of
GalCer—Galactosylceramide Synthesis in Oligodendrocytes
Effects of Hypoxia on Galactosylceramide Synthesis in Oligodendrocytes

TABLE I

<table>
<thead>
<tr>
<th>Condition</th>
<th>Time of hypoxia</th>
</tr>
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<tbody>
<tr>
<td>Serum-free media</td>
<td>100%</td>
</tr>
<tr>
<td>3% serum</td>
<td>100%</td>
</tr>
<tr>
<td>4%</td>
<td>50%</td>
</tr>
<tr>
<td>0 h</td>
<td>80%</td>
</tr>
<tr>
<td>6 h</td>
<td>81%</td>
</tr>
<tr>
<td>10 h</td>
<td>44%</td>
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</table>
Control OLG or OLG that had been subjected to 6 h of hypoxia in Botstein's media, 3% CS were fed oxygenated media containing 2 μCi/ml [3H]palmitic acid. The cells were allowed to label for 2 h, and then harvested. Lipids were extracted and identified by TLC as described under "Materials and Methods." Bands corresponding to specific lipids were scraped and quantitated by liquid scintillation counting. Data from a typical experiment is presented (in dpm/50 μg OLG protein) and is also given as percent labeling in hypoxic cells relative to that of controls. Percent values from a repeat experiment are given in the last column. PE, phosphatidylethanolamine; PA, phosphatidic acid; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine.

### Table II: Effect of Hypoxia on de Nova GalCer Synthesis in Oligodendrocytes

<table>
<thead>
<tr>
<th>Lipid species</th>
<th>Experiment 1</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>Experiment 2</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Hypoxic</td>
<td>% of control</td>
<td>% of control</td>
</tr>
<tr>
<td>PE + PA</td>
<td>160,837</td>
<td>201,490</td>
<td>125</td>
<td>129</td>
</tr>
<tr>
<td>PC</td>
<td>13,668</td>
<td>14,357</td>
<td>106</td>
<td>140</td>
</tr>
<tr>
<td>PI + PS</td>
<td>13,652</td>
<td>15,679</td>
<td>115</td>
<td>185</td>
</tr>
<tr>
<td>SM</td>
<td>1,633</td>
<td>1,384</td>
<td>85</td>
<td>118</td>
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<tr>
<td>v.l. NFA GalCer</td>
<td>3,417</td>
<td>1,202</td>
<td>35</td>
<td>22</td>
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<tr>
<td>NFA GalCer</td>
<td>3,260</td>
<td>1,782</td>
<td>55</td>
<td>38</td>
</tr>
<tr>
<td>HFA GalCer</td>
<td>2,377</td>
<td>1,027</td>
<td>43</td>
<td>36</td>
</tr>
</tbody>
</table>

**FIG. 2. Species of galactosylceramide (GalCer) synthesized by OLG.** This is a composite picture of OLG sphingolipid species separated on borate-impregnated thin layer chromatography plates (30) three times in chloroform/methanol/water (144:25:2.8) as described in Ref. 31 and under "Materials and Methods." The left-most lane depicts an autoradiograph of [H]palmitic acid-labeled OLG GalCer species, so designated due to their comigration with bovine brain GalCer standards (middle lane), according to Ref. 31. The right-most lane depicts an autoradiograph of OLG GalCer species labeled with [14C]lignoceric acid (24-carbon, saturated); this pattern confirms our designation of the top-most NFA-GalCer band as very long chain non-hydroxy-fatty acid-GalCer. While labeled lignoceric acid is not incorporated into the long chain non-hydroxy-fatty acid-GalCer (NFA-GalCer) band, [14C]stearic acid (18-carbon, saturated) is incorporated into NFA-GalCer (not shown), confirming the designation of this band. A single 2-hydroxy-fatty acid-GalCer (HFA-GalCer) band is labeled both with palmitic acid and lignoceric acid by OLG, suggesting a 24- (and possibly 18-) carbon fatty acid composition. Note that this system separates glucosylceramide (GlcCer) from GalCer; [14C]palmitate labels both GlcCer bands very weakly (10% of GalCer labeling), but [14C]lignoceric acid labels the upper, very long chain species quite well.

