THE ROLE OF FUMONISIN B_1 AND OTHER INHIBITORS OF *DE NOVO* SPHINGOLIPID BIOSYNTHESIS IN THE EXPRESSION OF p42 MAP KINASE (pERK2) IN LLC-PK_1 CELLS

by

SARAH SUZANNE RENTZ

(Under the direction of Dr. Ronald T. Riley)

ABSTRACT

FB_1 is a fungal toxin produced by *Fusarium verticillioides* that inhibits ceramide synthase, a key enzyme in the *de novo* sphingolipid biosynthesis pathway. The purpose of this study was to determine changes in expression of p42 MAP kinase (pERK2) in response to FB_1 in LLC-PK_1 cells. Significant inhibition of cell growth was first noted after 48 h exposure to FB_1, but pERK2 expression was decreased at 24 h. Serine palmitoyltransferase (SPT) inhibitors and the glucosylceramide synthase inhibitor, PDMP, did not reverse the decreased expression of pERK2 caused by FB_1. However, each SPT inhibitor alone or PDMP also caused a decreased expression of pERK2, indicating that FB_1-mediated changes in expression of pERK2 could be independent of alterations in sphingoid bases but dependent on *de novo* sphingolipid biosynthesis.

INDEX WORDS: Fumonisin B_1, Sphingolipids, LLC-PK_1, p42 MAP Kinase

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SARAH SUZANNE RENTZ

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SARAH SUZANNE RENTZ

Approved:

Major Professor: Dr. Ronald T. Riley

Committee: Dr. Cham Dallas
Dr. Raghubir Sharma

Electronic Version Approved:

Gordhan L. Patel
Dean of the Graduate School
The University of Georgia
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DEDICATION

Dedicated to my parents, Jim & Patrice Rentz.

Thanks for everything

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CHAPTER 1

LITERATURE REVIEW

I. History and Characteristics of Fumonisins

For centuries, molds have served in the production of food (such as in the ripening of cheese) and have provided numerous metabolites with medicinal uses, such as penicillin (Kotsonis *et al*., 1996). However, they may also produce metabolites that have the potential to produce severe health effects in mammals and humans.

Fungi cannot fix CO_2 as green plants do, and therefore must absorb carbon compounds with other nutrients to maintain growth (Yamazaki 1978). Most fungal metabolites are initially formed from carbon compounds during either the synthesis of essential chemical compounds or in releasing chemical energy; these reactions are termed "primary metabolism". Often during the formation of primary metabolites, part of these carbon compounds are not completely converted and become "intermediary metabolites" in the form of sugars, organic acids, and aromatic acids, leading to other compounds termed "secondary metabolites" or "natural products". The secondary metabolites are often specific to particular producing organisms and can be specific inhibitors in metabolic pathways, alter cell physiology, and induce cellular dysfunction in other organisms (Yamazaki 1978). *Mycotoxin* is the term used to designate these secondary fungal metabolites that can cause biochemical abnormalities in biological systems (Kurata 1978). Mycotoxicoses are the diseases caused by mycotoxins in animals and humans (Kotsonis *et al*., 1996; Pitt 1998).

Mycotoxins are inherently different from bacterial toxins. Generally, bacterial toxins are proteins that produce distinguishing symptoms after only a few hours, and

which are often recognized by the body's immune system resulting in antibody-mediated reactions. Conversely, mycotoxins are chemical compounds that are usually not detected by antibodies, and therefore produce few immediate immune responses. Nonetheless, mycotoxins can be both acutely and chronically toxic, depending on the dose and mechanism of toxicity. Acute animal disorders include liver and kidney damage, attack on the central nervous system, skin disorders such as necrotic lesions, or hormonal damage such as spontaneous abortions or swollen genitals. Toxic exposure at a lower level, when eaten in lesser quantities on a daily basis, can often produce chronic toxicity (Pitt 1998).

Recent interest in mycotoxins was spawned by a series of reports in 1960-1963 that associated the death of thousands of turkeys in England ("Turkey-X disease") and ducklings in Uganda and Kenya with consumption of peanut meal feeds. Scientists soon established the cause of these problems to be mold products, specifically aflatoxins, formed from *Aspergillus flavus* in the feeds (Yamazaki 1978; Kotsonis *et al*., 1996; Pitt 1998). The discovery of mycotoxins generated more research on previous outbreaks. Alimentary toxic aleukia (ATA) caused the deaths of thousands of people in the USSR in the 1940's during World War II. ATA causes fever, bleeding from the skin, nose, throat, and gums, necrosis, and suppression of the immune system. Later research in the 1970's found that ATA resulted from the consumption of poor quality grain that was allowed to remain in fields unharvested over the winter when labor was scarce; the toxin responsible was trichothecene T-2, derived from the *Fusarium* species (Pitt 1998).

Fusarium, one of the most important genera of plant pathogenic fungi, has a high level of infection in economically important plants. *Fusarium* are responsible for root rots, wilts, and cankers in legumes, pines, wheat, corn, other grasses, and many other crops (Hocking 1998). One of the more recently discovered mycotoxins of concern, fumonisin, develops primarily from the fungi *Fusarium verticillioides* (also called *F. moniliforme* Sheldon) and *Fusarium proliferatum*, common soil fungi that often

contaminate corn and other crops including rice, sugar cane, and sorghum (Riley *et al.*, 1993; Hocking 1998). *F. verticillioides* is found worldwide and is frequently found in soil or on corn grown in tropical and warm temperate zones (Hocking 1998). Fumonisin on corn is usually considered a field problem and in culture is produced under high oxygen levels and requires low pH for optimal production (Miller 2001). The most common naturally occurring fumonisins produced by *F. verticillioides* are fumonisins B_1, B_2, B_3, and B_4.

The current interest in fumonisin research that led to their isolation began in the early 1970's, when the fungus *F. verticillioides* was the predominant fungus isolated from moldy corn associated with a South African breakout of ELEM (equine leucoencephalomalacia), which is found in horses and characterized by liquefactive necrotic lesions in the brain (Hocking 1998; reviewed in Marasas 2001). In addition, other occurrences such as bile duct proliferation, increased numbers of mitotic figures, and multinucleated hepatocytes were found in the livers of these horses, indicating that *F. verticillioides* might be carcinogenic (reviewed in Marasas 2001).

Shortly after, another study indicated that fungal toxins might play a role in human esophageal cancer (EC) displayed in the Transkei region of South Africa. The rate of EC in males and females in this region of South Africa was one of the highest in the world, and the staple diet consisted of homegrown corn (reviewed in Marasas 2001). *F. verticillioides* was found to be the prevalent fungus in corn in this area. In addition, one strain of the fungus from this area (MRC 826) was also found to cause ELEM experimentally in horses, along with porcine pulmonary edema (PPE) in pigs, and was hepatotoxic and cardiotoxic in rats (Kriek *et al*., 1981).

The research effort on *F. verticillioides* on corn received increased attention when researchers in the United States reported that corn associated with outbreaks of ELEM containing high levels of *F. verticillioides* was also hepatocarcinogenic in rats (Wilson *et al*., 1985), just as it was in rats fed cultures of *F. verticillioides* strain MRC 826. This

evidence indicated that the unidentified carcinogens produced by *F. verticillioides* were present both in corn naturally contaminated with *F. verticillioides* and corn culture material produced using the Transkeian isolate of *F. verticillioides* (reviewed in Marasas 2001).

In 1988, the mycotoxins fumonisin B_1 (FB_1) and fumonisin B_2 (FB_2) were isolated from cultures of *F. verticillioides* MRC 826 in Tygerberg, South Africa (Bezuidenhout *et al*., 1988). Pure FB_1 was shown to cause ELEM in horses after intravenous injection and oral dosing, PPE in pigs by intravenous injection, and liver cancer in male rats during a dietary study (reviewed in Marasas 2001). In addition, homegrown corn from areas of Transkei with a high incidence of esophageal cancer contained significantly higher levels of FB_1 and FB_2 than areas with a low cancer incidence (reviewed in Marasas 2001). In 1989-1990, the United States experienced a widespread outbreak of ELEM and PPE after animals consumed mixed feeds containing fumonisin-contaminated corn, and another ELEM occurrence in 1995 in Virginia and Kentucky was attributed to fumonisins (Norred *et al*., 1996; reviewed in Marasas 2001). This international correlation between both the fungus *F. verticillioides* and its mycotoxin FB_1 and their roles in disease indicated a need for more research.

FB_1 is one of the most abundant of the fumonisin toxins, and among the most toxic. In assays for carcinogenicity, FB_1 lacked mutagenicity in the *Salmonella* mutagenicity assay, and lacked genotoxicity in DNA assays in primary rat hepatocytes, indicating that FB_1 might be a non-genotoxic carcinogen (reviewed in WHO 2000). The structure of FB_1, as determined by nuclear magnetic resonance, is $C_{34}H_{59}NO_{15}$ with a C_{20} hydroxylated long chain backbone and two propane-1,2,3-tricarboxylic side chains at carbons 14 and 15 with a molecular weight of 722 g/L (Meredith 2000; ApSimon 2001). It is very water soluble, poorly absorbed, rapidly eliminated, and not metabolized (reviewed in WHO 2000). The backbone of FB_1 is strikingly similar to the structures of sphingosine and sphinganine (Figure 1), sphingoid bases that are key components in

pathways of sphingolipid biosynthesis and turnover in cells. This structural correlation prompted the idea that the fumonisins and sphingolipids might be biosynthetically related (Wang *et al*., 1991).

II. Sphingolipid Metabolism

Sphingolipids and sphingoid bases are found in all eucaryotic cells and are especially plentiful in plasma membranes and related membranes such as lysosomes and Golgi membranes (reviewed in Merrill *et al*., 1997). Over 300 sphingolipids are known, and all possess a sphingoid base (long chain) backbone. Sphingoid bases are necessary for the formation of more complex sphingolipids and are known regulators of cellular growth, differentiation, and apoptosis. D-*erythro*-sphingosine and sphinganine, the most common sphingoid bases in animal cells, are present as free bases in low concentrations in cells, because they are quickly metabolized in the *de novo* pathway of sphingolipid biosynthesis and the pathway for sphingolipid turnover.

The *de novo* biosynthesis of sphingolipids requires several specific enzymes (Figure 2). Serine palmitoyltransferase (SPT) catalyzes the condensation of a fatty acyl CoA molecule (for example, palmitoyl-CoA) with serine to form α-ketosphinganine, which is subsequently reduced to form sphinganine. Sphinganine can be phosphorylated to sphinganine-1-phosphate by sphingosine kinase; however, it is normally rapidly N-acylated to form dihydroceramide by ceramide synthase. The addition of a double bond to the sphinganine moiety of dihydroceramide results in the *de novo* production of ceramide. Normally, ceramide produced *de novo* is rapidly converted into more complex sphingolipids, including sphingomyelin and glycosphingolipids, but it can be alternatively degraded to sphingosine and then to sphingosine-1-phosphate (Merrill *et al*., 1997; Riley *et al*., 1998).

Free sphingoid bases and more complex sphingolipids have the ability to regulate cell behavior at several levels of signal transduction. Previous research has shown that

sphingoid bases have the ability to inhibit transformation of cells. A model cell system for carcinogenesis demonstrated that sphingoid bases not only blocked the promotion phase by phorbol esters, but also reduced the number of cells initiated by γ-irradiation (reviewed in Merrill *et al*., 1997). In addition, sphingoid bases play a role in regulation of enzymes, including Na/K ATPase, insulin receptor tyrosine kinases, myosin light chain kinase, and diacylglycerol kinases (Spiegel *et al*., 1993). Sphingosine, in particular, has effects on regulating signaling molecules that play a role in proliferation, including protein kinase C (PKC), forms of phospholipases C and D, calcium, IP_3, cAMP, and Ras (Spiegel *et al*., 1993; Merrill *et al*., 1996b). While sphingosine is growth stimulatory at low concentrations, it has the ability to transverse the cell membrane and can be growth inhibitory and cytotoxic in most cell lines if accumulated and not converted to its 1-phosphate. At low concentrations, sphingosine has a mitogenic effect on Swiss 3T3 cells independently of protein kinase C, as seen by increased DNA synthesis (Spiegel *et al*., 1993; Schroeder *et al*., 1994). However, sphingosine has an inhibitory effect on protein kinase C in other cell lines in the protonated form (reviewed in Merrill 1991).

Ceramide is not only generated *de novo*, but also from the reacylation of sphingosine and turnover of sphingomyelin, or by the breakdown of complex glycosphingolipids (Merrill *et al*., 1996a; Kolesnick & Krönke 1998; Riley *et al*., 1998). Ceramide is one of the most biologically active, hydrophobic molecules and tends to remain within the lipid bilayer (Kolesnick & Krönke 1998). Ceramide has several biochemical targets, including activation of protein kinases and phosphatases, activation of MAP kinases and transcription factors, inhibition of protein kinase C, and dephosphorylation of the Rb gene product. While ceramide is known to induce proliferation in quiescent Swiss 3T3 fibroblasts, it is an apoptotic signal in several cell lines, and accumulation of ceramide very often causes a series of events that lead ultimately to increased cell death (reviewed in Merrill *et al*., 1997).

Similar to ceramide, the 1-phosphate metabolite of sphingosine, sphingosine-1-phosphate (SPP), is a regulator of cellular signaling through indirect activation of MAP kinases, regulation of transcription factors, and regulation of calcium homeostasis (Merrill *et al.*, 1996a). SPP stimulates growth in some cell types such as Swiss 3T3 fibroblasts, but inhibits growth in breast cancer cells (Van Brocklyn *et al*., 1998a; Hong *et al*., 1999; Spiegel *et al*., 1993). Increased conversion of sphingosine to SPP occurs concurrently with a decrease in ceramide (Perry 1999), and SPP is known to suppress ceramide-mediated apoptosis. In addition, activation of ceramide and apoptosis by cellular stresses can be prevented by addition of SPP, or factors that induce SPP such as protein kinase C (PKC) and platelet-derived growth factor (PDGF) (Van Brocklyn *et al*., 1998a; Spiegel 1999). Therefore, growth and apoptotic factors exerting opposing effects on the intracellular levels of ceramide and SPP are able to induce changes in the ratio of the concentrations of ceramide and SPP, thereby disrupting the balance of the so-called "ceramide/SPP rheostat" (Spiegel 1999).

Other complex sphingolipids such as sphingomyelin and glycosphingolipids regulate cellular growth in other ways. The agonist-induced generation of ceramide via turnover of sphingomyelin ("sphingomyelinase/ceramide cycle") serves as a lipid second messenger and induces cell cycle arrest (Merrill *et al*., 1996a). In addition, complex sphingolipids signal extracellular matrix proteins and structural components of membranes, while modulating cell-cell communication and cell adhesion. Complex sphingolipids also play a role in modulation of receptor kinases and expression of recognition sites for microorganisms and toxins (reviewed in Merrill 1991 and Merrill *et al*., 1997).

