

Sphingolipid Metabolism: Roles in Signal Transduction and Disruption by Fumonisin

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Sphingolipids have important roles in membrane and lipoprotein structure and in cell regulation as second messengers for growth factors, differentiation factors, cytokines, and a growing list of agonists. Bioactive sphingolipids are formed both by the turnover of complex sphingolipids and as intermediates of sphingolipid biosynthesis. Usually, the amounts are highly regulated; however, by inhibiting ceramide synthase, fumonisins block the biosynthesis of complex sphingolipids and cause sphinganine (and sometimes sphingosine) to accumulate. Where the mechanism has been studied most thoroughly, the accumulation of sphingoid bases is a primary cause of the toxicity of fumonisin B (FB). Nonetheless, the full effects of fumonisins probably involve many biochemical events. The elevations in sphingoid bases also affect the amounts of other lipids, including the 1-phosphates and *N*-acetyl derivatives of sphinganine. Furthermore, the aminopentol backbone of FB₁ (AP₁) is both an inhibitor and a substrate for ceramide synthase, and the resultant *N*-palmitoyl-AP₁ (PAP₁) is an even more potent inhibitor of ceramide synthase (presumably as a product analog). PAP₁ is 10 times more toxic than FB₁ or AP₁ for HT-29 cells in culture, and hence may play a role in the toxicity of nixtamalized fumonisins. All these processes—the effects of fumonisins on sphingolipid metabolism, the pathways altered by perturbation of sphingolipid metabolism, and the complex cellular behaviors regulated by sphingolipids—must be borne in mind when evaluating the pathologic effects of fumonisins. *Key words:* biomarker, C₂-ceramide, ceramide synthase, fumonisin B₁, lipid metabolism, sphinganine, sphingosine. — *Environ Health Perspect* 109(suppl 2):283–289 (2001).

<http://ehpnet1.niehs.nih.gov/docs/2001/suppl-2/283-289merrill/abstract.html>

Fumonisin is a category of toxic and carcinogenic mycotoxins that bear a remarkable structural similarity to sphingolipids (1,2). Many of the fumonisins, particularly of the B series such as fumonisin B₁ (FB₁), are potent inhibitors of ceramide synthase. This disruption of sphingolipid metabolism is a major contributor to the toxicity and carcinogenicity of FB₁, as described in an accompanying review (1). In this present article we summarize information about basic sphingolipidology that may be useful in understanding fumonisin action.

Sphingolipid Structures

Sphingolipids are defined by their characteristic 1,3-dihydroxy, 2-aminoalkane (sphingoid base) backbones (Figure 1) (2). Sphingosine is the prevalent backbone of mammalian sphingolipids and is sometimes used as a generic term for all sphingoid bases. However, sphingosine most often refers specifically to D-erythro-1,3-dihydroxy, 2-aminooctadec-4-ene or *trans*-4-sphinganine (abbreviated d18:1, for a dihydroxy-, 18-carbon sphingoid base with 1 double bond). Sphingoid bases vary in alkyl chain length, position (and number) of double bonds, and other functional groups such as a hydroxyl at position 4 (3). Complex sphingolipids (Figure 2) have a fatty acid attached in amide linkage (forming ceramides) and a polar headgroup (2). The fatty acids vary in chain length, degree of unsaturation (most are saturated), and presence or absence of a

hydroxyl group on the α or ω carbon atom. The headgroups range in complexity from simple phosphodiester to complex carbohydrates (Figure 2).

Mammalian sphingolipids (sphingomyelins, cerebrosides, globosides, gangliosides, sulfatides, etc.) have primarily sphingosine (d18:1^{A4}), sphinganine (d18:0) and 4-hydroxysphinganine (t18:0) as the sphingoid base backbones; amide-linked fatty acids of 16–30 carbon atoms in length, some (e.g., intestinal galactosylceramide) with an α -hydroxyl group and others (e.g., skin ceramides) with an ω -hydroxyl group; and a variety of headgroups (phosphocholine, glucose [Glc], galactose [Gal], *N*-acetylglucosamine, *N*-acetylgalactosamine, *N*-acetylneuraminic acid, fucose, and other carbohydrates) (2,4). In contrast, the complex sphingolipids of plants are mainly cerebrosides (mono- and oligohexosylceramides), with Glc (the most common hexose), Gal, mannose, and inositol (5). Fungi, yeast, and various microorganisms (but few prokaryotes) also have substantial amounts of sphingolipids (6,7). Additional structural substitutions include covalent attachment of sphingolipids to membrane proteins (8).

