INVOLVEMENT OF SPHINGOID BASES AND THEIR METABOLITES IN FUMONISIN B_1-INDUCED ALTERATIONS IN PROTEIN KINASE C-MEDIATED SIGNALING IN LLC-PK_1 CELLS

by

NEERA VINTRA GOPEE

(Under the Direction of Raghubir P. Sharma)

ABSTRACT

Fumonisin B_1 (FB_1), produced by the mycotoxin of *Fusarium verticillioides*, is a carcinogen and causes various species-specific toxicoses. FB_1 inhibits ceramide synthase and induces tumor necrosis factor α (TNFα) expression. Modulation of protein kinase C (PKC) by FB_1 may be involved in its cytotoxicity in porcine renal epithelial (LLC-PK_1) cells.

Temporal effects of 1 μM FB_1 revealed selective and transient increased cytosol to membrane translocation of PKCα exclusively at 5 min, which was correlated with an increase in PKC activity. FB_1-induced increased cytosol PKCα protein concentration at 15 min was not associated with increased activity and independent of protein biosynthesis. A concentration-dependent increase in membrane PKCα was observed on exposure to FB_1 concentrations of 0.1-1 μM. Intracellular sphinganine and sphingosine concentrations were unaffected by FB_1 up to 120 min. However, myriocin, the serine palmitoyltransferase inhibitor, did not prevent the short-term FB_1 effects on PKCα.

PKCα activation by 1 μM FB_1 was directly correlated with activation of the transcription factor, nuclear factor-kappa B (NF-κB) at 5 min. Sequential activation of TNFα mRNA expression at 15 min and caspase 3 at 60 min by 1 μM FB_1 via a PKC-dependent pathway was also observed in LLC-PK_1 cells.

A concentration-dependent inhibition of PKC-α, -δ, -ε and -ζ isoforms, NF-κB, and TNFα was observed on exposure to FB_1 concentrations of 1-50 μM at 24, 48, and 72 h in LLC-PK_1 cells. FB_1-induced apoptosis (≥ 10 μM) at 48 h was associated with an increase in caspase-3 activity. Intracellular sphinganine and sphingosine concentrations were increased in a concentration-dependent manner.

Exogenous sphinganine 1-phosphate stimulated cytosolic to membrane translocation of PKCα comparative to 10 μM FB_1 at 5 min. Inhibition of sphinganine kinase by *N*,*N*-dimethylsphingosine, prevented the FB_1-induction of PKCα at 5 min, suggesting that the selective and transient activation of PKCα is due to the FB_1-induced accumulation of sphinganine 1-phosphate. FB_1, sphinganine, sphingosine and ceramide repressed all PKC isoforms at 48 h. Co-exposure of myriocin prevented the inhibitory effects of FB_1 on PKC isoforms in LLC-PK_1 cells, suggesting that accumulation of sphinganine and its metabolite may be predominantly involved in the repression of PKC in LLC-PK_1 cells.

INDEX WORDS: Fumonisin, LLC-PK_1 cells, PKC, NF-κB, TNFα, Sphinganine 1-phosphate

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DEDICATION

To

My Husband

Nirmal Mohan

And

Parents

Mohan and Gloria Gopee

Who have been invaluable sources of inspiration and encouragement throughout my journey in life.

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assured me that anything was possible. They were always there to laugh with me in good times and comfort me in tough times. Without their love and support, I would not have the strength to continue.

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CHAPTER 1

INTRODUCTION

The discovery of the fumonisins, mycotoxins produced by the fungus *Fusarium verticillioides*, in 1988 by Marasas' group (Bezuidenhout *et al.*, 1988) provoked considerable interest as this is the most frequently reported mycotoxin associated with corn intended for human and animal consumption globally (Marasas, 1995). Fumonisin B_1 (FB_1), the most potent and toxicologically significant of the fumonisins, has been unambiguously linked to the etiology of several species-specific toxicoses in domestic and laboratory animals (Riley *et al*., 1993). Fungal infestation of plants result in diseases such as seedling blight, ear rot, and seedling wilts (Munkvold and Desjardins, 1997). Ingestion of FB_1 causes equine leukoencephalomalacia (Marasas *et al.*, 1988), porcine pulmonary edema (Harrison *et al*., 1990) and hepato- and nephro-toxicities in rats (Voss *et al*. 1996). FB_1 induces renal tumors in male rats and hepatic tumors in female mice (Howard *et al*., 2001) and male rats (Gelderblom *et al*., 1991). Consumption of FB_1-contaminated corn has been correlated with a high incidence of cancer of the esophagus and upper digestive tract in South Africa (Marasas *et al*., 1988) and China (Chu and Li, 1994).

FB_1 is a potent inhibitor of *de novo* sphingolipid biosynthesis and acts by interfering with a key pathway enzyme, sphinganine *N*-acyltransferase (ceramide synthase) (Wang *et al*., 1991; Yoo *et al*., 1992) leading to accumulation of sphingoid bases, sphingoid base metabolites, and depletion of more complex sphingolipids (Riley *et al*., 1996). Sphingolipids have been implicated as playing a role in cell contact, growth and differentiation. Subsequently, tumor necrosis factor α (TNFα) has been shown to modulate FB_1-induced toxicity *in vivo* in mice and *in vitro*. TNFα mRNA expression in livers was increased in FB_1-treated mice (Sharma *et al*., 2000a, b, 2001; 2002) and TNFα

induction was also observed in kidneys as early as 2 h following oral FB_1 treatment (Bhandari *et al*., 2000). *In vivo* effects of FB_1 were partially reversed by anti-TNFα antibodies (Dugyala *et al*., 1998), and mouse strains lacking p75 and p55 tumor necrosis factor receptors demonstrated tolerance against FB_1 toxicity (Sharma *et al*., 2000a, 2001). Additionally, the inhibitor of apoptosis protein (IAP), an inhibitor of TNFα signaling pathway, efficiently prevented the FB_1-induced apoptosis in African green monkey kidney (CV-1) cells (Ciacci-Zanella and Jones, 1999). A TNFα-like activity has also been observed in swine after FB_1 injection (Guzman *et al*., 1997) and FB_1 increased the TNFα expression at 4 h in LLC-PK_1 cells (He *et al*., 2001). In contrast, male transgenic mice expressing the human TNFα gene expressed increased TNFα on exposure to FB_1, but displayed reduced hepatotoxicity (Sharma *et al.*, 2000b). In addition, mice lacking both TNFα receptors showed reduced hepatotoxicity despite induction of TNFα expression (Sharma *et al*., 2002). These observations suggest that TNFα can also protect against FB_1-mediated toxicity.

The ability of FB_1 to alter signal transduction pathways, including that of protein kinase C (PKC), can play a role in its ability to induce apoptosis and carcinogenesis (Riley *et al*., 1998). As sphingolipids are the natural inhibitors of the phospholipid-dependent phosphorylating enzyme, PKC, it is conceivable that FB_1 may also affect PKC-regulated functions (Hannun *et al*., 1986; Kharlamov *et al*., 1993). PKC is a family of serine/threonine kinases that plays an important role in modulating a variety of biologic responses ranging from regulation of cell growth to cell death (Lee *et al*., 2000). In response to appropriate stimuli, PKC translocates from the cytosol to the membrane of cells (Kraft and Anderson, 1983). Following the redistribution and subsequent activation,

this enzyme is rapidly cleaved and is proteolytically degraded (Pontremoli *et al*., 1990). Incubation with FB_1 has been demonstrated to stimulate PKCγ cytosol to membrane translocation without affecting PKC enzyme activity (Yeung *et al*., 1996) in rat cerebrocortical slices. In contrast, Huang and coworkers (1995) have shown that exposure to FB_1 for 3-16 h resulted in a dose-dependent inhibition in PKC activity in a monkey kidney (CV-1) cell line.

PKC is known to activate nuclear factor-kappa B (NF-κB), which is composed of dimers of different members of the Rel protein family (Baeuerle and Henkel, 1994; Baldwin, 1996; Thanos and Maniatis, 1995). PKC is associated with an inhibitor protein, inhibitory kappa B (IκB), and is retained in the cytoplasm (Zabel and Baeuerle, 1990). Phosphorylation is an important event of NF-κB activation whereby the active NF-κB translocates to the nucleus where it binds to a NF-κB motif and functions as a transcriptional regulator (Kang *et al*., 2000). NF-κB is an essential transcription factor that regulates the gene expression of various cytokines, chemokines and growth factors (Barnes and Karin, 1997; Baldwin, 1996; Lenardo and Baltimore, 1989). The downstream events leading to the production of TNFα are regulated, in part, by NF-κB (Aggarwal *et al*., 1996; Shakhov *et al*., 1990; Yao *et al*., 1997).

Cultured pig renal epithelial, $LLC\text{-}PK_1$, cells are an excellent model to elucidate the FB_1-induced alterations in PKC since they are sensitive to the FB_1-induced disruption of sphingolipid biosynthesis (He *et al*., 2001; Yoo *et al*., 1996) and cytotoxicity (Yoo *et al*., 1996). In addition, it has been reported that FB_1 induces TNFα expression at 4 h in these cells (He *et al*., 2001). In the current project, the effect of FB_1 on PKC isoforms present in $LLC\text{-}PK_1$ cells in relation to the disruption of sphingolipid metabolism will be

investigated. I therefore propose to test the hypothesis that **"Fumonisin B_1-induced alterations in protein kinase C-mediated signaling are via accumulation of sphingoid bases and their metabolites in LLC-PK_1 cells."** The following specific aims will be attempted to accomplish the objectives:

1. **To determine the effects of short-term FB_1 exposure on PKC membrane translocation and activity of selective PKC isoforms in LLC-PK_1 cells.**
2. **To elucidate the PKC dependence of the FB_1-induced alterations in NF-kB and TNFa in LLC-PK_1 cells.**
3. **To associate the role of PKC, NF-kB and TNFa with delayed FB_1-induced apoptosis in LLC-PK_1 cells.**
4. **To relate FB_1-induced alterations in PKC translocation with disruption of sphingolipid metabolism.**

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CHAPTER 2

LITERATURE REVIEW

Fumonisin B_1: The mycotoxin

The fumonisins, a class of food borne carcinogenic mycotoxins were first isolated in 1988 from the phytopathogenic fungus *Fusarium verticillioides* Nirenberg, (synonym=*Fusarium moniliforme* Sheldon), a biological species of the mating populations within the *Gibberella fujikuroi* complex (Bacon *et al*., 2001; Marasas, 2001; Dutton, 1996). *F. verticillioides* is a facultative fungal endophyte (Seo *et al*., 1999) found globally in food and non-food commodities, namely corn and corn-based products. The association of this fungus in the corn plant is endophytic, while on corn kernels it is both external and systemic (Munkvold and Desjardins, 1997). The fungus is transmitted vertically and horizontally to the next generation of plants. The endophytic phase is vertically transmitted (Bacon *et al*., 2002, 2001).

During the biotrophic endophytic association with maize, as well as during saprophytic growth, *F. verticillioides* produces the toxic secondary metabolite, fumonisin. Fumonisins are a group of naturally occurring secondary mycotoxins comprised of at least 14 closely related structural analogues, fumonisin B_{1-4}, of which only B_1 and B_2 are of toxicological significance (Huang *et al*., 1995; Thiel *et al*., 1992). Procedures for detection and determination of fumonisins include thin layer chromatography, liquid chromatography (with fluorescence derivatization), post-hydrolysis gas chromatography, immunochemical assay, and mass spectrometry (Scott, 1993).

Fumonisin B_1: Prevalence

Fusarium verticillioides is found worldwide in corn and other agricultural commodities intended for human and animal consumption (Marasas, 1995). It occurs not only in the humid and sub-humid temperate zones, but also extending in to the subtropical and tropical zones and is uncommon in cooler temperate zones (Miller, 1994). It has been isolated from Argentina, Brazil, Benin, Canada, China, Croatia, Egypt, France, Honduras, Hungary, Italy, Japan, Korea, Nepal, Poland, Portugal, Romania, South Africa, Spain and the United States (Dutton, 1996; Marasas, 1995). A surveillance programme for mycotoxins in 1991 feed showed a mean level of 12.1 mg/kg FB_1 in United States maize screenings (Price *et al*., 1993). Notable high concentrations of fumonisin in corn were 38.5 mg/kg in Brazil, 50 mg/kg in France, 334 mg/kg in Hungary and 250 mg/kg in Sardinia (Dutton, 1996). Although approximately 90% of *F. verticillioides* is found in corn, it is commonly isolated from other cereal grains including wheat, barley and sorghum (Visconti and Doko, 1994). FB_1 is infrequently found in beans, beer and milk (Tseng *et al*., 1995, Hlywka and Bullerman, 1999; Maragos and Richard, 1994).

Fumonisin B_1: Control

Many infected plants do not show signs of infestations; therefore standard cleaning procedures are ineffective in controlling the fungal infestation and subsequent production of fumonisin. Fumonisin concentrations appear to decline as processing temperature increase (Bullerman *et al*., 2002). Thermal processing such as frying and extrusion cooking, where temperatures can exceed 175°C, result in greater fumonisin losses (90% or more) than baking and canning, where product temperatures rarely reach 175°C.

Adding glucose results in high losses of fumonisins during baking and extrusion processing. Nixtamalization, the alkaline processing of corn using 0.1 M calcium hydroxide at room temperature for 24 h, hydrolyzes fumonisins and results in loss of the major part of the FB_1 content (> 75%) (Dutton, 1996). To date, no completely effective detoxification process has yet been developed for use with fumonisin-contaminated feeds.

Breeding for increased tolerance and reduced mycotoxin levels, as well as transgenic approaches to ear mold/mycotoxin resistance, transgene-mediated control of the ability of *Fusarium* to infect and colonize maize and transgene approaches aimed at preventing mycotoxin biosynthesis, or detoxifying mycotoxins in planta are practical and feasible as well (Saunders *et al*., 2001). A biological system using an endophytic bacterium, *Bacillus subtilis*, has been developed that shows great promise for reducing mycotoxin accumulation during the endophytic growth phase (Bacon *et al*., 2001).

Fumonisin B_1: Structure

FB_1 is a water-soluble, polar metabolite structurally similar to sphinganine, but with side chains composed of tricarballylic acid (Fig. 2.1, Riley *et al*., 1998). Its structure is based on a long hydroxylated hydrocarbon chain (pentahydroxyeicosane) containing methyl and amino groups, and two propane-1,2,3-tricarboxylic acid molecules at carbon 14 and 15 (Dutton, 1996). FB_1 is hydroxylated at carbon positions 5 and 10 (Desjardins *et al*., 1996).

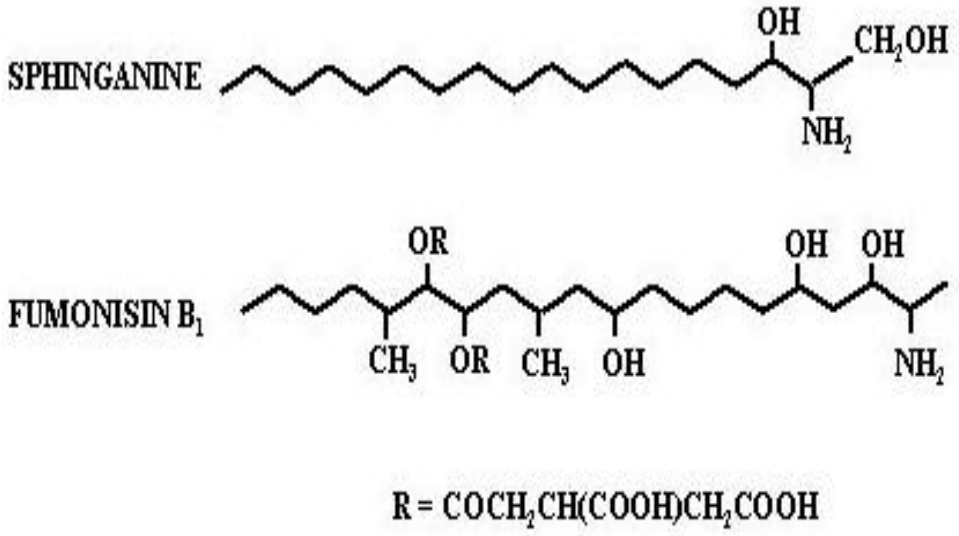

Figure 2.1. Structural resemblance of sphinganine and FB_1.

Fumonisin B_1: Pharmacokinetics

The pharmacokinetics of FB_1 in most species investigated involves poor absorption from the gastrointestinal tract, rapid clearance with low distribution and retention in certain tissues especially liver and kidneys (Norred *et al*., 1993; Shephard *et al*., 1995). Following intragastric administration of [^{14}C]FB_1 in rats, approximately 80% of the radiolabel was recovered in feces and up to 3% in urine with minimal radioactivity distributed in tissues, with the liver, kidney and blood having the highest percentages (Norred *et al*., 1993). The toxin that is absorbed persists especially liver and kidney, both been shown to be target organs for FB_1 (Gelderblom *et al*., 1991; Voss *et al*., 1993). The elimination half-lives for FB_1 was longer for liver (4.07 h) and kidney (7.07 h) than for plasma (3.15 h). Tissue accumulation of FB_1 was evidenced by the tissue/plasma area

under the concentration-time curve (AUC) ratios; the AUC tissue/AUC plasma for FB_1 was 2.03 in liver and 29.89 in kidney (Martinez-Larranaga *et al*., 1999).

Intravenous exposure to FB_1 in rats resulted in 10% of the intravenous dose being eliminated in the urine, with evidence of biliary excretion and 35 % recovered in feces (Norred *et al*., 1993) and the majority (66%) of a dose administered intraperitoneally was excreted in feces (Shephard *et al*., 1992). The elimination half-life of FB_1 after oral administration was longer (3.15) than that obtained following intravenous administration (1.03 h) (Martinez-Larranaga *et al*., 1999). The time interval for maximum drug concentration (T_{max}) in plasma was 1.02 h and bioavailablility 3.5% following oral administration of 10 mg/kg FB_1 in rats (Martinez-Larranaga *et al*., 1999).

At concentrations between 4 and 1000 μM of radiolabelled [U-^{14}C]FB_1, uptake by cultured renal epithelial (LLC-PK_1 cells) was linear following first order kinetics (Enongene *et al*., 2002). In addition, the amount of FB_1, based on high performance liquid chromatography, recovered from the aqueous extracts of LLC-PK_1 cells allowed to accumulate FB_1 for up to 4 h was the same as the calculated amount of accumulated FB_1 based on the specific activity of [U-^{14}C]FB_1 in the solution with which the cells were treated. The results of these experiments support the hypothesis that FB_1 is not metabolized to any appreciable extent.

Minimal degradation of FB_1 by ruminal microbes (about 10%) irrespective of concentration of FB_1 used indicated that microbial efficiency was unaffected by the presence of FB_1 (Gurung *et al*., 1999). Shephard and coworkers (1994) reported that the gut microflora of non-human primates is capable of removing one or both tricarboxylic acid groups from FB_1.

Fumonisin B_1: Toxicity

Plants

Fusarium verticillioides is phytotoxic, which is of particular interest for its potential role as a virulence factor to facilitate invasion of plant tissues by the fungus (Desjardins *et al.*, 1996). Droplets of FB_1 solution applied to the leaf surface of jimsonweed, black nightshade, and susceptible tomatoes caused necrosis, growth inhibition, and death (Abbas *et al*., 2000). A survey of listings of plant disease caused by *Fusarium verticillioides* indicates that it is either the primary or secondary incitant of seedling blights, foot rots, stem rots, pre- and post-harvest fruit rots, stunting and hypertrophies (Munkvold and Desjardins, 1997). The mycotoxin produces chlorosis, necrosis, tissue curling, stunting, and defoliation (Abbas and Boyette, 1992).

Horses

An early description for equine leukoencephalomalacia (ELEM) was given by Butler in 1902, also called, "hole in the head", blind staggers, foraging disease, corn stalk disease, moldy corn poisoning, leucoencephalitis, and cerebritis (Dutton, 1996). Clinical signs of neurotoxicosis, include nervousness followed by apathy, a wide-based stance, trembling, ataxia, reluctance to move, paresis of the lower lip and tongue, and an inability to eat or drink and tetanic convulsions (Marasas *et al*.; 1988; Dutton, 1996). The principal histological lesions are severe edema of the brain and early, bilaterally symmetrical, focal necrosis in the medulla oblongata. In severe cases, there may be a large liquefied cavity within the white matter of the right cerebral hemisphere, with the cerebrum posterior to the cavity enlarged and edematous with congested blood vessels (Dutton, 1996). The other organ affected is the liver, which often shows a mild swelling

with a color change to yellow-brown. In more severe cases, gross liver lesions may be seen with fibrosis of the centrilobular area. Hepatocytes on the edge of the fibrotic area have large fatty globules in their cytoplasm (Dutton, 1996).

FB_1-treated horses with clinical signs of neurologic disease have evidence of cardiovascular dysfunction. These include decreases in heart rate, cardiac output, right ventricular contractility, coccygeal artery pulse pressure, and pH and base excess in venous blood as well as increases in systemic vascular resistance, compared with values for control horses. FB_1-induced decreases in cardiovascular function may contribute to the pathophysiologic development of leukoencephalomalacia in horses (Smith *et al*. 2002). Donkeys (Haliburton *et al*., 1979) and ponies (Wang *et al*., 1992) of the equidae family were also reported to develop ELEM to varying degrees on exposure to FB_1. With what is known about FB_1 effects on animals, horses are the most sensitive species, and it has been recommended that a maximum fumonisin level of 5 μg/g should be allowed in horse feed to avoid the danger of inducing ELEM (Report of Joint Mycotoxin Committee, 1994).

Pigs

In 1990, an outbreak of an apparently species-specific disease in pigs, called porcine pulmonary edema (PPE), was investigated by Harrison and coworkers (1990). On necropsy, animals had pulmonary edema and hydrothorax, with the thoracic cavities being filled with a yellow liquid (Harrison *et al*., 1990). The hyperplastic nodules in the liver and the changes in the distal esophageal mucosa illustrate the unique chronic toxicity of this mycotoxin in pigs (Casteel *et al*., 1993). All pigs exposed to FB_1 show subacute hepatotoxicosis with individual hepatocellular necrosis, hepatomegalocytosis,

and increased numbers of mitotic figures (Osweiler *et al*., 1992) and pancreatic acinar cell degeneration (Haschek *et al*., 1992). Ultrastructural changes in orally dosed swine included loss of sinusoidal hepatocyte microvilli; membranous material in hepatic sinusoids; and multilamellar bodies in hepatocytes, Kupffer cells, pancreatic acinar cells and pulmonary macrophages. Thus, the target organs of FB_1 in the pig are the lung, liver, and pancreas. At lower doses, slowly progressive hepatic disease is the most prominent feature, while at higher doses, acute pulmonary edema is superimposed on hepatic injury and may cause death (Haschek *et al*., 1992; Motelin *et al*., 1994). Feeding FB_1 to lactating sows did not affect suckling pigs and there was no evidence of the toxin present in the milk (Becker *et al*., 1995). Cardiovascular function is altered by FB_1 and FB_1-induced PPE is caused by pulmonary hypertension caused by hypoxic vasoconstriction (Smith *et al*., 1996) and acute left-sided heart failure mediated by altered sphingolipid biosynthesis (Haschek *et al*., 2001) and not by altered endothelial permeability (Smith *et al*., 2000; Constable *et al*., 2000). For swine, a maximum level of 10 µg/g has been recommended to avoid PPE (Report of Joint Mycotoxin Committee, 1994).

Rodents

Hepato- and nephrotoxicities, disrupted sphingolipid metabolism, and liver cancer have been found in rodents fed FB_1 (Voss *et al*., 1998). The hepatocarcinogenic effects of FB_1 involve the development of hepatic nodules, adenofibrosis, hepatocellular carcinoma, cholangiofibrosis and cholangiocarcinoma (Voss *et al*., 1989). Diets containing FB_1 induced renal tubule carcinomas in male F344/N rats and hepatocellular carcinomas in female $B6C3F_1$ mice (Howard *et al*., 2001). FB_1 is hepatotoxic (Voss *et al*., 1993) and nephrotoxic (Suzuki *et al*., 1995; Voss *et al*., 1993). The kidneys are

considered to be the primary target organs in male rats (Bondy *et al*., 1996), but not mice. The liver lesions include apoptosis and hepatocellular and bile duct hyperplasia in both sexes, with the female rats being more responsive at lower doses. Lesions in the kidney, which include apoptosis and cellular degeneration, are predominately found in the outer medulla (Voss *et al*., 1996; Voss *et al*., 1989; Pozzi *et al*., 2001). FB_1 is immunosuppressive in mice; the magnitude of FB_1-induced immunosuppression is highly dependent on gender, females being more susceptible than males (Johnson and Sharma, 2001). FB_1 is fetotoxic to rats at 8 to 12 days of gestation by suppressing growth and fetal bone development (Lebepe *et al*., 1995).

Poultry

Purified FB_1 was incorporated into the diets of broiler chicks at 0, 20, 40, and 80 mg/kg, and fed to chicks from 0 to 21 d of age (Henry *et al*., 2000). Dietary FB_1, at concentrations of 80 mg/kg or less, did not adversely affect body weight, feed efficiency, or water consumption of broiler chicks. The relative weights of the liver, spleen, kidney, proventriculus, and bursa of Fabricius were also unaffected by any dietary concentration of FB_1. Histologically, chicks fed fumonisin has been shown to have multifocal hepatic necrosis, biliary hyperplasia, muscle necrosis, intestinal goblet-cell hyperplasia, thymic cortical atrophy, and rickets (Brown *et al*., 1992; Ledoux *et al*., 1992). FB_1 is also shown to be immunosuppressive in chicks (Li *et al*., 1999). Ducklings and turkeys, like other poultry, are relatively resistant to the toxic effects of FB_1 (Bermudez *et al*., 1995). Because of the greater resistance of poultry to FB_1 toxicity, maximum feed levels of 50 µg/g have been suggested (Report of Joint Mycotoxin Committee, 1994).

Non Human primates and humans

Classified as a Class 2b carcinogen (IARC, in press), consumption of FB_1-contaminated corn is correlated to a high incidence of cancer of the esophagus and upper digestive tract in eastern and southern Africa (Marasas *et al*., 1988; Rheeder *et al*., 1992), the Peoples Republic of China (Cheng *et al*., 1985; Chu and Li, 1994), coastal South Carolina (Brown *et al*., 1988; Fraumeni and Blot, 1977) and northern Italy (Franceschi *et al*., 1990; Rossi *et al*., 1982).

Apart from the adverse effects in the liver and kidneys, cholesterol and creatinine kinase were affected in vervet monkeys (Gelderblom *et al*., 2001). Several blood parameters, including white and red blood cells, also significantly decreased in the treated animals. Elevated liver enzymes gamma-glutamyl transferase, aspartate transaminase and alanine transaminase were also observed (van der Westhuizen *et al*., 2001). Baboons developed hepatic cirrhosis and intraventricular thrombosis (Kriek *et al*., 1981).

Other animals

One case of LEM symptoms in white-tailed deer has been described in the US (Howerth *et al*., 1989). Calves and Angora goats given feed containing FB_1 were unaffected in terms of feed intake or weight gain, although certain blood enzymes were elevated along with cholesterol (Osweiler *et al*., 1993), concluding that cattle and goats were less susceptible to FB_1 than other animals (Gurung *et al*., 1998). Because of the greater resistance of cattle to FB_1 toxicity, maximum feed levels of 50 µg/g have been suggested (Report of Joint Mycotoxin Committee, 1994).

In rabbits, microscopic examination revealed focal small hemorrhages in cerebral white matter, with malacia and hemorrhage also present in the hippocampus (Bucci *et al*.,

1996). The lesions were bilateral. Both animals also had marked degeneration of renal tubule epithelium and of hepatocytes. Apoptosis was the dominant degenerative change in kidney and liver. Studies were done on catfish fed with culture material containing FB_1, and it was concluded that levels of 20 mg/kg or above are toxic to both 1- and 2-year old fish (Brown *et al*., 1994).

Cell culture

FB_1 is cytotoxic (Norred *et al*., 1991) and can induce apoptosis in several mammalian cell lines, including rat hepatoma, dog kidney epithelial cells (Shier *et al*., 1991) and African green monkey kidney fibroblasts (CV-1) (Jones *et al*., 2001). FB_1 selectively affected glial cells; in particular, FB_1 delayed oligodendrocyte development and impaired myelin formation and deposition in glial cells (Monnet-Tschudi *et al*., 1999). Both the elevation of free sphingoid bases and the decrease in complex sphingolipids contribute to the decreased cell growth and cytolethality of FB_1 by inducing apoptosis in pig kidney LLC-PK_1 cells (Yoo *et al*., 1996). Cytotoxic concentrations of FB_1 induce cellular cycle arrest in phase G_2/M in rat C6 glioma cells possibly in relation with genotoxic events (Mobio *et al*., 2000). FB_1 has been described as a mitogen in Swiss 3T3 cells based on stimulation of [^{3}H]thymidine incorporation (Tolleson *et al*., 1996). COS-1 cells, Chinese Hamster Ovary (CHO) cells, primary rat hepatocytes and liver slices are relatively resistant to FB_1 cytotoxicity (Yu *et al*., 2001, Ciacci-Zanella *et al*., 1998; Riley *et al*., 1998).

Fumonisin B_1 and sphingolipids:

The pathway of *de* novo sphingolipid biosynthesis begins with the condensation of serine with palmitoyl-CoA at the endoplasmic reticulum and proceeds rapidly to the biosynthesis of ceramide and more complex sphingolipids (Riley *et al*., 2001, Fig. 2.2). The turnover of more complex sphingolipids results in the production of ceramide, sphingosine and its metabolites (Riley *et al*., 2001).

FB_1 is known to be a potent inhibitor of sphingolipid biosynthesis, specifically inhibiting the key enzyme *N*-acyltransferase (synonym=ceramide synthase), which catalyzes the acylation of sphinganine and sphingosine to ceramides (Norred *et al*., 1996; Wang *et al*., 1991; Yoo *et al*., 1992). Ceramide synthase appears to recognize both the amino group (sphingoid binding domain) and the tricarballylic acid side chains (fatty acyl CoA domain) of FB_1 (Merrill *et al*., 1996) and FB_1 exhibits competitive-type inhibition with respect to both substrates of this enzyme (sphinganine and fatty acyl-CoA) having IC_{50} of 50-100 nM (Wang *et al*., 1991).

The FB_1-induced disruption of sphingolipid metabolism includes inhibition of dihydroceramide biosynthesis, a depletion of more complex sphingolipids, an increase in free sphinganine, a decrease in reacylation of sphingosine derived from complex sphingolipid turnover (and degradation of dietary sphingolipids), an increase in sphingoid base degradation products (i.e. sphingosine (sphinganine)-1-phosphate, ethanolamine phosphate, and fatty aldehydes), and alterations in other lipid pools (Merrill *et al*., 1996, Fig. 2.2). The FB_1-induced disruption of sphingolipid metabolism is an important event in the cascade of events leading to altered cell growth, differentiation, and cell injury observed in both *in vitro* and *in vivo* (Riley *et al*., 1994). The accumulation of these

bioactive compounds, as well as the depletion of complex sphingolipids, may account for the toxicity, and perhaps the carcinogenicity, of fumonisins (Merrill *et al*., 1996; Wolf, 1994).

Studies with diverse types of cells (hepatocytes, neurons, kidney cells, fibroblasts, macrophages, and plant cells) have established that FB_1 not only blocks the biosynthesis of complex sphingolipids; but also causes sphinganine to accumulate and be released, therefore it can be used as a biomarker for exposure (Merrill *et al*., 1996). Changes in free sphingoid base concentrations have been demonstrated *in vivo* in horses/ponies (Wang *et. al*., 1992), pigs (Riley *et al*., 1993), rats (Riley *et al*., 1994), and mice (Tsunoda *et al*., 1998), rabbits, and non-human primates and chickens (Gumprecht *et al*., 1995; Riley *et al*., 1994; Shephard *et al*., 1996; Henry *et al.*, 2000).

Sphingolipids:

The "sphingosin" backbone of sphingolipids was so named by J. L. W. Thudichum in 1884 for its enigmatic ("Sphinx-like") properties (Merrill *et al*., 1997). Until the late 1970's, sphingolipids were primarily thought to serve as inert structural compounds; the suggestion that sphingolipids might be directly implicated in intracellular signaling pathways followed the discovery that sphingosine, a product of complex sphingolipid metabolism, inhibited protein kinase C (PKC) activity (Hannun *et al*., 1986; Hannun and Bell, 1989).

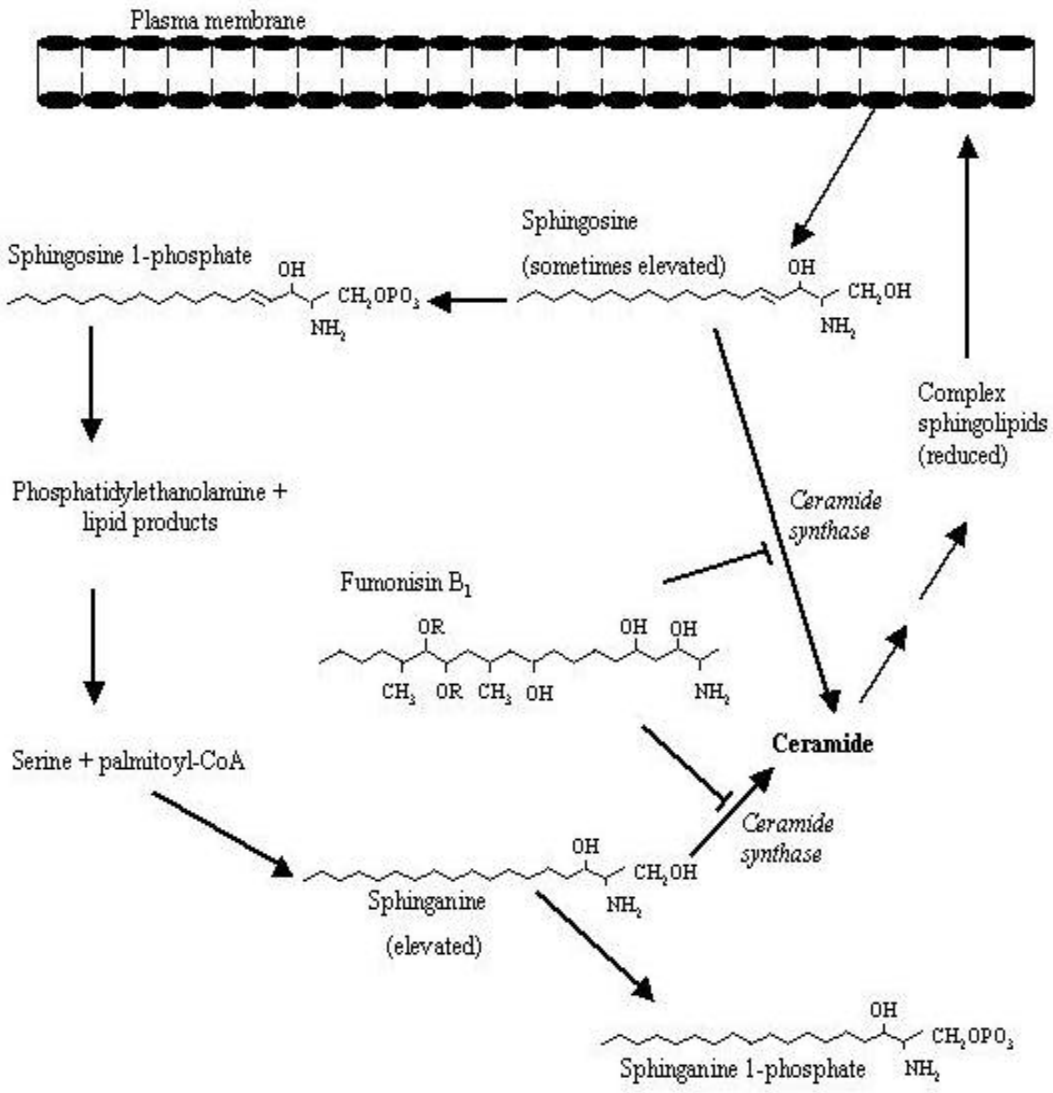

Figure 2.2. A schematic illustration of the inhibition of ceramide synthase by FB_1.

