Fumonisins B1 Inhibits Sphingosine (Sphinganine) N-Acyltransferase and de Novo Sphingolipid Biosynthesis in Cultured Neurons in Situ*

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Fumonisins, mycotoxins produced by Fusarium moniliforme and a number of other fungi, cause neuronal degeneration, liver and renal toxicity, cancer, and other injury to animals. Recent work with rat hepatocytes (Wang, E., Norred, W. P., Bacon, C. W., Riley, R. T., and Merrill, A. H. Jr. (1991) J. Biol. Chem. 266, 14486–14490) found that fumonisins block sphingosine biosynthesis by inhibiting the conversion of sphinganine to dihydroceramides, which precedes introduction of the 4,5-trans-double bond of sphingosine. The current study utilized mouse cerebellar neurons in culture to evaluate how this affects the distribution of newly synthesized ceramides among different complex sphingolipids. Fumonisins B1, inhibited ceramide synthase in mouse brain microsomes with a competitive-like kinetic behavior with respect to both sphinganine and stearoyl-CoA. Fumonisins B1, inhibited sphingolipid biosynthesis in cultured cerebellar neurons in situ as reflected by accumulation of free sphinganine, a reduction in the mass of total sphingolipids, and injury to animals. Recent work with rat hepatocytes (Wang, E., Norred, W. P., Bacon, C. W., Riley, R. T., and Merrill, A. H. Jr. (1991) J. Biol. Chem. 266, 14486–14490) found that fumonisins block sphingosine biosynthesis by inhibiting the conversion of sphinganine to dihydroceramides, which precedes introduction of the 4,5-trans-double bond of sphingosine. The current study utilized mouse cerebellar neurons in culture to evaluate how this affects the distribution of newly synthesized ceramides among different complex sphingolipids. Fumonisins B1, inhibited ceramide synthase in mouse brain microsomes with a competitive-like kinetic behavior with respect to both sphinganine and stearoyl-CoA. Fumonisins B1, inhibited sphingolipid biosynthesis in cultured cerebellar neurons in situ as reflected by accumulation of free sphinganine, a reduction in the mass of total sphingolipids, reductions in the incorporation of [14C]serine into glycosphingolipids, and gangliosides (Gm3, Gd2a, Gd1a, Gd1b, Gd2, and Gd1), and inhibition of the incorporation of [14C]galactose and [3H]sphinganine into complex sphingolipids. Dose-response studies revealed that the labeling of sphingomyelin (IC50 of 0.7 μM) was more sensitive to inhibition by fumonisins B1 than was glycolipid formation (IC50 of ~7 μM) in these cells. A similar effect was seen when β-fluoroceramide was added to inhibit the activity of serine palmitoyltransferase, the first enzyme of the pathway. The inhibition of complex sphingolipid synthesis was reversible, and nearly normal labeling profiles were obtained 48 h after removing the mycotoxin. These studies establish that fumonisins B1 inhibit de novo sphingolipid biosynthesis by neuronal cells and, moreover, that limiting ceramide synthesis differentially affects the formation of sphingomyelin versus glycosphingolipids.

Fumonisins are mycotoxins produced by Fusarium moniliforme (Sheldon) and Fusarium proliferatum, fungi that are prevalent on maize, other grains, and agricultural commodities in the U.S. and throughout the world (Marasas et al., 1984a; Ross et al., 1990; Sydenham et al., 1990, 1991). Contamination of food with these molds is a major agricultural problem, because fumonisins cause a neurodegenerative disease of horses called equine leukoencephalomalacia (Marasas et al., 1988) as well as pulmonary edema in pigs (Harrison et al., 1990), liver and renal toxicity in numerous animals (Kriek et al., 1981; Voss et al., 1990), and liver cancer in rats (Marasas et al., 1984b; Gelderblom et al., 1988, 1991). Consumption of corn contaminated with F. moniliforme has been correlated with human esophageal cancer in areas of southern Africa and China (Lin et al., 1980; Yang, 1980; Marasas, 1982), and fumonisins have been found in low amounts in corn meal and grits in many parts of the world, including the U.S. (Sydenham et al., 1991).

Recent studies with rat hepatocytes (Wang et al., 1991) and a renal cell line (LLC-PK1 cells) (Yoo et al., 1992) found that fumonisins block the incorporation of [14C]serine into the sphingosine backbone of cellular sphingolipids. The site of inhibition is ceramide synthase, which catalyzes the conversion of [14C]sphinganine to N-acyl-14C]sphinganines, a step that is thought to precede the introduction of the 4,5-trans-double bond of sphingosine (Ong and Brady, 1973; Stoffel and Bister, 1974; Merrill and Wang, 1986; Rother et al., 1992). The IC50 for inhibition of ceramide synthase in vitro has been estimated to be 0.1 μM for rat liver microsomes (Wang et al., 1991), and cellular effects of fumonisins are also seen with micromolar fumonisins (Wang et al., 1991; Yoo et al., 1992). The inhibition apparently arises from the remarkable structural similarities between the fumonisins (Bezuidenhout et al., 1988) and long-chain bases, such as sphinganine and sphingosine (Fig. 1). Subsequent studies have shown that free sphinganine accumulates in tissues (Riley et al., 1993) and serum (Wang et al., 1992) from animals given fumonisins or feed contaminated with F. moniliforme. Therefore, it is clear that disruption of sphingolipid metabolism is a major biochemical target in the action of the fumonisins (Norred et al., 1992; Merrill et al., 1993), and that this can serve as a biomarker for exposure to these mycotoxins (Riley et al., 1993).