Functions of Complex Sphingolipids

Sphingolipids are located in cellular membranes, lipoproteins (especially low-density

lipoproteins), and other lipid-rich structures. Complex sphingolipids are critical for the maintenance of membrane structure (especially microdomains such as caveolae) (9,10), serve as binding sites for extracellular matrix proteins as well as for some microorganisms, microbial toxins, and viruses (11,12), and modulate the behavior of growth factor receptors (9). Complex sphingolipids also function as precursors for second messengers that mediate cell responses to growth factors, cytokines, differentiation factors, 1 α ,25-dihydroxy-vitamin D₃, and a growing list of agonists (including stress and toxic insults such as γ radiation) (12–16).

This signaling function of sphingolipids is illustrated in Figure 3. As shown, some agonists, such as platelet-derived growth factor (PDGF), activate a panel of enzymes that hydrolyze sphingomyelin to ceramide (sphingomyelinases), ceramide to sphingosine (ceramidases), and sphingosine to sphingosine 1-phosphate (sphingosine kinases) (17). Each of these intermediates is a bioactive compound that can affect protein kinases, phosphoprotein phosphatases, and other cell regulatory pathways. Sphingosine 1-phosphate is an additionally intriguing compound because it has both extracellular and intracellular functions (16).

Other agonists, such as tumor necrosis factor- α and interleukin-1 β , usually activate only sphingomyelinase, which results in ceramide accumulation (13,14). These distinctions are not universal, even within a single cell type, because interleukin-1 β can activate the entire pathway (sphingomyelinase through sphingosine kinase) at low concentrations but only sphingomyelinase (with inhibition of ceramidase) at high concentrations in rat hepatocytes (17). The subtle regulation of these steps is clearly important because the products have such profound effects on cell behavior: ceramide and sphingosine are usually growth inhibitory and cytotoxic (often via apoptosis) (13,14,18–21), whereas sphingosine

This article is based on a presentation at the International Conference on the Toxicology of Fumonisin held 28–30 June 1999 in Arlington, Virginia, USA.

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Received 10 April 2000; accepted 18 July 2000.

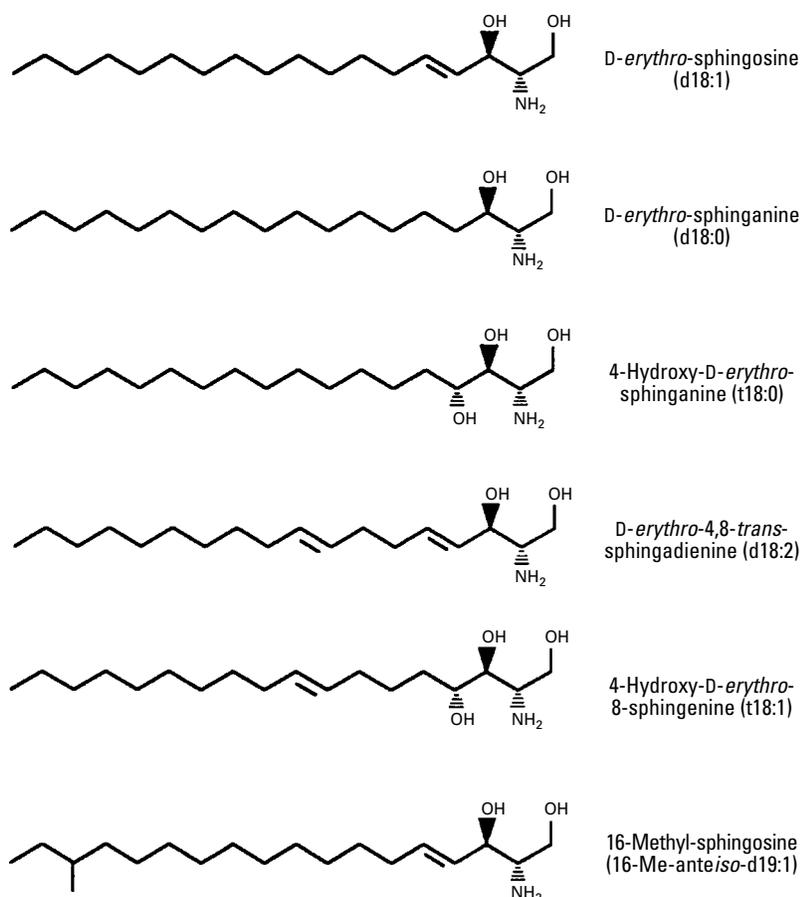


Figure 1. Structures of long-chain (sphingoid) bases from various sources. The common name and abbreviation for each sphingoid base are given. The latter is based on the number of hydroxyl groups (d, two; t, three), the number of carbon atoms (e.g., 18) and double bonds (0, 1, or 2).