Different sphingolipids modulate PKC activity and/or PKC-mediated events differently in a variety of experimental models. Sphingosine 1-phosphate is known to be ineffective effect on PKC activity (Sadahira *et al*., 1992) or increase PKC expression (Lampasso *et al*., 2001) and PKC activity (Meacci *et al*., 1999) in various cells. Ganglioside GM_3 activated PKC when substituted for phosphatidylyserine (Momoi, 1986). Ceramide has been reported to both activate (Limatola *et al*., 1997; Lozano *et al*.,

1994) and prevent activation of PKC depending on cell type and exposure conditions (Lee *et al*., 1996; Jones and Murray, 1995; Chmura *et al*., 1996; Bourbon *et al*., 2001). In contrast, PKC activation can be prevented by several gangliosides, as demonstrated in *in vitro* systems, intact neural cells and neural tissues (Hakomori, 1990). Sphingosine and lysosphingolipids are potent and reversible inhibitors of PKC *in vitro* (Hannun *et al*., 1986; Hannun and Bell, 1987). Smith *et al*. (1997) showed that elevation of free sphingoid bases, either by the act of changing J774A.1 macrophages to fresh growth medium or by addition of FB_1 caused a significant decrease in phorbol dibutryrate binding and an increase in cytosolic PKC activity.

Sphingolipids and their metabolites can mediate either mitogenic or apoptotic effects depending on the cell type and exposure conditions (Hannun, 1996; Kolesnick and Fuks, 1995; Spiegel and Milstien, 1995). The biological functions of sphingosine, sphingosine 1-phosphate and sphinganine are paradoxical in effect; they can induce cell proliferation (Davaille *et al.*, 2002; Merrill *et al*., 1997; Linn *et al*., 2001) or trigger apoptosis (Davaille *et al*., 2002; Gennero *et al*., 2002; Linn *et al*., 2001). In addition to the newly discovered role of ceramide as an intracellular second messenger for tumor necrosis factor α (TNFα) and other cytokines, it has been reported to induce both apoptosis (Hsu *et al*., 1998; Tepper *et al*., 1997) and stimulate cell proliferation (Olivera and Spiegel, 1992).

Fumonisin B_1 and tumor necrosis factor a

In various studies tumor necrosis factor α (TNFα) has also been shown to modulate FB_1 toxicity. FB_1 increased TNFα expression in several strains of mice *in vivo* (Sharma

et al., 2000a, b, 2001) and *in vitro* (Dugyala *et al*., 1998; He *et al*., 2001). The *in vivo* hematological effects of FB_1 were partially reversed by anti-TNFα antibodies (Dugyala *et al*., 1998). Mouse strains lacking p75 or p55 tumor necrosis factor receptor also showed reduced liver toxicity following FB_1 exposure (Sharma *et al*., 2000a, 2001). Macrophages derived from mice treated with FB_1 produced higher amounts of TNFα when stimulated by the mitogenic endotoxin, lipopolysaccharide (LPS), compared to untreated cells (Dugyala *et al*., 1998). A TNFα-like activity has also been observed in swine after FB_1 injection (Guzman *et al*., 1997). In addition FB_1 increased TNFα expression at 4 h in LLC-PK_1 cells (He *et al*., 2001). FB_1 toxicity in CV-1 (African green monkey kidney fibroblasts) was prevented by inhibitor of apoptosis protein (IAP), an inhibitor of the TNF pathway (Ciacci-Zanella and Jones, 1999). Expression of tumor necrosis factor type 1 receptor associated protein 2 was induced in FB_1-sensitive CV-1 cells but repressed in FB_1-resistant COS-7 cells demonstrating the involvement of TNFα-induced death pathway in FB_1-cell death (Zhang *et al*., 2001).

In contrast, male transgenic mice expressing the human TNFα gene expressed increased TNFα on exposure to FB_1, but displayed reduced hepatotoxicity (Sharma *et al.*, 2000b). In addition, mice lacking both TNFα receptors showed reduced hepatotoxicity despite induction of TNFα expression (Sharma *et al.*, 2002). These observations suggest that TNFα can also protect against FB_1-mediated toxicity.

Tumor necrosis factor a :

The pro-inflammatory cytokine tumor necrosis factor (TNFα) is recognized as a key mediator of inflammation with actions directed towards tissue destruction and recovery

from damage (Balkwill and Mantovani, 2001; Balkwill, 2002). The biological activities of TNFα are mediated by two structurally related but functionally distinct receptors, designated p55 and p75 TNFα receptors (Arnott *et al*., 2002). It has become apparent that a complex array of signaling events are initiated in response to TNF receptor activation, giving rise to the pleiotropic effects of TNFα on cells (Baud and Karin, 2001; Locksley *et al*., 2001). The cytokine has pardoxical roles in the evolution and treatment of malignancy, where high local administration of TNFα is anticarcinogenic (Lejeune *et al*., 1998), but TNFα appears to act as an endogenous tumor promoter when chronically produced (Balkwill and Mantovani, 2001).

Fumonisin B_1 and protein kinase C:

FB_1 is an inhibitor of ceramide synthase, a key enzyme in *de* novo sphingolipid biosynthesis and reacylation of free sphingosine. As sphingolipids are natural inhibitors of the calcium (Ca^{2+})-activated, phospholipid-dependent phosphorylating enzyme, protein kinase C (PKC) (Hannun *et al*., 1986; Hannun and Bell, 1987), FB_1 also affects PKC-regulated functions. Huang and coworkers (1995) demonstrated that FB_1 decreased steady state levels of specific PKC isoforms in African green monkey kidney cells (CV-1 cells). Repression of PKC activity was evident following exposure to 0.01 to 10 μM FB_1 for 3 and 16 hr. In contrast, Yeung and co-workers (1996) showed that FB_1 added *in vitro*, did not affect total PKC activity at concentrations of 1-1000 nm in rat cerebrocortical slices. However, a 20-min incubation with FB_1 (1-1000 nm) resulted in a concentration-dependent reduction in cytosolic PKCγ protein and activity and a comparable increase in membrane-associated PKCγ. It was also found that in a crude

cerebrocortical membrane preparation, both FB_1 and exogenous sphingosine inhibited phorbol dibutryrate binding in short term incubations. The results suggested that both FB_1 and sphingoid bases interacted directly with the diacylglycerol binding site in the membrane preparation.

Since most cells express more than one type of PKC, differences among the isozymes with respect to activation conditions and subcellular locations suggest that individual PKCs mediate distinct biological processes (Gopalakrishna and Jaken, 2000). Experiments in which activities of indivual isozymes are upregulated or downregulated confirm that individual PKCs mediate distinct cellular events (Nishizuka, 1992, Stabel and Parker, 1991).

Protein kinase C:

The discovery of protein kinase C (PKC) in 1977 by Nishizuka and co-workers represented a major breakthrough in the signal transduction field (Takai *et al*., 1977). PKC is a multigene family that encodes at least 12 phospholipid-dependent serine/threonine kinases, composed of 3 major classes, that are involved in a variety of pathways that regulate cell growth, death, differentiation, neoplastic transformation and stress responsiveness (Jeong *et al*., 2001; Buchner, 2000). The discovery that PKC represents a large family of isoforms differing remarkably in their structure and expression in different tissues, in their mode of activation and in substrate specificity may enable us to elucidate the key role of PKC isoenzymes in signal transduction and to link PKC isoform action to the modulation of gene expression necessary for changes in the proliferative and differentiation status of cells (Hug and Sarre, 1993).

PKC isozymes have been grouped into three subclasses according to their regulatory properties, which are conferred by specific domains of the proteins (Ron and Kazanietz, 1999). The 'conventional' or 'classical' PKCs (cPKCs) include PKC α, βI, βII and γ are Ca^{2+}-dependent and stimulated by second-messenger diacylglycerol (DAG) (Gopalakrishna and Jaken, 2000; Ron and Kazanietz, 1999). The 'novel' PKCs (nPKCs) δ, ε, θ, and η can also be activated by DAG and phorbol esters but are Ca^{2+} independent (Gopalakrishna and Jaken, 2000; Ron and Kazanietz, 1999). The 'atypical' PKCs (aPKCs), which include PKCζ and PKCι (its mouse homologue has been named PKCλ), are unresponsive to Ca^{2+} and DAG/phorbol esters (Ron and Kazanietz, 1999; Gopalakrishna and Jaken, 2000). Each PKC isoform is composed of a single polypeptide chain of 77-83 kDa (Azzi *et al*, 1992) having two structurally well-defined domains: the amino-terminal regulatory domain and the carboxy-terminal catalytic domain (Ron and Kazanietz, 1999). PKC isoforms show a markedly different tissue distribution as most cell types express only a subset of PKC isoforms, which are differentially activated upon appropriate stimulation (Dekker and Parker, 1994).

Conventional and novel PKCs have been identified as the cellular receptor for the lipid messenger diacylglcerol (DAG), and is therefore a key enzyme in the signaling mechanisms by activation of receptors coupled to phospholipase C, which leads to a transient elevation in DAG levels (Ron and Kazanietz, 1999). Activation of conventional and novels PKCs by Ca^{2+} and/or DAG or phorbol esters is thought to involve the redistribution of the enzyme from a cytosolic location in resting cells to a membrane-associated site during stimulation (Kraft and Anderson, 1983). The discovery that PKC is a high-affinity receptor for the phorbol ester tumor promoters established the basis of

its involvement in multistage carcinogenesis and has provided us with powerful pharmacological tools with which to manipulate PKC both *in vitro* and in cellular systems (Blumberg, 1988). It has been shown that there are two populations of membrane-associated PKC, a reversible bound form and a membrane-inserted form (Bazzi and Nelsestuen, 1988a, b, c). The connection between PKC activation and its cellular responses is mediated by proteins, which become physiologically phosphorylated by PKC (Woodgett *et al*., 1987) many of which are involved in the signal transduction that influence cytokine production, growth, differentiation, apoptosis and mitogenesis (Jeong *et al*., 2001). Some of the putative substrates of PKC include the myristoylated alanine-rich C-kinase substrate (MARCKS) (Isacke *et al*., 1986), the epidermal growth factor (EGF) receptor (Lin *et al*., 1986), inhibitory-kappa B (IκB) (Lenardo and Baltimore, 1989), the Na^+/H^+ exchanger (Sardet *et al*., 1990), mitogen-activated protein (MAP) kinase (Ray and Sturgill, 1988), neurogranin (Baudier *et al*., 1991) and lamin B (Hornbeck *et al*., 1988).

Activation of PKC has been documented to result in the activation of specific transcription factors (La Porta and Comolli, 1998) and subsequent production of TNFα, Interleukin-1β (IL-1β) and Interleukin-6 (IL-6) in human monocytes (Kontny *et al*., 1999). In addition, PKC is the major high-affinity intracellular receptor for phorbol esters, a class of potent tumor promoters (Buchner, 2000), and its activation has been documented to be involved in involvement in multistage carcinogenesis (La Porta *et al*., 1997; Benzil *et al*., 1992; Liu *et al*., 1992; Bamberger *et al*., 1996). Paradoxically, repression of PKC is also linked to its ability to induce malignant transformation (Craven and DeRubertis, 1992; Guillem *et al*., 1987; Kopp *et al*., 1991; Kusunoki *et al*., 1992;

Kahl-Rainer *et al*., 1994; Suga *et al*., 1998; Morris and Smith, 1992; Scaglione-Sewell *et al*., 1998) and induction of apoptosis (Reyland *et al.*, 1999; Chmura *et al.*, 1996).

Following translocation to the membrane and activation of PKC, there is a rapid and eventual proteolytic degradation or down-regulation of the enzyme (Pontremoli *et al*., 1990). Calpain, a calcium-dependent cysteine proteinase, releases the catalytic domain of PKC yielding the irreversibly activated Ca^{2+} (Inoue *et al*., 1977; Takai *et al*., 1977), and the lipid-cofactor-independent fragment known as PKM. Conversion of PKC to PKM may represent a physiological regulatory mechanism (Pontremoli *et al*., 1990).

Protein kinase C and sphingolipids:

Until the late 1970's, sphingolipids were primarily thought to serve as inert structural compounds; the suggestion that sphingolipids might be directly implicated in intracellular signaling pathways followed the discovery that sphingosine, a product of complex sphingolipid metabolism, inhibited PKC activity (Hannun *et al.*, 1986). The discovery that breakdown products of cellular sphingolipids are biologically active and can modulate PKC activity has generated great interest in the role of these molecules in cell signaling (Hannun and Bell, 1989) as different sphingolipids are able to affect PKC activity and/or PKC-mediated events in a variety of experimental models. Sphingosine, sphinganine and lysosphingolipids are potent and reversible inhibitors of PKC *in* vitro (Hannun *et al*., 1986; Hannun and Bell, 1987). Sphingosine 1-phosphate is stimulatory (Lampasso *et al.*, 2001; Banno *et al.*, 1999) to PKC and ceramide, has been reported to both activate (Limatola *et al.*, 1997; Lozano *et al.*, 1994) and prevent activation of PKC depending on cell type and exposure conditions (Lee *et al*., 1996; Jones and Murray,

1995; Chmura *et al.*, 1996; Bourbon *et al.*, 2001). Sphingolipids and their metabolites can mediate either mitogenic or apoptotic effects depending on the cell type and exposure conditions (Hannun, 1996; Kolesnick and Fuks, 1995; Spiegel and Milstien, 1995).

Nuclear factor-kappa B:

Nuclear factor-kappa B (NF-κB) is a prototypic transcription factor involved in the regulation of numerous genes (Baeuerle and Henkel, 1994; Baeuerle and Baltimore, 1989) involved in the regulation of a variety of biological processes, such as immune response, inflammatory response, cell adhesion, growth control and cell death, depending on cell type and stimulus (Baldwin, 1996). Many stimuli induce NF-κB activity, including TNFα, Interleukin-1 (IL-1), activators of PKC, viruses, bacterial LPS, ionizing radiation, and oxidants (Jeon *et al*., 2000). NF-κB activation is subject to several levels of control, involving phosphatase and kinase activity (Fernandez and Dobbelaere, 1999).

NF-κB is composed of homo- or heterodimers of the members of the Rel protein family of transcription factors (Baeuerle and Henkel, 1994; Baldwin, 1996; Thanos and Maniatis, 1995), which is associated at rest with an inhibitor protein, inhibitory-kappa B (IκB), and is retained in the cytoplasm (Zabel and Baeuerle, 1990). Phosphorylation of IκB at two serine residues, Ser 32 and Ser 36 is followed by ubiquitin-dependent processing of IκB in the 26S proteosome. NF-κB rapidly translocates to the nucleus (Vertegaal *et al*., 2000) where it binds to a NF-κB motif and functions as a transcriptional regulator (Kang *et al*., 2000).

Nuclear factor-kB and tumor necrosis factor a :

NF-κB plays an important role in the transcriptional activation of TNFα gene expression (Sweet and Hume, 1996; Aggarwal *et al*., 1996). TNFα expression following LPS stimulation is dependent on the activation of the transcription factor NF-κB (Shakhov *et al*., 1990; Yao *et al*., 1997).

Protein kinase C and nuclear factor-kB:

NF-κB has been shown to be activated by various members of the PKC family (Signorelli *et al*., 2001). Activators of PKC activates several transcription factors, including NF-κB, are involved in cytokine synthesis (Drouet *et al*., 1991). Vertegaal and coworkers (2000) have demonstrated that PKC is an upstream activator of the IκB complex in the 12-*O*-tertradecanoyl phorbol 13-acetate (TPA) signal transduction pathway to NF-κB in U2OS cells. Sanz and coworkers (1999) have demonstrated that two members of the atypical protein kinase C subfamily (PKC-ζ and PKC-λ) are involved in the control of NF-κB. Lallena *et al*. (1999) reported that the atypical PKCs and PKCα seem to be important intermediaries in the phosphorylation of inhibitory kappa B by TNFα and PMA, respectively. It has been found that PMA activates NF-κB through direct activation of classical and/or novel PKC isoforms and was inhibited by PKC inhibitors (Vertegaal *et al*., 2000; Lallena *et al*., 1999; Trushin *et al*., 1999). A role for PKC in the regulation of NF-κB activation pathway has also been evidenced by the demonstration that PKCζ associates with IκBα kinase activity and that over-expression

of a dominant-negative mutant of PKCζ blocked NF-κB activation (Diaz-Meco *et al*., 1993; Folgueira *et al*., 1996).

Protein kinase C, nuclear factor-kB and tumor necrosis factor a :

The activation of NF-κand TNFα via a PKC-dependent pathway is shown is Figure 2.3. Exposure of macrophages to LPS activates PKC (Shapira *et al*., 1994) and experiments using various PKC activators and inhibitors indicated that PKC activity is required for the expression of several macrophage functions, including TNFα and IL-1 secretion, nitric oxide production and tumoricidal activity (Shapira *et al*., 1994; Taniguchi *et al*., 1989; Kovacs *et al*., 1988). PKC inhibitors inhibited LPS-induced TNFα synthesis and secretion in murine peritoneal macrophages and human monocytes (Kovacs *et al*., 1988; Taniguchi *et al*., 1989). *Norcardia* water-soluble mitogen (NWSM) and *Norcadia* lysozyme digest (NLD) was shown to stimulate TNFα secretion by human monocytes in a PKC-dependent manner as sphingosine, staurosporine and calphostin C markedly inhibited TNFα secretion in response to NWSM and NLD (Mege *et al*, 1993). Studies in human monocytes, utilizing staurosporine, H7, calphostin, and chelerythrin, determined that PKC activation was required for TNFα production and NF-κB DNA binding (Shapira *et al*., 1994; Prabhakar *et al*., 1993). Stimulation of PKC directly with PMA resulted in NF-κB activation and TNFα production by human monocytes (Shames *et al*., 1999). Shames and coworkers (1999) found that stimulation of PKC directly with PMA resulted in NF-κB activation and TNFα production by human monocytes. TNFα production in murine alveolar and peritoneal macrophages in response to lipopolysaccharide occurred via PKC activation (Meldrum *et al*., 1998; West *et al*.,

1997). Angiotensin II provokes TNFα biosynthesis in the adult heart through a pathway that involves the sequential activation of PKC, followed by activation of NF-κB (Kalra *et al*., 2002).

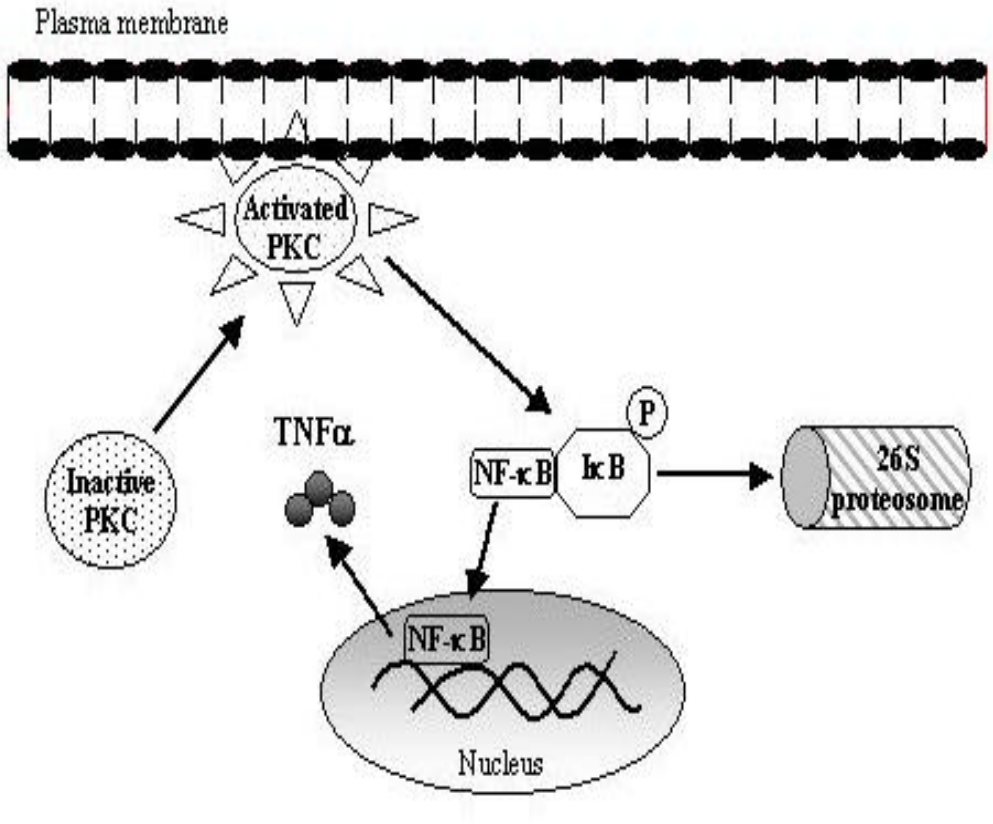

Figure 2.3. PKC cell signaling.

Porcine renal epithelial (LLC-PK$_1$) cells:

The origin, development and special characteristics of the stable epithelial-like pig kidney cell strain, LLC-PK$_1$ cells, were first described by Hull *et al*. (1976). This strain has been carried through more than 300 serial passages, has remained free of microbial and viral contaminants, and has retained a near diploid number of chromosomes (Hull *et*

al., 1976). These cells are epithelial-like, with rather large round-to-slightly-oval-shaped nuclei. Most nuclei contain one to three nucleoli. Multinucleated cells are rare, but occasional binucleated cells may be observed. Several morphological features have been observed in these cultures including ring-like structures that are three-dimensional and described as "domes" (indicated by arrow in Fig. 2.4). The fluid-filled domes of confluent cultures of LLC-PK_1 cells arise from the unidirectional transport of solutes and water from the culture media bathing the apical side of the cells (Clarke *et al*, 2000). The presence of intact tight-junctional seals between the epithelial cells is necessary to maintain these domes. Exposure to the PKC activator, phorbol ester, TPA, increases leakiness of tight junctions and result in collapse and disappearance of the domes (Clarke *et al*., 2000). In addition, when cultures are grown on serum-free medium, but not in the presence of fetal bovine serum, many small circular bodies or "globules" appear attached to the cell surface (Hull *et al*., 1976), which may be pieces of the cell membrane pinched off during periods of high activity and then reattach firmly to cell surface.

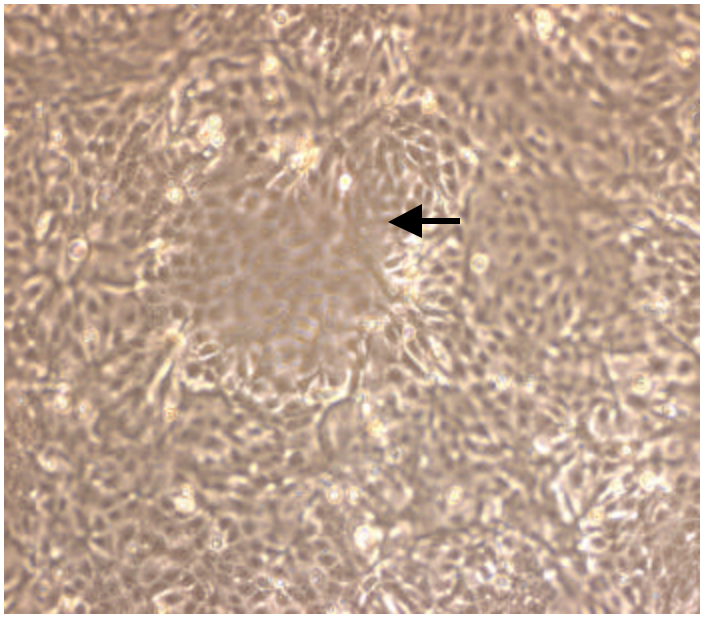

Figure 2.4. Fluid-filled "dome" in confluent LLC-PK_1 cells.

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CHAPTER 3

SELECTIVE AND TRANSIENT ACTIVATION OF PROTEIN KINASE C a BY THE MYCOTOXIN FUMONISIN B_1, A CERAMIDE SYNTHASE INHIBITOR, IN CULTURED RENAL EPITHELIAL LLC-PK_1 CELLS[1]

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ABSTRACT

Fumonisin B_1, a potent and naturally occurring mycotoxin produced by the fungus *Fusarium verticillioides*, has been implicated in fatal and debilitating diseases in animals and humans. Fumonisin B_1 affects a variety of cell signaling proteins including protein kinase C (PKC); a serine/threonine kinase involved in a number of signal transduction pathways, including those involved with cytokine induction, carcinogenesis and apoptosis. The aim of this study was to investigate the short-term temporal and concentration-dependent effects of fumonisin B_1 on PKC isoforms present in LLC-PK_1 cells in relation to the fumonisin B_1-induced accumulation of sphinganine and sphingosine utilizing various inhibitors and activators. Our studies demonstrated that fumonisin B_1 ($\leq$ 1 μM) caused selective and transient activation of PKCα, but did not affect PKC -δ, -ε and -ζ isoforms. At higher fumonisin B_1 concentrations and later time points (15-120 min), PKCα membrane concentrations declined to untreated levels. An increase in cytosolic PKCα protein expression at 15 min was not associated with an increase in activity or protein biosynthesis. Calphostin C, a PKC inhibitor, abrogated the fumonsin B_1-induced translocation of PKCα. Preincubation with the PKC activator, phorbol 12-myristate 13-acetate (PMA) resulted in an additive effect of membrane translocation as compared to PMA or fumonisin B_1 alone. Intracellular sphinganine and sphingosine concentrations were not altered at all time points tested. Myriocin, the specific inhibitor of serine palmitoyltransferase, the first enzyme in *de novo* sphingolipid biosynthesis, did not prevent the fumonisin B_1-induced PKCα translocation. Altering PKC and its signal transduction pathways may be of importance in the ability of fumonisin B_1 to induce apoptosis and/or carcinogenesis.

[1]The abbreviations used are: PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; TNFα, tumor necrosis factor α; DAG, diacylglycerol; Ca^{2+}, calcium; SPT, serine palmitoyltransferase; PKM, catalytic fragment of PKC; Myr; myriocin; Cal-C, calphostin C and CHX, cycloheximide.

INTRODUCTION

Fumonisins are the principal mycotoxins, produced by the fungus *Fusarium verticillioides*, associated with corn intended for human and animal consumption globally (*1*). Fumonisin B_1, the most potent and toxicologically significant of the fumonisins, is unambiguously linked to the etiology of several species-specific toxicoses in domestic and laboratory animals (*2*). Fumonisin B_1 causes equine leukoencephalomalacia (*3*), porcine pulmonary edema (*4*) and hepato- and nephro-toxicity in rats (*5*). Fumonisin B_1 induces renal tumors in male rats and hepatic tumors in female mice (*6*) and male rats (*7*). Consumption of fumonisin B_1 has also been correlated with a high incidence of cancer of the esophagus and upper digestive tract in humans (*3*).

Fumonisin B_1 bears remarkable structural similarity to sphinganine and is the first known naturally occurring inhibitor of *de novo* sphingolipid biosynthesis via its ability to inhibit the enzyme sphinganine (sphingosine) *N*-acetyltransferase (ceramide synthase) (*8*) (Fig. 3.1). TNFα also modulates fumonisin B_1-induced toxicity where repeated fumonisin B_1 exposure has been shown to increase TNFα in several strains of mice (*9;10*) and anti-TNFα antibodies partially reversed the fumonisin B_1 toxicity *in vivo (11)*.

Fumonisin B_1-induced sphingolipid disruption depletes complex sphingolipids and increases free sphingoid bases, sphinganine and sphingosine, and their degradation

products (e.g. sphinganine 1-phosphate, sphingosine 1-phosphate) (*12*) (Fig 3.1). The disruption of sphingolipid metabolism is an early event that is closely related with the onset and progression of proliferation and increased cell death, both in primary cell culture and cell lines (*13*). Sphingolipid breakdown products are potent modulators of protein kinase C (PKC) activity *in vitro* (*14*). Fumonisin B_1 for 20 min stimulated cytosolic to membrane translocation of PKCγ and increases PKC activity in the presence of phosphatidylserine and Ca^{2+} in rat brain (*15*). In contrast, at later time points, Huang *et al.* (*16*) repressed PKC expression in African green monkey kidney cells. To date, the acute temporal effects of fumonisin B_1-induced alterations on various PKC isoforms utilizing known PKC activators and inhibitor in relation to disruption of sphingolipid metabolism have not been discerned.

The PKC multigene family encodes at least 12 phospholipid-dependent serine/threonine kinases, composed of 3 major classes, that are involved the regulation of cell growth, death, differentiation, neoplastic transformation, and stress responses (*17;18*). Conventional PKCs (cPKCs such as α, βI, βII and γ) are activated by diacylglycerol (DAG) or phorbol ester in a calcium (Ca^{2+})-dependent manner (*19;20*). In contrast, activation of novel PKCs (nPKCs such as δ, ε, θ, and η) is Ca^{2+}-independent (*19;20*). Neither Ca^{2+} nor DAG is required for the activation of atypical PKCs (aPKCs) represented by the isoforms λ and ζ (*21*). In response to DAG or the tumor promoters, e.g., phorbol esters, PKC translocates from the cytosol to the membrane of cells (*22*). Following redistribution and subsequent activation, this enzyme is rapidly cleaved and is proteolytically degraded (*23*).

Cultured pig renal epithelial LLC-PK_1 cells are an excellent paradigm to elucidate the fumonisin B_1-induced alterations in PKC since an elevation of free sphingoid bases or a decrease in complex sphingolipids contributes to decreased cell growth and cytolethality of fumonisin B_1 in these cells (*24*). In addition, TNFα expression occurs following exposure to fumonisin B_1 (*25*). The aims of these studies were to determine the effect of short-term exposure to fumonisin B_1 on PKC isoforms present in LLC-PK_1 cells and to confirm the effects of fumonisin B_1 on PKC using PKC inhibitors and activators. We further attempted to define the mechanism by which fumonisin B_1 modulates PKC utilizing the serine palmitoyltransferase (SPT) inhibitor, myriocin (Fig. 3.1). Here we also demonstrate that fumonisin B_1 selectively and transiently activates PKCα and that the responses are not prevented by myriocin.

EXPERIMENTAL PROCEDURES

Materials

Fumonisin B_1 (> 98% purity) was obtained from Programme on Mycotoxins and Experimental Carcinogenesis (PROMEC, Tygerberg, South Africa). Cycloheximide (CHX), phorbol 12-myristate 13-acetate (PMA) and horseradish peroxidase (HRP)-conjugated goat secondary anti-mouse antibody were obtained from Sigma (St. Louis, MO). Calphostin C was obtained from Calbiochem (San Diego, CA). Myriocin was obtained from Biomol Research Laboratory (Plymoth, PA). Primary mouse polyclonal anti-PKCα, rabbit polyclonal anti-PKCδ, rabbit polyclonal anti-PKCε, fluorescein isothiocyanate (FITC)-conjugated secondary anti mouse IgG and HRP donkey anti-rabbit secondary antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz,

CA). Primary anti-PKCζ antibody was obtained from Upstate Biotechnology (Lake Placid, NY).

Cell cultures and Treatment

Porcine kidney epithelial cells (LLC-PK_1, CRL 1392, passage 197) were obtained from American Type Culture Collection (ATCC, Rockville, MD). Cells were grown in 75 cm^2 culture flasks containing Dulbecco's modified Eagle's medium/Ham's F12 (1:1) supplemented with 5% fetal bovine serum (FBS) and maintained at 37° C in 5% CO_2 air atmosphere. Cells were subcultured in 100 × 15 mm sterile petri dishes and allowed to attach and grow for 2 days. The media was replaced with serum-free media 18 h prior to treatment on the third day. To prevent the burst of free sphingoid bases after addition of fresh medium (*26*), 0.1 ml medium from each well was taken out, and 0.1 ml of appropriate chemical dissolved in serum-free medium, was added. LLC-PK_1 cells were treated with 0.01 μM PMA diluted in 0.1% dimethyl sulfoxide (DMSO) (Fisher Scientific, Pittsburgh, PA) in serum-free medium, for 30 min. Calphostin C represents a class of kinase inhibitors specific for PKC (*27*). Inhibition of PKC by calphostin C is photo-dependent (*28*) therefore we preincubated LLC-PK_1 cells with 100 nM calphostin C diluted in serum-free medium for 60 min in the presence of light to ensure adequate inhibition of PKC. For the myriocin study, LLC-PK_1 cells were incubated 200 nM myriocin for 4 h prior to treatment with fumonisin B_1 to ensure adequate inhibition of SPT and subsequent inhibition of intracellular free *de novo* sphinganine biosynthesis.

Immunoblot analysis for PKC isoforms

Cytosolic and membrane fractions were obtained using modifications to the methods as described by of Clarke *et al*. (*29*). Briefly, cells were washed 3 times in cold phosphate-buffered saline (PBS) and scraped into 100 µl of lysis buffer A (20 mmol/L Tris-HCl, pH 7.5, 0.25 mol/L sucrose, 10 mmol/L EGTA, 2 mmol/L EDTA, 20 µg/mL leupeptin, 10 µg/mL aprotinin and 200 µmol/L phenylsulfonyl fluoride) at 4°C and sonicated on ice. Samples were centrifuged at 42,000 × *g* for 2 h at 4°C and the supernatants (′cytosolic fraction′) was transferred to separate tubes. Lysis buffer A (50 µl) containing 1% TritonX-100 at 4°C was added to each pellet were mixed for 1 h at 4°C. The solutions were centrifuged at 42,000 × *g* for 2 h and the supernatants (′membrane fraction′) were transferred to separate tubes. Protein concentrations were determined using a Bio-Rad protein assay (Bio-Rad, Hercules, CA) using bovine serum albumin (BSA) (Sigma, St. Louis, MO) as the standard. Equal amounts of protein from each sample (10 µg/ lane) were then loaded on 8% polyacrylamide minigels, electrophoresed and transblotted (99 V) to nitrocellulose membranes. Nonspecific binding was blocked in 5% milk protein for 1 h prior to incubating with appropriate primary antibodies. Membranes were then rinsed 5 times and incubated with HRP-conjugated secondary antibody. The blots were rinsed 5 times and protein bands were visualized by Pierce Super Signal® chemiluminescent substrate (Pierce, Rockford, IL). Blots from each treatment group were exposed on film (Kodak X-OMAT AR, Rochester, NY) and images were acquired with a scanner and analyzed with UN-SCAN-IT software (Silk Scientific Inc., Orem, UT). Following appropriate visualization, membranes were

washed, stripped utilizing Pierce Restore™ Western Blot Stripping Buffer (Pierce, Rockford, IL), blocked and reprobed with appropriate primary and secondary antibodies.

PKC activity in total cell lysates

To obtain total protein, cells were washed 3 times in cold PBS and scraped in 100 µl lysis buffer (20 mmol/L Tris-HCl, pH 7.4, 1 mmol/L EGTA, 1 mmol/L dithiothreitol (DTT), 1 mmol/L PMSF, 0.2 mmol/L leupeptin, and 10 µg/mL aprotinin). The lysates were sonicated on ice and centrifuged for 5 min at 600 $\times$ *g* and the supernatants were used for the PepTag® assay for non-radioactive detection of PKC (Promega Corp., Madison, WI). To confirm the specificity of isoform activation, lysates were incubated for 2 h at room temperature in 96 well assay plates (Becton Dickinson, Franklin Lakes, NJ) coated with anti-PKC antibodies of specific isoforms to immunoprecipitate out the PKC isoform being tested. The fluorescence of phosphorylated substrate was determined using a Spectramax Gemini fluorescent plate reader (Molecular Devices, Irvine, CA) and the fluorescence signal was digitized and analyzed using SoftMax Pro™ (version 3.1.1, Molecular Devices, Irvine, CA). The amount of protein added to the kinase assay was standardized using the Bio-Rad assay.

Membrane localization of PKCα by immunofluorescence

LLC-PK_1 cells were grown to subconfluence in sterile 4-chambered slides (Nalge Nunc International, Naperville, IL) and treated with PMA or calphostin C in the absence or presence of fumonisin B_1. The slides were washed 3 times in cold PBS and fixed and permeabilized for 30 min in 1:1 cold methanol:acetone followed by 2 washes with cold

PBS. Slides were incubated for 50 min with 1% normal goat serum in PBS containing 0.1% Triton X-100 followed by an overnight incubation with mouse polyclonal anti-PKCα antibody diluted in PBS containing 2 mg/ml fatty acid free BSA and 0.1% Trion X-100. Slides were then washed 3 times with PBS followed by 2 h incubation with FITC-conjugated anti-mouse IgG antibody. The slides were washed and mounted and viewed with an Olympus IX71 inverted fluorescence microscope (Melville, NY) microscope equipped with appropriate optics. Images from the microscope were recorded by Olympus digital camera using the Olympus Magnifier SP software.

Quantitation of intracellular free sphingoid bases

Free sphinganine and sphingosine concentrations were determined by the method of He *et al.* (*25*). Briefly, cells were washed once with ice-cold PBS and then were scraped into 1 ml ice-cold PBS. An aliquot (0.1 ml) of cell suspension in PBS was transferred to another tube, spun at 2,000 × *g* at 4°C for 5 min. The cell pellet was solubilized using lysis buffer and analysis of total protein was performed as described above. Free sphingoid bases were extracted from the remainder of cells by using the modified method of Yoo *et al. (30)*. The relative amounts of free sphinganine and sphiongosine in base-treated cell extracts were determined by high-performance liquid chromatography (HPLC) utilizing a modification (*30*) of the method originally described by Merrill *et al.* (*31*). Sphingoid bases were quantitated based on the recovery of a C_{20} sphinganine internal standard. The instrument limit of detection for C_{20} was 26.8 fmol/assay (equivalent to 1 fmol/ mg protein).