It remains to be determined, however, how inhibition of de novo ceramide biosynthesis affects the formation of specific complex sphingolipids. This study examined the incorporation of [14C]serine into sphingomyelin and glycosphingolipids by mouse cerebellar neurons in culture, a well characterized model of complex sphingolipid biosynthesis (van Echten and Sandhoff, 1989; van Echten et al., 1990). The results demonstrate that fumonisin B1 blocks sphingolipid formation from [14C]serine, [3H]galactose, and [3H]sphinganine in neuronal cells, that sphinganine accumulates in cells treated with this mycotoxin; and that sphingomyelin biosynthesis from [14C]
EXPERIMENTAL PROCEDURES

Materials—Six-day-old NMRI mice were obtained from Prof. Dr. K. Karzel of the Pharmacological Institute in Bonn. Culture medium (Dulbecco’s modified Eagle’s medium), trypsin, deoxyribonuclease, bovine serum albumin, and horse serum were purchased from Life Technologies, Inc., and plastic petri dishes were obtained from Falcon (Heidelberg, Germany).

Fumonisin B1 was purchased from Sigma, L-[3-14C]serine (2.13 GBq/mmol) and D-[3-14C]galactose (1.86 GBq/mmol) were from Amersham Corp., and [3H]serine was made according to Mandon et al. (1991) using [14C]serine and palmitoyl-CoA as substrates. Amersham Corp., and 3H-labeled sphinganine was made according to Schwarzmann and Sandhoff (1987) with a specific activity of 12.5 μCi/μg. All other chemicals were of analytical grade and obtained from Sigma or Merck. Silica gel 60 plates were supplied by Merck. All other chemicals were obtained from Sigma or Merck.

Cell Culture—Granule cells were cultured from the cerebella of 6-day-old mice as described in detail before (van Echten and Sandhoff, 1990). In brief, cells were isolated by mild trypsinization (0.05%, mass/volume) followed by trituration on the tissue in a deoxyribonuclease solution (0.1% mass/volume) using fire-polished Pasteur pipettes with decreasing pore size; the resultant cell suspension was then centrifuged and resuspended in Dulbecco’s modified Eagle’s medium containing 10% heat-inactivated horse serum and plated onto poly(L-lysine)-coated 35 mm-diameter petri dishes (5 × 10^5 cells/dish). 24 h after plating, cytosine arabinoside was added to the medium (0.04 mM) to arrest the division of non-neuronal cells. At day 4 of culture, cells were incubated in medium supplemented with 0.5% heat-inactivated horse serum and incubated overnight with the indicated concentration of fumonisin.

Sphingolipid Biosynthesis—The sphingolipids were labeled by feeding cells with [3H]serine for 24 h and then isolated and identified as previously described (van Echten et al., 1990) (other specific details are given in the legends to the tables and figures). In brief, after the indicated times cells were harvested and lipids extracted from cell pellet with 3 ml of chloroform/methanol/water/pyridine (60:30:6:1, by volume) for 48 h at 50°C. The lipid extracts were desalted by reversed-phase chromatography on silica gel LiChroprep RP18, applied to TLC and chromatographed with chloroform/methanol/aqueous CaCl2 (0.22%), (60:35:8, by volume).

Mass measurements of the long-chain bases were conducted by HPLC as previously described (Merrill et al., 1988) with C<sub>24</sub>-sphinganine as an internal standard. To quantitate total sphingolipids, the extracts were acid hydrolyzed according to Gaver and Sweeley (1966) before preparing the samples for HPLC and corrected, if necessary, for any losses during hydrolysis or extraction by spiking replicate samples with a known amount of ceramide or sphingomyelin.

The incorporation of radiolabel into other lipids was determined by separating the phospholipids by TLC using silica gel H plates developed with CHCl3/methanol/formic acid/water (56:30:2:1, by volume), visualization of the spots and standards with I<sub>2</sub> vapor, and quantitation by scintillation counting.

Isolation of Microsomes—Mice were killed by decapitation. Cerebella were immediately removed and placed in ice-cold, 50 mM Hepes pH 7.4 at 4°C containing 0.32 m sucrose. The material was weighed and gently homogenized in the same solution at a mass to volume ratio of 1:3. Nuclei, debris, and mitochondria were removed by centrifugation at 10,000 × g for 20 min. The microsomal fraction was then sedimented at 105,000 × g for 1 h. It was washed with 50 mM Hepes pH 7.4 at 4°C, recentrifuged, and finally taken up in 50 mM Hepes and 5 mM dithiothreitol (~1 ml/g of original tissue). The microsomal preparations could be stored for at least a month in aliquots at ~80°C without loss of activity.