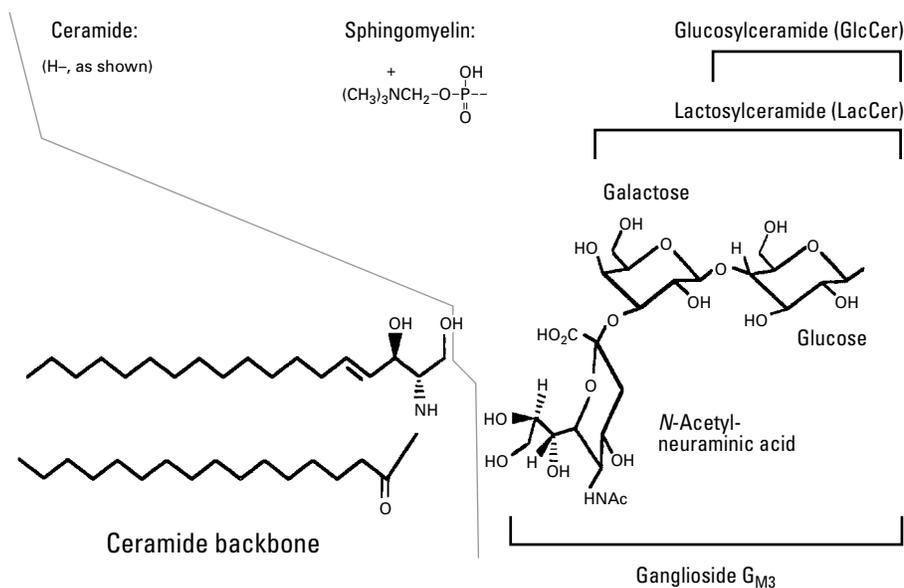


Figure 2. Representative complex sphingolipids. Shown is a ceramide (*N*-palmitoylsphingosine) and the headgroups for sphingomyelin and three glycosphingolipids.

1-phosphate is a potent mitogen and an inhibitor of apoptosis (16,22).

Sphingolipid Biosynthesis in Cell Regulation

The intermediates of *de novo* sphingolipid biosynthesis (sphingosine, dihydroceramides, and ceramides) (Figure 4) are also highly bioactive, and under normal conditions, the amounts of these compounds are kept low (23). However, various forms of cell stress can induce *de novo* sphingolipid synthesis and perturb cell behavior due to increases in these compounds (24–27). The best characterized among these insults are the fumonisins, which inhibit ceramide synthase, the enzyme that acylates sphingoid bases (sphinganine from *de novo* synthesis and sphingosine from sphingolipid turnover), as illustrated in Figure 5. Inhibition of ceramide synthase causes sphinganine to accumulate, as shown, and sometimes increases sphingosine; the latter usually occurs later, when there is sufficient cell injury to trigger membrane degradation (1,28–32).

Inhibition of Ceramide Synthase by Fumonisins

The nature of the inhibition of ceramide synthase by FB₁ is consistent with the model shown in Figure 6 (33). That is, FB₁ appears to interact with the binding sites for sphinganine and fatty acyl-coenzyme A (CoA) because its potency is influenced by the concentrations of both substrates (34). This model is further supported by the finding (35) that removal of the tricarballic acid sidechain decreases the potency of ceramide synthase inhibition *in vitro* by approximately 10-fold. Moreover, FB₁ is not acylated by ceramide synthase, but removal of the tricarballic acid sidechains (producing the aminopentol or AP₁) converts this inhibitor into a substrate, as indicated in Figure 7 (36). This is only possible if AP₁ occupies the sphingoid-base binding site and leaves the fatty acyl-CoA binding site accessible.

The product of this reaction (which we have abbreviated PAP₁ for *N*-palmitoyl-AP₁) has interesting properties. It is also an inhibitor of ceramide synthase *in vitro* and causes a greater increase in sphinganine in HT-29 cells than FB₁ or AP₁ causes at comparable concentrations (Figure 8, right panel) (36). Although the mechanism for the inhibition of ceramide synthase by PAP₁ is not known, the simplest explanation is that PAP₁ interacts with the hydrophobic binding domains for the substrates and product (compare Figures 6 and 7). As would be predicted from the increase in sphinganine, PAP₁ is more toxic than FB₁ or AP₁ for HT-29 cells (Figure 8, left panel) (36).

Together, these findings suggest that when AP₁ is taken up by cells, it can be converted to a more potent (toxic) inhibitor of ceramide synthase. This may account for the previously puzzling observation that although AP₁ per se is much less inhibitory for ceramide synthase than FB₁, feeding AP₁ to rats causes lesions in liver and kidney that are indistinguishable from those caused by FB₁ (21). In addition, the organ-specific effects of feeding nixtamalized *Fusarium moniliforme* culture material (which contains AP₁) to rats are similar to those of the diet prepared from untreated (FB₁-containing) culture material (38). AP₁ also appears to have the same liver cancer-promoting activity as FB₁ (39).

