Inhibition of Sphingolipid Biosynthesis by Fumonisins

IMPLICATIONS FOR DISEASES ASSOCIATED WITH FUSARIUM MONILIFORME

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Elaine Wang‡, William P. Norred‡, Charles W. Bacon‡, Ronald T. Riley‡, and Alfred H. Merrill, Jr.‡‡

From the ‡Department of Biochemistry, Rollins Research Center, Emory University School of Medicine, Atlanta, Georgia 30322 and the ‡Toxicology and Mycotoxins Research Unit/United States Department of Agriculture-Agricultural Research Service, Athens, Georgia 30613

Culture materials and grains contaminated with certain isolates of Fusarium moniliforme cause equine leukoencephalomalacia, porcine pulmonary edema syndrome, and liver cancer in rats. The causative agents are thought to be a family of compounds called fumonisins, which bear considerable structural similarity to the long-chain (sphingoid) base backbones of sphingolipids. Incubation of rat hepatocytes with fumonisins inhibited incorporation of [1-14C]serine into the sphingosine moiety of cellular sphingolipids with an IC50 of 0.1 μM for fumonisin B1. In contrast, fumonisin B1 increased the amount of the biosynthetic intermediate sphinganine, which suggests that fumonisins inhibit the conversion of [1-14C]sphinganine to N-acyl-1-[14C] sphinganines, a step that is thought to precede introduction of the 4,5-trans double bond of sphingosine (Merrill, A. H., Jr. and Wang, E. (1986) J. Biol. Chem. 261, 3764–3769). In agreement with this mechanism, fumonisin B1 inhibited the activity of sphingosine N-acyltransferase (ceramide synthase) in rat liver microsomes with 50% inhibition at approximately 0.1 μM and reduced the conversion of [1-3H]sphingosine to [3H]ceramide by intact hepatocytes. As far as we are aware, this is the first discovery of a naturally occurring inhibitor of this step of sphingolipid metabolism. These findings suggest that disruption of the de novo pathway of sphingolipid biosynthesis may be a critical event in the diseases that have been associated with consumption of fumonisins.

Fusarium moniliforme (Sheldon) is one of the most prevalent fungi on maize, other grains, and agricultural commodities in the United States and throughout the world (1). Culture materials from certain isolates of (and grains naturally contaminated with) F. moniliforme have been shown to be toxic and carcinogenic for animals (2–5); furthermore, consumption of contaminated maize has been correlated with esophageal cancer in areas of southern Africa, China, and other countries (6–8). Several mycotoxins, termed fumonisins, have been isolated from extracts of F. moniliforme (9) and naturally contaminated corn (10, 11). Fumonisin B1 has been shown to cause equine leukoencephalomalacia (12), porcine pulmonary edema (13), and promotion of liver tumors in rats (14). Recent findings indicate that high levels of fumonisin B1 are present in United States feeds associated with field cases of these animal diseases (15).

The molecular mechanism of action of the fumonisins is not known; however, these compounds bear a remarkable structural similarity to sphingosine (Fig. 1), the long-chain (sphingoid) base backbone of sphingomyelin, cerebroside, sulfatides, gangliosides, and other sphingolipids. Sphingolipids are thought to be involved with the regulation of cell growth, differentiation, and neoplastic transformation through participation in cell-cell communication and cell-substratum interactions and possible interactions with cell receptors and signaling systems (16–20). Hence, we hypothesized that disruption of sphingosine metabolism might be a target of fumonisins. This manuscript reports that fumonisins inhibit de novo sphingolipid biosynthesis by rat liver hepatocytes and identifies an important site of inhibition as the reaction catalyzed by sphingosine N-acyltransferase (ceramide synthase).