Modulation of the *de novo* sphingolipid biosynthesis and turnover pathway alters levels of sphingoid bases and other sphingolipids within cells, and these changes are involved in cellular signaling. The balance between certain sphingolipids can either induce cell proliferation (survival) or induce cell death (apoptosis). Thus, toxin

disruption of sphingolipid metabolism via effects on biosynthetic enzymes can alter the expected outcome of critical signaling pathways and ultimately cell function (Riley *et al*., 1996; Riley *et al*., 1999; Riley *et al*., 2001).

III. Disruption of Sphingolipid Biosynthesis, Turnover, and Downstream Signaling

Since the structure of FB_1 is similar to that of sphingoid bases, it was proposed that FB_1 might alter some sphingolipid-dependent activity. It was discovered that FB_1 was an inhibitor of *de novo* sphingolipid biosynthesis (Wang *et al*., 1991). The earliest direct action of FB_1 is the inhibition of ceramide synthase, also known as sphinganine/sphingosine *N*-acyltransferase (Riley *et al*., 1998). Ceramide synthase recognizes both the sphingoid binding domain (amino group) and the fatty acyl CoA domain (tricarballylic acid side chains) of FB_1. This disruption of sphingolipid metabolism results in an inhibition of ceramide biosynthesis, a subsequent increase in intracellular sphinganine, and a decrease in more complex sphingolipids, though these are not concurrent or synonymous. It can also result in an increase in free sphingosine concentrations, which if metabolized also increases SPP levels. However, when toxicity is evident, approximately 95% of the increase in free sphingoid bases is sphinganine (Riley *et al*., 1998; Riley *et al*., 2001).

The toxicity of FB_1 in the farm animal diseases, ELEM and PPE, is well documented and closely correlated to altered sphingolipid biosynthesis (reviewed in Riley *et al*., 1996). The aforementioned symptoms of ELEM in horses, including necrotic lesions in the brain and liver toxicity, are preceded by elevation in the serum sphinganine: sphingosine ratio, along with an increase in serum enzymes, indicating that sphingolipid biosynthesis has been disrupted. In addition, free sphinganine and sphingosine are both elevated; the sphingosine is elevated due to its inability to be metabolized to ceramide. Previous research indicated that the minimum toxic dose of FB_1 from contaminated corn that causes sphingoid base alteration could be as low as 22 ppm (Wang *et al*., 1992).

The species-specific reaction to FB_1 by swine is pulmonary edema (PPE), or severe respiratory distress. PPE occurs only at high levels of exposure to FB_1 from naturally contaminated corn; previous research indicated that levels ≥92 ppm caused PPE within 4-7 days of dosing (Haschek *et al*., 2001). However, pigs fed lower dosages (10-40 ppm) for longer times (4 weeks) showed no clinical toxicity. PPE is correlated with dose- and time-dependent increases in free sphinganine, smaller increases in free sphingosine, and an increased sphinganine: sphingosine ratio in serum and lung, liver, and kidney tissues (Haschek *et al*., 2001).

In rats, FB_1 causes decreased weight and feed consumption, and is also hepatotoxic and carcinogenic in long-term feeding studies (Gelderblom *et al*., 1991). FB_1 is also hepatocarcinogenic in female B6C3F mice and nephrocarcinogenic in male Fisher 344 rats (NTP 1999). In the NTP tumor studies in both rats and mice there was a close correlation between elevation of free sphingoid bases and tumorigenicity (Delongchamp *et al*., 2001; Howard *et al*., 2002). In addition, Wang *et al*. (1991) also demonstrated increases in sphinganine and the sphinganine: sphingosine ratio in hepatocytes after incubation with FB_1.

While many *in vivo* studies correlate FB_1 with animal toxicity, *in vitro* studies have also shown that disruption of sphingolipid metabolism by FB_1 is involved in the control of apoptosis and proliferation, along with alteration of several downstream signals. In fumonisin-treated cells and in animals and plants dosed with fumonisins, there is a close correlation between the accumulation of sphinganine, the decrease in complex sphingolipids, and changes in cell growth, apoptosis, and toxicity. However, SPT inhibitors such as myriocin or β-chloroalanine combined with FB_1 reduce or prevent the resulting sphinganine accumulation but contribute to a decrease in more complex sphingolipids (Riley *et al*., 1998). In both fumonisin-induced apoptosis and increased proliferation, several studies have shown that the cell-specific response to FB_1 could be prevented using SPT inhibitors (Schroeder *et al*., 1994; Yoo *et al*., 1996; Riley *et al*.,

1999; Tolleson *et al*., 1999). The sites of action of FB_1 and SPT inhibitors are depicted in Figure 3.

Hypotheses of fumonisin-induced changes in cells include both repressed expression and increased activity of protein kinase C (Huang *et al*., 1995; Smith *et al*., 1997), activation of MAP kinases, inhibition of serine/threonine phosphatases, inhibition of apoptosis, increased tumor necrosis factor (TNF)-α, altered calcium homeostasis, and increased lipid peroxidation. Current studies focus on how these alterations contribute to cell death and proliferation and their role in increased cancer risk in the liver and kidney (Riley *et al*., 2001).

A. Apoptosis

For many years, the death of a cell was thought to be an uncontrolled failure of homeostasis in response to cellular injury. However, it has been discovered that not only is apoptosis a normal response to toxic injury, it is a biochemically regulated programmed cell death that maintains the integrity of the organism. Apoptosis occurs throughout development (such a removal of interdigital cells during digit formation) and is important to the immune system (during deletion of lethal B- and T-lymphocytes). Apoptosis is also advantageous to the organism as it provides a means of disposal of damaged cells without disturbing the surrounding tissue environment. It plays a substantial role in diseases such as cancer, AIDS, and neurological disorders including Alzheimer's disease (Davis 2000; Gill & Dive 2000).

Apoptosis is composed of regulated stages, including a loss of cell contact with neighboring cells, chromatin condensation, and shrinkage of the organelles. These are followed by a breakdown of the cell into "apoptotic bodies", or membrane-bound fragments. The fragments are then rapidly engulfed and degraded by phagocytes, keeping cellular elements such as hydrolases and lysosomes from spilling into the

environment. This process develops without an inflammatory response, as occurs in oncotic necrosis (Gill & Dive 2000).

Many studies have shown that ceramide is a second messenger in response to many apoptotic stress signals, including TNF-α, retinoic acid, hydrogen peroxide, nitric oxide, UV light, irradiation, heat shock, and anti-tumor agents (reviewed in Okazaki *et al*., 1998). In addition, several studies correlate increased levels of cellular ceramide with cell cycle arrest and/or programmed cell death.

Previous research has shown that delivery of synthetic (C2) ceramide to HL-60 cells results in inhibition of cell growth and induced apoptosis with nuclear condensation and DNA fragmentation, indicating the role of ceramide in apoptosis (reviewed in Okazaki *et al*., 1998). In another study with Molt-4 cells, both elevations in natural ceramide and delivery of synthetic C6-ceramide correlated with induction of cell cycle arrest and apoptosis (Jayadev *et al*., 1995). In a study involving cellular senescence in human diploid fibroblasts (HDF), ceramide levels remained stable as long as cells were proliferating, but increased with the decline in cell proliferation. Addition of ceramide to young HDF also showed the ability of ceramide to inhibit DNA synthesis and cell growth (Venable *et al*., 1995).

In *de novo* sphingolipid biosynthesis, if palmitate is blocked from beta-oxidation by mitochondrial inhibitors, higher concentrations will flow into the sphingolipid pathway, generating an increase in ceramide (reviewed in Riley *et al*., 2001). Ceramide has been shown to increase the permeability of membranes by forming large stable pores via an extensive hydrogen-bonding network (Siskind & Colombini 2000). Increased ceramide levels have been shown to have an effect on various aspects of mitochondrial function, including induction of cyctochrome *c* release, alteration of calcium homeostasis, enhanced generation of reactive oxygen species, and ATP depletion (Siskind & Colombini 2000).

However, other studies have shown that depletion, not accumulation, of ceramide also results in apoptosis. Tolleson *et al*. (1999) found that treatment of human keratinocytes with FB_1 resulted in increased DNA fragmentation that correlated with ceramide depletion. In LLC-PK_1 cells, it was also found that FB_1 blockage of ceramide synthase resulted in apoptosis, indicating that ceramide accumulation *de novo* was not the signal for apoptosis in these lines (Riley *et al*., 1999). These findings indicate that apoptosis may be induced when intracellular ceramide levels are both above and below the tolerable limits.

The mechanisms by which ceramide affects apoptosis are still unclear, though there are many plausible pathways. One target of ceramide, the MAP kinases, can be drastically affected due to disruption of signaling, leading to the hypothesis that ceramide disrupts the phosphorylation and activation of these kinases (reviewed in Okazaki *et al.*, 1998).

B. MAP Kinases

MAP kinases, or mitogen-activated protein kinases, sustain upstream signals for cell growth and differentiation by cascades of either tyrosine or serine/threonine phosphorylations (Seger & Krebs 1995). Once a MAP kinase is activated, it relays signals downstream by phosphorylating various proteins in a cell, including other kinases, transcription factors, and gene regulatory proteins (Alberts *et al*., 1994).

The family of MAP kinases includes the subfamily of extracellular-signal-regulated kinases (ERKs), which include p44 MAP kinase (ERK1) and p42 MAP kinase (ERK2), p44/42 representing 44 kDa and 42 kDa, respectively, and the "p" indicating phosphorylation (Hayakawa *et al*. 1999). Cloning of ERK1 and ERK2 revealed a 90% identity between them, indicating functional redundancy (Seger & Krebs 1995). The MAP kinases are phosphorylated by MAP kinase/ERK kinases (MEK), which in turn is activated by MEK kinase (MEKK).

Upstream of the ERK cascade, many signals including growth factors, hormones, and cellular stresses activate Ras, a small GTP binding protein. Ras in turn activates Raf-1, a member of the MEKK family. Raf-1 is known to activate MEK 1/2, which then activates ERKs 1 and 2 (Figure 4) (Whitmarsh & Davis, 1996). Following ERK phosphorylation is the activation of several proteins in both the cytoplasm and nucleus including EGF receptor, microtubule-associated proteins, and cytoplasmic phospholipase A2 to release arachidonic acid. It also regulates the activity of transcription factors such as Elk, c-Myc, c-Fos, and c-Jun (Seger & Krebs 1995; Whitmarsh & Davis 1996). Usually, ERKs respond to growth factors, cytokines, and phorbol esters and are associated with proliferative signals and protection from apoptosis (Wang *et al*., 2000). Still other studies have reported that ERK responds weakly to the apoptosis-associated cytokine TNF-α (Westwick *et al*., 1995). It is reported that the ERK cascade is essential in promoting cell proliferation and opposing cell death stimuli, and that its interruption or absence of activation can sensitize cells to apoptosis (Xia *et al*., 1995; Schlesinger *et al*., 1998; Hayakawa *et al*., 1999).

However, other studies have demonstrated an increase in phosphorylated ERKs when cells are treated with known apoptotic agents. 2,3,5-*tris*-(glutathion-*S*-yl)hydroquinone (TGHQ) causes death in renal cells in a reactive oxygen species (ROS) manner and correlates with increased pERK expression (Huang *et al*., 2000). Hydrogen peroxide (H_2O_2) is also known to induce oxidative stress, correlated with increased phosphorylation of ERKs in cell lines including LLC-PK_1 (pig kidney epithelial) and MCT (mouse proximal tubular) cells (Andreoli *et al*., 1998; Hannken *et al*., 2000). In addition, the potent mitochondrial inhibitor carbonyl cyanide m-chlorophenylhydrazone is associated with inducing pERK (Bloom *et al*., 2001). Still other agents can have a biphasic effect on ERK activation; cadmium, a human carcinogen, decreases pERK in CL3 cells at low concentrations (15-80 μM) but induces the phosphorylation at higher doses (Chuang *et al*., 2000).

Therefore, ERK could be involved in both cell proliferation and apoptosis. The complexities of the role of ERKs may be due to the balances and duration of activation in combination with other MAP kinases (Xia *et al*., 1995). While transient ERK activation leads to proliferation in some cell lines, persistent phosphorylation may induce growth arrest (Chen *et al*., 1996; Chuang *et al*., 2000).

The complexity of the pathways that regulate cell physiology are centered on numerous biochemical components upstream of the signal transduction pathways, as it is possible for several different signals to activate a single downstream factor; likewise, a single upstream signal may play a role in activation of many downstream ones (Alberts *et al*., 1994). Of particular interest is the response of ERK to the levels of ceramide in a cell. One primary mechanism that links ceramide induction of apoptosis to ERKs involves a phosphorylation-dephosphorylation pathway. Ceramide directly targets and activates ceramide-activated protein (CAP) kinase. CAP kinase, in turn, is known to phosphorylate and activate Raf-1 kinase upstream of the MEK proteins (Kolesnick & Krönke 1998). Other studies have shown that ceramide itself binds to Raf-1 to activate MAP kinases (reviewed in Okazaki *et al*., 1998; Huwiler *et al*., 1996).

Ceramide has been linked to activation of ERKs in different cell lines. In a study by Raines *et al*. (1993), addition of C2 and C6 analogs of ceramide to serum-deprived myeloid HL-60 cells resulted in a rapid phosphorylation of p42 MAP kinase within 30 s, but was not sustained and returned to a dephosphorylated state by 5 min. Studies by Blazquez *et al*. (2000) with astrocytes showed that addition of palmitate to generate *de novo* ceramide resulted in an increase of apoptotic DNA fragmentation and ERK activity. Palmitate-induced apoptosis was prevented by the presence of PD098059, a selective inhibitor of the ERK cascade, indicating that ceramide-generated apoptosis involves MAP kinases.

MAP kinases are likely to be a target for FB_1 because they are central enzymes in the signal transduction pathways, activated by many different types of agents that

regulate cell growth such as proto-oncogenes, other kinases, and growth factors. Previous research by Wattenberg *et al*. (1996) studied the effect of FB_1 on the ERK pathway in Swiss 3T3 cells. When cells were incubated at various times with 75 μM FB_1, activation of ERK occurred four minutes later, up to 30 minutes, demonstrating that phosphorylation of ERK is an early response to FB_1 treatment in correlation with increased DNA synthesis. The effects of FB_1 in Swiss 3T3 cells may be due to the accumulation of sphingosine-1-phosphate, which acts upstream of the MAP kinases and is known to activate ERK2 in a pertussis toxin-sensitive manner (Merrill *et al.,* 1997; Van Brocklyn *et al.,* 1998b).