Assays of Ceramide Synthase—For assaying ceramide synthase (Merrill and Wang, 1992), D-erythro-[4,5-3H]-sphinganine and stearoyl-CoA were used as substrates, except where otherwise noted. The reaction mixture in a total volume of 100 μl contained 0.1 M Hepes pH 7.4 at 25°C, 1 mM dithiothreitol, varying concentrations of sphinganine and stearoyl-CoA, and 20–100 μg of microsomal protein. To achieve the required concentrations of sphinganine, a stock mixture of sphinganine and egg phosphatidylcholine (1:10, mol:mol) was dried, 0.1 M Hepes buffer was added, and the sample was sonicated for several minutes (for some of the kinetic analyses, the [3H]sphinganine was diluted with unlabeled sphingamine at a molar ratio of 1:2.5). Varying amounts of this mixture were added to each assay tube, and a similarly prepared stock of phosphatidylcholine alone was added to maintain a single phospholipid concentration (1 mM). After incubation for 15 min at 37°C, the lipids were extracted, separated on TLC (CHCl3/methanol, 90:30:6:1, by volume), and the radiolabel in the ceramide was determined by scanning or cutting out the regions of interest and scintillation counting.

Serine palmitoyltransferase was assayed as described previously (Mandon et al., 1991) using [3H]serine and palmitoyl-CoA as substrates.

RESULTS

Inhibition of Ceramide Synthase in Vitro—To determine whether fumonisin B1 inhibits ceramide synthase in brain microsomes, the activity of this enzyme was assayed with varying concentrations of fumonisin B1 (Fig. 2A). Fumonisin B1 inhibited ceramide synthase activity when palmitoyl-CoA, stearoyl-CoA, or lignoceroyl-CoA were used as the co-substrate (Fig. 2B). Because stearoyl-CoA yielded the greater activity as had earlier been reported by Morell and Radin (1970), the kinetics were analyzed further with this substrate (Fig. 2, C and D). Under the assay conditions employed, the apparent Km was 35 μM for sphinganine (Fig. 2C) and 3 μM for stearoyl-CoA (Fig. 2D). In the presence of fumonisin B1, the kinetic lines intersected with the controls for both sphinganine (Fig. 2C) and stearoyl-CoA (Fig. 2D) as the variable substrate, which is suggestive of competitive inhibition. The results show that fumonisin B1 is a potent inhibitor of ceramide synthase in mouse brain microsomes, with ≥50% inhibition by 75 nM fumonisin B1 when both substrates were present at concentrations ≤ their apparent Km.

Fumonisin B1 Inhibits Complex Sphingolipid Synthesis from [3H]Serine by Neuronal Cells in Situ—After 24 h of incubation with [3H]serine, cultured mouse cerebellar neurons exhibited a sphingolipid labeling profile typical of neuronal tissue (Fig. 3, left lane), i.e., with labeling of sphingomyelin, glucosyl- and lactosyl-ceramides, and primarily gangliosides

1The abbreviation used is: HPLC, high performance liquid chromatography.
Fumonisins B₁ and Cultured Neurons

**FIG. 2. Inhibition of (dihydro)ceramide synthase by fumonisin B₁ in vitro.**

A, assays of (dihydro)ceramide synthase with 1 μM [³H]sphinganine, 100 μM palmitoyl-CoA, and varying concentrations of fumonisin B₁. B, comparison of the activity of (dihydro)ceramide synthase (pmol/min/mg of microsomal protein) with palmitoyl-CoA (Pal), stearoyl-CoA (St), and lignoceroyl-CoA (Lig), each at 100 μM, in the presence (+) and absence (−) of 1 μM fumonisin B₁ (FB₁). C, double-reciprocal plot of (dihydro)ceramide synthase activity (nmol/min/mg of microsomal protein) with 100 μM stearoyl-CoA and the shown concentrations of [³H]sphinganine (●). Fumonisin B₁ was added at 75 nM (○). D, double-reciprocal plot of (dihydro)ceramide synthase activity (pmol/min/mg of microsomal protein) with 1 μM [³H]sphinganine and the shown concentrations of stearoyl-CoA (●). Fumonisin B₁ was added at 75 nM (○). For C and D, the protein concentration was kept low to ensure that substrate did not become limiting at the lower concentrations.

**FIG. 3. Sphingolipid biosynthesis from [¹⁴C]serine by cultured cerebellar neurons and inhibition by fumonisin B₁.** Cultured cells were incubated in the absence (left lane) or presence (right lane) of 25 μM fumonisin B₁ for 24 h and then new medium of this same composition containing 2 μCi/ml of [¹⁴C]galactose was added. After 24 h, the sphingolipids were extracted, analyzed on silica gel thin-layer chromatography in the solvent system chloroform/methanol/CaCl₂ (0.02%) (60:35:8, by volume), and the radioactivity detected by radiometric scanning. Abbreviations are as in Fig. 3.