EXPERIMENTAL PROCEDURES

Materials—Fumonisins B1 and B2 were purchased from Division of Food Sciences and Technology, Council for Scientific and Industrial Research, Pretoria, South Africa. Tissue culture media were purchased from GIBCO, and collagenase (Type IV), collagen (Type III), and palmityl-CoA were from Sigma. Matrigel (from Engelbreth-Holm-Swarm mouse tumor) were obtained from Collaborative Research Inc. (Bedford, MA). The [2-14C]serine and the [3H]sphingosine (prepared by catalytic exchange) were from Du Pont-New England Nuclear. The sphingolipid standards were purchased from Sigma or prepared synthetically (21).

Hepatocyte Culture—Hepatocytes were prepared by a collagenase perfusion method (22) from male Sprague-Dawley rats (125–200 g) fed a chow diet (Ralston Purina, St. Louis, MO) ad libitum. The hepatocytes were isolated using aseptic procedures and plated in 60-mm tissue culture dishes coated with collagen for short term (~24 h) or with Matrigel for longer term (~3–4 days) experiments. After plating for 4 h, the medium was changed to 2 ml of Dulbecco’s modified Eagle’s medium (with 3.7 g/l NaHCO3) containing 10 μg/ml insulin, 10 g/l streptomycin sulfate, and varying concentrations of fumonisins (added from a 1 mM solution in Dulbecco’s phosphate-buffered saline). The cells were maintained in a tissue culture incubator at 37 °C and an atmosphere of 5% CO2; the medium was changed every 24 h. Cell viabilities were assessed by the ability of the cells to exclude 0.1% trypan blue and were greater than 96%.

Lipid Analyses—The de novo biosynthesis of sphingosine was measured as described before (23, 24). Briefly, cells that had been

‡ To whom correspondence should be addressed: Dept. of Biochemistry, Rollins Research Ctr., Emory University School of Medicine, Atlanta, GA 30322. Tel.: 404-727-5978; Fax: 404-727-2738 or Dr. R. T. Riley, Toxicology and Mycotoxins Research Unit, USDA/ARS, P.O. Box 5677, Athens, GA 30613. Tel.: 404-546-3377; Fax: 404-546-3567.

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The abbreviations used are: l, liter; HPLC, high performance liquid chromatography.
Acyltransferase—A microsomal fraction was isolated from rat liver
N-acyltransferase (30) in the presence of varying concentrations of
whereas, to determine total sphingolipids, the extracts were acid-
were base-treated to remove glycerolipids before analysis (28);
HPLC as previously described (28) with C2O-sphinganine as an
of radiolabel was determined by scintillation counting.
ether:methanol (99:1, v/v), visualized with I2 vapor, and the amount
sphingosine using silica gel H TLC plates developed with diethyl
hydrolyzed 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.

Furthermore, sphingosine biosynthesis de novo by rat hepatocytes is relatively easy to follow using [14C]serine and appears to proceed via the reactions shown in Fig. 2 (24) like in other cells (23, 32).

Fumonisin B1 (1 μM) caused an almost complete inhibition of [14C]sphingosine formation by hepatocytes (Figs. 2 and 3). Similar inhibition occurred when [14C]serine and fumonisin B2 were added together for short term (2 h) or overnight (16 h) incubations and when the cells were incubated overnight with fumonisin before adding [14C]serine (Fig. 3A, the 16 h + 2 h group). Hence, fumonisin B1 appears to act fairly quickly, and the inhibition is persistent. The IC50 for inhibition of [14C]serine incorporation into [14C]sphingosine was approximately 0.1 μM for fumonisin B1 (Fig. 3B). A similar degree of inhibition was obtained with fumonisin B3 (Fig. 3A and data not shown), another mycotoxin often produced in substantial amounts by F. moniliforme (10).

