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MOLECULAR MECHANISM OF FUMONISIN-INDUCED KIDNEY TOXICITY

By

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ABSTRACT

MOLECULAR MECHANISM OF FUMONISIN-INDUCED KIDNEY TOXICITY

By

Min Sun Kim

Fumonisin B_1 is a toxic and carcinogenic mycotoxin with renal tubular epithelial cells being the most sensitive target. Due to structural similarities with sphingoid bases, fumonisin B_1 inhibits ceramide synthase in the *de novo* biosynthetic pathway of sphingolipids causing depletion of complex sphingolipids and accumulation of sphingoid bases. The goals of this research were to use LLC-PK_1 renal tubular epithelial cells to: 1) Determine whether fumonisin B_1 kills cells by inducing apoptosis and evaluate the role of disruption of sphingolipid metabolism in fumonisin-induced cell death; 2) Identify genes affected by fumonisin B_1 and investigate the molecular mechanism whereby fumonisin B_1 induces gene expression; and 3) Determine whether fumonisin B_1 affects signaling pathways involved in cell proliferation and cell death.

Fumonisin B_1 produced morphological changes and time-dependent increases in fragmented DNA indicative of apoptosis within 16 hours and simultaneously caused accumulation of sphinganine. To investigate the role of sphinganine in fumonisin B_1-induced apoptosis, β-fluoroalanine (βFA) was used to inhibit serine palmitoyltransferase, which catalyzes an earlier step in the sphingolipid biosynthetic pathway. βFA blocked sphinganine accumulation and prevented fumonisin B_1-induced DNA fragmentation, confirming that fumonisin B_1-induced apoptosis depends on accumulation of sphinganine.

Fumonisin B_1 selectively induced several genes including calmodulin; whereas, the mycotoxin did not affect the *bcl-2* family genes *bcl-2*, *bcl-x*, and *bax*. Fumonisin B_1

increased both calmodulin mRNA and protein in concentration-dependent manners, and the calmodulin antagonist W7 blocked fumonisin B_1-induced DNA fragmentation, supporting a role for calmodulin in fumonisin B_1-induced apoptosis. Fumonisin B_1 did not influence stability of calmodulin mRNA and appears to induce calmodulin by increasing transcription. βFA added together with fumonisin B_1 blocked sphinganine accumulation and blunted induction of calmodulin, suggesting a role for sphinganine or the 1-phosphate metabolite. Exogenous addition of sphinganine had no effect on calmodulin expression, but addition of the 1-phosphate significantly increased calmodulin mRNA, indicating sphinganine 1-phosphate mediates fumonisin B_1 induction of calmodulin.

To examine signaling pathways involved in fumonisin-induced cell death, LLC-PK_1 cells were cultured with either fumonisin B_1 or sphinganine. Exogenously added D-*erythro*-sphinganine acted more rapidly than fumonisin B_1, inhibiting growth and causing cell death. Both fumonisin B_1 and sphinganine reduced phosphorylation of MEK and ERK without affecting phosphorylation of Raf. Fumonisin B_1 and sphinganine also decreased phosphorylation of Akt (protein kinase B). Furthermore, addition of βFA together with fumonisin B_1 blocked fumonisin-induced dephosphorylation, suggesting that sphinganine mediates the effect of fumonisin B_1 on phosphorylation of MEK, ERK, and Akt proteins.

Taken together, fumonisin B_1 appears to cause kidney toxicity by disrupting sphingolipid metabolism and causing accumulation of sphinganine and sphinganine 1-phosphate. These sphingolipid metabolites induce selected genes including calmodulin, and alter signaling through cell proliferation and cell death pathways leading to renal tubular epithelial cell apoptosis

This dissertation is dedicated to my devoted parents for their love and encouragement.

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ABBREVIATIONS

Abbreviations	Meaning
μg	Microgram
AGP	α_1-acid glycoprotein
BSA	Bovine serum albumin
Ca	Calcium
CAPP	Ceramide-activated protein phosphatase
CYP2C11	Cytochrome P450 2C11
CytoC	Cytochrome C
EDTA	Ethylenediamine tetraacetic acid
ERK	Extracelluar receptor activated kinase
FADD	Fas-associated death domain
FBS	Fetal bovine serum
g	Gram
G3PDH	Glyceraldehyde 3-phosphate dehydrogenase
hrs	Hours
IκB	Inhibitory κ B
IL	Interleukin
iv	Intravenous
kg	Kilogram
MAPK	Mitogen-activated protein kinase
MEK	MAPK kinase
mg	Milligram
min	Minute
ml	Milliliter
NFκB	Nuclear factor κ B
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PI3K	Phosphatidylinositol 3-kinase
PKB	Protein kinase B
PKC	Protein kinase C
pmol	Picomole
RT	Reverse transcriptase
ser	Serine
SK	Sphingosine kinase
thr	Threonine
TNF	Tumor necrosis factor

INTRODUCTION

Fumonisins are toxic and carcinogenic mycotoxins produced by *Fusarium moniliforme*, the most common mold found on corn throughout the world (Marasas, 1996). Fumonisins cause animal diseases (Wilson *et al.*, 1990; Harrison *et al.*, 1990) and have been associated with esophageal (Marasas *et al.*, 1988), liver (Ueno *et al.*, 1997) and stomach (Groves *et al.*, 1999) cancers in humans.

The mechanism of action of fumonisins is not clear, but appears to involve disruption of sphingolipid metabolism. Due to the structural similarity with the sphingoid base backbones of sphingolipids, fumonisins inhibit ceramide synthase (sphingoid *N*-acyltransferase) which acylates sphinganine to form ceramide in the *de novo* biosynthetic pathway of sphingolipids. Inhibition by fumonisins results in accumulation of sphinganine and depletion of complex sphingolipids (Wang *et al.*, 1991; Merrill *et al.*, 1993a). Complex sphingolipids mediate cell-cell, cell-substratum, and cell-microbial interactions and serve as substrates for agonist-induced cell signaling; while, sphingolipid metabolites serve as bioactive molecules which affect cellular behavior by acting as second messengers for many growth factors and cytokines. Sphingoid bases, which include sphinganine, have been shown to arrest cell growth and induce apoptosis, early responses in tissues exposed to fumonisins (Voss *et al.*, 2001). Apoptosis may mediate fumonisin-induced toxicities in liver and kidney, the latter being the most sensitive organ (Bondy *et al.*, 1996; Bucci *et al.*, 1998).

Sphingoid bases modulate expression of *bcl-2* family genes (Sakakura *et al.*, 1996; Shirahama *et al.*, 1997) and decrease activity of MAPK (Raeder *et al.*, 1999) to suppress cell proliferation and induce apoptosis, suggesting that these pathways may be affected by fumonisins. In addition, the PI3K/Akt pathway is a probable target of fumonisins because ceramide (Schubert *et al.*, 2000) and sphingosine (Chang *et al.*, 2001) inactivate Akt to induce apoptosis.

In the present study, LLC-PK$_1$ porcine renal tubular epithelial cells were used as a model system to examine the type of cell death, the role of disruption of sphingolipid metabolism and effects on gene expression and cell signaling in the molecular mechanism of fumonisin-induced kidney toxicity.

CHAPTER I

LITERATURE REVIEW

A. Fumonisins

A. 1. Introduction

First discovered in 1988 (Gelderblom *et al.*, 1988), the fumonisins are a family of cytotoxic and carcinogenic mycotoxins produced by *Fusarium moniliforme* and other related fungi which are common contaminants of corn and other agricultural commodities occurring in the field and during storage in the United States and throughout the world (Marasas, 1996). More than ten types of fumonisins have been isolated and characterized with the 'B' series representing the most common of these mycotoxins. Fumonisin B_1 is the most prevalent form and has been shown to cause all of the animal diseases associated with consumption of *F. moniliforme* culture materials (Riley *et al.*, 1993b); whereas, fumonisin B_2 which is less prevalent exhibited a higher toxicity and more specific binding to rat hepatocytes than fumonisin B_1 (Cawood *et al.*, 1994).

The primary concern about fumonisins is due to the prevalence of *Fusarium moniliforme* as a common contaminant of corn. Naturally occurring fumonisins have been documented in corn (Sydenham *et al.*, 1990) and corn-based animal feeds (Plattner *et al.*, 1991). The extent of contamination of raw corn with fumonisins varies with geographic location, agronomic and storage practices, and the vulnerability of the plants to fungal invasion during all phases of growth, storage, and processing. High concentrations of fumonisins are associated with hot and dry weather followed by periods of high humidity (Shelby *et al.*, 1994). Numerous studies have reported that fumonisins naturally contaminate corn at concentrations ranging from <1 to 330 μg/g for fumonisin B_1 and concentrations ranging from <1 to 48 μg/g for fumonisin B_2 which were associated with outbreaks of porcine pulmonary edema in the United States from 1989 to 1990 (Colvin and Harrison, 1992; Haschek *et al.*, 1992; Osweiler *et al.*, 1992; Ross *et al.*, 1992; Ross *et al.*, 1991). Furthermore, recent analyses of food-grade corn and corn-based food products of United States origin for human consumption showed that 71% of the samples contained fumonisin B_1, ranging from 43 to 1642 µg/kg (Gutema *et al.*, 2000). The results from this report suggest that United States consumers are at risk for fumonisin-induced health problems.

A. 2. Toxicity

Fumonisins are associated with a variety of adverse health effects in livestock animals. Incidence of leukoencephalomalacia in horses has been linked with mold-contaminated feeds (Wilson *et al.*, 1990) and was reproduced by feeding a horse naturally contaminated corn screenings (Marasas *et al.*, 1989a) and by orally dosing fumonisin B_1 (Kellerman *et al.*, 1990). Fumonisin B_1 also causes pulmonary edema in pigs (Harrison *et al.*, 1990; Osweiler *et al.*, 1992). Pulmonary edema which was preceded by respiratory distress was induced in pigs fed corn screenings containing 155 mg/kg fumonisin B_1 (Harrison *et al.*, 1990; Motelin *et al.*, 1994) and in one pig by intravenous injection of 11.3 mg fumonisin B_1 (Colvin and Harrison, 1992). In addition to lung, both liver and kidney are target organs of fumonisin B_1 in swine (Haschek *et al.*, 1992).

Fumonisins also are toxic for laboratory animals. Progressive toxic hepatitis characterized by hepatocellular necrosis, bile duct proliferation and fibrosis was observed in rats treated with fumonisin B_1 in short-term toxicity tests (Gelderblom *et al.*, 1991). The chromatin of exposed cells is irregularly condensed and marginated or may be fragmented, a common feature of apoptotic cells. Also, necrotic hepatocytes are present hours after exposure to the mycotoxin. Serum chemical indications of hepatocellular injury, including increased alanine and aspartate transaminase, alkaline phosphatase, and lactate dehydrogenase activities as well as increased cholesterol and triglyceride concentrations are routine, early findings (Voss *et al.*, 2001).

In addition, fumonisin-induced kidney toxicity was reported in rats (Bondy *et al.*, 1996), lambs (Edrington *et al.*, 1995), and rabbits (Bucci *et al.*, 1998). Whereas hepatotoxicity was observed with a diet containing 150 mg/kg, renal toxicity was observed even at concentrations as low as 15-50 mg/kg of fumonisin B_1 (Voss *et al.*, 1993), suggesting that kidney is more sensitive to fumonisin-induced toxicity than liver (Bondy *et al.*, 1996; Bucci *et al.*, 1998; Voss *et al.*, 2001). In Sprague-Dawley and Fischer 344 rats fed fumonisin B_1, males were more sensitive than females (Voss *et al.*, 2001). As in liver, apoptosis is the initial microscopic finding in the kidney and apoptotic cells are initially found exclusively in tubules of the outer medulla. Mitotic figures appear and the number of apoptotic cells increases in the tubule epithelium as tissue injury progresses (Voss *et al.*, 2001). It should be noted that simultaneous cell loss and replacement has been observed including apoptosis and mitosis at the cellular level, tubular atrophy and hyperplasia on the histologic level, and grossly by decreased kidney weight (Voss *et al.*, 2001). The replacement or regeneration process accompanying apoptosis may mediate, at least in part, fumonisin-induced carcinogenicity.

A. 3. Carcinogenicity

Fumonisin B_1 is carcinogenic in animals. This mycotoxin causes primary hepatocellular carcinoma and cholangiocarcinoma in rats at a dietary concentration of 50 mg/kg fed for 18-26 months (Gelderblom *et al.*, 1991). A recent carcinogenicity study by the National Toxicology Program demonstrated that fumonisin B_1 induces renal tubule tumors in male F344 rats and hepatic tumors in female B6C3F mice (Howard *et al.*, 2001).

Fumonisin B_1 may affect human health in certain areas of the world where corn is a staple of the diet. Epidemiological studies suggest that there is a strong correlationship between consumption of fumonisin B_1 and the incidence of human esophageal cancer in certain areas of South Africa (Marasas *et al.*, 1988b) and liver and stomach cancers in China (Ueno *et al.*, 1997; Groves *et al.*, 1999). The high incidence of esophageal cancer in the Transkei region of South Africa was associated with the consumption of beer brewed with moldy corn and a nonalcoholic fermented drink made with corn (Rose, 1981). In a sample of home-grown corn from a high incidence area of esophageal cancer in the Transkei, visibly healthy kernels contained 44 μg/g fumonisin B_1 and *Fusarium*-infected kernels contained 83 μg/g fumonisin B_1 (Marasas, 1996). Esophageal basal cell hyperplasia has been demonstrated in rats by feeding culture material of *F. moniliforme* (Marasas *et al.*, 1984), providing a partial explanation for the esophageal cancer epidemic in certain regions of the world.

The mechanism(s) by which fumonisins cause cancer is not clear. Fumonisins were neither mutagenic in the *Salmonella* mutagenicity assay (Gelderblom *et al.*, 1991) nor genotoxic in DNA repair assays using primary rat hepatocytes (Norred *et al.*, 1992). Fumonisin B_1 was found to be a weak cancer-initiating agent when rats were fed fumonisins at a level of 100 mg/kg diet for 21 days, for which the intact molecule and the presence of a free amino group was essential (Gelderblom *et al.*, 1993). In addition, fumonisin B_1 was demonstrated to be a strong cancer-promoter for rat liver when diethylnitrosamine was used as a cancer initiator (Gelderblom *et al.*, 1996b). Fumonisin B_1 exhibited a cancer-promoting effect in the absence of adverse hepatotoxicity and at dietary levels that did not show any sign of cancer-initiation. The carcinogenic effects of fumonisins may be due to their mitogenicity indicated by stimulated DNA synthesis as shown in Swiss 3T3 cells (Schroeder *et al.*, 1994). Recent advances in the molecular genetics of cancer suggest many nongenotoxic carcinogens may increase the risk of various types of genetic errors by stimulating cell division *per se* (Preston-Martin *et al.*, 1990). For example, cell division is necessary for conversion of adducts or other single stranded DNA damage to gaps or mutations (Ames and Gold, 1990; Cohen and Ellwein, 1990). In addition, cell division allows for mitotic recombinations that result in changes that are more profound than those from a single mutation (Ramal, 1988). Therefore, it is possible that fumonisins might promote tumor formation by stimulating cellular proliferation.

A. 4. Structure

Fumonisins are categorized as members of either the 'A', 'B', or 'C' series. The 'B' series is the most abundant and toxic of the fumonisins. Fumonisin B_1 is the diester of propane-1,2,3-tricarboxylic acid and 2-amino-12,16-dimethyl-3,5,10,14,15-pentahydroxyicosane (Figure 1.1). The propanetricarboxylic acid moieties are esterified at the C-14 and C-15 positions of the 20-carbon aminopentol backbone. Less is known about fumonisins in the 'A' and 'C' series. Fumonisins in the 'A' series have an amino group which has been acetylated, and they appear to be relatively innocuous (Gelderblom *et al.*, 1991). The 'C' series fumonisins lack the 1-methyl group. *Alternaria alternata lycopersici* (AAL) toxins are structurally related compounds and share similar biological activity with fumonisin B_1 (Van Der Westhuizen *et al.*, 1998; Wang *et al.*, 1996).

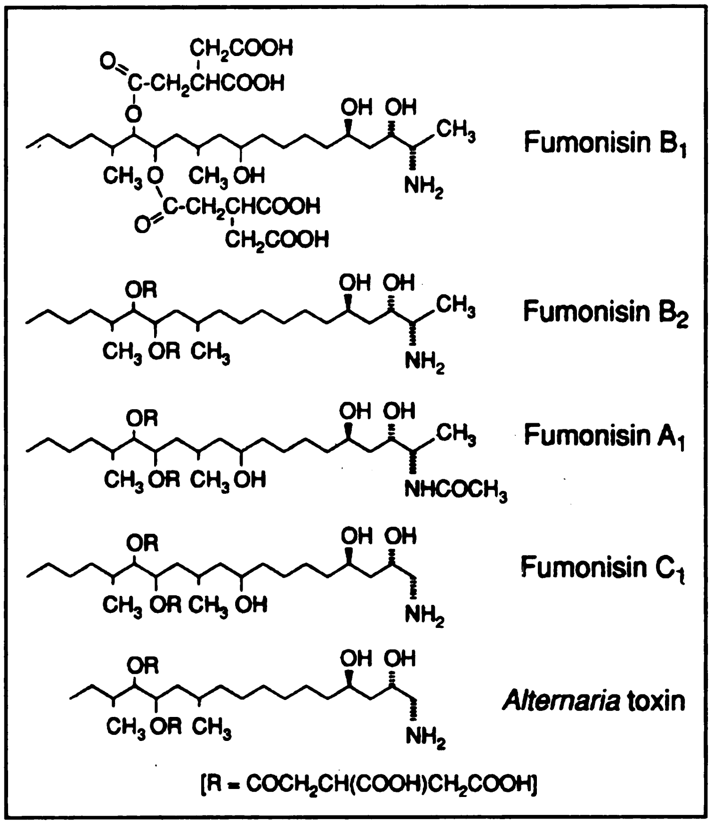


Figure 1.1. Structures of fumonisins and related mycotoxins.

A. 5. Toxicokinetics

To investigate the distribution and excretion of fumonisins, ^{14}C-labeled fumonisin B_1 was prepared by the addition of ^{14}C-methionine to a culture of *Fusarium moniliforme* and used in rats (Norred *et al.*, 1993; Shephard *et al.*, 1994a), swine (Prelusky *et al.*, 1994), and laying hens (Vudathala *et al.*, 1994). In rats, fumonisins appear to be absorbed poorly and excreted rapidly (Norred *et al.*, 1993; Shephard *et al.*, 1994a; Shephard *et al.*, 1995). In laying hens, fumonisin B_1 is poorly absorbed and is rapidly eliminated with the level of residues near negligible (Vudathala *et al.*, 1994). In swine, fumonisin B_1 is primarily excreted in feces (~58%) and to a lesser extent in urine (~20%) (Prelusky *et al.*, 1994). Fumonisin B_1 is rapidly cleared from plasma and showed wide distribution with residues in liver > kidney > large intestine > brain > lung, heart and adrenal gland (Shephard *et al.*, 1992; Prelusky *et al.*, 1994). In bile-cannulated pigs, bile recovery was ~71% with absence of γ phase, indicating enterohepatic circulation (Prelusky *et al.*, 1994). Results from both rat and pig studies indicate the possible enterohepatic circulation of fumonisin B_1. Thus, it is possible that the true bioavailability of fumonisin B_1 was underestimated because bile cannulation may have interrupted normal enteroheptatic circulation of fumonisin B_1 and increased fecal excretion of the mycotoxin. Importantly, fumonisin residues accumulated in liver and to a lesser extent in kidney when pigs were fed fumonisins at dietary concentrations of 2-3 ppm for 24 days (Prelusky *et al.*, 1996). Therefore, consumption of fumonisin-contaminated diets over extended periods may result in the accumulation of toxic residues in liver and kidney.

A. 6. Metabolism

Studies with ^{14}C-labeled fumonisin B_1 also showed that fumonisin B_1 is not metabolized by cytochrome P-450 monooxygenase or esterases, and is not a substrate for hepatic lipases (Cawood *et al.*, 1994). However, partially hydrolyzed (hydrolysis of one of the ester groups) fumonisin B_1 was identified by mass spectrometry and nuclear magnetic resonance spectroscopy (Shepherd *et al.*, 1994c). A study focused on the structure-activity relationships of the fumonisins indicated that the intact molecule is responsible for the toxic and carcinogenic activity rather than a metabolite (Cawood *et al.*, 1994; Gelderblom *et al.*, 1993).

A.7. Inhibition of Ceramide Synthase

The chemical structure of fumonisin B_1 is strikingly similar to that of the sphingolipid sphinganine including the toxin's long carbon chain ("backbone"), amine group at the C-2 position, and hydroxyl groups attached to the backbone (Figure1.2). Due to this relationship, fumonisin B_1 inhibits sphinganine (sphingosine) N-acyltransferase (ceramide synthase). Ceramide synthase is responsible for the acylation of sphinganine in the *de novo* biosynthetic pathway of sphingolipids as well as the re-acylation of sphingosine that is released from turnover of complex sphingolipids (Merrill *et al.*, 1993a; Wang *et al.*, 1991). The IC_{50} for inhibition of ceramide synthase is ~0.1 μM and fumonisin B_1 is competitive with both sphinganine and the fatty acyl CoA, suggesting that portions of fumonisin B_1 occupy both the sphingoid base and the fatty acyl CoA binding sites on ceramide synthase (Wang *et al.*, 1991).

Inhibition of ceramide synthase by fumonisins causes accumulation of sphingoid

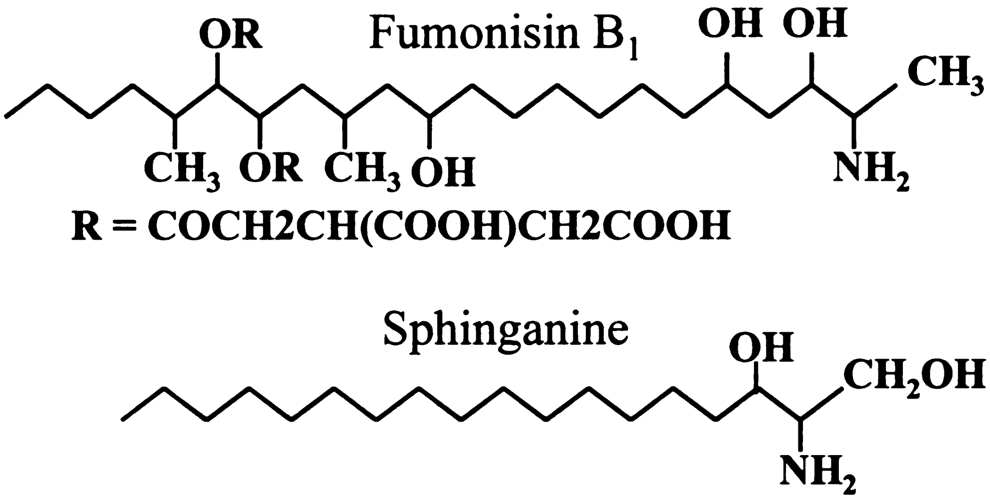


Figure 1.2. Structures of fumonisin B_1 and sphinganine.

bases, especially, sphinganine. Fumonisin-induced sphinganine accumulation was shown in the tissues of mice (Martinova and Merrill, 1995), rats (Kwon *et al.*, 1997; Riley *et al.*, 1994a), mink (Restum *et al.*, 1995) and pigs (Riley *et al.*, 1993a). Some of the sphinganine is metabolized to the 1-phosphate and degraded to hexadecanal and ethanolamine phosphate, the latter being incorporated into phosphatidylethanolamine (Smith and Merrill, 1995). Sphinganine is also released from cells and appears in the blood and urine of rats (Riley *et al.*, 1994a), ponies (Wang *et al.*, 1992), mink (Morgan *et al.*, 1997), pigs (Riley *et al.*, 1993a) and monkeys (Shephard *et al.*, 1996). The sphinganine/sphingosine ratio in blood (Wang et al., 1992; Riley *et al.*, 1993a) and in urine (Morgan *et al.*, 1997; Riley et al.,1994) has been proposed as a sensitive indicator of fumonisin exposure.

Inhibition of ceramide synthesis by fumonisins also eventually depletes complex sphingolipids such as ceramide and sphingomyelin (Riley *et al.*, 1993a; Wang *et al.*, 1992). However, complex sphingolipids differentially respond to fumonisin B_1. For example, sphingomyelin was more rapidly depleted than glycosphingolipids (Meivar Levy and Futerman, 1999; Merrill *et al.*, 1993a). The fumonisin B_1-induced alterations in complex sphingolipids may be responsible for various morphological changes seen in cell culture. Fumonisin B_1 disrupted axonal growth in cultured hippocampal neurons (Harel and Futerman, 1993) by affecting the formation or stabilization of axonal branches (Schwarz *et al.*, 1995). Long-term culture with fumonisin B_1 affected fibroblast morphology and proliferation with a concomitant decrease in the synthesis of ganglioside GM_3, the major glycosphingolipid in 3T3 fibroblasts and of sphingomyelin (Meivar Levy *et al.*, 1997). All of the effects of fumonisin B_1 on cell morphology were reversed by addition of ganglioside GM_3 even in the presence of fumonisin B_1; whereas, the bioactive intermediates sphinganine, sphingosine, and ceramide had no effect. These data suggest that disruption of complex sphingolipid synthesis by fumonisin B_1 can impair the structural integrity of cells.