In another study, Pinelli *et al*. (1999) found that human bronchial epithelial cells (W126 VA) exhibited phosphorylation of ERKs within one hour in response to 10 μM FB_1, but returned to a dephosphorylated state by 24 h. The activated MAP kinases, in turn, signaled activation of several downstream proteins including cytoplasmic phospholipase A_2 ($cPLA_2$) and cyclic AMP (cAMP) in response to cellular addition of FB_1. These relationships between FB_1 and ERK activation lead to speculation of similar signaling pathways in another cell line, LLC-PK_1. Currently, the phosphorylation of MAP kinases in LLC-PK_1 cells in response to FB_1 is unknown.

IV. Previous Research

A. LLC-PK_1 Cells

Research on several cell types, including human fibroblasts, colonic cells, esophageal epithelial cells, rat fibroblasts, pig renal cells, and monkey renal cells, has shown alterations in cell growth and apoptosis after exposure to the mycotoxin FB_1 (for example, Tolleson *et al*., 1996; Riley *et al.,* 1999). Since free sphingoid bases, their degradation products, and ceramide can affect cell signal transduction, and with differing effects on proliferation and apoptosis, the results of disrupted sphingolipid metabolism are cell-line specific. As part of both cell survival and death involving highly regulated

signal transduction pathways, inhibition of ceramide synthase by FB_1 can initiate different sphingolipid-modulated downstream signals.

Previous research by Yoo *et al*. (1992) has studied the effects of fumonisins on LLC-PK_1 (porcine renal epithelial tubule cells), a proliferating cell line; the LLC-PK_1 cell line was chosen for initial studies because of its easy maintenance in culture and is well characterized in terms of its biochemistry, physiology, and response to toxins (Yoo *et al*., 1996; Riley *et al*., 1999). The objectives of initial studies were to determine (i) the cytotoxicity of FB_1 to LLC-PK_1 cells, (ii) the effect of FB_1 on *de novo* sphingolipid biosynthesis, and (iii) the dose-response relationship between cytotoxicity in LLC-PK_1 cells and the effects on *de novo* sphingolipid biosynthesis.

FB_1 inhibited proliferation and induced cytotoxicity in LLC-PK_1 cells. Cell proliferation was measured by increases in protein content, and began to decrease at concentrations between 10 and 35 μM FB_1. The rate of increase in protein content became negative at concentrations above 35 μM, indicating cytotoxicity. In addition, a lag period of 24-48 h, during which cells appeared normal, preceded the FB_1-induced decrease in cell proliferation. After the lag period, cells treated with 35 μM FB_1 began to develop loss of cell-cell contact and elongation of cell processes along the periphery of growing colonies, indicating cytotoxicity. Cells treated with levels of FB_1 that were not cytotoxic (0.5 to <10 μM) grew at the same rate as controls but began to die after five days, suggesting that FB_1 is not a mitogen in LLC-PK_1 cells.

To determine the effect of FB_1 on *de novo* sphingolipid biosynthesis in LLC-PK_1 cells, the amounts of free sphingoid bases and the ratio of free sphinganine (Sa) to sphingosine (So) after exposure to 35 μM FB_1 was determined by incorporation of [^{3}H]serine (Yoo *et al*., 1992). Increasing FB_1 concentrations along with increasing exposure time resulted in a 128- and 87-fold increase in free sphinganine after 24 and 48 h, respectively. Free sphingosine increased 2.7-fold at 24 h and returned to control

values after 48 h. The free Sa: So ratio increased 21- and 84-fold after 24 and 48 h, respectively, in response to 35 μM FB_1, but showed changes as early as 6 h. This data suggests that FB_1, in a time- and concentration-dependent manner, disrupts the *de novo* sphingolipid pathway. The incorporated levels of [^{3}H]serine in free sphingoid bases is consistent with the fact that sphinganine is made *de novo*, while sphingosine results from the turnover of more complex sphingolipids (reviewed in Merrill *et al*., 1997).

FB_1-induced cytotoxicity occurred at higher concentrations (35 μM) after three days' exposure, while sphingolipid changes in the *de novo* pathway occurred as early as 6 h after exposure. This suggests that inhibition of *de novo* sphingolipid biosynthesis is an early event leading to cytotoxicity, and that the effects of FB_1 on the two events are correlated and related to toxicity in LLC-PK_1 cells (Yoo *et al*., 1992).

Subsequent research by Yoo *et al*. (1996) on LLC-PK_1 cells further studied the role of elevated sphingoid bases and depletion of more complex sphingolipids in cytotoxicity. Based on the earlier study, it was hypothesized that early fumonisin-induced disruption of sphingolipid metabolism was the cause of cytotoxicity and decreased cell proliferation in LLC-PK_1 cells, and that altered sphingolipid metabolism was not just a coincidental effect. To test this hypothesis, the changes in free sphingoid bases and more complex sphingolipids were determined by inhibiting cell growth using both FB_1 and exogenous sphinganine. In addition, the ability of β-chloroalanine (an inhibitor of serine palmitoyltransferase) to prevent the fumonisin-induced sphingolipid changes and cell death was evaluated.

After treatment with FB_1 in a concentration- and time-dependent manner, 50 μM FB_1 was found to be an optimal concentration for cytotoxicity. At 50 μM, FB_1 caused a significant increase in free sphingoid bases as early as 7 h (as determined by HPLC), with free sphinganine accounting for over 95% of the increase. These changes preceded the decrease in more complex sphingolipids, which was not significantly observed until 24 h.

Both alterations in sphingolipid metabolism preceded the significant decrease in cell growth and onset of cell death at 48 h. This indicated that a low rate of sphingolipid biosynthesis at this FB_1 concentration contributes to inhibited proliferation and cell death.

Adding exogenous free sphinganine (1 to 3 μM) resulted in elevation of free sphinganine and mimicked the cytotoxic effects of fumonisin-induced elevation of free sphinganine in LLC-PK_1 cells, but without depletion of complex sphingolipids. Addition of β-chloroalanine (25-100 μM) minimally decreased protein content and increased cell death but significantly depleted complex sphingolipids in a concentration-dependent manner. However, cells began to show a marked increased in detachment when treated with concentrations of β-chloroalanine higher than 100 μM.

Simultaneous addition of β-chloroalanine (100 μM) and FB_1 (50 μM) was used to determine the extent to which the fumonisin-induced increases in free sphingoid bases (especially free sphinganine) correlated to the fumonisin-induced effects on cell growth and death. The concentration of free sphingoid bases in cells treated only with FB_1 was 4.4 nmol/mg protein at 48 hours, and reduced to 0.3 nmol/mg protein when β-chloroalanine and FB_1 were simultaneously added, but this was still significantly greater than control cells. In addition, the combination of β-chloroalanine and FB_1 was more effective at inhibiting sphingolipid biosynthesis (as shown by decreased complex sphingolipids) than either alone.

Less morphological changes were seen in the cells with the addition of both agents together than when either was added alone; β-chloroalanine only partially prevented the effects of FB_1 on cell growth and cell death. The rate of cell growth (based on total protein) between 48 and 72 h was 0.77 and 0.27 μg/hr for β-chloroalanine- and FB_1-treated cultures, respectively. The combination of β-chloroalanine and FB_1 resulted in a cell growth rate of 0.53 μg/hr.

Both elevation of free sphinganine (by exogenous addition) and depletion of complex sphingolipids (by β-chloroalanine) were correlated with a slight decrease in cell growth. In addition, the observation that there is a lag time of 24-48 h after treatment with FB_1 before changes in cell proliferation occurs, but complex sphingolipids are depleted by 24 h (Yoo *et al*., 1996), indicates that cell division dilutes the cell's complex sphingolipid pool present, and that this depletion is also elevated by treatment with β-chloroalanine alone. These results indicate that changes in the *de novo* sphingolipid pathway due to FB_1 contribute to decreased cell growth and lethality in LLC-PK_1 cells.

Subsequent studies on LLC-PK_1 cells by Riley *et al*. (1999) determined the ability of myriocin (ISP-1), a specific SPT inhibitor, to prevent the anti-proliferative and apoptotic effects of FB_1. In earlier studies, β-chloroalanine only partially reversed FB_1 effects on cell growth and cell death (Yoo *et al.,* 1996), presumably because it also inhibited other pyridoxal 5'-phosphate-dependent enzymes. The purpose of the study with myriocin was to determine the ability of a specific and more potent SPT inhibitor (myriocin) to prevent the anti-proliferative and cytotoxic effects of FB_1.

Initial concentration-response studies with myriocin showed that concentrations ≤170 nM had no significant effect on cell growth at 48 h, but concentrations equal to or greater than 190 nM inhibited cell growth. Myriocin prevented the fumonisin (50 μM)-induced inhibition of cell growth at concentrations ≥16 nM, and mean protein content from those treatments were not significantly different from treatments of myriocin alone or controls that were not treated with either FB_1 or myriocin. The addition of myriocin (IC_{95}=22 nM) also reversed the FB_1-induced increase in free sphingosine and free sphinganine.

Proliferating cells treated with FB_1 displayed significant decreases in mitotic figures and significant increases in apoptotic bodies. These FB_1-induced changes were prevented by myriocin treatment (170 nM). While DNA laddering was not increased in

attached FB_1-treated cells at 48 h, the number of detached cells (floaters) in the growth medium was significantly increased and these cells exhibited classical DNA laddering indicative of apoptotic cell growth. The findings indicate that myriocin prevented apoptosis in FB_1-treated cells. However, it should be noted that while myriocin reversed the effects of fumonisin on cell growth, detachment, and apoptosis, it did cause subtle morphological changes in the cells.

Similar results were seen by Yu *et al*. (2001) in LLC-PK_1 cells after treatment with 0.5 mM L-cycloserine, another SPT inhibitor, to reverse the anti-proliferative effects of FB_1. The concentration of intracellular free sphinganine in cells treated with 50 μM for 72 h was 1450 pmol/mg protein, and was reduced to 140 pmol/mg protein after treatment with a combination of L-cycloserine and FB_1. Treatment with L-cycloserine also lowered the free Sa: So ratio induced by FB_1 by approximately 5-fold, while treatment with L-cycloserine alone resulted in a free Sa: So ratio that was comparable to control cells. In addition, L-cycloserine reversed the fumonisin-induced apoptosis by approximately 4-fold, as determined by distribution of apoptotic cells.

The facts that inhibitors of SPT are protective against FB_1 and that exogenous addition of sphinganine imitates FB_1 effects strongly support the conclusion that the FB_1-induced increase in free sphinganine leads to later inhibition of cell growth and increased apoptosis in LLC-PK_1 cells. However, while the use of SPT inhibitors is significant in initially assessing FB_1 toxicity to proliferating LLC-PK_1 cells, long-term use has several consequences to *de novo* sphingolipid biosynthesis and overall cell function. The use of fumonisins and SPT inhibitors simultaneously gives an additive effect on depletion of more complex sphingolipids, and these changes could contribute to changes in morphology seen in LLC-PK_1 cells treated with either FB_1 or SPT inhibitors and the increased cell death seen in LLC-PK_1 cells exposed to SPT inhibitors for periods longer than 48 h.

B. MDCK Cells

Research by Shayman *et al*. (1991) showed that the growth of Madin-Darby canine kidney (MDCK) cells, another renal epithelial cell line comparable to LLC-PK_1 cells, could be modulated in response to inhibitors of glycosphingolipids downstream of ceramide. Glucosylceramide (GlcCer), in particular, has been shown to induce proliferation in a number of cell lines, and inhibition of GlcCer synthase has been shown to reduce cell growth and block proliferation. The inhibition of GlcCer synthase by PDMP (D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol) (Figure 3) results in a decrease in glucosylceramide and an increase in intracellular ceramide, sphingomyelin, and sphingosine. MDCK cells were treated with 20 μM PDMP for 48 h in order to analyze the changes in endogenous GlcCer and other sphingolipids. The results showed a 75% decrease in the amount of endogenous GlcCer when compared to controls, while a 30% increase in ceramide levels occurred. In addition, PDMP treatment produced time- and concentration-dependent decreases in cell number, protein, and DNA after 48 h. Cells treated with 20 μM PDMP for 24 h also exhibited a decrease in protein kinase C activity. The study indicated that the growth of MDCK cells is associated with cellular GlcCer content, and that cell death could be associated with increased ceramide or sphingosine. These findings lead to the hypothesis that, in addition to sphingoid bases, the comparable LLC-PK_1 cell line could rely on glycosphingolipids for signal transduction pathways necessary for cell growth, apoptosis, and response to FB_1.

V. Rationale for Research

The rodent carcinogenicity and cancer promotion of fumonisin B_1 is closely correlated with increased apoptosis and subsequent regenerative cellular proliferation (NTP, 1999; Dragan *et al*., 2001). Both the fumonisin-induced increased apoptosis and cell proliferation are closely related with the degree of disruption of sphingolipid metabolism as evidenced by the increase in free sphingoid bases (Delongchamps *et al*.,

2001; Howard *et al*., 2002). Many cancer promoters are activated when mitogenic signals are turned on. The fact that MAP kinases are mitogenic signals affected by fumonisin inhibition rationalizes that cancer promoters can be turned on due to disruption of sphingolipid metabolism and other signals related to disruption of sphingolipid metabolism. Therefore, this research will be beneficial in understanding the cellular processes regulating the tumorigenicity of FB_1 and the role of disrupted sphingolipid metabolism in these processes in both animals and humans.

VI. Objectives and Hypothesis

The primary objective of this project is to determine the biochemical and molecular mechanisms of action of fumonisin B_1 on sphingolipid pathways and subsequently on MAP kinases in cell signal transduction pathways. The purpose of this study is to determine, in LLC-PK_1 cells, changes in expression of p42 MAP kinase (pERK2) in response to FB_1, SPT inhibitors, and PDMP in order to better understand the downstream signals associated with sphingoid bases and more complex sphingolipids. We hypothesize that the response (proliferation or apoptosis) of a particular cell type to fumonisins is determined by the cell-specific downstream targets that are acted on by the fumonisin-induced accumulation or depletion of specific bioactive molecules in the sphingolipid pathways.