Since hypoxia caused a reduction in OLG ATP content (by 19%, see Table I) as well as a reduction in oxygen available to the cells, it was of interest to determine whether an ATP decrease alone was capable of mimicking the effect of hypoxia on GalCer synthesis. The mitochondrial respiratory inhibitor oligomycin was chosen to address this question because oligomycin acts directly on the mitochondrial ATPase enzyme and does not interfere with electron transport processes in the cell. OLG were treated with 12 nM oligomycin for 4 h in serum-free media; otherwise labeling conditions and lipid analysis procedures were exactly the same as in the hypoxia experiments. Oligomycin (12 nM) was used because this dose had a small effect on OLG ATP content (approximately a 10% decrease in ATP), while still actively inhibiting GalCer metabolism. Higher doses of oligomycin caused greater ATP depletion (36 nM caused a 50% depletion in cells) but inhibited GalCer synthesis completely (not shown). Fig. 3B shows that oligomycin (12 nM) caused a global inhibition of GalCer synthesis relative to controls, with v.l. NFA-GalCer reduced by 70 ± 6%, NFA-GalCer by 58 ± 9%, and HFA-GalCer by 58 ± 6%. Oligomycin also inhibited SM synthesis by 46%. These results suggested that 12 nM oligomycin did not mimic the selective effect of hypoxia on de novo HFA-GalCer synthesis. All oligomycin experiments henceforth were performed under hypoxia.
Effect of Hypoxia on Galactosylceramide Synthesis in Oligodendrocytes

Cultured OLG were labeled as described under "Materials and Methods" with [3H] or [14C]palmitate during 6 h of hypoxia or 4 h of 12 nm oligomycin at 37 °C (HFA-ceramide could be detected only with [14C]palmitate labeling, as described in the text). The cells were extracted and the ceramides analyzed by TLC as described. Bands corresponding to HFA or NFA-ceramides were scraped and quantitated by liquid scintillation counting. The results were normalized to cell protein and expressed as % of control labeling. All values are averages of measurements made in two or three independent experiments. Typical values for control cells were NFA-ceramide, 8000 dpm/50 pg protein, with both isotopes (4000 in oligomycin experiments), and HFA-ceramide, 500 dpm/50 pg (14C only).

Table III

<table>
<thead>
<tr>
<th>Condition</th>
<th>Ceramide</th>
<th>Incorporation % of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypoxia</td>
<td>HFA</td>
<td>108 ± 5%</td>
</tr>
<tr>
<td></td>
<td>NFA</td>
<td>194 ± 11%</td>
</tr>
<tr>
<td>Oligomycin</td>
<td>HFA</td>
<td>103 ± 0%</td>
</tr>
<tr>
<td></td>
<td>NFA</td>
<td>210 ± 70%</td>
</tr>
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</table>

Fig. 3. Effect of hypoxia and oligomycin on de novo OLG GalCer synthesis. A, 25-day-old rat OLG cultures were labeled with [3H]palmitic acid (2 µCi/ml) in Bottenstein's defined media, 3% calf serum continuously during 6 h of gradual hypoxia. Cells were harvested and extracted as described under "Materials and Methods," the glycolipids chromatographed, autoradiographed, scraped, and quantitated by liquid scintillation. The results, once normalized to 50 µg of protein, are represented as % label incorporation (relative to controls) per species of GalCer and saptiunguolinye (SM). Standard errors are included. Student's t test indicated that the labeling during hypoxia of HFA-GalCer was significantly different than the NFA GalCer labeling, with p < 0.01. Typical values for control incorporation are 10,275 dpm/50 µg protein for v.l. NFA-GalCer, 5000 dpm/50 µg for NFA-GalCer, and 5100 dpm/50 µg for HFA-GalCer species. B, OLG were labeled with [3H]palmitate (2 µCi/ml) in serum-free media in the presence of 12 nm oligomycin for 4 h. Cells were then harvested and their lipids analyzed as in A. Results are expressed as % of control ± range. Typical control values are 2100 dpm/50 µg for v.l. NFA-GalCer, 1800 dpm/50 µg for NFA, and 2100 dpm/50 µg for HFA-GalCer.

in serum-free EMS and all hypoxic treatments in EMS, 3% serum.