G₃M₁, G₁D₈, G₂D₁b, G₇T₁b, and G₉Q₁b (van Echten et al., 1990). The band just above sphingomyelin was attributed to free sphinganine and sphingosine (which was confirmed by HPLC, as described below) and a radiochemical contaminant of the [¹⁴C]serine that has been seen previously (Merrill and Wang, 1986). Addition of 25 μM fumonisin B₁ reduced the labeling of all of the sphingolipids (Fig. 3, right lane) but increased the amount in the region where free long-chain bases migrate. This behavior is similar to the results with rat hepatocytes (Wang et al., 1991) and LLC-PK1 cells (Yoo et al., 1992), where fumonisin B₁ blocked the total sphingolipid labeling but increased the amount of [¹⁴C]sphinganine.

**FIG. 4. Sphingolipid biosynthesis from [¹⁴C]galactose by cultured cerebellar neurons and inhibition by fumonisin B₁.** Cultured cells were incubated in the absence (upper profile) and presence (lower profile) of 25 μM fumonisin B₁ for 24 h, and then new medium of this same composition containing 2 μCi/ml of [¹⁴C]galactose was added. After 24 h, the sphingolipids were extracted, analyzed on silica gel thin-layer chromatography in the solvent system chloroform/methanol/CaCl₂ (0.02%) (60:35:8, by volume), and the radioactivity detected by radiometric scanning. Abbreviations are as in Fig. 3.
When cerebellar neurons were incubated with \(^{3}H\)sphinganine (Fig. 5A), about half of the radiolabel migrated in the same region of the chromatoplate (Rf ~0.5) as sphingosine, sphinganine, and unesterified fatty acids. The remainder was distributed among polar compounds at the origin and less polar compounds near the solvent front, where the fatty acid standard was found. Further incubation with \(25 \mu M\) fumonisin B\(_1\) for 24 h, approximately 1 \(\mu Ci\) of \(^{3}H\) sphinganine (ca. 0.1 nmol/ml) was added, and the cells were incubated for 24 h. Then the sphingolipids were extracted and analyzed by silica gel thin-layer chromatography using a solvent system for long-chain bases (chloroform, methanol, 2 N NH\(_4\)OH, 40:10:1) for panels A and B or the ganglioside system described in Fig. 3 for panels C and D. The radiolaabeled regions of the chromatoplates were detected by radiometric scanning of the plates and the compounds identified by comparison with standards. Complex SL, mixed complex sphingolipids; FA, fatty acids; Cer, ceramide; Deg, degradation products. Other abbreviations are as in Fig. 3.

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When 25 \(\mu M\) fumonisin B\(_1\) was added (Fig. 5, B and D), there was little formation of complex sphingolipids, and essentially all of the radiolabel migrated in the region of sphinganine and its catabolites. These findings confirm that fumonisin B\(_1\) has blocked the acylation of \(^{3}H\)sphinganine without preventing degradation of free long-chain bases, which proceeded via the phosphorylation of sphingosine and sphinganine at position 1. This implies that fumonisin B\(_1\) does not inhibit this kinase, which has been confirmed experimentally by assays of sphingosine kinase.\(^2\)

**TABLE I**

| Treatment (long-chain base) | Period of incubation |
|-----------------------------|----------------------|
|                            | 24 h                 | 96 h                 | 168 h                |
| Control                     |                      |                     |                      |
| Free sphingosine            | 1.30 ± 0.10          | 1.33 ± 0.08         | 1.21 ± 0.28          |
| Free sphinganine            | 0.34 ± 0.02          | 0.80 ± 0.05         | 0.44 ± 0.02          |
| Total sphingosine           | 244 ± 3              | 207 ± 16            | 244 ± 11             |
| Total sphinganine           | 23 ± 3               | 23 ± 4              | 26 ± 6               |
| 1 \(\mu M\) Fumonisin B\(_1\)|                      |                     |                      |
| Free sphingosine            | 1.80 ± 0.10          | 2.04 ± 0.14         | 2.07 ± 0.08          |
| Free sphinganine            | 1.05 ± 0.01          | 1.96 ± 0.14         | 3.73 ± 0.12          |
| Total sphingosine           | 183 ± 10             | 170 ± 10            | 224 ± 32             |
| Total sphinganine           | 22 ± 1               | 23 ± 5              | 27 ± 2               |
| 25 \(\mu M\) Fumonisin B\(_1\)|                      |                     |                      |
| Free sphingosine            | 2.15 ± 0.21          | 1.50 ± 0.07         | 1.42 ± 0.20          |
| Free sphinganine            | 6.24 ± 0.17          | 10.0 ± 0.2          | 7.66 ± 1.77          |
| Total sphingosine           | 214 ± 20             | 150 ± 6             | 194 ± 2              |
| Total sphinganine           | 29 ± 2               | 19 ± 2              | 26 ± 2               |

*Mean ± range of two experiments.