B. SPHINGOLIPIDS

B. 1. Introduction

Sphingolipids are characterized by a common structural feature, i.e., a sphingoid base backbone such as D-*erythro*-1,3-dihydroxy-2-aminooctadec-4-ene (sphingosine). The "sphingosine" backbone was named by J. L. W. Thudichum in 1884 for its enigmatic ("Sphinx-like") properties while he was studying the chemical constituents of brain (Thudichum, 1884). Sphingolipids are found in all eucaryotic cells, where they are especially rich in the plasma membrane and the membranes of organelles responsible for their synthesis (endoplasmic reticulum and Golgi) and degradation (endosomes and lysosomes). The sphingolipids of mammalian tissues, lipoproteins, and milk include ceramides, sphingomyelins, cerebrosides, gangliosides and sulfatides.

Sphingolipids are constituents of most foods. The sphingolipid content of pork, beef, and chicken is ~0.3 - 0.5 μmol/g (Blank *et al.*, 1992). Whole milk, butter, and cheese contain ~0.5 - 1.0 μmol/g (Zeisel *et al.*, 1986; Ahn and Schroeder, 2002). The sphingolipid content is relatively low in plant foods with the exception of soybean which contains ~2 μmol/g (Ohnishi and Fujino, 1982; Ahn and Schroeder, 2002). Dietary sphingolipids are hydrolyzed throughout the intestine (Nilsson, 1969; Schmelz *et al.*, 1994).

Sphingolipids are involved in the signal transduction pathways that mediate cell growth, differentiation and cell death. Hydrolysis of sphingomyelin by sphingomyelinase generates ceramide as a cellular second messenger for tumor necrosis factor-α (TNF-α), IL-

1β, and other cytokines. Subsequently, ceramide can be hydrolyzed by multiple ceramidases to generate sphingosine in response to IL-1β (Nikolova-Karakashian *et al.*, 1997) and may be further phosphorylated to form sphingosine 1-phosphate in response to PDGF (Olivera *et al.*, 1999a). Sphingosine and sphingosine 1-phosphate modulate a variety of targets, mobilize cellular calcium, and regulate cell proliferation (Merrill *et al.*, 1997a). Perturbation of sphingolipid metabolism by key enzyme inhibitors such as fumonisins appears to cause abnormal cell functions and certain diseases.

B. 2. *De novo* Biosynthesis

Sphingolipids are synthesized *de novo* from the amino acid serine and palmitoyl CoA (Figure1.3). In the endoplasmic reticulum, serine palmitoyltransferase condenses the two molecules to form 3-ketosphinganine which NADPH-dependent reductase rapidly reduces to form sphinganine, a free sphingoid base. Sphinganine is *N*-acylated with a long-chain fatty acid to form *N*-acyl-sphinganine (dihydroceramide) by ceramide synthase. Subsequently, a 4,5-*trans* double bond is introduced to form ceramide (*N*-acyl-sphingosine) (Merrill and Wang, 1992; Rother *et al.*, 1992). Additional reactions take place in the Golgi apparatus where head groups are added. For example, the transfer of a phosphorylcholine head group from phosphatidylcholine to the 1 position of ceramide yields sphingomyelin (Futerman and Pagano, 1991). Currently there are more than 300 known sphingolipids with distinct head groups (Bell *et al.*, 1993). All contain a long-chain (sphingoid) base backbone with D-*erythro*-sphingosine being the most prevalent backbone. However, there are more than 60 different sphingoid base backbones that vary in lengths of alkyl chain from 14 to 22 carbon atoms, degree of saturation and position of the double bonds, presence of a hydroxyl

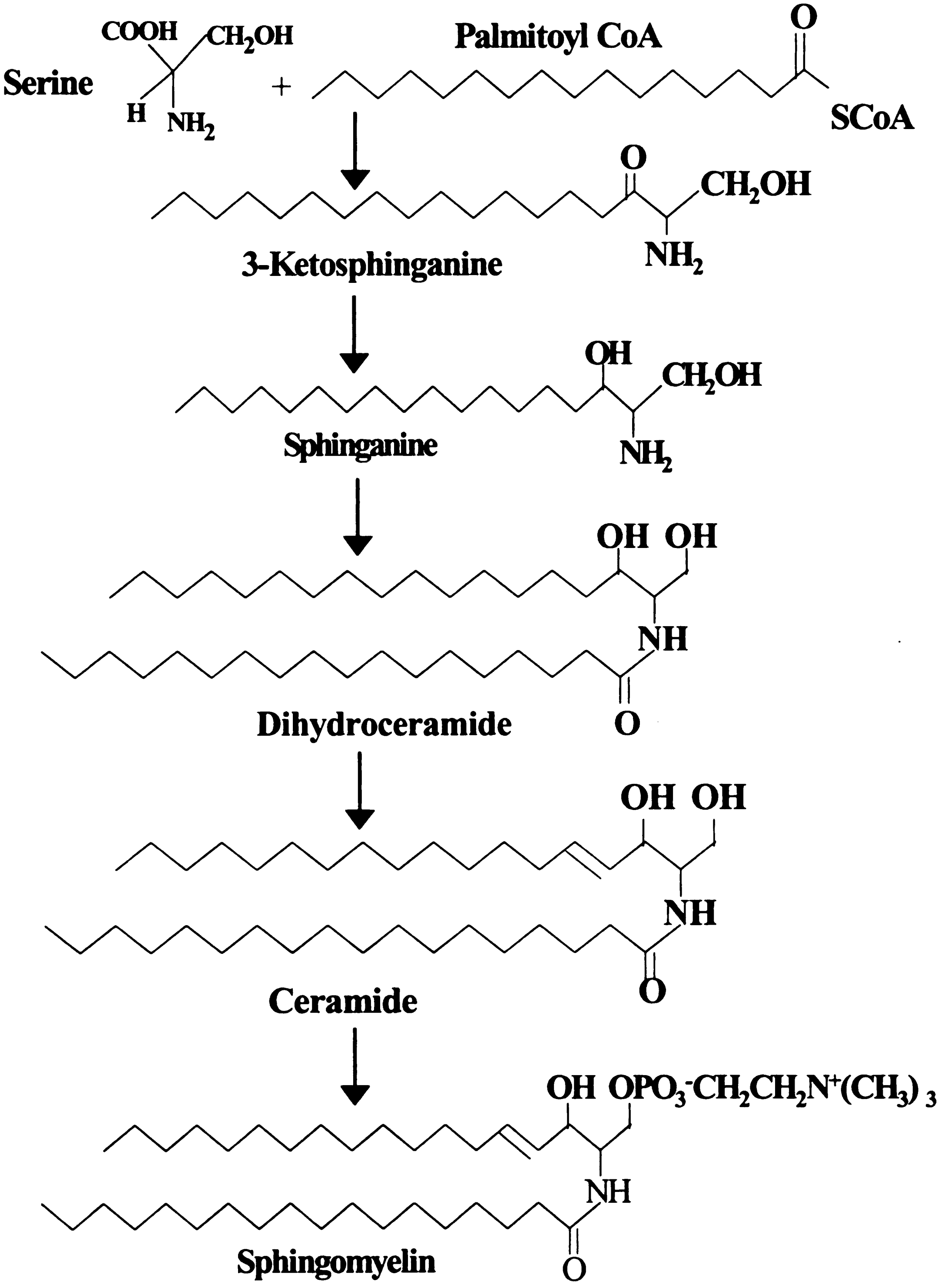


Figure 1.3. *De novo* sphingolipid biosynthesis

group at position 4 and branching of the alkyl chain (Karlsson, 1970). Sphinganine and other intermediates in the *de novo* biosynthetic pathway of sphingolipids are highly bioactive and, under normal conditions, the cellular concentrations of these compounds are kept very low (Merrill *et al.*, 1986).

B. 3. Turnover

The ordinary turnover of sphingolipids involves removal of the head groups such as phosphorylcholine from sphingomyelin by sphingomyelinases in the lysosomes (Figure1.4). Ceramidase catalyzes the cleavage of the amide-linked fatty acid of ceramide to form free sphingosine. Sphingosine can then be either reacylated to form ceramide or phosphorylated via sphingosine kinase to form sphingosine 1-phosphate (Stoffel *et al.*, 1968, 1970). Subsequently, sphingosine 1-phosphate is cleaved to form ethanolamine phosphate and *trans*-2-hexadecanal by a pyridoxal phosphate-dependent lyase located in endoplasmic reticulum (Stoffel *et al.*, 1968). The ethanolamine phosphate may be used for the synthesis of phosphatidylethanolamine and phosphatidylcholine. Another possibility for the metabolism of sphingosine has been suggested to involve methylation to form di- and trimethyl sphingosine (Igarashi *et al.*, 1990; Hakomori and Igarashi, 1993).

B. 4. Sphingolipid Signaling

In addition to the normal turnover of sphingolipids, the metabolism of sphingolipids has evolved as an important metabolic pathway which plays essential roles in the production of cellular messengers to regulate cell behavior. That is, complex sphingolipids in membranes may also serve as substrates for agonist-induced signaling. The resulting

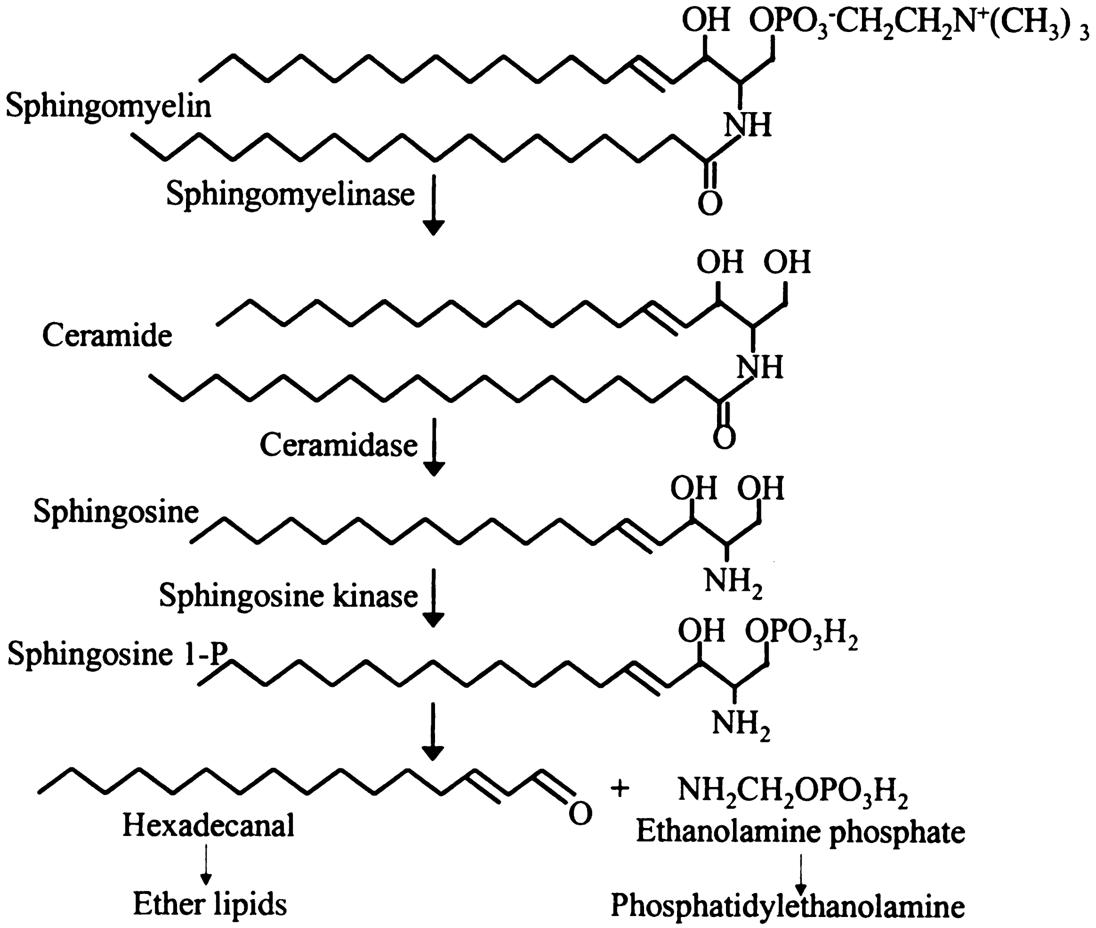


Figure 1.4. Sphingolipid turnover

sphingolipid metabolites mediate a variety of cellular processes (Figure1.5). TNF-α, interferon-γ, (Kim *et al.*, 1991) and 1α-25-dihydroxyvitamin D_3 (Okazaki *et al.*, 1989, 1990) induces sphingomyelin hydrolysis by sphingomyelinase to produce ceramide which triggers monocytic differentiation of human premyelocytic leukemia HL-60 cells. A number of studies indicate that ceramide may regulate cell growth, either by inhibiting proliferation of normal fibroblasts (Hannun, 1994; Venable *et al.*, 1994) or inducing proliferation of confluent, quiescent 3T3 fibroblasts (Olivera and Spiegel, 1993).

Ceramide is a well-known mediator of programmed cell death or apoptosis, induced by TNF-α, Fas ligand, and ionizing radiation (Cifone *et al.*, 1994; Haimovitz-Friedman *et al.*, 1994; Kolesnick and Golde, 1994; Obeid *et al.*, 1993; Hannun, 1994). TNF-α is coupled to sphingomyelinase to produce ceramide (Kim *et al.*, 1991), but the mechanism is not clearly understood. Exogenously added ceramide also induces apoptosis in various cell lines (Bielawska *et al.*, 1997; Charles *et al.*, 2001; Dawson *et al.*, 1998; Hannun and Obeid, 1995; Jarvis *et al.*, 1996; Saba *et al.*, 1996). However, anti-apoptotic *bcl-2* antagonizes ceramide action and prevents apoptosis (Pinton *et al.*, 2001; Wieder *et al.*, 1997; Zhang *et al.*, 1996).

Ceramide appears to act by affecting signaling molecules involved in the regulation of cell proliferation and apoptosis. Raf1 is phosphorylated by a ceramide-activated protein kinase, activating the MAPK cascades (Raines *et al.*, 1993; Yao *et al.*, 1995). Also, a cytosolic ceramide-activated protein phosphatase (CAPP) has been identified as a molecular target for the action of ceramide (Dobrowsky and Hannun, 1992; Hannun, 1994). Okadaic acid, a phosphatase inhibitor, prevented ceramide-induced apoptosis and CAPP activity correlates with growth inhibition in numerous cell types (Hannun, 1994). Recently, ceramide was shown to induce dephosphorylation of Akt (protein kinase B) at serine 473, suggesting

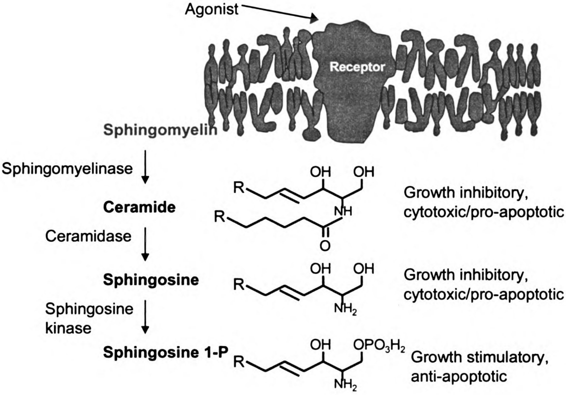


Figure 1.5. Sphingolipid signaling and cellular effects

activation of a phosphatase (Schubert *et al.*, 2000). Since Akt is involved in the activation of the cell proliferation pathway and regulation of apoptosis (Krasilnikov, 2000; Zhou *et al.*, 2000; Zundel and Giaccia, 1998), inhibition of Akt activity by ceramide-induced dephosphorylation may be one of the mechanisms by which ceramide inhibits growth and induces apoptosis.

Some of the actions of ceramide in cellular processes appear to be mediated by deacylation to sphingosine which also is a putative second messenger. For example, IL-1β induces the hydrolysis of sphingomyelin to ceramide in primary cultures of rat hepatocytes and ceramide has been suggested to play a role in the down regulation of cytochrome p450 2C11 (CYP2C11) and induction of α_1-acid glycoprotein (AGP) (Chen *et al.*, 1995). Further, IL-1β increased not only sphingomyelinase activity, but also ceramidase with a concomitant increase of cellular sphingosine. Sphingosine was more potent than C2-ceramide in down-regulation of CYP2C11 (Nikolova-Karakashian *et al.*, 1997), suggesting that sphingosine is a mediator of the regulation of CYP2C11 by IL-1β. The sphingoid bases, sphingosine and sphinganine (Figure1.6), are produced from the metabolism of ceramide by ceramidase and during the *de novo* biosynthesis of sphingolipids, respectively (Figure1.3 and 1.4), and have received significant attention due to their inhibitory action on protein kinase C (PKC) (Hannun *et al.*, 1986; Hannun and Bell, 1987) and PKC-dependent cellular processes in platelets (Hannun *et al.*, 1986), neutrophils (Wilson *et al.*, 1986), and HL-60 cells (Merrill *et al.*, 1986). Sphingosine is competitive with diacylglycerol, phorbol dibutyrate, and Ca^{++}, and also blocks PKC activation by unsaturated fatty acids and other lipids (el Touny *et al.*, 1990; Oishi *et al.*, 1988; Wilson *et al.*, 1986).

Like ceramide, sphingoid bases have been shown to be growth inhibitory (Merrill *et*

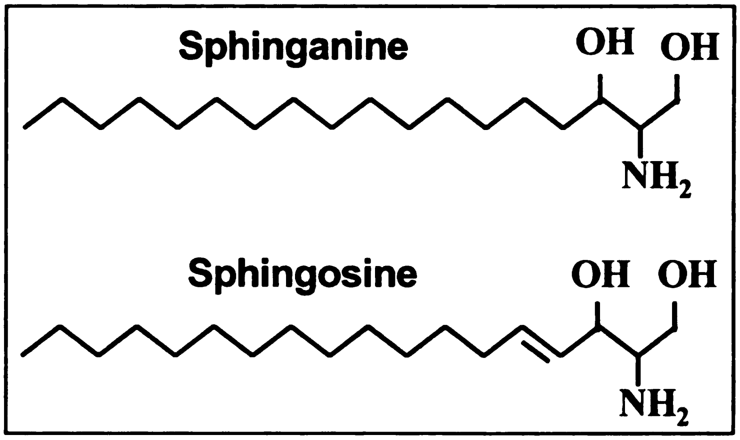


Figure 1.6. Structures of sphingoid bases.

al., 1986; Chao *et al.*, 1992; Dbaibo *et al.*, 1995; Spiegel *et al.*, 1993) and to induce apoptosis (Smyth *et al.*, 1997; Dawson *et al.*, 1998; Ohta *et al.*, 1995; Shirahama *et al.*, 1997; Jarvis *et al.*, 1996; Sakakura *et al.*, 1998). Sphingoid bases may cause cell death by affecting key signaling molecules that are involved in regulating cell growth and apoptosis. In addition to inhibiting PKC, sphingosine has been shown to inhibit phorbol dibutyrate binding (Merrill *et al.*, 1986; Hannun *et al.*, 1986), inhibit cell growth by arresting the cell cycle in the G_0/G_1 phase (Chao *et al.*, 1992; Dbaibo *et al.*, 1995), activate the tumor suppressor, retinoblastoma protein, by inducing dephosphorylation (Pushkareva *et al.*, 1995), inhibit p42/p44 MAPK activity (Sakakura *et al.*, 1998) and induce release of Ca^{++} from intracellular stores (probably via conversion to sphingosine 1-phosphate [SPP])(Ghosh *et al.*, 1990). Recently, sphingosine also has been shown to dephosphorylate Akt during induction of hepatoma cell apoptosis (Chang *et al.*, 2001) (Figure 1.7). In addition, sphingosine suppresses expression of *bcl-2* in human leukemic HL-60 cells (Sakakura *et al.*, 1996) and *bcl-x* in human prostatic carcinoma DU-145 cells (Shirahama *et al.*, 1997) during apoptosis.

Addition of a phosphate group on sphingoid bases (sphingosine or sphinganine) by sphingosine kinase produces sphingoid base 1-phosphates (SPP), the phosphorylated metabolites of sphingoid bases. In contrast to sphingoid bases, in most cases, SPP have been found to be growth stimulatory and anti-apoptotic (Conway *et al.*, 1997; Olivera *et al.*, 1999b; Spiegel, 1999). Platelet-derived growth factor (PDGF) stimulates sphingosine kinase activity and SPP acts as a second messenger in cell proliferation induced by PDGF and fetal calf serum (Olivera *et al.*, 1999a; Olivera and Spiegel, 1993). Rapid activation of the Raf/ERK pathway (Wu *et al.*, 1995) and increased DNA binding activity of activator protein-1 (AP-1) (Su *et al.*, 1994) also may mediate the mitogenic effects of SPP, resulting in cell

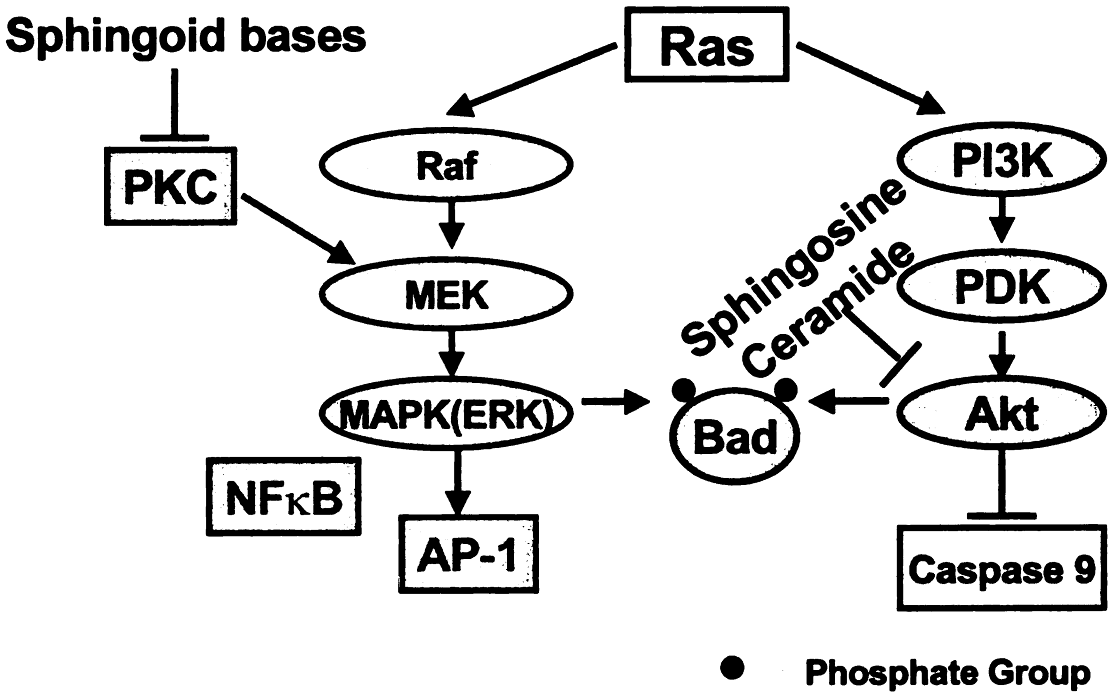


Figure 1.7. Effects of sphingolipids on cell proliferation signaling pathways

proliferation. SPP mediates nerve growth factor-induced neuronal survival and differentiation and has been shown to inhibit caspase activation during Fas- and ceramide-mediated apoptosis in Jurkat T lymphocytes (Cuvillier *et al.*, 1998). In rat periosteal RP-11 cells, SPP mediates the inhibition of apoptosis by forskolin-induced cAMP via stimulation of sphingosine kinase activity (Machwate *et al.*, 1998). Recent work by Olivera *et al.* (1999b) demonstrated that overexpression of sphingosine kinase markedly increased the concentration of SPP in NIH 3T3 fibroblasts and HEK293 cells and is sufficient to promote growth in low-serum media, expedite the G_1/S transition, and increase DNA synthesis. Furthermore, overexpression of sphingosine kinase in NIH 3T3 cells protected against ceramide-induced apoptosis (Olivera *et al.*, 1999b). Since SPP can be produced from the metabolism of ceramide (Figure1.4), it has been suggested that the balance between intracellular ceramide and SPP may determine the cell fate (Cuvillier *et al.*, 1996). It should be noted, however, SPP inhibits proliferation of human hepatic myofibroblasts (Davaille *et al.*, 2000). Therefore, SPP may be growth inhibitory and/or apoptotic under certain conditions.