In contrast, there was no reduction in the radiolabeling of phosphatidylserine, phosphatidylethanolamine, or phospho-

FIG. 2. Sphingosine biosynthesis and inhibition by fumonisins B1. Free long-chain bases (3-ketosphinganine, sphinganine, and sphingosine) have not been found to accumulate as detectable intermediates of this pathway under normal conditions (23, 24, 29); therefore, sphingosine formation is assessed by hydrolyzing the N-acyl products and analyzing the labeled long-chain bases as depicted in this figure. The chromatograms illustrate the labeling of sphinganine and sphingosine obtained by acid hydrolysis of the lipid extracts from hepatocytes incubated for 2 h with [14C]serine with and without 1 μM fumonisin B1. The large amount of radiolabel that migrates with the solvent front is primarily fatty acids.

RESULTS AND DISCUSSION

Rat hepatocytes were selected as a model for the effects of fumonisins because F. moniliforme culture materials, now known to contain the cancer-promoting fumonisin B2 (9, 15), are hepatotoxic and hepatocarcinogenic in the rat (4, 10, 31). Furthermore, sphingosine biosynthesis de novo by rat hepatocytes is relatively easy to follow using [14C]serine and appears to proceed via the reactions shown in Fig. 2 (24) like in other cells (23, 32).

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tidylcholine from \([^{14}\text{C}]\)serine (Table I) nor in the mass of these phospholipids under conditions where sphingolipid labeling (Figs. 2 and 3) and mass (Table I) were reduced significantly. Fatty acid biosynthesis from \([^{14}\text{C}]\)acetic acid was not altered (Table I). Hence, the inhibition of sphingolipid biosynthesis does not appear to be due to the inability of the cells to take up \([^{14}\text{C}]\)serine and to incorporate it into lipids in general nor to affect the ability of the cell to form the other biosynthetic precursors (i.e., fatty acids).

Although \textit{de novo} sphingolipid biosynthesis was completely blocked by 10 \textmu M fumonisin B\(_1\), there was only a slight reduction in the mass of total sphingolipids after 1 day (Table I). This is probably due to the generally slow turnover of sphingolipids (32). A greater effect was seen when the cells were plated on Matrigel to allow incubation with 1 \textmu M fumonisin B\(_1\) for 4 days, which reduced the level of total sphingolipids by about half (i.e., from 4.6 \pm 0.7 nmol/dish to 2.1 \pm 0.1 nmol/dish). There was no decrease in the number of viable cells despite treatment with 1 \textmu M fumonisin for 4 days.

There are several potential sites at which fumonisins might affect sphingosine metabolism (Fig. 2), hence, these were investigated further. Fumonisins do not appear to act at the first step of this pathway, because even a fairly high concentration (25 \textmu M) of fumonisin B\(_1\) did not reduce the activity of serine palmitoyltransferase \textit{in vitro} (i.e., the activities in the presence and absence of 25 \textmu M fumonisin B\(_1\) were 88 \pm 3 and 61 \pm 15 pmol 3-ketosphinganine formed per min/mg of microsomal protein, respectively). Furthermore, treatment of hepatocytes with fumonisin B\(_1\) followed by assays of this enzyme in disrupted cells revealed no inhibition (i.e., the activities were 7.7 \pm 0.2 and 8.2 \pm 0.8 pmol/min/mg of cellular protein in the presence and absence of 2.5 \textmu M fumonisin B\(_1\), respectively). Inhibition at the second step of the pathway is also unlikely, because 3-ketosphinganine was not seen to accumulate (Fig. 2), and there was no reduction in the formation of \([^{14}\text{C}]\)sphinganine (which will be discussed below).