*Turnover of Radiolaabeled Sphingolipids in Cultured Cerebellar Neurons—Before conducting more detailed analyses of the effects of fumonisin B\(_1\) on sphingolipid biosynthesis, its possible effect on turnover was evaluated by incubating cells with \(^{14}C\)serine for 24 h; then unlabeled serine was added, and the sphingolipids were analyzed after chase periods of 0, 24, and 48 h. There was essentially no change in the labeling profile for any of the sphingolipids (cf. Figs. 3 and 6, left panel), nor was there a change when fumonisin B\(_1\) was added during the chase period (Fig. 6). Thus, the effects of fumonisin B\(_1\) on sphingolipid labeling is due to inhibition of synthesis, not increased turnover.

Unlike for the sphingolipids, there were major changes in the amount of radiolabel in phosphatidylserine and phosphatidylethanolamine during the chase period, with phosphatidylserine decreasing by 3,000 dpm over 48 h and phosphatidylethanolamine increasing by approximately this amount. These changes appear to reflect the decarboxylation of phosphatidylserine to phosphatidylethanolamine. Fumonisin B\(_1\) caused no noticeable inhibition of the incorporation of \(^{14}C\) serine into phosphatidylserine or phosphatidylethanolamine (see time 0 in Fig. 6, right panel), nor did it alter the changes during the chase period (Fig. 6, right panel).

**Mass Measurements of Total Sphingolipids and Free Sphingosine and Sphinganine—Fumonisin B\(_1\) had little or no effect on the total sphingolipid amounts after 24 h of incubation; however, there was some reduction at 96 h (28%) and 168 h (21%) (Table I). These numbers may underestimate the effect of fumonisins on sphingolipid mass, because, although the cells that remained on the dishes were viable (based on morphology and trypan blue exclusion, data not shown), there

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\(^2\) R. M. Bell, personal communication.
was a small reduction (10–30%) in the amount of protein in the dishes that had been treated with fumonisin B₁. Therefore, if a reduction in mass leads to cell death, the results would be biased by the remaining, viable cells.

In contrast to the slight reduction in overall sphingolipid mass, free sphinganine increased significantly when the cells were treated with fumonisin B₁ (Fig. 7 and Table I). Within 24 h, sphinganine increased 20-fold, and this elevation was maintained over 168 h of incubation with 25 μM fumonisin B₁. Free sphingosine initially increased about 2-fold over the control; however, it was the same as for the controls at 96 and 168 h. The accumulation of sphinganine could be seen even when the fumonisin B₁ concentration was reduced to 1 μM (Table I and Fig. 7), although the effect was not as pronounced.

Fumonisin B₁ caused the accumulation of another long-chain base that migrated at 9.5 min, after sphinganine but before the [C2]sphinganine internal standard (Fig. 7). This compound may be the same as the faint band that is visible above sphinganine and sphingosine for the fumonisin-treated cells in Fig. 3. The nature of this compound is not known, but we have begun to observe it in other cell lines treated with fumonisins (data not shown). It does not appear to be one of the well known long-chain bases, such as phytosphingosine (which elutes before sphingosine) or 3-ketosphinganine (which was ruled out by comparison with a synthetic standard and because treatment of extracts with sodium borohydride did not alter the mobility of the unknown) (not shown), but the mobility was similar to that for [C2]sphingosine.

Time Course of Inhibition and Reversal—Because these cells are maintained in culture for up to a week for the longer term incubations (and long-chain bases change in rat cerebellar neurons during culture) (Valsecchi et al., 1993), the labeling pattern was examined over this period. When the cerebellar neurons were maintained in culture for 24, 72, or 144 h, [14C]serine was added, and the sphingolipids analyzed after an additional 24 h (Fig. 8, left panels), there were some differences in the labeling pattern, but the cells clearly continued to synthesize a full profile of complex gangliosides. Furthermore, the labeling profiles of cells treated with fumonisin B₁ over these time periods were also similar (Fig. 8, right panels).

![Fig. 7. Analysis of the free long-chain bases of cerebellar neurons treated with fumonisin B₁ for varying times. Shown are the elution profiles of the o-phthalaldehyde derivatives of the free long-chain bases with [C2]sphinganine as an internal standard (eluting at 12 min). The elution times for sphingosine (So) and sphinganine (Sa) were 5.5 and 6.8 min, respectively.](image)

![Fig. 8. Labeling profiles for cerebellar neurons incubated with [14C]serine after varying periods in culture. Cultured cerebellar neurons were placed in culture for 24, 72, or 144 h, and [14C]serine was added to label over the period shown. After incubation with the [14C]serine for this 24-h period, the sphingolipids were extracted and separated by TLC as described in the legend to Fig. 3, and the radiolabel was detected by radiometric scanning. Abbreviations are as in Fig. 3.](image)

![Fig. 9. Reversibility of fumonisin B₁ inhibition of sphingolipid biosynthesis from [14C]serine. Cultured cerebellar neurons were incubated with 25 μM fumonisin B₁ for the times shown (48 or 96 h), the medium was removed, and, after the times shown on the right column, the incorporation of [14C]serine into sphingolipids was determined as described in the legend to Fig. 3. Abbreviations are as in Fig. 3.](image)

The stability of the cells to long-term incubations allowed us to determine whether cells treated with fumonisin B₁, followed by removal of the mycotoxin, recovered the ability to incorporate [14C]serine into sphingolipids. This experiment (Fig. 9) was conducted by incubating cells with fumonisin for the times shown and then removing the fumonisin for the periods indicated and analyzing the incorporation of [14C]serine into sphingolipids during the last 24 h. Although there are qualitative differences in these profiles, the cells have the capacity to make each of the complex sphingolipids after removal of the mycotoxin. The most prominent difference from the controls (cf. Figs. 8 and 9) was an elevation in the amount of label at Rf 0.5, which is where free long-chain bases migrate. This may be due to partial inhibition of ceramide synthase by residual fumonisin B₁, because such a tight binding inhibitor might not be removed completely even after multiple changes of the medium.