SPP may mediate the mitogenic effects of sphingoid bases. In Swiss 3T3 cells, sphingosine stimulated cellular proliferation via a protein kinase C-independent pathway (Zhang *et al.*, 1990). Sphingosine induced a rapid increase in SPP levels in a concentration-dependent manner and there was a strong correlation with sphingosine's effect on DNA synthesis (Zhang *et al.*, 1991). When added together, there was no additive or synergistic effect, suggesting that sphingosine and SPP modulate cellular proliferation through a common pathway (Zhang *et al.*, 1991). Both sphingosine and SPP potently mobilize calcium from internal stores with SPP acting more rapidly than sphingosine (Mattie *et al.*, 1994;

Olivera *et al.*, 1994; Zhang *et al.*, 1991) and both activate phospholipase D (Desai *et al.*, 1992), well known events in the control of cellular proliferation.

B. 5. Disruption of Sphingolipid Metabolism as a Causative Factor in Fumonisin-induced Toxicity

Fumonisins are cytotoxic and carcinogenic and this appears to be due to their cell-type specific effects on cellular behavior. Fumonisin B_1 is apoptotic in some cells while being mitogenic in others. The mechanism of action of fumonisins is not clearly understood but inhibition of ceramide synthase by fumonisins and the consequent depletion of complex sphingolipids and accumulation of sphinganine and metabolites may mediate the actions of fumonisins. Inhibition of acylation of sphinganine by fumonisins ultimately depletes complex sphingolipids and their role in fumonisin-induced toxicity is not clearly understood. Complex sphingolipids have been suggested to be involved in the regulation of cell growth and differentiation. Their profiles change as cells grow (Hakomori, 1981) and the majority of the glycosphingolipids and sphingomyelin are located in the external leaflet of the plasma membrane (Miller-Podraza *et al.*, 1982), where they interact with cell surface receptors and mediate cell-cell, cell substratum, and cell-microbial interactions. This is further supported by results from *in vitro* studies which demonstrated that exogenously added gangliosides G_{M3} or G_{M1} inhibited cell growth by delaying the G_1 phase of the cell cycle (Laine and Hakomori, 1973) and can make cells refractory to growth stimulation by growth factors (Bremer and Hakomori, 1982). Also, addition of ganglioside G_{M3} induced HL-60 cells to differentiate into monocyte-like cells (Saito *et al.*, 1985). On the other hand, removal of ganglioside G_{M1} by adding the β-subunit of cholera toxin or antibody which binds to this ganglioside is mitogenic (Spiegel *et al.*, 1985) and potentiates the response to the growth factors (Spiegel and Fishman, 1987). Therefore, complex sphingolipids including gangliosides may play crucial roles in the regulation of cell growth and differentiation and their depletion during long-term exposure to fumonisins could contribute to carcinogenicity and other toxicities caused by fumonisins. Further investigations are needed to determine the role of depletion of complex sphingolipids in fumonisin-induced toxicities.

The most well known consequence of fumonisin exposure is the accumulation of sphingoid bases which have been shown to inhibit cell growth and induce apoptosis (Figure1.5). Therefore, fumonisins may exert their toxicity via accumulation of sphinganine. The cytotoxic and apoptotic effects of sphingoid bases could be due to their inhibitory effect on protein kinase C (PKC), an important regulator of cell proliferation (Hannun and Bell, 1986). One of the major downstream targets of PKC is p44/p42 MAPK (or ERK) of which phosphorylation leads to phosphorylation and activation of activator protein-1 (AP-1). AP-1 consists of dimers of *fos* and *jun* and regulates expression of genes involved in cell growth. Inhibition of ERK activity by sphingosine has been suggested to be a partial mechanism of action in sphingosine-induced apoptosis in various tumor cell lines (Sakakura *et al.*, 1998). Therefore, fumonisin-induced sphinganine accumulation and down-regulation of ERK activity may contribute to fumonisin-induced cell death.

Apoptosis often accompanies the suppression of anti-apoptotic signals such as PI3K/Akt (Jarvis *et al.*, 1997). The PI3K/Akt signaling pathway has been shown to mediate both Ras- and cytokine-induced protection from apoptosis. Activation of PI3K recruits Akt to the plasma membrane where Akt is phosphorylated at serine 473 and threonine 308 (Stephens *et al.*, 1998; Stokoe *et al.*, 1997). Akt is a serine/threonine kinase involved in the regulation of apoptosis via Bad and caspase-9 (Figure 1.8) (Krasilnikov, 2000; Zhou *et al.*, 2000; Zundel and Giaccia, 1998). Phosphorylation of Akt activates its kinase function so that it may add a phosphate group on substrates such as the *bcl-2* family protein Bad and caspase 9 (Krasilnikov, 2000). Phosphorylation of Bad serves as an anti-apoptosis signal by allowing *bcl-x* to inhibit release of cytochrome C from the mitochondria into the cytosol, which is a critical event in apoptosis. Also, phosphorylation of caspase 9 inhibits cleavage which is an activation process, preventing further activation of caspase 3, the executive protease protein in apoptosis. Recently, ceramide has been shown to dephosphorylate Akt and Bad during apoptosis (Basu *et al.*, 1998; Schubert *et al.*, 2000). Inhibition of Akt activity by ceramide-induced dephosphorylation may be one of the mechanisms of ceramide action in its effect on growth inhibition and apoptosis induction. Importantly, sphingosine appears to mediate the action of ceramide on Akt because sphingosine also has been shown to reduce phosphorylation of Akt with concomitant reduction of its activity during sphingosine-induced apoptosis in human hepatoma cells (Chang *et al.*, 2001). Moreover, expression of constitutively active Akt prevented sphingosine-induced apoptosis (Chang *et al.*, 2001). Thus, fumonisin-induced sphinganine accumulation could induce Akt dephosphorylation and trigger cascades of molecular events to induce cell death.

Sphingoid bases also may affect expression of genes involved in the regulation of cell survival and apoptosis. Numerous apoptotic agents depend on gene expression for their effects. Those genes in which expression has been documented to play a role in the apoptotic process include p53 tumor suppressor gene (Attardi *et al.*, 1996; Bouvet *et al.*, 1998), *bcl-2* family member genes (Bargou *et al.*, 1995a; Bargou *et al.*, 1995b; Bruckheimer *et al.*, 1998; Ealovega *et al.*, 1996; Kondo *et al.*, 1994), *c-myc* (Desbarats *et al.*, 1996; Donzelli *et al.*,

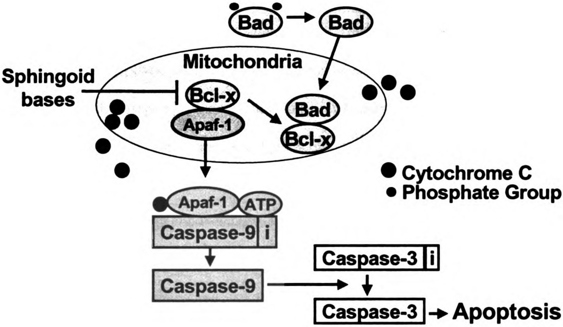


Figure 1.8. Effects of sphingolipids on apoptotic signaling pathway

1999; Galderisi *et al.*, 1999; Kihara Negishi *et al.*, 1998; Wang *et al.*, 1997), calmodulin (Dowd *et al.*, 1991), heat-shock protein 70 (Garcia Bermejo *et al.*, 1997), E1A (Putzer *et al.*, 2000), and poly (ADP-ribose) polymerase (Putzer *et al.*, 2000). Sphingoid bases suppress expression of *bcl-2* (Sakakura *et al.*, 1996) and *bcl-x* (Shirahama *et al.*, 1997) which are involved in regulating apoptosis (Figure 1.8). Therefore, it is possible that fumonisin-induced sphinganine accumulation affects expression of these genes to induce apoptosis.

Fumonisin B_1 was mitogenic and induced DNA synthesis in Swiss 3T3 cells (Schroeder *et al.*, 1994). Fumonisin-induced mitogenicity was sphinganine-dependent because prevention of sphinganine accumulation using β-fluoroalanine inhibited fumonisin-induced DNA synthesis. Also, the exogenous addition of sphingoid bases stimulates DNA synthesis in Swiss 3T3 cells (Zhang *et al.*, 1990), suggesting that fumonisin-induced DNA synthesis is due to sphinganine accumulation. Therefore, it appears that fumonisin-induced sphinganine accumulation has bimodal effects on cell growth depending on cell type. Both the apoptotic and proliferative processes may be regulated by a similar signaling pathway (Desbarats *et al.*, 1996) and may involve a similar pattern of early gene responses (Grassilli *et al.*, 1992). Therefore, fumonisin B_1 may affect expression of genes that are involved in both apoptosis and cell proliferation, inducing either cytotoxicity and carcinogenicity.

C. LLC-PK$_1$ RENAL TUBULAR EPITHELIAL CELLS AS A MODEL

LLC-PK$_1$ renal epithelial cells appear to be a good model system for the study of the mechanism of action of fumonisins. Kidney is the most sensitive organ to fumonisin-induced toxicity (Gumprecht *et al.*, 1995; Riley *et al.*, 1994a) with the primary target appearing to be the renal tubular epithelial cell. Fumonisin B_1 inhibited growth and increased death in LLC-PK_1 cells with an early increase of intracellular sphinganine (Yoo *et al.*, 1996). The dose of fumonisin B_1 which caused sphinganine accumulation was similar to the dose which caused toxicity, suggesting a possible relationship between sphinganine accumulation and fumonisin-induced toxicity (Yoo *et al.*, 1992).

D. HYPOTHESIS AND SPECIFIC AIMS

This study is based on the hypothesis that fumonisin-induced kidney toxicity is mediated by disruption of sphingolipid metabolism. In this study, β-fluoroalanine, an inhibitor of serine palmitoyltransferase, will be used to dissect and differentiate the role of sphinganine accumulation and complex sphingolipid depletion in fumonisin-induced kidney toxicity. This study with LLC-PK_1 cells would help to better understand the role of disruption of sphingolipid metabolism in fumonisin-induced cytotoxicity and carcinogenicity in kidney and other tissues. The specific aims are:

(1) Determine whether fumonisin B_1 kills cells by inducing apoptosis and evaluate the role of disruption of sphingolipid metabolism in fumonisin-induced cell death

(2) Identify genes affected by fumonisin B_1 and investigate the molecular mechanism whereby fumonisin B_1 induces gene expression

(3) Determine whether fumonisin B_1 affects signaling pathways involved in cell proliferation and cell death

CHAPTER II

FUMONISIN B_1 INDUCES APOPTOSIS IN LLC-PK_1 RENAL EPITHELIAL CELLS VIA A SPHINGANINE- AND CALMODULIN-DEPENDENT PATHWAY

A. ABSTRACT

Fumonisins are a family of mycotoxins produced by *Fusarium moniliforme*, which is the most common mold found on corn throughout the world. These compounds are both toxic and carcinogenic for animals, and perhaps humans, with the kidney being the most sensitive organ to fumonisin toxicity. The molecular mechanism of fumonisin toxicity appears to involve disruption of *de novo* biosynthesis of sphingolipids and accumulation of sphinganine. The goals of this study were to determine whether fumonisin B_1 kills LLC-PK_1 renal kidney epithelial cells by inducing apoptosis and to identify genes affected by sphinganine that mediate fumonisin B_1-induced cell death. Fumonisin B_1 produced morphological changes (i.e., cell shrinkage, membrane blebbing) and time-dependent increases in DNA fragmentation demonstrating that the toxin induces apoptosis. Simultaneously, fumonisin B_1 blocked sphingolipid biosynthesis and caused accumulation of sphinganine. To further investigate the role of sphinganine in fumonisin B_1-induced apoptosis, β-fluoroalanine (βFA) was used to inhibit serine palmitoyltransferase, which catalyzes an earlier step in the sphingolipid biosynthetic pathway. βFA blocked sphinganine accumulation and prevented fumonisin B_1-induced DNA fragmentation, confirming that apoptosis induced by fumonisin B_1 is dependent upon accumulation of sphinganine. To examine gene expression, differential display reverse transcriptase polymerase chain reaction (DDRT-PCR) was applied to RNA isolated after 16 h of exposure to fumonisin B_1. Differential expression in response to fumonisin B_1 of a gene identified as calmodulin has been verified by Northern analysis. Sphinganine appears to mediate the effect because βFA reduces induction of calmodulin mRNA by fumonisin B_1. Fumonisin B_1 also increases calmodulin protein in a concentration-dependent manner and the calmodulin antagonist W7

blocks fumonisin B_1-induced DNA fragmentation, supporting a role for calmodulin in fumonisin B_1-induced apoptosis. In contrast, fumonisin B_1 has no effect on expression of *bcl-2* family genes (*bax*, *bcl-2*, and *bcl-x*). These findings demonstrate that fumonisin B_1 kills LLC-PK_1 kidney cells by inducing apoptosis. Further, the results establish a sequence of events for fumonisin B_1-induced apoptosis involving initial disruption of sphingolipid metabolism and accumulation of sphinganine (or a metabolite), which, in turn, induces expression of calmodulin.

B. INTRODUCTION

Fumonisins are a family of cytotoxic and carcinogenic mycotoxins produced by *Fusarium monilifome,* one of the most common molds found on corn and other agricultural commodities in the United States and throughout the world (Marasas, 1996). Interest in the fumonisin mycotoxins arises, in part, because of their adverse effects on animal health. Fumonisins cause equine leukoencephalomalacia and hepatotoxicity (Marasas *et al.*, 1988), porcine pulmonary edema (Harrison *et al.*, 1990), and renal toxicity, hepatotoxicity, and liver cancer in rats (Gelderblom *et al.*, 1988), with the kidney being the most sensitive organ to fumonisin toxicity (Bucci *et al.*, 1998; Gumprecht *et al.*, 1995). Fumonisins also may pose a threat to human health, as consumption of contaminated maize has been correlated with esophageal cancer in areas of southern Africa, China, and other countries (Marasas, 1996).

Fumonisin toxicity appears to be mediated by disruption of sphingolipid metabolism (Merrill *et al.*, 1997a). Fumonisins bear a remarkable structural resemblance to sphingosine and sphinganine, the long-chain (sphingoid) base backbones of more complex sphingolipids, and block *de novo* biosynthesis of sphingolipids by potently inhibiting sphingosine (sphinganine) N-acyltransferase (Norred *et al.*, 1992; Wang *et al.*, 1991; Yoo *et al.*, 1992). The inhibition causes depletion of more complex sphingolipids and accumulation of sphinganine (and sometimes sphingosine) in a variety of cultured cells (Norred *et al.*, 1992; Wang *et al.*,1991; Yoo *et al.*,1992; Schroeder *et al.*, 1994; Tolleson *et al.*, 1999) and in the sera, liver, kidney, and urine of animals fed contaminated grains (Merrill *et al.*, 1997b; Morgan *et al.*, 1997; Riley *et al.*, 1993a, 1994b; van der Westhuizen *et al.*, 2001; Wang *et al.*, 1992). Studies using a renal kidney epithelial cell line (LLC-PK_1) have demonstrated a strong association between accumulation of sphinganine caused by fumonisin B_1 and inhibition of cell growth. morphological changes, and increased cell death (Yoo *et al.*, 1992, 1996).

The mode by which fumonisin B_1 kills LLC-PK_1 kidney cells is not known; however, the mycotoxin induces apoptosis in other cell types and *in vivo* (Lim *et al.*, 1996; Tolleson *et al.*, 1996a, 1996b, 1999; Sharma *et al.*, 1997; Wang *et al.*, 1996). Moreover, Tolleson *et al.* (1996a, 1996b, 1999) have shown that exogenous sphinganine induces apoptosis in human keratinocytes and that addition of β-haloalanine blocks fumonisin B_1-induced sphinganine accumulation and DNA fragmentation, suggesting that accumulation of sphinganine plays a key role in apoptosis induced by fumonisin B_1. Apoptosis induced by sphingosine in HL-60 cells is accompanied by a decrease in expression of *bcl-2*, an apoptosis suppresser gene (Sakakura *et al.*, 1996). In addition, sphingosine induces apoptosis in androgen-independent human prostatic carcinoma DU-145 cells by down-regulating *bcl-xL* (Shirahama *et al.*, 1997). Thus, sphingoid bases may mediate fumonisin-induced cell death by altering expression of a key gene(s).

This study used LLC-PK_1 renal kidney epithelial cells as a model system to test the hypothesis that fumonisin B_1 kills the cells by inducing apoptosis. Further, this study also tested the hypothesis that sphinganine accumulation caused by fumonisin B_1 mediates cell death by altering gene expression. Based on previous findings that sphingoid bases alter the expression of *bcl-2* family genes, we have examined the effect of fumonisin B_1 on expression of members of this gene family. In addition, we have used differential display reverse transcriptase PCR as an unbiased approach to examine the influence of fumonisin B_1 on expression of other genes.

C. MATERIALS AND METHODS

C.1. Reagents and cell culture α-^{32}P-dCTP was obtained from DuPont NEN and C20-sphinganine was obtained from Matreya Co. All other reagents were obtained from Sigma Chemical (St. Louis, MO) unless otherwise noted. Porcine kidney epithelial LLC-PK_1 cells were obtained from American Type Culture Collection (ATCC) and cultured in Dulbecco's Modified Eagle Medium/F-12K nutrient solution (Gibco BRL) (1:1) with 5% fetal bovine serum (FBS), 100 units/ml penicillin, 100 µg/ml streptomycin sulfate, and 100 µM of L-serine (Gibco BRL). Cells were treated when they were 70-80 % confluent.

C.2. Analysis of DNA fragmentation by agarose gel electrophoresis assay Following treatments, fragmented DNA from LLC-PK1 cells was extracted as described (Sellins and Cohen, 1987). In brief, cells from 100-mm dish were harvested using rubber policeman and collected by centrifugation (5 min, 500 g). The medium was removed and the cell pellet was resuspended in phosphate-buffered saline (PBS). After another centrifuge, the pellet was suspended in 0.1 ml hypotonic lysis buffer (10 mM Tris, 10 mM EDTA, 0.5% Triton X-100, pH 8.0). Cells were incubated for 10 min at 4°C and the lysate was centrifuged (30 min, 13,000 g, 4 °C). The supernatant containing fragmented DNA was treated with RNase A (0.4 μg/μl) for 1 h at 37 °C and then incubated with proteinase K (0.4 μg/μl) for 1 h at 37 °C. DNA was precipitated overnight by incubation at -20 °C in 50% isopropanol and 0.5 M NaCl. The DNA precipitate was pelleted by centrifugation (30 min, 13,000 g, 4 °C), dissolved in distilled H_2O and then electrophoresed in a 2 % agarose gel containing ethidium bromide (0.5 μg/ml) in 0.5X Tris-borate-EDTA buffer. After electrophoresis, DNA was visualized with a UV transilluminator and photographs were taken using a Polaroid system.

C.3. Flow cytometric analysis of apoptotic cells. Flow cytometric analysis was performed as described by Telford *et al.* (1994) with modifications. Briefly, treated cells were collected by trypsinization and resuspended into single cell suspension in culture media and fixed with 70% ethanol, reaching final concentration of 50-53%.. Cells were pelleted and resuspended in PBS (pH 7.4) containing 0.1% Triton X-100, 0.1 mM EDTA. After pelleting the cells again, cells were resuspended with DNA staining solution (50 μg/mL propidium iodide, 0.1% Triton X-100, 100 μM EDTA, and 0.05 mg/mL RNase A) and analyzed for the percentage of cells in the sub-G0/G1 using a FACS Vantage Flow Cytometer (Becton Dickinson, San Jose, CA).

C.4. Mass measurement of sphinganine The cellular concentration of sphinganine was determined using HPLC method (Merrill *et al.*, 1988). Briefly, cells were harvested in PBS and the unnatural sphingoid base, C20-sphinganine, was added as an internal standard. The sphingoid bases were extracted with chloroform and methanol and treated with base to remove interfering glycerolipids. After preparation of the *o*-phthalaldehyde derivatives, the long-chain bases were separated by reverse-phase HPLC using a C18 column and eluted isocratically with methanol:5mM potassium phosphate, pH 7.0 (90:10).

C.5. RNA isolation Total RNA was isolated by the method of Chomczynski and Sacchi (1987) using RNA STAT 60 (Tel-Test). The integrity and the equal amount of the RNA was verified by examining 28S and 18S bands on 1.0% formaldehyde-agarose gel containing ethidium bromide (0.5 µg/mL).

C.6. Quantitation of bcl-2, bcl-x and bax mRNA levels Competitive RT-PCR was used to quantitate *bcl-2*, *bcl-x* and *bax* mRNA levels (Wang *et al*, 1995). This method is based on a competitive PCR approach using non-homologous internal standards called PCR MIMICs with the use of the PCR-MIMIC construction kit (Clontech Laboratories, Palo Alto, CA). MIMICs are DNA fragments constructed for use in competitive PCR amplification for quantitation of target mRNA levels. Each PCR MIMIC consists of a heterologous DNA fragment with primer templates that are recognized by a pair of gene-specific primers. Thus, these templates "mimic" the target and are amplified during PCR. The procedure used one set of primers to amplify both target cDNA and an externally added MIMIC of known concentration. MIMICs for *bcl-2*, *bcl-x* and *bax* quantitation were constructed following the manufacturer's direction. The primer pairs used for amplification of *bcl-2*, *bcl-x* and *bax* mRNA. 1 µg of RNA was used routinely for cDNA synthesis (Clontech). After first-strand cDNA synthesis reaction, the cDNA was made into 100 µl final volume, and 4 µl were used for PCR. A typical PCR consisted of 0.2 µM dNTP, 2 µl MIMIC, 4 µl cDNA, 0.2 µM of each primer, PCR buffer, and Taq polymerase. The PCR profile was 95 °C for 45 s, 60 °C for 45 s, and 72 °C for 2 min for 30 cycles, followed by 72 °C for 7 min. After PCR, aliquots of the reaction were analyzed on 1.8 % agarose gel (0.5 µg/ml ethidium bromide). The amount of mRNA was quantitated by comparing relative intensities of the amplified MIMIC and specific message bands. All results were normalized against glyceraldehyde 3-phosphate dehydrogenase (G3PDH) mRNA quantitated with the use of identical PCR conditions. Results are expressed as level of expression relative to G3PDH. The primer pairs and competitor for G3PDH were purchased from Clontech.

C.7. Densitometry Analysis Western and Northern blot images were subjected to densitometry analysis using Quantity One (Bio-Rad, Hercules, CA).

C.8. Differential Display Reverse Transcription Polymerase Chain Reaction (DD RT-PCR) Differential display gel kits including RTase (Genhunter Corp., Nashville, TN) were used for this study. RNA from control and Fumonisin B_1-treated cells were isolated and used for RT reaction using poly T primer with A, G or C as outlined by the supplier. RT products were labeled with 25 μCi α-^{35}S dATP and amplified with 15 different combinations using 3 different down stream primers and 5 different upstream primers in a thermocycler (GeneAmp, PCR System 2400, Perkin Elmer Norwalk, CT) in the presence of DNA polymerase (Perkin Elmer Norwalk, CT). Labeled cDNAs were fractionated by size on a 6% DNA sequencing gel at a constant 60 W at 50 °C for 2 h. Gels were transferred to Whatman 3 filter paper and dried at 80 °C for 1 h. The dried gel was exposed to X-ray film for 1-2 days to obtain an autoradiograph. The target cDNA was isolated from a dried gel and reamplified in the absence of the radioactive dATP. These cDNAs were used for cloning and as probes for northern analyses.

C.9. Northern analysis Northern analyses of total RNA was performed as previously described (Schroeder and Cousins, 1990) using PCR-amplified partial cDNAs labeled with 50 μCi α-^{32}P-dCTP (DuPont NEN) by the random oligolabeling method (Prime-a-Gene Labeling System from Promega).

C.10. cDNA cloning After screening for the false positives, the PCR-amplified partial cDNA was inserted into the pCR2.1-TOPO plasmid vector using TOPO TA Cloning Kit (Invitrogen). This plasmid was used to transform TOP 10 ONE shot™ Cells with One Shot kit (Invitrogen).

C.11. DNA sequencing The transformed competent cells were propagated in LB-media and the plasmid was isolated (QIAprep Spin Miniprep Kit, QIAGEN, Germany) for sequencing at the Michigan State University Core Sequencing Facility. DNA sequences were obtained and submitted to the GenBank.

C.12. Immunoblot analysis for calmodulin Cellular protein was extracted from LLC-PK_1 cells with lysis buffer (20 mM Tris-Cl, pH 8.0, 150 mM NaCl, 10 % glycerol, 1 % Triton X-100, 2 mM EDTA, 1 mM penylmethylsulfonyl fluoride, 10 µM of leupeptin and aprotinin cocktail solution), sonicated, and centrifuged. The supernatant protein was denatured by incubating for 5 min at 95 °C in Laemmli buffer. The sample was applied to 15 % SDS-bis acrylamide denaturing gel and transferred onto nitrocellulose membrane. The protein on the membrane was blocked by overnight incubation in 5 % non-fat dry milk/TBS-Tween 20 (0.1%), pH 7.4. The membrane was probed with anti-calmoduin antibody (Santa Cruz) raised against a goat calmodulin for 1 hour at room temperature and treated with anti-goat IgG conjugated with horseradish peroxidase for additional 1 hour at room temperature. Chemiluminescence solution from Santa Cruz was used to develop the immunoblot.