Statistical analysis

Data presented here represent a minimum of three experiments and where appropriate are expressed as mean ± SE. Differences among the various treatment groups were determined by one-way analysis of variance followed by Duncan's multiple range tests utilizing Version 8 of the Statistical Analysis Software (SAS) (SAS Institute Inc., Cary, NC). A P value ≤ 0.05 was taken to denote statistical difference.

RESULTS

Fumonisin B_1 induced transient membrane translocation and activation of PKCα in LLC-PK$_1$ cells: In response to external stimuli, PKC translocates from the cytosol to the membrane of cells (24) Accordingly, immunoblot analyses were conducted using cytosolic and membrane fractions prepared from cells following incubation with fumonisin B_1 for 5, 15, 30 and 120 min (Fig. 3.2). Treatment of LLC-PK$_1$ cells with 1 μM fumonisin B_1 resulted in a transient and significant increase at 5 min in the membrane fraction PKCα exclusively (Fig. 3.2A), followed by its down-regulation and return to control levels at later time points (15-120 min). Cytosolic PKCα was decreased in response to 1 μM fumonisin B_1 at 5 min followed by a significant increase at 15 min and eventual return to untreated levels at later time points (Fig. 3.2B).

To determine whether the changes in PKC distribution corresponded to altered enzyme activity, total PKC activity using the PepTag® assay for non-radioactive detection of protein kinase C was measured from cells treated as described in experimental procedures above. Total PKC activity increased in response to 1 μM fumonisin B_1 at 5 min, followed by a reduction to control levels at 15, 30 and 120 min

(Fig. 3.2C). To confirm specificity of PKCα activation by fumonisin B_1, cell lysates were incubated with anti-PKCα antibodies to precipitate out PKCα proteins from the lysates. Fumonisin B_1 increased PKC activity in the absence of anti-PKC antibodies, whereas lysates incubated anti-PKCα antibody which immunoprecipitated out PKCα proteins, prevented the effects of fumonisin B_1 on PKC activation (Fig. 3.2D). To further confirm the activation of PKCα by fumonisin B_1, lysates obtained from fumonisin B_1 treated cells were incubated with a mixture of anti-PKC-δ, -ε and -ζ antibodies, thus these PKC isoforms were precipitated out, resulting in increased PKC activity. This finding confirms that PKCα cytosolic to membrane translocation in response to fumonisin B_1 positively correlates with a comparative increase in its activity, and not that of the other isoforms tested, in LLC-PK_1 cells.

PKC isoforms -d, -e, and -z were insensitive to acute exposure to fumonisin B_1: In addition to PKCα, Huwiler and coworkers (*32;33*) demonstrated the presence of PKC -δ, -ε, and -ζ in renal mesangial cells. Immunoblot analysis of cytosolic and membrane fractions indicated that LLC-PK_1 cells express PKC-δ, -ε, and -ζ proteins (Fig. 3.3). Protein concentrations of PKC- isoforms-δ, and -ζ were present predominantly in the cytosolic fraction whereas PKCε was more abundant in the membrane of both fumonisin B_1-treated and untreated LLC-PK_1 cells. At all time points tested, fumonisin B_1 did not alter the cytosolic to membrane redistribution of these PKC isoforms in LLC-PK_1 cells.

Fumonisin B_1-induced overexpression of cytosolic PKCa was not associated with increased activity or protein biosynthesis in LLC-PK_1 cells: The increased PKCα

cytosolic protein concentration following 15 min exposure to 1 μM fumonisin B_1 was not associated with an increase in PKC activity (Fig. 3.4A). In addition, the increase in PKCα protein levels in the cytosol at 15 min was not associated with protein biosynthesis, since pretreatment with 10 μg/ml cycloheximide, a potent protein synthesis inhibitor, did not prevent the fumonisin B_1-induced increase in the cytosolic PKCα protein concentration (Fig. 3.4B).

Fumonisin B_1 induced a concentration-dependent increase in membrane translocation of PKCα at concentrations £ 1 mM in LLC-PK_1 cells: In order to discern whether fumonisin B_1 alters PKCα membrane translocation in a concentration-dependent manner, LLC-PK_1 cells were exposed increasing concentrations of fumonisin B_1 (0.1-10 μM) for 5 min. A concentration-dependent increase and concurrent decrease in PKCα membrane and cytosolic protein concentrations was observed with fumonisin B_1 concentrations of 0.1-1 μM, respectively (Fig. 3.5). Fumonisin B_1 concentrations of 0.3 and 1 μM induced a significant increase in PKCα membrane protein expression. This increase in membrane translocation of PKCα at fumonisin B_1 concentrations of ≤ 1 μM, was followed by subsequent down-regulation and return to control levels at concentrations of > 1 μM. Cytosolic PKCα protein concentrations were reduced compared to the untreated LLC-PK_1 cells at all fumonisin B_1 concentrations administered.

PKCα membrane translocation by fumonisin B_1 was sensitive to the PKC activator, PMA, and inhibitor, calphostin C: Fumonisin B_1 induced a significant increase in the

membrane fraction of PKCα (Fig. 3.6A). Preincubation of LLC-PK_1 cells with 0.01 μM PMA for 30 min, resulted in an additive effect in membrane protein PKCα concentrations as compared to PMA or fumonisin B_1 alone. This concentration of PMA was the lowest concentration that elicited significant changes in PKCα redistribution in LLC-PK_1 cells (data not shown). Additionally, cytosolic PKCα protein concentration was significantly decreased on exposure to PMA alone or in combination with fumonisin B_1. In contrast, the fumonisin B_1-induced redistribution of PKCα was abrogated by preincubating LLC-PK_1 cells for 60 min with 100 nM of the photo-activated calphostin C (Fig. 3.6B).

Membrane translocation of PKCα by fumonisin B_1 was corroborated by immunostaining: Fumonisin B_1-induced redistribution of PKCα from cytosol to membrane was also analyzed by immunofluorescence. Immunostaining for PKCα using specific antibody to this isoform revealed diffuse cytpoplasmic staining of untreated control cells (Fig. 3.7A). Following exposure to 1 μM fumonisin B_1 for 5 min, PKCα staining appeared to be intensified within the plasma membrane compartments (Fig. 3.7B), indicative of membrane translocation. PMA (0.01 μM) alone (Fig. 3.7C) and in the presence of fumonisin B_1 (Fig. 3.7D) showed intensified staining at the cellular membrane for PKCα. In contrast, treatment with calphostin C (100 nM) displayed similar diffuse cytosolic staining as untreated LLC-PK_1 cells (Fig. 3.7E). Exposure to calphostin C prior to fumonisin B_1 treatment prevented the fumonisin B_1-induced intensified plasma membrane staining and displayed similar diffuse cytosolic staining as untreated LLC-PK_1 cells (Fig. 3.7F).

Short-term fumonisin B_1 exposure did not significantly alter sphingoid base concentration in LLC-PK_1 cells: Figures 3.8A and 3.8B show the effect of 1 μM fumonisin B_1 on the intracellular sphinganine and sphingosine concentrations in LLC-PK_1 cells as function of time of exposure. The sphinganine concentrations were not significantly increased in both untreated cells and following exposure to 1 μM fumonisin B_1 at all times tested (Fig. 3.8A). In contrast, in both untreated and fumonisin B_1-treated LLC-PK_1 cells, there was an observable reduction in intracellular sphingosine concentrations (Fig. 3.8B). Overall, The sphinganine and sphingosine concentrations in the cells remained relatively constant compared to the untreated cells for all the times of fumonisin B_1 exposure. More than 2 h was necessary for fumonisin B_1 was necessary to efficiently inhibit ceramide synthase and increase in sphinganine and sphingosine levels.

The serine palmitoyltransferase inhibitor, myriocin, did not prevent the fumonisinB_1-induce translocation of PKCα: Fumonisin B_1 is a potent inhibitor of *de novo* sphingolipid biosynthesis and acts by interfering with a key pathway enzyme, N-acyl-transferase (11). As such, inhibition of SPT, an enzymatic step that occurs before N-acylation of sphinganine should prevent the fumonisin B_1-induced accumulation of sphinganine. Fumonisin B_1 caused an increase in membrane protein PKCα concentration and a concomitant decrease in cytosolic protein (Fig. 3.9). Exposure of LLC-PK cells to 200 nM of the SPT inhibitor, myriocin, did not alter PKCα subcellular redistribution and showed similar cytosolic and membrane protein expressions similar to untreated cells.

Preincubation of LLC-PK$_1$ cells with 200 nM myriocin did not prevent the fumonisin B$_1$-induced increase in PKCα cytosol to membrane redistribution.

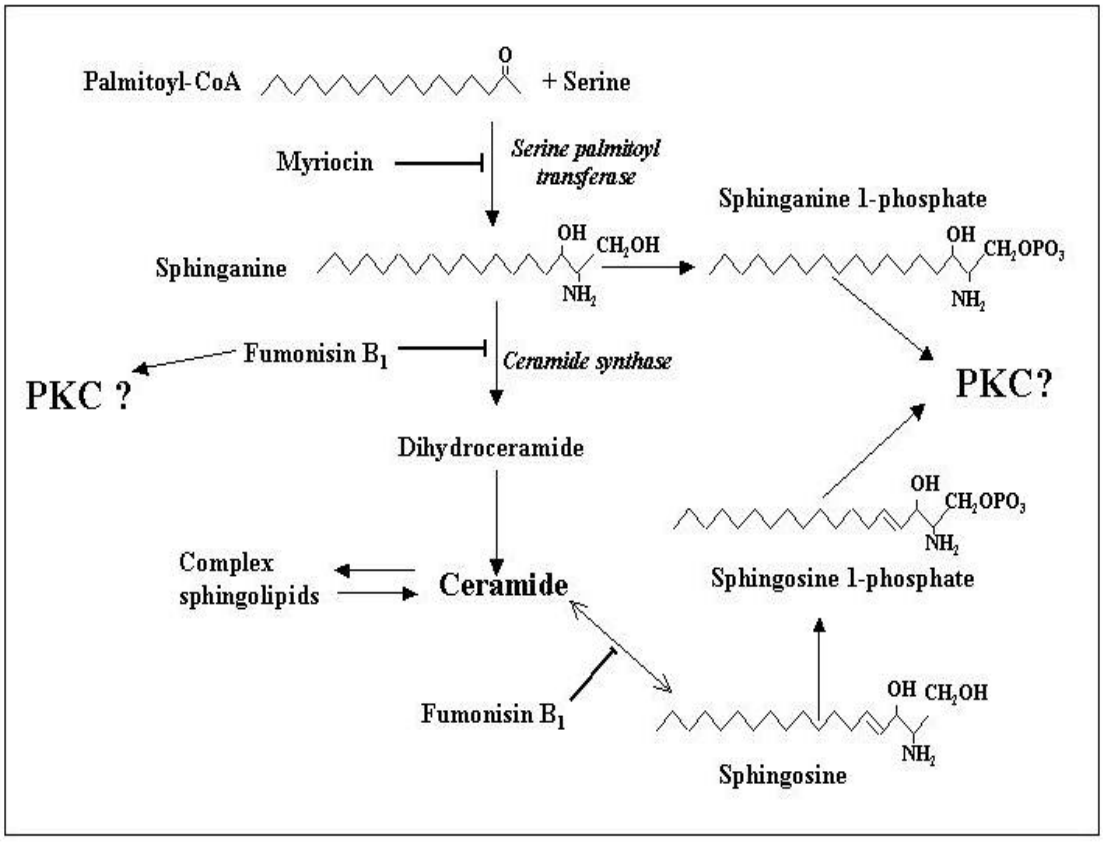

Figure 3.1. Schematic diagram of inhibitors in the *de novo* biosynthetic pathway and the sphingolipid turnover pathway. Fumonisin B$_1$ and myriocin may incompletely inhibit their respective enzymes resulting in the possible formation of sphingoid bases and their degradation products that may be involved in PKC cell signaling such as sphingaine, sphingosine, sphinganine 1-phosphate and sphingosine 1-phosphate.

Figure 3.2. Temporal effect of fumonisin B_1 on cytosol to membrane translocation of PKCα and PKC activity in LLC-PK_1 cells. LLC-PK_1 cells were exposed to 1 μM fumonisin B_1 at the times indicated. Cell lysates were obtained and immunoblot and PKC activity assays were performed as described under "Experimental Procedures". The effect of 1 μM fumonisin B_1 on PKCα membrane (A) and cytosol (B) concentrations in response to fumonisin B_1 at the times indicated are shown. Representative photographs are shown above each graph where the bands correspond to the same order as the bar graph. PKC assay of total cell lysates (20 μg) on exposure to fumonisin B_1 at the times indicated (C). A representative photograph is shown above the graph where lanes 1, 3, 5 and 7 and lanes 2, 4, 6 and 8 correspond to the untreated and treated samples at 5, 15, 30 and 120 min, respectively. PKC assay of total cell lysates incubated exposed to fumonisin B_1 (FB_1) and incubated with anti-PKCα antibody (anti-PKCα) (D) and a mixture of anti-PKC-δ, -ε and -ζ antibodies (anti-PKCδ, ε, ζ). Data represents the mean ± standard error of triplicate determinations (n=3) from a representative experiment that was repeated at least twice with similar results. * Significantly different from the respective control at $P \leq 0.05$.

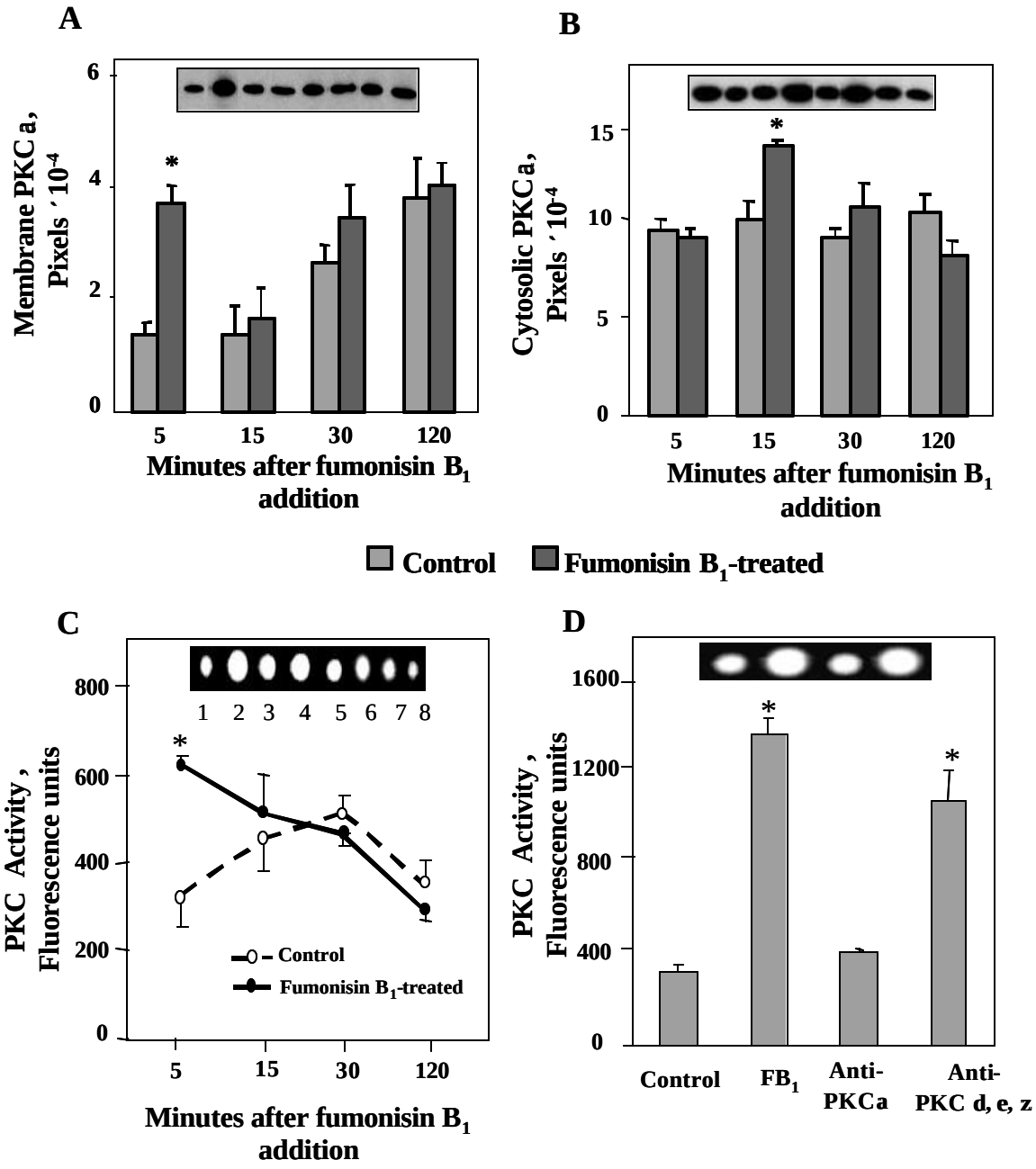

Figure 3.2. Temporal effect of fumonisin B_1 on cytosol to membrane translocation of PKCa and PKC activity in LLC-PK_1 cells.

Figure 3.3. Effect of fumonisin B_1 on cytosol and membrane protein concentrations of PKC-d, -e, and -z isoforms in LLC-PK_1 cells. LLC-PK_1 cells were grown for 2 days and replaced with serum-free HDMX 18 h prior to treatment. The cells were then exposed to 1 μM fumonisin B_1 for 5, 15, 30 and 120 min. Cell lysates were then obtained and 10 μg of protein was resolved by 8% SDS-PAGE gel, electroblotted to a nitrocellulose membrane and visualized autoradiographically using chemiluminescent reagents. Isoform protein cytosolic (A, C and D) or membrane (B, E and F) distributions for PKCδ (A and B), PKCε (C and D), and PKCζ (E and F) are displayed. Representative photographs of one experiments are above each graph and the bands correspond to the bars on the graph. Results are expressed as the mean ± standard error of triplicate determinations (n=3) from a representative experiment that was repeated at least twice with similar results. * Significantly different from the control at $P \leq 0.05$.

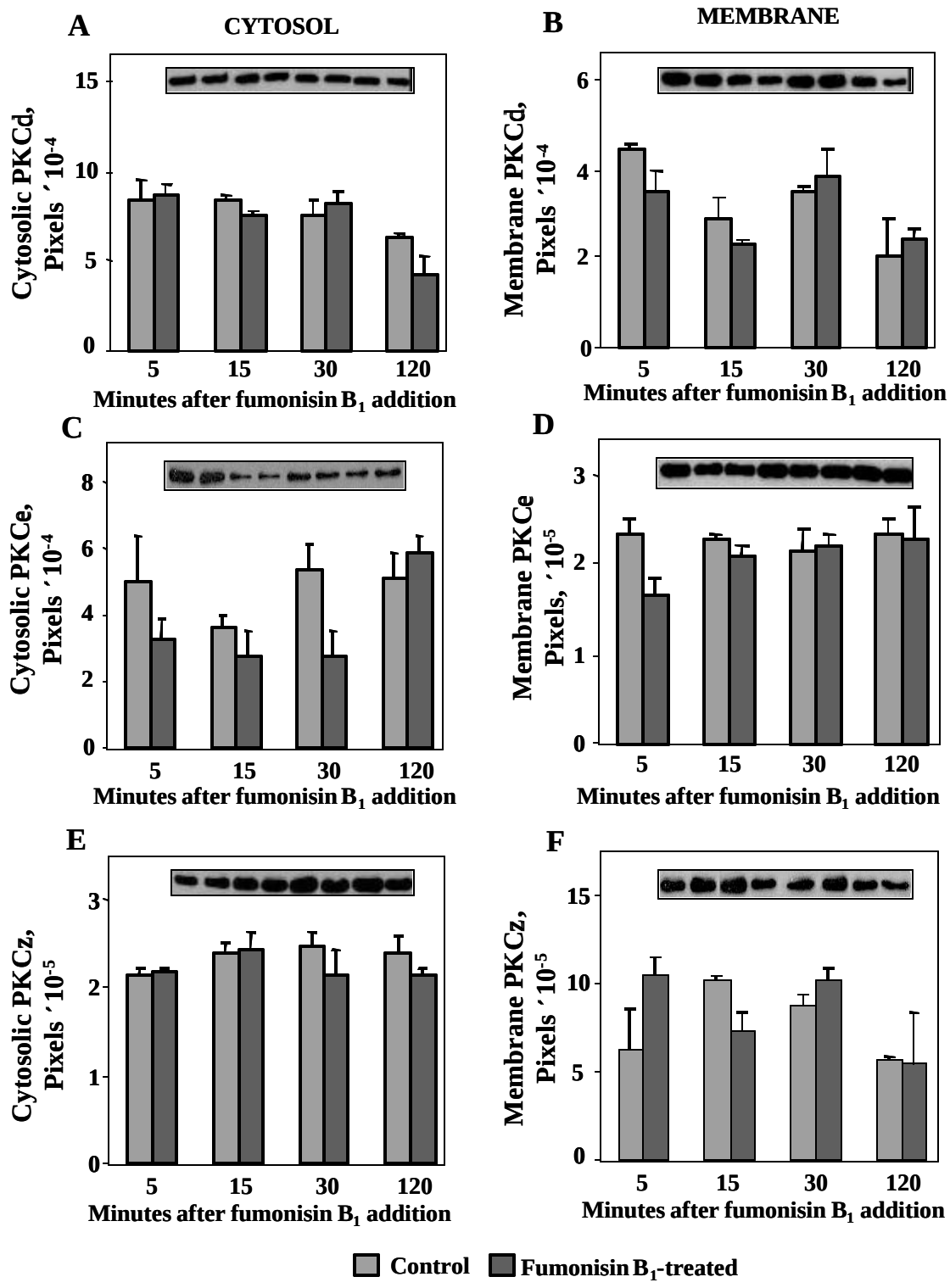

Figure 3.3. Effect of fumonisin B_1 on cytosol and membrane protein concentrations of PKC-d, -e, and -z isoforms in LLC-PK_1.

Figure 3.4. Effect of fumonisin B_1 on PKC activity and cycloheximide on PKCα cytosol protein concentrations at 15 min in LLC-PK_1 cells. A, LLC-PK_1 cells were grown for 2 days and replaced with serum-free HDMX 18 h prior to treatment. Following 15 min exposure to fumonisin B_1 (1 μM), cells were lysed, and phosphorylation of a synthetic colored substrate assessed cytosolic PKC activity. B, Immunoblot analysis of the cytosolic PKCα protein expression in response to fumonisin B_1 (FB_1) and the protein synthesis inhibitor, cycloheximide (CHX) were performed. LLC-PK_1 cells were grown for 2 days and replaced with serum-free HDMX 18h prior to treatment. They were subsequently preincubated with or without, 10 μg/ml CHX for 120 min prior to exposure 1 μM FB_1 for 15 min. Denatured proteins (10 μg) from the cytosolic fraction were separated by 8% SDS-PAGE. Following SDS-PAGE and transfer of proteins to nitrocellulose membranes, immunodetection was performed. Representative photographs are present above each graph and the bands correspond to the bars of the graph. Data represents the mean ± standard error of triplicate determinations (n=3) from a representative experiment that was repeated at least twice with similar results. * Significantly different from the control at $P \leq 0.05$.

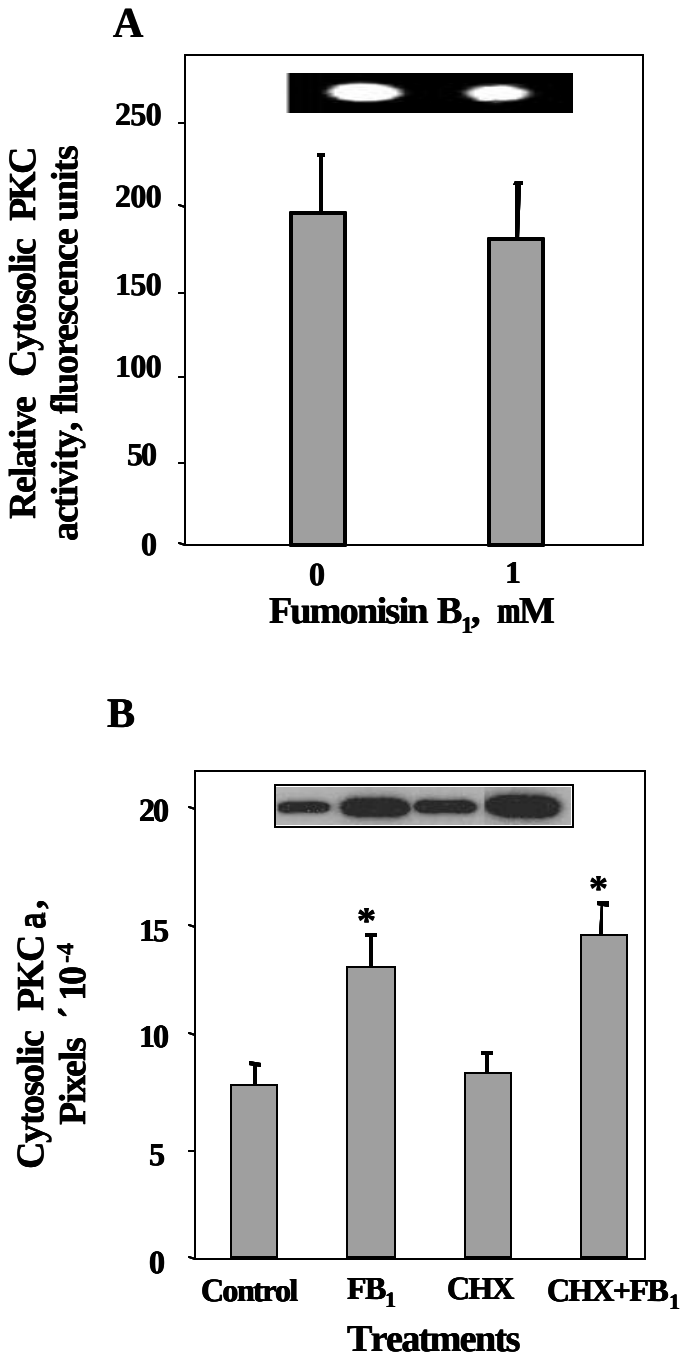

Figure 3.4. Effect of fumonisin B_1 on PKC activity and cycloheximide on PKC a cytosol protein concentrations at 15 min in LLC-PK_1 cells.

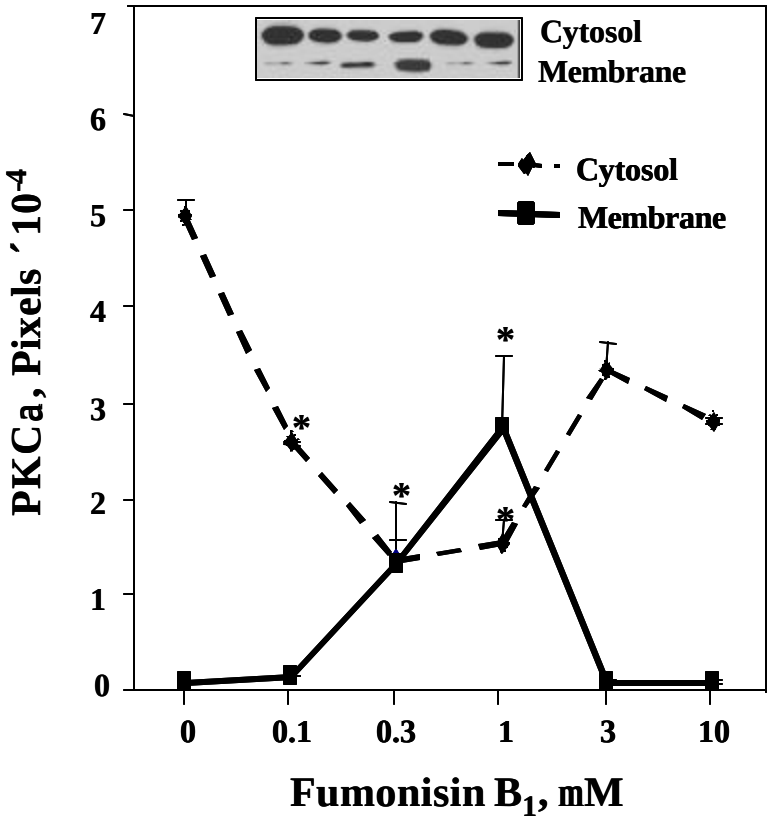

Figure 3.5. Fumonisin B_1 induces a concentration-dependent increase in PKCa membrane protein at 5 min in LLC-PK_1 cells. LLC-PK_1 cells were grown for 2 days and replaced with serum-free HDMX 18 h prior to treatment. The cells were then exposed to 0.1-10 μM fumonisin B_1 for 5 min. Cell lysates were obtained and 10 μg of protein was resolved on an 8% SDS-PAGE gel, electroblotted to a nitrocellulose membrane and visualized autoradiographically using chemiluminescent reagents. Representative photograph is present above the graph and the bands correspond to the points on the line. Results are expressed as the mean ± standard error of triplicate determinants (n=3) from a representative experiment that was repeated at least twice with similar results. * Significantly different from the respective control at $P \leq 0.05$.

Figure 3.6. Effect of the PKC activator and inhibitor on the fumonisin B_1-induced membrane translocation of PKCa in LLC-PK_1 cells. Cells were treated with PMA (0.01 μM) for 30 min or 100 nM calphostin C (Cal-C) for 60 min in the presence or absence of fumonisin B_1 (FB_1) (1 μM for 5 min). Membrane and cytosolic fractions were obtained and immunoblots were performed as described in "Experimental Procedures". Representative photographs are presnt above each graph and the bands correspond to the bars on the graphs. Values are mean ± standard error of triplicate determinants (n=3). The experiment is repeated at least 3 times. * Significantly different from the respective control at $P \leq 0.05$.

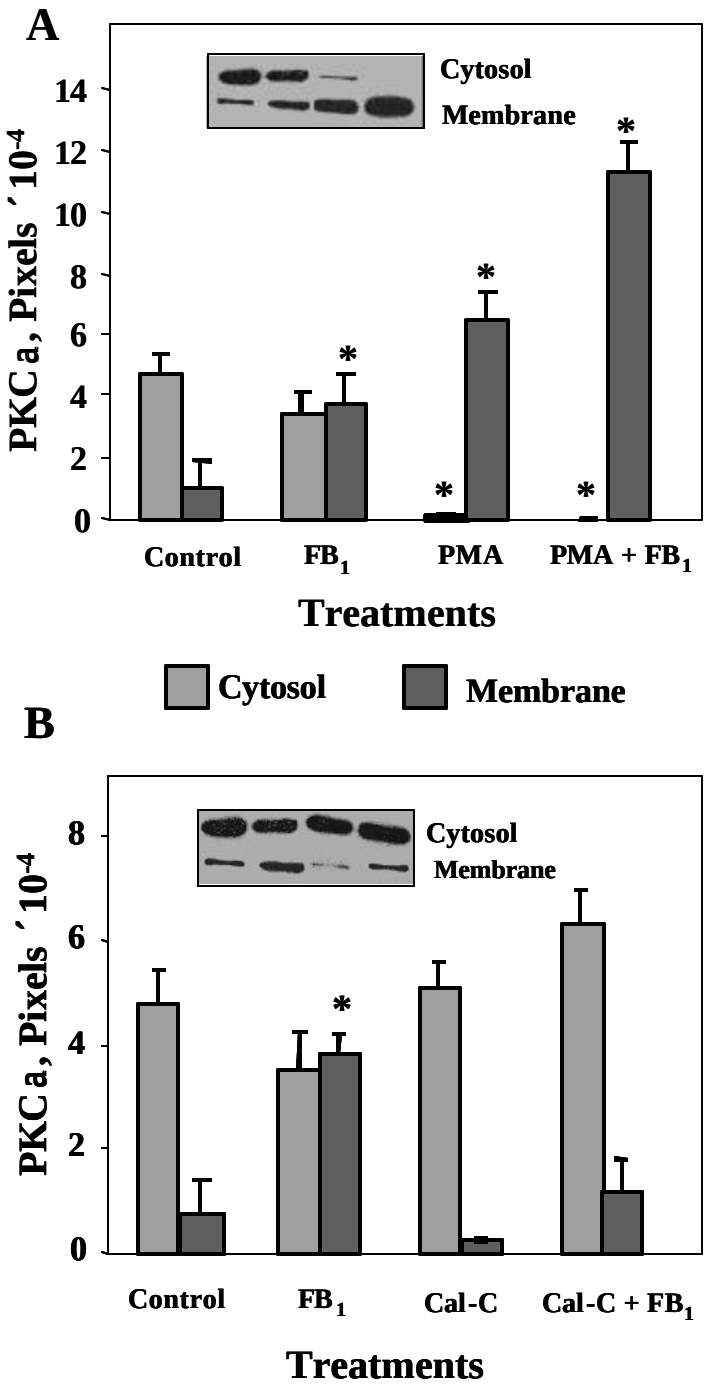

Figure 3.6. Effect of the PKC activator and inhibitor on the fumonisin B_1-induced membrane translocation of PKC a in LLC-PK_1 cells.

Figure 3.7. Fumonisin B_1-induced cytosol to membrane redistribution of PKCa is corroborated with immunostaining. Immunofluorescent staining of PKCα isoform in LLC-PK_1 cells treated with 100 nM calphostin C (Cal-C) for 60 min in the light or PMA (0.01 μM) (D) for 30 min in the presence or absence of fumonisin B_1 for 5 min. Untreated LLC-PK_1 cells displayed diffuse cytosolic stiaing at 5 min (A). Intensified staining at the plasma membrane is observed in LLC-PK_1 cells treated with fumonisin B_1 (B) compared to untreated cells. PMA, in the absence (C) or presence of fumonisin B_1 (D), displayed intensified plasma membrane staining Calphostin C displayed similar diffuse cytosolic staining (E) to untreated cells and inhibited the fumonisin B_1 membrane staining (F) as a reduction in cellular membrane staining indicates a reduction in isoform PKCα translocation. A representative photograph of three similar experiments is displayed. Increased plasma membrane staining is indicated by arrows

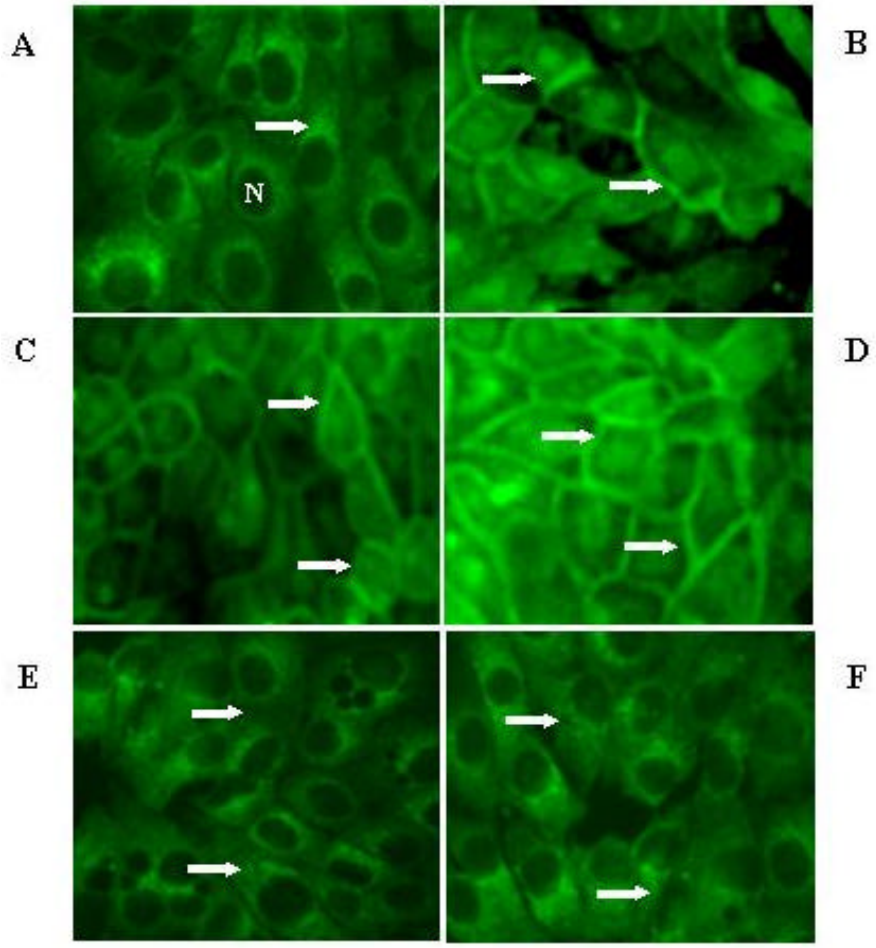

Figure 3.7. Fumonisin B_1-induced cytosol to membrane redistribution of PKCa is corroborated with immunostaining.