**Dose Response of Sphingolipid Labeling**—The scans of a chromatoplate from a representative dose-response experiment are shown in Fig. 10. The striking feature of these scans...
ponents, one for sphingomyelin that is <1

changes are clearly not the same for all of the sphingolipid

times shown

B1 (51.0

evident. To characterize this on a more quantitative basis,

each region of the chromatogram was scraped into  scintilla-

tion vials and  the dpm determined. These results  are  pre-

radiolabel in sphingomyelin at concentrations of fumonisin

is that  there  are substantial decreases in  the amount of

sphingolipid biosynthesis is approximately 1

Inhibition of sphingolipid biosynthesis is inhibited, experiments were also conducted with

preferentially inhibits sphingomyelin synthesis over glycolipid formation.

Inhibition of Sphingolipid Biosynthesis by β-Fluoroala-

Assays of Serine Palmitoyltransferase—Recent  work  has shown that adding sphingosine to mouse cerebellar neurons in culture causes a decrease in the incorporation of [14C]serine into sphingolipids, apparently due to down-regulation of serine palmitoyltransferase (van Echten et al., 1990; Mandon et al., 1991). Because fumonisin B1 caused cellular sphinga-

is that there are substantial decreases in the amount of

radioactivity in sphingomyelin at concentrations of fumonisin

FB1 (≤1.0 μM) where little change in glycolipid labeling is evident. To characterize this on a more quantitative basis, each region of the chromatogram was scraped into scintilla-

tion vials and the dpm determined. These results are pre-

sented in Fig. 11 to show the relationship between the dpm in total and individual sphingolipids (Fig. 11A) and the dpm in sphingomyelin versus the sum of all of the glycolipids (Fig. 11B). By this analysis, the IC50 for inhibition of total sphingolipid biosynthesis is approximately 1 μM; however, the changes are clearly not the same for all of the sphingolipid classes (Fig. 11A). The overall IC50 is comprised of two com-

ponents, one for sphingomyelin that is <1 μM and a higher IC50 for glycolipids (>5 μM) (Fig. 11B).

This observation was further confirmed by plotting the

results of two experiments (not shown) and calculation of the

100% inhibition was found for all of the glycolipids at fumonisin B1 concentrations of 7 ± 3 μM, whereas the IC50 for sphingomye-

lin was 10-fold lower (0.7 μM). These results indicate that fumonisin B1 preferentially inhibits sphingomyelin synthesis over glycolipid formation.

FIG. 10. Dose response of fumonisin B1 inhibition of [14C] serine incorporation into sphingolipids. Cerebellar neurons were incubated with the shown concentrations of fumonisin B1 for the times shown (48 or 96 h), the medium was removed, and the incor-

poration of [14C]serine into sphingolipids was determined as described in the legend to Fig. 3. Abbreviations are as in Fig. 3.

FIG. 11. Graphical analyses of the concentration depend-

ence of the inhibition of sphingolipid biosynthesis by fumon-

isin B1. The dpm in the major sphingolipids identified in Fig. 10 are graphed versus the concentration of fumonisin B1 (panel A) and as the dpm in sphingomyelin compared with that for total glycolipids (panel B). The results shown are the mean of two experiments that differed by ±10%. Abbreviations are as in Fig. 3.

FIG. 12. Labeling of sphingomyelin as a percent of total labeled sphingolipid at different concentrations of β-fluoroala-

niprogressed by the dpm in sphingomyelin as a percent of the total sphingolipid

labeling.

| Sphingolipid | Concentrationa | μM |
|-------------|----------------|----|
| Sphingomyelin| 0.7 ± 0.3      |    |
| Glucosylceramide | 7 ± 1 | |
| Lactosylceramide | 7 ± 1 | |
| Ganglioside GM1 | 7 ± 1 | |
| Ganglioside GD1b | 4 ± 3 | |
| Ganglioside GD2b | 10b     | |
| Ganglioside GT1b | 6 ± 1 | |
| Ganglioside GQ1b | 4      | |

a Mean ± S.D. from two experiments conducted in duplicate.

b Average of duplicate analyses from a single experiment.

TABLE II

Concentrations of fumonisin B1, for 50% inhibition of sphingolipid labeling

The results shown are the mean of two experiments that

-10-fold lower (0.7 μM). These results indicate that fumonisin B1 preferentially inhibits sphingomyelin synthesis over glycolipid formation.