C.13. Cell protein measurement. Total cell protein was determined by the method of Lowry *et al.* (1995) using bovine serum albumin as a standard.

C.14. Statistical analysis. Data from sphinganine were subjected to ANOVA and Bonferroni's all pairwise comparison tests. The Student t test was used to analyze flow cytometric data. Differences were considered statistically significant at $p \leq 0.05$

D. RESULTS

LLC-PK$_1$ renal kidney epithelial cells were used as a model systemfor these studies because the kidney is the most sensitive organ to fumonisin toxicity (Bucci *et al.*, 1998; Gumprecht *et al.*, 1995) and Yoo *et al.* (1992, 1996) have shown a strong association between death of LLC-PK$_1$ cells caused by fumonisin B$_1$ and accumulation of sphinganine.

Fumonisin B$_1$ induces apoptosis and causes sphinganine accumulation in LLC-PK$_1$ cells. Morphological changes characteristic of apoptosis, including cell shrinkage, membrane blebbing, chromatin condensation, and the formation of apoptotic bodies were observed in LLC-PK$_1$ cells cultured with 50 μM fumonisin B$_1$ (data not shown). To further investigate the mechanism of death, LLC-PK$_1$ cells cultured with fumonisin B$_1$ were examined for the presence of DNA fragmentation, a hallmark of apoptosis. Control cultures (without toxin) showed no evidence of DNA fragmentation for up to 48 h (data not shown). In contrast, fumonisin B$_1$ produced time-dependent increases in a 200-bp DNA ladder indicative of apoptosis (Figure 2.1A). DNA fragmentation was detectable within 24 h of exposure to fumonisin B$_1$, with a maximal response at 48 h. Fumonisin B$_1$ increased the percentage of cells undergoing apoptosis by about ~6.5-fold at 48 h (Figure 2.1B). Fumonisin B$_1$ at 50 μM also produced time-dependent increases in sphinganine. Fumonisin B$_1$ caused sphinganine accumulation within 6 h (Figure 2.2). Maximal accumulation of ~1.7 nmol/mg protein (~50-fold that of controls) was achieved by 48 h. Thereafter, sphinganine in fumonisin-treated cultures decreased to ~1.2 nmol/mg protein by 72 h.

β-Fluoroalanine blocks sphinganine accumulation caused by fumonisin B$_1$. The findings demonstrate that fumonisin B$_1$ induces kidney cell apoptosis over a time course that is consistent with dependence upon accumulation of sphinganine but do not prove a cause

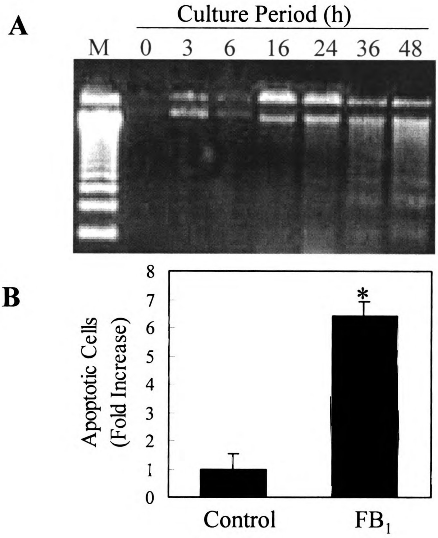


Figure 2.1. Fumonisin B_1 induces internucleosomal DNA fragmentation in LLC-PK$_1$ kidney cells. (A) LLC-PK$_1$ cells were cultured in the presence of fumonisin B_1 at 50 μM for various lengths of time and DNA fragmentation was assessed by agarose gel electrophoresis as described under Materials and Methods Results are from a representative experiment. M: 123-bp DNA markers. (B) LLC-PK$_1$ cells were cultured in the absence or presence of fumonisin B_1 at 50 μM and the percentage of cells in the sub-G0/G1 apoptotic region was determined by flow cytometric analysis as described under Materials and Methods.

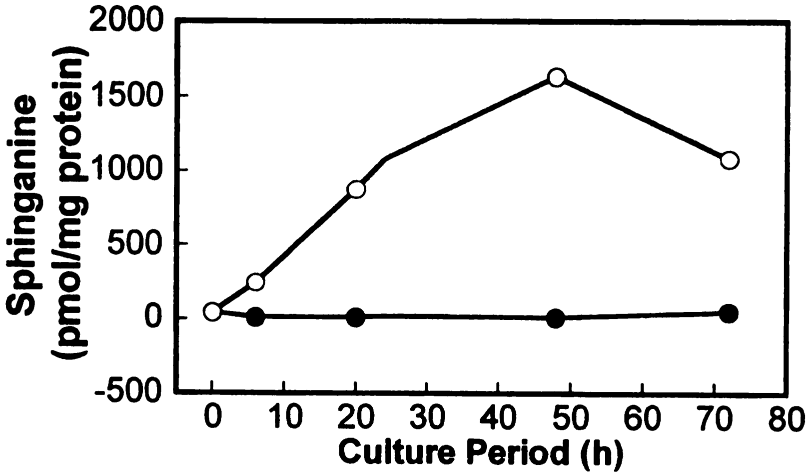


Figure 2.2. Fumonisin B_1 causes sphinganine accumulation in LLC-PK_1 kidney cells. LLC-PK_1 cells were cultured either in the absence (●) or presence (○) of fumonisin B_1 at 50 μM for various lengths of time. Cellular concentrations of sphinganine were determined by HPLC as described under Materials and Method. Each value is the mean ± SD (n=3) from a representative experiment. Error bars that are not visible are hidden by the symbols. * value is significantly different ($p \leq 0.05$) from corresponding control value.

and effect relationship. To obtain more information about the mechanism, studies were conducted using β-fluoroalanine. β-haloalanines act as mechanism-based irreversible inhibitors of serine palmitoyltransferase by substituting for the substrate serine during Schiff base formation (Medlock and Merrill, 1988). β-fluoroalanine is ~1000-fold more potent than β-chloroalanine for inhibition of serine palmitoyltransferase. Because serine palmitoyltransferase catalyzes the initial, rate-limiting step of sphingolipid biosynthesis, β-haloalanines block *de novo* synthesis of sphingolipids but would not be expected to cause accumulation of sphinganine.

LLC-PK$_1$ cells were cultured with combinations of fumonisin B$_1$ (50 μM) and β-fluoroalanine (50 μM) to determine the effects on sphinganine accumulation and DNA fragmentation. The addition of fumonisin B$_1$ alone increased sphinganine (Figure 2.3) and stimulated DNA fragmentation (Figure 2.4); whereas, β-fluoroalanine alone had no effect on sphinganine (Figure 2.3) or DNA fragmentation (Figure 2.4). In contrast, the stimulatory effects of fumonisin B$_1$ on sphinganine accumulation and DNA fragmentation were completely blocked when β-fluoroalanine was added together with fumonisin B$_1$ (Figures 2.3 and 2.4). These results establish that the *de novo* biosynthesis (and, presumably, accumulation) of sphinganine or a metabolite such as the 1-phosphate is required for the stimulation of DNA fragmentation by fumonisin B$_1$.

Fumonisin B$_1$ does not affect expression of bax, bcl-2, and bcl-x. Since altered expression of *bcl-2* family genes has been implicated in apoptosis induced by free sphingoid bases, the effects of fumonisin B$_1$ on mRNA of these genes was examined using competitive RT-PCR. Figure 2.5 shows *bax*, *bcl-2*, and *bcl-x* mRNA after 16 h of exposure to an apoptotic concentration of fumonisin B$_1$ (50 μM). Densitometric analysis indicated the toxin

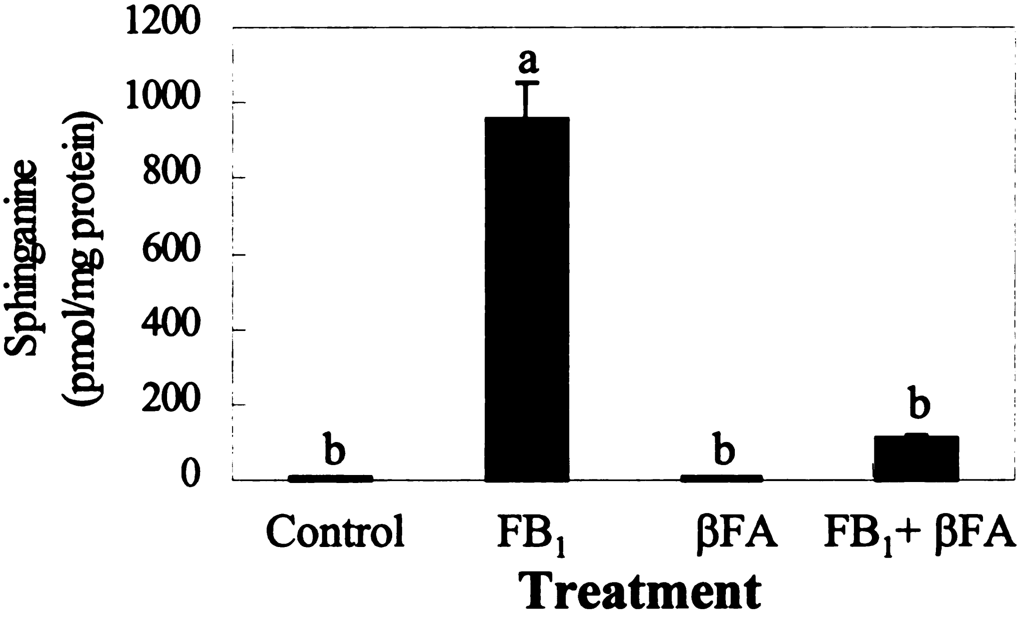


Figure 2.3. Inhibition of fumonisin B$_1$-stimulated sphinganine accumulation in LLC-PK$_1$ cells by β–fluoroalanine. LLC-PK$_1$ cells were cultured in the absence or presence of fumonisin B$_1$ (FB$_1$) at 50 μM and β–fluoroalanine (βFA) at 50 μM as indicated. After 16 hours, cellular concentrations of sphinganine were determined by HPLC as described under Materials and Methods. Each value is the mean ± SD from a representative experiment. Means with differing letters are significantly different ($p \leq 0.05$).

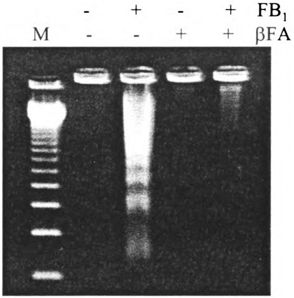


Figure 2.4. Inhibition of fumonisin B_1-induced DNA fragmentation in LLC-PK_1 cells by β–fluoroalanine. LLC-PK_1 cells were cultured in the absence or presence of fumonisin B_1 (FB_1) at 50 μM and/or β–fluoroalanine (βFA) at 50 μM. After 72 hours, cells were harvested and DNA fragmentation was assessed by agarose gel electrophoresis as described under "Materials and Methods". Results are from a representative experiment. M: 123 base-pair DNA markers.

had no effect on expression of these genes. These results suggest that alterations in *bcl-2* family gene expression are not necessary for fumonisin B_1-induced apoptosis in LLC-PK_1 kidney cells.

Fumonisin B_1 induces expression of calmodulin mRNA by a sphinganine-dependent pathway. As an alternative approach to studying individual mRNAs, we have further examined RNA isolated after 16 h of culture with 50 μM fumonisin B_1 using DDRT-PCR. This method provides an unbiased approach to analyze changes in gene expression associated with a treatment without DNA sequencing information (Liang and Pardee, 1992). Using DDRT-PCR we have identified ~40 cDNAs that are differentially displayed in response to fumonisin B_1. Figure 2.6 shows examples of three up-regulated kidney cell cDNAs (K1 from control cells and K2 and K3 from cells cultured with fumonisin B_1. Northern analysis using the amplified cDNAs as probes confirmed differential expression of K3 (Figure 2.7); whereas, K1 and K2 were false positives (data not shown). The sequence of the K3 insert was determined and submitted to GenBank. The highest homology corresponds to human and mouse calmodulin, with 98% base sequence identity and 100% amino acid identity to human calmodulin (Figure 2.8).

These findings establish that calmodulin mRNA is increased in LLC-PK_1 cells after 16 h of exposure to fumonisin B_1 and are consistent with, but not proven, to be dependent upon prior accumulation of sphinganine. To obtain information about the role of sphinganine in fumonisin-induced calmodulin expression, β-fluoroalanine was used to block sphinganine accumulation. As shown in Figures 2.9A and 2.9B, the addition of fumonisin B_1 alone stimulated calmodulin mRNA ~6.5-fold. In contrast, the stimulatory effect of fumonisin B_1 on calmodulin expression was reduced by about 50% when β-fluoroalanine

Figure 2.5. Fumonisin B_1 does not affect gene expressions of *bcl-2* family genes in LLC-PK_1 cells. LLC-PK_1 cells were cultured in the absence (C) or presence (F) of fumonisin B_1 at 50 μM. After 16 hr, total RNA were isolated and *bax*, *bcl-2*, and *bcl-x* mRNA was quantitated by RT-PCR as described under Materials and Methods. G3PDH, glyceraldehyde 3-phosphate dehydrogenase. Results are from a representative experiment.

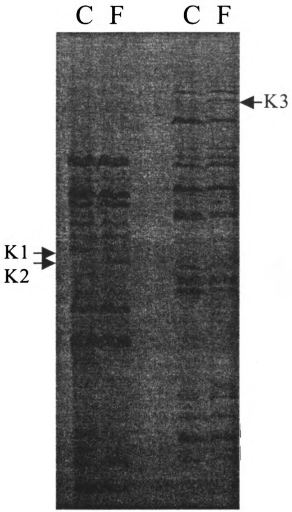


Figure 2.6. Differential display of LLC-PK$_1$ kidney cell cDNAs. LLC-PK$_1$ cells were cultured in the absence (C) or in the presence (F) of fumonisin B$_1$ at 50 μM. After 16 hours, total RNA was isolated and used for DDRT-PCR as described under Materials and Methods. The two primer combinations that were used for this experiment were: 5'-T11-G-3' and 5'-GCAATCGATG-3' (lanes 1 and 2) and 5'-T11-A-3' and 5'-CCGAAGGAAT-3' (lanes 3 and 4).

Figure 2.7. Fumonisin B_1 induces expression of K3 mRNA. LLC-PK_1 cells were cultured in the absence (C) or presence of fumonisin B_1 (FB_1) at 50 μM. After 16 hr, total RNA was isolated and examined via Northern analysis as described under Materials and Methods.

K3	tgtgtttgataaggatggcaatggctatattagtgc**a**gcaga**g**cttcgccatgtgatgac 60
Human CaM	tgtgtttgataaggatggcaatggctatattagtgc**t**gcaga**a**cttcgccatgtgatgac 400
K3	aaaccttggagagaagttaacagatgaaga**g**gttgatgaaatgatcagggaagcagatat 120
Human CaM	aaaccttggagagaagttaacagatgaaga**a**gttgatgaaatgatcagggaagcagatat 460
K3	tgatggtgatggtcaagtaaactatgaagagtttgtacaaatgatgacagcaaagtgaag 180
Human CaM	tgatggtgatggtcaagtaaactatgaagagtttgtacaaatgatgacagcaaagtgaag 520
K3	ac**g**ttgtacagaatgtgttaaatttcttgtacaaaattgtttatttgccttttctttgtt 240
Human CaM	ac**c**ttgtacagaatgtgttaaatttcttgtacaaaattgtttatttgccttttctttgtt 580

Figure 2.8. Sequence homology of K3 DNA insert with human calmodulin. The K3 cDNA insert was cloned and sequenced as described under Materials and Methods and submitted to GenBank for identification. Shown are the K3 cDNA insert and the corresponding sequence of human calmodulin (human CaM). K3 cDNA bases shown in bold are different from the corresponding base of human calmodulin.

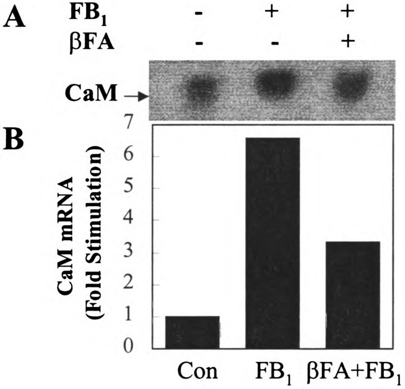


Figure 2.9. Inhibition of fumonisin B_1-induced calmodulin expression in LLC-PK_1 cells by β-fluoroalanine. LLC-PK_1 cells were cultured in the absence or presence of fumonisin B_1 (FB_1) at 50 μM and/or β–fluoroalanine (βFA) at 50 μM. After 16 hr, total RNA was isolated. (A) Northern analysis using K3 cDNA as a probe as described under Materials and Methods. (B) Densitometric analysis of the Northern blot as described under Materials and Methods. Results are from a representative experiment.

was added together with fumonisin B_1. This finding establishes that fumonisin B_1-induced expression ofcalmodulin mRNA is dependent on accumulation of sphinganine.

Fumonisin B_1 increases calmodulin protein in LLC-PK_1 kidney cells. Additional studies were conducted to examine the effect of fumonisin B_1 on calmodulin protein. LLC-PK_1 cells were cultured with fumonisin B_1 for 16 h and calmodulin protein was assessed by Western analysis. Fumonisin B_1 produced concentration-dependent increases in calmodulin protein with a maximun response of ~4-fold at 50 μM (the highest dose tested) (Figures 2.10A and 2.10B)

Calmodulin antagonist blocks fumonisin-induced DNA fragmentation. The findings to this point establish that fumonisin B_1 induces calmodulin expression as well as apoptosis in LLC-PK_1 cells via a sphinganine-dependent mechanism. To determine whether calmodulin plays a causal role in fumonisin-induced apoptosis, studies were conducted using W7. W7 acts as a calmodulin antagonist by competitively binding to calmodulin at the same site to which calmodulin-dependent enzymes bind (Osawa *et al.*, 1998). LLC-PK_1 cells were cultured with combinations of fumonisin B_1 and W7 to determine their effects on DNA fragmentation. The addition of fumonisin B_1 alone increased DNA fragmentation, while W7 alone at either 10 or 20 μM had no effect (Figure 2.11). However, the stimulatory effect of fumonisin B_1 on DNA fragmentation was almost completely eliminated when W7 was added together with fumonisin B_1. These results establish that fumonisin B_1 induces apoptosis in LLC-PK_1 kidney cells by a calmodulin-dependent mechanism.

E. DISCUSSION

Fumonisin mycotoxins are of growing concern because of their prevalence in corn

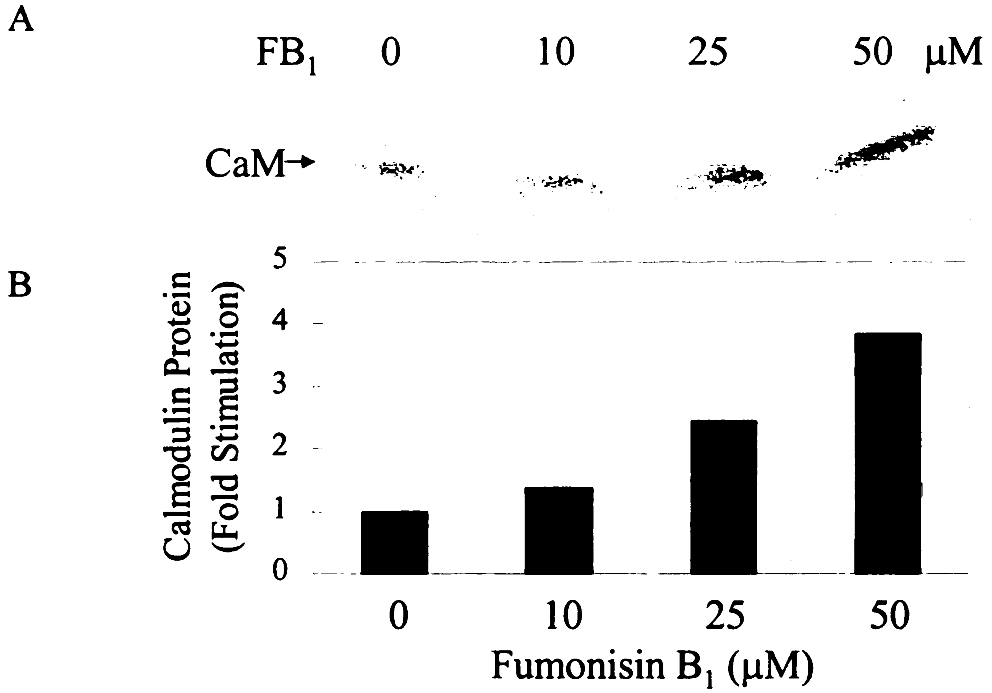


Figure 2.10. Concentration of fumonisin B$_1$ induction of calmodulin protein in LLC-PK$_1$ kidney cells. LLC-PK$_1$ cells were cultured with various concentrations of fumonisin B$_1$ (FB$_1$). After 16 hr, cellular protein was isolated. (A) Immunoblot analysis of calmodulin as described under Materials and Methods. (B) Densitometric analysis of the immunoblot as described under Materials and Methods. Results are from a representative experiment.

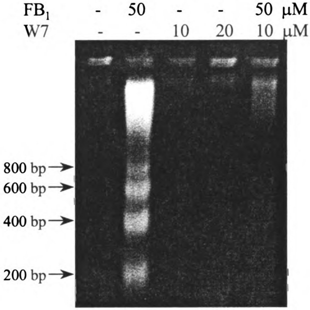


Figure 2.11. Inhibition of fumonisin-induced DNA fragmentation in LLC-PK$_1$ cells by calmodulin antagonist, W7. LLC-PK$_1$ cells were cultured in the absence or presence of fumonisin B$_1$ as indicated. W7 was also added to some cultures 16 hr after the addition of fumonisin B$_1$. After 48 hr, DNA fragmentation was assessed by agarose gel electrophoresis as described under Materials and Methods. Results are from a representative experiment.

and corn-based products (Shephard *et al.*, 1996) as well as other agricultural commodities and because of their toxic and carcinogenic properties for animals and, perhaps, humans (Marasas, 1996). Across species, the most sensitive organ to fumonisin toxicity is the kidney (Bucci *et al*, 1998; Gumprecht *et al*, 1995). The primary response of kidney to consumption of feed containing fumonisin B_1 is tubular epithelial cell apoptosis (Bucci *et al.*, 1998; Gumprecht *et al*, 1995). Yoo *et al.* (1992, 1996) have reported that fumonisin B_1 is cytotoxic for LLC-PK_1 porcine renal tubular epithelial cells, suggesting that this cell line may be an appropriate model to further investigate the molecular mechanism of renal toxicity caused by fumonisins. A major finding of the current study is that fumonisin B_1 kills LLC-PK_1 cells by inducing apoptosis. The concentration of fumonisin necessary to induce apoptosis, 50 μM, is identical to that previously reported to be toxic for these cells (Yoo *et al.*, 1992, 1996) and is similar to that which induces apoptosis in a variety of other cultured cells, including human keratinocytes, fibroblasts, esophageal epithelial cells, and hepatoma cells (Tolleson *et al.*, 1996b).

Numerous studies have demonstrated that the mechanism of action of fumonisins appears to involve disruption of sphingolipid metabolism via inhibition of sphinganine *N*-acyltransferase (Merrill *et al.*, 1997a). The inhibition causes accumulation of sphingoid bases in a variety of cell types (Norred *et al.*, 1992; Wang *et al.*, 1991; Yoo *et al.*, 1992; Schroeder *et al.*, 1994; Tolleson *et al.*, 1999) and in the tissues and urine of animals fed fumonisin-contaminated grain (Merrill *et al.*, 1997b; Morgan *et al.*, 1997; Riley *et al.*, 1993a, 1994; van der Westhuizen *et al.*, 2001; Wang *et al.*, 1992). In the present study, an apoptotic concentration of fumonisin B_1 caused cellular sphinganine to increase to ~1.0 nmol/mg of protein at least 8 h prior to evidence of DNA fragmentation. There are several possible mechanisms whereby disruption of sphingolipid metabolism could cause apoptosis. First, fumonisin B_1 might block the formation of a complex sphingolipid(s) that is required for growth and cell survival. Hanada *et al.* (1992) showed in mutant Chinese hamster ovary cells (which can not synthesize complex sphingolipids) that addition of exogenous complex sphingolipids restored the ability of the cells to proliferate. Moreover, the addition of ceramide derivative reversed fumonisin inhibition of axonal growth in hippocampal neurons (Harel and Futtemlan, 1993) and the addition of *N*-acetylsphinganine partially protected human keratinocytes from fumonisin B_1-induced apoptosis (Tolleson *et al.,* 1999). Moreover, Yoo *et al.* (1996) demonstrated that, when β-chloroalanine was used to inhibit *de novo* synthesis of sphingolipids in LLC-PK_1 cells to a similar degree as fumonisin B_1, it both inhibited cell growth and increased cell death. In contrast, when *de novo* synthesis of sphingolipids was blocked in the present study using a more specific inhibitor of serine palmitoyltransferase, β-fluoroalanine, there was no effect on DNA fragmentation. Thus, reduced synthesis of complex sphingolipids does not appear to explain the apoptotic effect of fumonisin B_1 in LLC-PK_1 cells.