Figure 3.8. Temporal effect of fumonisin B_1 on accumulation of intracellular sphinganine and sphingosine in LLC-PK_1 cells. LLC-PK_1 cells were serum starved overnight in HDMX-F12 defined media prior to exposure to 1 μM fumonisin B_1 (FB_1). At the indicated times appropriate HPLC was performed as described under "Experimental Procedures" in order to determine the intracellular sphinganine (A) and shphingosine (B) concentrations. Values are mean ± standard error of triplicate determinants (n=3) from a representative experiment that was repeated at least twice with similar results. * Significantly different from the respective control at $P \leq 0.05$.

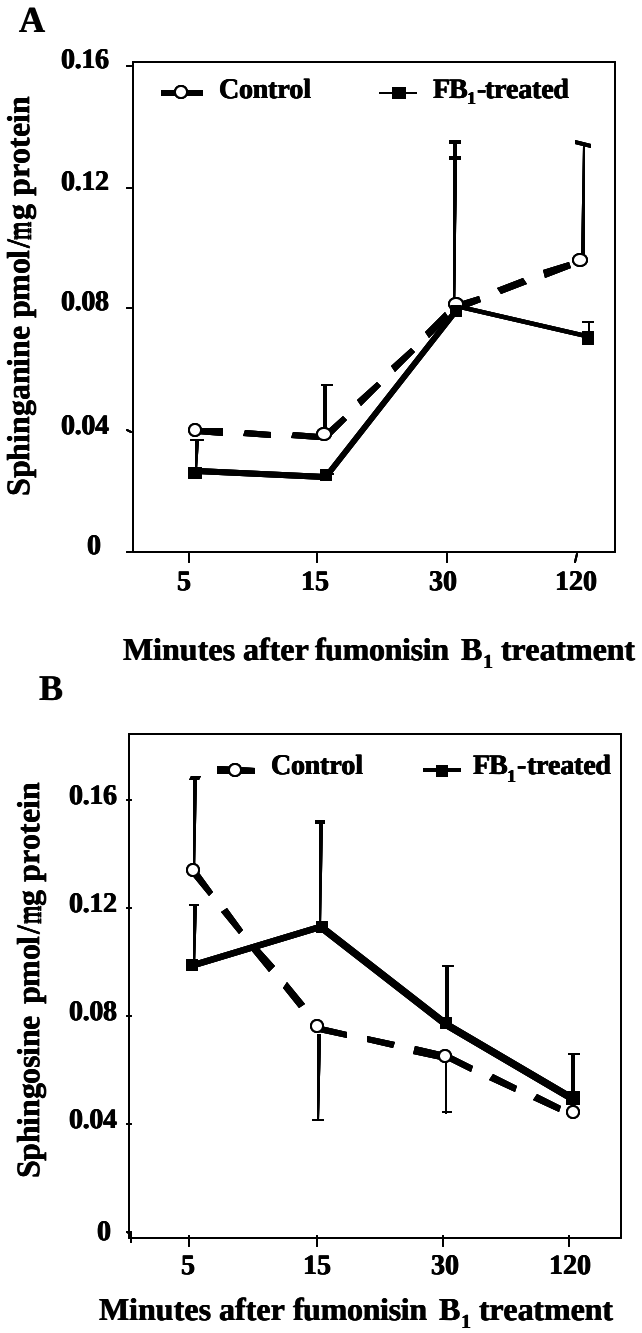

Figure 3.8. Temporal effect of fumonisin B_1 on accumulation of intracellular sphinganine and sphingosine in LLC-PK_1 cells.

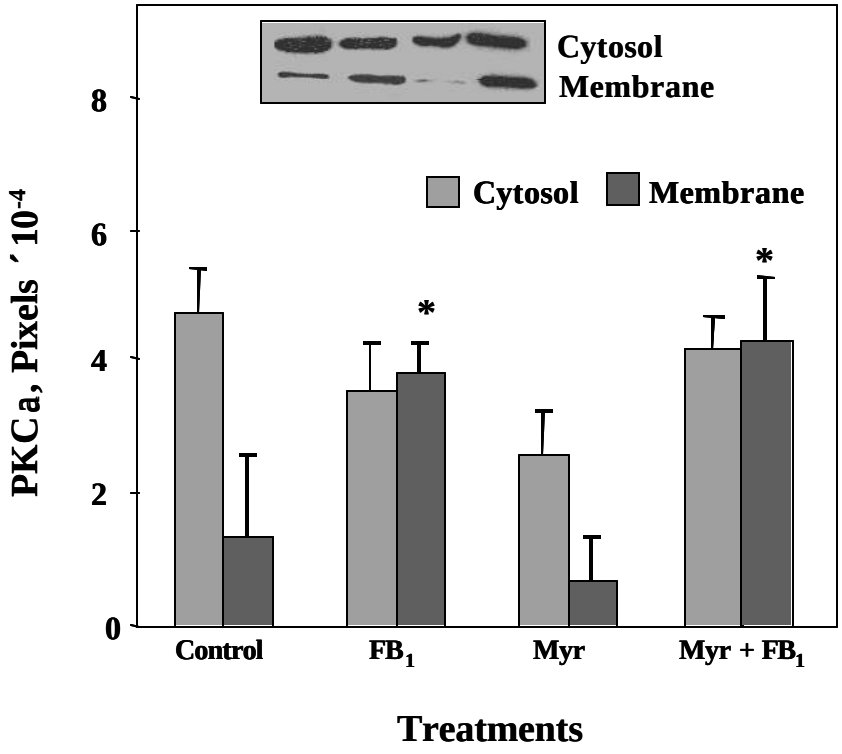

Figure 3.9. The effect of myriocin on fumonisin B_1-induced membrane translocation of PKCa in LLC-PK_1 cells. LLC-PK_1 cells were grown for 2 days and replaced with serum-free HDMX 18 h prior to treatment. The cells were then preincubated with 200 nM myriocin (Myr) for 4 h prior to addition of 1 μM fumonisin B_1 (FB_1) for 5 min. Cell lysates were then obtained and 10 μg of protein was resolved by 8% SDS-PAGE gel, electroblotted to a nitrocellulose membrane and visualized autoradiographically using chemiluminescent reagents. Results are expressed as the mean ± standard error of triplicate deteminants (n=3) from a representative experiment that was repeated at least twice with similar results. * Significantly different from the respective control at $P \leq 0.05$.

DISCUSSION

The aim of the present study was to examine the effects of short-term fumonisin B_1 exposure on PKC isoforms present in LLC-PK_1 cells and to relate these effects to the disruption of sphingolipid biosynthesis utilizing an SPT inhibitor. To this end, we have shown that fumonisin B_1 selectively and transiently activates PKCα in LLC-PK_1 cells as documented by immunoblot, immunofluorescent localization and enzyme activity. The fumonisin B_1-induced membrane translocation of PKCα was prevented by calphostin C and additive with PMA. Intracellular sphinganine and sphingosine concentrations were not altered in response to fumonisin B_1 and myriocin did not prevent the fumonisin B_1-induced activation of PKCα in LLC-PK_1 cells.

PKC isoforms show a markedly different tissue distribution as most cell types express only a subset of PKC isoforms, which are differentially activated upon appropriate stimulation (*34*). We therefore investigated the subcellular distribution of various PKC isoforms present in LLC-PK_1 cells and found that these cells express PKC-α, -δ, -ε, and -ζ isoforms. As in most cell lines investigated, PKC-α, -δ, and -ζ in LLC-PK_1 cells was predominantly expressed in the cytosol (*32;33;35-37*). PKCε is predominantly membrane-associated in LLC-PK_1 cells as it is in U937 cells (*38*), rat6 fibroblasts (*36*) and renal mesangial cells (*32*).

Taking into account the possibility that only some isoforms might be affected upon stimulation (*21*) the subcellular distribution of PKC-α, -δ, and -ζ isoforms after fumonisin B_1 treatment in LLC-PK_1 cells was examined. The data presented here showed that 5 min exposure to fumonisin B_1 concentrations of 0.1-1 μM exclusively activates and induces cytosolic to membrane translocation of PKCα, a Ca^{2+} and DAG dependent

isoform, followed by its rapid down-regulationat higher concentrations and later time points. In agreement with our findings, Yeung *et al*. (*15*) also reported similar results for PKCγ in cerebrocortical slices on exposure to fumonisin B_1 (≤ 1 μM), however higher concentrations were not addressed in this study. The results suggest that distinct PKC isoforms regulate different functions within different cells lines. Fumonisin B_1 may be responsible for selectively and transiently activating the reversible form of PKCα in the present study (*39*). Transient PKC activation is a phenomenon that is physiologically relevant and has also been reported to occur in many cell lines (*40;41*). Rapid down-regulation of PKC is due to calpain, a calcium-dependent cysteine proteinase, which releases the catalytic domain of PKC yielding independent fragment of PKC known as PKM (*42*).

The ability of fumonisin B_1 to alter signal transduction pathways, including that of PKC, can play a role in its ability to induce apoptosis and carcinogenesis (*43*). The cytosol-to-membrane translocation of PKCα with subsequent activation of various transcription factors represent critical steps in the induction of signaling cascade leading to the production of proinflammatory cytokines, including TNFα (*44*), which is known to modulate fumonisin B_1 toxicity (*9;10*). The fumonisin B_1-induced modulation of PKC, the major high-affinity intracellular receptor for potent tumor promoters (*17*) may also establish the basis of its involvement in multistage carcinogenesis (*45*). While the physiological significance of the effects of fumonisin B_1 on PKCα activation is still not known, the role of PKC in signal transduction may play a role in the mechanisms involved in mycotoxin-induced mitogenesis and/or apoptosis.

The fumonisin B_1 increase in the stabilization cytosolic PKCα protein following at 15 min observed in the present study may be due to retardation in the degradation of the activated PKCα by fumonisin B_1. Alternatively, Ramljak *et al*. (*46*) demonstrated that fumonisin B_1 treatment causes post-translational modification of cyclin D1 in such a way as to make it less negatively charged (possibly less phosphorylated) resulting in an increase its cytosolic stability in response to fumonisin B_1. A similar mechanism may also be responsible for the increase cytosolic PKCα observed in the present study.

We found that PMA (0.01 μM) had an additive effect on the fumonisin B_1-induced membrane translocation of PKCα in LLC-PK_1 cells. In contrast, Yeung and coworkers (*15*) demonstrated that the effects of fumonisin B_1 and 1 μM PMA is neither additive nor synergistic in cerebrocortical slices. The discrepancy may be due to the lower concentration of PMA used in the respective studies. In addition, the inhibition of PKCα membrane translocation in response to fumonisin B_1 by calphostin C may be explained by the fact that calphostin C specifically interacts with the regulatory DAG domain of PKC and it is hypothesized that the action of fumonisin B_1 is likely mediated by its interaction with this DAG site of PKC (*15*).

To correlate the effects of fumonisin B_1 on PKC activity and altered sphingolipid metabolism, we investigated the effect of acute fumonisin B_1 exposure on sphingolipid turnover. No significant changes in the levels of free sphinganine or sphingosine in response to 1 μM fumonisin B_1 in LLC-PK_1 cells were observed over the time periods tested. This finding is consistent with that of Pinelli *et al*. (*47*) who demonstrated that in human bronchial W126 VA cells, both sphinganine and sphingosine concentrations did not significantly increase until 2 h following exposure to 10 μM fumonisin B_1. The

complete inhibition of ceramide synthase by fumonisins causes the intracellular sphinganine concentration to increase rapidly (*8*). However, before this can occur, the capacity of sphingosine kinase to degrade free sphinganine must be exceeded. Free sphingosine concentration may also increase through fumonsin B_1 inhibition of reacylcation of sphingosine derived from sphingolipid turnover or dietary sources/growth medium. It is possible that partial inhibition of ceramide synthase could increase the rate of sphingoid base 1-phosphate biosynthesis, and activate PKC, without any apparent increase in the free sphinganine or sphingosine concentrations (Fig. 3.1). In our studies (unpublished data) we have observed sphinganine 1-phosphate to produce a similar response as 1 μM fumonisin B_1 at 5 min in LLC-PK_1 cells.

Myriocin, a specific inhibitor of SPT, a key enzyme responsible for the *de novo* biosynthesis of sphinganine (*48*) did not prevent the fumonisin B_1-induced activation of PKCα in LLC-PK_1 cells. Although He *et al* (*25*) demonstrated that myriocin concentrations 0.15-1.0 μM efficiently prevented the accumulation of free sphingoid bases as a result of fumonisin B_1 treatment without affecting LLC-PK_1 cell viability. The rate of inhibition of fumonisin B_1 on ceramide synthase may be much faster than that of myriocin on SPT, thereby generating bioactive breakdown products of cellular sphingolipids that can activate PKC (*14*) (Fig. 3.1).

We hypothesize several contingent mechanisms by which fumonisin B_1 can activate PKCα. The transient activation of PKCα by fumonisin B_1 may be due to generation of bioactive sphingoid base metabolites, e.g. sphinganine 1-phosphate, that may or may not directly interact with PKC (*14*). Yeung and coworkers (*15*) demonstrated that fumonisin B_1-induced PKC translocation from cytosol to membrane is likely mediated by its action

of fumonisin B_1 on the DAG site. Alternatively, it is also possible that fumonisin B_1 may interact with another distinct site on PKC or through another mechanism. Fumonisin B_1 has been demonstrated to induce lipid peroxidation (*49-51*) and several lines of evidence demonstrate that PKC is an upstream target for both oxidants and chemopreventive antioxidants (*19*).

In conclusion, our data contributes significantly to the understanding of the ability of fumonisin B_1 to selectively and transiently activate PKCα activation in LLC-PK_1 cells. Alterations in PKC signal transduction pathways may have implications in the ability of fumonisin B_1 to produce both apoptosis and carcinogenesis. Further studies are being undertaken to determine the downstream effectors of PKCα, chronic effects of fumonisin B_1 on PKC isoforms as well as relating these changes in fumonisin B_1 to the disruption in sphingolipid biosynthesis in LLC-PK_1 cells.

ACKNOWLEDGEMENTS

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CHAPTER 4

INHIBITION OF CERAMIDE SYNTHASE BY FUMONISIN B_1 TRANSIENTLY ACTIVATES NUCLEAR FACTOR-kB AND SUBSEQUENTLY TUMOR NECROSIS FACTOR a EXPRESSION IN PORCINE RENAL CELLS VIA ACTIVATION OF PROTEIN KINASE C a [1]

[1]Gopee, N.V. and Sharma, R.P. Submitted to *Biochemistry*, 11/12/02.

ABSTRACT

Fumonsin B_1 (FB_1) is a toxic metabolite produced by the phytopathogenic fungus *Fusarium verticillioides* predominantly present in corn. The principal biochemical responses of FB_1 involve disruption of sphingolipid metabolism from the inhibition of ceramide synthesis and induction of tumor necrosis factor α (TNFα). The ability of FB_1 to modulate signal transduction pathways also plays a role in its toxicity. Two important observations underlie the aim of this study, namely (1) FB_1 selectively and transiently activates protein kinase C α (PKCα) in porcine renal epithelial cells (LLC-PK_1) and (2) PKC can activate nuclear factor-kappa B (NF-κB), an important transcription factor for the proinflammatory cytokine, TNFα. The aim of this study was to investigate the effect of PKCα activation by FB_1 on NF-κB activation and subsequently on TNFα gene expression and caspase induction in LLC-PK_1 cells. FB_1 (1 μM for 5 min) transiently activated PKCα and increased nuclear translocation of NF-κB, followed by their down-regulation at later time points. Preincubating LLC-PK_1 cells with the PKC inhibitor, calphostin C, prevented the activation of NF-κB by FB_1. TNFα mRNA expression was increased following 15 min exposure to either FB_1 or the PKC activator, phorbol 12-myristate 13-acetate (PMA). In addition, an increase in caspase 3 activity was observed after addition of FB_1 for 1 h. Calphostin C prevented the FB_1-induced increase in TNFα gene expression and caspase 3 activation. In summary, the PKCα-dependent activation of NF-κB with the subsequent induction of TNFα and caspase 3 puts forward a plausible mechanism involved in FB_1-induced toxicity in LLC-PK_1 cells.

[1]Abbreviations used are: FB_1, fumonisin B_1; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; TNFα, tumor necrosis factor-α; NF-κB, nuclear factor-kappa B; IκB, inhibitory kappa B; Cal-C, calphostin C; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

INTRODUCTION

Toxigenic isolates of the ubiquitous fungus *Fusarium verticillioides* Nirenberg frequently contaminates corn and produce a group of mycotoxins known as the fumonisins (*1*). In domestic animals, consumption of fumonisin B_1 (FB_1) causes various species-specific syndromes, including equine leukoencephalomalacia (*2*) and pulmonary edema in pigs (*3*). In laboratory animals, FB_1 has been shown be hepatotoxic and nephrotoxic (*4*). In addition, FB_1 is a hepatic carcinogen in rats and female mice and a renal carcinogen in male rats (*5, 6*). There has also been a high correlation between the consumption of FB_1-contaminated corn and human esophageal cancer in South Africa and China (*7*).

FB_1 is a potent inhibitor of sphinganine *N*-acyl-transferase (ceramide synthase), the important rate limiting enzymatic step in *de novo* sphingolipid biosynthesis (*8*). The FB_1-induced disruption of sphingolipid biosynthesis and turnover can alter lipid second messengers resulting in altered cell signaling and cytokine production. Additionally, there is evidence that tumor necrosis factor α (TNFα) may also be a contributing factor to FB_1-induced apoptosis and other observed toxic effects *in vivo* and *in vitro* (*9-12*).

FB_1 alters the subcellular redistribution and activity of protein kinase C (PKC) (*13, 14*) and we have found that a 5 min exposure to FB_1 transiently activated the conventional

calcium- and diacylglycerol-dependent PKC isoform, PKCα, in porcine renal epithelial cells (LLC-PK_1) cells. PKC is a family of phospholipid-dependent serine/threonine kinases that activates nuclear factor-kappa B (NF-κB). NF-κB is composed of dimers of different members of the Rel protein family (*15*), which is associated at rest with an inhibitor protein, IκB, and is retained in the cytoplasm (*16*). Phosphorylation is an important event of NF-κB activation whereby the active NF-κB translocates to the nucleus and binds to a NF-κB motif, functioning as a transcription factor (*15*). NF-κB regulates the gene expression of chemokines, growth factors and various cytokines (*17*), including TNFα (*18-20*). TNFα is a pleotropic proinflammatory cytokine reported to modulate proliferation, differentiation, and apoptotic or necrotic cell death in a number of cell types (*21*). Activation of caspases, a group of cysteine proteases, by TNFα which contribute to the degradation of various cellular components thereby inducing apoptosis (*22*). Thus, the influence of TNFα may be a contributing factor to FB_1-induced apoptosis and other observed toxic effects (*23*).

The aim of this study was to provide evidence for the FB_1 activation of NF-κB, TNFα and caspase 3 via a PKCα-dependent pathway in LLC-PK_1 cells. LLC-PK_1 is a renal epithelial cell line, which has several characteristics of proximal tubular cells and is sensitive to FB_1-induced alterations in sphingolipid biosynthesis, TNFα induction and apoptosis (*24-26*). Specifically, we investigated the effect of PKCα activation by FB_1 on NF-κB and TNFα induction and we found that FB_1 transiently activated PKCα resulting in the sequential activation of NF-κB and induction of TNFα. The FB_1 induction of TNFα resulted in an increase in caspase 3 activity. The results confirm that FB_1-induced

NF-κB activation and TNFα expression leading to an increase in caspase 3 activity in LLC-PK_1 cells are dependent on PKCα activation.

MATERIALS AND METHODS

Materials. FB_1 (98% > purity) was obtained from Programme on Mycotoxins and Experimental Carcinogenesis (PROMEC, Tygerberg, South Africa). Phorbol 12-myristate 13-acetate (PMA) and horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibody was obtained from Sigma (St. Louis, MO). Calphostin C was procured from Calbiochem (San Diego, CA). Primary mouse polyclonal anti-PKCα, primary mouse anti-NF-κB p65 subunit and fluorescein isothiocyanate (FITC)-conjugated anti mouse IgG secondary antibodies were obtained from (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Primary mouse monoclonal anti-NF-κB p65 subunit antibody for immunofluorescence was purchased from Boehringer Mannheim (Indianapolis, IN). NF-κB consensus sequences were purchased from Promega (Madison, WI).

Cell culture. LLC-PK1 cells (CRL 1392, passage 197) were obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured at 37°C in a humidified atmosphere with 5% CO_2 in Dulbecco's modified Eagle's Medium/Ham's F12 (1:1) medium supplemented with 5% fetal bovine serum. For all experiments, the cells were subcultured in 100 x 15 mm sterile Petri dishes. Cells were allowed to attach and grow for 2 days and the media was replaced with serum-free media 18 h prior to appropriate treatment. To prevent the burst of free sphingoid bases after addition of fresh medium (*27*), 0.1 ml medium from each well was taken out prior to adding 0.1 ml of

appropriate chemical dissolved in serum-free medium. For appropriate experiments, LLC-PK_1 cells were treated with 0.01 μM PMA diluted in 0.1 % dimethyl sulfoxide in serum-free medium, for 30 min. Inhibition of PKC by calphostin C is photo-dependent (*28*), therefore LLC-PK_1 cells were preincubated with 100 nM calphostin C for 1 h in the presence of light to ensure adequate inhibition of PKC.

Subcellular fractionation. Following appropriate treatments, LLC-PK_1 cells were washed 3 times in cold phosphate-buffered saline (PBS). Modifications to the method described by Clarke *et al.* (*29*) were used to extract cytosolic and membrane fractions for PKC. Briefly, cells were scraped into 100 μl cold homogenization buffer containing 20 mmol/L Tris-HCl, pH 7.5, 0.25 mol/L sucrose, 10 mmol/L EGTA, 2 mmol/L EDTA, 20 μg/mL leupeptin, 10 μg/mL aprotinin and 200 μmol/L phenylsulfonyl fluoride (PMSF) at 4°C and sonicated on ice. The resulting homogenate was centrifuged at 42,000 × *g* for 2 h at 4°C and the supernatant (′cytosolic fraction′) was collected. To the pellet 50 μl of homogenization buffer containing 1% Triton X-100 at 4°C was added and the samples were mixed for 1 h at 4°C, followed by centrifugation at 42,000 × *g* for 2 h at 4°C. The supernatant (′membrane fraction′) was transferred to separate tubes.

Nuclear and cytoplasmic extraction for NF-κB involved washing of cells 3 times in cold PBS and homogenization in 100 μl buffer (0.6% IGEPAL, 150 mM NaCl, 10 mM HEPES, 1 mM EDTA, 0.5 mM PMSF, 0.5 mM dithiothreitol (DTT), 5 μg/ml aprotinin, 5 μg/ml leupeptin, 1 μM sodium orthovanadate). Samples were sonicated and incubated for 5 min on ice while shaking prior to centrifugation at 5,000 rpm for 5 min at 4°C. The supernatant was collected (′cytoplasmic fraction′) and stored at -80°C. To the pellet, 30 μl of lysis buffer B (25% glycerol, 0.5 mM DTT, 20 mM HEPES, 420 mM NaCl, 1.2

mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF, 5 μg/ml leupeptin, 5 μg/ml aprotinin) was added and incubated on ice while shaking for 20 min. Samples were centrifuged at $13{,}000 \times g$ for 15 min and the supernatant ('nuclear fraction') stored at -80°C.

Gel Electrophoresis and Western blotting. Protein concentrations were determined by Bio-Rad protein assay (Bio-Rad, Hercules, CA) with bovine serum albumin (BSA) (Sigma, St. Louis, MO) as a standard. Proteins were separated by electorphoresis on 8% SDS-PAGE gels and transblotted to nitrocellulose membranes. The protein bound nitrocellulose membranes were incubated in 5% milk protein for 1 h prior to incubating with appropriate primary antibodies. Membranes were then rinsed 5 times and then incubated with HRP-conjugated mouse secondary antibody. Protein bands were visualized by Pierce Super Signal® chemiluminescent substrate (Pierce, Rockford, IL). In each experiment, blots from each treatment group were exposed on the same piece of film (Kodak X-OMAT AR, Rochester, NY). Images were acquired with a scanner and analyzed with UN-SCAN-IT software (Silk Scientific Inc., Orem, UT).

Immunofluorescence and microscopy. Cells grown to subconfluence in sterile 4-chambered slides (Nalge Nunc International, Naperville, IL) were treated with 0.01μM PMA or 100 nM calphostin C in the absence or presence of 1 μM FB_1. After washing, cells were fixed and permeabilized in 1:1 cold methanol:acetone for 30 min, washed twice with PBS, and blocked for 50 min with 1% normal goat serum in PBS containing 0.1% Triton X-100. Mouse monoclonal anti-NF-κB p65 subunit primary antibody diluted in PBS containing 2 mg/ml fatty acid free BSA (Sigma, St. Louis, MO) and 0.1% Trion X-100 was applied the slides and incubated overnight at 4°C in a humidified chamber. Cells were washed 3 times with PBS followed by 2 h incubation with FITC-

conjugated anti-mouse secondary antibody. Slides were washed 3 times with PBS and then twice with distilled water, cover slips mounted and viewed with an Olympus IX71 inverted fluorescence microscope (Melville, NY) equipped with appropriate optics. Images from the microscope were recorded by Olympus digital camera using the Olympus Magnifier SP software.

Fluorescent Electrophoretic Mobility Shift Assay. Nuclear extracts were obtained as described by Weber *et al*. (*30*). Briefly, LLC-PK_1 cells were collected and lysed in a HEGD buffer (25 mM HEPES, pH 7.6, 1.5 mM EDTA, 10% glycerol, 1 mM DTT, and 0.1 mg/ml PMSF) in a sonicator. Homogenates were centrifuged at 12,000*g* for 5 min at 4°C, and the supernatant discarded. The remaining pellet was centrifuged for 10 s, and the residual supernatant was aspirated. The pellet was extracted with 30 µl HEGDK buffer (25 mM HEPES, pH 7.6, 1.5 mM EDTA, 10% glycerol, 1 mM DTT, 0.1 mg/ml PMSF and 0.5 M KCl) for 1 h on ice. Extracted pellets were centrifuged at 16,000 × *g* for 20 min at 4°C, and the supernatant was designated as the nuclear extract. Protein concentration was determined as described above.

For the fluorescent mobility shift assays, oligonucleotides were annealed by heating for 10 min at 70°C in 10 X annealing buffer (200 mM Tris-HCl, pH 7.6, 50 mM $MgCl_2$, 1mM DTT and 0.1 mM EDTA) followed by incubation at 36.5°C for 30 min. The DNA binding procedure involved coincubating 4 µg of protein with the oligonucleotide duplex for 0.5 h on ice. Bound DNA was separated on a 6% polyacrylamide nondenaturing gel for 1.5 h at 120 V at 4°C. Specificity for the binding reaction was confirmed by addition of NF-κB p65 subunit to one of the binding reactions. Following electrophoresis, the gel sandwich was separated and stained with SYBR Gold (Molecular Probes, Eugene, OR).

The gel was rinsed and bands were detected on a UV transilluminator (Ultra Lum Inc., Carson, CA) and photographed.

Semiquantitative analysis of TNFα mRNA by reverse transcriptase polmerase chain reaction (RT–PCR). The amplification of TNFα mRNA by RT-PCR was performed as described by He *et al.* (*25*). Briefly, total RNA was isolated from LLC-PK_1 cells using TRI reagent (Molecular Research Center, Cincinnati, OH). Total RNA was transcribed into cDNA, which was then amplified by PCR. PCR reactions were performed in a thermal cycler (Coy Inc., Ann Arbor, MI). The thermo amplification program consisted of an initial denaturation (5 min at 95°C), followed by 32 cycles (for TNFα) or 24 cycles (for GAPDH) of 0.5 min denaturation (94°C), 0.5 min annealing (50°C), and 1 min elongation (72°C), with a final extension period of 1 min at 72°C. For relative measurements, the number of cycles within a linear increase in the both products was selected (as indicated by arrows in Figure 4.6 A). The sense and antisense primers were 5'-AAT GGC AGA GTG GGT ATG-3' and 5'-CTT GAT GGC AGA GAG GAG-3' for TNFα, and 5'-TCC CTG CTT CTA CTG GTG CT-3', and 5'-TGA GCT TGA CAA AGT GGT CG-3' for GAPDH, respectively (chosen by Primer3 program; Whithead Institute, Cambridge, MA). PCR products were separated on 2% agarose gel containing 0.476 mM ethidium bromide and detected by a UV transilluminator (Ultra Lum Inc., Carson, CA) and photographed. The photographs were scanned and bands were quantified by using UN-SCAN-IT software (Silk Scientific Inc., Orem, UT). TNFα was normalized to the housekeeping GAPDH gene.

Determination of caspase 3 activation. Caspase 3 activity was determined using the CaspACE™ fluorometric activity assay (Promega) with modifications as follows.

Briefly, cells were treated in 24 wells following which Triton X-100 was added and repeatedly pipetted to lyse the cells. The homogenates were centrifuged at 10,000*g* for 10 min to remove cell to remove cell debris. The supernatant was assayed for caspase 3 activity using the CaspACE™ system according to the manufacturer's instructions. The fluorescence of cleaved substrate was determined using a Spectramax Gemini fluorescent plate reader (Molecular Devices, Irvine, CA). The fluorescence signal was digitized and analyzed using SoftMax Pro™ (version 3.1.1, Molecular Devices, Irvine, CA).

Statistical analysis. The results are expressed as mean ± standard error. Differences among the various treatment groups were determined by one-way analysis of variance followed by Duncan's multiple range test utilizing Version 8 of the Statistical Analysis Software (SAS). The level of $P \leq 0.05$ was taken to denote statistical significance.

RESULTS

FB_1 induced membrane translocation of PKCα. FB_1 concentrations of ≤ 1 μM induced a concentration-dependent increase in cytosolic to membrane translocation of PKCα, which positively correlated with an increase in PKC activity (data presented elsewhere). The concentration of FB_1 and time of PKCα activation was optimized as 1 μM at 5 min, respectively. Exposure of LLC-PK_1 cells to 1 μM FB_1 at 5 min resulted in an increase in the membrane protein concentration and a concurrent decrease in the cytosolic fraction PKCα (Figure 4.1).

Temporal effect of FB_1 on NF-κB nuclear translocation. Exposure of LLC-PK_1 cells to 1 μM FB_1 for 5 min significantly decreased NF-κB cytosolic protein concentrations,

with concomitant increased nuclear protein concentrations (Figure 4.2). At later time points, cytosolic and nuclear protein concentrations returned to control levels.

Fumonisin B_1-induced NF-kB nuclear translocation was inhibited by the PKC inhibitor calphostin C. To confirm that PKC activation was directly related to the NF-κB nuclear translocation in LLC-PK_1 cells, the PKC inhibitor calphostin C was added to cells in the absence or presence of FB_1. Cells exposed to calphostin C alone displayed cytosolic and nuclear protein levels comparable to those of untreated cells (Figure 4.3). Fumonisin B_1 caused an approximate 4-fold increase in nuclear protein concentration and a concomitant reduction in the cytosolic protein levels. However, preincubation with calphostin C, prevented the FB_1-induction of cytosolic to nuclear translocation suggesting that the activation of NF-κB by FB_1 is dependent upon PKC stimulation.

Effect of FB_1, PMA and calphostin C on NF-kB activation. NF-κB activity increased at 5 min in both FB_1 (Figure 4.4, lane 2) and PMA-treated cells (Figure 4.4, lane 4), compared to untreated controls (Figure 4.4, lane 1) as indicated by the intensity of the shifted bands. Pretreatment with 100 nM calphostin C for 55 min prevented the FB_1-induced activation of NF-κB (Figure 4.4, lane 3). Supershift studies confirmed that the p65 NF-κB subunit was involved in the FB_1-induced NF-κB DNA binding (Figure 4.4, lane 5).

Nuclear localization of NF-kB cells on exposure to FB_1 is corroborated by immunofluorescence. To further confirm the PKC dependent activation of NF-κB by FB_1, immunostaining using specific anti-NF-κB primary antibody was conducted in LLC-PK_1 cells. Exposure of LLC-PK_1 cells to FB_1 (1 μM for 5 min) (Figure 4.5B) or 0.01 μM PMA (Figure 4.5D), showed intensified nuclear staining compared to the diffuse

cytosolic staining of untreated cells (Figure 4.5A). Preincubation of cells with calpostin C prevented the FB_1-induced nuclear localization of NF-κB, as can be seen in Figure 4.5C, these cells displayed similar diffuse cytosolic staining as untreated cells.

Temporal effect of FB_1 on TNFα mRNA expression. The temporal effect of FB_1 on TNFα mRNA expression was determined by the semi-quantitative RT-PCR and found that TNFα mRNA expression was significantly increased following 15 min exposure to FB_1 in LLC-PK_1 cells (Figure 4.6). TNFα levels were equivalent to control at later time points.

Effects of FB_1 on TNFα expression in response to PMA and calphostin C. Confirmation of the PKC-dependence of the FB_1-induced increase in TNFα expression was performed using PMA or calphostin C. A two fold increase in TNFα expression was observed after 15 min exposure to FB_1, whereas there was a four fold induction in TNFα mRNA expression on exposure to PMA (Figure 4.7A). However, co-exposure of cells with PMA and FB_1 were neither additive nor synergistic. Calphostin C alone did not affect TNFα expression in LLC-PK_1 cells (Figure 4.7B); however, preincubation of cells with calphostin C abrogated the FB_1-induced stimulation of TNFα expression.

FB_1 activation of caspase 3 is inhibited by calphostin C. A significant increase in caspase 3 activity was observed on exposure to 1 μM FB_1 for 1 h (Figure 4.8A). The activity returned to untreated levels at later time points tested. The reversible caspase 3 inhibitor, Ac-DEVD-CHO® (Promega), prevented the effects of FB_1 where the enzyme's activity fell to untreated levels (data not shown). Preincubation with calphostin C prevented the FB_1-induced increase in caspase 3 activity (Figure 4.8B).

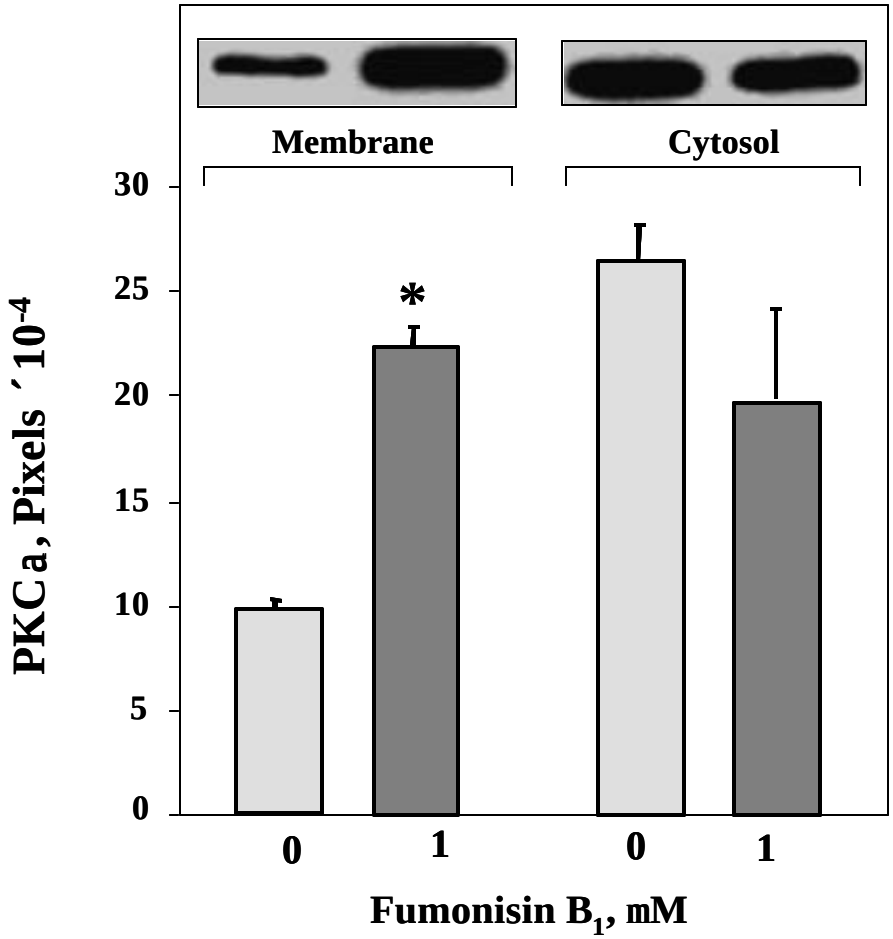

Figure 4.1. FB_1 increases cytosol to membrane translocation of PKCa in LLC-PK_1 cells at 5min. LLC-PK_1 cells were grown for 2 days and replaced with serum-free HDMX 18 h prior to treatment with 1 μM FB_1 for 5 min. Cell lysates were then obtained and 10 μg of protein was resolved by 8% SDS-PAGE gel, electroblotted to a nitrocellulose membrane and visualized autoradiographically using chemiluminescent reagents. Results are expressed as the mean ± S.E. (n=3). * Significantly different from the respective control at $P \leq 0.05$.