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Legends for Figures

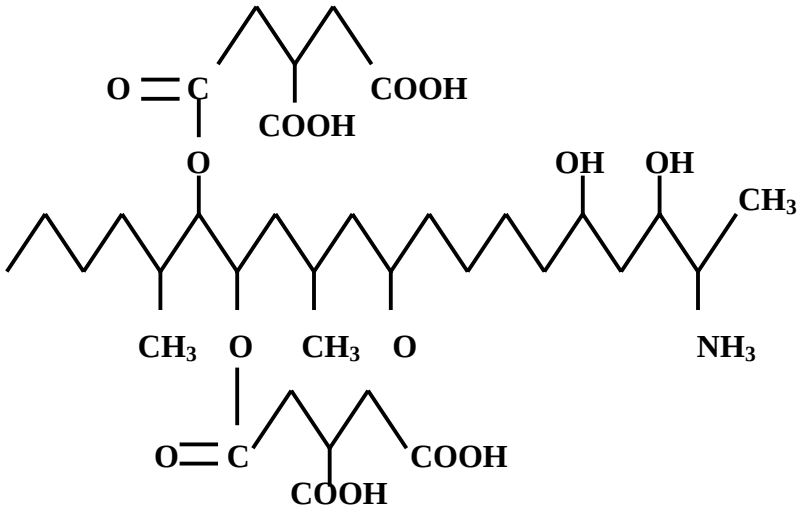
Figure 1: Structures of fumonisin B_1 (A), sphinganine (B), and sphingosine (C).

Figure 2: The *de novo* pathway of sphingolipid biosynthesis and turnover.

Figure 3: The sites of action of SPT (serine palmitoyltransferase) inhibitors, fumonisin B_1, and PDMP (D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol) in the *de novo* sphingolipid biosynthesis and turnover pathway.

Figure 4: The MAP kinase phosphorylation cascade activated by Ras. The specific kinases that are phosphorylated in the ERK cascade (Raf-1, MEK 1/2, and ERK1/2) are indicated (adapted from Alberts *et al*., 1994).

A.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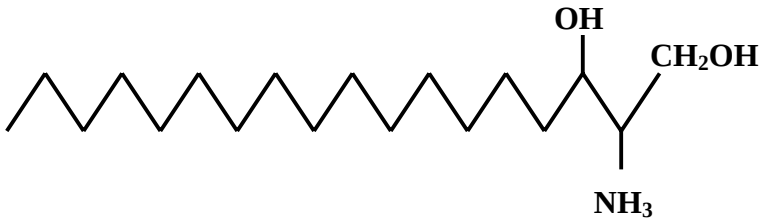
B.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C.

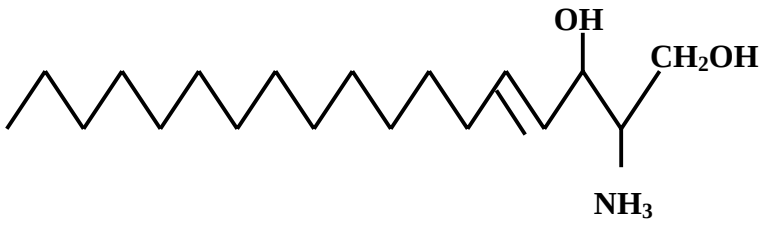
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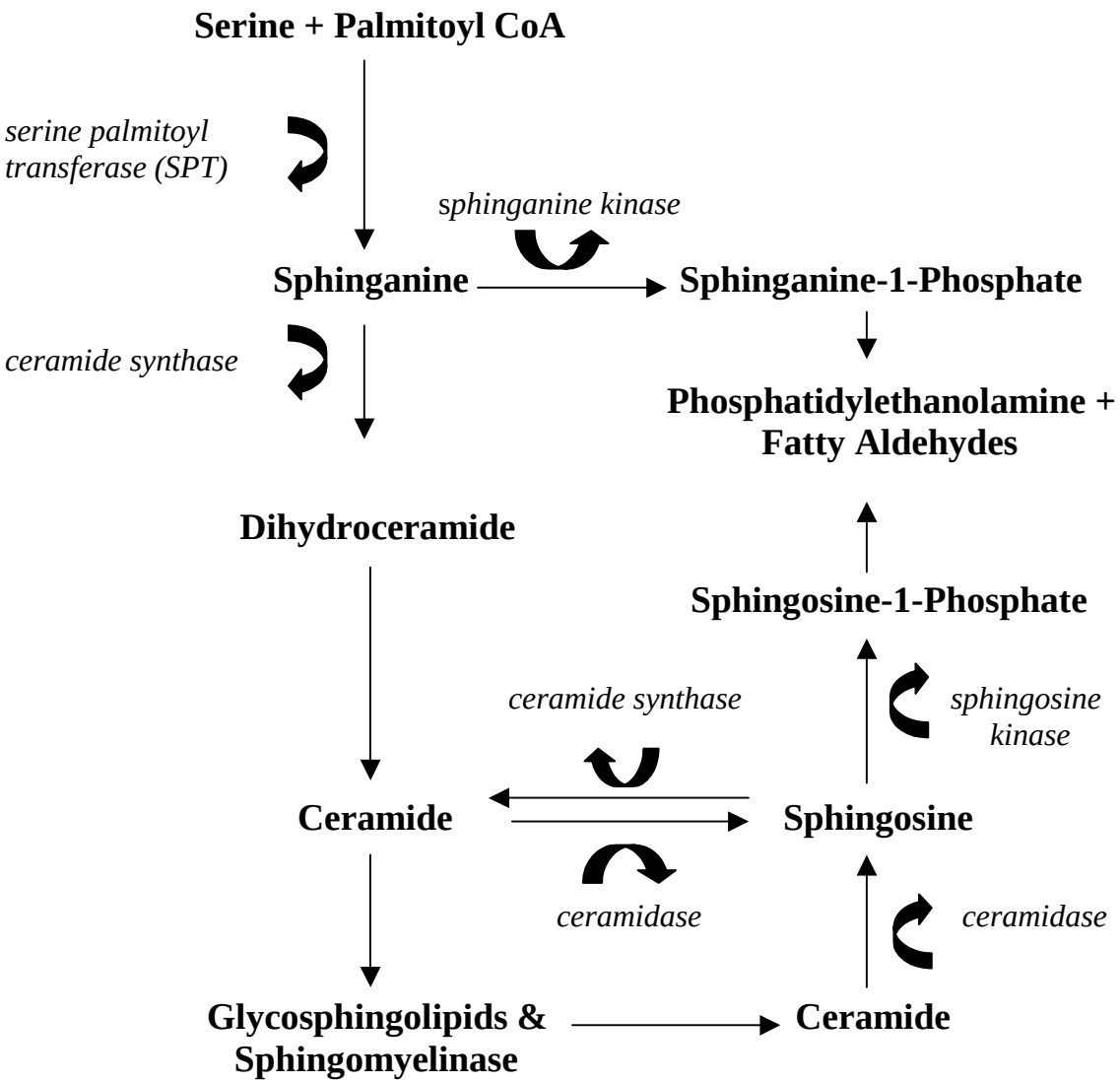
Figure 1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sphinganine kinase
Sphinganine
Sphinganine-1-Phosphate
ceramide synthase
Phosphatidylethanolamine + Fatty Aldehydes
Dihydroceramide
Sphingosine-1-Phosphate
ceramide synthase
sphingosine kinase
Ceramide
Sphingosine
ceramidase
ceramidase
Glycosphingolipids & Sphingomyelinase
Ceramide

Figure 2

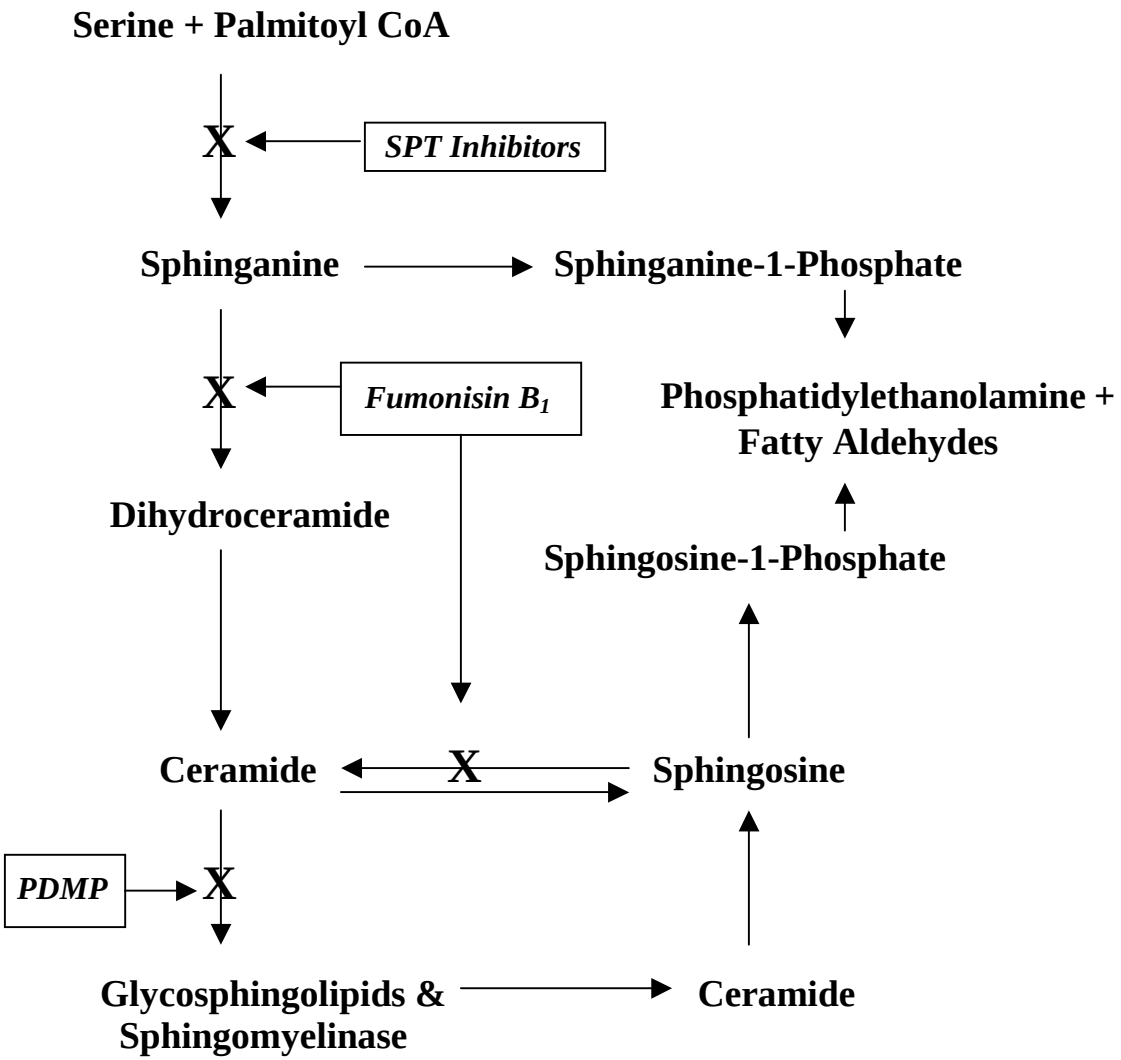
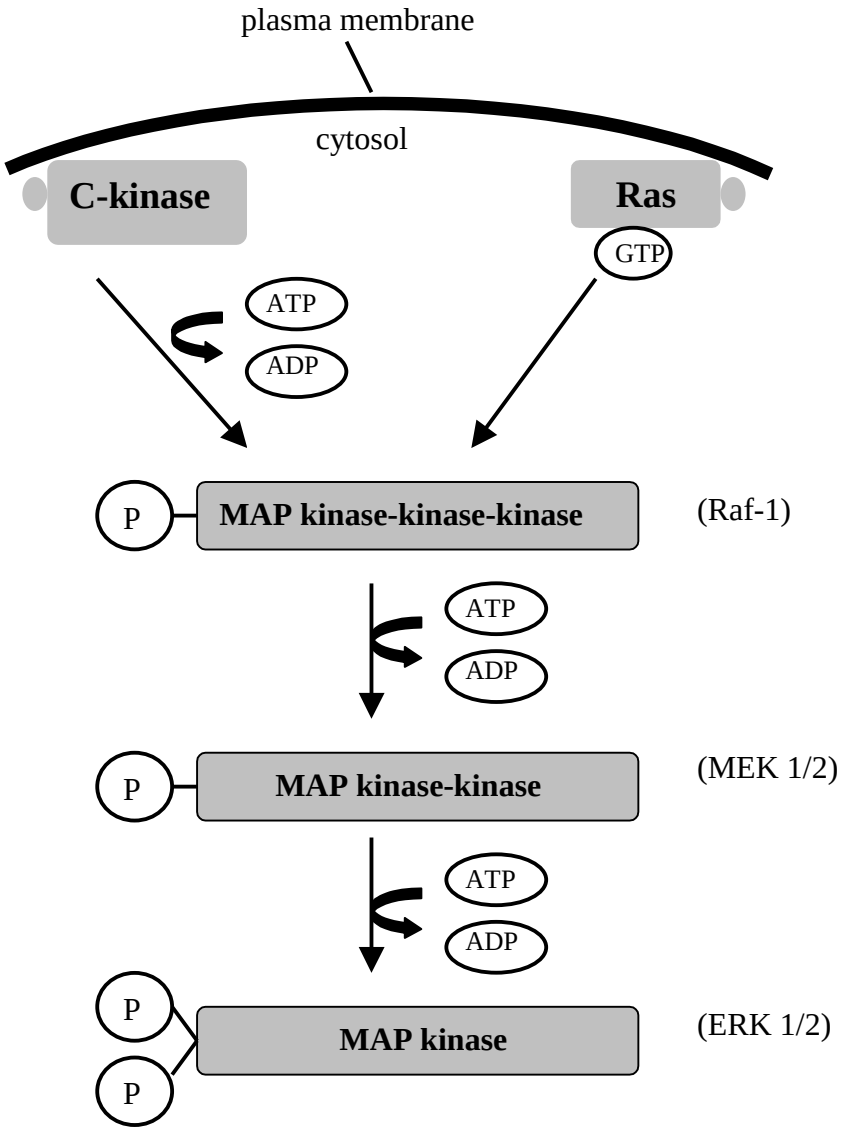

Serine + Palmitoyl CoA
X
SPT Inhibitors
Sphinganine
Sphinganine-1-Phosphate
X
Fumonisin B1
Phosphatidylethanolamine +
Fatty Aldehydes
Dihydroceramide
Sphingosine-1-Phosphate
Ceramide
X
Sphingosine
PDMP
X
Glycosphingolipids &
Sphingomyelinase
Ceramide

Figure 3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cytosol
C-kinase
Ras
GTP
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P
MAP kinase-kinase-kinase
(Raf-1)
ATP
ADP
P
MAP kinase-kinase
(MEK 1/2)
ATP
ADP
P
P
MAP kinase
(ERK 1/2)

Figure 4

CHAPTER 2

INHIBITION OF SPHINGOLIPID BIOSYNTHESIS DECREASES PHOSPHORYLATED ERK2 EXPRESSION IN LLC-PK_1 CELLS[1]

[1] Rentz, S.S., Meredith, F.I., Showker, J.A., and Riley, R.T. To be submitted to *Toxicology & Applied Pharmacology*.