The second possible mechanism whereby disruption of sphingolipid metabolism could mediate fumonisin B_1 induction of apoptosis is by causing accumulation of the sphingoid base sphinganine (or a metabolite). Free sphingoid bases have been shown to be both growth inhibitory and cytotoxic for a variety of cell systems (Hannun *et al.,* 1991; Stevens *et al.,* 1990). Moreover, Yoo *et al.* (1996) reported that sphingoid base accumulation was, in part, responsible for fumonisin toxicity in LLC-PK_1 kidney cells. In support of a causal relationship between sphinganine accumulation and apoptosis, our study shows that fumonisin B_1-induced DNA fragmentation was virtually eliminated upon the addition of β-fluoroalanine, which blocked sphinganine synthesis and reduced the cellular sphinganine to a concentration similar to that of control cultures. These results provide compelling evidence that fumonisin B_1 induces apoptosis in LLC-PK_1 kidney cells by a pathway that involves disruption of sphingolipid metabolism and requires accumulation of sphinganine.

Sphinganine could mediate the apoptosis induced by fumonisin B_1 either by a direct action or via conversion to a metabolite. Exogenous sphingoid bases can be converted to ceramide, a sphingolipid that induces apoptosis (Obeid *et al.,* 1993). However, a role for ceramide in mediating fumonisin-induced apoptosis seems unlikely because the conversion of sphinganine to ceramide is blocked by fumonisins. Another sphinganine metabolite that could mediate apoptosis is the 1-phosphate metabolite. Though sphingoid base 1-phosphate is most often considered to be mitogenic (Zhang *et al.,* 1991), recent studies have demonstrated that this molecule also has antiproliferative properties (Bornfeldt *et al.,* 1995; Davaille *et al.,* 2000).

The mechanism by which fumonisins induce apoptosis via sphinganine is not clear; however, recent studies demonstrate that apoptosis induced by sphingosine in HL-60 cells is accompanied by a decrease in expression of *bcl-2,* an apoptosis suppresser gene (Sakakura *et al.,* 1996). In addition, sphingosine induces apoptosis in androgen-independent human prostatic carcinoma DU-45 cells by down-regulating *bcl-x_L* (Shirahama *et al.,* 1997). Therefore, sphingoid bases may mediate fumonisin-induced cell death by regulating expression of a *bcl-2* family gene(s). However, in our study, an apoptotic concentration of fumonisin B_1 had no effect on *bax, bcl-2,* or *bcl-x* mRNA after 16 h of exposure. These results indicate that apoptosis of LLC-PK_1 cells is not due to an effect on *bcl-2* family gene expression. As an alternative to studying individual mRNAs, the present study used DDRT-PCR as an unbiased approach to analyze changes in gene expression associated with fumonisin B_1 treatment. A major finding of our study using this technique is that fumonisin B_1 induces calmodulin expression. The mycotoxin induces calmodulin expression in a concentration-dependent manner with maximal effects of ~6.5-fold at the mRNA level and ~4-fold at the protein level with an apoptotic concentration of fumonisin B_1. Fumonisin B_1 could be envisioned to increase calmodulin expression either via a mechanism that is independent of its effects on sphingolipid metabolism or by a mechanism that is mediated via disruption of sphingolipid metabolism. In support of a causal role of sphinganine, fumonisin B_1 stimulation of calmodulin expression was reduced by ~50% upon the addition of β-fluoroalanine, which blocked sphinganine synthesis. This result provides convincing evidence that fumonisin B_1 induces calmodulin expression by a pathway that requires accumulation of sphinganine.

The mechanism by which fumonisins stimulate calmodulin expression via sphinganine is not known. Transcriptional regulation of calmodulin is still poorly understood. However, Toutenhoofd *et al.* (1998) have identified possible AP-1 binding sites and TATA-like sequences 27 nucleotides upstream of the transcriptional start site in the 5' flanking sequence of human calmodulin 2 and the sphingoid base sphingosine has been shown to induce DNA binding activity of AP-1 (Su *et al.*, 1994). In addition, Wang *et al.* (1996) have hypothesized that fumonisin-induced growth arrest and apoptosis in African green monkey kidney cells was a result of AP-1 repression via inhibition of protein kinase C. No attempt was made to relate changes to fumonisin disruption of sphingolipid metabolism; however, the effects on protein kinase C are consistent with fumonisin-induced sphinganine accumulation, since sphingoid bases are well-established inhibitors of protein kinase C (Hannun *et al.,* 1991).

In support of a causal role of calmodulin induction in apoptosis, another major finding of our study is that DNA fragmentation induced by fumonisin B_1 was virtually eliminated upon the addition of the calmodulin antagonist W-7. This finding provides strong evidence that fumonisin B_1 induces apoptosis in LLC-PK_1 kidney cells by a pathway that requires calmodulin. This discovery is consistent with the results of several recent studies that indicate a role for calmodulin in apoptosis. For example, Rosenthal *et al.* (1998) have reported that W-7 blocked apoptosis induced by sulfur mustard. In addition, Micoli *et al.* (2000) have demonstrated that the proapoptotic effect of HIV-1 envelope glycoprotein gp160 is blocked by the calmodulin antagonist trifluoroperazine. The mechanism by which fumonisin induction of calmodulin leads to apoptosis has not been examined but could involve activation of calcineurin (Stemmer and Klee, 1994), which, in turn, has been shown to dephosphorylate BAD, a proapoptotic member of the *bcl-2* family (Wang et al., 1999). Activation of calcineurin by calmodulin is achieved by lowering the threshold concentration of calcium needed for activation and calcium mobilizing agents such as ionomycin and A23187 induce dephosphorylation of BAD, indicating activation of calcineurin (Wang *et al.*, 1999). Since fumonisin B_1 has been shown to increase sphinganine 1-phosphate in some cell types (Smith and Merrill, 1995) and sphingosine 1-phosphate mobilizes cellular calcium (Olivera *et al.,* 1994), fumonisins may trigger bimodal regulation of calcineurin to dephosphorylate BAD and induce apoptosis.

Taken together, the findings of this study using LLC-PK_1 cells provide a plausible molecular mechanism to explain toxicity of fumonisins to kidney renal tubular epithelial cells. Fumonisin B_1 was found to disrupt sphingolipid metabolism and cause apoptosis by a pathway that involved initial accumulation of sphinganine, which, in turn, stimulated induction of calmodulin.

CHAPTER III

SPHINGANINE 1-PHOSPHATE MEDIATES CALMODULIN EXPRESSION INDUCED BY FUMONISIN B_1 IN LLC-PK_1 PORCINE RENAL TUBULAR EPITHELIAL CELLS

A. ABSTRACT

Fumonisins are a family of mycotoxins produced by *Fusarium moniliforme*, which is the most common mold found on corn throughout the world. These compounds are both toxic and carcinogenic for animals, and perhaps humans, with the kidney being the most sensitive organ to fumonisin toxicity. Fumonisin B_1, the most prevalent of these mycotoxins, induces calmodulin expression in LLC-PK_1 porcine kidney epithelial cells and causes apoptosis via a calmodulin-dependent pathway (Kim *et al.*, 2001). The molecular mechanism of calmodulin induction by fumonisin B_1 is not known, but appears to involve disruption of sphingolipid metabolism. Fumonisin B_1 inhibits ceramide synthase in the *de novo* biosynthetic pathway of sphingolipids and causes depletion of complex sphingolipids and accumulation of sphingoid bases. In the present study, LLC-PK_1 porcine renal tubular epithelial cells were used to assess the influence of fumonisin B_1 on calmodulin mRNA stability and to determine whether induction of calmodulin by fumonisin B_1 is mediated by the depletion of complex sphingolipids or accumulation of the sphingoid base sphinganine or the 1-phosphate metabolite. Fumonisin B_1 increased both calmodulin mRNA and calmodulin protein in concentration-dependent manners, but did not influence stability of calmodulin mRNA, suggesting that fumonisin B_1 induces calmodulin by increasing transcription. Moreover, the transcription inhibitor actinomycin D blocked fumonisin-induced apoptosis. Neither the addition of exogenous sphinganine nor β-fluoroalanine (an inhibitor of serine palmitoyltransferase which depletes complex sphingolipids without causing sphinganine accumulation) induced calmodulin expression; whereas, sphinganine 1-phosphate increased calmodulin mRNA. Taken together, these findings suggest that fumonisin B_1 induces calmodulin in LLC-PK_1 kidney cells by disrupting sphingolipid

metabolism and causing accumulation of the sphinganine metabolite sphinganine 1-phosphate which, in turn, increases transcription of the calmodulin gene.

B. INTRODUCTION

Fumonisins are toxic and carcinogenic toxins produced by *Fusarium moniliforme*, the most common mold found on corn in the United States and throughout the world (Marasas, 1996). Fumonisin B_1 is the most abundant of these mycotoxins and has been correlated with several animal diseases with kidney being the most sensitive organ (Bondy *et al.*, 1996; Bucci *et al.*, 1998). In rats, consumption of feed contaminated with fumonisin B_1 causes apoptosis in renal tubular epithelial cells within 48 hr (Voss *et al*, 2001) and renal tubular adenomas and carcinomas within two years (Howard *et al.*, 2001). In addition to kidney carcinogenicity, fumonisin B_1 consumption has been linked to esophageal (Marasas *et al.*, 1988), liver (Ueno *et al.*, 1997) and stomach (Groves *et al.*, 1999) cancers in humans. The molecular mechanism of fumonisin-induced apoptosis is not clear, but appears to involve calmodulin induction (Kim *et al.*, 2001). In LLC-PK_1 renal tubular epithelial cells, fumonisin B_1 induces calmodulin expression and calmodulin activity is required for fumonisin B_1-induced apoptosis (Kim *et al.*, 2001).

The mechanism by which fumonisin B_1 induces calmodulin is not clear. Due to structural similarity with sphingoid bases, fumonisin B_1 inhibits ceramide synthase (sphinganine *N*-acyltransferase) (Figure 3.1). The inhibition causes depletion of complex sphingolipids as well as accumulation of sphingoid bases and/or the 1-phosphate metabolite (Smith and Merrill, 1995; Norred *et al.*, 1992; Wang *et al.*, 1991; Yoo *et al.*, 1992; Schroeder *et al.*, 1994; Tolleson *et al.*, 1999) and in the tissues and urine of animals fed fumonisin-

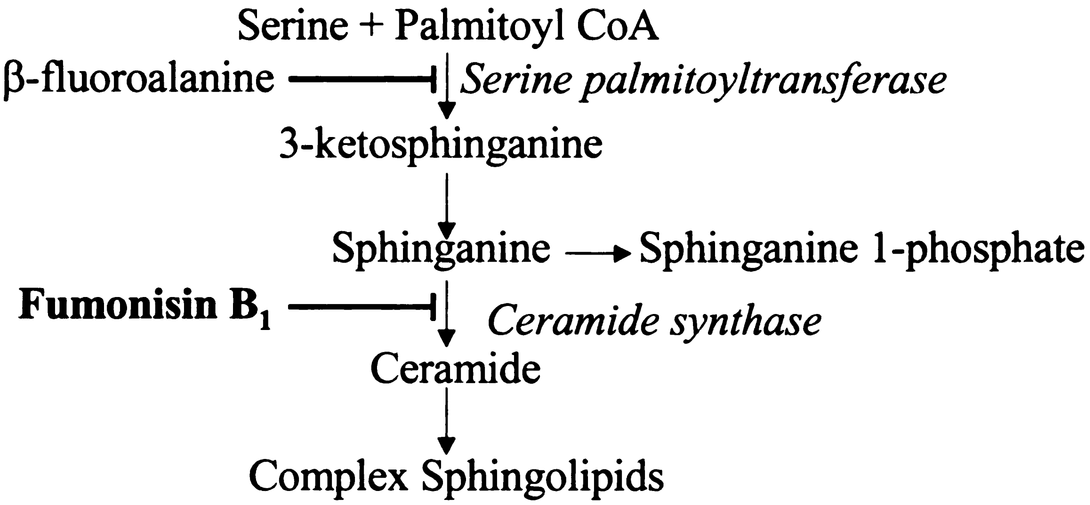


Figure 3.1. ***De novo*** **biosynthetic pathway of sphingolipids and inhibition of ceramide synthase by fumonisin B_1**

contaminated grain (Merrill *et al.*, 1997b; Morgan *et al.*, 1997; Riley *et al.*, 1993a, 1994; van der Westhuizen *et al.*, 2001; Wang *et al.*, 1992). Fumonisin-induced toxicities both *in vitro* and *in vivo* are dependent on disruption of sphingolipid metabolism (Martinova and Merrill, 1995; Restum *et al.*, 1995; Yoo *et al.*, 1996; Yoo *et al.*, 1992) and fumonisin-induced calmodulin expression in LLC-PK$_1$ porcine renal epithelial cells was blunted by β-fluoroalanine, an inhibitor of *de novo* biosynthesis of sphingolipids which blocks at an earlier step than fumonisins and prevents sphinganine accumulation (Kim *et al.*, 2001). Therefore, fumonisin-induced calmodulin expression is, at least in part, dependent on accumulation of sphinganine and/or a metabolite such as the 1-phosphate (Kim *et al.*, 2001).

In the present study, LLC-PK$_1$ porcine renal tubular epithelial cells were used to test the hypothesis that the induction of calmodulin mRNA by fumonisin B$_1$ is mediated by the depletion of complex sphingolipids or accumulation of sphinganine and/or sphinganine 1-phosphate. In addition, the transcription inhibitor actinomycin D was used to examine the effect of fumonisin B$_1$ on the stability of calmodulin mRNA as well as the requirement for novel gene transcription in fumonisin-induced apoptosis.

C. MATERIALS AND METHODS

C.1. LLC-PK$_1$ cell culture Porcine kidney epithelial LLC-PK$_1$ cells were obtained from American Type Culture Collection (ATCC) and cultured in Dulbecco's Modified Eagle Medium/F-12K nutrient solution (Gibco BRL) (1:1) containing 5% (v/v) fetal bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin sulfate, and 100 μM of L-serine (Gibco BRL) in a 5 % CO_2 incubator at 37°C. Cells (~8 x 10^5) were plated on 100-mm diameter tissue culture dishes (Corning, NY) and cultured for ~48 hr before addition of treatments.

C.2. Materials Fumonisin B_1 used in this study was purchased from PROMEC (South Africa) and actinomycin D (4 ng/ml) was obtained from Sigma (St. Louis, MO). Sphinganine and sphinganine 1-phosphate were purchased from Biomol (Plymouth Meeting, PA). Sphinganine was prepared by forming a 1:1 complex with Bovine Serum Albumin (cell culture grade, GibcoBRL). Sphinganine 1-phosphate was dissolved in methanol:tetrahydrofuran:water (60:30:10) at 65°C, evaporated, and dissolved in 0.4% BSA solution. RNA Stat-60 was from Tel-Test, Inc (Friendswood, TX).

C.3. Analysis of DNA fragmentation by agarose gel electrophoresis assay Following treatments, fragmented DNA from LLC-PK1 cells was extracted as described (Sellins and Cohen, 1987). In brief, cells from 100-mm dish were harvested using rubber policeman and collected by centrifugation (5 min, 500 g). The medium was removed and the cell pellet was resuspended in phosphate-buffered saline (PBS). After another centrifuge, the pellet was suspended in 0.1 ml hypotonic lysis buffer (10 mM Tris, 10 mM EDTA, 0.5% Triton X-100, pH 8.0). Cells were incubated for 10 min at 4°C and the lysate was centrifuged (30 min, 13,000 g, 4°C). The supernatant containing fragmented DNA was treated with RNase A (0.4 μg/μl) for 1 hr at 37°C and then incubated with proteinase K (0.4 μg/μl) for 1 h at 37°C. DNA was precipitated overnight by incubation at -20°C in 50% isopropanol and 0.5 M NaCl. The DNA precipitate was pelleted by centrifugation (30 min, 13,000 g, 4°C), dissolved in distilled H_2O and then electrophoresed in a 2 % agarose gel containing ethidium bromide (0.5 μg/ml) in 0.5X Tris-borate-EDTA buffer. After electrophoresis, DNA was visualized with a UV transilluminator and photographs were taken using a Polaroid system.

C.4. Differential display reverse transcription polymerase chain reaction (DDRT-

PCR) Differential display gel kits including RTase (Genhunter Corp., Nashville, TN) were used for this study as described previously (Kim *et al.*, 2001).

C.5. Immunoblot analysis for calmodulin Cellular protein was extracted from LLC-PK$_1$ cells with lysis buffer (20 mM Tris-Cl, pH 8.0, 150 mM NaCl, 10 % glycerol, 1 % Triton X-100, 2 mM EDTA, 1 mM penylmethylsulfonyl fluoride, 10 μM of leupeptin and aprotinin cocktail solution), sonicated, and centrifuged. The supernatant protein was denatured by incubating for 5 min at 95 °C in Laemmli buffer. The sample was applied to 15 % SDS-bis acrylamide denaturing gel and transferred onto nitrocellulose membrane. The protein on the membrane was blocked by overnight incubation in 5 % non-fat dry milk/TBS-Tween 20 (0.1%), pH 7.4. The membrane was probed with anti-calmoduin antibody (Santa Cruz) raised against a goat calmodulin for 1 hour at room temperature and treated with anti-goat IgG conjugated with horseradish peroxidase for additional 1 hour at room temperature. Chemiluminescence solution from Santa Cruz was used to develop the immunoblot.

C.6. Northern blot analysis Total cellular RNA was extracted from LLC-PK$_1$ cells by the modification of the one-step method (Chomczynski and Sacchi, 1987) using RNA Stat-60 reagent (Tel-test "B" Inc., Friendswood, TX). 20 μg of total RNA was separated by electrophoresis on a 1.2% (wt/vol) agarose gel (Gibco BRL) containing 0.6 M formaldehyde (Sigma), 0.02 M MOPS (Gibco BRL) at 80 Volt for ~3 hr. The gel was stained with ethidium bromide to verify the integrity and equal RNA loading, and the RNA was transferred to a nylon membrane (NEN, Boston, MA) with 20X SSC. PCR-amplified calmodulin cDNAs (Kim *et al.*, 2001) labeled with 50 μCi α-^{32}P-dCTP (DuPont NEN) were prepared by random oligo-labeling method (Prime-a-Gene Labeling System, Promega). After cross-linking, the membrane was pre-hybridized at 42°C in Denhardt's hybridization buffer (Kingston, 1997) for 2 hr. Blots were hybridized at the same temperature overnight in a hybridization bag containing radio-labeled cDNA probe described earlier. The membrane was then washed in low and high stringency washing buffer and exposed to the K-screen (Kodak, Rochester, NY) for phospho-imager analysis. Density of each band was quantified using the FX software program after background was subtracted.

C.7. Statistical Analysis The statistical analysis was performed by using Sigma-Stat Analysis system (Jandel Scientific, San Rafael, CA). The half lives for the calmodulin mRNAs were calculated using regression analysis. The student t-test was used to analyze calmodulin mRNA for statistical significance. Differences were considered statistically significant at $p \leq 0.05$.

D. RESULTS

LLC-PK$_1$ renal kidney epithelial cells were used as a model system for these studies because the kidney is the most sensitive organ to fumonisin toxicity (Bucci *et al.*, 1998; Gumprecht *et al.*, 1995). Furthermore, fumonisin B$_1$ induced expression of calmodulin in LLC-PK$_1$ cells and apoptosis induced by the mycotoxin was dependent on calmodulin (Kim *et al.*, 2001).

Fumonisin B$_1$ increases calmodulin mRNA and calmodulin protein in concentration-dependent manners. Fumonisin B$_1$ induces calmodulin protein in a concentration-dependent manner (Kim *et al.*, 2001). To compare the response of calmodulin mRNA to that of calmodulin protein, kidney cells were cultured with various concentrations of fumonisin B$_1$. Fumonisin B$_1$ induced both calmodulin mRNA (Figure 3.2A) and calmodulin protein (Figure 3.2B) in concentration-dependent manners. At 25 μM, fumonisin B$_1$ induced

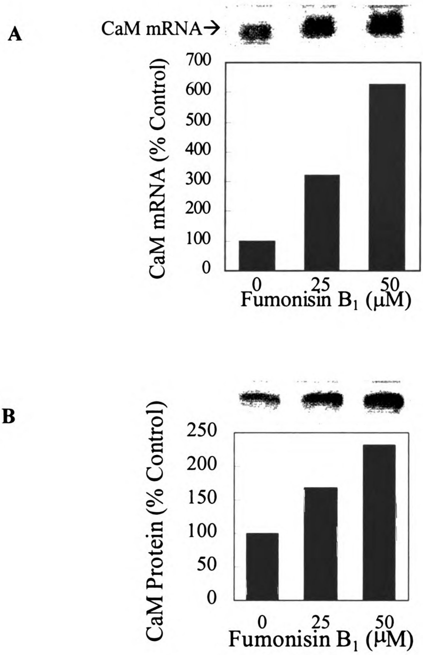


Figure 3.2. Fumonisin B_1 increases calmodulin mRNA and calmodulin protein in concentration-dependent manners. 10^6 LLC-PK_1 kidney cells were plated in 100 mm dishes and grown for 48 hrs. Cells were then added with fumonisin B_1 at 0, 10, 25, and 50 μM in a fresh culture medium and cultured for 16 hr. At the end of the culture period, RNA (A) and cellular protein (B) were harvested and Northern and Western analysis were performed respectively as described under "Materials and Methods".

calmodulin mRNA ~3-fold, while increasing calmodulin protein ~70%. At 50 μM, fumonisin B_1 increased calmodulin mRNA ~5-fold and caused a corresponding increase in calmodulin protein of ~2.4 fold.

Fumonisin B_1 does not affect stability of calmodulin mRNA. Increased abundance of calmodulin mRNA by fumonisin B_1 could be either due to increased gene transcription or increased mRNA stability. To examine the effect of the mycotoxin on stability of calmodulin mRNA, the transcription inhibitor actinomycin D was used to prevent transcription. In the absence of the mycotoxin, the concentration of calmodulin mRNA was initially increased up to 6 hr and then decreased steadily until 36 hr (Figure 3.3). Regression analysis applied to the data from 6 to 36 hour indicates that calmodulin mRNA has a half life of ~30 hr. A similar pattern of calmodulin mRNA was observed in response to fumonisin B_1 with an mRNA half life of ~36 hr; however, regression analysis indicates that the slopes are not significantly different between the two groups, indicating that fumonisin B_1 does not affect the stability of calmodulin mRNA.

Novel gene transcription is required for fumonisin B_1-induced apoptosis. Since fumonisin B_1 appears to increase calmodulin expression by increasing transcription rather than by increasing mRNA stability and apoptosis is dependent on calmodulin, dependence of fumonisin-induced apoptosis on novel gene transcription was examined using actinomycin D. Fumonisin B_1 alone at 50 μM caused DNA fragmentation (Figure 3.4); whereas, actinomycin D at 2 ng/ml has no effect. However, addition of actinomycin D in the presence of fumonisin B_1 prevented fumonisin-induced DNA fragmentation.

Fumonisin B_1 selectively induces calmodulin. To determine whether fumonisin B_1 induces calmodulin in a selective manner, DDRT-PCR was conducted as previously

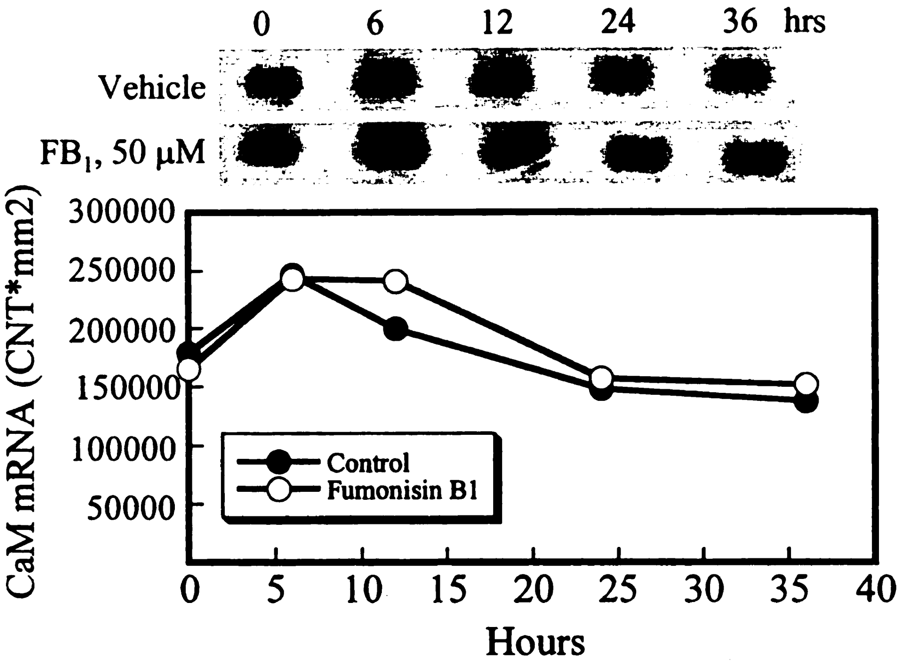


Figure 3.3. Fumonisin B_1 does not affect stability of calmodulin mRNA. 10^6 LLC-PK_1 kidney cells were plated in 100 mm dishes and grown for 48 hrs. Actinomycin D (4 ng/ml) was then added to culture media in the presence or absence of fumonisin B_1 and kidney cells were cultured at various hours. At the end of the culture, total RNA was harvested and Northern analysis was performed as described under "Materials and Methods".