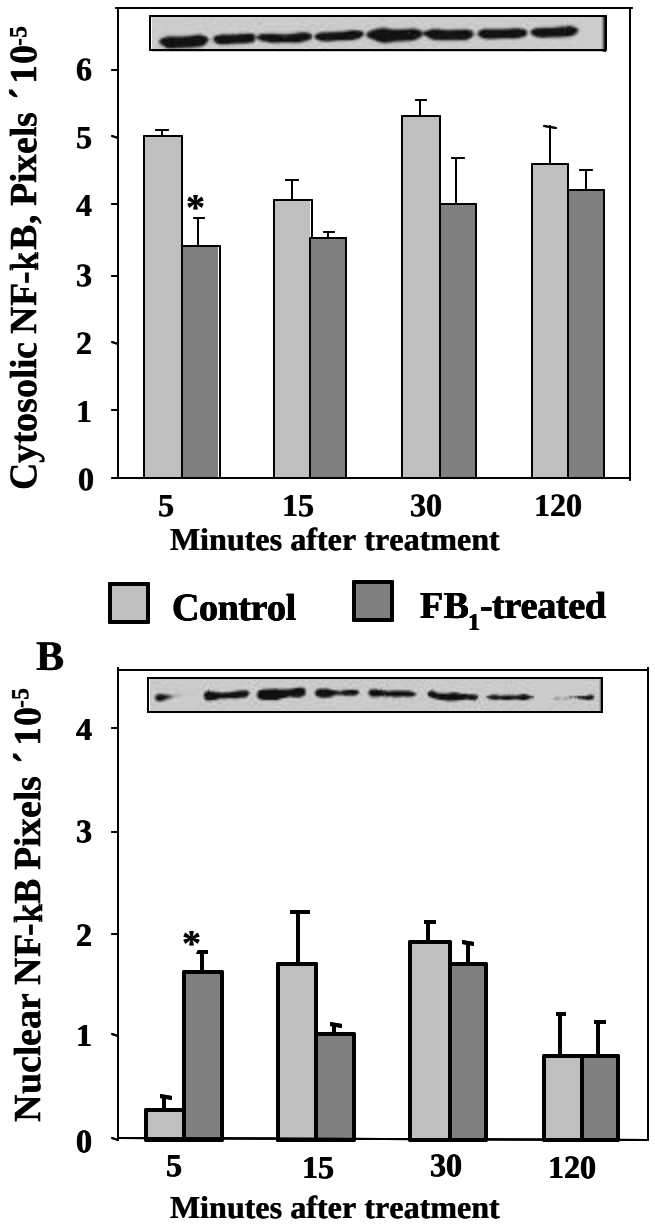

Figure 4.2. FB_1 stimulates NF-kB cytosol to nuclear translocation in LLC-PK_1 cells at 5 min. LLC-PK_1 cells were exposed to FB_1 (1 μM) for 5, 15, 30 and 120 min. Cytosolic (A) and nuclear (B) proteins were resolved by 8% SDS-PAGE gel, electroblotted to a nitrocellulose membrane and visualized autoradiographically using chemiluminescent reagents. Results are expressed as the mean ± S.E. (n=3). * Significantly different from the respective control at $P \leq 0.05$.

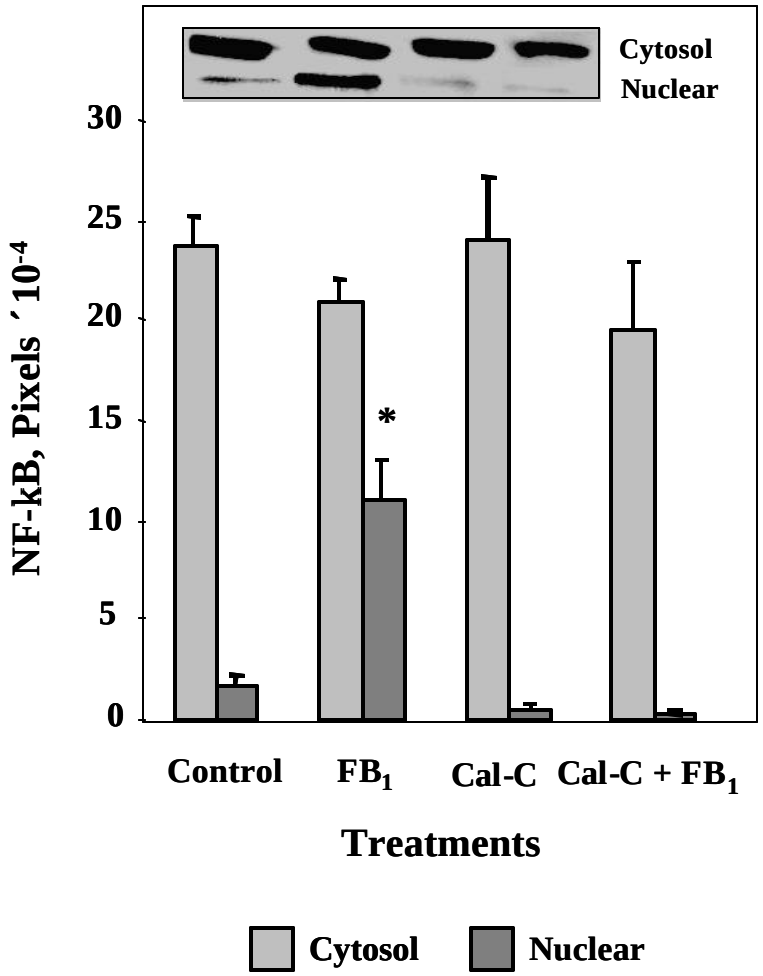

Figure 4.3. Calphostin C inhibits the FB_1-induced nuclear translocation of NF-kB in LLC-PK_1 cells. Following 5 min exposure to 1 μM FB_1, LLC-PK_1 cells were lysed, and cytosolic and nuclear protein concentrations were determined by Western blot analysis. Results are expressed as the mean ± S.E. (n=3). * Significantly different from the respective control at $P \leq 0.05$.

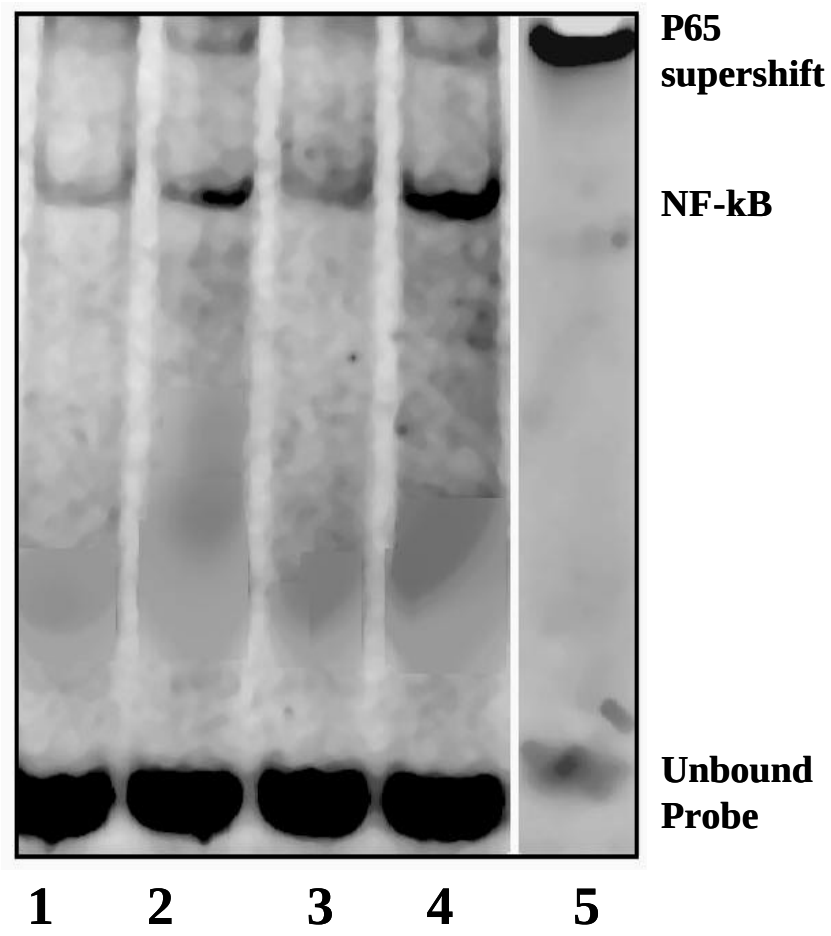

Figure 4.4. Effect of FB_1, PKC activator and inhibitor on NF-kB activation in LLC-PK_1 cells. LLC-PK_1 cells were treated with PMA or FB_1 in the absence or presence of calphostin C prior to isolation of nuclear proteins as described under ′Experimental Procedures′. FB_1 induced an increase in NF-κB activation (lane 2) as compared to untreated cells (lane 1). Pretreatment with calphostin C attenuated the FB_1-induced NF-κB activation (lane 3). PMA also increased NF-κB activation comparative to that of fumonisin B_1 (lane 4). The results of supershift experiments where addition of antibody to p65 caused a supershift in the DNA/protein complex is shown in lane 5. A representative gel is shown of three experiments which showed similar results.

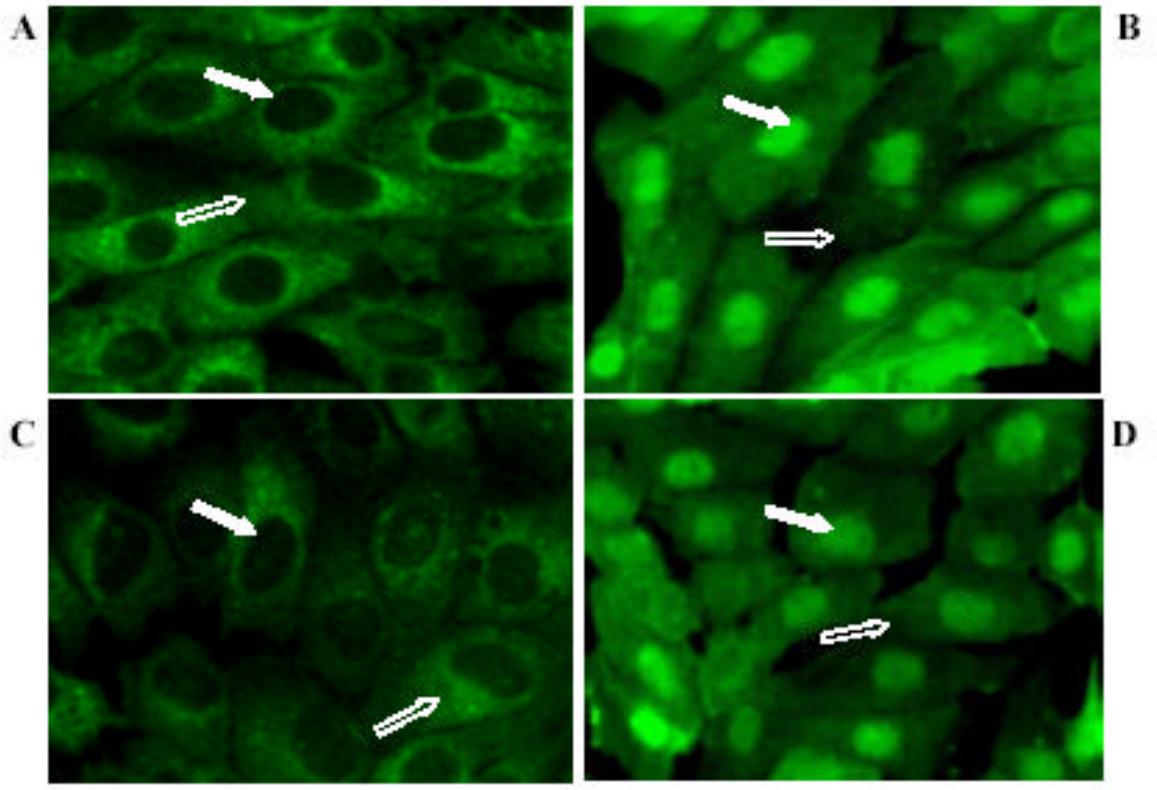

Figure 4.5. Immunofluorescent staining of NF-kB in LLC-PK$_1$ cells treated with FB$_1$ in the absence or presence of calphostin C or PMA. Untreated LLC-PK$_1$ cells display diffuse cytosolic staining (A), whereas intensified staining in the nucleus is observed in LLC-PK$_1$ cells treated with FB$_1$ (B) is indicative of NF-κB nuclear translocation. Calphostin C abrogated the FB$_1$-induced nuclear translocation of NF-κB and displayed diffuse cytosolic staining similar to untreated cells (C). An intensified nuclear staining indicative of NF-κB nuclear translocation was displayed in response to PMA alone (D). Representative photographs are shown. Bold arrows indicate nuclear staining and blank arrows indicate cytosolic staining.

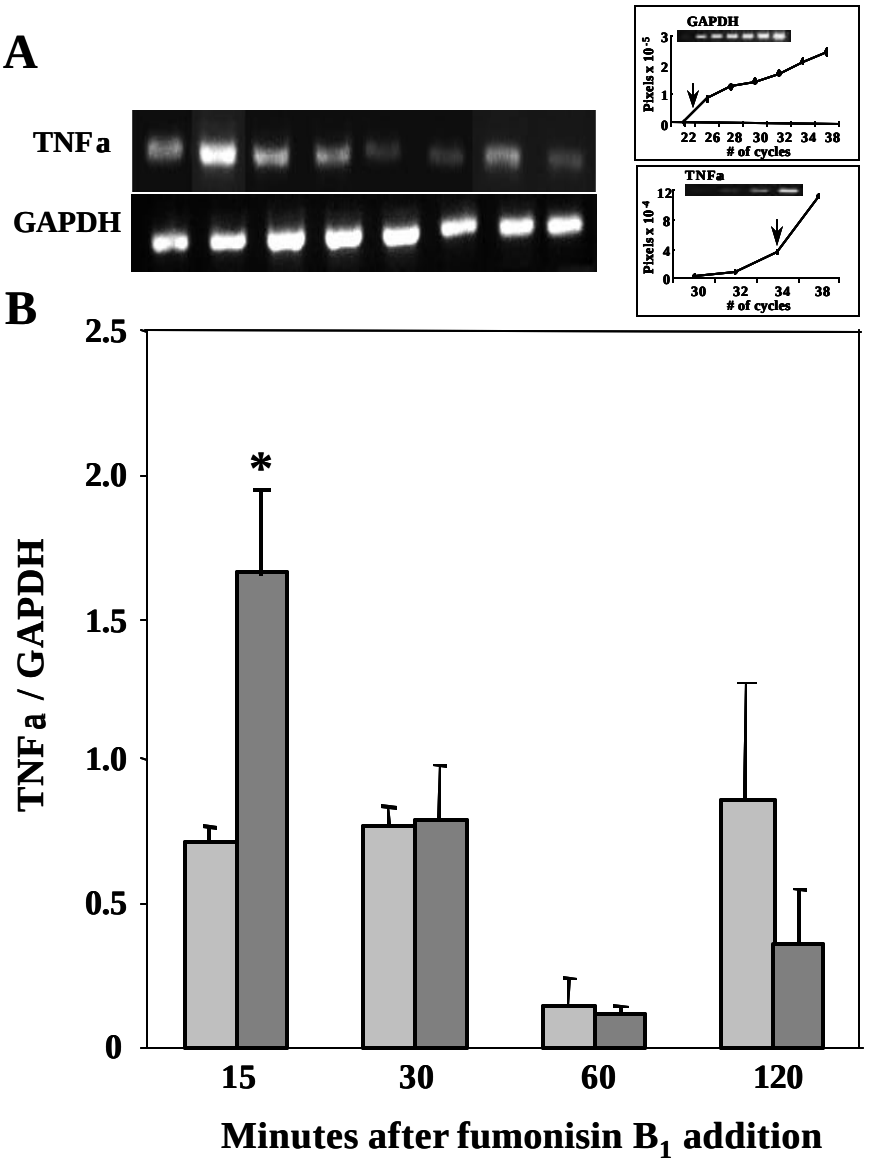

Figure 4.6. Temporal effect of FB_1 on TNFa mRNA expression in LLC-PK_1 cells. LLC-PK_1 cells were treated with FB_1 (1 μM) for the times indicated and RT-PCR performed. Representative gels are shown, as well as the use of 24 cycles for GAPDH and 32 cycles for TNFα (A). The photographs were scanned and bands were quantified and TNFα was normalized to the house keeping GAPDH (B). Values are mean ± S.E. (n=3). * Significantly different from the control at $P \leq 0.05$.

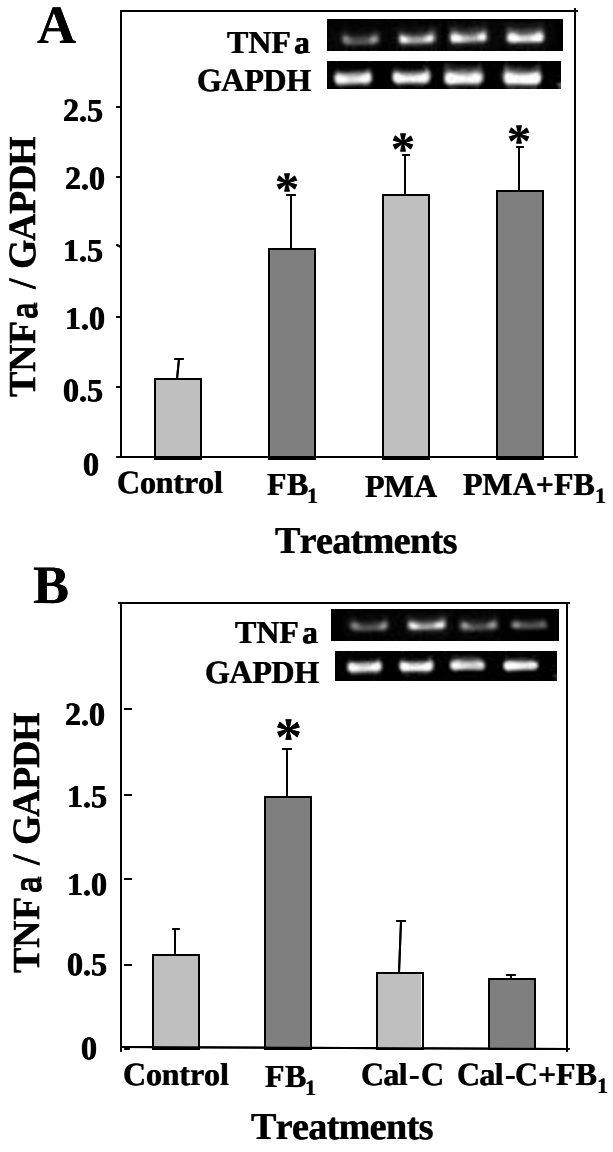

Figure 4.7. TNFa mRNA expression in LLC-PK$_1$ cells treated with PMA or calphostin C in the presence or absence of FB$_1$ for 15 min. LLC-PK$_1$ cells were preincubated with 0.01 μM PMA (A) or 100 nM calphostin C (Cal-C) (B) in the light prior to exposure to 1 μM FB$_1$ for 15 min and RT-PCR performed. Representative gels correspond to the order of the bars. Values are expressed as mean ± S.E. (n=3). * Significantly different from the control at $P \leq 0.05$.

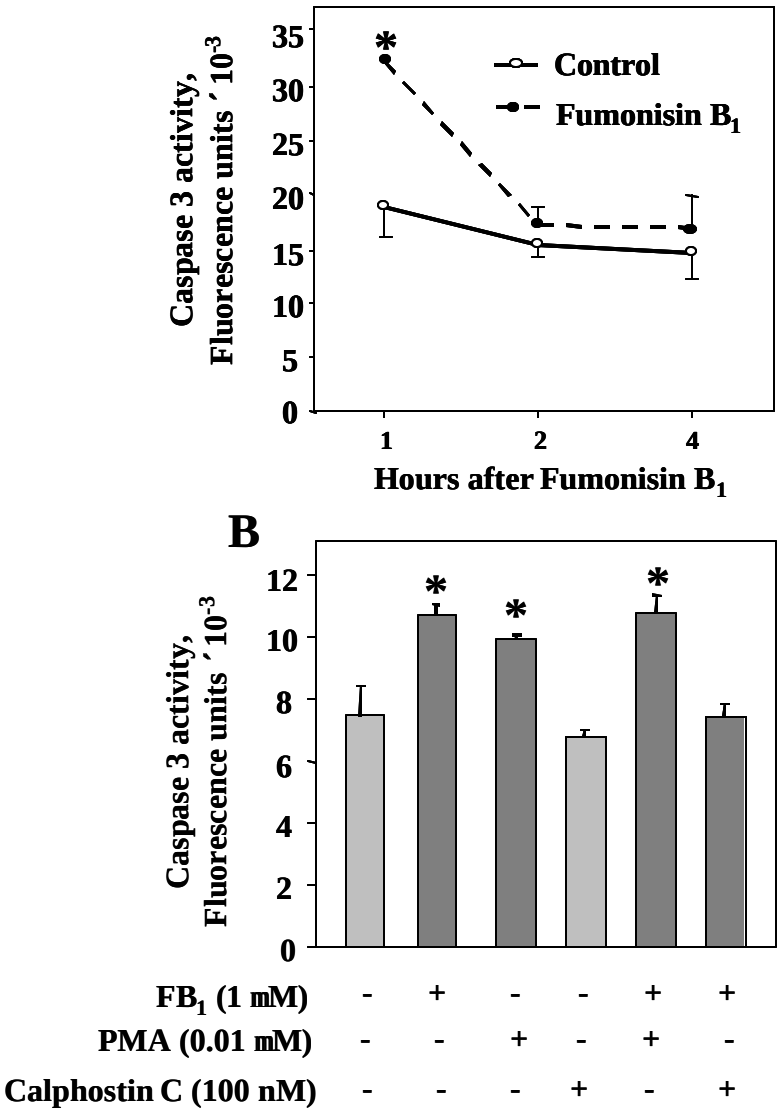

Figure 4.8. FB_1-induced increase in caspase 3-activity is prevented by calphostin C in LLC-PK_1 cells. Cells were treated with 1µM FB_1 at the times indicated and the fluorescence of cleaved substrate was determined (A). LLC-PK_1 cells were preincubated with 100 nM calphostin C for 60 min in the presence of light prior to the addition of 1 µM FB_1 for 1 h and assay for caspase 3-activity (B). Values are expressed as mean ± S.E. (n=3). * Significantly different from the control at $P \leq 0.05$.

DISCUSSION

The aim of this study was to determine the implications of transient activation of PKCα by FB_1 on induction of NF-κB, TNFα and caspase 3 in LLC-PK_1 cells. The results demonstrate that FB_1 (1 μM) transiently activates PKCα and NF-κB in LLC-PK_1 cells and this FB_1-induced activation of NF-κB appears to be PKC-dependent since the PKC inhibitor, calphostin C, prevented the effects of FB_1. In addition, activation of NF-κB by FB_1 induced TNFα mRNA expression by a probable PKC-dependent mechanism, since PMA induces TNFα expression, and calphostin C prevented the FB_1-induced TNFα expression. Subsequent to TNFα induction was an increase in caspase 3 activity which was also dependent upon PKCα activation by FB_1. The major conclusion to be drawn from these findings is that FB_1-induced NF-κB activation and subsequent TNFα expression and increase in caspase 3 activity in LLC-PK_1 cells is dependent on the selective and transient activation of PKCα in LLC-PK_1 cells.

The ability of FB_1 to alter signal transduction pathways of PKC can play a role in its ability to induce apoptosis and carcinogensis (*31*). The present study showed that FB_1 selectively and transiently activated PKCα in LLC-PK_1 cells. This finding is in agreement with Yeung and coworkers (*13*) who also showed that FB_1, by possibly binding to the diacylglycerol binding site of PKC, increased membrane translocation and activity of PKC in brain slices. Alternatively, cellular sphingolipids (sphinganine and sphingosine) and their metabolites (sphinganine- or sphingosine 1-phosphate) resulting from the inhibition of ceramide synthase by FB_1 are biologically active in modulating PKC activity (*32*) and are either mitogenic or apoptotic depending on the cell type and exposure conditions (*33-35*). The cytosol-to-membrane translocation of PKCα may

exposure conditions (*33-35*). The cytosol-to-membrane translocation of PKCα may represent critical steps in the induction of signaling cascade leading to the production of proinflammatory cytokines, including TNFα, which is known to be involved FB_1 toxicity (*36*). In addition, PKC is the major high-affinity intracellular receptor for phorbol esters, a class of potent tumor promoters (*37*) may mediate a variety of intracellular signaling establishing the basis of its involvement in multistage carcinogenesis (*38*).

NF-κB is activated by various members of the PKC family (*39*) and since FB_1 activates PKCα, we sought to determine whether FB_1 could also activate NF-κB in LLC-PK_1 cells. We found that FB_1 (1 μM) stimulated rapid and transient translocation of NF-κB at 5 min in LLC-PK_1 cells. Although this is the first study to demonstrate increase cytosolic to nuclear translocation and hence activation of NF-κB *in vitro* in response to FB_1, it has been reported that FB_1 increased cytosolic NF-κB in C56BL/6J (*9*) and in male transgenic mice expressing human TNFα gene (*10*).

The PKC inhibitor, calphostin C, prevented the FB_1 activation of NF-κB, further suggesting that FB_1 activates NF-κB via a PKC dependent-pathway. NF-κB activation is subject to several levels of control, involving phosphatase and kinase activity (*40*) and PMA is reported to activate NF-κB through direct activation of conventional PKC isoforms in several cell lines (*41, 42*). In LLC-PK_1 cells, albumin exposure induces a rapid increase in NF-κB protein activity in renal proximal tubule cells of different species via a protein kinase C-dependent pathway, and this albumin-induced increase in NF-κB DNA-binding activity was inhibited by bisindolylmaleimide, a protein kinase C inhibitor (*43*).

NF-κB is a prototypic transcription factor involved in the regulation of numerous genes (*44*) including the transcriptional activation of TNFα (*19*). The results of the present study demonstrate that FB_1 induces TNFα expression at 15 min in LLC-PK_1 cells. Induction of TNFα in response to FB_1 has been documented in LLC-PK_1 cells (*25*), in liver and in lipopolysaccharide-stimulated macrophages from mice (*9-12*). The FB_1-induced toxicity may be due to the induction of this potent pleiotropic proinflammatory cytokine as the *in vivo* hematological effects of FB_1 were partially reversed by anti-TNFα antibodies (*12*), and mouse strain lacking p75 or p55 TNFα receptor showed reduced hepatotoxicity following FB_1 treatment (*9, 11*).

We found that the increase in TNFα expression in response to FB_1 was prevented by calphostin C, whereas PMA significantly stimulated TNFα expression in LLC-PK_1 cells at 15 min, supporting the role of PKCα in the FB_1-induction of TNFα expression in LLC-PK_1 cells. In monocytes and macrophages, the induction of TNFα have been reported to be prevented by the use of PKC inhibitors in a number of cells (*45-48*). In addition, activation of PKC has also been shown to sequentially activate NF-κB and TNFα (*42, 47, 49, 50*).

Subsequent to TNFα induction at 15 min, an increase in caspase 3 activity was observed at 1 h in LLC-PK_1 cells. The increase in caspase 3 activity was dependent upon PKC activation of TNFα since calphostin C prevented the effects of FB_1. Transient activation of caspases by TNFα has been extensively documented (*51, 52*). NF-κB modulates TNFα and the modulation of TNFα-induced apoptosis by NF-κB has been

LLC-PK_1 cells. To our knowledge, this is the first study, which describes the PKC-dependent signal transduction pathway that may be involved in the FB_1-induced apoptosis in LLC-PK_1 cells.

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CHAPTER 5

FUMONISIN B_1-INDUCED APOPTOSIS IS ASSOCIATED WITH DELAYED INHIBITION OF PROTEIN KINASE C, NUCLEAR FACTOR-kB AND TUMOR NECROSIS FACTOR a IN LLC-PK_1 CELLS[1]

[1]Gopee, N.V., He, Q. and Sharma, R.P. Submitted to *Toxicological Sciences*, 11/14/02.

ABSTRACT

Fumonisin B_1, the most potent of the newly described fumonisin mycotoxins, is a carcinogen and causes a wide range of species-specific toxicoses. Fumonisin B_1 modulates the activity of protein kinase C (PKC), a family of phospholipid-dependent serine/threonine kinases that play important roles in modulating a variety of biologic responses ranging from regulation of cell growth to cell death. In this study, we investigated the mechanism of fumonisin B_1-induced cytotoxicity at 24, 48, and 72 h and related this to PKC activity and its downstream targets, namely nuclear factor-kappa B (NF-κB) and tumor necrosis factor α (TNFα) in porcine renal epithelial (LLC-PK_1) cells. In cells exposed to > 1 μ M fumonisin B_1, viability was decreased, due in part to apoptosis as demonstrated by an increase in caspase 3 activity and nuclear fragmentation. Increasing concentrations of fumonisin B_1 repressed cytosolic to membrane translocation of PKC-α, -δ, -ε, and -ζ isoforms at 24, 48, and 72 h. The fumonisin B_1-induced PKC membrane protein repression was corroborated by a concentration-dependent decrease in total PKC activity. Exposure to fumonisin B_1 (1-50 μM) was also associated with a concentration-dependent inhibition of NF-κB nuclear translocation and TNFα gene expression at 24, 48, and 72 h. At all time points, intracellular free sphinganine and sphingosine concentrations were elevated upon exposure to fumonisin B_1 in a concentration-dependent manner. These results demonstrate that fumonisin B_1-induced apoptosis is associated with repression of PKC isoforms, NF-κB and TNFα, events that may be involved in the toxicity of this mycotoxin.

Keywords: fumonisin B_1; protein kinase C; nuclear factor-kappa B; tumor necrosis factor α; caspase 3

INTRODUCTION

Corn, overtly healthy or diseased, harbors the endophytic fungus *Fusarium verticillioides* (Stockenstrom *et al.*, 1998). Fumonisin B_1, the most potent of the newly described fumonisin mycotoxins, is the causative agent of the species-specific toxicoses, equine leukoencephalomalacia (Marasas *et al.*, 1988) and porcine pulmonary edema (Harrison *et al.*, 1990). Fumonisin B_1 is carcinogenic in laboratory animals (Howard *et al.*, 2001) and a suspected human carcinogen, being linked to a higher incidence of esophageal cancer in rural areas of South Africa and China (Sydenham *et al.*, 2000).

Fumonisin B_1 is structurally similar to the sphingolipids, sphingosine and sphinganine (Wang *et al*., 1991) and is believed to evoke its toxicity by blocking the enzyme, *N*-acyltransferase (ceramide synthase), which converts sphinganine (or sphingosine) and fatty acyl CoA to ceramide (Norred *et al.*, 1999). Disruption of sphingolipid metabolism is an early event that is closely correlated with alterations in cell proliferation and increased cell death in most primary cell cultures and cell lines examined (Riley *et al.*, 1998). In addition, tumor necrosis factor α (TNFα) is shown to play an important role in fumonisin B_1 toxicity *in vivo* and *in vitro* (Dugyala *et al.*, 1998; Sharma *et al.*, 2000a,b, 2001).

Protein kinase C (PKC) represents a family of more than 12 phospholipid-dependent serine/threonine kinases, modulating a variety of biologic responses ranging from regulation of cell growth to cell death (Lee *et al.*, 2000). The PKC family consists of calcium (Ca^{2+})-dependent conventional (cPKCα, $\beta_{I/II}$, and γ), Ca^{2+}-independent or novel (nPKCδ, ε, θ, η and μ) and Ca^{2+} and dioleoyl-*sn*-glycerol (DAG)-independent or atypical (aPKCζ, ι and λ) isoenzymes (Mellor and Parker, 1998). Activation of PKC usually

results in the translocation of the enzyme from cytoplasm to plasma membrane and this is often followed by the rapid cleavage and down-regulation of this protein (Pontremoli *et al.*, 1990).

Activation of PKC results in the phosphorylation and activation of downstream effectors; including the transcription factor nuclear factor-kappa B (NF-κB) (Kontny *et al.*, 2000). When activated NF-κB translocates from the cytosol into the nucleus where it binds to a NF-κB motif and functions as a transcription factor (Kang *et al*., 2000), regulating the gene expression of various cytokines, chemokines and growth factors (Barnes and Karin, 1997), including TNFα (Aggarwal *et al.*, 1996). The pathogenic effects fumonisin B_1 are paradoxical and involve stimulation of mitogenesis or induction of apoptosis (Riley *et al.*, 2001). *In vitro* and *in vivo,* cells exposed to fumonisin B_1 have undergone a mixture of necrotic and apoptotic cell death (Tolleson *et al.*, 1996; Voss *et al.*, 1996, Sharma *et al.*, 1997), which may be followed by compensatory regeneration (Howard *et al*., 2001). Caspases are a group of cysteine proteases that play a critical role in the execution of apoptosis (Koriyama *et al.*, 1999; Au *et al.*, 1997).

Sphingolipids are potent and reversible modulators of PKC *in vitro* and in cell systems (Hannun *et al.*, 1986; Hannun and Bell, 1987). Moreover, Huang and coworkers (1995) have demonstrated that exposure of African green monkey kidney cells for 3 or 16 h resulted in dose-dependent repression of PKC. In contrast, Yeung *et al.* (1996) demonstrated fumonisin B_1 activated PKCγ at 20 min in brain slices. However, the effect of long-term fumonisin B_1 exposure to increasing concentrations of fumonisin B_1 on PKC isoforms and its downstream targets namely NF-κB and TNFα in porcine renal epithelial (LLC-PK_1) cells have not been addressed.

Porcine renal epithelial, LLC-PK_1, cells are sensitive to the fumonisin B_1-induced disruption of sphingolipid biosynthesis (He *et al.*, 2001; Yoo *et al.*, 1996a) and cytotoxicity (Yoo *et al.*, 1996a). In addition, we have observed that 1 μM fumonisin B_1 selectively and transiently activated PKCα at 5 min with repression at later time points and higher fumonisin B_1 concentrations (unpublished data), in addition to induction of TNFα expression (He *et al.*, 2001) via a PKC-dependent pathway (unpublished data). The hypothesis driving this study was that the fumonisin B_1–induced apoptosis is associated with repression of PKC isoforms, NF-κB and TNFα. The aims of the present study were to: (1) to determine whether caspase 3 and apoptosis are involved in the cytotoxicity of fumonisin B_1 at 24, 48, and 72 h, (2) to explore and identify the PKC isoforms affected following exposure to varying concentrations of fumonisin B_1 and (3) to relate the fumonsin B_1-induced alterations in PKC to downstream signal transduction pathways, namely NF-κB and TNFα, in LLC-PK_1 cells.

MATERIALS AND METHODS

Materials. Fumonisin B_1 (> 98% purity) was purchased from Programme on Mycotoxins and Experimental Carcinogenesis (PROMEC, Tygerberg, South Africa). Horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibody was purchased from Sigma (St. Louis, MO). Primary mouse polyclonal anti-PKCα, rabbit polyclonal anti-PKCδ, rabbit polyclonal anti-PKCε, mouse anti-NF-κB p65 subunit and HRP donkey anti-rabbit secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Primary anti-PKCζ antibody was procured from Upstate Biotechnology (Lake Placid, NY).

Cell culture. Porcine renal epithelial, LLC-PK_1, cells (American Type Culture Collection, Rockville, MD; CRL 1392, passage 197) were cultured in Dulbecco's modified Eagle's Medium/ Ham's F12 (1:1), supplemented with 5% fetal bovine serum in a 5% CO_2-humidified incubator at 37°C. LLC-PK_1 cell number and viability were assessed by trypan blue exclusion using a hematocytometer. Cells were subcultured at approximately 5 x 10^5 viable cells in 100 x 15 mm sterile petri dishes and allowed to attach and grow for 72 h prior to appropriate treatment. To prevent the burst of free sphingoid bases after addition of fresh medium (Smith *et al.*, 1997), 0.1 ml medium from each well aspirated, and 0.1 ml of appropriate chemical diluted in medium was added.