Fumonisins apparently inhibit at the step where \([^{14}\text{C}]\)sphinganine is converted to N-acyl-\([^{14}\text{C}]\)sphinganine, because the amount of radiolabel in \([^{14}\text{C}]\)sphinganine increased when hepatocytes were treated with fumonisin B\(_1\) (Fig. 4A). This was accompanied by an increase in the mass of free sphinganine (Fig. 4B); when hepatocytes were incubated with 1 \textmu M fumonisin B\(_1\) for 4 days, sphinganine increased 110-fold (i.e., to 1499 \pm 18 pmol/dish compared with 13.6 \pm 0.4 pmol/dish for the control). There was only a small reduction in the amount of free sphinganine within the first 2 h (Fig. 4B); however, free sphinganine decreased significantly after 4 days (i.e., to 52 \pm 1 pmol/dish compared with 233 \pm 14 pmol/dish for the control). These findings are consistent with the view that the free sphinganine found in hepatocytes is not an intermediate of the \textit{de novo} biosynthetic pathway (23, 24) (hence, will be affected little by short term treatment with fumonisin B\(_1\)) but arises from the turnover of complex sphingolipids (27, 32) and would, therefore, be most affected when there is a decrease in total sphingolipids due to longer term exposure to these compounds.

Inhibition at this step of the pathway was demonstrated directly by \textit{in vitro} assays of sphingosine N-acyltransferase, which has been reported to acylate both sphinganine and sphingosine (30) (Fig. 5A) and by following the conversion of \([^{3}\text{H}]\)sphingosine to \([^{3}\text{H}]\)ceramide by intact cells (Fig. 5A). The

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**TABLE I**

Effects of fumonisin B\(_1\) on selected lipids of rat hepatocytes

Hepatocytes were incubated for 16 h with \([^{14}\text{C}]\)serine and the concentrations of fumonisin B\(_1\) shown, using the conditions described in Fig. 2. The lipids were extracted, separated by thin-layer chromatography, and quantitated as described in the text. The groups that are significantly different (p < 0.05) from the control (no fumonisin) group are designated by asterisks.

| Parameter | Fumonisin B\(_1\) |
|-----------|------------------|
| 0 \textmu M | 1 \textmu M | 10 \textmu M |
| **A. Radiolabeling** | | |
| Phosphatidylethanolamine | 103 \pm 8.3 | 163 \pm 4.4* | 153 \pm 10* |
| Phosphatidylserine | 44.9 \pm 9.5 | 488 \pm 2.9 | 523 \pm 3.6 |
| Phosphatidylcholine | 2270 \pm 226 | 2640 \pm 264 | 2740 \pm 196* |
| Base labile fatty acids* | 2055 \pm 261 | 2075 \pm 54 | 2075 \pm 103 |
| **B. Mass measurements** | | |
| Phosphatidylethanolamine | 44.1 \pm 2.9 | 44.9 \pm 1.4 | 44.4 \pm 0.9 |
| Phosphatidylserine | 13.8 \pm 2.8 | 15.5 \pm 2.8 | 12.3 \pm 1.9 |
| Phosphatidylcholine | 118 \pm 3.6 | 105 \pm 1.8 | 112 \pm 3.4 |
| Total sphingolipids | 8.9 \pm 0.9 | 6.7 \pm 0.3* | 6.5 \pm 0.7* |

* Determined after incubation with 10 \textmu Ci of \([^{14}\text{C}]\)acetic acid.

* Determined by phosphate analyses (27) with the exception of the total sphingolipids, which were estimated by acid hydrolysis of the lipid extracts and analysis of the amount of sphingosine by HPLC (28).
FIG. 4. Increases in sphinganine upon incubating hepatocytes with fumonisin B1. A, amounts of label detected in [14C]sphinganine; the conditions for these analyses were the same as in Figs. 2 and 3. B, mass amounts of sphingosine and sphinganine after incubation of hepatocytes with fumonisin B1, for 2 h, with analysis of the free long-chain bases by HPLC (28). The inset to B shows an HPLC profile of the free long-chain bases of rat liver hepatocytes after incubation with 1 μM fumonisin B1, for 2 h (for comparison with the usual HPLC profile of untreated liver, see Ref. 28). The abbreviations are: So, sphingosine; Sa, sphinganine; and C20, the C20-sphinganine, the internal standard.