The toxicity of AP₁ warrants further investigation because nixtamalization of maize is a common practice in some regions, such as Central and South America, and as much as 185 µg AP₁ has been found per gram of tortillas (40). If significant amounts of PAP₁ are formed (and play a role in the toxic effects of AP₁), it might be easy to detect this compound because it is more hydrophobic than other fumonisins and might accumulate in fatty tissues.

Reversibility of Inhibition of Ceramide Synthase

Inhibition of ceramide synthase is reversed upon dilution of the inhibitor from *in vitro* assays, removal of FB₁ from the medium of cells in culture, and changing animals from fumonisin-contaminated to fumonisin-free feed (32,41,42). In studies with intact cells, the inhibition of ceramide synthase has been evaluated indirectly by the amounts of sphinganine in the cells, culture medium, or biologic fluids (blood and urine). Because FB₁ appears to inhibit ceramide synthase through noncovalent interactions, such reversibility would be predicted upon reduction of the concentration of FB₁ in the cytosol. Other factors that probably affect reversibility are cellular concentrations of the substrates for ceramide synthase and rate of removal of the accumulated sphinganine and sphingosine, as discussed in Riley et al. (1).

In vivo reversibility is illustrated in Figure 9 for rats fed 10 µg FB₁/g feed and then changed to diets containing 0, 1, or 10 µg FB₁/g (42). Urinary sphinganine returned to basal levels within 10 days when the rats were changed to 0 µg of FB₁/g; however, if they were changed to 1 µg FB₁/g (an amount that alone did not elevate sphinganine), urinary sphinganine remained elevated (Figure 9). This finding raises the possibility that amounts of FB₁ that are not toxic when consumed alone (e.g., 1 µg of FB₁/g) may be more damaging if there is occasional consumption of higher amounts (e.g., 10 µg/g).

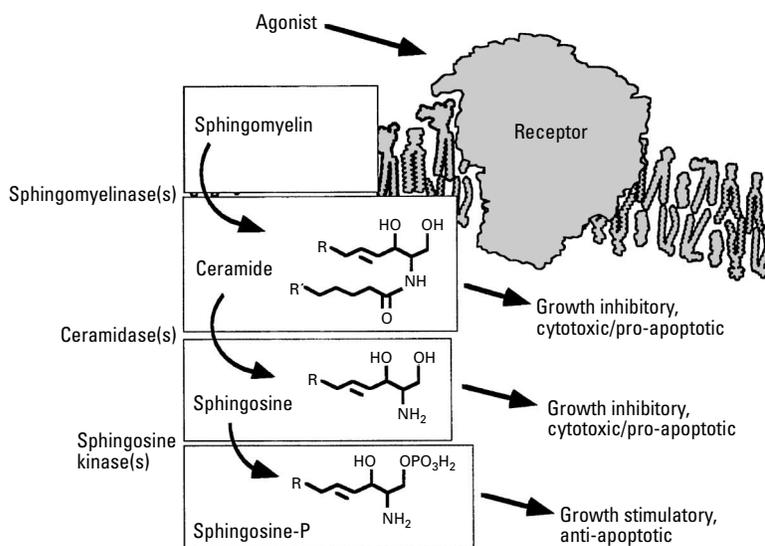


Figure 3. Schematic representation of sphingolipid turnover in response to agonists, and the types of responses that can be achieved depending on the enzymes that are activated. For example, a growth factor may activate all the shown enzymes, whereas a growth inhibitory (or pro-apoptotic) factor may activate only sphingomyelinase and ceramidase.