MTT assay for cell viability. Cell viability was determined using 3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay as described by Mossman *et al.* (1983). Briefly, LLC-PK_1 cells were seeded in 96-well plates at a density of ~1,000 cells per well. The cultures were grown for 72 h, prior to addition of varying concentrations of fumonisin B_1 (1-50 μM) for 24, 48, and 72 h. MTT solution (20 μl of 5 mg/ml in medium) was added to the 96-well plates 4 h prior to the respective endpoints and incubated at 37 °C. Following aspiration of 150 μl of the medium, the cell and dye crystals were solubilized by adding 100 μ1 of HCl-isopropanol and the absorption was measured at 570 nm.

Measurement of cell proliferation by [^{3}H]thymidine incorporation. Cells were seeded in 96-well microtiter plates at a density of ~1,000 cells per well. The cultures were grown for 72 h, prior to addition of increasing concentrations of fumonisin B_1 (1-50 μM) for 24, 48, and 72 h. Each well was pulsed with 20 μl of [methyl-^{3}H]thymidine (25

μCi/ml, 6.7 Ci/mmol, DuPont NEN products, Boston, MA) 6 h prior to the 24, 48, and 72 h endpoints. Following incubation, cells were harvested onto glass fiber filter strips (Cambridge Technology, Watertown, MA) using a cell harvester (PHD, Cambridge Technology) and radioactivity was counted using a liquid scintillation counter. Proliferative responses were represented as disintegrations per minute (DPM).

Quantitation of intracellular free sphingoid bases. Free sphinganine and sphingosine concentrations were determined by the method described by He *et al.* (2001). Briefly, cells were washed once with ice-cold PBS and then were scraped into 1 ml ice-cold PBS. An aliquot (0.1 ml) of cell suspension in PBS was transferred to another tube, spun at 2000 X *g*, 4°C for 5 min. To obtain total protein, the cell pellet was solubilized in lysis buffer (20 mmol/L Tris-HCl, pH 7.4, 1 mmol/L EGTA, 1mmol/L DTT, 1 mmol/L PMSF, 0.2 mmol/L leupeptin, and 10 μg/mL aprotinin) and the lysates centrifuged for 5 min at 600 × *g*. Protein concentrations in each sample were determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA) with bovine serum albumin (Sigma, St. Louis, MO) as a standard. Free sphingoid bases were extracted from the remainder of cells by using the modified method of Yoo *et al.* (1996b). The relative amounts of free sphinganine and sphingosine in base-treated cell extracts were determined by high-performance liquid chromatography (HPLC) utilizing a modification (Yoo *et al.*, 1996b) of the method originally described by Merrill *et al.* (1988). Sphingoid bases were quantitated based on the recovery of a C_{20} sphinganine internal standard. The instrument limit of detection for C_{20} was 26.8 fmol/assay (equivalent to 1 fmol/mg protein).

Subcellular fractionation for PKC and NF-kB. The procedure for cytosolic and membrane protein extraction for PKC was performed as described by Clarke *et al*. (2000), with centrifugation at 42,000 × g for 2 h as a modification. To obtain cell cytosol and nuclear fractions for NF-κB, cells were washed 3 times in cold phosphate-buffered saline (PBS) and scraped into 100 µl of lysis buffer A (0.6% IGEPAL, 150 mM NaCl, 10 mM HEPES, 1 mM EDTA, 0.5 mM PMSF, 0.5 mM dithiothreitol (DTT), 5 µg/ml aprotinin, 5 µg/ml leupeptin, 1 µM sodium orthovanadate). Samples were sonicated and incubated for 5 min on ice while shaking prior to centrifugation at 5,000 rpm for 5 min at 4°C. The supernatant was collected ('cytoplasmic fraction') and stored at -80°C. To the pellet, 30 µl of lysis buffer B (25% glycerol, 0.5 mM DTT, 20 mM HEPES, 420 mM NaCl, 1.2 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF, 5 µg/ml leupeptin, 5 µg/ml aprotinin) was added and incubate on ice while shaking for 20 min. Samples were centrifuged at 13,000 × *g* for 15 min and the supernatant ('nuclear fraction') stored at -80°C.

Western blot analysis for PKC and NF-kB translocation. Protein concentrations were determined as described above using the Bio-Rad assay. Proteins were fractionated on 8% gels and then electrophoretically transferred to a nitrocellulose membrane followed by blocking in 5% milk protein for 1 h prior to incubating with appropriate primary and secondary antibodies. Protein bands were visualized utilizing Pierce Super Signal® chemiluminescent substrate (Pierce, Rockford, IL) on film (Kodak X-OMAT AR, Rochester, NY). Images were acquired with a scanner and analyzed with UN-SCAN-IT software (Silk Scientific Inc., Orem, UT). Following visualization,

membranes were washed, stripped utilizing Pierce Restore™ Western Blot Stripping Buffer (Pierce, Rockford, IL), blocked and reprobed with appropriate primary and secondary antibodies and digitized as above.

Assay for PKC activity. Total protein was obtained and their concentrations were determined as described. The lysates were sonicated on ice and centrifuged for 5 min at 600 × *g* and the supernatants were used for the PepTag® assay for non-radioactive detection of protein kinase C (Promega Corp., Madison, WI). The amount of protein added to the kinase assay was standardized Bio-Rad protein assay. The fluorescence of phosphorylated substrate was determined using a Spectramax Gemini fluorescent plate reader (Molecular Devices, Irvine, CA). The fluorescence signal was digitized and analyzed using SoftMax Pro™ (version 3.1.1, Molecular Devices, Irvine, CA).

Semiquantitative analysis of TNFα mRNA by reverse transcriptase polmerase chain reaction (RT–PCR). This procedure was conducted according to He *et al*. (2001). Total RNA was isolated from LLC-PK_1 cells using TRI reagent (Molecular Research Center, Cincinnati, OH), cDNA was synthesized and amplified by PCR. PCR reactions were performed in a thermal cycler (Coy Inc., Ann Arbor, MI). The thermoamplification program consisted of an initial denaturation (5 min at 95°C), followed by 32 cycles (for TNFα) or 24 cycles (for GAPDH) of 30 s denaturation (94°C), 30 s annealing (50°C), and 1 min elongation (72°C), with a final extension period of 1 min at 72°C. For relative measurements, the numbers of cycles within a linear increase in the both products were selected (as indicated by arrows in Fig. 9A). The sense and antisense primers were 5'-AAT GGC AGA GTG GGT ATG-3' and 5'-CTT GAT GGC AGA GAG GAG-3' for

TNFα, and 5'-TCC CTG CTT CTA CTG GTG CT-3', and 5'-TGA GCT TGA CAA AGT GGT CG-3' for GAPDH, respectively (chosen by Primer3 program; Whithead Institute, Cambridge, MA). PCR products were separated on 2% agarose gel containing 0.476 mM ethidium bromide and detected by a UV transilluminator (Ultra Lum Inc., Carson, CA) and photographed. The photographs were scanned and bands were quantified by using UN-SCAN-IT software (Silk Scientific Inc., Orem, UT). TNFα was normalized to GAPDH, the housekeeping gene.

Determination of apoptosis by caspase 3 activity and Hoechst staining nuclear morphology. Caspase 3 activity was determined using the CaspACE™ fluorometric activity assay (Promega Corporation, Madison, WI). Briefly, cells were treated in 24 wells following which Triton X-100 was added and repeatedly pipetted to lyse the cells. The homogenates were centrifuged at 10,000 $\times$ *g* for 10 min to remove cell debris. The supernatant was assayed for caspase 3 activity by measuring the fluorescence of cleaved substrate using Spectramax Gemini fluorescent plate reader (Molecular Devices, Irvine, CA). The fluorescence signal was digitized and analyzed using SoftMax Pro™ (version 3.1.1, Molecular Devices, Irvine, CA). Nuclear morphology for detection of apoptotic bodies was determined using epifluorescence following staining with Hoechst 33258 (H33258) (Sigma, St. Louis, MO). Cells were plated at a density of 3,000 cells/cm^2 in 24-well plates. Following 24, 48, and 72 h exposure, cells were stained with H33458 (10 μg/ml in PBS) for 5 min and fluorescence microscopy was performed using the Olympus IX71 inverted microscope (Olympus America Inc., Melville, NY). Digital images were acquired using the Magnafire SP® digital camera.

Statistical analysis. The results were expressed as mean ± standard error. Differences among the various treatment groups were determined by one-way analysis of variance followed by Duncan's multiple range test utilizing Version 8 of the Statistical Analysis Software (SAS). The level of $P \leq 0.05$ was taken to denote statistical significance.

RESULTS

Fumonisin B_1 is cytotoxic to LLC-PK_1 cells in a concentration-dependant manner at 24, 48, and 72 h. LLC-PK_1 cell injury in response to increasing concentrations of fumonisin B_1 was estimated by examining cellular metabolism using the MTT assay. Overall, there was a concentration-dependent reduction in cell viability and proliferative capacity over time. At all time points tested, fumonisin B_1 concentrations ≥ 10 μM significantly decreased cell viability (Fig. 5.1A). The reduction in viability was associated with a decrease in the proliferative capacity of these cells at 24 and 48 h at fumonisin B_1 concentration of 50 μM (Fig. 5.1B), confirming the cytotoxicity of fumonisin B_1 and sensitivity of LLC-PK_1 cells to increasing concentrations of fumonisin B_1 at 24, 48, and 72 h.

Fumonisin B_1 increased intracellular sphinganine and sphingosine concentrations. Intracellular sphinganine and sphingosine concentrations were increased in a concentration-dependent manner on exposure to fumonisin B_1 concentrations of > 1μM at 24, 48, and 72 h (Fig. 5.2A). At 72 h, intracellular sphinganine concentrations were significantly increased at fumonisin B_1 concentrations of ≥ 10 μM. However, at 24 and 48 h sphinganine concentrations were significantly increased exclusively at ≥ 50 μM fumonisin B_1. To a lesser extent, intracellular sphingosine concentrations were increased

in a concentration-dependent manner on exposure to increasing concentrations of fumonisin B_1 (Fig. 5.2B). Fumonisin B_1 concentrations ≥ 10 μM significantly increased intracellular sphingosine concentrations at 24, and 48, and 72 h.

Concentration-dependent repression of PKC isoforms by fumonisin B_1 in LLC-PK_1 cells. Immunoblot analyses were conducted using cytosolic and membrane fractions prepared from LLC-PK_1 cells since the cytosolic to membrane redistribution of PKC upon stimulation represents an important step in the activation of this enzyme (Kraft and Anderson, 1983). Overall, treatment of LLC-PK_1 cells with increasing concentrations of fumonisin B_1 resulted in a concentration-dependent increase in the cytsolic fraction and a concomitant decrease in the particulate fraction of PKC-α, -δ, -ε, and -ζ isoforms at 24, 48, and 72 h (Fig. 5.3-5.6).

A significant increase in the PKCα (Fig. 5.3) cytosolic fraction compared to the untreated control was observed at fumonisin B_1 concentrations ≥10 μM at 24 h and at 50 μM at all time points tested. The membrane protein concentration was significantly decreased on exposure to all concentrations of fumonisin B_1 at 24 h and 1 and 10 μM at 48 h, respectively. For PKCδ (Fig. 5.4), a significant increase in the cytosolic fraction was observed at 48 and 72 h on exposure to fumonisin B_1 concentrations ≥10 μM and ≥ 1 μM, respectively. Concomitant with the increase in cytosolic PKCδ was a significant reduction in membrane protein levels at fumonisin B_1 concentrations ≥ 10 μM at 24 and 48 h.

In contrast to the other PKC isoforms tested, PKCε was predominantly present in the membrane fraction of treated and untreated LLC-PK_1 cells (Fig. 5.5). There was an

overall increase in cytosolic protein and a concomitant decrease in membrane protein PKCε concentrations at 24 h, 48 h and 72 h. A significant decrease in the membrane protein concentration at ≥ 1 μM fumonisin B_1 was accompanied by increases, although not significant, in cytosolic protein levels at 24 h. The decrease in PKCε membrane protein concentration on exposure to 10 μM fumonisin B_1 and the increase in cytosolic PKCε protein level at 50 μM fumonisin B_1 at 48 h, were both statistically significant. Overall, PKCζ cytosolic protein levels were accompanied by a concurrent decrease in membrane protein expression at 24, 48, and 72 h (Fig. 5.6). A significant increase in cytosolic protein levels following exposure fumonisin B_1 concentrations ≥ 1 μM at 24 h was associated with significant reduction in membrane protein levels at fumonisin B_1 concentrations ≥ 10 μM. Membrane protein concentration for this isoform was significantly reduced at 48 and 72 h following exposure to ≥ 10 μM fumonisin B_1, whereas the increment in cytosolic protein of PKCζ was statistically significant on exposure to 50 μM fumonisin B_1 at 48 h exclusively.

Fumonisin B_1-induced PKC protein repression in membrane translocation was correlated with a concentration-dependent reduction in PKC activity. Expression level and activity may or may not correlate, as isoforms can be present in an inactive form. In order to determine whether the changes in PKC distribution corresponded to altered enzyme activity, total PKC activity using the PepTag® assay for non-radioactive detection of protein kinase C was measured. In agreement with the above findings there was a concentration-dependent decrease in PKC on exposure to ≥ 1 μM fumonisin B_1 at 24, 48, and 72 h (Fig. 5.7). This finding confirms that the repression in the cytosolic to

membrane translocation of PKC isoforms in response to fumonisin B_1 positively correlates with a comparative decrease in the enzyme's activity in LLC-PK_1 cells.

Fumonisin B_1 induces a concentration-dependent decrease in NF-kB nuclear translocation. NF-κB lies downstream of PKC and PKC is known to regulate the activation of NF-κB, we investigated the effects of fumonisin B_1 on NF-κB activation. Since all concentrations of fumonisin B_1 used repressed PKC, we tested the effects of 1 and 10 μM fumonisin B_1 on NF-κB subcellular redistribution in LLC-PK_1 cells. Increasing concentrations of fumonisin B_1 resulted in a concentration-dependent decrease in NF-κB nuclear protein concentration and a concomitant increase in the cytosolic fraction at all time points tested (Fig. 5.8). At 24 h, exposure to fumonisin B_1 concentrations of ≥ 1 μM resulted in significant increase in cytosolic NF-κB protein levels at 24 h, whereas only 10 μM fumonisin B_1 concentrations significantly increased cytosolic fraction at 48 and 72 h. NF-κB nuclear protein levels were significantly decreased on exposure to 10 μM fumonisin B_1 at 72 h.

Fumonisin B_1 induced a concentration-dependent decrease in TNFα mRNA expression. Fumonisin B_1 (1-10 μM) resulted in a concentration-dependent inhibition of TNFα mRNA expression in LLC-PK_1 cells at 24, 48, and 72 h (Fig. 5.9). At 24 and 48 h, TNFα expression was significantly inhibited at both fumonisin B_1 concentrations used.

Fumonisin B_1 activated caspase 3 in LLC-PK_1 cells. Caspase 3 activity increased in a concentration-dependent manner in response at 24, 48, and 72 h (Fig. 5.10). However, a significant increase in caspase 3 activity was observed only on exposure to ≥ 1 μM

fumonisin B_1 at 48 h. The reversible caspase 3 inhibitor, Ac-DEVD-CHO™ (Promega), reversed the effects of fumonisin B_1 where the enzyme's activity fell to untreated levels (data not shown), indicating the specificity of inhibition of caspase 3 by fumonisin B_1.

Fumonisin B_1 induced nuclear fragmentation in LLC-PK$_1$ cells, indicative of apoptosis. The nuclear morphology of cells exposed to fumonisin B_1 concentrations ≥ 10 μM displayed increased number of cells stained with H33458 (Fig. 5.11), mainly at 48 and 72 h. In addition, fumonisin B_1 (≥10 μM) increased the number of fragmented nuclei, a hallmark of apoptosis, notably at 48 and 72 h (Fig. 5.11).

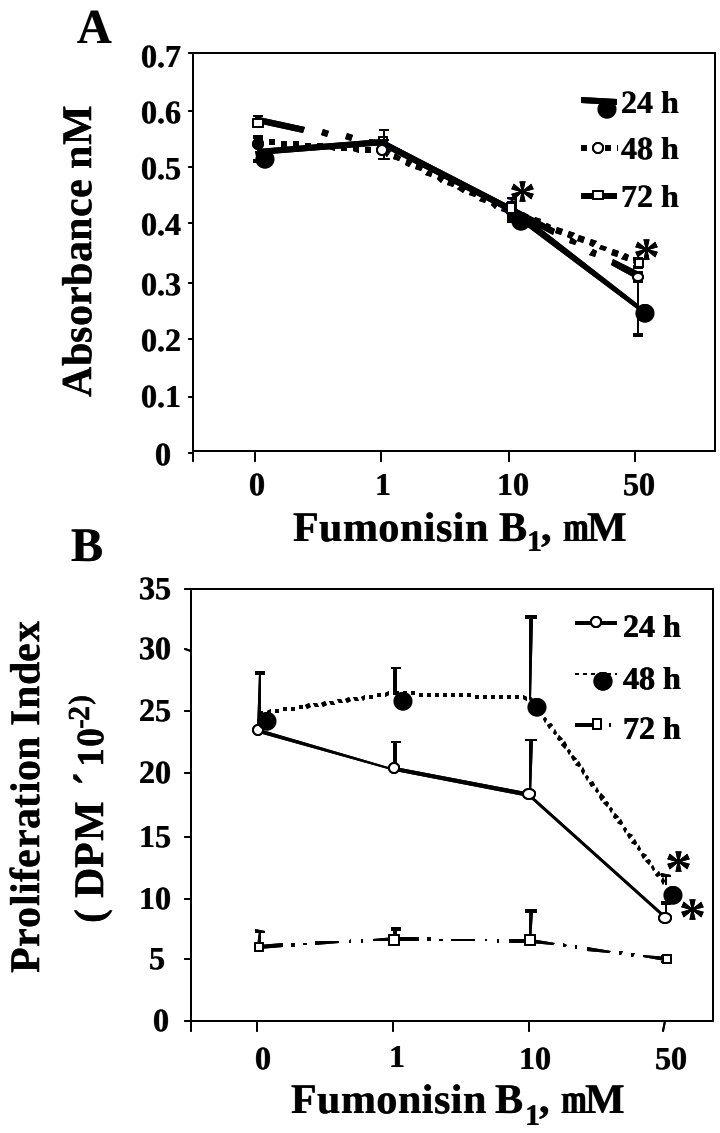

Figure 5.1. The effects of fumonisin B_1 on cell viability and proliferation in LLC-PK_1 cells. LLC-PK_1 cells were added to 96-well microtiter plates and exposed to increasing concentrations of fumonisin B_1 (1-50 μM) for 24, 48, and 72 h. Cell viability was determined by MTT assay (A). For proliferation, cells were pulsed with 20 μl of [methyl-^{3}H]thymidine for 6 h prior to the respective endpoints (B). Results are representative of 3 independent experiments, the mean ± standard error (n=3) are presented. *Significantly different from the respective control at $P \leq 0.05$.

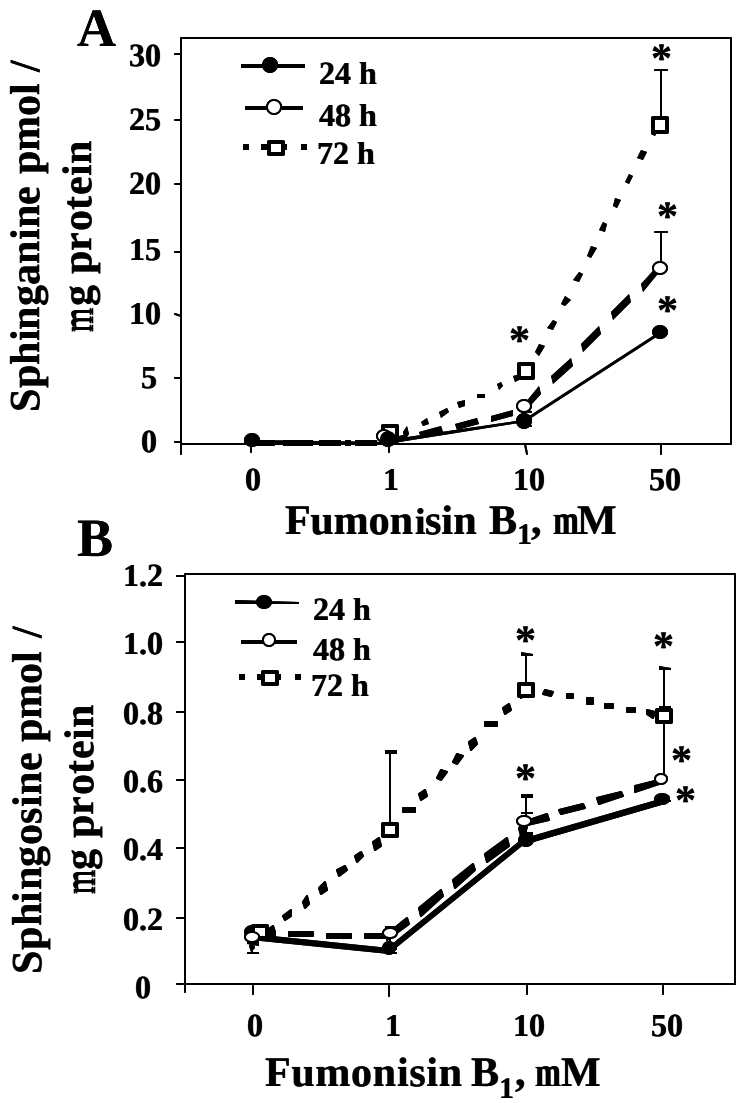

Figure 5.2. Fumonisin B_1 increases intracellular sphinganine and sphingosine in LLC-PK_1 cells. Cells were grown for 72 h prior to exposure to increasing concentrations of fumonisin B_1 (1-50 μM). At the times indicated, HPLC was performed as described in ′Materials and Methods′ to determine the intracellular sphinganine (A) and sphingosine (B) concentrations. Results are representative of 3 independent experiments, the mean ± standard error (n=3) are presented. *Significantly different from the respective control at $P \leq 0.05$.

Figure 5.3. Fumonisin B_1 inhibits cytosol to membrane translocation of PKCa in LLC-PK_1 cells. Cells were grown for 72 h and exposed to fumonisin B_1 (1-50 μM) at the times indicated. Cell lysates were obtained and 10 μg of protein was resolved on an 8% gel, electroblotted to a nitrocellulose membrane and visualized autoradiographically using chemiluminescent reagents. Results are representative of 3 independent experiments, the mean ± standard error (n=3) are presented. *Significantly different from the respective control at $P \leq 0.05$. Representative PKCα cytosolic and membrane protein concentrations at 24 (A), 48 (B) and 72 h (C) are displayed.

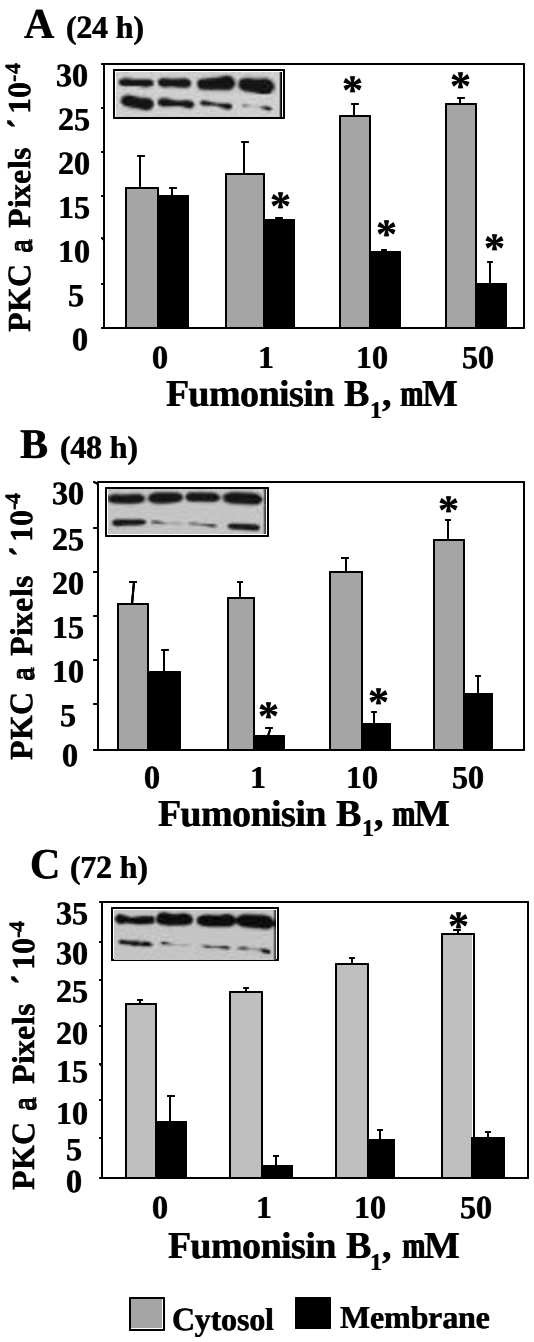

Figure 5.3. Fumonisin B_1 inhibits cytosol to membrane translocation of PKCa in LLC-PK_1 cells.

Figure 5.4. Fumonisin B_1 inhibits cytosol to membrane translocation of PKCd in LLC-PK_1 cells. Cells were grown for 72 h and exposed to fumonisin B_1 (1-50 μM) at the times indicated. Cell lysates were obtained and 10 μg of protein was resolved on an 8% gel, electroblotted to a nitrocellulose membrane and visualized autoradiographically using chemiluminescent reagents. Results are representative of 3 independent experiments, the mean ± standard error (n=3) are presented. *Significantly different from the respective control at $P \leq 0.05$. Representative cytosolic and membrane protein concentrations for PKCδ at 24 (A), 48 (B) and 72 h (C) are displayed.

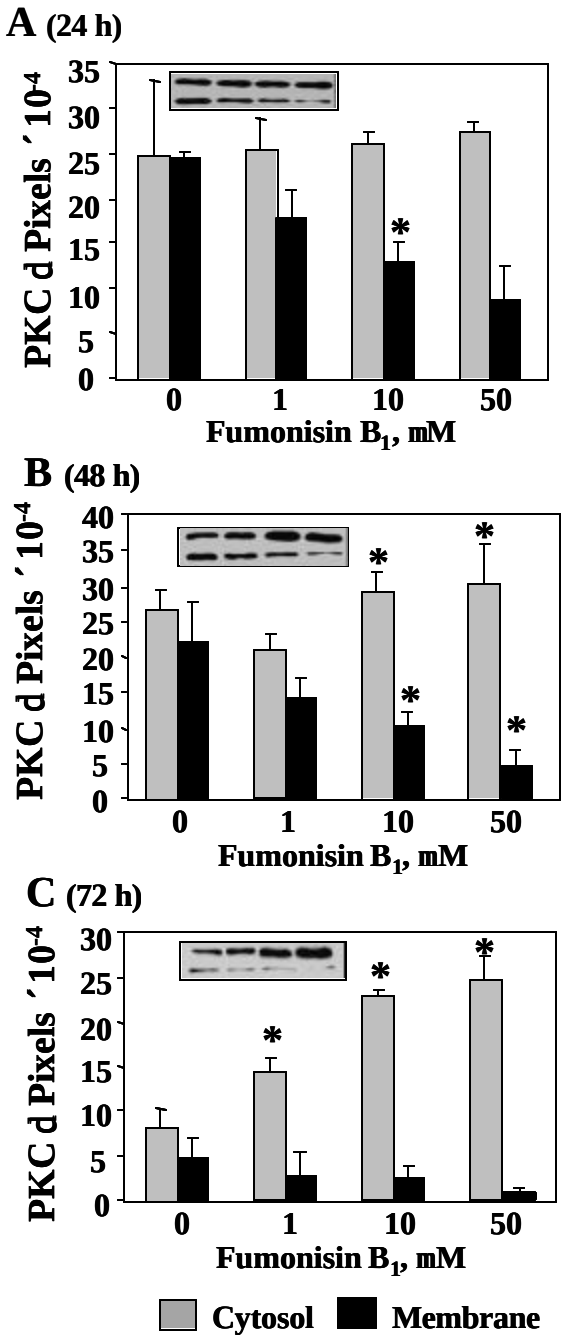

Figure 5.4. Fumonisin B_1 inhibits cytosol to membrane translocation of PKCd in LLC-PK_1 cells.

Figure 5.5. Fumonisin B_1 inhibits cytosol to membrane translocation of PKCe in LLC-PK_1 cells. LLC-PK_1 cells were grown for 72 h and exposed to fumonisin B_1 (1-50 μM) at the times indicated. Cell lysates were obtained and 10 μg of protein was resolved on an 8% gel, electroblotted to a nitrocellulose membrane and visualized autoradiographically using chemiluminescent reagents. Results are representative of 3 independent experiments, the mean ± standard error (n=3) are presented. *Significantly different from the respective control at $P \leq 0.05$. Representative cytosolic and membrane protein concentrations for PKCε at 24 (A), 48 (B) and 72 h (C) are displayed.

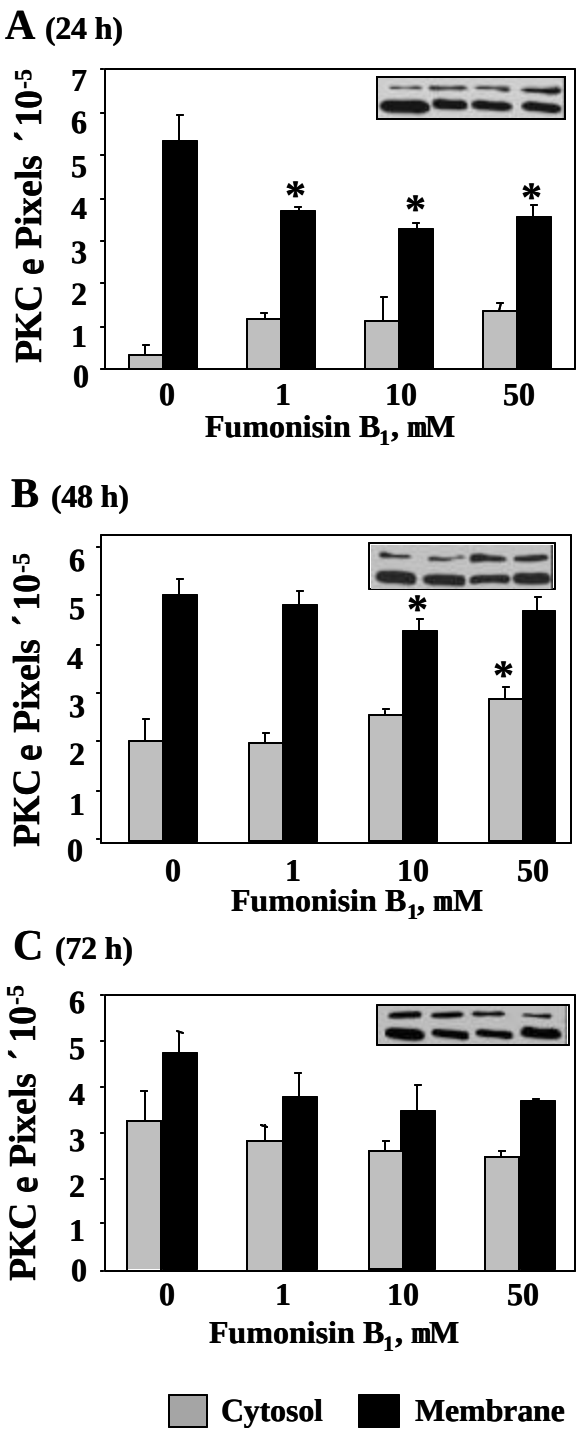

Figure 5.5. Fumonisin B_1 inhibits cytosol to membrane translocation of PKCe in LLC-PK_1 cells.

Figure 5.6. Fumonisin B_1 inhibits cytosol to membrane translocation of PKCz in LLC-PK_1 cells. LLC-PK_1 cells were grown for 72 h and exposed to fumonisin B_1 (1-50 μM) at the times indicated. Cell lysates were obtained and 10 μg of protein was resolved on an 8% gel, electroblotted to a nitrocellulose membrane and visualized autoradiographically using chemiluminescent reagents. Results are representative of 3 independent experiments, the mean ± standard error (n=3) are presented. *Significantly different from the respective control at $P \leq 0.05$. Representative cytosolic and membrane protein concentrations for PKCζ at 24 (A), 48 (B2) and 72 h (C) are displayed.

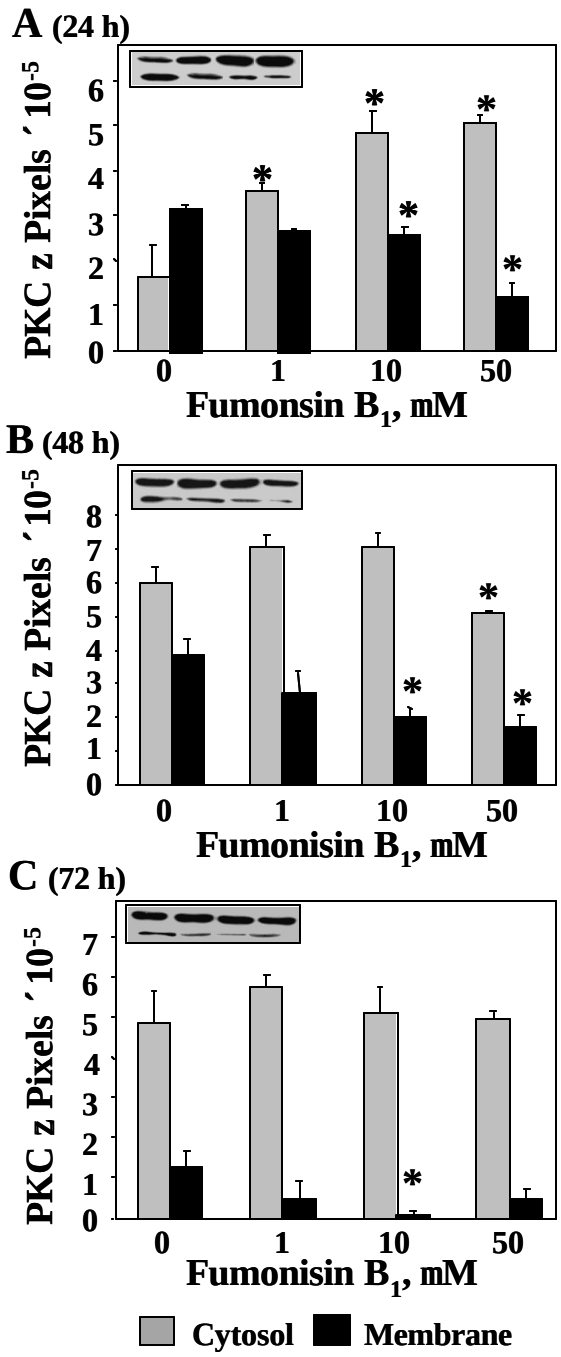

Figure 5.6. Fumonisin B_1 inhibits cytosol to membrane translocation of PKCz in LLC-PK_1 cells.

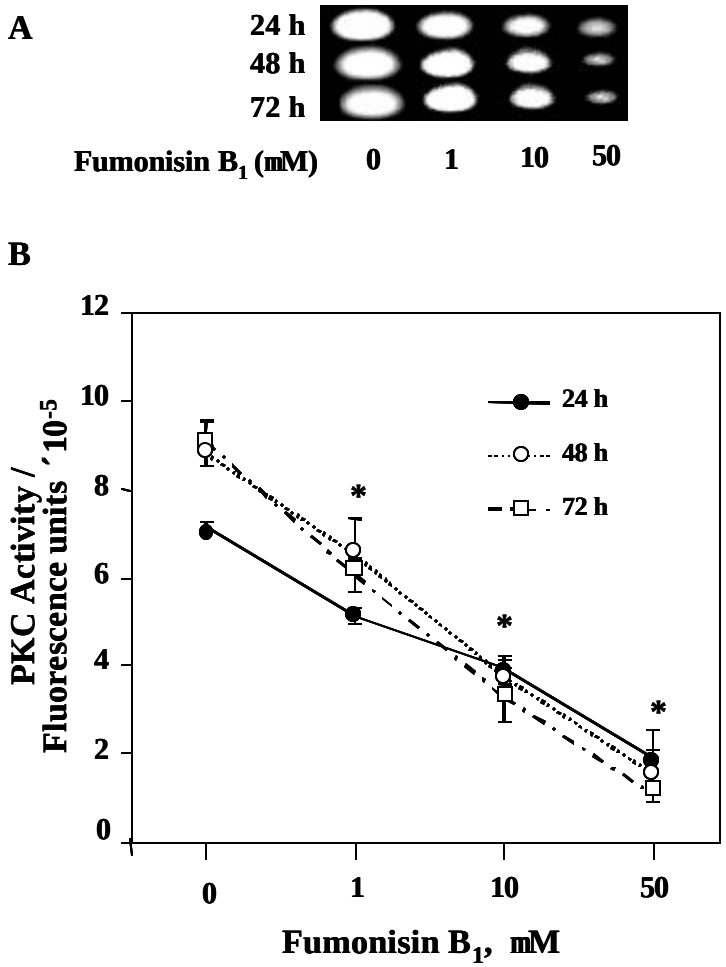

Figure 5.7. Fumonisin B_1 decreases PKC activity in a concentration-dependent manner in LLC-PK_1 cells. LLC-PK_1 cells were grown 72 h prior to exposure to 0, 1, 10 and 50 μM fumonisin B_1. At the indicated times total protein was obtained and PepTag® assay for non-radioactive detection of PKC activity assay was performed. A representative gel is shown in (A) and quantitative determination of PKC activity is shown in (B). Data shown are representative of 3 independent experiments, the mean ± standard error (n=3) are presented. *Significantly different from the respective control at $P \leq 0.05$.