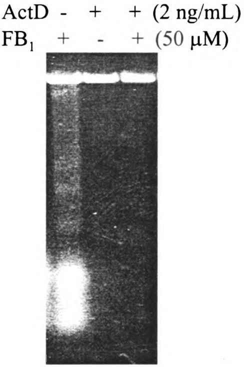


Figure 3.4. Novel gene transcription is required for fumonisin B$_1$-induced apoptosis 10^6 LLC-PK$_1$ kidney cells were plated in 100 mm dishes and grown for 48 hrs. Cells were then added with 50 μM fumonisin B$_1$ (FB$_1$) and/or 2 ng/ml actinomycin D (ActD) in a fresh culture medium and cultured for ~48 hr. At the end of the culture, fragmented DNA was harvested and analyzed by agarose gel electrophoresis as described under "Materials and Methods".

described (Kim *et al.*, 2001). The results demonstrate that an apoptotic concentration of fumonisin B_1 affected expression of only a few genes in LLC-PK_1 cells (Figure 3.5). Those genes include calmodulin (CaM).

Depletion of complex sphingolipids does not affect expression of calmodulin. Since fumonisin-induced kidney cell apoptosis requires calmodulin activity (Kim *et al.*, 2001) and appears to be partially dependent on depletion of complex sphingolipids (Yoo *et al.*, 1996; Riley *et al.*, 1999), the effect of depletion of complex sphingolipids on calmodulin mRNA was examined by using β-fluoroalanine. β-fluoroalanine blocks de novo sphingolipid biosynthesis similar to fumonisin B_1 but without causing accumulation of sphinganine (3.1). The concentration of calmodulin mRNA was not altered by β-fluoroalanine (Figure 3.6), suggesting that depletion of complex sphingolipids does not play a role in fumonisin-induced calmodulin expression.

Sphinganine does not mediate fumonisin-induced calmodulin expression. β-fluoroalanine blocks sphinganine accumulation caused by fumonisin B_1 and partially prevents calmodulin expression (Kim *et al.*, 2001). To determine whether sphinganine acts directly to induce calmodulin expression, exogenous sphinganine at 10 μMwas added to LLC-PK_1 cultures. As shown in Figure 3.7, addition of an apoptotic concentration of exogenous sphinganine had no effect on calmodulin mRNA.

Sphinganine 1-phosphate induces calmodulin expression. Since sphinganine 1-phosphate accumulates in the presence of fumonisin B_1 (Smith and Merrill, 1995), the effect of this sphinganine metabolite on calmodulin expression was also examined. Sphinganine 1-phosphate at 2 μM increased the concentration of calmodulin mRNA ~50% in LLC-PK_1 kidney cells (Figure 3.8).

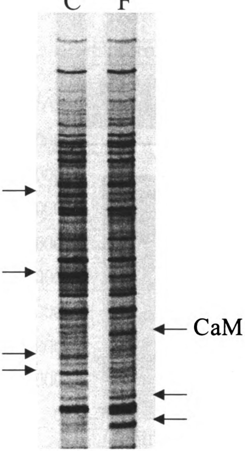


Figure 3.5. Fumonisin B_1 selectively induces calmodulin. Cells were cultured in the absence (C) or presence (F) of 50 μM fumonisin B_1 for 16 hr and DD RT-PCR was performed as described under "Materials and Methods". Arrows indicate differential expression caused by fumonisin B_1 (CaM=calmodulin). The primers used for this figure were 5'-T12-A-3' and 5'-TAGCAAGTGC-3'.

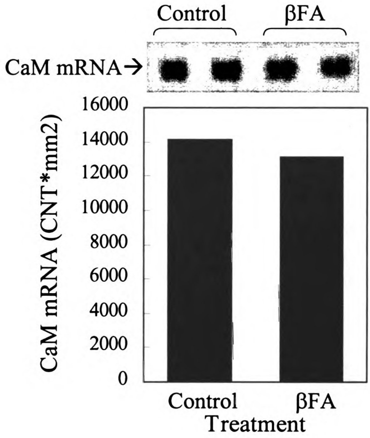


Figure 3.6. Depletion of complex sphingolipids does not affect expression of calmoduolin. LLC-PK1 cells were cultured in the presence of 50 μM β-fluoroalanine (βFA) for 16 hr. At the end of 16 hr culture, total RNA was harvested and Northern analysis was performed as described under "Materials and Methods".

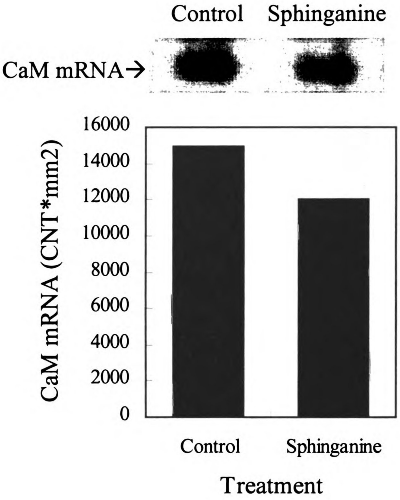


Figure 3.7. Sphingoid bases does not mediate fumonisin-induced calmodulin expression. LLC-PK$_1$ cells were cultured in the presence of 10 μM sphinganine. At the end of culture, total RNA was harvested and Northern analysis was performed as described under "Materials and Methods".

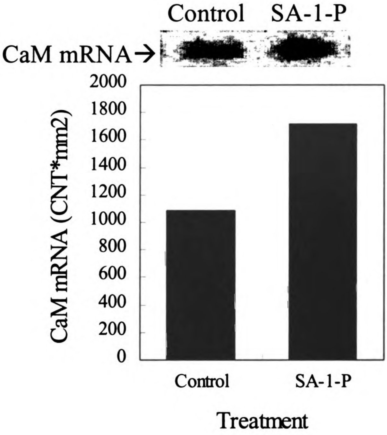


Figure 3.8. Sphinganine 1-phosphate induces calmodulin expression. LLC-PK$_1$ cells were cultured in the presence of 2 μM sphinganine 1-phosphate (SA-1-P) for 3 hr. At the end of culture, total RNA was harvested and Northern analysis was performed as described under "Materials and Methods".

E. DISCUSSION

The mechanism of toxicity of fumonisins has not been well elucidated, but an apoptotic concentration of fumonisin B_1 was shown to induce calmodulin expression in LLC-PK_1 renal tubular epithelial cells (Kim *et al.*, 2001). Moreover, fumonisin-induced kidney cell apoptosis was dependent on calmodulin activity (Kim *et al.*, 2001). Calmodulin is the major Ca^{++} sensor protein and plays an essential role in regulating many cellular processes including cell proliferation (Rasmussen and Means, 1987; Rasmussen and Means, 1989), cell-cycle progression (Chafouleas *et al.*, 1982; Chafouleas *et al.*, 1984; Rasmussen *et al.*, 1992), transformation (Chafouleas *et al.*, 1981) and apoptosis (Dowd *et al.*, 1991; Kim *et al.*, 2001; Rosenthal *et al.*, 1998). Calmodulin is ubiquitous and structurally conserved across species and regulates more than 30 enzymes in mammalian cells via the Ca^{++}/calmodulin complex (Stoclet *et al.*, 1987). The activity of calmodulin was long thought to be regulated primarily by changes in the net flux or distribution of Ca^{++} rather than by changes in the cellular concentration of the protein because expression of calmodulin is tightly regulated (Chafouleas *et al.*, 1981). Results of the present study show that fumonisin B_1 induces both calmodulin mRNA and calmodulin protein in LLC-PK_1 kidney cells in concentration-dependent manners. Furthermore, DDRT-PCR clearly demonstrates that fumonisin B_1 causes differential expression of calmodulin and only a few other genes. This is consistent with our previous findings indicating specificity in response to fumonisin B_1 (Kim *et al.*, 2001) and with studies showing early induction of calmodulin gene expression in lymphocytes undergoing glucocorticoid-mediated apoptosis (Dowd *et al.*, 1991). In addition, changes in the concentration of calmodulin have been reported in differentiated BC3H-1 mouse myoblast cells (Epstein *et al.*, 1989), in NGF-induced differentiation of neuronal cells

(Bai and Weiss, 1991), in CHO-K1 cells released from stationary phase to re-enter into the cell cycle (Chafouleas *et al.*, 1984), in transformed chicken embryo fibroblasts (Watterson *et al.*, 1976), and in several malignancies including those of liver (Wei *et al.*, 1982), lung (Liu *et al.*, 1996) and breast (Singer *et al.*, 1976; Tuccari *et al.*, 1993). Increased concentrations of calmodulin caused by fumonisin B_1 may stimulate a variety of calmodulin-dependent enzymes (Heist and Schulman, 1998; Van Eldik *et al*, 1979) which could, in turn, mediate the kidney toxicity of fumonisins by causing renal tubular cell apoptosis.

Increases in calmodulin mRNA caused by fumonisin B_1 could be due to either increased transcription of the gene or increased stability of the mRNA. The present study supports a role for fumonisin B_1 in increasing calmodulin transcription since fumonisin B_1 did not alter the stability of calmodulin mRNA. The half life of calmodulin mRNA in LLC-PK_1 cells is ~30 hr and is not significantly altered by fumonisin B_1. Bai and Weiss (1991) have reported comparable calmodulin mRNA half lives in PC12 neuronal cells ranging from ~10 to 29 hr depending on the size of the transcripts. This is consistent with the results of other studies which suggest that the steady state level of cellular calmodulin is increased by a higher rate of transcription rather than changes in mRNA stability (Chafouleas *et al.*, 1981; Epstein *et al.*, 1989). The mechanism by which fumonisin B_1 increases calmodulin transcription may involve disruption of sphingolipids. Numerous studies both *in vivo* and using a variety of cell types have demonstrated that the mechanism of toxicity of fumonisins requires disruption of sphingolipid metabolism via inhibition of sphinganine *N*-acyltransferase (Merrill *et al.*, 1997a).

There are several possible mechanisms by which disruption of sphingolipid metabolism could cause induction of calmodulin. First, fumonisin B_1 could increase calmodulin expression by depleting complex sphingolipids. Several studies have demonstrated that depletion of complex sphingolipids mediate, at least in part, fumonisin-induced toxicities (Yoo *et al.*, 1996; Riley *et al.*, 1999). When β-chloroalanine was used to inhibit serine palmitoyltransferase and block *de novo* synthesis of sphingolipids in LLC-PK_1 cells to a similar degree as fumonisin B_1, it both inhibited cell growth and increased cell death (Yoo *et al.*, 1996). Also, the addition of ceramide derivative reversed fumonisin inhibition of axonal growth in hippocampal neurons (Harel and Futerman, 1993) and the addition of *N*-acetylsphinganine partially protected human keratinocytes from fumonisin B_1-induced apoptosis (Tolleson *et al.*, 1999). Riley *et al* (1999) demonstrated that prevention of fumonisin-induced sphinganine accumulation by inhibitors of serine palmitoyltransferase blocks fumonisin-induced apoptosis but does not abolish the morphological changes caused by the mycotoxin, suggesting that depletion of sphingolipids could play a role in fumonisin toxicity. To examine the role of depletion of complex sphingolipids in fumonisin-induced calmodulin induction, β-fluoroalanine was used to block *de novo* sphingolipid biosynthesis. However, the findings demonstrate no effect of β-fluoroalanine on calmodulin mRNA and, therefore, suggest that depletion of complex sphingolipids does not mediate fumonisin-induced calmodulin expression in LLC-PK_1 kidney cells.

A second possible mechanism whereby disruption of sphingolipid metabolism could mediate fumonisin-induced calmodulin expression is by causing accumulation of the sphingoid base sphinganine. β-fluoroalanine together with fumonisin B_1 blocks fumonisin-induced sphinganine accumulation and partially prevents fumonisin-induced calmodulin expression in LLC-PK_1 kidney cells (Kim *et al.*, 2001). However, the addition of exogenous sphinganine did not affect calmodulin mRNA. Therefore, sphinganine does not appear to directly mediate fumonisin-induced calmodulin expression.

Another possible mechanism by which disruption of sphingolipid metabolism could mediate fumonisin-induced calmodulin expression is *via* the sphinganine metabolite, sphinganine 1-phosphate. Smith and Merrill (1995) showed that in the presence of fumonisin B_1, sphinganine is converted to the 1-phosphate metabolite by sphingosine (sphinganine) kinase. The present study demonstrates that exogenous sphinganine 1-phosphate induces calmodulin expression and suggests that increased formation of sphinganine 1-phosphate in the presence of fumonisin B_1 in kidney cells, at least in part, mediates stimulation of calmodulin expression. Studies have demonstrated that intracellularly generated sphingoid base 1-phosphate can be released into extracellular space in a variety of cells (Goetzl and An, 1998; Hla, 2001; Spiegel and Merrill, 1996). Therefore, it is possible that both endogenously produced sphinganine 1-phosphate as a result of fumonisin B_1 exposure as well as exogenously added sphinganine 1-phosphate may act through interaction with G-protein coupled sphingoid base 1-phosphate receptor EDG family members to induce calmodulin expression. The lower effect of exogenous sphinganine 1-phosphate on calmodulin expression than fumonisin B_1 may be due to complete degradation of exogenously added sphinganine 1-phosphate to hexadecanal and ethanolamine phosphate (Merrill *et al.*, 1996); whereas, in the presence of fumonisin B_1, endogenously generated sphinganine 1-phosphate would continue to be produced.

The mechanism by which sphinganine 1-phosphate increases calmodulin expression is not known, but may involve mobilization of intracellular Ca^{++}. Changes in intracellular Ca^{++} have been shown to affect changes in nuclear functions including gene expression (Bito *et al.*, 1997). Sphingoid base 1-phosphate has been shown to increase cytosolic Ca^{++} by mobilizing Ca^{++} from internal stores (Ghosh *et al.*, 1994; Mattie *et al.*, 1994; Tornquist *et al.*, 1997). Elevated cytosolic Ca^{++} is accompanied by significant elevation of nuclear Ca^{++}, producing a signal which may directly activate Ca^{++}-dependent proteins within the nucleus (Heist and Schulman, 1998; Himpens *et al.*, 1994). However, Ca^{++} mobilization by sphinganine 1-phosphate is not likely to be the mechanism of calmodulin induction by fumonisin B_1 because addition of intracellular Ca^{++} chelator BAPTA AM did not affect fumonisin-induced calmodulin increase (data not shown).

Another possible mechanism by which sphinganine 1-phosphate might induce calmodulin is *via* activation of the MAPK pathway and transcription factor Sp-1. Toutenhoofd *et al* (1998) demonstrated that human calmodulin is regulated at the transcriptional level, that the 5' untranslated region of the calmodulin gene was necessary, and that it contains multiple Sp-1 sites (Toutenhoofd *et al.*, 1998). The Sp transcription factor family is necessary for basal calmodulin gene transcription and for regulation of calmodulin expression (Pan *et al.*, 2000; Solomon *et al.*, 1997). Furthermore, Zhang *et al* (1999) demonstrated that fumonisin B_1 activates transcription of CDK inhibitor p21 *via* Sp-1binding sites. It is not clear if sphingoid base 1-phosphate activate Sp-1 binding to DNA; however, the 1-phosphate has been shown to activate mitogen-activated protein kinase (MAPK) which, in turn, activates a variety of transcription factors including Sp-1 (Davis *et al.*, 1996). Therefore, it is plausible that fumonisin B_1 causes elevated sphinganine 1-phosphate which, in turn, activates the MAPK pathway and transcription factors such as Sp-1, resulting in stimulation of calmodulin transcription.

In the cell, the Ca^{++}/calmodulin complex plays a wide variety of roles and several studies reported evidence relating calmodulin to apoptosis, especially those induced by sustained increases in cellular calcium concentration (Kim *et al.*, 2001; Bellomo *et al.*, 1992; Dowd *et al.*, 1991). Inhibition of calmodulin activity by W7 protects LLC-PK$_1$ cells from fumonisin-induced apoptosis (Kim *et al.*, 2001). In addition, treatment of certain lymphoma cell lines and thymocytes with glucocorticoid activates a programmed cell death pathway (McConkey *et al.*, 1989) and calmodulin inhibitors also protected lymphocytes from glucocorticoid-induced apoptosis (Dowd *et al.*, 1991). Also, calmodulin antagonists inhibited Fas-mediated apoptosis of CD4+ T-cells from patients with AIDS (Pan *et al.*, 1998) and sulfur mustard-induced apoptosis of keratinocytes (Rosenthal *et al.*, 1998). Despite these findings, the mechanism by which calmodulin is involved in the signaling of the cell-death pathway is not understood. A recent study discovered a new calcium/calmodulin-dependent protein kinase called death-associated protein kinase 2 that is believed to signal apoptosis through its catalytic activity (Kawai *et al.*, 1999). Further studies are needed to determine if this novel protein kinase is involved in fumonisin-induced kidney cell apoptosis.

Taken together, the findings of the present study provide a possible mechanism by which fumonisin B$_1$ induces calmodulin expression in LLC-PK$_1$ porcine renal tubular epithelial cells. Fumonisin B$_1$ disrupts sphingolipid metabolism and causes accumulation of sphinganine 1-phosphate which activates transcription of calmodulin. Further studies are necessary to elucidate the underlying mechanism of calmodulin induction by sphinganine 1-phosphate and the role of increased concentration of calmodulin in fumonisin-induced toxicity.

CHAPTER IV

FUMONISIN B_1 AND SPHINGANINE REDUCE PHOSPHORYLATION OF MAPK/ERK AND PKB/AKT IN LLC-PK_1 PORCINE RENAL TUBULAR EPITHELIAL CELLS

A. ABSTRACT

Fumonisins are a family of mycotoxins produced by *Fusarium moniliforme* which is the most common mold found on corn throughout the world. These compounds are both toxic and carcinogenic for animals, and perhaps humans, with the kidney being the most sensitive organ to fumonisins. Fumonisin B_1, the most prevalent of these mycotoxins, induces apoptosis in LLC-PK_1 renal tubular epithelial cells (Kim *et al.*, 2001). The molecular mechanism of fumonisin toxicity is not clear, but appears to involve disruption of *de novo* biosynthesis of sphingolipids. Fumonisin B_1 inhibits ceramide synthase causing depletion of complex sphingolipids and accumulation of the bioactive sphingoid base, sphinganine. In the present study, LLC-PK_1 cells were used to examine the effects of disruption of sphingolipid metabolism on signaling pathways responsible for determining cell survival and cell death. The studies used β-fluoroalanine as a tool to dissect the relative contribution of depletion of complex sphingolipids and accumulation of sphinganine. β-fluoroalanine is an inhibitor of serine palmitoyltransferase which, when added alone, blocks complex sphingolipid biosynthesis to a similar degree as fumonisin B_1 , but without causing accumulation of sphinganine. When added together with fumonisin B_1, β-fluoroalanine blocks sphinganine accumulation. Fumonisin B_1 alone had no apparent effect on kidney cell growth or death within the first 16 hrs of culture, but inhibited kidney cell growth and caused cell death with 24-36 hrs of culture, consistent with dependence on disruption of sphingolipid metabolism. Similarly, the exogenous addition of sphinganine inhibited growth and caused death, but within only 1-2 hrs of culture. Fumonisin B_1 had no effect on ERK phosphorylation at 2 hrs of culture, but reduced phosphorylation of MEK and ERK at 16 hrs. In contrast, β-fluoroalanine did not decrease phosphorylation of MEK or ERK together with

fumonisin B_1. Similar to fumonisin B_1, sphinganine alone and in the presence of a sub-optimal concentration of fumonisin B_1 reduced phosphorylation of both MEK and ERK. Neither fumonisin B_1, β-fluoroalanine, nor sphinganine affected either total ERK or phosphorylation of Raf-1. Both fumonisin B_1 and sphinganine decreased phosphorylation of Akt at both serine 473 and threonine 308; whereas, β-fluoroalanine did not decrease phosphorylation of Akt and blocked the effect of fumonisin B_1. The findings indicate that growth arrest and apoptosis induced by fumonisin B_1 in LLC-PK_1 porcine renal tubular epithelial cells is mediated by sphinganine which inhibits phosphorylation of Akt and of ERK by a Raf-1 independent pathway.

B. INTRODUCTION

Fumonisins are cytotoxic and carcinogenic mycotoxins produced by *Fusarium moniliforme*, the most common mold contaminant of corn and other agricultural commodities in the United States and throughout the world (Marasas, 1996). Fumonisin B_1, the major fumonisin, causes several animal diseases including equine leukoencephalomalacia (Marasas *et al.*, 1988a) and porcine pulmonary edema (Osweiler *et al.*, 1992). Early studies with impure culture material containing fumonisins indicated that the mycotoxins cause liver tumors (Gelderblom *et al.*, 1996a; Gelderblom *et al.*, 1996b). The National Toxicology Program has confirmed the carcinogenicity of fumonisin B_1 with a two year study which demonstrated that fumonisin B_1 induces hepatic tumors in female B6C3F mice and renal tubule tumors in male F344 rats (Howard *et al.*, 2001). In addition, consumption of *Fusarium*-contaminated corn has been associated with human esophageal cancer in certain areas of the world (Marasas *et al.*, 1988b). Fumonisin B_1 is wide spread in variable amounts among food-grade corn and corn-based food products of U.S. origin (Gutema *et al.*, 2000) suggesting that human populations are at significant risk of exposure to fumonisin B_1 through their normal diet. This raises concerns regarding possible health implications that can be caused by chronic exposure to these mycotoxins.

The toxicity caused by fumonisin B_1 is mediated by disruption of sphingolipid metabolism. Due to its structural similarity with sphingolipids (Figure 4.1), fumonisin B_1 potently and competitively (with both sphinganine and acyl CoA) inhibits sphinganine (sphingosine) *N*-acyltransferase (ceramide synthase), the enzyme that acylates sphinganine to form ceramide in the *de novo* biosynthetic pathway of sphingolipids (Merrill *et al.*, 1993a; Wang *et al.*, 1991). Inhibition of ceramide synthase causes accumulation of sphinganine and simultaneous depletion of complex sphingolipids including sphingomyelin as shown in mice (Martinova and Merrill, 1995), rats (Kwon *et al.*, 1997; Riley *et al.*, 1994), mink (Restum *et al.*, 1995), ponies (Wang *et al.*, 1992) and pigs (Riley *et al.*, 1993a). Since sphingolipids are bioactive compounds that regulate cellular behavior, i.e., cell proliferation, terminal differentiation, and apoptosis (Hannun and Bell, 1989; Spiegel *et al.*, 1994; Zhang *et al.*, 1991), fumonisin-induced disturbance of the balance among these lipid molecules has significant impact on the fate of cells, leading to degenerative diseases. Sphingoid bases like sphinganine are growth-inhibitory and apoptotic (Jarvis *et al.*, 1996; Ohta *et al.*, 1994, 1995) and accumulation of sphinganine and its 1-phosphate metabolite appear, at least in part, to be responsible for fumonisin-induced toxicity (Kim *et al.*, 2001; Kim and Schroeder, 2002). Fumonisin B_1 induces apoptosis in LLC-PK_1 porcine renal tubular epithelial cells in a sphinganine-dependent manner (Kim *et al.*, 2001).