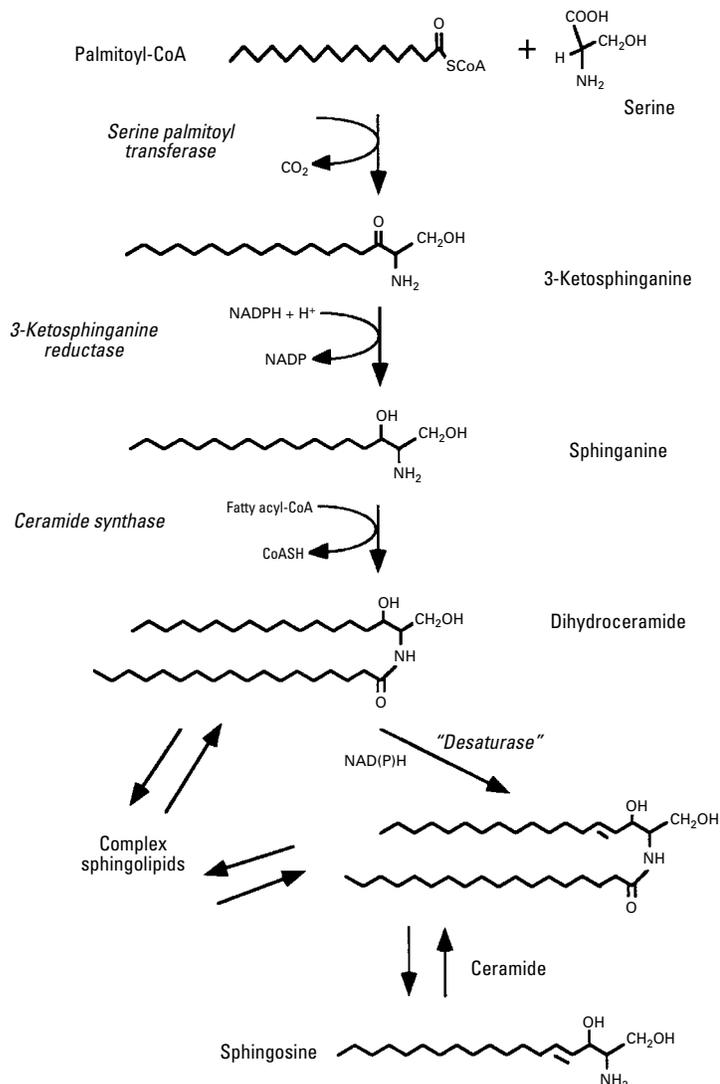


Figure 4. The *de novo* biosynthetic pathway for the ceramide backbone of complex sphingolipids. Note that sphingosine is not a direct intermediate of the pathway but is formed only after turnover of ceramide.

There have been no systematic studies of the toxicity of fumonisins when consumed in this manner.

Elevations in Sphingoid Bases Also Increase Formation of Other Metabolites

Inhibition of ceramide synthase not only results in accumulation of sphinganine, but it also changes in other lipids that have important cell functions, as summarized in Figure 10. These include:

Depletion of More Complex Sphingolipids

Fumonisin can completely block synthesis of new sphingolipids and deplete the total mass of cellular sphingolipids (1,28–30,34,43). However, the magnitude of these responses depends on many factors, such as whether the cells are growing or are confluent (effects are usually greatest in growing cells) (37) and the rate of sphingolipid turnover. For example, in mouse cerebellar neurons, *de novo* synthesis is

blocked completely, but there is little change in total sphingolipids for several days because turnover is slow (34).

Changes in the amounts of cellular sphingolipids can disrupt cell functions dependent on complex sphingolipids. For example, FB₁ treatment of intestinal cells in culture blocks folate uptake because the folate transporter is a glycosylphosphatidylinositol-anchored protein, which typically requires sphingolipids and cholesterol to function normally (44). One would predict that depletion of complex glycolipids would alter the behavior of growth-factor receptors because these proteins are often modulated by gangliosides (4,9,10). Accumulation of sphingoid bases also perturbs membrane structure and has been shown to inhibit protein kinase C (45).

Accumulation of Sphingoid Base 1-Phosphates and Downstream Metabolites (e.g., Fatty Aldehydes, Ethanolamine Phosphate)

Sphingoid bases are catabolized by phosphorylation and lytic cleavage to a fatty aldehyde and ethanolamine phosphate (2). FB₁ increases the amounts of cellular sphingoid base 1-phosphates (highly bioactive compounds, as discussed earlier in this review) and increases the amount of sphingolipid-derived ethanolamine phosphate incorporated into phosphatidylethanolamine (46). Perturbation of fatty acid metabolism would be predicted because of the accumulation of fatty aldehydes as well as very long-chain fatty acids (a large portion of which is found in sphingolipids). However, as far as we are aware, this has not been studied in mammalian systems.

Alteration of Other Lipid Metabolic Pathways

Phosphatidic acid phosphatase is one of the enzymes of other lipid pathways and is highly sensitive to cellular amounts of free sphingoid bases (47–49); FB₁ has been shown to alter this and other pathway(s) in yeast (50). This enzyme is a key component of cell signaling via the phospholipase D, phosphatidic acid, and diacylglycerol pathway, but it is not the only enzyme in this pathway that is affected. Sphingoid bases (51,52) and their 1-phosphates (53) can also activate phospholipase D. Additionally, an enzyme of diacylglycerol synthesis (monoacylglycerol acyltransferase) is inhibited by sphingoid bases (54). Cholesterol metabolism would also be expected to be altered by disruption of sphingolipid metabolism because of the interrelationships between these pathways (55).