ABSTRACT

Fumonisin B_1 (FB_1) is a fungal toxin produced by *Fusarium verticillioides* that inhibits ceramide synthase (CS), a key enzyme in the *de novo* sphingolipid biosynthesis pathway. In LLC-PK_1 cells, FB_1 inhibits cell proliferation and induces apoptosis, which can be prevented by inhibitors of serine palmitoyltransferase (SPT). Inhibition of SPT prevents the FB_1-induced accumulation of free sphinganine, a precursor of ceramide biosynthesis. However, not all of the effects of FB_1 in LLC-PK_1 cells can be explained solely by the increase in free sphingoid bases. The downstream signaling pathways that are affected by FB_1-induced disruption of sphingolipid biosynthesis are not well understood. This study determined, in LLC-PK_1 cells, changes in expression of p42 MAP kinase (phosphorylated ERK2 [pERK2]) in response to various inhibitors of key enzymes of the *de novo* sphingolipid biosynthesis pathway (CS, SPT, and glucosylceramide synthase [GlcCer synthase]). The results show that inhibition of any of the three enzymes caused a similar decrease in pERK2 expression with no reduction in total ERK2. The co-treatment of FB_1 (CS inhibitor) with SPT inhibitors or the GlcCer synthase inhibitor had no effect on the FB_1-induced reduction in pERK2 expression, indicating that FB_1-mediated changes in expression of pERK2 were independent of the FB_1-induced increases in free sphinganine or reduction in ceramide. Nonetheless, the decrease in pERK2 expression was dependent on inhibition of *de novo* sphingolipid biosynthesis. Decreased pERK2 expression could contribute to the physiological effects of FB_1 in LLC-PK_1 cells that are not due to alteration in pathways modulated by free sphingoid bases and their metabolites.

INTRODUCTION

Fumonisins are a group of mycotoxins derived from *Fusarium verticillioides* (= *F. moniliforme*), a prevalent fungal contaminant in corn (reviewed in WHO, 2000). Fumonisin B_1 (FB_1) is the most abundant of the fumonisins and consumption of FB_1 causes domestic animal diseases including equine leucoencephalomalacia and porcine pulmonary edema (reviewed in WHO, 2000). In domestic and laboratory animals, FB_1 is hepato- and nephrotoxic and hepatocarcinogenic in male BD IX rats and female B6C3F mice and nephrocarcinogenic in male Fisher 344 NCTR rats (Gelderblom *et al*., 1991; NTP 1999). Exposure to fumonisins is also correlated with human cancers in southern Africa and China (Ueno *et al*., 1997; reviewed in Marasas 2001).

FB_1 is structurally similar to sphingoid bases produced in the *de novo* sphingolipid biosynthesis and turnover pathways (Fig 1). FB_1 is known to inhibit ceramide synthase, a key enzyme in *de novo* ceramide biosynthesis and the re-acylation of sphingosine generated by the breakdown of complex sphingolipids (e.g. glycosphingolipids and sphingomyelin) (Wang *et al*., 1991). The disruption of sphingolipid metabolism by FB_1 results in an increase in intracellular free sphinganine, inhibition of *de novo* sphingosine and ceramide biosynthesis, depletion of more complex sphingolipids (glycosphingolipids and sphingomyelin), and increased phosphatydlethanolamine (reviewed in Merrill *et al*., 2001 and Riley *et al*., 2001).

The effects of fumonisin on sphingolipid biosynthesis have been studied extensively in the porcine renal epithelial cell line LLC-PK_1 (Yoo *et al*., 1992; Yoo *et al*., 1996; Riley *et al*., 1999; He *et al*., 2001; Kim *et al*., 2001; He *et al*., in press; Enongene *et al*., in press). Inhibition of ceramide synthase causes free sphinganine and sphingosine to accumulate in LLC-PK_1 cells, whereas, inhibition of either serine palmitoyltransferase (SPT), the first and rate-limiting step in *de novo* sphingolipid biosynthesis (Fig 1), or ceramide synthase inhibits the biosynthesis of complex sphingolipids (including ceramide). Simultaneous inhibition of SPT and ceramide synthase inhibits ceramide

biosynthesis but prevents the FB_1-induced accumulation of free sphinganine (Riley *et al*., 1999) (Fig 1). Glycosphingolipid biosynthesis can also be inhibited using PDMP (D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol), an inhibitor of glucosylceramide (GlcCer) synthase (Shayman *et al*., 1991). Thus, it is possible to dissect the contribution of free sphingoid bases, ceramide, and glycosphingolipid biosynthesis in FB_1-treated LLC-PK_1 cells using the various inhibitors of *de novo* sphingolipid biosynthesis.

Many short-term acute studies (minutes to a few hours) using cultured cells correlate the accumulation of ceramide with cell death (for review see Riley *et al*., 2001). If the source of ceramide is via the *de novo* sphingolipid biosynthesis pathway, then FB_1 inhibition of ceramide synthase protects cells, including LLC-PK_1 cells, from cell death (Ueda *et al*., 1998; Riley *et al*., 2001). However, with longer FB_1 exposure (hours to days) the accumulation of free sphinganine, a precursor of ceramide, induces cell death in LLC-PK_1 cells (Yoo *et al*., 1996; Riley *et al*., 1999; Yu *et al*., 2001; He *et al*., in press). Inhibition of GlcCer synthase using PDMP causes a decrease in GlcCer, an increase in ceramide and sphingosine, and inhibition of cell growth in Madin-Darby canine kidney (MDCK) epithelial cells (Shayman *et al*., 1991).

In LLC-PK_1 cells, treatment with micromolar concentrations of FB_1 leads to sphinganine-induced inhibition of cell growth and apoptosis (Yoo *et al*., 1996; Riley *et al*., 1999; He *et al*., 2001; Kim *et al*., 2001; He *et al*., in press;). While sphinganine accumulation is evident within a few hours after exposure (Yoo *et al*., 1992; He *et al*., 2001; Kim *et al*., 2001), there is a lag time of approximately 12 to 24 hours before morphological changes and biochemical changes associated with increased cell death and inhibition of cell growth become apparent (Yoo *et al*., 1996; Riley *et al*., 1999; Kim *et al*., 2001; He *et al*., in press). Treatment of cells with SPT inhibitors blocks the FB_1-induced accumulation of free sphinganine, increased apoptosis and inhibition of cell growth. However, the SPT inhibitors and GlcCer synthase inhibitors also inhibit cell

growth and alter cell morphology based on light microscopic examination (Shayman *et al*., 1991; Yoo *et al*., 1996; Riley *et al*., 1999), and depletion of complex sphingolipids, including ceramide, has effects independent of those due to elevation of free sphinganine (Yoo *et al*., 1996; Riley *et al*., 1999; Tolleson *et al*., 1999).

While the effects of ceramide synthase, SPT, and GlcCer synthase inhibitors on sphingolipids have been well studied in renal cell lines, the downstream effects are not well understood. FB_1 has been shown to induce sphinganine-dependent changes in expression of calmodulin (Kim *et al*., 2001) and caspase III activity (Yu *et al*., 2001) in LLC-PK_1 cells. Conversely, FB_1 induces a transient increase in expression of TNF-α in LLC-PK_1 cells that is independent of the elevation in free sphinganine (He *et al*., 2001). Several studies have reported FB_1-induced increase in MAP kinase activity, specifically extracellular-signal regulated kinases (ERKs) activity, in various cell lines (Wattenberg *et al*., 1996; Pinelli *et al*., 1999). ERKs are usually associated with cellular growth, and it is reported that the ERK cascade is essential in opposing cell death stimuli and that its interruption or absence of activation can sensitize cells to apoptosis (Xia *et al*. 1995; Schlesinger *et al*. 1998; Hayakawa *et al*. 1999).

In MDCK cells, PDMP inhibition of GlcCer synthase inhibits cell growth and protein kinase C activity (PKC), whereas, stimulation of GlcCer activity increases PKC activity and has a proliferative effect in these cells (Shayman *et al*., 1991). One mechanism for MAP-kinase-kinase-kinase phosphorylation is through PKC (Alberts *et al*., 1994). It is possible that some of the FB_1-induced cellular effects in LLC-PK_1 cells, that are not sphinganine-dependent, may be a result of other consequences of disrupted sphingolipid metabolism. Currently, the role of ERKs in cellular growth and death in FB_1-treated LLC-PK_1 cells is unknown. The purpose of this study was to determine, in LLC-PK_1 cells, changes in expression of p42 MAP kinase (pERK2) in response to FB_1, SPT inhibitors, and PDMP in order to better understand the downstream signals associated with sphingoid bases and more complex sphingolipids.

MATERIALS AND METHODS

Reagents: FB_1 was prepared as described in Meredith *et al*. (1996) and the purity verified to be greater than 95%. ISP-1 was prepared as described in Riley and Plattner (2000). β-chloroalanine, L-cycloserine, PMSF (phenylmethylsulfonyl fluoride), and Tween-20 were purchased from Sigma (St. Louis, MO). D-threo-1-phenyl-2-decanoylamine-3-morpholino-1-propanol (PDMP) was purchased from Matreya, Inc. (State College, PA). Dulbecco's phosphate-buffered saline (PBS), Dulbecco's modified Eagle medium (DMEM), Ham's F12, fetal calf serum (FCS), Hanks' balanced salt solution, and trypsin were obtained from Gibco (Rockville, MD). EDTA (ethylenediaminetetracetic acid) was obtained from Calbiochem (La Jolla, CA). Reagents and membranes used for protein assay, electrophoresis, and Western blotting were obtained from Bio-Rad (Hercules, CA). BSA (bovine serum albumin) and antibodies for total ERK2 (rabbit polyclonal) and phospho-specific ERK2 (mouse monoclonal) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Biotinylated anti-rabbit and anti-mouse IgG antibodies and a biotinylated detector reagent (R.T.U. ABC Reagent) were purchased from VectorLabs (Burlingame, CA).

Cell Culture: Pig kidney epithelial cells (LLC-PK_1, CL 101, passage 196 or 127) were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cells were grown in DMEM/Ham's F12 (HDMX) plus 5% FCS and incubated at 37°C/ 95% humidity/ 5% CO_2. For all experiments, cells were seeded in 25 cm^2 flasks and allowed to attach and grow for 48 h, at which time they were approximately 50%-confluent. Before treatment, growth medium was removed and cells washed with cold phosphate-buffered saline (PBS). Media was centrifuged in order to remove cellular debris, and poured back onto cells. Fresh growth medium was not used in order to avoid "bursts" of sphingosine and sphinganine that could affect cell function (Smith *et al*. 1995, 1997). Working solutions of FB_1, β-chloroalanine, and L-cycloserine were dissolved in sterile glass distilled water and added directly into growth medium (≤ 10 μl/ml). ISP-1

and PDMP were dissolved first in ethanol (1 mg/ ml and 5 mg/ml respectively) and then diluted (approximately 1:1000 and 1:10, respectively) into sterile glass distilled water which was then added into growth medium (approximately 10 μl/ml).

Harvesting Cells and Protein Analysis: After treatment, cells were washed twice with ice-cold 1X PBS, the flasks placed on ice, and cells removed from the surface of the flask using a rubber scraper. The cells detached by scraping were collected in PBS and centrifuged for 10 min at 16,000 x RCF at 4°C. The cell pellets were lysed in 125 μl of a buffer containing 60 mM Tris pH 6.8, 35 mM SDS, and 2 mM PMSF. The lysates were boiled for 5 to10 minutes to denature proteins. Samples were then sonicated for 5 minutes, allowed to cool, and centrifuged for 10 min at 16,000 RCF at 4°C. The lysates were assayed for total protein content using the Bio-Rad protein assay.

Western Blotting: Laemmli sample buffer with 5% β-mercaptoethanol was added to protein samples in a 1:2 dilution for gel loading purposes. Lysates (10 μg total protein) were applied to a 12.5% SDS gel, proteins separated by electrophoresis, and transferred onto a nitrocellulose membrane at 30 V overnight at 4°C. The membrane was blocked in Tris-buffered saline with 0.1% Tween-20 containing 5% BSA, probed with a primary antibody (for either total or phospho ERK2) at 4°C overnight, treated with a secondary antibody (either anti-rabbit or anti-mouse IgG) for an additional hour at room temperature, and treated with biotinylated detector reagent for 30 minutes. Incubation with chemiluminescence reagents and detection by Hyperfilm (Amersham/Pharmacia, Piscataway, NJ) allowed for visualization of proteins, which were then quantitated by strip densitometry using the TNImage computational program (T.J. Nelson, Rockville, MD).

Concentration response and time course experiments: LLC-PK_1 cultures (four 25 cm^2 flasks/concentration) were harvested after treatment with 0, 1, 10, or 50 μM FB_1 for 24 h. The time course of changes in pERK2 expression was determined after exposure to

50 μM FB_1 for 0 min, 10 min, 30 min, 1 h, 2 h, 4 h, 8 h, and 24 h (two 25 cm^2 flasks/ time point), and in a separate experiment at 24, 48 and 72 h (three 25 cm^2 flasks/time point). For all experiments, total protein content, pERK2, and total ERK2 expression were determined as described above.

SPT Inhibitors and PDMP experiments: For treatment with ISP-1, LLC-PK_1 cultures (nine 25 cm^2 flasks/treatment) were treated with vehicle only (control-treated), 50 μM FB_1, 150 nM ISP-1, or a combination of 50 μM FB_1/150 nM ISP-1. For treatment with β-chloroalanine, LLC-PK_1 cultures (three 25 cm^2 flasks/treatment) were treated with vehicle only (control-treated), 50 μM FB_1, 100 μM β-chloroalanine, or a combination of 50 μM FB_1/100 μM β-chloroalanine. For treatment with L-cycloserine, LLC-PK_1 cultures (three 25 cm^2 flasks/treatment) were treated with vehicle only (control-treated), 50 μM FB_1, 500 μM L-cycloserine, or a combination of 50 μM FB_1/ 500 μM L-cycloserine. The concentrations of SPT inhibitors were based on previous findings by Yoo *et al*. (1996), Riley *et al*. (1999), and Yu *et al*. (2001). For treatment with PDMP, LLC-PK_1 cultures (five 25 cm^2 flasks/treatment) were treated with vehicle only (control-treated), 50 μM FB_1, 20 μM PDMP, or a combination of 50 μM FB_1/ 20 μM PDMP. The concentration of PDMP was based on previous findings by Shayman *et al*. (1991). For all experiments, cells were harvested after treatment for 24 h, and protein content, pERK2, and total ERK2 expression determined as described above.