The growth inhibitory and apoptotic effects of fumonisin B_1 could be mediated by

Fumonisin B_1

Sphinganine

Sphingosine

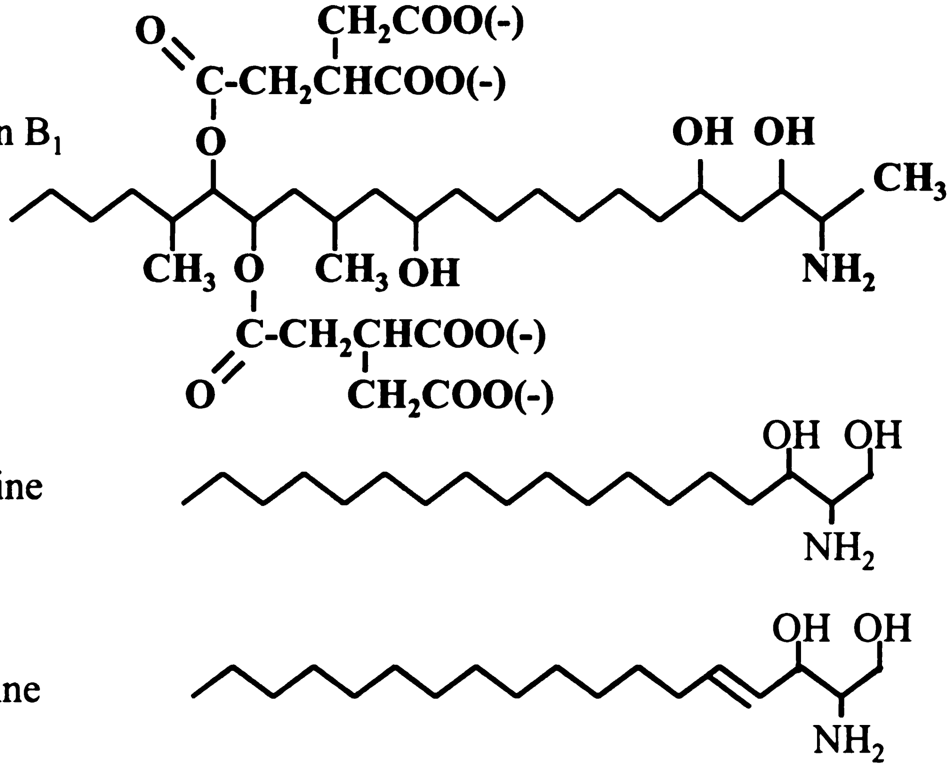


Figure 4.1. Structures of fumonisin B_1, sphinganine, and sphingosine.

sphinganine via inhibition of the mitogen-activated protein kinase (ERK) pathway, a crucial regulator of growth, survival, and maintenance of various kidney cells (di Mari *et al.*, 1999; Dudley *et al.*, 1995; Ishizuka *et al.*, 1999; Kinane *et al.*, 1997; Schramek *et al.*, 1997). Fumonisin B_1 (Huang *et al.*, 1995) and sphingoid bases (Hannun and Bell, 1986; Smith *et al.*, 2000) potently inhibit protein kinase C (PKC), an important activator of MEK and p44/p42 MAPK (or ERK), via either Raf-1 or MEK kinase. Inhibition of ERK activity by sphingoid bases has been suggested to be a partial mechanism of action in sphingoid base-induced growth arrest and apoptosis in various tumor cell lines (Sakakura *et al.*, 1998; Jarvis *et al.*, 1997). Fumonisin-induced sphinganine accumulation may suppress ERK activity and contribute to fumonisin-induced growth inhibition and cell death via inhibition of PKC.

Alternatively, the growth inhibitory and apoptotic effects of fumonisin B_1 may be mediated by sphinganine via suppression of PI3K/Akt signaling, an important anti-apoptotic pathway (Krasilnikov, 2000; Zhou *et al.*, 2000). PI3K/Akt signaling has been shown to mediate both Ras- and cytokine-induced protection from apoptosis. Activation of PI3K phosphorylates Akt (Stephens *et al.*, 1998; Stokoe *et al.*, 1997), a serine/threonine kinase which, in turn, suppresses apoptosis by phosphorylating Bad and caspase-9 (Krasilnikov, 2000; Zhou *et al.*, 2000; Zundel and Giaccia, 1998). The sphingoid base sphingosine has been shown to reduce phosphorylated Akt with concomitant reduction of Akt activity during sphingosine-induced apoptosis in human hepatoma cells (Chang *et al.*, 2001). Also, constitutively active Akt prevents sphingosine-induced apoptosis (Chang *et al.*, 2001). Thus, sphinganine accumulation caused by fumonisin B_1 may suppress Akt phosphorylation and trigger a molecular cascade leading to growth inhibition and apoptosis.

The present study used LLC-PK_1 renal tubular epithelial cells to examine the effects of disruption of sphingolipid metabolism on ERK and PI3K/Akt phosphorylation cascades leading to LLC-PK$_1$ kidney cell growth arrest and apoptosis. To determine the relative contributions of depletion of complex sphingolipids and accumulation of sphinganine on ERK and Akt phosphorylation, β-fluoroalanine, an inhibitor of an earlier step in *de novo* sphingolipid biosynthesis than fumonisin B$_1$, was used both alone and together with fumonisin B$_1$.

C. MATERIALS AND METHODS

C.1. Materials. Fumonisin B$_1$ was purchased from Sigma and dissolved in phosphate buffered saline (PBS). D-*erythro*-sphinganine was purchased from Biomol Research Laboratories, Inc. and prepared for cell culture treatment as previously described (Schroeder *et al.*, 1994). Rabbit antibodies against ERK, pERK, Akt, and pAkt proteins were obtained from New England BioLabs (Beverly, MA) and anti-rabbit IgG was obtained from Santa Cruz.

C.2. Cell Culture. Porcine kidney tubular epithelial LLC-PK$_1$ cells were obtained from American Type Culture Collection and cultured in Dulbecco's Modified Eagle Medium/F-12K nutrient solution (Gibco BRL) (1:1) with 5% fetal bovine serum (FBS) and 100 μM L-serine (Gibco BRL) until they reach the appropriate confluency for chemical treatments in each experiment.

C.3. Total Nucleic Acid Measurement. Briefly, 10^5 cells were cultured in 6-well plates for 24 hr and treated for appropriate times. Then, cells were washed with ice-cold PBS twice and air-dried. Cellular nucleic acid was harvested by lysing cells with 0.1 N NaOH and quantitated by measuring absorbance at 260 nm.

C.4. Western blot Analysis. After treatment as indicated in the figure legend, cells were washed in ice-cold PBS once and cellular protein was extracted from LLC-PK$_1$ cells with lysis buffer (20 mM Tris-Cl, pH 8.0, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μM of leupeptin and aprotinin cocktail solution) and sonicated for 15-20 sec followed by centrifuge. The protein extract was denatured by incubating for 5 min at 95°C in Laemmli buffer. The sample was applied to SDS-bisacrylamide denaturing gel and transferred onto PVDF membrane (NEN Life Sciences). The proteins on the membrane were blocked for an hour in 5% non-fat dry milk/TBS-0.1% Tween 20 solution. The membrane was incubated in 3% BSA/TBS-T containing primary antibody by overnight followed by 1 hr incubation in blocking solution containing anti-rabbit IgG conjugated with horseradish peroxidase and developed with chemiluminescence solution from Santa Cruz.

C.5. Statistical Analysis. The statistical analysis was performed by using Sigma-Stat Analysis system (Jandel Scientific, San Rafael, CA). The student t-test was used to analyze data for total nucleic acids for statistical significance. Differences were considered statistically significant at $p \leq 0.05$.

D. RESULTS

Fumonisin B$_1$ inhibits LLC-PK$_1$ cell growth and induces apoptosis. To investigate the effect of fumonisin B$_1$ on proliferation, LLC-PK$_1$ cells were cultured in the absence or presence of 50 μM fumonisin B$_1$ for 48 hr and total nucleic acids were measured as an index of cell number (Figure 4.2). In control cultures, total nucleic acids increased at a consistent rate throughout the entire 48 hr culture period with a doubling time of ~18 hr. Fumonisin

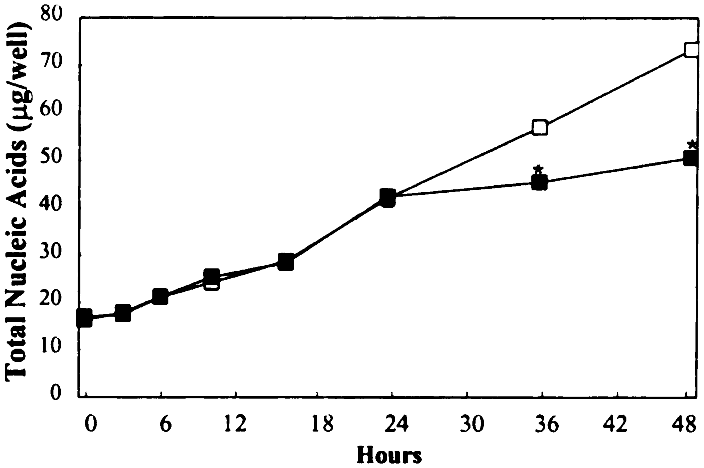


Figure 4.2. Fumonisin B_1 inhibits growth and induces death of LLC-PK_1 kidney cells. ~10^5 LLC-PK_1 cells per well were cultured in a six-well plate in the absence (☐) or presence of 50 μM fumonisin B_1 (■) at various times up to 48 hrs. At each time point, cells were harvested and total nucleic acids were measured as described under "Materials and Methods". * indicates statistical difference between two groups (P<0.0001).

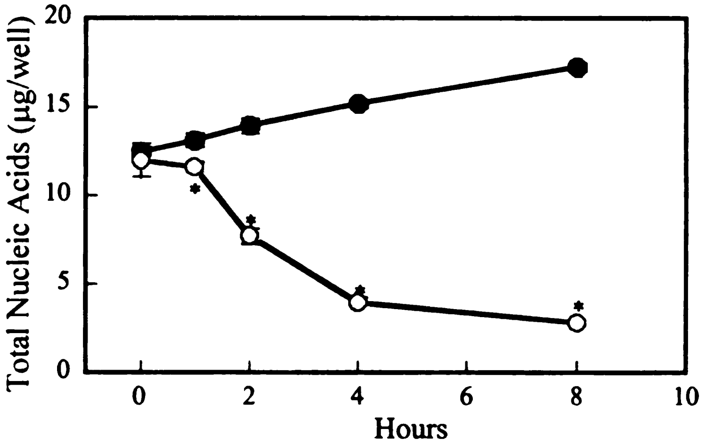


Figure 4.3. Sphinganine inhibits growth and induces death of LLC-PK_1 kidney cells. ~10^5 LLC-PK_1 cells per well were cultured in a six-well plate in the absence (●) or presence of 20 μM D-*erythro*-sphinganine (○) at various times. At each time point, cells were harvested and total nucleic acids were measured as described under "Materials and Methods". * indicates statistical difference between two groups (P<0.0001).

B_1 produced morphological changes characteristic of apoptosis within 16-24 hr, including chromatin condensation, cell shrinkage, membrane blebbing, and formation of apoptotic bodies (data not shown). Total nucleic acids in cells cultured with fumonisin B_1 increased at a similar rate to that of control cultures up to 24 hr. Thereafter, total nucleic acids did not change during the final 24 hr of culture.

Sphinganine inhibits LLC-PK$_1$ cell growth and induces apoptosis. To determine if sphinganine causes similar effects on kidney cell growth to that of fumonisin B_1, LLC-PK$_1$ cells were cultured in the presence of 20 μM sphinganine and total nucleic acids were measured as an index of cell number. In the presence of sphinganine, LLC-PK$_1$ cells showed morphological changes characteristic of apoptosis similar to those of the cells cultured with fumonisin B_1, but as early as 1 hr after addition of the sphingoid base (data not shown). Simultaneously, sphinganine significantly decreased total nucleic acids within 1-2 hrs and killed 70% of the cells within 4 hr (Figure 4.3).

Fumonisin B_1 inhibits ERK and MEK phosphorylation in a sphinganine-dependent manner. Fumonisin B_1 does not cause significant sphinganine accumulation in LLC-PK$_1$ cells until 6 hr of culture (Kim *et al.*, 2001). To determine the effect of fumonisin B_1 on ERK phosphorylation prior to sphinganine accumulation, LLC-PK$_1$ kidney cells were cultured in the presence of 50 μM fumonisin B_1 for various times up to 2 hr and cellular protein was lysed and used for Western blot analysis with an antibody specific for phosphorylated ERK (pERK). Fumonisin B_1 increased pERK rapidly with maximum phosphorylation at 10 min. Thereafter, phosphorylation decreased to the basal level within 30 min (Figure 4.4A). To determine if the effect was specifically caused by fumonisin B_1, pERK of cells cultured with fumonisin B_1 was compared to that of control cultures. Time-

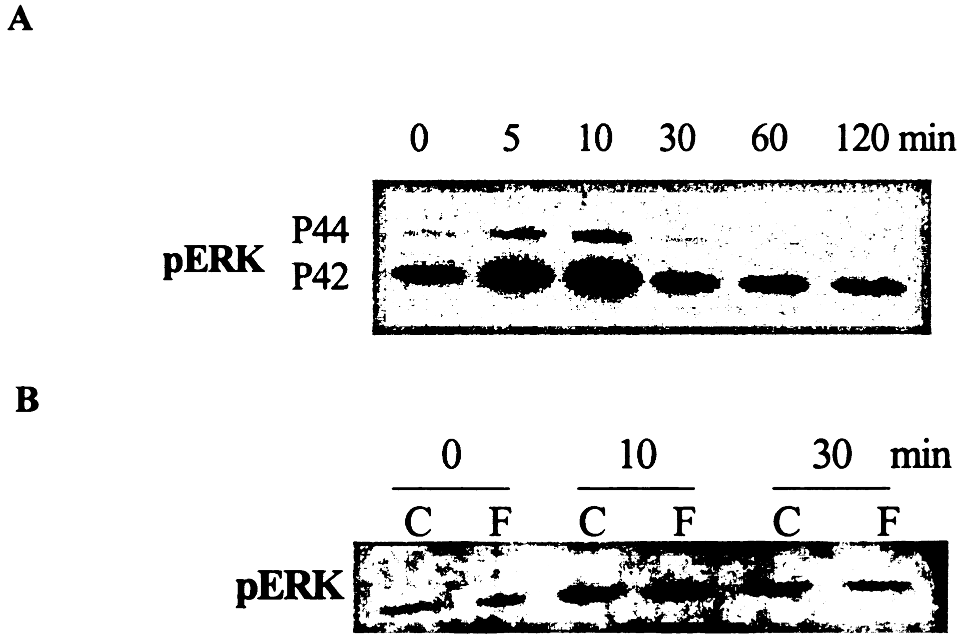


Figure 4.4. Time-dependent changes in the phosphorylation of ERK in control and fumonisin B_1-treated LLC-PK_1 cells. LLC-PK_1 cells were grown in DMEM/F12-K growth medium containing 5% Fumonisin B_1 for 48 hours in 100 mm dishes. A. Kidney cells were treated with 50 μM of fumonisin B_1 for 5, 10, 30, 60 and 120 minutes and harvested for western blot analysis against phosphorylated form of ERK (pERK) protein as described under "Materials and Methods". B. Comparison between control (C) and fumonisin B_1 (F) at 0, 10, and 30 min.

dependent changes in phosphorylation of ERK (pERK) were similar for both control cultures and fumonisin-treated cultures (Figure 4.4B).

To further examine the effect of fumonisin B_1 on ERK phosphorylation and the role of sphinganine accumulation, additional studies were conducted for 16 hr. This is the point at which cellular sphinganine increases to ~1 nmol/mg protein and DNA fragmentation is evident (Kim *et al.*, 2001). β-fluoroalanine, which inhibits the first step of *de novo* sphingolipid biosynthesis catalyzed by serine palmitoyltransferase and prevents fumonisin-induced sphinganine accumulation, was used as a tool to further dissect the roles of complex sphingolipid depletion and of sphinganine accumulation on the ERK pathway. As shown in Figure 4.5, the total amount of ERK protein was not affected by any treatments (A). However, phosphorylation of ERK and MEK (B and C, respectively) was reduced in cells cultured with 50 μM fumonisin B_1 for 16 hr. Importantly, addition of 50 μM β-fluoroalanine alone did not reduce phosphorylation of ERK or MEK and β-fluoroalanine added together with 50 μM fumonisin B_1 for 16 hr restored phosphorylated ERK and MEK. Phosphorylation of Raf-1 (D) was not affected by any of the treatments.

Sphinganine significantly reduces phosphorylation of ERK and MEK, but not Raf-1. To further investigate the possibility that sphinganine mediates the fumonisin-induced decrease in ERK phosphorylation, LLC-PK_1 cells were cultured in the absence or presence of a growth inhibitory and apoptotic concentration of D-*erythro*-sphinganine. Since ceramide is also growth inhibitory and apoptotic, a subtoxic and non-apoptotic dose of fumonisin B_1 (10 μM) was used to prevent acylation of sphinganine to form ceramide. As shown in Figure 4.6, 10 μM fumonisin B_1 alone had no effect; whereas, 20 μM sphinganine, either alone or in the presence of 10 μM fumonisin B_1 potently reduced phosphorylation of

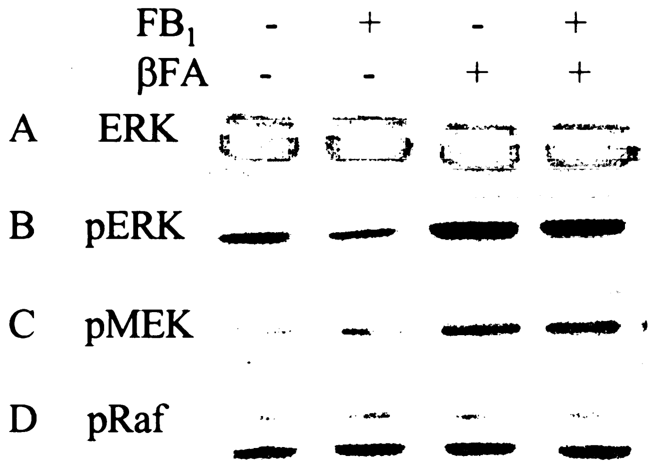


Figure 4.5. Fumonisin B_1 reduces phosphorylated ERK and MEK, but not Raf-1. LLC-PK_1 cells were cultured in the absence or presence of fumonisin B_1 (FB_1) at 50 μM and/or β–fluoroalanine (βFA) at 50 μM. After 16 hr, cellular protein was isolated and used for Western blot analysis as described under "Materials and Methods". Above blots are probed against total ERK (A), phosphorylated ERK (B), phosphorylated MEK (C) and phosphorylated Raf-1 (D).

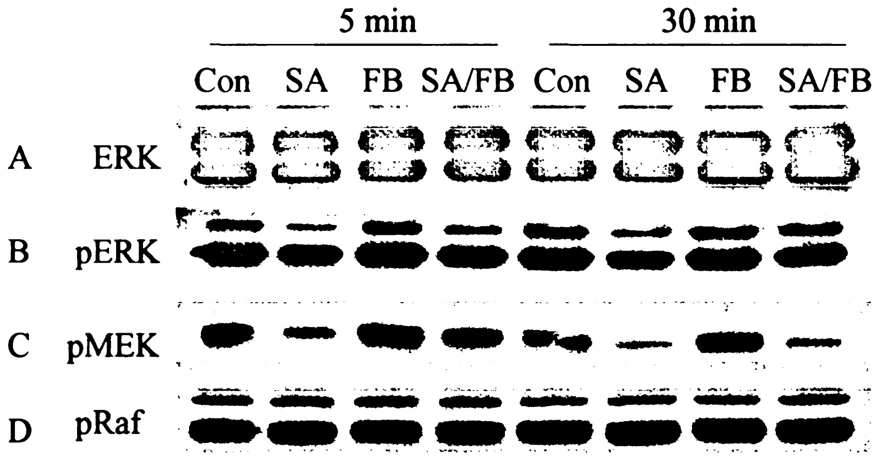


Figure 4.6. Sphinganine reduces phosphorylated ERK and MEK, but not Raf-1. LLC-PK_1 cells were cultured in the presence of vehicle (Con), sphinganine at 20 μM (SA), fumonisin B_1 at 10 μM (FB), and sphinganine (20 μM) together with fumonisin B_1 (10 μM)(SA/FB). After 5 or 30 min, cellular protein was isolated and used for Western blot analysis as described under "Materials and Methods". Above blots are probed against total ERK (A), phosphorylated ERK (B), phosphorylated MEK (C) and phosphorylated Raf-1 (D)

ERK (B) and MEK (C) without affecting the total amount of ERK (A). The effects of sphinganine persisted ~30 min, and subsided by ~1 hr (data not shown). Raf-1 was not affected by any treatment.

Fumonisin B_1 suppresses Akt phosphorylation. To determine the effect of fumonisin B_1 on the Akt pathway and to dissect the role of sphinganine accumulation, Western blot analysis was conducted using antibodies to total Akt and to Akt phosphorylated at thr308 and at ser473. As shown in Figure 4.7, total Akt (C) was not affected by any treatment. However, culture with 50 μM fumonisin B_1 for 16 hr reduced both pAkt (ser473) (A) and pAkt (thr308) (B). Culture with β-fluoroalanine alone had no effect and addition of β-fluoroalanine together with alone or in the presence of fumonisin B_1 restored pAkt (thr308) and pAkt (ser473) to that of controls.

Sphinganine significantly reduces phosphorylation of Akt. To determine if sphinganine has similar effects as fumonisin B_1 on Akt phosphorylation, LLC-PK_1 cells were cultured in the absence or presence of sphinganine. Again, a subtoxic and non-apoptotic concentration of fumonisin B_1 (10 μM) was used to prevent acylation of sphinganine to form ceramide. Cultures of LLC-PK_1 cells with 10 μM fumonisin B_1 had no effect; whereas, culture with 20 μM sphinganine both alone and in the presence of 10 μM fumonisin B_1 reduced the phosphorylation of Akt at both thr308 (A) and ser473 (B) at both 5 or 30 min of culture (Figure 4.8).

E. DISCUSSION

Inhibition of ceramide synthase by fumonisin B_1 causes accumulation of sphingoid bases and depletion of complex sphingolipids (Norred *et al.*, 1992; Wang *et al.*, 1991; Schroeder

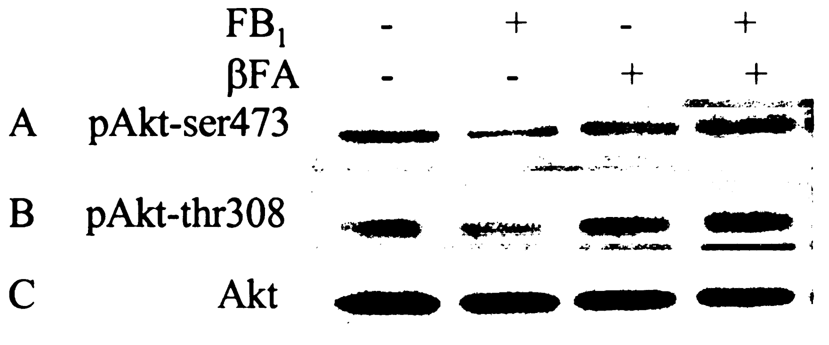


Figure 4. 7. Fumonisin B$_1$ reduces phosphorylated Akt. LLC-PK$_1$ cells were cultured in the absence or presence of fumonisin B$_1$ (FB$_1$) at 50 μM and/or β–fluoroalanine (βFA) at 50 μM. After 16 hr, cellular protein was isolated and used for Western blot analysis as described under "Materials and Methods". Above blots are probed against phosphorylated Akt at serine 473 (pAkt-ser473) (A) and threonine 308 (pAkt-thr308)(B), and total Akt (C).

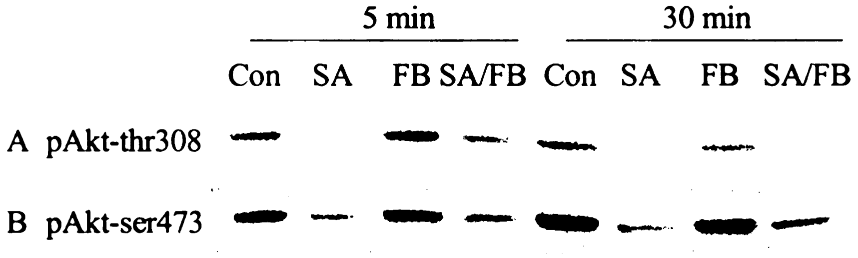


Figure 4.8. Sphinganine reduces phosphorylated Akt. LLC-PK$_1$ cells were cultured in the presence of vehicle (Con), sphinganine at 20 μM (SA), fumonisin B$_1$ at 10 μM (FB), and sphinganine (20 μM) together with fumonisin B$_1$ (10 μM)(SA/FB). After 5 or 30 min, cellular protein was isolated and used for Western blot analysis as described under "Materials and Methods". Above blots are probed against phosphorylated Akt at serine 473 (pAkt-ser473) (A) and threonine 308 (pAkt-thr308) (B).

et al., 1994). Sphingoid bases are growth inhibitory and cytotoxic for many cell types (Merrill *et al.*, 1993b) and complex sphingolipids also have been implicated in the regulation of cell growth and differentiation (Hakomori and Igarashi, 1993; Laine and Hakomori, 1973; Bremer and Hakomori, 1982; Saito *et al.*, 1985). Consistent with previous findings (Kim *et al.*, 2001), fumonisin B_1 caused morphological changes indicative of apoptosis within 16 hr and inhibited growth of LLC-PK_1 cells within 24 hr of culture. This coincides with the time when endogenous sphinganine reaches a concentration of ~1 nmol/mg protein which is growth inhibitory and apoptotic (Kim *et al.*, 2001). The potent anti-proliferative effect and concomitant morphological changes characteristic of apoptosis in LLC-PK_1 cells as early as 1 hr after the addition of exogenous sphinganine support a mechanism of fumonisin toxicity involving sphinganine accumulation. The ability of sphinganine to diffuse rapidly through cell membranes (Hannun *et al.*, 1991) explains the more rapid action of exogenously added sphinganine. This cytotoxicity of sphinganine in kidney cells is consistent with other reports where sphingoid bases inhibited cell growth and induced apoptosis (Ohta *et al.*, 1995, 1994; Stevens *et al.*, 1990). The growth inhibitory and cytotoxic effects of sphinganine in LLC-PK_1 cells explain fumonisin's interruption of regeneration after mechanical injury and enlargement of swiped area in renal proximal tubule cell culture (Counts *et al.*, 1995) as well as the apoptotic features shown in the tubular epithelial region of the kidney (Voss *et al.*, 2001; Tolleson *et al.*, 1996a; Bondy *et al.*, 1996; Bucci *et al.*, 1998).