Formation of *N*-Acetyl Derivatives of Sphingoid Bases (C₂-Ceramides)

We recently addressed the hypothesis that accumulation of free sphingoid bases leads to

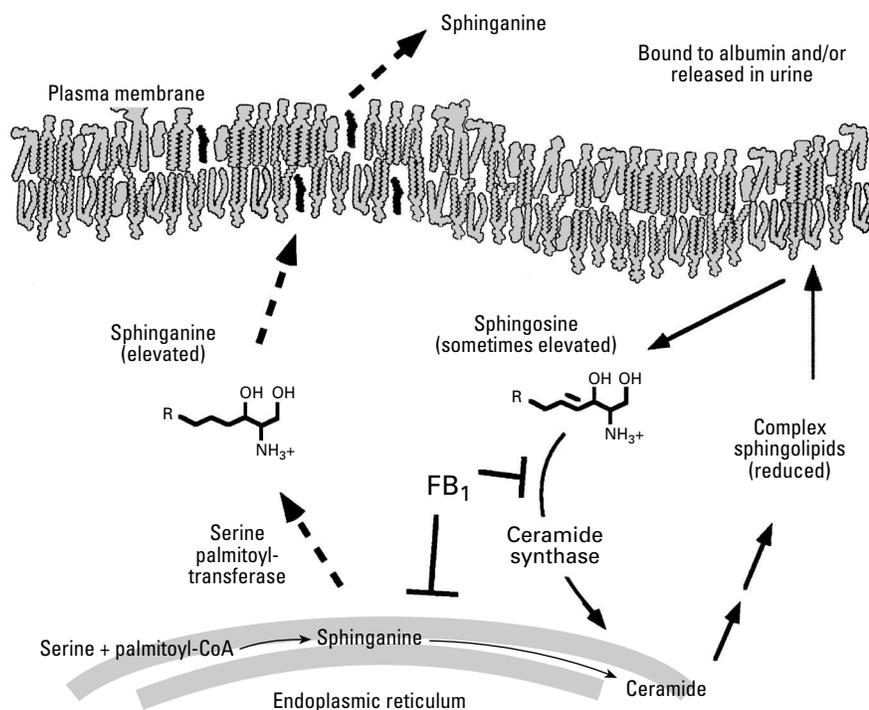


Figure 5. A schematic illustration of the inhibition of the ceramide synthase by FB₁ and the consequences for acylation of sphinganine (from *de novo* biosynthesis pathway) and sphingosine (from sphingolipid turnover) and/or complex sphingolipid formation.

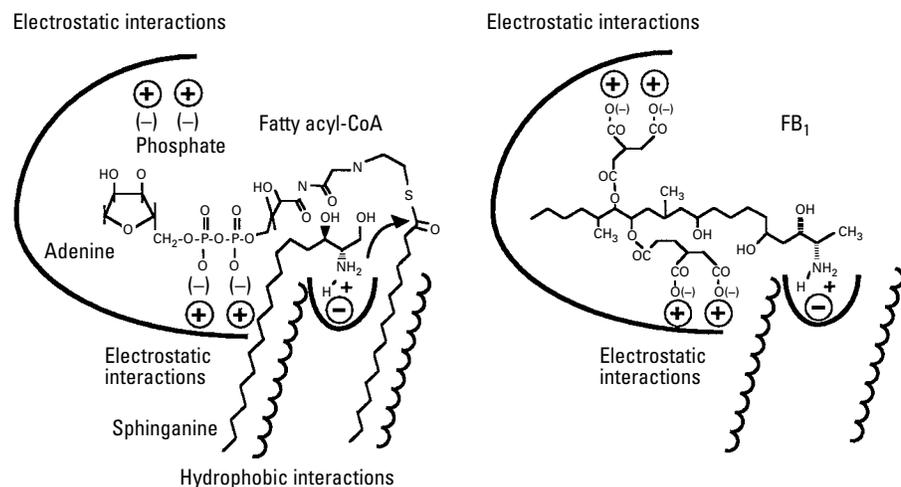


Figure 6. A model for the active site of ceramide synthase and how FB₁ mimics regions of both the sphingoid base and the fatty acyl-CoA substrates. The likely contact points between ceramide synthase and substrates/inhibitors are depicted by the dark lines.

their metabolism by the *N*-acetyltransferases that participate in xenobiotic metabolism (56) or a transacetylase that transfers the acetyl group from platelet-activating factor to sphingosine (57). C_2 -ceramides (*N*-acetyl-sphingosine and *N*-acetylsphinganine) were measured in livers from rats fed a fumonisin-free diet and rats fed 150 μg FB_1 per gram of diet. As shown in Figure 11, control livers contained approximately 0.6 nmol *N*-acetyl-sphingosine/g and 0.3 nmol of *N*-acetylsphinganine. FB_1 feeding had no effect on the amount of *N*-acetylsphingosine, but increased *N*-acetylsphinganine by 4-fold.