Effect of Hypoxia on de Novo Synthesis of Ceramide—Table III shows the effects of both hypoxia and oligomycin on the incorporation of [3H]palmitic acid or [14C]palmitic acid into ceramide, the ungalactosylated precursor of GalCer (Fig. 1). Hypoxia resulted in an increase in labeled NFA-ceramide in OLG of 34 ± 10% over controls. The labeled NFA-ceramide pool in OLG is very large, containing about as many dpm of [3H]palmitic acid as v.l. NFA-GalCer and NFA-GalCer combined (approximately 8000 dpm/50 µg protein/6 h labeling), in agreement with previous observations that a large NFA-ceramide pool exists in white matter (11). The actual dpm lost from NFA-GalCer species in hypoxia were more than accounted for by the increase in dpm in the NFA-ceramide pool, suggesting that NFA-ceramide was labeled with [3H] palmitic acid normally or at an enhanced rate during hypoxia, but that its metabolism to GalCer was somehow blocked. Oligomycin treatment (12 nM) resulted in a larger and more variable increase in labeling of the large NFA-ceramide pool (by 110 ± 70%). HFA-ceramide, the precursor to HFA-GalCer, could only be detected upon autoradiography when labeled with [14C]palmitic acid, as it labeled so weakly (at approximately 5% of NFA-ceramide labeling). Previous studies have detected only trace amounts of HFA-ceramide in white matter (11, 39). Table III shows that labeling of this small HFA-ceramide pool did not appear to be affected by hypoxia. However, since the HFA-ceramide pool did not accumulate the dpm lost from HFA-GalCer (up to 2000 dpm/50 µg protein) this suggested that HFA-ceramide synthesis did not proceed at its normal rate during the inhibition of HFA-GalCer synthesis. Oligomycin (12 nM) similarly resulted in no detectable change in HFA-ceramide labeling (Table III).

Pulse-Chase Studies on [3H] or [14C]Palmitic Acid-labeled Cells—The results in Table III suggested that the increased dpm in the labeled NFA-ceramide pool during hypoxia or oligomycin treatment represented molecules of ceramide that were not being converted into NFA-GalCer. To confirm that this labeled ceramide pool was actually available for conversion into GalCer, pulse-chase experiments were performed as follows: OLG were treated with 12 nm oligomycin or hypoxia for 4 or 6 h, respectively, in the presence of [3H] or [14C] palmitate. The cells were then washed twice with media, fed fresh (oxygenated) medium and incubated for 18 h. Fig. 4A shows the results of a typical experiment with oligomycin (12 nm). Labeling of the NFA-ceramide pool increased dramatically during treatment (from 3580 to 10,420 dpm/50 µg protein), far beyond a value corresponding to dpm lost from NFA-GalCer (1595 dpm/50 µg), suggesting that an enhanced incorporation of label into NFA-ceramide was occurring in addition to an accumulation of ungalactosylated NFA-ceramide. Chasing for 18 h reduced the NFA-ceramide pool, however, along with a simultaneous increase in NFA-GalCer by approximately the same amount as chased from ceramide (approximately 5500 dpm/50 µg). Thus, it appeared that the accumulated labeled NFA-ceramide was readily converted into GalCer upon restoring the cells to aerated conditions. The same pulse-chase results were obtained with hypoxia, although the increase in NFA-ceramide labeling in hypoxia was not as dramatic (see Table III). The increase in labeling of NFA-ceramide in hypoxia or oligomycin treatment beyond a value corresponding to dpm lost from GalCer raised the possibility that the apparent inhibition of the conversion of NFA-ceramide to GalCer may have been an artifact due to a change in the specific activity of the NFA-ceramide pool. To investigate this possibility, an experiment was done to compare the rate of chaising of NFA-ceramide into GalCer in
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Fig. 4. Pulse-chase studies on [3H]/[14C]Palmitate-labeled OLG. OLG cultures were treated with hypoxia (6 h) or 12 nM oligomycin (4 h) as described in Fig. 2, in the presence of radiolabeled palmitate (2 μCi/ml). The cultures were then washed twice, fed fresh (aerated) media, and allowed to incubate a designated time before harvesting. Ceramide and GalCer analysis was carried out as described under “Materials and Methods,” in Table III and Fig. 3. The experiments below depict typical data for 12 nM oligomycin; similar results were obtained with hypoxia, though the increase in NFA-ceramide was not as dramatic as with oligomycin (Table III).