In various cell lines including LLC-PK_1 cells, the ERK pathway plays a critical role in determining cellular growth (di Mari *et al.*, 1999; Dudley *et al.*, 1995; Kinane *et al.*, 1997; Schramek *et al.*, 1997). Phosphorylation and activation of ERK activates transcription factors such as NF-κB and AP-1 via a series of signaling events and leads to transcription of genes involved in cell proliferation (Cowley *et al.*, 1994; Hunter, 1995; Marshall, 1995). Previously, fumonisin B_1 was reported to activate ERK at ~10 min after addition of fumonisin B_1 to cultures of human bronchial epithelial cells (Pinelli *et al.*, 1999) and Swiss 3T3 cells (Wattenberg *et al.*, 1996). However, in the present study, ERK phosphorylation increased as early as 5-10 min for both control and fumonisin B_1 cultures and then decreased to the initial level by 30 min, indicating ERK phosphorylation is not rapidly altered in response to fumonisin B_1. The reason for the disparity in results may be because appropriate controls were not included in the previous studies (Pinelli *et al.*, 1999; Wattenberg *et al.*, 1996). Alternatively, the discrepancy in results may be due to distinct metabolic responses to sphingoid bases of different cell types.

In contrast to the lack of an early response to fumonisin B_1, the present study demonstrates that the ERK pathway was down-regulated within 16 hr of culture with fumonisin B_1. Again, this is the point at which endogenous sphinganine has increased to ~1 nmol/mg protein (Kim *et al*, 2001). To our knowledge, this is the first study demonstrating down-regulation of ERK by fumonisin B_1. Fumonisin B_1 also produced similar effects when ERK was stimulated by insulin or inhibited by PD98059, a MEK inhibitor (data not shown). These observations indicate that fumonisin B_1 inhibits kidney cell proliferation, at least in part, by suppressing the ERK pathway. Further, the time-dependence for inhibition of ERK phosphorylation by fumonisin B_1 suggests a possible role of sphinganine accumulation.

Exogenously added sphinganine also significantly decreased phosphorylation of ERK and MEK, but even more rapidly than fumonisin B_1. This is consistent with previous studies which demonstrated that potent inhibition of ERK plays an important role in sphingoid base-mediated death of U937 human monoblastic leukemia cells (Jarvis *et al.*, 1997). A causal role for sphinganine in mediating suppression of the ERK pathway by fumonisin B_1 is further supported by the finding that addition of β-fluoroalanine (which prevents sphinganine accumulation caused by fumonisin B_1) abolished the inhibiting effect of fumonisin B_1 on phosphorylation of ERK and MEK. The possibility of complex sphingolipid depletion as a cause for fumonisin's effect on the ERK pathway is unlikely because β-fluoroalanine alone, which depletes complex sphingolipid such as sphingomyelin to a similar degree as fumonisin B_1 (Merrill *et al.*, 1993a), did not decrease ERK or MEK phosphorylation. ERK and MEK phosphorylation were inhibited to a similar extent by both sphinganine alone or sphinganine together with a suboptimal concentration of fumonisin B_1 (which prevents formation of ceramide from exogenous sphinganine), indicating that sphinganine, not ceramide, inhibits phosphorylation of ERK and MEK. Furthermore, neither fumonisin B_1 nor sphinganine altered phosphorylation of Raf-1, a serine threonine kinase which mediates signaling from Ras to MEK (Dent *et al.*, 1992; Howe *et al.*, 1992; Kyriakis *et al.*, 1992). These findings demonstrate that down-regulation of ERK phosphorylation by fumonisin B_1 is mediated by sphinganine accumulation via a mechanism which is independent of Raf-1 and which may involve MEK kinase, the other MEK activator.

Fumonisin B_1 and sphinganine may regulate MEK and ERK via MEK kinase by either inhibiting PKC or by affecting heterodimeric G-protein-coupled receptors. PKC regulates the ERK pathway via either MEK kinase or Raf-1 (Hill *et al.*, 1993; Nishida and Gotoh, 1993; Pelech *et al.*, 1993). Furthermore, sphingoid bases are potent inhibitors of PKC (Hannun and Bell, 1986) and fumonisin B_1 has been shown to inhibit PKC activity in a concentration-dependent manner (Huang *et al.*, 1995). Alternatively, fumonisin B_1 and sphingoid bases also interact with G-protein-coupled receptors (Ho *et al.*, 1996) and G-protein-coupled receptors regulate ERK activity via MEK kinase, rather than Raf-1 (Cobb and Goldsmith, 1995; Lavoie *et al.*, 1996). Moreover, in LLC-PK$_1$ cells G$\alpha_{i\text{-}2}$ has been shown to mediate growth via Raf-independent activation of ERK (Kinane *et al.*, 1997). Fumonisin B$_1$ and sphinganine may regulate MEK and ERK via MEK kinase by either inhibiting PKC or by affecting heterotrimeric G protein-coupled receptors.

Akt (also known as protein kinase B), a serine/threonine kinase activated by PI3K, plays an important role in the regulation of cell death (Krasilnikov, 2000; Zhou *et al.*, 2000). Akt kinase protects cells from apoptosis induced by a variety of extracellular stresses including loss of cell adhesion, serum withdrawal, and UV-B irradiation (Kennedy *et al.*, 1997; Khwaja *et al.*, 1997; Kulik *et al.*, 1997). Further, increased activity of Akt contributes to carcinogenesis in various tissues (Graff *et al.*, 2000; Krasilnikov, 2000; Tsatsanis and Spandidos, 2000; Yuan *et al.*, 2000). Recently, Ramljak *et al.* (2000) showed Akt was activated in a neoplastic liver specimen obtained from a long-term feeding study of fumonisin B$_1$ in rats and suggested that fumonisin-induced Akt activation may be a mechanism by which fumonisin B$_1$ acts as a carcinogen. In contrast, the present study demonstrates down-regulation of Akt phosphorylation by fumonisin B$_1$. The disparity in results could be due to differences in species (rat *vs* pig), organ (liver *vs* kidney), model system (*in vivo vs. in vitro*), and/or study period (chronic *vs*. acute). Treatment of LLC-PK$_1$ cells with PI3K inhibitor LY294002 significantly reduced Akt phosphorylation and the metabolic capacity (data not shown), indicating the importance of Akt in normal LLC-PK$_1$ cellular metabolism. Fumonisin-induced decreases in Akt phosphorylation may suppress the cell survival signal and shift the balance toward cell death, providing an additional mechanism by which fumonisin B$_1$ inhibits cell growth and induces apoptosis.

Similar to the ERK pathway, the mechanism by which fumonisin B_1 down-regulates Akt phosphorylation appears to involve sphinganine accumulation rather than complex sphingolipid depletion. Exogenously added sphinganine significantly reduced phosphorylated Akt on both ser473 and thr308 and β-fluoroalanine prevented the fumonisin-induced decreases in Akt phosphorylation. The possibility that complex sphingolipid depletion mediates the effect of fumonisin B_1 on Akt phosphorylation is unlikely because β-fluoroalanine alone, which depletes complex sphingolipids but without causing sphinganine accumulation, did not decrease Akt phosphorylation at either ser 473 nor thr308. Down-regulation of Akt and induction of cell death by sphinganine are consistent with those of Chang *et al.* (2001) who demonstrated that sphingosine, another sphingoid base, induces dephosphorylation of Akt during apoptosis in human hepatoma (Hep3B) cells. Sphingosine also caused cytochrome C release and caspase-3 activation (Chang *et al.*, 2001) which are critical events in the regulation of apoptosis (Kluck *et al.*, 1997). Attenuation of sphingosine-induced cytochrome C release and caspase 3 activation by overexpression of activated Akt kinase (Chang *et al.*, 2001) implies that suppression of Akt by sphingoid bases could cause cell death. Thus, the cytotoxic effect of sphinganine in the present study is, at least in part, due to the suppression of signals via the PI3K/Akt pathway and this property appears to contribute to the growth-inhibitory and cytotoxic effects of fumonisin B_1 in LLC-PK_1 cells, leading to fumonisin-induced kidney cell death.

Taken together, the findings of this study suggest that fumonisin-induced growth inhibition and cell death in LLC-PK_1 porcine renal tubular epithelial cells is mediated by sphinganine which suppresses ERK and Akt signaling. Moreover, down-regulation of the ERK phosphorylation cascade is independent of Raf-1.

CHAPTER V

SUMMARY AND CONCLUSIONS

The present study investigated how fumonisin B_1, the most common contaminant of corn, can cause toxicity in the kidney, the most sensitive target organ, using an *in vitro* culture system with LLC-PK_1 porcine renal tubular epithelial cells. LLC-PK_1 cells cultured with fumonisin B_1 underwent growth arrest and exhibited increased DNA fragmentation, cell shrinkage, and membrane blebbing indicative of apoptosis similar to renal tubular epithelial cells *in vivo*. Flow cytometric analysis revealed that fumonisin B_1 produced a 7-fold increase in the number of cells in the sub-G_0/G_1 range which represents apoptotic cells. Therefore, LLC-PK_1 cells appear to be an excellent model to study the molecular mechanism of toxicity caused by fumonisins.

Studies which examined the role of disruption of sphingolipid metabolism in fumonisin-induced apoptosis demonstrated that apoptosis was highly dependent on sphinganine accumulation. Since sphinganine accumulation is also evident in kidney and other tissues of animals fed fumonisins, *in vivo* examination of known targets of sphingoid bases and their 1-phosphate metabolites may provide insight into the mechanism of action of these mycotoxins.

Fumonisin B_1 also increased calmodulin mRNA and calmodulin protein, apparently by increasing calmodulin gene transcription via sphinganine 1-phosphate. Fumonisin B_1 and sphinganine 1-phosphate are the first two examples of chemical inducers of calmodulin. Thus, both fumonisin B_1 and sphinganine 1-phosphate provide novel tools to study transcriptional regulation of calmodulin genes.

Since apoptosis induced by fumonisin B_1 was dependent on calmodulin activity, overexpression of the calmodulin gene either by activating the calmodulin gene promoter or via calmodulin gene transfection may lead to a better understanding of the mechanism of action of fumonisins. In addition, calmodulin antisense cDNA and calmodulin antagonists as well as calmodulin knockout animals may be useful tools to further elucidate the mechanism of fumonisin toxicity. Calmodulin antisense cDNA and calmodulin antagonists also may serve as drugs to treat animals and/or humans exposed to fumonisins. Moreover, calmodulin mRNA and calmodulin protein may provide sensitive biomarkers for exposure to the mycotoxin.

Sphinganine 1-phosphate appears to mediate fumonisin-induced calmodulin induction and, thereby, contributes to fumonisin-induced apoptosis. This observation is contradictory to dogma which contends that sphinganine 1-phosphate is "mitogenic" as opposed to "apoptotic." The full growth inhibitory and apoptotic response to fumonisin B_1 appears to be dependent not only on sphinganine 1-phosphate and calmodulin, but also on sphinganine and regulation of key signaling pathways. The results underscore that changes in cellular behavior (in this case apoptosis) are the result of a number of mediators acting on a variety of targets rather than a single mediator acting on only one or very few targets.

Fumonisin B_1 decreased phosphorylation of both ERK and Akt proteins via sphinganine accumulation. The more potent effects on ERK and Akt of exogenous sphinganine than fumonisin B_1 may be due to accumulation of a different profile of mediators. Exogenous sphinganine causes accumulation of sphinganine and sphinganine 1-phosphate as well as ceramide; whereas, fumonisin B_1 prevents formation of ceramide and causes accumulation of sphinganine and sphinganine 1-phosphate. Ceramide has been shown to inhibit ERK and Akt, while sphinganine 1-phosphate has been reported to activate ERK and the effect on Akt is not know.

The molecular mechanism by which fumonisin B_1 suppresses ERK and Akt signaling may involve sphinganine inhibition of protein kinase C (PKC). Though the effects of sphinganine and the mycotoxin on PKC were not measured in this study, PKC is an upstream regulator of ERK and sphingoid bases potently inhibit PKC. Future studies should examine the role of PKC in fumonisin-induced suppression of ERK. If inhibition of PKC does play a role, activators of PKC may serve as drugs to counteract the action of fumonisin B_1 and/or sphinganine

There are interconnections between sphinganine 1-phosphate and calmodulin which implicate these molecules not only in fumonisin-induced apoptosis, but also in carcinogenesis caused by fumonisins. Sphinganine 1-phosphate induces calmodulin expression and mobilizes intracellular calcium and calmodulin is dependent on calcium for some regulatory activities. In addition, the concentration of both sphinganine 1-phosphate and calmodulin appear to be increased by common stimuli including serum and neural growth factor and both have been shown to stimulate DNA synthesis, expedite cell cycle progression through G_1/S, and play roles in activation of cyclin-dependent kinases. Moreover, calmodulin is elevated in various forms of tumors. These facts suggest that sphinganine 1-phosphate and calmodulin may be key not only in the initial acute response to fumonisin exposure which is apoptosis, but also in carcinogenesis which is the result of chronic exposure to fumonisins. Therefore, future studies should examine the roles of sphinganine 1-phosphate and calmodulin in regenerative growth of surviving cells surrounding tissue initially killed by fumonisin exposure, which could contribute to fumonisin-induced carcinogenesis in various tissues including kidney and liver.

The findings of this study, taken together with the results of other investigations, provide a plausible molecular mechanism for renal toxicity caused by fumonisins (Figure

5.1). Under conditions of exposure to fumonisins via the diet, ceramide synthase is inhibited causing depletion of complex sphingolipids and accumulation of sphinganine and sphinganine 1-phosphate. The specific contributions of depletion of complex sphingolipids to fumonisin-induced apoptosis are not clear. Accumulation of sphinganine inhibits PI3K and blocks ERK signaling probably by inhibiting protein kinase. Accumulation of sphinganine 1-phosphate stimulates calmodulin gene transcription and calcium mobilization which act to increase Ca^{++}/CAM and activate the phosphatase calcineurin. Inactivation of ERK and Akt and activation of calcineurin converge to cause dephosphorylation of Bad which triggers apoptosis.

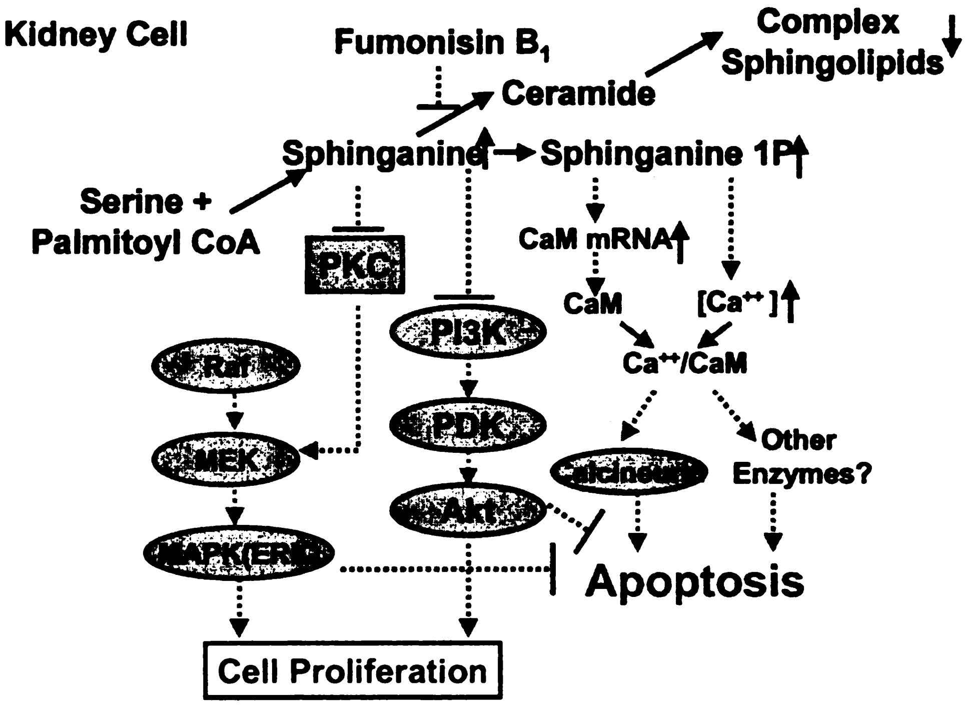


Figure 5.1. Summary of possible molecular mechanism of fumonisin-induced kidney toxicity. (Solid line with arrow, conversion; dotted line with perpendicular line, inhibition; and dotted line with arrow, activation/stimulation)

APPENDIX

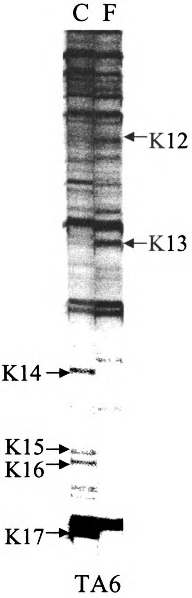
Appendix 1. Primer pairs for amplification of *bcl-2*, *bcl-x* and *bax* mRNA (Chapter II)

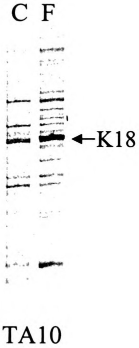
***bcl-2* mRNA**
Forward
5'-ACTTGTGGCCCAGATAGGCACCCAG-3'
Reverse
5'-CGACTTCGCCGAGATGTCCAGCCAG-3'

***bax* mRNA**
Forward
5'-CAGCTCTGAGCAGATCATGAAGACA-3'
Reverse
5'-GCCCATCTTCTTCCAGATGGTGAGC-3'

***bcl-x* mRNA**
Forward
5'-AATGTCTCAGAGCAACCGGGAGCTG-3'
Reverse
5'-TCATTTCCTACTGAAGAGTGAGCCCA-3'

Appendix 2. Differential display RT-PCR.





C, Control; **F**, 50 uM fumonisin B_1
Primers: **TA6**; 5'-T12-A-3' and 5'-GCAATCGATC-3'
TA10; 5'-T12-A-3' and 5'-TAGCAAGTGC-3'

Sequences of cDNAs

K 12

1 GAATCNCCNT NGCTTGGGTA CCGAGCTCGG ATCCACTAGT
AACGGGCCGC CAGTGTGCTG
61 GAATTCGCCC TTGCAATCGA TGTAACATTA TTATTGCCAC
ACACATTTTT ATACTAGATC
121 CTAACACTTC GTCTACATTG CTTTTAAATA ATGTTCGTAT
GTCACTGTGT GTTATAAATC
181 TGCATCAATT TCCGTTTTAA TACCAGTATC CTTTTTCCTC
TATACGAATC CCTTGAATCT
241 TAGGGTAATG TACCTTACAA AATTTTAGGT TCTACAGATG
CCATATTGTC TGAATCTCAC AAGGATTTCA ATATGGCTAT

K 13

1 ATTGGGCCCT CTAGATGCAT GCTCGAGCGG CCGCCAGTGT
GATGGATATC TGCAGAATTC
61 GNCCTTGCAA TCGATGACGG CAAAGAGCAT TGTGATCAAA
TCCACATGAG TAAAGTAACA
121 TTTGGAGCCC AGCAGGGGAT AAAAAGGAAG GTATTGTTGC
TCGGAGTGCC ACAGTATTGT
181 CCTTTACCAT TTTAGGATGT ATTGAAATGC ATAAGTAATA
GTTGTAATTG CATATATGAG
241 AAAGCACCAA GCAAGCCCTA GCTCCTTTGA AAAGGCCGGG
CTGGGAGGTG AAAATCATAA
301 ATCTGGGCGC CTTGAAACAG CATTCTTTAG GGAAGGAGAC
TATGTGATCG CTGTGCAGCA
361 GGTTCGAATC CATAATAAGA CAGATCAGAA TCTGAGCCAC
TTGAGATGGA TAGGCTCACT
421 TGTGCATACT ACATCGATTG CAAGGGCGAA TTCCAGCACA
CTGGCGGCCG TACTAGTGGA

K 14

1 CGGGCGAATT GGGCCCTCTA GATGCATGCT CGAGCGGCCG
CCAGTGTGAT GGATATCTGC
61 AGAATTCGTC CTTGCAATCG ATGGAAATCT GAATGATGGA
TGTCAAAGAG CACTGCAGGC
121 AACGGGGGAC AATTATTTAC CTAGAAAATA TCTACAGTAG
ACACGTTCGT CTGTAGTATC
181 TAAAGGTCTT AATTCTGCTG CCGCTCTGCC ACTTACTGCA
TGAATCTGTG TGGGTTGAAT
241 TCATTGCTCT GAGGTTTCAT TTGTCCATTT AAAATGCATG
ATCTGCATTA TCCACCTCAG

301 GAGCTTGTAT TGAAGCTCAA GTGTCAAAAA TGCATCAAAA TGCTAAAGCG TTACCATTAT
361 AAAATGACTG AAATTGTTAC TCATCGATTG CAAGGGCGAA TTCCAGCACA CTGGCGGCCG

K 15

1 ACCCTTNGCT TGGTACCCGA GCTCGGATCC ACTAGTAACG GGCCGCCAGT GTGCTGGAA
61 TTCGCCCTT GCAATCGATG TATAAAGACA NTTTTAATAC TCTGCAAATT CAGGGAGTTT
121 TTTCTCCTAT CTCTAGTATC TTTTCACTAT CCAATTACAT CAAAGCCACC CCTGAGCACT
181 GCTCCCTTCA TATTGNCATT GTGGTAACTT ACTTTAAAAC TTCTTTAGAA TAAAGAGGTG
241 ATATGTGTGC AAATTAGCTT TCATCTGGAC CTCAAGGGAG

K 16

1 ACNCTTNCTA CTATCCGGCG AATTGNGCCC TCTAGATGCA TGCTCGAGCG GCCGCCAGTG
61 TGATGGATAT CTGCAGAATT CGCCNTTGCA ATCGATGGGC AGGCACGCCA GCCAGACTTC
121 TTAAGAATCC TGGCAAGAGT TAATACAACA GAAGAAACTG AGAACCATAA CTAGCAGCTG
181 TGTCTTTCCT GATTTGTTTG GGTTTTTTAG TAGCCGGCAT ATAATTATGT CTTCATCTTT
241 CAAGAACTGT CATTTTGTCA GTTGCCCAGT TTGATTTATG TGATAAAGNT GAGGATTTGG
301 AAACATACAA TTCAGTTCTA TTTTTATCAT GTCAAAGAAT TAAATTAATT AAAACAATTG

K 17

1 CCGGGCGAAT TGGGCCCTCT AGATGCATGC TCGAGCGGCC GCCAGTGTGA TGGATATCTG
61 CAGAATTCGN CCTTGCAATC GATGCAGATC CACAGGCTAA TGCTACCTCT GGTTTAGGAA
121 TAGAGGAAGT AAATTATTCT ACCTATAATC TATTGGAGCA CAGTGCAGAT GTGAAAGATT
181 GTATCCAGAA AACTTCTTCT CCAAATTTAG ACCTTGTACC TTCTCATATT GATTTGGTAG
241 CAGCGGAAAT TGAGTTAGTA GACCGTGACA AGAGAGAATA TATGCTTAAA AAAGCATTAG
301 AAGAGGTGAA ATCTGAGTAT GACTACATCA TCATCGATTG CAAGGGCGAA TTCCAGCACA

K 18

1 TTGGGCCCTC TAGATGCATG CTCGAGCGGC CGCCAGTGTG
ATGGATATCT GCAGAATTCG
61 TCCTTTAGCA AGTGCTGCAG CTGACAAAAT CCCCGGGTTG
TTAGGTGTCT TTCAGAAGCT
121 GATTGCATCC AAAGCCAATG ACCACCAAGG TTTTTATCTT
CTAAACAGTA TAATAGAGCA
181 CATGCCTCCT GAGTCAGTTG ACCAGTCAGG AAGCAAATCT
TCATTCTGCT ATTCCAAAGA
241 CTTCAGAATT CCAAAACAAC CAAGTTTATC AAGAGTTTCT
TAGTCTTTAT TAATTTGNAT
301 TGCATAAAAT ACGGGGCACT AGCACTGCAA GAAATATTTG
ACGGTATACA ACCAAAAATG
361 GTTGGAATGG GTTTGGAAAA AATCATTATT CCTGGAATTC
AGAAAGTATC TGGGAATGTA

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