More studies are needed to determine if elevations in *N*-acetylsphinganine, and in some cases, *N*-acetylsphingosines, are common in animals exposed to fumonisins. If confirmed, C_2 -ceramides may be useful as biomarkers for long-term exposure to fumonisins (vs sphingoid bases, which reflect recent exposure), as they are lipophilic and are likely to be stored in fatty tissue.

Additional Issues That Should Be Borne in Mind

Most of the studies to date using cells in culture or animals have shown a good correlation between exposure to fumonisins and accumulation of sphinganine, often expressed as the sphinganine:sphingosine ratio (1). This ratio is often used because endogenous sphingosine can serve as an internal standard to correct for losses of sphingoid bases during lipid extraction, derivitization, and analysis by high-performance liquid chromatography. However, there are circumstances where sphingosine is also elevated, for example, when there is extensive cell death and sphingolipid turnover; hence, the amounts of both of these sphingoid bases, as well as the ratio, should be considered.

This correlation alone is not proof of a cause-and-effect relationship between disruption of sphingolipid metabolism and the toxicity and carcinogenicity of fumonisins. The best evidence that such a relationship exists is that inhibition of serine palmitoyl-transferase—thereby blocking sphinganine formation at an earlier step of sphingolipid biosynthesis—reverses the toxicity of fumonisins, at least temporarily (37,43,58). Nonetheless, based on the diversity of the pathologic effects of fumonisins, one suspects that they are due to a combination of accumulations of sphingoid bases, reductions in key complex sphingolipids, and possibly interactions with additional targets.

A few studies have concluded that changes in cell behavior without (or before) increases in sphinganine prove that disruption of sphingolipid metabolism is not involved in the action of fumonisins. This interpretation cannot be made without an in-depth analysis

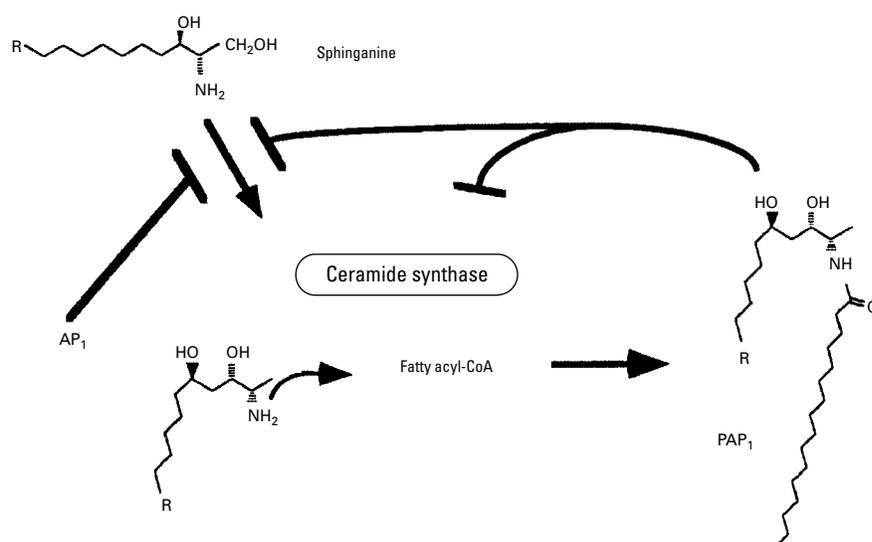


Figure 7. A depiction of the triple effects of AP_1 : inhibition of the acylation of sphinganine by AP_1 , acylation of AP_1 , and inhibition of ceramide synthase by the product PAP_1 .

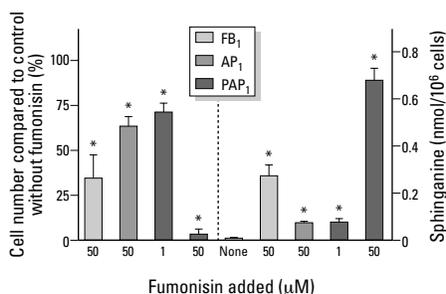


Figure 8. Comparison of the effects of FB_1 , AP_1 , and PAP_1 on HT-29 cells. Cells were incubated with the shown concentrations of fumonisins for 24 hr, then the number of viable cells were counted using a hemocytometer and expressed as the percentage of the matched control (left panel) and the amounts of sphinganine were determined (right panel). Results are shown as mean \pm SE ($n = 3$); groups significantly different from the control are designated by an asterisk (*). The data in this figure were compiled from references Humpf et al. (36) and Schmelz et al. (37).