Statistical Analysis. Statistical analysis was done using Sigma Stat software (Jandel Scientific, San Rafael, CA). One way analysis of variance (ANOVA) was used followed by tests for post hoc multiple comparison. All data were expressed as mean $\pm$ SD, and differences among means were considered significant if the probability was <0.05. For the time course study the data were analyzed by non-linear regression analysis and ANOVA.

RESULTS

Fumonisin B_1 induced concentration- and time-dependent decreases in phosphorylated ERK2 expression. Exposure of LLC-PK_1 cells to FB_1 for 24 h resulted in a significant concentration-dependent decrease in phosphorylated ERK2 (pERK2) expression (Fig 2). Expression of pERK2 was decreased on average 40%, 75%, and 90% at 1, 10, and 50 μM FB_1, respectively, relative to the control-treated cells (Fig 2B). The estimated IC_{50} for inhibition of pERK2 expression was between 1 and 10 μM FB_1 (Fig 2B). Cells from all treatments displayed similar amounts of total ERK2 (Fig 2A and data not shown), indicating that the amount of ERK2 in the cells had not changed as a result of FB_1 treatment. In LLC-PK_1 cells exposed to 50 μM FB_1 for various periods of time (0 to 72 h), there was a time-dependent decrease in pERK2 expression (Fig 3 and Fig 4A and B) which was maximal at 24 to 48 h and then increased slightly, but significantly, between 48 and 72 h (Fig 4A and B).

Fumonisin B_1 inhibition of cell growth and morphological changes. While pERK2 was maximally inhibited after 24 h at 50 μM FB_1, there was no effect (p=0.19) on cell growth for the first 24 h of exposure based on the total protein content of the 25 cm^2 flasks (Fig 4C). After 24 h there were changes in cell morphology (Fig 5) similar to those that have been reported previously (Yoo *et al*., 1996; Riley *et al*., 1999). Cells treated with 50 μM FB_1 for periods greater than 24 h exhibited a significant decrease in cell growth (Fig 4C) and an increase in detached cells that were seen floating in the growth medium (data not shown). Previous studies have shown that the nature of cell death in the detached cells was apoptotic based on the pattern of DNA fragmentation (Riley *et al*., 1999).

ISP-1 and L-cycloserine decrease pERK2 expression. Treatment with 150 nM ISP-1 for 24 h resulted in a 5-fold decrease in expression of pERK2 that was similar to treatment with FB_1 alone or the combination of FB_1 plus ISP-1 (Fig 6A and 6B). L-

cycloserine, another SPT inhibitor, demonstrated similar results (Fig 6C). β-chloroalanine treatment induced a similar reduction in expression of pERK2 (data not shown). None of the SPT inhibitors caused increased cytotoxicity over the 24 h exposure period based on visual observations, a finding consistent with earlier studies (Yoo *et al.*, 1996; Riley *et al*., 1999).

PDMP decreases pERK2 expression. Previous research with the Madin-Darby canine kidney (MDCK) cell line found 20 μM PDMP to optimally inhibit GlcCer synthase (Shayman *et al*., 1991). LLC-PK_1 cells treated with 20 μM PDMP for 24 h exhibited a 4-fold decrease in pERK2 expression that was significantly different from the controls but was not significantly different from FB_1 alone or the combination of FB_1 plus PDMP (Fig 7). Based on light microscopy, LLC-PK_1 cells treated with 20 μM PDMP or PDMP plus FB_1 showed no signs of increased cytotoxicity.

DISCUSSION

The results of this study show that FB_1 and other inhibitors of *de novo* sphingolipid biosynthesis cause a decrease in pERK2 expression, indicating that sphingoid bases and more complex sphingolipids are necessary in modulating downstream ERK pathways. The sphingolipid biosynthetic pathway is required for normal cell growth and development. This has been proven using mutants lacking SPT, the first and rate limiting enzyme in *de novo* sphingolipid biosynthesis (Hanada *et al*., 1990 and 1992). Numerous studies using inhibitors of *de novo* sphingolipid biosynthesis have demonstrated the importance of various sphingolipid intermediates and end-products in regulating a multitude of physiological functions that are necessary for normal cell function and cell survival (reviewed in WHO, 2000).

In LLC-PK_1 cells, inhibition of ceramide synthase inhibits cell growth, induces increased apoptotic and oncotic cell death, and causes morphological changes that include an apparent loss of cell-cell contact and an increased fibroblast appearance (Riley

et al., 1999). Similar FB_1-induced effects have been reported in numerous studies using primary cell cultures, cells lines, and in liver and kidney *in vivo* (for review see Riley *et al.*, 2001 and Merrill *et al.*, 2001). In short term experiments (< 48 h) with LLC-PK_1 cells, inhibitors of SPT have little effect on cell growth or cell death, but longer exposure (> 48 h) or higher concentrations will cause morphological changes that are similar to those caused by FB_1, and can inhibit cell growth and induce cell death (Yoo *et al*., 1996; Riley *et al.*, 1999). In the present study, FB_1 caused a concentration- and time-dependent decrease in pERK2 expression that occurred before there was any evidence of a decrease in cell growth as measured by the total protein content in the 25 cm^2 flasks. At later time points (> 24 h) there was a decrease in cell growth that has been shown in other studies to occur at the same time as increased cell death (Riley *et al*., 1999; Kim *et al*., 2001; Yu *et al*., 2001). These cytotoxic effects in LLC-PK_1 cells have been totally or partially prevented by co-treatment with SPT inhibitors and are therefore known to be dependent on the accumulation of free sphinganine or its metabolites (Riley *et al*., 1999; Kim *et al*., 2001; Yu *et al*., 2001; He *et al*., in press).

In this study, the ceramide synthase inhibitor (FB_1), SPT inhibitors, and the glucosylceramide synthase inhibitor (PDMP) caused similar decreases in pERK2. Concurrent treatment with SPT inhibitors plus FB_1 did not prevent the FB_1-induced decrease in pERK2. This indicates that the decrease was not due to accumulation of free sphinganine or its metabolites but did require inhibition of *de novo* sphingolipid biosynthesis.

Similarly, GlcCer synthase inhibition with PDMP also inhibited pERK2 expression, and the combination of FB_1 plus PDMP did not reduce the extent of the decrease. In the MDCK cell line, PDMP has been shown to cause both a decrease in glucosylceramide biosynthesis and an increase in ceramide (Shayman *et al*., 1991). It is unlikely that the PDMP-induced decrease in pERK2 expression in LLC-PK_1 cells could be a consequence of increased levels of ceramide. The combination of FB_1 (ceramide

synthase inhibition) plus PDMP would prevent the PDMP-induced ceramide accumulation, and increased ceramide would be expected to increase pERK2 since ceramide is a signal in the CAPK/Raf/MEKK/MEK pathway (Merrill *et al*. 1997; Kolesnick & Krönke 1998). It is also unlikely that the PDMP decrease in pERK2 expression in LLC-PK_1 cells could be a consequence of increased levels of sphingosine or sphingosine 1-phosphate since these compounds are known to activate ERK2, not decrease its expression (Spiegel, 1999). In MDCK cells, PDMP caused a decrease in protein kinase C (PKC) activity that was attributed to decreased glucosylceramide levels (Shayman *et al.*, 1991). PKC activity has been shown to activate MAP kinase pathways (Alberts *et al*., 1994). Thus, the glucosylceramide biosynthesis-dependent decrease in pERK2 expression could be mediated by changes in glycosphingolipid-dependent expression or activity of protein kinase C.

While previous research has shown an increased phosphorylation of ERK2 in other cell lines treated with FB_1 (Wattenberg *et al*., 1996; Pinelli *et al*., 1999), in LLC-PK_1 cells at concentrations known to inhibit cell proliferation and increase cell death, FB_1 induced a persistent decrease in pERK2 expression relative to control-treated cells growing in the presence of 5% fetal calf serum. It is possible that the decreased pERK2 expression plays a role in the FB_1-induced effects in LLC-PK_1 cells that are not sphinganine-dependent. For example, a decrease in pERK2 may affect the loss of cell-cell contact and other morphological effects due to FB_1 that are not prevented by SPT inhibitors, or that can also be induced by prolonged exposure to SPT inhibitors alone (Yoo *et al*., 1996; Riley *et al*., 1999). It is also possible that persistent pERK2 inhibition sensitizes LLC-PK_1 cells to sphingoid base-induced apoptosis (Hayakawa *et al*., 1999; Chuang *et al*., 2000).

In summary: (i) FB_1 causes a decrease in the phosphorylation of ERK2 in LLC-PK_1 cells, (ii) inhibitors of serine palmitoyltransferase do not reverse the FB_1-induced decrease in pERK2, and SPT inhibitors alone also cause a decrease in pERK2, (iii) the

inhibition of GlcCer synthase by PDMP also causes a decrease in pERK2 expression, indicating that the decreased glucosylceramide biosynthesis can modulate ERK2 phosphorylation.

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Legends for Figures

Figure 1. The *de novo* sphingolipid biosynthesis and turnover pathway. The sites of action of serine palmitoyltransferase (SPT) inhibitors (ISP-1, L-cycloserine, and β-chloroalanine), the ceramide synthase inhibitor fumonisin B_1 (FB_1), and the glucosylceramide synthase inhibitor D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) are also shown.

Figure 2. Concentration-dependent changes in ERK2-expression in LLC-PK_1 cells treated with 0, 1, 10, or 50 μM FB_1 for 24 h. The expression of pERK2 and total ERK2 was analyzed as described under Materials and Methods. (A) Examples of Western blots showing both total and phosphorylated ERK2 in response to various concentrations of FB_1. (B) The intensities (pixels/10 μg total protein) of the pERK2 bands were quantitated by strip densitometry and expressed as the mean ± SD from four 25 cm^2 flasks of cells. Different letters indicate significant differences ($p<0.05$) among treatments, as determined by analysis of variance and Bonferroni's method of pairwise comparison.

Figure 3. Time-dependent changes in pERK2-expression in LLC-PK_1 cells treated with 50 μM FB_1 at early time points (0 to24 h). The expression of pERK2 was analyzed as described under Materials and Methods. Each point is the mean intensity (pixels/10 μg total protein) of the pERK2 bands from two 25 cm^2 flasks of cells quantified by strip densitometry. The line is the predicted response from the least squares regression analysis of all the points ($r^2=0.53$, n=16, p=0.007).

Figure 4. Time-dependent changes in pERK2-expression (A and B) and total protein content (C) in 25 cm^2 flasks of LLC-PK_1 cells treated with 50 μM FB_1 for 24, 48, or 72 h. Total protein content and pERK2 expression were analyzed as described in Materials and

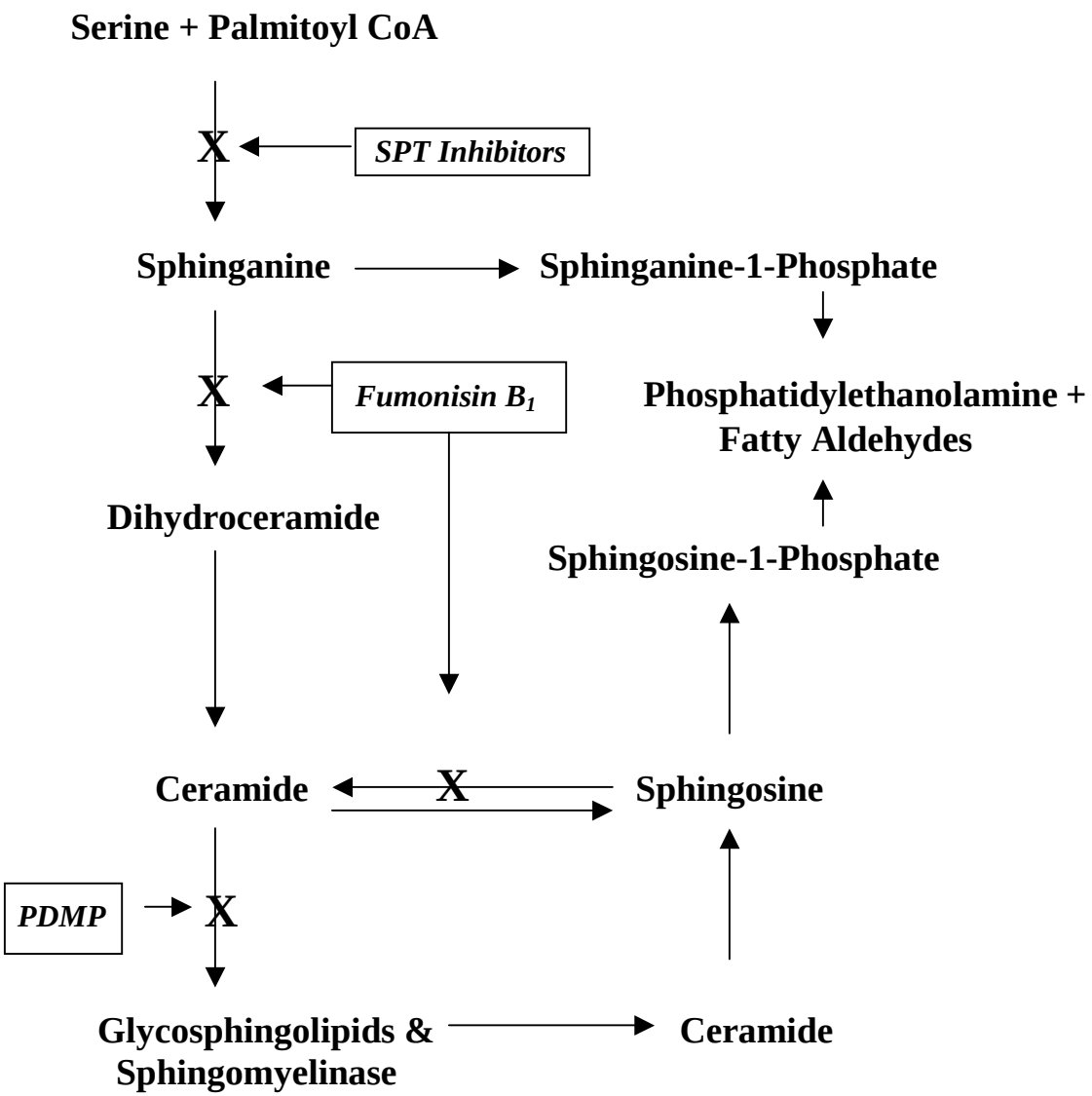
Methods. Values in (B) are the mean ± SD intensities (pixels/10 μg total protein) of the pERK2 bands and values in (C) are the mean ± SD for the total protein from 3 flasks at 24 and 48 h and 2 flasks at 72 h.. Different letters indicate significant differences ($p<0.05$) among treatments, as determined by analysis of variance and Student-Newman-Keuls Method of pairwise comparison.