Figure 5.8. Fumonisin B_1 inhibits NF-kB cytosol to nuclear translocation in LLC-PK_1 cells. LLC-PK_1 cells were grown for 72 h and treated with increasing concentrations of fumonisin B_1 (1-10 μM). Cytosolic (A) and nuclear (B) proteins (2 μg) were resolved on an 8% gel, electroblotted to a nitrocellulose membrane and visualized autoradiographically using chemiluminescent reagents. Results are representative of 3 independent experiments, the mean ± standard error (n=3) are presented. *Significantly different from the respective control at $P \leq 0.05$.

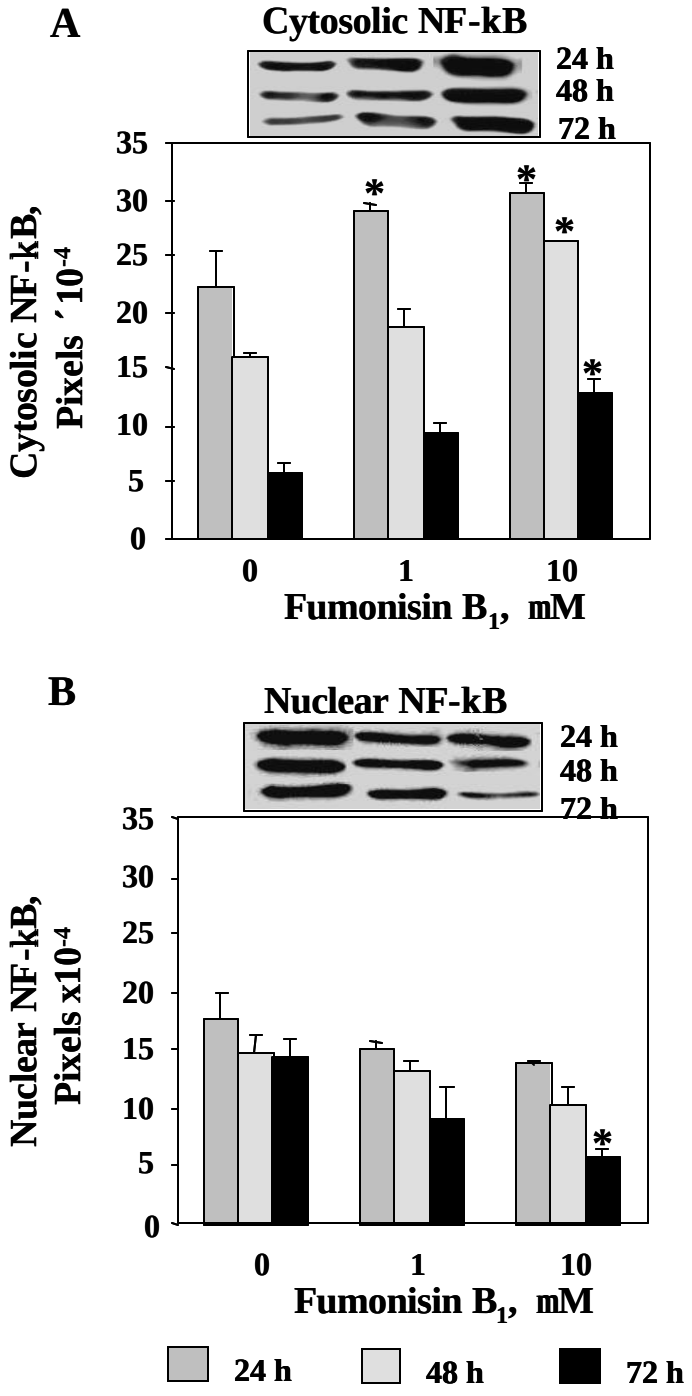

Figure 5.8. Fumonisin B_1 inhibits NF-kB cytosol to nuclear translocation in LLC-PK_1 cells.

Figure 5.9. Effect of fumonisin B_1 on TNFa mRNA expression in LLC-PK_1 cells at 24, 48 and 72 h. LLC-PK_1 cells were treated with increasing fumonisin B_1 (1-10 μM) concentrations at the times indicated and RT-PCR performed. RNA was extracted and cDNA synthesized and amplified. PCR products were separated on 2% agarose gel and detected by a UV transilluminator and photographed. A representative gel at 24, 48, and 72 h and the optimization of the cycle number for TNFα and GAPDH is shown (A). The photographs were scanned, bands were quantified and TNFα was normalized to the house keeping GAPDH (B). Values are mean ± standard error for 3 independent experiments each performed in triplicate. * Significantly different from the respective control at $P \leq 0.05$.

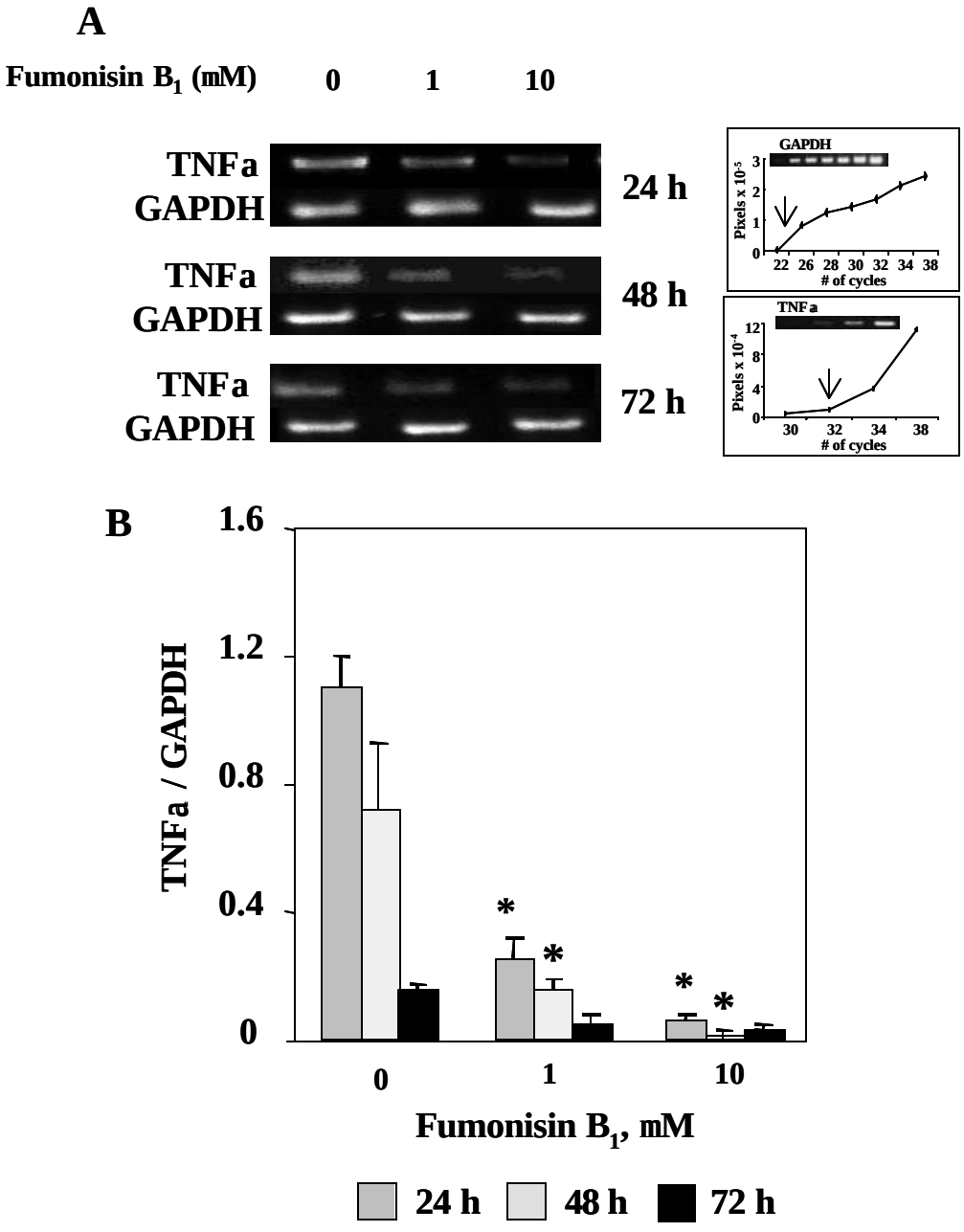

Figure 5.9. Effect of fumonisin B_1 on TNFa mRNA expression in LLC-PK_1 cells at 24, 48 and 72 h.

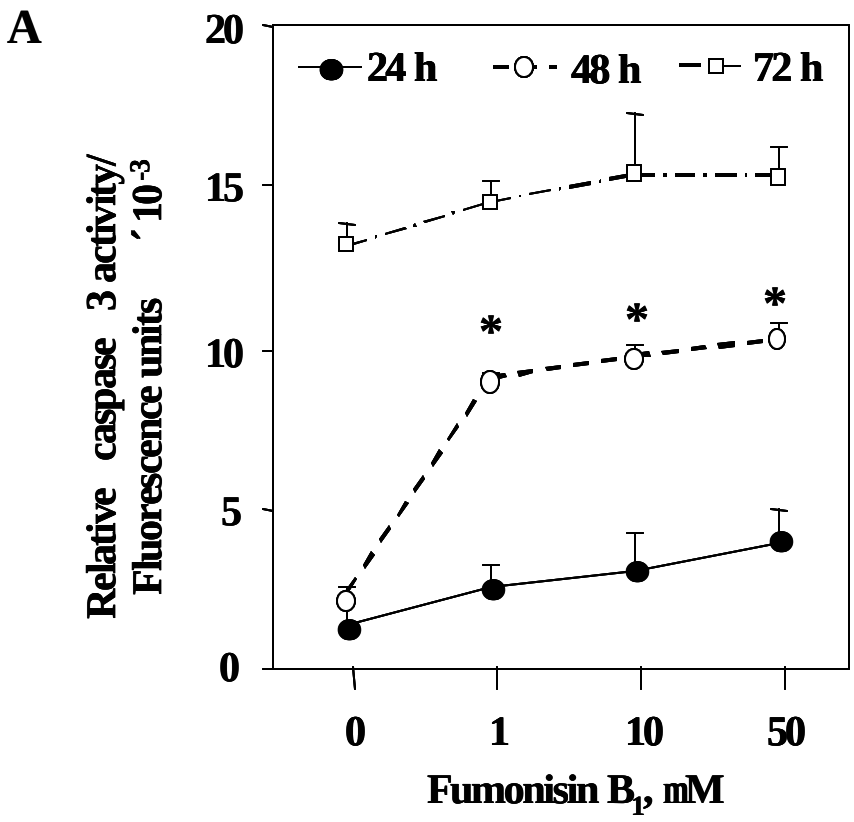

Figure 5.10. Effect of increasing concentrations of fumonisin B_1 on caspase 3 activity in LLC-PK_1 cells at 24, 48, and 72 h. Cells were treated with fumonisin B_1 (1-50 μ) for 24, 48, and 72 h in 24 wells and the supernatant was assayed for caspase 3 activity using the CaspACE™ system. The fluorescence of cleaved substrate was determined using a Spectramax Gemini fluorescent plate reader. Values are mean ± standard error for 3 independent experiments each performed in triplicate. * Significantly different from the respective control at $P \leq 0.05$.

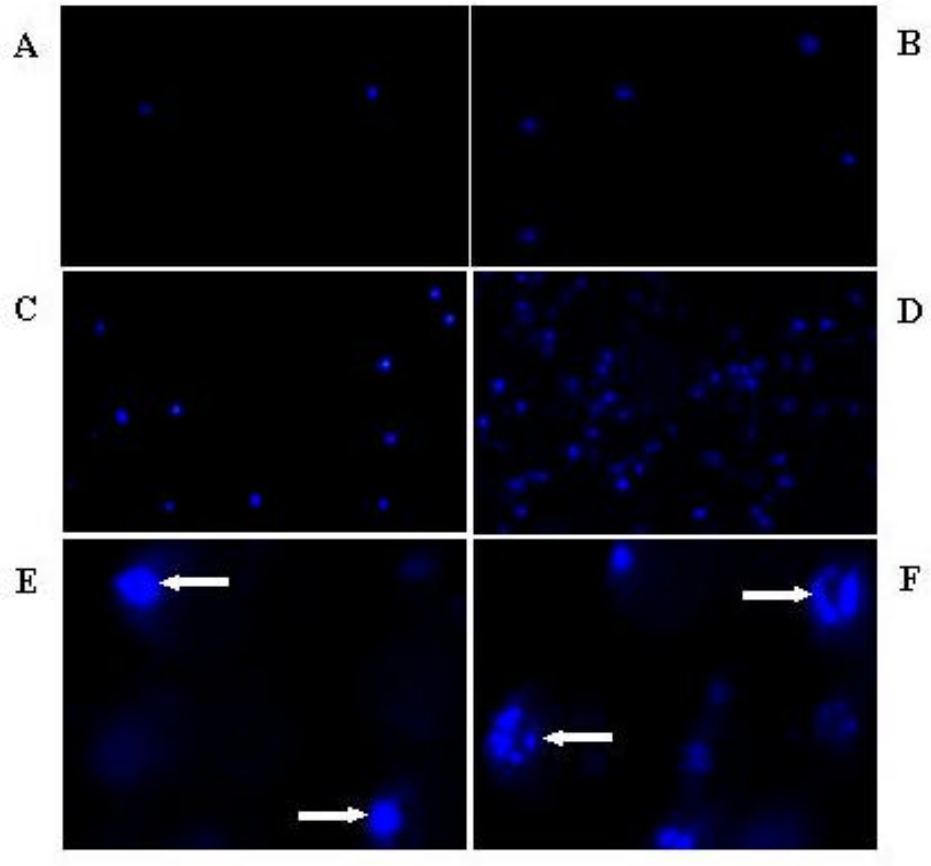

Figure 5.11. Effect of increasing concentrations of fumonisin B_1 on nuclear morphology at 48 h in LLC-PK_1 cells. Cells were grown in 24-well plates for 72 h prior to exposure to fumonisin B_1 (1- 50 μM) for 24, 48, and 72 h. Cells were stained with H33458 and visualized via fluorescence microscopy. Representative photographs show increased nuclear staining with H33458 at 48 h in cells exposed to 0 μM fumonisin B_1 (A), 1 μM fumonisin B_1 (B), 10 μM fumonisin B_1 (C) and 50 μM fumonisin B_1 (D) at 48 h. Arrows indicate intact nuclei in untreated cells (E) and fragmented nuclei in cells exposed to 50 μM fumonisin B_1 (F) at 48 h.

DISCUSSION

This study demonstrated that exposure to increasing concentrations of fumonisin B_1 resulted in a concentration-dependent decrease in cell viability, which was associated with an increase in caspase 3 activity and nuclear fragmentation indicative of apoptosis at 24, 48, and 72 h in LLC-PK_1 cells. Increase in intracellular sphinganine and sphingosine concentrations were also increased in a concentration-dependent manner at these time points. In addition, a concentration-dependent repression PKC was observed as determined by a reduction in cytosolic to membrane translocation and PKC activity. The global inhibition of PKC was associated with a concomitant reduction in NF-κB cytosolic nuclear translocation and TNFα gene expression. The results demonstrate that fumonisin B_1-induction of apoptosis is associated with an overall inhibition of PKC, NF-κB and TNFα in LLC-PK_1 cells.

We found that fumonsin B_1 concentrations ≥10 μM increased intracellular sphinganine, and to a lesser extent sphingosine, in a concentration-dependent manner. In agreement with our findings, Yoo *et al* (1992) determined the EC_{50} for alterations in sphingolipid biosynthesis in LLC-PK_1 cells to be 10 to 15 μM fumonisin B_1 where levels of free sphinganine, and to a lesser extent sphingosine, increased in fumonisin-treated cells in a dose-dependent manner. Both elevation of free sphingoid bases and the decrease in complex sphingolipids preceed and contribute to the decreased cell growth and lethality of fumonisin B_1 in LLC-PK_1 cells (Yoo *et al*., 1996a).

Similarly, we showed that cytotoxicity and inhibition of proliferation of LLC-PK_1 cells exposed to fumonisin B_1 concentrations ≥10 μM. In addition to LLC-PK_1 cells (Yoo *et al.,* 1992), the rat hepatoma H4TG and dog kidney MDCK cell lines (Shier *et al*.,

1991) are highly sensitive to the inhibitory and cytotoxic effects by fumonisin B_1. In the present study, apoptosis was found to be partially responsible for the fumonisin B_1 cytotoxicity as determined by an increase in caspase 3 activity and nuclear fragmentation. The ability of fumonisin B_1 to induce apotosis via increase in caspase 3 activity was also reported to occur in astrocytes and LLC-PK_1 cells (Galvano *et al.*, 2002; Yu *et al.*, 2001).

Huwiler *et al.* (1991, 1992) have demonstrated the presence of PKC -α, -δ, -ε, and -ζ in renal mesangial cells and taking into account the possibility that only some isoforms might be affected (Schnaper, 2000), the protein concentrations of these isoforms were examined in LLC-PK_1 cells. Fumonisin B_1 concentrations ≥ 1 μM represses cytosolic to membrane translocation of PKC-α, -δ, -ε, and -ζ, as well as total PKC activity, at 24, 48, and 72 h in LLC-PK_1 cells. In agreement with these findings, Huang *et al.* (1995) demonstrated that fumonisin B_1 (0.01-10 μM) repressed PKC activity in a dose-dependent manner in African green monkey CV-1 kidney cells. Induction of apoptosis by fumonisin B_1 may be linked to its repression of PKC activity, since PKC inhibition can induce apoptosis in a number of cell lines (Reyland *et al.*, 1999; Chmura *et al.*, 1996). In addition, the ability of fumonisin B_1 to act as a carcinogen may be linked to its ability to repress specific PKC isoforms and consequently alter signal transduction pathways (Suga *et al.*, 1998; Scaglione-Sewell *et al.*, 1998). Therefore, down-regulation of PKC expression could be correlated with malignant transformation and/or apoptotic activity in response to fumonisin B_1.

There are several possible mechanisms by which fumonisin B_1 could down-regulate PKC. Sphingolipids, namely sphingosine, sphinganine and lysosphingolipids, are potent and reversible inhibitors of PKC *in vitro* and in cell systems (Hannun *et al.*, 1986;

Hannun and Bell, 1987). Therefore, it would appear that these sphingolipids could serve as natural antagonists to the lipid activators of PKC (Merrill *et al*., 1993), which could explain the fumonisin B_1-induced repression of PKC in LLC-PK_1 cells. Second, chronic exposure of PKC to phorbol esters leads to overall down-regulation of PKC levels in the cell (Clemens *et al.*, 1992). Yeung and coworkers (1996) suggested that the action of fumonsin B_1 on PKC activity is likely to be mediated by its interaction with the DAG sites that also bind phorbol esters. Therefore, long-term fumonisin B_1 exposure could result in PKC down-regulation via a mechanism similar to that of chronic phorbol ester exposure. Another potential mechanism to explain the fumonisn B_1-induced down-regulation of PKC may the depletion of DAG, the physiological activator of PKC. Fumonisin B_1 decreases the DAG content of GF17 cells in a time dependent manner (Baron and Malhotra, 2002). Thus, lowering the DAG levels by fumonisin B_1 treatment may be responsible, in part, for the PKC repression observed.

In additional experiments, we demonstrated that transient fumonisin B_1 activation of PKC directly activates NF-κB leading to TNFα induction in LLC-PK_1 cells (unpublished data). In the present study we found that in addition to PKC repression, fumonisin B_1 inhibited the activation of NF-κB in a concentration- dependent manner in LLC-PK_1 cells. It is conceivable that inhibition or down-regulation of PKC by fumonisin B_1 in LLC-PK_1 cells is associated with inhibition of NF-κB, as demonstrated in this study. Subsequently, the fumonisin B_1-induced global down-regulation of PKC isoforms and NF-κB in LLC-PK_1 cells was associated with a concentration-dependent reduction in TNFα gene expression at 24, 48, and 72 h. Other reports have demonstrated that down-

regulation and eventual depletion of PKC inhibit TNFα (Mege *et al.*, 1993; Kontny *et al.*, 1999).

In conclusion, the fumonisin B_1induced repression of PKC and its downstream targets, NF-κB and TNFα, is associated with its capacity to induce apoptosis. Taken together, these findings suggest that fumonisin B_1 disrupts PKC cell signaling events in LLC-PK_1 cells, causing cells to engage an apoptotic program. Presently, studies are being undertaken to determine whether the observed response on PKC is due to disrupted sphingolipid metabolism induced by fumonisin B_1.

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CHAPTER 6

SPHINGOID BASES AND THEIR PHOSPHATES: TRANSIENT ACTIVATION AND DELAYED REPRESSION OF PROTEIN KINASE C ISOFORMS AND THEIR POSSIBLE INVOLVEMENT IN FUMONISIN B_1 CYTOTOXICITY[1]

[1]Gopee, N.V. and Sharma, R.P. Submitted to *Toxicology*, 11/15/02.

ABSTRACT

Fumonisin B_1, an inhibitor of ceramide synthase, leads to accumulation of sphinganine, and later, sphingosine *in vivo* and *in vitro*. Fumonisin B_1 modulates the activity of protein kinase C (PKC), however, which metabolite of disrupted sphingolipid metabolism is involved, has not been ascertained. In the present study, we evaluated the modulation of PKC by sphingoid bases and their metabolites using exogenous sphingolipid analogues in porcine renal epithelial (LLC-PK_1) cells. Previously, we found that fumonisin B_1 (1 μM) selectively and transiently activated PKCα, whereas fumonisin B_1 concentrations of 1-50 μM at 24, 48, and 72 h repressed PKC-α, -δ, -ε and -ζ isoforms in a concentration-dependent manner. Addition of exogenous sphinganine 1-phosphate (1 μM for 5 min) alone stimulated cytosolic to membrane translocation of PKCα comparative to fumonisn B_1. Co-exposure fumonisin B_1 with *N*,*N*-dimethylsphingosine (DMS), an inhibitor of sphingosine/sphinganine kinase, prevented the effects of fumonisin B_1 on PKCα. Sphinganine, sphingosine, sphingosine 1-phosphate and ceramide added exogenously, did not alter PKCα cytosolic to membrane translocation at 5 min. Fumonisin B_1 (10 μM), sphinganine, sphingosine and ceramide significantly repressed PKC-α and -δ isoforms at 48 h, whereas all the exogenously added sphingolipids significantly repressed PKC-ε and ζ similar to fumonisin B_1. Co-exposure of myriocin with fumonisin B_1 prevented the inhibitory effects of fumonisin B_1 on PKC isoforms in LLC-PK_1 cells. DMS was cytotoxic to LLC-PK_1 cells at 48 h even at very low concentrations. This study demonstrated that selective and transient activation of PKCα may be due to the fumonisin B_1-induced accumulation of the bioactive sphinganine 1-phosphate, whereas the long-term repression of PKC isoforms may be

predominantly due to the accumulation of sphinganine or its phosphate, and to a lesser extent sphingosine or its metabolite in LLC-PK_1 cells. These findings suggest that the direct or indirect modulation of PKC by these sphingolipids is involved at least in part in the action of fumonisin B_1.

Keywords: Fumonisin B_1, Protein kinase C, Sphingolipids, Sphinganine, Sphinganine 1-phosphate, Myriocin

INTRODUCTION

Fumonisin B_1 is a ubiquitous toxic secondary metabolite produced by the fungus *Fusarium verticillioides* present predominantly on corn. Consumption of contaminated corn leads to leukoencephalomalacia in horses, pulmonary edema in pigs, and liver and kidney toxicity in domestic and laboratory animals (Riley *et al.*, 1998). This mycotoxin has been reported to be carcinogenic in laboratory animals (Howard *et al.*, 2001) and it has been correlated to the incidence of human esophageal cancer (Rheeder *et al.*, 1992).

Fumonisin B_1 is a water soluble, polar metabolite which posses a lipophilic (sphingoid-like) backbone and structurally related to the sphingoid base, sphinganine (Wang *et al.*, 1991; Yin *et al.*, 1996). Based on the remarkable structural similarity of fumonisins to sphingoid bases, it has been established that fumonisin B_1 is a potent and reversible inhibitor of sphinganine or sphingosine *N*-acyltransferase (ceramide synthase), located in the endoplasmic reticulum (Wang *et al.*, 1991), resulting in disruption of sphingolipid metabolism (Fig. 6.1). Fumonisin B_1-induced disruption of sphingolipid metabolism leads to inhibition of ceramide biosynthesis, an increase in free sphinganine and sphingosine, a decrease in reacylation of sphingosine derived from complex

sphingolipid turnover and degradation of dietary sphingolipids, an increase in sphingoid base degradation products i.e. sphingosine and sphinganine 1–phosphate, ethanolamine phosphate, and fatty aldehydes, and alterations in other sphingoid lipid pools (Riley *et al*., 1998).

Until the late 1970's, sphingolipids were primarily thought to serve as inert structural compounds; the suggestion that sphingolipids might be directly implicated in intracellular signaling pathways followed the discovery that sphingosine, a product of complex sphingolipid metabolism, inhibited protein kinase C (PKC) activity (Hannun *et al.*, 1986). The discovery that breakdown products of cellular sphingolipids are biologically active and can modulate PKC activity has generated great interest in the role of these molecules in cell signaling (Hannun and Bell, 1989) as different sphingolipids are able to affect PKC activity and/or PKC-mediated events in a variety of experimental models. Sphingolipids and their metabolites can mediate either mitogenic or apoptotic effects depending on the cell type and exposure conditions (Hannun, 1996; Spiegel and Milstien, 1995).

We recently observed that 1 μM fumonisin B_1 selectively and transiently activates PKCα at 5 min in porcine renal epithelial cells (LLC-PK_1 (unpublished data). Exogenously added myriocin, an inhibitor of serine palmitoyltransferase (SPT), the first enzyme in *de novo* sphingolipid biosynthesis (Yoo *et al.*, 1996) (Fig. 6.1), did not prevent the fumonisin B_1 activation of PKCα at 5 min. In contrast, a concentration-dependent repression of PKC-α, -δ, -ε and -ζ isoforms were observed on exposure to fumonisin B_1 concentrations of 1-50 μM at 24, 48, and 72 h in these cells. The purpose of this study was to elucidate the mechanistic relationship between sphingoid base and their

metabolites-induced alterations in PKC activity and relate these to the fumonisin B_1 effects in LLC-PK_1 cells. In order to carry out this, LLC-PK_1 cells were treated with fumonisin B_1 in the absence or presence of various inhibitors or exogenous sphingolipid analogues PKC cytosol to membrane translocation at 5 min and 48 h.

MATERIAL AND METHODS

Materials

Fumonisin B_1 (purity > 98%) was obtained from Programme on Mycotoxins and Experimental Carcinogenesis (PROMEC, Tygerberg, South Africa). D-*erythro*-sphinganine (purity > 99%), D-*erythro*-sphingosine (purity > 99%), sphinagnine 1-phophate (purity > 99%), sphingosine 1-phosphate (purity > 99%), *N*,*N*-dimethylsphingosine (DMS) (purity > 99%) were obtained from Avanti® Polar Lipids, Inc. (Alabaster, AL). Myriocin was obtained from Biomol Research Laboratory (Plymoth, PA). Cell permeable C_6-ceramide, horseradish peroxidase (HRP)-conjugated goat secondary anti-mouse antibody, bovine serum albumin (BSA) and free fatty acid BSA were obtained from Sigma (St. Louis, MO). Primary mouse polyclonal anti-PKCα, rabbit polyclonal anti-PKCδ, rabbit polyclonal anti-PKCε and HRP donkey anti-rabbit secondary antibody were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Primary anti-PKCζ antibody was obtained from Upstate Biotechnology (Lake Placid, NY).

Preparation stock solutions of sphingolipids, myriocin and DMS

The specific chemical was added to methanol at 0.5 mg/ml, the mixture heated to 45°C-65°C and sonicated until the chemical dissolves. The methanol stock solution was aliquotted to a glass tube and the methanol was removed using a stream of nitrogen. The glass tube containing the dried chemical was stored at -20°C until ready to use. Prior to addition to cultures, fatty acid free BSA (4 mg/ml) dissolved in deionized water was added to the glass tube containing the dried chemical and incubated for 30 min at 37°C, vortexing occasionally. The solution was filtered and used for subsequent experiments.

Cell culture and treatment

LLC-PK_1 cells (CRL 1392, passage 197), originally derived from porcine renal cortex (Hull *et al.*, 1976) were obtained from American Type Culture Collection (ATCC, Rockville, MD). Cells were maintained at 37° C in a humidified atmosphere with 5% CO_2 in Dulbecco's modified Eagle's Medium/Ham's F12 (1:1) supplemented with 5% fetal bovine serum, penicillin (100 mg/ml), and streptomycin (100 mg/ml). The cells were subcultured in 100 × 15 mm sterile petri dishes. For the short-term 5 min studies, cells were allowed to attach and grow for 2 days and the media replaced with serum-free media 18 h prior to treatment on the third day. For the long-term 48 h studies, cells were allowed to attach and grow for 3 days prior to treatment. To prevent the burst of free sphingoid bases after addition of fresh medium (Smith *et al.*, 1997), 0.1 ml medium from each well was taken out, and 0.1 ml of appropriate chemical dissolved in serum-free medium, was added.

For all studies, cells were treated with 1 μM of sphinganine, sphingosine, sphinganine 1-phosphate, sphingosine 1-phosphate or ceramide, for 5 min or 48 h. The concentrations were optimized in preliminary experiments. In appropriate experiments, cells were preincubated with 200 nM myriocin for 4 h prior to addition of 1 μM fumonisin B_1 for 5 min, to ensure adequate inhibition of SPT and prevent intracellular free sphinganine accumulation (He *et al.*, 2001). Myriocin (20 nM) and fumonisin B_1 (10 μM) were added together for 48 h, this concentration of myriocin was shown to adequately inhibit SPT (Riley *et al.*, 1999). Cells were preincubated for 1 h with DMS (1 μM), the sphingosine/sphinganine kinase inhibitor (Fig. 6.1), for 1 h prior to adding fumonisin B_1 for 5 min. The concentrations of sphingolipids and enzyme inhibitors used in these studies produced minimal cytotoxicity at 48 h as determined by MTT assay (data not shown).

Immunoblot analysis for PKC isoforms

Cytosolic and membrane fractions were obtained using modifications to the methods as described by of Clarke *et al*. (2000). Briefly, cells were washed 3 times in cold phosphate-buffered saline (PBS) and scraped into 100 μl of lysis buffer A (20 mmol/L Tris-HCl, pH 7.5, 0.25 mol/L sucrose, 10 mmol/L EGTA, 2 mmol/L EDTA, 20 μg/mL leupeptin, 10 μg/mL aprotinin and 200 μmol/L phenylsulfonyl fluoride) at 4°C and sonicated on ice. Samples were centrifuged at 42, 000 × *g* for 2 h at 4°C and the cytosolic fractions (supernatants) transferred to separate tubes. Lysis buffer A (50 μl) containing 1% Triton X-100 at 4°C was added to each pellet were mixed for 1 h at 4°C.

The solutions were centrifuged at 42, 000 × *g* for 2 h at 4°C and the membrane fractions (supernatants) were transferred to separate tubes.

Protein concentrations were determined using a Bio-Rad protein assay (Bio-Rad, Hercules, CA) using bovine serum albumin (BSA) (Sigma, St. Louis, MO) as a standard. Equal amounts of protein from each sample (10 µg/ lane) were loaded on 8% polyacrylamide minigels, electrophoresed and transblotted to nitrocellulose membranes. Nonspecific binding was blocked in 5% milk protein for 1 h prior to incubating with appropriate primary and HRP-conjugated secondary antibodies. Immunoreactive bands were visualized by Pierce Super Signal® chemiluminescent substrate (Pierce, Rockford, IL). Images were acquired with a scanner and analyzed with UN-SCAN-IT software (Silk Scientific Inc., Orem, UT). Following appropriate visualization, membranes were washed, stripped utilizing Pierce Restore™ Western Blot Stripping Buffer (Pierce, Rockford, IL), blocked and reprobed with appropriate primary and secondary antibodies.

Statistical analysis

Data presented here represent a minimum of three experiments and where appropriate are expressed as mean ± SE. Differences among the various treatment groups were determined by one-way analysis of variance followed by Duncan's multiple range tests utilizing Version 8 of the Statistical Analysis Software (SAS) (SAS Institute Inc., Cary, NC). A P value ≤ 0.05 was taken to denote statistical difference

RESULTS

Fumonisin B_1 and sphinganine 1-phosphate significantly increased cytosolic to membrane translocation of PKCα at 5 min. Fumonisin B_1 (1 μM) significantly increased PKCα membrane protein concentration at 5 min in LLC-PK_1 cells (Fig. 6.2). A comparative increase in PKCα membrane protein level was observed on exposure to exogenous sphinganine 1-phosphate (1 μM). The increase in PKCα membrane levels was associated by a decrease in cytosolic PKCα protein concentrations. Addition of exogenous sphinganine (1 μM), sphingosine (1 μM), sphingosine 1-phosphate (1 μM) and ceramide (1 μM) had no effect on PKCα membrane or cytosolic protein concentrations at 5 min.

DMS prevented the fumonisin B_1 increase in PKCα membrane protein concentrations at 5 min. Since sphinganine 1-phosphate stimulated cytosolic to membrane translocation of PKCα at 5 min, we employed DMS, which inhibits sphingosine/sphinganine kinase. Exposure of LLC-PK_1 cells to fumonisin B_1 (1 μM for 5 min) stimulated a significant increase in PKCα membrane protein concentrations (Fig. 6.3). This was associated with a decrease in cytosolic PKCα protein levels. DMS (1 μM) alone had no effect on PKCα cytosolic or membrane protein levels. However, preincubation with DMS prevented the fumonisin B_1 effects on cytosolic and membrane PKCα concentrations.

Effect of fumonisin B_1 and exogenous sphingolipids on membrane protein concentrations of PKC-α, -δ, -ε and -ζ isoforms at 48 h. Fumonisin B_1 (10 μM),

sphinganine (1 μM), sphingosine (1 μM) and ceramide (1 μM) significantly repressed PKC-α and -δ membrane protein concentrations at 48 h in LLC-PK_1 cells, whereas sphinganine 1-phosphate (1 μM) and sphingosine 1-phosphate (1 μM) had no effect on the membrane protein levels of these isoforms. In contrast, all these sphingolipids significantly repressed PKC-ε and -ζ membrane protein concentrations at 48 h (Fig. 6.4). The cytosolic protein concentrations of these isoforms were not significantly altered upon any of the treatments, therefore only membrane protein concentrations are shown.

Myriocin reversed the fumonisin B_1 repression of PKC-α, -δ, -ε and -ζ membrane protein concentrations at 48 h in LLC-PK_1 cells. PKC-α, -δ, -ε and -ζ membrane protein concentrations in response to myriocin (20 nM) was similar to untreated control (Fig. 6.5), whereas fumonisin B_1 (10 μM) significantly decreased membrane protein levels at 48 h. However, co-exposure of LLC-PK_1 cells with myriocin and fumonisin B_1 reversed the inhibitory effects of fumonisin B_1 on PKC-α, -δ, -ε and -ζ membrane protein concentrations at 48 h.

Figure 6.1. Structures of sphinganine and fumonisin B_1, sphingolipid metabolism pathway and sites of inhibition by fumonisin B_1, DMS and myriocin. Fumonisin B_1 structurally resembles the sphingoid base, sphinganine (A) Inhibition of ceramide synthase by fumonisin B_1 results in prevention of complex sphingolipid biosynthesis, accumulation of sphinganine and to a lesser extent sphingosine, and diversion of sphinganine and sphingosine to sphinganine- and sphingosine-1-phosphate and related metabolites (B). Mryiocin inhibits serine palmitoyltransferase, the first enzyme in *de novo* sphingolipid biosynthesis, preventing the accumulation of sphinganine induced by fumonisin B_1. Dimethylsphingosine (DMS) inhibition of sphingosine/sphinganine kinase inhibits phosphorylation of sphingosine and sphinganine preventing the accumulation of sphingosine- and sphinganine-1-phosphate.