FIG. 5. Inhibition of sphingosine N-acyltransferase by fumonisin B1. A shows the dose dependence of inhibition of sphingosine N-acyltransferase in uitro. The ratio of conversion of [1H]sphingosine to [1H]ceramide was determined with palmitoyl-CoA and varying concentrations of fumonisin B1, using rat liver microsomes as described in the text. B shows the inhibition of [1H]sphingosine conversion to ceramides by fumonisin B1, by intact hepatocytes that were incubated with 1 μM fumonisin B1, for 1 h and then approximately 1 μCi of [1H]sphingosine was added, and the cells were incubated for the times shown. The lipids were extracted and analyzed by TLC as described in the text. Asterisks designate the groups that were different from control (no fumonisin treatment) with p < 0.05.

apparent IC50 for inhibition of this activity in uitro was approximately 0.1 μM for fumonisin B1, but the inhibition may be biphasic (Fig. 5B). All together, these findings suggest strongly that the inhibition of sphingosine N-acyltransferase accounts for the disruption of de novo sphingolipid biosynthesis by fumonisins, although these compounds may have additional effects on other enzymes of sphingolipid metabolism.

The structural basis for this inhibition is unknown; however, one can speculate that similarities between the fumonisins and long-chain (sphingoid) bases (Fig. 1) allow them to be recognized as substrate (or transition state or product) analogues by sphingosine N-acyltransferase. The absence of a hydroxymethyl group at carbon 1 may alter their orientation in the active site of this enzyme and preclude acylation or, if acylated, result in an inhibitory ceramide that cannot be removed by addition of a sphingolipid headgroup at that position. The lack of a hydroxymethyl group at position 1 is shared by a number of other fumonisins-like compounds, such as the host-specific phytoxins produced by another fungus Alternaria alternata (33); hence, more naturally occurring inhibitors of this pathway may exist. These compounds are also unable to follow the usual pathway of long-chain base catabolism, which proceeds via phosphorylation at position 1 (32), and this may contribute to the persistence of the inhibition.

These findings with rat hepatocytes provide the first identification of a biochemical target for the action of fumonisins and imply that inhibition of de novo sphingolipid biosynthesis in uitro may underlie the hepatotoxicity and hepatocarcinogenicity of this mycotoxin in viv o. The hepatic levels of fumonisins under these conditions are not known; however, if all the fumonisin given in feeding studies with rats (1-10 mg) (3-5, 31) was distributed uniformly in the body, the concentrations could be between 10 and 100 μM. This is surely an over- (or under-) estimation of the amount that reaches liver in viv o, but certainly considerably higher than the IC50 for inhibition of sphingolipid biosynthesis by hepatocytes (0.1 μM). In preliminary studies, we have noted a small (10 and 35%) reduction in [14C]sphingosine biosynthesis by hepatocytes isolated from two rats given fumonisin B1 (5 mg/200 g body weight) by gavage for just 2 days. Perhaps more interestingly, there was a pronounced reduction in the ratio of free [14C]sphingosine to [14C]sphinganine from 2.6 ± 0.14 (a typical ratio for control hepatocytes) to 0.5 ± 0.12. Disruption of this pathway is an attractive mechanism for aspects of cell growth, differentiation, and transformation (18-20). For example, the degeneration of neuronal cells seen in equine leukoencephalomalacia may be due to inhibition of sphingolipid biosynthesis because brain contains high levels of sphingolipids (17). One can also speculate that an accumulation of sphinganine in cells exposed to fumonisins might lead to cell death since long-chain bases can be highly cytotoxic (34, 35) or to cell proliferation since these compounds are mitogenic to some cell types (36) and affect diverse cell systems (20), including protein kinase C (37), the epidermal growth factor receptor (38), the Na+/K+-ATPase (39), and phosphatidic acid phosphohydrolase (40), inter alia. Although toxic, fumonisins (or related compounds) might also be therapeutically useful in diseases where defects in sphingolipid turnover cause cells to accumulate high levels of sphingolipids.

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