The bar depicts dpm normalized to 50 μg of cell protein and is the average of two experiments done. [3H]Palmitate is the label required to detect HFA-ceramide into HFA-GalCer. This experiment is representative of four experiments done. [14C]Palmitate is the label; each bar depicts dpm normalized to 50 μg of cell protein and is the average of duplicates that were within 5–10% of each other.

control and hypoxic cells. The results of this study showed that control OLG chased labeled NFA-ceramide into NFA-GalCer at a rate of 8745 dpm/50 μg protein/6 h chase, and hypoxic OLG at a rate of 7792 dpm/50 μg protein/6 h chase. These numbers, which are 11% apart, suggest that the specific activities of labeled NFA-ceramide pools in hypoxic cells were virtually the same as in controls. NFA-ceramide was therefore as available for galactosylation in hypoxic or appeared that the inhibition of galactosylation of de novo synthesized ceramide in hypoxic cells was not due to a depletion of UDP-Gal.

Fig. 4B indicates that the small pool of labeled HFA-ceramide (about 500 dpm; note difference in scales in Fig. 4) was not affected by either hypoxia or oligomycin, or the decrease in labeling of HFA-GalCer. However, upon reoxygination, this pool is readily chased into HFA-GalCer in only 6 h of chasing. Thus, it seemed that the limited amount of HFA-ceramide synthesized during hypoxic or oligomycin-treated OLG was in controls, but its conversion to GalCer was blocked.

Galactosylation of Ceramide Pools: [3H]Galactose Labeling of GalCer—The inhibition of conversion of both HFA- and NFA-ceramides into GalCer in hypoxic or oligomycin-treated OLG prompted an investigation of the possibility that these conditions inhibited the galactosylation of ceramide, and so blocked GalCer synthesis. A possible mechanism for such an inhibition would be a decrease in cytosolic levels of UDP Gal, whose synthesis depend on ATP. [3H]Galactose was incorporated by OLG into the three GalCer species described in Fig. 2, at a ratio of approximately 5:1 (total NFA/HFA, corresponding to 1000 dpm/50 μg protein in NFA and 200 dpm/50 μg in HFA). Because galactosylation is the terminal event in GalCer synthesis, the incorporation of [3H]galactose into GalCer may reflect galactosylation (via a UDP-[3H]Gal intermediate) of preexisting pools of ceramide in the cell as well as ceramide made de novo during the labeling period. [3H]Galactose was not incorporated to any significant extent into ceramides (data not shown), and so is a reflection only of galactosylation of ceramide, and not de novo GalCer synthesis. Fig. 5 shows the results of labeling OLG with [3H]galactose during 6 h of hypoxia. The incorporation of label into v.1. NFA and NFA-GalCer species was enhanced more than 2-fold (223% of control for v.1. NFA-GalCer and 251% for NFA-GalCer), suggesting increased galactosylation of some NFA-ceramide pool. The labeling of HFA-GalCer was not increased by hypoxia; however, and so suggested that the apparent galactosylation observed was not an artifact due to a change in the specific activity of UDP-[3H]Gal in the cytosol during hypoxia. This was confirmed by studies on the incorporation of [3H]galactose into trichloroacetic acid-precipitable material (glycoproteins) in hypoxic cells (described under “Materials and Methods”). Results indicated that galactosylation of proteins in hypoxic cells was not different than in control cells (2000 dpm/50 μg protein). Thus, it appeared that the inhibition of galactosylation of de novo synthesized ceramide in hypoxic cells was not due to a depletion of UDP-Gal. To test the possibility that the observed galactosylation of pre-existing ceramide pools was due to an enhanced UDP-Gal:ceramidegalactosyltransferase activity in the cells, in vitro activities of homogenates of control and hypoxic cells were determined as described under “Materials and Methods”. Table IV indicates that no change in activity could be detected by this assay.