Figure 5. Photomicrographs of growing cultures depicting morphological differences of (A) control-treated and (B) FB_1-treated (50 μM) cells at 48 h. Similar differences were seen as early as 24 h after exposure to FB_1 but to a lesser extent (Adapted from Riley *et al.*, 1999).

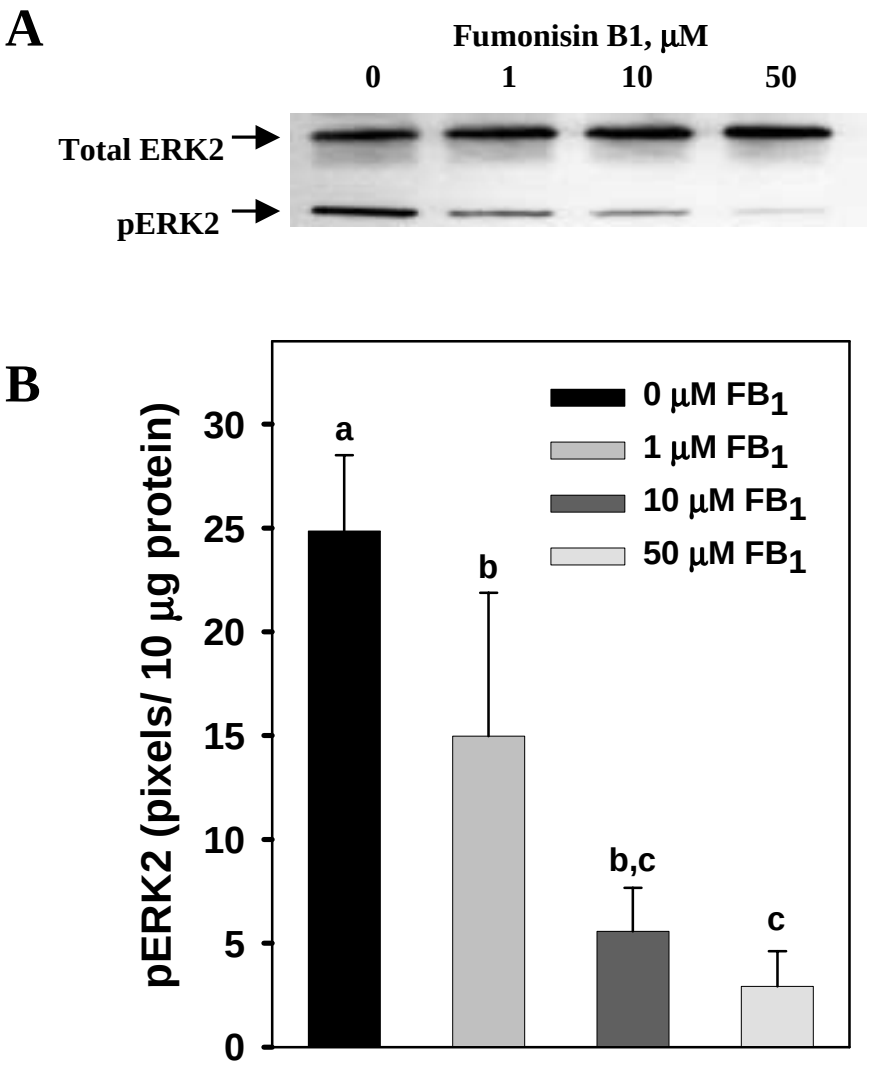
Figure 6. Changes in pERK2 expression in LLC-PK_1 cells treated with serine palmitoyltransferase inhibitors. pERK2 expression in control-treated LLC-PK_1 cells and in cells treated with 50 μM FB_1, 150 nM ISP-1, or FB_1 plus ISP-1(A and B), and 50 μM FB_1, 500 μM L-cycloserine, or FB_1 plus L-cycloserine for 24 h (C). The expression of pERK2 was analyzed as described under Materials and Methods. Example of Western blots for the ISP-1 experiments are shown in (A) and the results for L-cycloserine were similar (data not shown). Values in (B) and (C) are the intensities (pixels/10 μg total protein) of the pERK2 bands quantified by strip densitometry and expressed as the mean ± SD from nine and three 25 cm^2 flasks of cells for the experiments with ISP-1 (B) or L-cycloserine (C), respectively. Different letters indicate significant differences ($p<0.05$) among treatments, as determined by analysis of variance and Bonferroni's method of pairwise comparison.

Figure 7: Changes in pERK2 expression in LLC-PK_1 cells treated with the glucosylceramide synthase inhibitor, PDMP. pERK2 expression in control-treated LLC-

PK_1 cells and in cells treated with 50 μM FB_1, 20 μM PDMP, or FB_1 plus PDMP for 24 h. The expression of pERK2 was analyzed as described under Materials and Methods. Example of a Western blot showing pERK2 response for each treatment (A) and the intensities (pixels/10 μg total protein) of the pERK2 bands quantified by strip densitometry and expressed as the mean ± SD from five 25 cm^2 flasks of cells (B). Different letters indicate significant differences ($p<0.05$) among treatments, as determined by analysis of variance and Bonferroni's method.



Rentz *et al*., Figure 1



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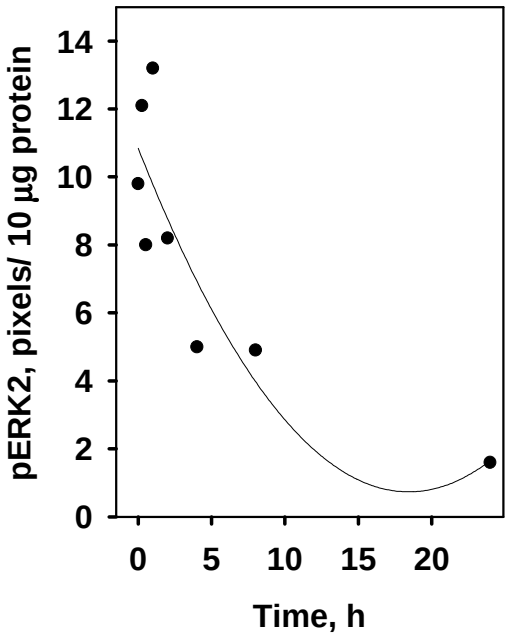

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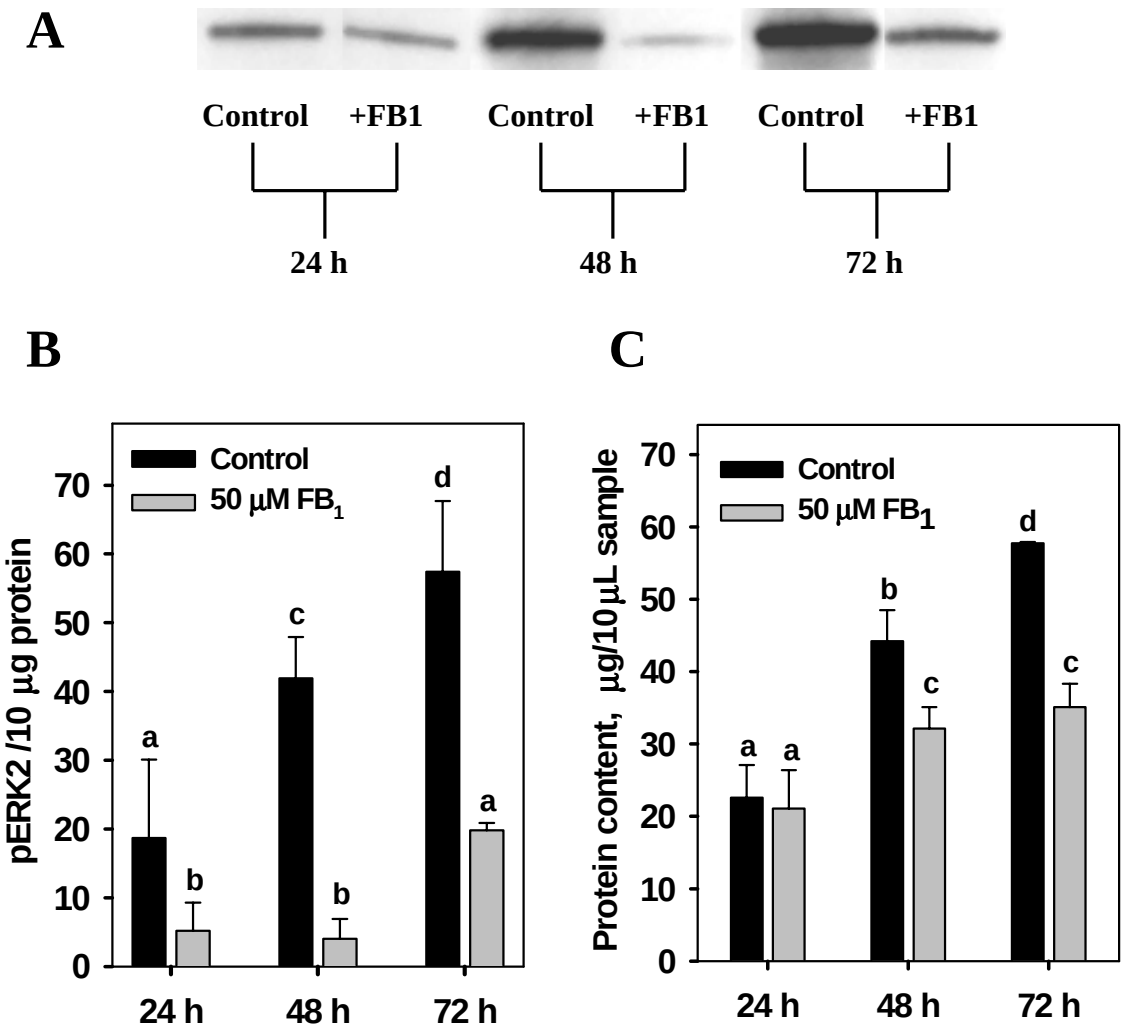

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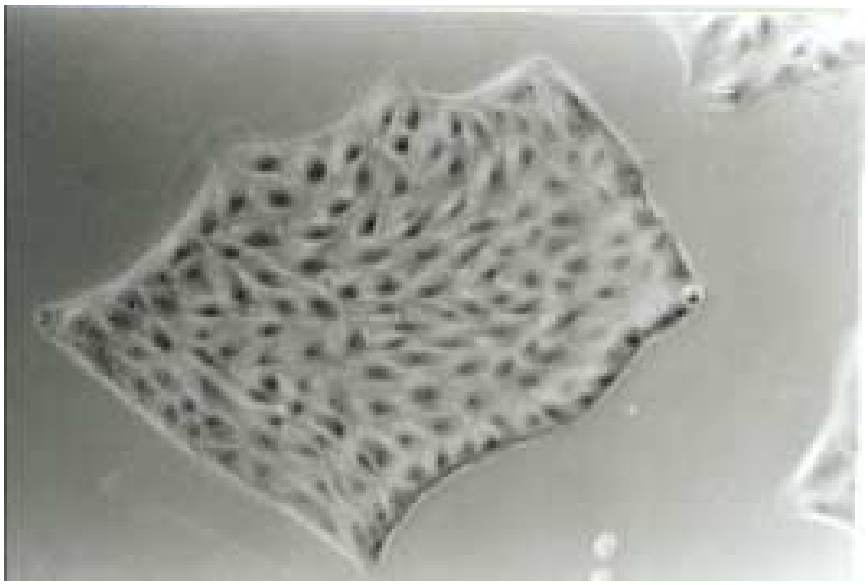

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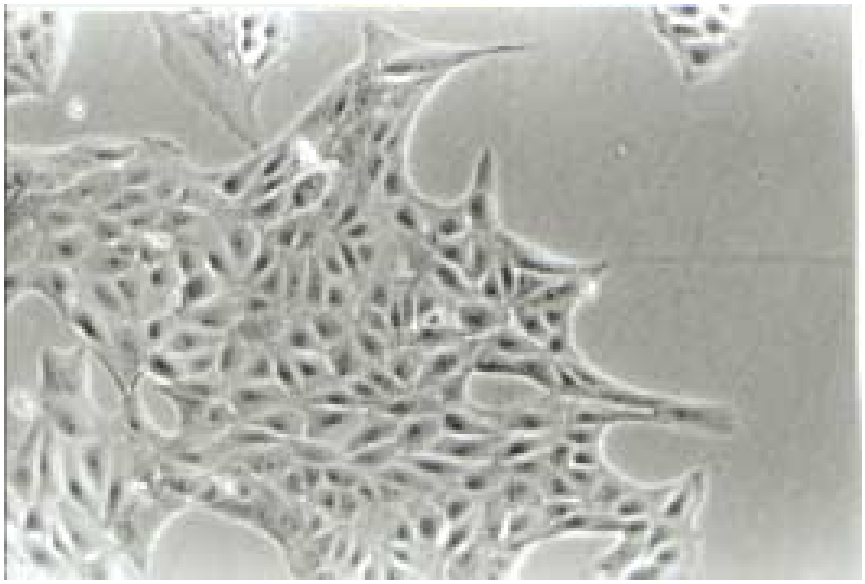

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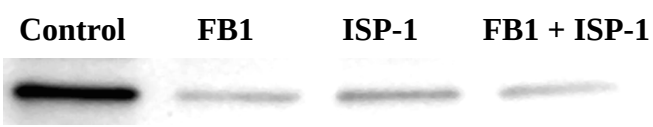

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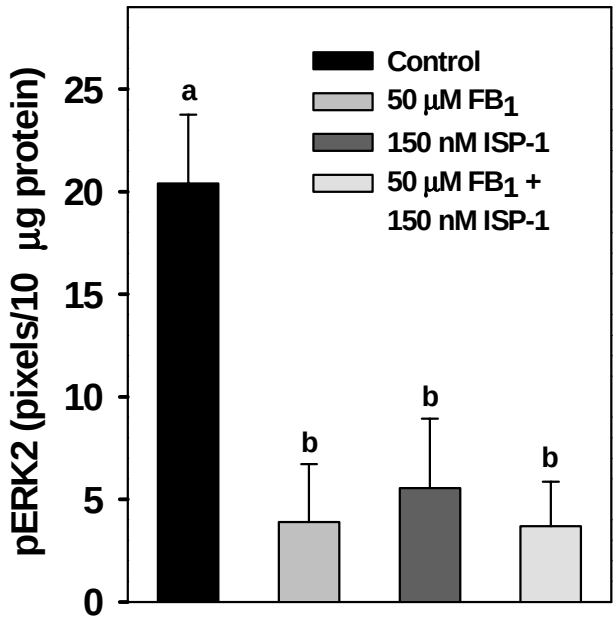