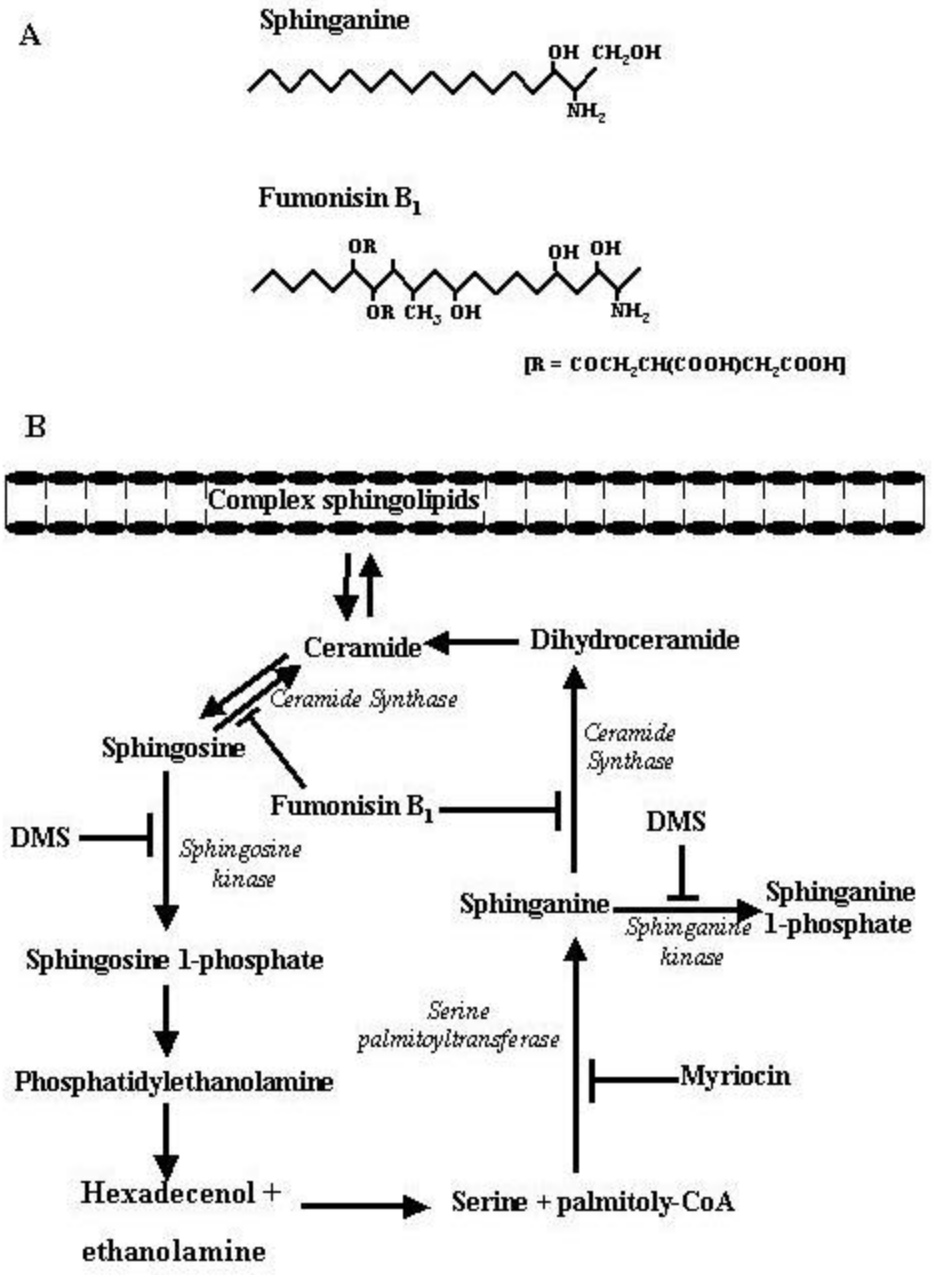

Figure 6.1. Structures of sphinganine and fumonisin B_1, sphingolipid metabolism pathway and sites of inhibition by fumonisin B_1, DMS and myriocin.

Figure 6.2. Effect of fumonisin B_1 and exogenous sphingolipids on PKCa membrane protein concentrations at 5 min. LLC-PK_1 cells were grown serum-free media 18 h prior to addition of fumonisin B_1 (FB_1) (1 μM), exogenous sphinganine (Sa) (1μM), sphingosine (So) (1 μM), sphinganine 1-phosphate (Sa-1-P) (1 μM), sphingosine 1-phosphate (So-1-P) (1 μM) and ceramide (Cer) (1μM) for 5 min. Membrane (A) and cytosolic (B) PKCα proteins visualized autoradiographically using chemiluminescent reagents. Similar findings were noted in 3 independent experiments, results from a representative experiment (mean ± standard error, n=3) have been shown above. *Significantly different from the respective control at $P \leq 0.05$.

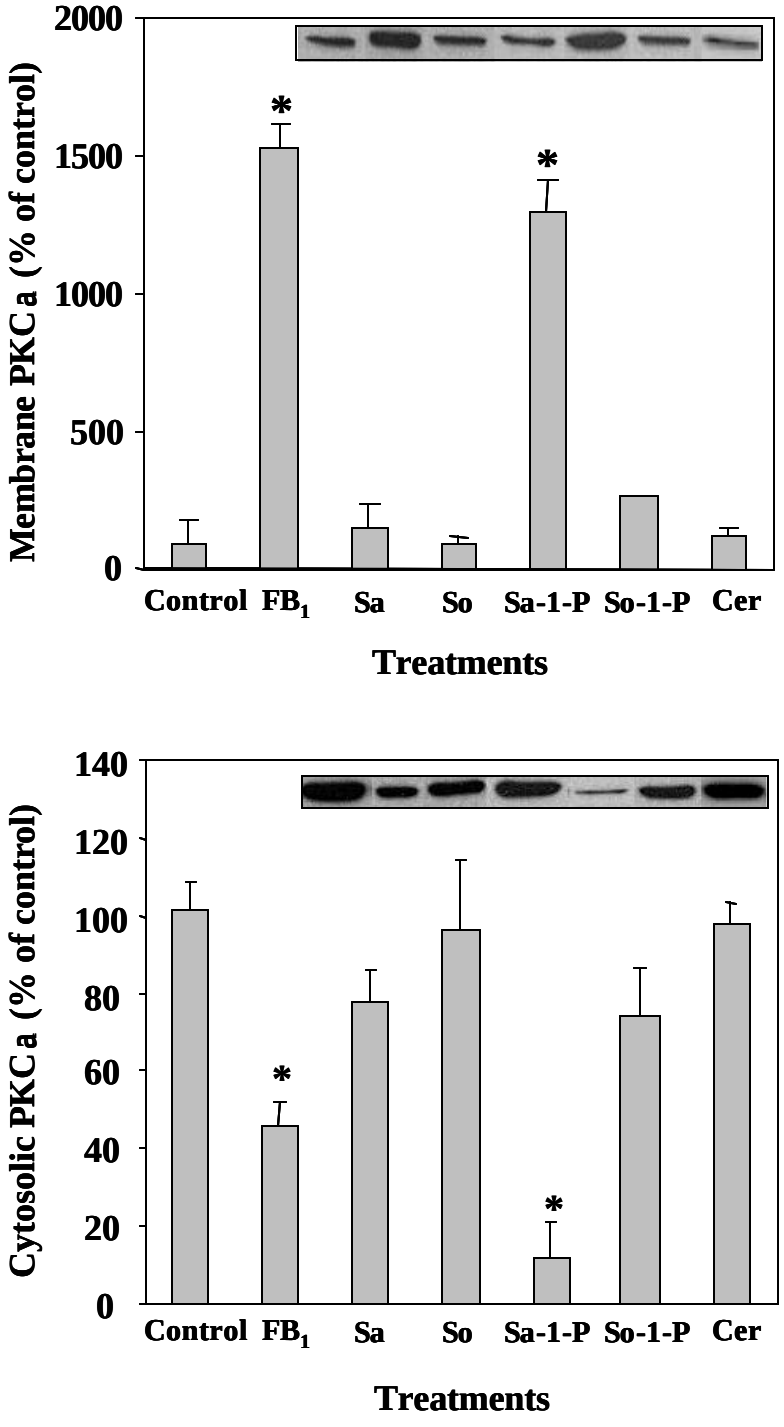

Figure 6.2. Effect of fumonisin B_1 and exogenous sphingolipids on PKCa membrane protein concentrations at 5 min.

Figure 6.3. Effect of dimethylsphingosine on fumonisin B_1 induction of PKCa cytosol to membrane redistribution at 5 min in LLC-PK_1 cells. Cells were preincubated with 1 μM dimethylsphingosine (DMS) for 60 min prior to the addition of fumonisin B_1 (FB_1) (1 μM) for 5 min. Membrane (A) and cytosolic (B) proteins were obtained and immunoblot assays were performed as described under ′Materials and Methods′. Results presented are mean ± standard error (n=3) from a representative experiment independently repeated three times. *Significantly different from the respective control at $P \leq 0.05$.

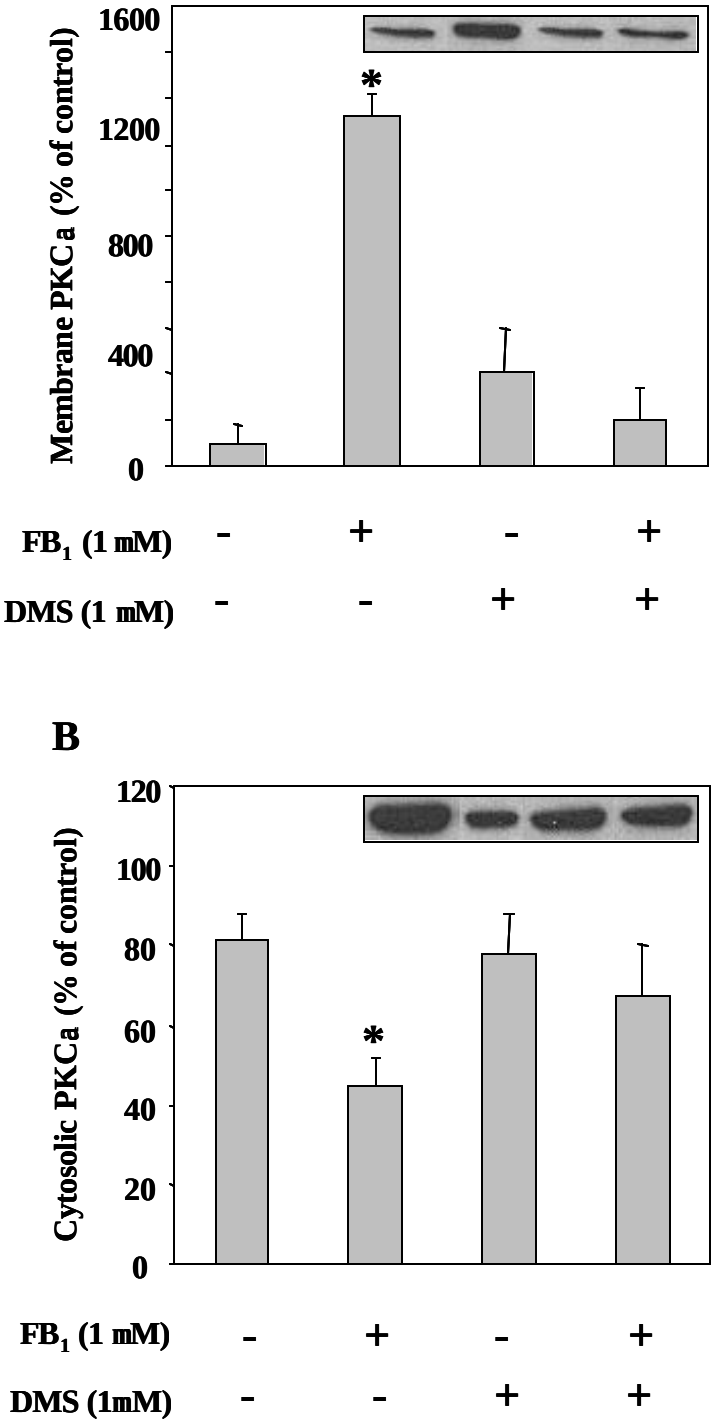

Figure 6.3. Effect of dimethylsphingosine on fumonisin B_1 induction of PKCa cytosol to membrane redistribution at 5 min in LLC-PK_1 cells.

Figure 6.4. Effect of fumonisin B_1 and exogenous sphingoid bases on membrane protein concentrations of PKC-a, -d, -e and -z isoforms in LLC-PK_1 cells at 48 h. LLC-PK_1 cells were grown for 72 h prior to addition of fumonisin B_1 (FB_1) (10 μM), exogenous sphinganine (Sa) (1μM), sphingosine (So) (1 μM), sphinganine 1-phosphate (Sa-1-P) (1 μM), sphingosine 1-phosphate (So-1-P) (1 μM) and ceramide (Cer) (1μM) for 48 h. Membrane protein for PKC-α (A), -δ (B), -ε (C) and -ζ (D) were visualized autoradiographically using chemiluminescent reagents. Results (mean ± standard error, n=3) are from a representative experiment of 3 independent trials. *Significantly different from the respective control at $P \leq 0.05$.

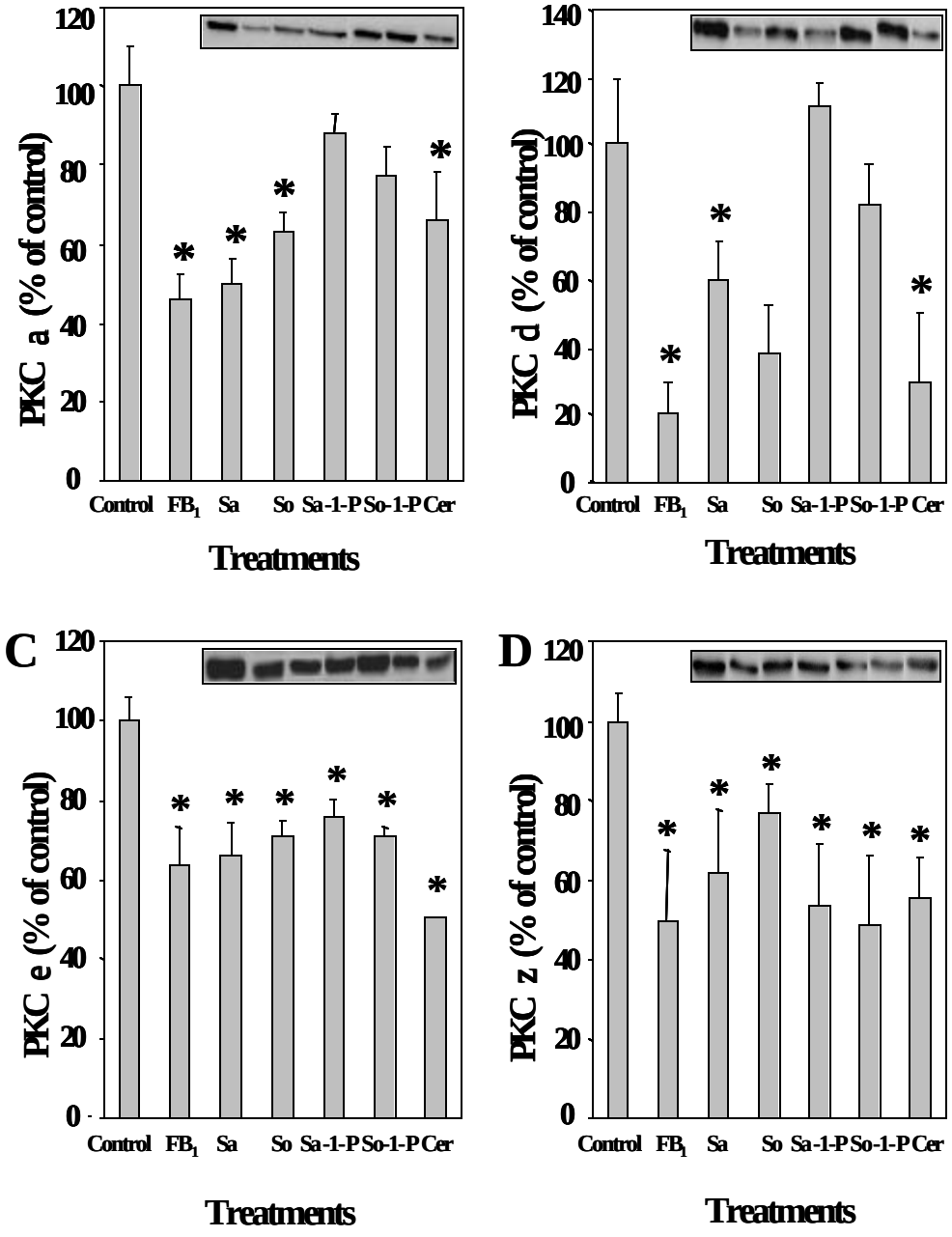

Figure 6.4. Effect of fumonisin B_1 and exogenous sphingoid bases on membrane protein concentrations of PKC-a, -d, -e and -z isoforms in LLC-PK_1 cells at 48 h.

Figure 6.5. Myriocin reversed the effects of fumonisin B_1 inhibition on PKC-a , -d , -e and -z membrane protein concentrations at 48 h in LLC-PK_1 cells. LLC-PK_1 cells were grown for 72 h prior to co-exposure of 10 μM fumonisin B_1 (FB_1) and 20 nM myriocin (Myr) for 48 h. Membrane proteins for PKC-α (A), -δ (B), -ε (C) and -ζ (D) isoforms were visualized autoradiographically using chemiluminescent reagents. Similar findings were noted in 3 independent experiments, results from a representative experiment (mean ± standard error, n=3) have been presented. *Significantly different from the respective control at $P \leq 0.05$.

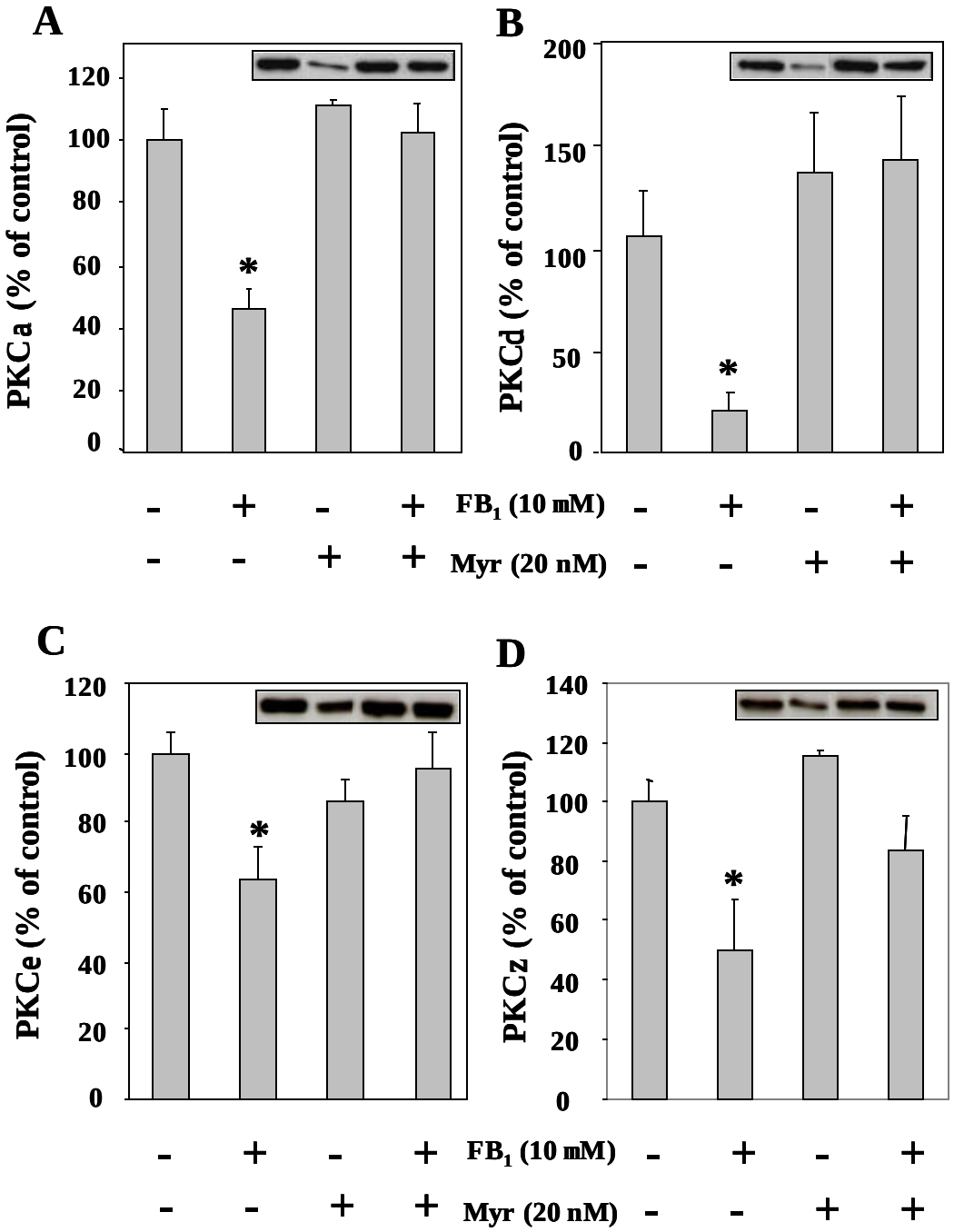

Figure 6.5. Myriocin reversed the effects of fumonisin B_1 inhibition on PKC-a, -d, -e and -z membrane protein concentrations at 48 h in LLC-PK_1 cells.

DISCUSSION

The present study supported the hypothesis that fumonisin B_1-induced alterations in PKC were due to accumulation of sphingoid bases or their metabolites. Previously we have shown that fumonisin B_1 (1 μM for 5 min) selectively and transiently activates PKCα min followed by its down-regulation at later time points in LLC-PK_1 cells (unpublished). We also found that preincubation with myriocin did not prevent the transient activation of PKCα by fumonisin B_1. In current study, exogenous sphinganine 1-phosphate, stimulated cytosolic to membrane translocation of PKCα similar to that of 1 μM fumonisin B_1 at 5 min. the effect was prevented by preincubating cells with sphingosine/sphinganine kinase inhibitor, DMS. At later times fumonisin B_1 (10 μM) repressed the membrane protein translocation of all isoforms of PKC examined here (-α, -δ, -ε and -ζ) in LLC-PK_1 cells. All of the exogenous sphingolipids used here produced a delayed inhibition of different PKC isoforms; co-exposure of cells with myriocin reversed the inhibitory effects of fumonisin B_1 on the PKC.

The results of the present study suggested that sphinganine 1-phosphate (1 μM for 5 min) stimulates cytosolic to membrane translocation of PKCα in a manner similar to fumonisin B_1. The transient activation of PKCα by fumonisin B_1 may be involved in the activation of transcription factors, such as nuclear factor-kappa B (NF-κB, La Porta and Comolli, 1998) leading to the production of proinflammatory cytokines, including tumor necrosis factor-α (TNFα, Kontny *et al*., 1999) or malignant transformation (La Porta *et al*., 1997; Bamberger *et al*., 1996). The complete inhibition of ceramide synthase by fumonisin B_1, which has an IC_{50} of 0.05-0.1 μM, increases the rate of sphingoid base 1-phosphate biosynthesis without any apparent increase in the free sphinganine

concentration (Wang *et al.*, 1991; Yoo *et al*., 1996) (Fig. 1). In fumonisin B_1-treated cultured neurons half of the exogenously applied sphinganine was metabolized to polar compounds, including sphinganine-1-phosphate (Merrill *et al.*, 1993).

He *et al* (2001) demonstrated that myriocin concentrations 0.15-1 μM efficiently prevented the accumulation of free sphingoid bases as a result of fumonisin B_1 treatment. However, the rate of inhibition of fumonisin B_1 on ceramide synthase may be faster than that of myriocin on SPT, generating sufficient sphinganine to be rapidly phosphorylated to sphinganine 1-phosphate and activate PKCα. To our knowledge, this is the first study, which documents the effects of sphinganine 1-phosphate on PKC. The biological roles of sphinganine 1-phosphate remains enigmatic; however, it may be involved in growth stimulation (Linn *et al.*, 2001), inhibition of apoptosis (Linn *et al.*, 2001), heat stress resistance (Skrzypek *et al.*, 1999), regulation of intracellular calcium homeostasis and cell motility (Merrill *et al.*, 1997).

The role of sphinganine 1-phosphate in the transient activation of PKCα was confirmed by preincubation with DMS, which prevented the effects of fumonisin B_1 on PKCα at 5 min. DMS is a specific competitive inhibitor of sphingosine/sphinganine kinase and has no effect on protein kinase C activity or its membrane translocation as demonstrated here and in other studies (Edsall *et al.*, 1998). Since sphingosine and sphinganine differ only by a double bond, sphinganine 1-phosphate acts as an effective substrate for sphingosine kinase, to produce sphinganine 1-phosphate (Edsall *et al.*, 1998). Inhibition of sphinganine kinase by DMS inhibits the phosphorylation of sphinganine and hence the formation of sphiganine 1-phosphate thereby preventing the stimulatory action of this sphinganine metabolite on PKCα.

Fumonisin B_1 (10 μM) repressed the membrane protein translocation of PKC-α, -δ, -ε and -ζ isoforms at 48 h. Malignant transformation (Suga *et al*., 1998; Scaglione-Sewell *et al*., 1998) and induction of apoptosis have both been reported to be associated to PKC inhibition in a number of cell lines (Reyland *et al*., 1999; Chmura *et al*., 1996). This effect of fumonisin B_1 was reversed when LLC-PK_1 cells were co-exposed with myriocin. Approximately 95% of the fumonisin B_1-induced increase in free sphingoid bases is due to the increase in sphinganine (Yoo *et al*., 1992) and inhibition of SPT by myriocin adequately prevents the fumonisin B_1-induced accumulation of sphinganine (Riley *et al*., 1999). However, myriocin may not prevent the accumulation of free sphingosine (~5%) and its metabolites through fumonsin B_1 inhibition of reacylcation of sphingosine derived from sphingolipid turnover prior to the development of cytotoxicity (Fig. 1). In another unpublished study we estimated the accumulation of sphingosine by 10 μM fumonisin B_1 at ~0.1 μM in LLC-PK_1 cells at 48 h, an order of magnitude lower at which we observed inhibitory effects of exogenous sphingosine or its phosphate (1 μM). Therefore, the fumonisin B_1-induced down-regulation of PKC isoforms, which is prevented by myriocin, suggests that sphinganine or its metabolites may be predominantly involved in the fumonisin B_1-induced delayed repression of the above PKC isoforms. However, sphingosine and its phosphate may also contribute, to a lesser extent, to the delayed repression of PKC by fumonisin B_1. DMS was found to be cytotoxic to LLC-PK_1 cells, as well as other cells (Edsall et al., 1988), at all concentrations tested and therefore was not used to elucidate its effect on PKC at this time.

Addition of exogenous sphinganine, sphingosine, sphingosine 1-phosphate and ceramide had no effect on PKCα membrane protein concentrations at 5 min in LLC-PK_1 cells. In addition, sphinganine 1-phosphate and sphingosine 1-phosphate did not produce any effect on PKC-α and -δ proteins at 48 h. These sphingolipid analogues may not be effective at such exposure times possibly due to their extremely low cellular uptake, instability of these sphingolipids (Riboni et al., 1997), and/or the PKC isoform involved. At 48 h, sphinganine, sphingosine and ceramide were inhibitory to membrane PKC-α and -δ proteins, whereas PKC-ε and -ζ membrane protein concentrations were significantly decreased on exposure to all the exogenous sphingolipids used in these studies at 48 h.

Sphingosine, sphinganine and other long–chain sphingoid bases inhibit PKC *in vivo* and in cell systems (Merrill *et al.*, 1986; Spiegel *et al*., 1993; Hannun and Bell, 1989; Hannun and Linardic, 1993). There is a paucity of information on the inhibitory role of sphingosine 1-phosphate on PKC; however, it has been reported to have no effect (Sadahira *et al.*, 1992) or may be stimulatory (Lampasso *et al.*, 2001; Banno *et al.*, 1999) to PKC. To our knowledge, inhibition of PKC by sphingosine 1-phosphate has not been documented, as observed in our studies on the inhibition of membrane protein of PKC-ε and -ζ isoforms. The biological functions of sphingosine, sphingosine 1-phosphate and sphinganine are paradoxical as they can induce mitogenesis (Davaille *et al.*, 2002; Merrill *et al.*, 1997; Zhang *et al.*, 1991; Linn *et al.*, 2001) or trigger apoptosis (Davaille *et al.*, 2002; Gennero *et al.*, 2002; Linn *et al.*, 2001).

Ceramide, documented to induce both apoptosis (Hsu *et al.*, 1998; Tepper *et al.*, 1997) and mitogenesis (Hauser *et al.*, 1994), has been reported to activate (Limatola *et al.*, 1997; Lozano *et al.*, 1994) and prevent activation of PKC (Bourbon *et al.*, 2001;

Chmura *et al.*, 1996). The use of fumonisin B_1 in combination with myriocin should block all *de novo* fatty acyl CoA-dependent sphingolipid biosynthesis and ceramide synthesis that use free sphingoid bases derived from sphingolipid turnover or dietary sources (Riley e*t al.*, 2001). It has been demonstrated that at 48 h, fumonisin B_1 inhibited TNFα expression in LLC-PK_1 cells (unpublished data), therefore generation of ceramide from TNFα will be minimal and the ceramide will eventually be converted to sphingosine in the sphingolipid catabolic pathway. This may result in the eventual decrease in intracellular ceramide concentrations, suggesting that ceramide may not be directly involved in the global repression of PKC isoforms by fumonisin B_1 at later times in LLC-PK_1 cells.

This is the first study that demonstrates that sphinganine 1-phosphate stimulated cytosol to membrane translocation of PKCα in a similar to fumonisin B_1 at 5 min. The role of sphinganine 1-phosphate was confirmed by the use the sphinganine 1-phosphate analogue and DMS, an inhibitor of sphingosine/sphinganine kinase. In addition, myriocin reversed the fumonisin B_1 repression of PKC isoforms at 48 h in LLC-PK_1 cells, suggesting sphinganine, and to a lesser extent sphingosine, or their metabolites may be involved in the modulation of PKC by this mycotoxin.

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CHAPTER 7

SUMMARY AND CONCLUSIONS

Fumonisin B_1 (FB_1) belongs to a group of mycotoxins produced primarily by the fungus *Fusarium verticillioides*, which commonly contaminates corn and other agricultural commodities intended for consumption. FB_1 causes a wide range of species-specific diseases in animals and is a known carcinogen. Inhibition of ceramide synthase by FB_1 leading to disruption of sphingolipid metabolism is one mechanism by which FB_1 produces its toxicity. In addition, the induction of tumor necrosis factor α (TNFα) by FB_1has been shown to modulate the FB_1 toxicity. The ability of FB_1 to alter protein kinase C (PKC), a multigene family of serine/threonine kinases involved in diverse regulatory processes, and may be part of the cascade of events through which FB_1 exerts its toxicity and carcinogenic potential.

FB_1 (≤ 1 μM) caused selective and transient activation of PKCα but did not affect PKC -α, -δ, -ε and -ζ isoforms at 5 min in LLC-PK_1 cells. An increase in the stabilization of cytosolic protein expression of PKCα at 15 min was not associated with a concomitant increase in activity or protein biosynthesis. Calphostin C, a potent inhibitor of PKC, abrogated the fumonsin B_1-induced translocation of PKCα. Preincubation with the PKC activator phorbol 12-myristate 13-acetate (PMA), a PKC activator, resulted in an additive effect of membrane translocation as compared to PMA or fumonsin B_1 alone. Myriocin, the specific inhibitor of serine palmitoyltransferase, the first enzyme in *de novo* sphingolipid biosynthesis, did not prevent the transient fumonisin B_1-induced PKCα translocation. Altering PKC and its signal transduction pathways may be of importance in the ability of fumonisin B_1 to induce apoptosis and/or carcinogenesis.

Fumonisin B_1 (1 μM for 5 min) transiently activated PKCα and increased nuclear translocation of NF-κB, followed by their down-regulation at later time points.

Preincubating LLC-PK_1 cells with the PKC inhibitor, calphostin C, prevented the activation of NF-κB by FB_1. TNFα mRNA expression was increased similarly following 15 min exposure to either FB_1 or the PKC activator, phorbol 12-myristate 13-acetate (PMA). Calphostin C prevented the FB_1-induced increase in TNFα gene expression. The PKCα-dependent induction of TNFα via NF-κB activation puts forward a plausible mechanism involved in FB_1-induced toxicity in LLC-PK_1 cells.

In cells exposed to > 1 μ M fumonisn B_1, viability was decreased and due in part to apoptosis as demonstrated by an increase in caspase 3 activity and nuclear fragmentation by Hoechst staining at 24, 48, and 72 h. Increasing concentrations of fumonisin B_1 repressed cytosolic to membrane translocation of PKC-α, -δ, -ε, and -ζ isoforms at 24, 48, and 72 h. The fumonsin B_1-induced PKC repression was corroborated by a concentration-dependent decrease in total PKC activity. Exposure to fumonisin B_1 (1-50 μM) was associated with a concentration-dependent inhibition of nuclear translocation of NF-κB and TNFα gene expression at 24, 48, and 72 h. At all time points, intracellular free sphinganine and sphingosine concentrations were elevated upon exposure to fumonisin B_1 in a concentration-dependent manner. These results demonstrate that fumonisin B_1-induced apoptosis is associated with repression of PKC isoforms and consequently NF-κB and TNFα, events that may be involved in the toxicity of this mycotoxin.

Addition of exogenous sphinganine 1-phosphate (1 μM for 5 min) alone stimulated cytosol to membrane translocation of PKCα similar to fumonisn B_1 alone. Co-exposure of fumonisin B_1 with *N*,*N*-dimethylsphingosine (DMS), an inhibitor of sphingosine/sphinganine kinase, prevented the fumonisin B_1-induction of PKCα.

Sphinganine, sphingosine, sphingosine 1-phosphate and ceramide added exogenously, did not alter PKCα cytosolic to membrane translocation at 5 min. Fumonisin B_1 (10 μM), sphinganine, sphingosine and ceramide significantly repressed PKC-α and -δ isoforms at 48 h, whereas all the exogenously added sphingolipids significantly repressed PKC-ε and ζ similar to fumonisin B_1. Sphinganine 1-phosphate and sphingosine 1-phosphate had no effect on PKC-α and -δ membrane levels at 48 h. Addition of myriocin alone at 48 h had no effect on the membrane protein translocation of all isoforms teated. However, co-exposure of myriocin with fumonisin B_1 prevented the inhibitory effects of fumonisin B_1 on PKC isoforms in LLC-PK_1 cells. DMS was cytotoxic to LLC-PK_1 cells at 48 h even at very low concentrations. This study demonstrated that selective and transient activation of PKCα may be due to the fumonisin B_1-induced accumulation of the bioactive sphinganine 1-phosphate. The long-term repression of PKC isoforms by fumonisin B_1 can be prevented by the serine palmitoyltransferase inhibitor, suggesting that accumulation of sphinganine or its metabolite may be involved in the repression of PKC in LLC-PK_1 cells. These findings suggest that the direct or indirect modulation of PKC by these sphingolipids is involved at least in part in the action of fumonisin B_1.

In summary, the results of these findings suggest that the accumulation of sphingoid bases and their metabolites is in part responsible for the fumonisin B_1-induced modulation of PKC activity in LLC-PK_1 cells. Brief treatment with fumonisin B_1 results in the rapid production of sphinganine 1-phosphate, which in turn selectively and transiently activate PKCα. PKCα then activates NF-κB, which ultimately transcribes TNFα. Sphinganine and its metabolites may be predominantly responsible for the fumonisin B_1 inhibition of PKC, NF-κB and TNFα at 24, 48, and 72 h. Although, to a

lesser extent, sphingosine and its phosphate may also play a role in the repression of PKC by fumonisin B_1, if elevated to high enough concentrations. The inhibition of PKC and its downstream signaling pathway by fumonisin B_1 was associated with increased apoptosis. In summary, modulation of PKC and its signal transduction pathways as a result of the accumulation of sphingoid bases and their metabolites by fumonisin B_1 are of importance fumonisin B_1-induced apoptosis and/or carcinogenesis.