Inhibition of Sphingolipid Biosynthesis by β-Fluoroala-

nine—To determine whether this differential effect of fumon-

isin B1 on sphingomyelin synthesis is peculiar to this myco-

toxin or can occur more generally when the rate of ceramide synthesis is inhibited, experiments were also conducted with

β-fluoroalanine, a "suicide" inhibitor of serine palmitoyltrans-

ferase (Medlock and Merrill, 1988). The IC50 for inhibition of sphingolipid labeling from [14C]serine was 25 μM (data not shown). As with fumonisin B1, there was a greater effect on sphingomyelin labeling than for glycolipid synthesis, as ex-

pressed by the dpm in sphingomyelin as a percent of the total radioactivity in sphingomyelin (Fig. 12). Therefore, a decrease in sphingomyelin formation over glycolipid synthesis occurs as a general response to reduced ceramide biosynthesis.

Assays of Serine Palmitoyltransferase—Recent work has shown that adding sphingosine to mouse cerebellar neurons in culture causes a decrease in the incorporation of [14C]serine into sphingolipids, apparently due to down-regulation of serine palmitoyltransferase (van Echten et al., 1990; Mandon et al., 1991). Because fumonisin B1 caused cellular sphinga-

nine to accumulate, this mycotoxin might induce a secondary inhibition of the first step of the pathway as well as at ceramide synthase. However, in experiments in which the
cells were treated overnight with 25 μm fumonisin B₁ and the microsomes were isolated and assayed for serine palmitoyltransf erase activity, the activity of the fumonisin-treated cells (3,740 ± 890 dpm/mg of protein) was not significantly different from controls (4,460 ± 900 dpm/mg of protein).

**DISCUSSION**

Fumonisins have been shown to inhibit overall sphingolipid biosynthesis in cultured hepatocytes (Wang et al., 1991) and a renal cell line (Yoo et al., 1992), but this is the first characterization of the effects of this mycotoxin on neuronal cells and on individual sphingolipid classes. As would be predicted for an inhibitor of (dihydro)ceramide synthase, fumonisin B₁ caused accumulation of free long-chain bases. Fumonisin B₁ increased the amount of free sphinganine by about 20-fold within 24 h, but further increases were not seen at 96 or 168 h. This might be due to down-regulation of serine palmitoyltransferase, as has been seen when exogenous long-chain bases have been added to these cells (van Echten et al., 1990; Mandon et al., 1991); however, the activity of this enzyme was not changed when assayed in vitro. It is possible that regulatory factors have been lost or diluted in the in vitro assays; however, based on the extensive degradation of [3H] sphinganine that occurred when this radiolabeled long-chain base (alone or with fumonisin B₁) was added to the cells, it is probable that cellular levels of free long-chain bases are also governed by their rate of degradation, which is not affected by fumonisin B₁.

This study also provided information about the pathways that lead to the appearance of free long-chain bases in cells. The greater accumulation of sphinganine than sphingosine provides further evidence that introduction of the 4,5-trans-double bond of sphingosine occurs after acylation of sphinganine (Ong and Brady, 1973; Stoffel and Bister, 1974; Merrill and Wang, 1986; Rother et al., 1992). A corollary to this hypothesis is that free sphingosine arises from the turnover of cellular sphingolipids rather than as an intermediate of the de novo biosynthetic pathway (Merrill and Wang, 1986; Slife et al., 1989; Wang et al., 1991, 1992); therefore, increases in sphingosine caused by fumonisin B₁ are probably due to inhibition of the reacylation of this long-chain base (Wang et al., 1991, 1992). The cultured cerebellar neurons exhibited a slow turnover of the sphingolipids based on the labeling profile and on the small change in overall sphingolipid mass when treated with fumonisin B₁. This agreed, therefore, with the small increases in free sphingosine compared with sphinganine.

Based on the slow turnover of the sphingolipids and the lack of an increase in the overall sphingolipid mass, it appears that these differentiated cells are engaged in relatively little de novo sphingolipid biosynthesis. It is not possible to compare the sphingolipid mass with the amounts of sphingolipids made de novo from [³⁵S]serine or [¹⁴C]galactose, because the cellular specific radioactivities of these precursors are not known. Nonetheless, based on the specific activity of the starting [³⁵S]serine, the amount of newly synthesized sphingolipids would be at least 0.25 nmol/mg of protein in 24 h, or 0.1% of the total cellular sphingolipid. It appears that relatively little remodeling of existing sphingolipids is occurring, because fumonisin B₁ reduced the labeling of sphingolipids by [¹⁴C]galactose as well as from [³⁵S]serine or [¹⁴C]sphinganine.