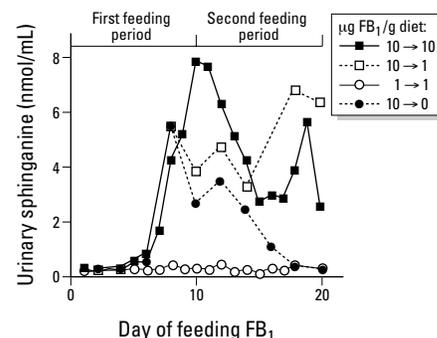


Figure 9. Reversibility of elevations in urinary sphingosine upon ceramide synthase inhibition by FB_1 *in vivo*. Rats were fed 1 or 10 μg FB_1 per gram of diet for 10 days, then one group on 10 μg FB_1 per gram was changed to 0 μg FB_1 per gram, and another was changed to 1 μg FB_1 per gram. Data redrawn from reference Wang et al. (42).

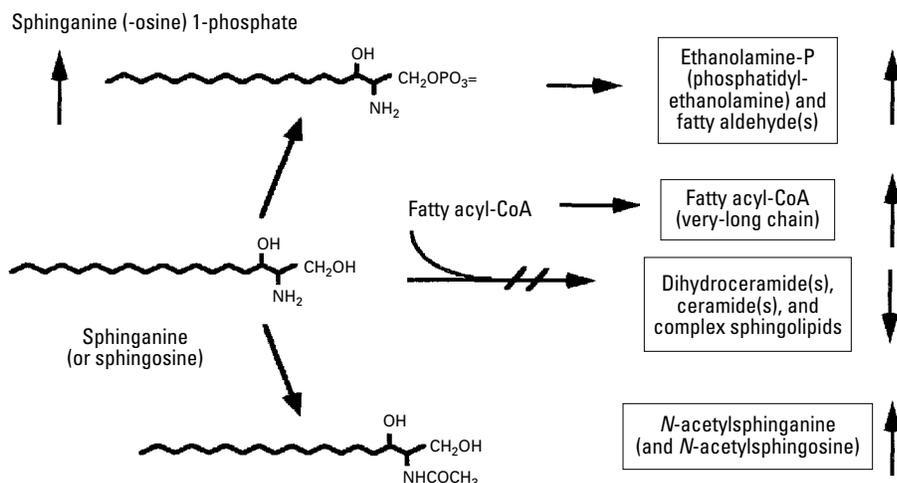


Figure 10. Summary of other (sphingo) lipid metabolites that are increased (up arrow) or decreased (down arrow) when ceramide synthase is inhibited by fumonisins.

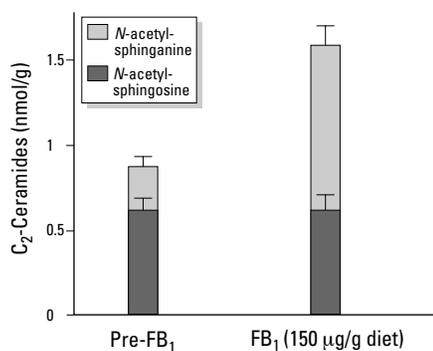


Figure 11. Amounts of C₂ ceramides in rat liver before and after consumption of 150 µg FB₁ per gram. The C₂ ceramides (*N*-acetylsphingosine and *N*-acetylsphinganine) were determined in lipid extracts of rat liver by separating the sphingolipids by thin-layer chromatography, extraction of the region that comigrates with synthetic C₂ ceramide standards, and quantitation of the sphingoid base backbones by high-performance liquid chromatography after acid hydrolysis (42).

of other sphingolipid metabolites because, as discussed in this review, free sphingoid bases can be metabolized to other bioactive metabolites. The situation may be even more complex *in vivo* because sphingoid bases produced in one tissue may be transported to another (such as liver and kidney). This might account for the hepatotoxicity and nephrotoxicity of fumonisins, as these organs are probably both sources of sphingoid bases and recipients of sphingoid bases produced elsewhere in the body.

Another complicating factor that has received little attention is the possibility that other components of the diet might alter the toxicity of fumonisins. There is considerable variation in the amounts of sphingolipids in food (55), as well as in the amounts of precursors for *de novo* sphingolipid biosynthesis (46,59); therefore, the impact of fumonisins might be greatest when these other components of the diet increase the amounts of sphingoid bases that accumulate.

Fumonisin may have *in vivo* effects in addition to disruption of sphingolipid metabolism; however, it is most probable that direct or secondary changes in cell-signaling pathways that involve sphingolipids are major contributors to the toxicity and carcinogenicity of these mycotoxins. Sorting through these complex interrelationships to identify the most important remains a daunting challenge.

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