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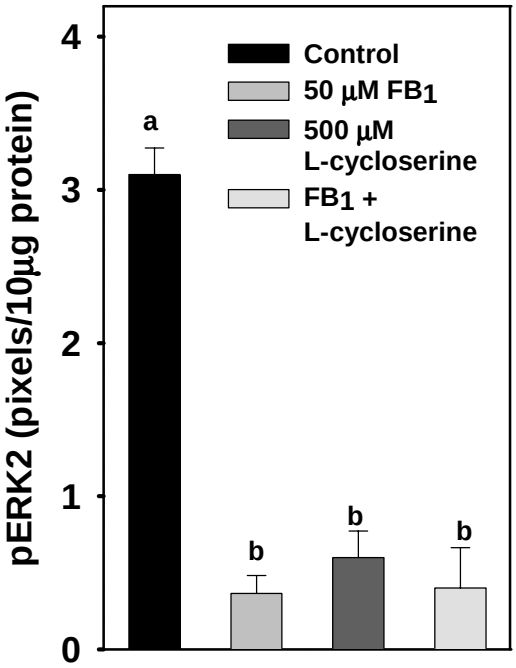

Rentz *et al*., Figure 6

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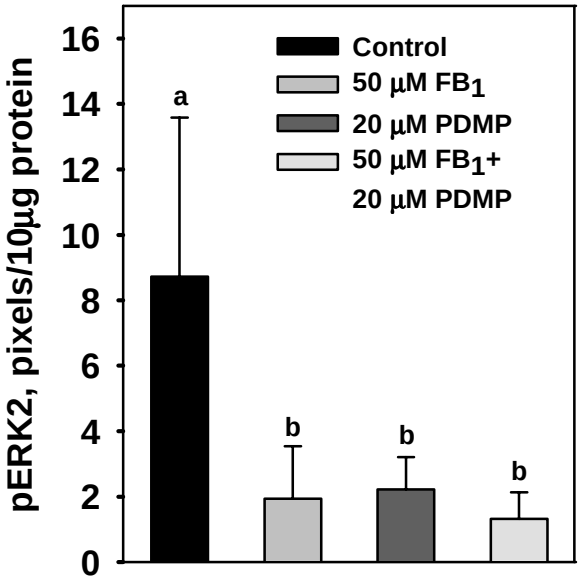


Rentz *et al*., Figure 7

CHAPTER 3

SUMMARY AND CONCLUSIONS

Fumonisin B_1 (FB_1) is a fungal toxin that is derived from *Fusarium verticillioides*, a prevalent fungal contaminant of corn that is associated with several diseases in humans and farm animals. The mode of action of FB_1 is inhibition of ceramide synthase, a key enzyme in the *de novo* sphingolipid biosynthesis and turnover pathways. FB_1 induces accumulation of sphinganine and sphingosine, a decrease in *de novo* Sphingosine biosynthesis, depletion of ceramide, and depletion of more complex sphingolipids. Many cellular signals that are downstream of the *de novo* sphingolipid biosynthesis pathway are dependent on sphingoid bases and/or more complex sphingolipids. The FB_1-induced alterations in levels of sphingoid bases and more complex sphingolipids often leads to disruptions in downstream signaling pathways, leading to induction or inhibition of cellular growth/proliferation and cellular death/apoptosis pathways.

The effects of FB_1 are cell-line specific; in LLC-PK_1 (pig renal) cells, the downstream signals associated with FB_1-induced apoptosis are not fully understood. In particular, the role of MAP kinases cellular growth or death signaling has not been fully explored. In this study, the phosphorylation (and therefore activation) of MAP kinases, specifically ERK2, was analyzed in LLC-PK_1 cells after treatment with FB_1 in a concentration known to cause inhibition of cell proliferation and apoptosis.

Cells treated with 50 μM FB_1 exhibited a decrease in phosphorylation of ERK2 after 24 h, while there was not a decrease in cell growth until 48 h, as analyzed by protein content. FB_1-induced cell death is correlated with an increase in free sphinganine; in

order to determine whether sphinganine was the cellular signal of pERK2, cells were treated with serine palmitoyltransferase (SPT) inhibitors and/or FB_1. Cells treated with FB_1, SPT inhibitors, or a combination of FB_1 and SPT inhibitor all exhibited a decrease in pERK2, indicating that levels of sphinganine have no direct effect on ERK2 phosphorylation. These findings led to the hypothesis that the sphingolipid signal for ERK2 was most likely further down the *de novo* sphingolipid biosynthesis pathway.

In order to determine if ceramide signals pERK2, cells were treated with PDMP, an inhibitor of the glucosylceramide synthase pathway that causes an accumulation of ceramide, *de novo* sphingosine, and consequently sphingosine-1-phosphate (SPP). Cells treated with FB_1, PDMP, or a combination of FB_1 and PDMP all exhibited a decrease in pERK2. These findings suggest that ceramide does not directly signal pERK2 in LLC-PK_1 cells, despite previous studies that ceramide can activate CAP kinase and lead to ERK activation in other cell lines. The findings also suggest that SPP is also most likely not a direct signal for pERK2 in LLC-PK_1 cells, though SPP is known to activate cellular growth through ERKs in other cell lines. The decrease in pERK2 must be dependent on more complex sphingolipids that are still further down the *de novo* pathway.

It is hypothesized that the (PDMP-induced) decrease in glucosylceramide is correlated with the decrease in pERK2, as inhibition of glucosylceramide has been shown to cause a decrease in cellular growth in a similar renal cell line. In addition, any of the previous inhibitions (SPT inhibitors and FB_1) of the *de novo* sphingolipid biosynthesis pathway will also cause a decrease in glucosylceramide, strengthening the hypothesis that glucosylceramide signals pERK2. More research in this area will be beneficial in understanding the cellular processes regulating the tumorigenicity of FB_1 and the role of disrupted sphingolipid metabolism in these processes in both animals and humans.

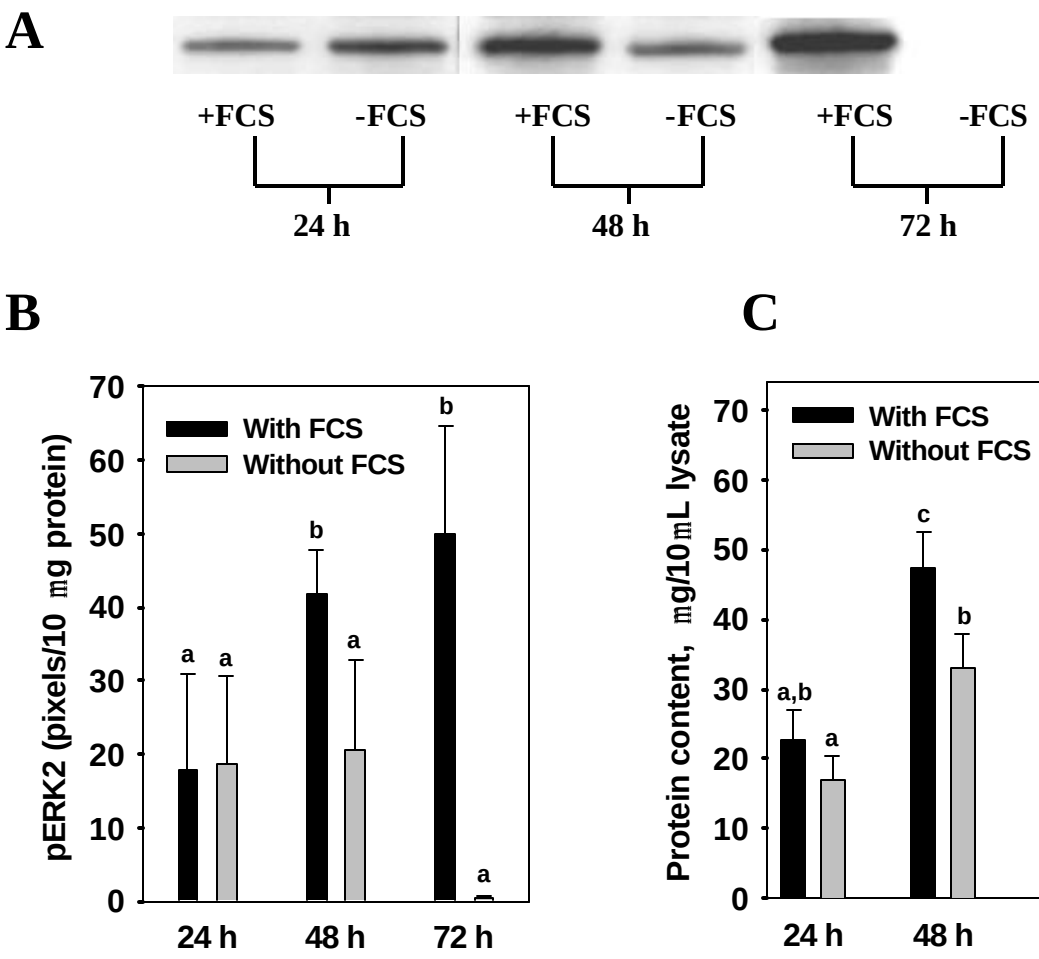
APPENDICES

1. LLC-PK_1 cells were seeded in 25 cm^2 flasks (32 flasks in three experiments) and allowed to attach and grow in DMEM/Ham's F12 (HDMX) plus 5% fetal calf serum (FCS) incubated at 37°C/ 95% humidity/ 5% CO_2 for two days at which time they were approximately 50% confluent. After two days the growth medium in sixteen flasks was removed and replaced with DMEM/Ham's F12 (HDMX) without FCS and the expression of pERK2 and the total protein content of the flasks with and without FCS and with and without 50 μM FB_1 was determined after 24, 48, and 72 h. The methods for Western blotting and assay of total protein were the same as described in the Materials and Methods section of Chapter 2.

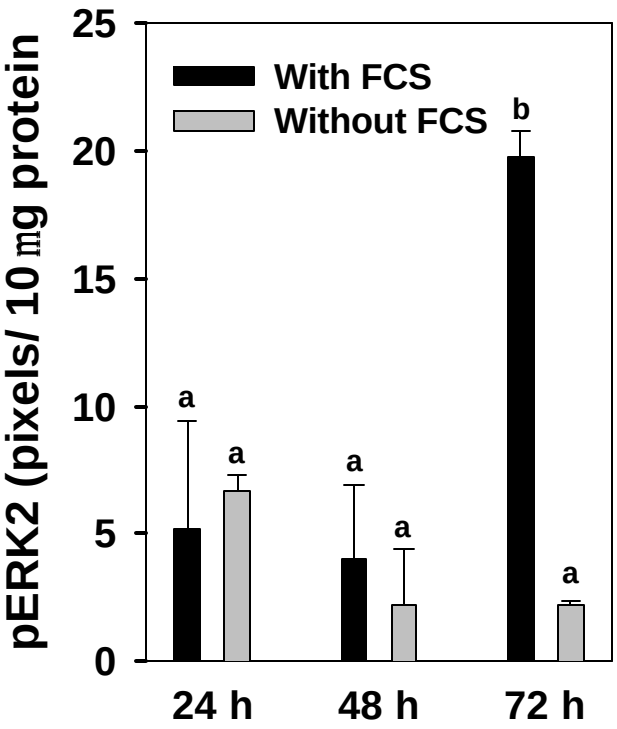
In the absence of 50 μM FB_1, significant differences in both cell growth (as determined by total protein content) and pERK2 expression did not occur until >24 h of serum deprivation (Fig 1). The absence of serum for 72 h resulted in almost no expression of pERK2 (Fig 1) and a significant decrease in total protein due to an increase in the number of detaching cells. As shown in Figure 4 of Chapter 2, pERK2 expression is decreased by 50 μM FB_1 at 24 h in the presence of FCS. pERK2 expression in FB_1-treated cells was significantly greater in the presence of FCS compared to the FB_1 treatment without FCS (Fig 2). The results of the studies with and without FCS indicate that serum provides crucial growth and survival signals that can influence ERK2 phosphorylation, and the effect of FB_1 on pERK2 expression may involve altered growth factor signaling pathways.

2. Silymarin is a flavonoid antioxidant that has been shown to prevent FB_1-induced cell death in LLC-PK_1 cells through a mechanism that does not reduce the FB_1-induced increase in free sphingoid bases (He *et al*., in press). Silymarin was added to growth medium of FB_1-treated LLC-PK_1 cells in order to determine if silymarin could prevent the FB_1-induced decrease in pERK2. Silymarin (100 μM) did not prevent the

decrease in pERK2 expression at 24 h caused by 50 μM FB_1 (Fig 3). However, unlike the SPT and GlcCer inhibitors, silymarin alone did not cause decreased expression of pERK2 (Fig 3). These findings further indicate that products of the *de novo* sphingolipid biosynthesis pathway are necessary signals for modulating ERK2 phosphorylation and silymarin prevention of FB_1-induced effects does not involve modulation of the ERK2 signaling pathway.



Appendices, Figure 1



Appendices, Figure 2

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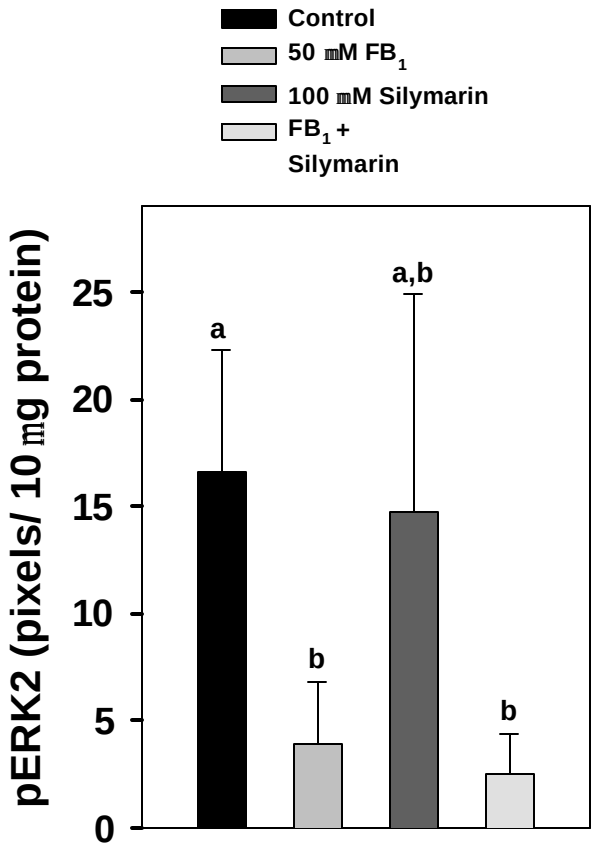

Appendices, Figure 3