It was somewhat surprising that fumonisin B₁ had a more potent effect on sphingomyelin formation than on glycolipid biosynthesis. This is probably due to a greater sensitivity of sphingomyelin synthesis to a reduction in ceramide formation, because it was also seen with another inhibitor of de novo ceramide biosynthesis (β-fluoroleucine). Such selectivity may be due to a number of causes. 1) The Km for ceramide of glucosylceramide synthase may be lower than that of sphingomyelin synthase; thus, a decrease in ceramide synthesis will not affect glycosphingolipid formation until the ceramide concentration drops below the effective Km. 2) The intracellular localization of sphingomyelin and glycosphingolipid biosynthesis (Hoekstra and Kok, 1992) might account for these differences. For example, glycosphingolipid synthesis may begin in a cellular compartment that is more proximal to the site of ceramide synthesis; therefore, these enzymes would have first access to ceramide. This explanation agrees with recent evidence that ceramide synthesis is on the cytoplasmic face of the endoplasmic reticulum (Mandon et al., 1992) and glucosylceramide synthesis (Coste et al., 1985, 1986; Futerman and Pagano, 1991; Jeckel et al., 1992) occurs on the cytoplasmic surface of various Golgi membranes, whereas sphingomyelin synthase is thought to be on the luminal side of the cis-Golgi (Futerman et al., 1990; Jeckel et al., 1990, 1992), although some cells appear to have more than one intracellular site of sphingomyelin synthesis (Kalnen et al., 1993). 3) The inhibitors (fumonisin B₁ and β-fluoroleucine) may inhibit a ceramide "flipase" that moves this substrate from the cytosolic to the luminal side of the Golgi membrane. 4) The inhibitors may affect sphingomyelin synthase and glycosyltransferase(s) directly but more potently inactivate the former. In our view, the similar findings with two very structurally different compounds, fumonisin B₁ and β-fluoroleucine, make these last two explanations less likely. Thus, the greater sensitivity of sphingomyelin synthesis is probably due to the differences in the Km and/or subcellular localization of the pertinent enzymes.

If inhibition of ceramide formation in vivo also has a selective effect on sphingomyelin synthesis, this might be clinically useful in diseases caused by defective sphingomyelin turnover (such as Niemann-Pick disease), because it might be possible to reduce the accumulation of sphingomyelin without disrupting cell functions dependent on other sphingolipids. Inhibition of ceramide synthesis might also provide information about the proposed association between sphingomyelin and cholesterol metabolism (as discussed in Merrill and Jones, 1990).

These studies with cultured mouse cerebellar neurons also provide information with a possible model for the neurotoxicity of fumonisin B₁. Ponies that consume fumonisins exhibit behavioral changes and other signs of leukoencephalomalacia within days of consumption of fumonisins (Wang et al., 1992). Considering the diverse functions of sphingolipids (Merrill et al., 1993a), it is possible that these responses are due to inhibition of de novo sphingolipid metabolism by the brain. More work is needed to determine whether this is due to loss of important complex sphingolipid(s) or to the accumulation of free long-chain bases, which are highly bioactive molecules when added to cells. Long-chain bases have been shown to affect multiple cell regulatory pathways, including inhibition of protein kinase C (Hannun et al., 1986), phosphatidic acid phophatase (Lavie and Liscovitch, 1990; Jamai et al., 1991; Mullman et al., 1991), the (Na⁺⁺/K⁺)-ATPase (Oishi et al., 1990), c-src and v-src kinases (Igarashi et al., 1989), and other systems (Hannun and Bell, 1989; Merrill et al., 1993a) including activation of the EGF receptor (Faucher et al., 1988; Wedegaertner and Gill, 1989). At low concentrations, long-chain bases are mitogenic to some cell types (Zhang et al., 1990) and, for other cells, highly cytotoxic (Merrill et al., 1993a; Stevens et al., 1990). Sphingosine-1-phosphate, an intermediate of long-chain base degradation, has been impli-
cated as a mediator of the release of calcium from intracellular stores (Ghosh et al., 1990; Zhang et al., 1991). Therefore, the changes in neuronal cell function may be triggered by the high levels of free long-chain bases or metabolites.

Comments on the Inhibition of Ceramide Synthase by Fumonisin B1—These kinetic profiles for inhibition of ceramide synthase by fumonisin B1 are difficult to interpret mechanistically, because the assay contains not only crude membrane preparations of the enzyme but also two amphiphatic substrates (attempts to simplify the analyses by using detergent micelles were unsuccessful, because activity is labile in detergents). Nonetheless, the degree of inhibition was affected by the concentrations of both substrates with a competitive behavior that suggests that fumonisin B1 may interact with the binding sites for both substrates. Fumonisin B1 may have two modes of interaction with ceramide synthase. The "sphingamine-like" domain may interact with the sphingamine binding site, and the negatively charged tricarboxylic acid groups may interact with the fatty acyl-CoA binding site (by mimicking the polyanionic phosphate groups of the CoA). The latter interaction is probably not the most important, because fumonisin B1 may interact with the fatty acyl-CoA binding site (by mimicking the polyanionic phosphate groups of the CoA). The latter interaction is probably not the most important, because fumonisin B1 does not inhibit another CoA-dependent enzyme (serine palmitoyltransferase) or labeling of glycolipids (Wang et al., 1991), and removal of the tricarboxylic acid groups increases the IC50 only about 10-fold (Merrill et al., 1993b). The ability of fumonisin B1 to interact with both binding sites might account for its potency.

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