DISCUSSION

We present evidence that the synthesis of the major myelin glycolipid galactosylceramide (GalCer) by developing OLG is compromised by hypoxia. Recent work in cell injury has shown that while the terminal events of cell injury and death are common to many cell types (Ca2+ influx and membrane lipid peroxidation, for example) (38, 40), different cell types respond to injurious stimuli in unique, specific ways that occur well before injury. These responses may represent de
Effects of Hypoxia on Galactosylceramide Synthesis in Oligodendrocytes

TABLE IV

<table>
<thead>
<tr>
<th>GalCer species</th>
<th>Control</th>
<th>Hypoxia</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>dpm</td>
<td>Activity</td>
</tr>
<tr>
<td>NFA</td>
<td>0.180</td>
<td>3.0</td>
</tr>
<tr>
<td>HFA</td>
<td>1650.0</td>
<td>16.0</td>
</tr>
</tbody>
</table>

The effect of 6 h of hypoxia on UDP-
Galceramide:galactosyltransferase activity in OLG

OLG were harvested after 6 h in either hypoxic or control conditions, sonicated, and 20 μg of protein was incubated with NFA and HFA ceramide substrates and UDP-[3H]Gal in buffer for 1 h at 37 °C (as under “Materials and Methods”). The mixture was extracted with chloroform/methanol and the lipids separated by TLC as described. Typical results are expressed as dpm of [3H]GalCer produced by 20 μg of OLG protein (an average of duplicates), and also as enzyme activity (pmol × 10−12) of UDP-Gal incorporated into GalCer/20 μg protein/h.

fense mechanisms, such as neuronal heat shock protein expression (41) and ATP conservation maneuvers in hypoxic skeletal muscle (42), or unfortunate vulnerabilities, such as excitatory amino acid release by ischemic hippocampal neurons (43), or prostaglandin release by complement-injured OLG (44). GalCer, in particular HFA-GalCer, is considered an important ingredient for proper compaction of myelin (7). The early and specific inhibition of GalCer synthesis by hypoxia presented here may be the reason why myelination in the developing brain is so sensitive to energy impairment, such as occurs in carbon monoxide poisoning (4). The work presented here provides evidence that 6 h of progressive hypoxia in the GasPak apparatus did not injure or metabolically impair developing rat OLG, as shown by morphologic examination, minimal effects on ATP content (in the presence of serum), and lipid metabolism studies (Table II). OLG did not appear to be injured until 12 h of hypoxia, and this places them well within the injury time scale reported for other cultured brain cells, with neurons at 8 h (45) and astrocytes at 18 h (46). It is unclear why cultured cells are so resistant to hypoxia, although it is likely that the relatively uncrowded nature of the cell monolayer allows for an excess of nutrients and oxygen/cell. Such reserves, coupled to the gradual increase in hypoxia over time, stretch out the time course of cell injury. This is very useful, as it allows study of the cell's early and specific responses to hypoxia.

6 h of hypoxia did not inhibit the de novo synthesis ([3H]palmitic acid incorporation) of glycerophospholipids or the phosphosphingolipid SM but did dramatically inhibit the de novo synthesis of HFA-GalCer (by 55%) and less so the synthesis of NFA-GalCer (v.1. NFA by 27%, NFA by 39%) (Fig. 3). The depletion of molecular O2 by hypoxia could explain the selective inhibition of HFA-GalCer relative to NFA-GalCer species, since the 2-hydroxylation of fatty acids has been shown to be dependent on molecular oxygen (10).

We then determined whether the small (19%) decrease in ATP during hypoxia could be responsible for the observed inhibition of NFA-GalCer synthesis. The mitochondrial inhibitor oligomycin acts directly on the F1-ATPase in mitochondria to decrease ATP synthesis without interfering with electron transport processes. A low (12 nM) dose of oligomycin decreased cell ATP levels by 10% and was found to inhibit synthesis of all species of GalCer by 60–70%, and SM by 50%. Balch et al. (47) have previously shown that the transport of vesicular stomatitis protein protein from the endoplasmic reticulum of cells to the Golgi apparatus for processing is completely and reversibly blocked by an equally small (15%) lowering of cellular ATP content. To test the hypothesis that the 10–20% decrease in ATP caused by both hypoxia and 12 nM oligomycin was preventing the transport of NFA-ceramide from its site of synthesis to its site of galactosylation, the labeling of NFA-ceramide (normally present as a large precursor pool in OLG) in hypoxia and oligomycin treatment was examined. [3H]Palmitate labeling of NFA-ceramide was indeed increased by 34% in hypoxic cells, and this increase was more than large enough to account for the decrease in dpm from the v.i. NFA and NFA-GalCer species combined (Table 3). A similar accumulation of ceramide has been reported in BHK-21 cells treated with 2-deoxyglucose, a competitive inhibitor of glycosylation (48). An increase in NFA-ceramide labeling was also seen following oligomycin treatment. Since the increase in labeling of the NFA-ceramide was often considerably larger than the dpm lost from the GalCer species (Fig. 4A), it appeared that both hypoxia and oligomycin were causing an enhanced incorporation of [3H]palmitic acid into NFA-ceramide, beyond a value corresponding to an accumulation of unconverted ceramide. Pulse-chase studies showed that this accumulated pool of labeled NFA-ceramide was certainly available for galactosylation, as it was converted into GalCer upon chasing with aerated media (Fig. 4A). Similarly, the rate of chosing of NFA-ceramide into GalCer was virtually the same in previously hypoxic cells as in controls, indicating that the apparent inhibition of conversion could not be explained by a change in the specific activity of the NFA-ceramide pool available for galactosylation. Studies on the incorporation of [3H]galactose into GalCer species (Fig. 5) and into OLG glycoproteins indicated that the inhibition of conversion of NFA-ceramide to NFA-GalCer was not due to a decrease in available UDP-Gal. Further, it was determined that VDP-Gal:ceramide:galactosyltransferase activity in hypoxic OLG homogenates (Table IV) was not decreased relative to controls.

It is hypothesized that the likely site of accumulation of NFA-ceramide in hypoxic OLG is the endoplasmic reticulum. Studies on the cellular distribution and metabolism of a fluorescent ceramide analogue, C6-NBD-ceramide (14, 15), have shown that C6-NBD-ceramide accumulates in perinuclear structures when fed to cells at 0 °C. With warming of the cells to 20 or 37 °C, fluorescence moves to the Golgi apparatus. This step is associated with the conversion of ceramide to its natural anabolic products, which in fibroblasts are glucosylceramide and SM. We propose that while synthesis of ceramide in OLG is localized to the endoplasmic reticulum (corresponding to the perinuclear or “pre-Golgi” compartment observed in Refs. 14 and 15), the galactosylation of ceramide occurs in the Golgi apparatus, and that transport of ceramide to the Golgi is required for galactosylation. It is this ATP-dependent transport that we suggest is inhibited by hypoxia and oligomycin treatment, resulting in the accumulation of NFA-ceramide.

The observation that ATP depletion alone (by oligomycin treatment) did not mimic the selective effect of hypoxia on HFA-GalCer synthesis (Fig. 3) suggests that depletion of O2 in hypoxia was dramatically inhibiting the 2-hydroxylation of fatty acids, a rate-limiting step in HFA-GalCer synthesis (Fig. 1). The data presented in Table 3 support this hypothesis: it indicates that synthesis of HFA-ceramide was inhibited by hypoxia, since it did not accumulate during the inhibition of HFA-GalCer synthesis. Oligomycin treatment also appeared to inhibit HFA-ceramide synthesis, however, and so indicates that an ATP depletion alone may block fatty acid 2-hydroxylation, although not as effectively as O2 depletion. It may be that ATP depletion in oligomycin treatment results in cellular conservation of O2 for respiration, a process which may involve down-regulation of fatty acid hydroxylation. Results in
Fig. 4 suggest that the small pool of HFA-ceramide synthesized in hypoxic OLG accumulates in a pre-Golgi compartment, as was seen for NFA-ceramide. This small HFA-ceramide pool was also readily chased into HFA-GalCer in 6 h after reoxygenation (Fig. 4B).

Evidence in the literature suggests that compartments of ceramide galactosylation other than microsomal do in fact exist in OLG. A number of researchers have presented evidence that galactosylation may occur in myelin itself (49-51), and since myelin is an extension of OLG processes, this suggests galactosylation may occur in OLG plasma membrane. Similarly, both plasma membrane and Golgi apparatus preparations have been shown to be capable of synthesizing SM (14, 15, 52). Our apparently paradoxical data indicating that the incorporation of [3H]galactose into NFA-GalCer (labeled HFA-ceramide) was not detectable in Golgi preparations (Fig. 4A), and since myelin is an extension of OLG processes, this suggests galactosylation may occur in OLG plasma membrane.

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REFERENCES