MODIFICATION OF FUMONISIN B_1 HEPATOTOXICITY IN MICE: IMPLICATION OF FREE SPHINGOID BASE ACCUMULATION AND CYTOKINE SIGNALING

by

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(Under the Direction of Raghubir P. Sharma)

ABSTRACT

Fumonisin B_1 (FB_1) is a toxic and carcinogenic mycotoxin produced by *Fusarium verticillioides* present on corn worldwide. Fumonisin B_1 disrupts sphingolipid metabolism by inhibiting ceramide synthase and induces expression of cytokines including tumor necrosis factor α (TNFα) in liver leading to perturbation of cell signaling. We hypothesized that FB_1 hepatotoxicity in mice can be modulated by interfering with cell signaling factors.

Myriocin prevented hepatic free sphinganine accumulation and overexpression of TNFα superfamily cytokines. Myriocin did not alter FB_1-induced liver damage; indeed longer treatments with myriocin and FB_1 were lethal. This study suggests that additive inhibition of sphingolipid biosynthesis by myriocin and FB_1 induces greater toxicity. Free sphinganine and /or its metabolites contributed to the induction of hepatic cytokines by FB_1.

Kupffer cells are an important source of hepatic cytokine production. Gadolinium chloride depleted Kupffer cells and attenuated increases in circulating enzyme activities, hepatocyte apoptosis, and free sphinganine following FB_1 treatment. Both gadolinium and FB_1 individually increased expression of selected cell signaling factors in liver; gadolinium did not alter FB_1-induced expression of the above genes. Results indicate that Kupffer cells play a role in FB_1

liver injury. Decrease of sphinganine accumulation and induction of protective TNFα signaling may partly account for gadolinium's ameliorating effect on FB_1-induced hepatotoxicity.

Inhibition of TNFα signaling by either anti-TNFα antibodies or pentoxifylline exacerbated FB_1-induced liver damage. Anti-TNFα antibodies did not alter FB_1-induced accumulation of free sphingoid bases and expression of TNFα, interleukin (IL)-12, and interferon (IFN) γ; pentoxifylline significantly reduced free sphinganine accumulation and TNFα expression without altering IL-12 and IFNγ expression induced by FB_1. These findings suggest a partially protective role of TNFα signaling activation in FB_1 hepatotoxicity.

Silymarin significantly diminished FB_1-induced hepatocyte apoptotic death, while it augmented hepatocyte proliferation. Silymarin dramatically potentiated FB_1-induced accumulation of free sphinganine and sphingosine in both liver and kidney. Silymarin itself slightly increased expression of hepatic TNFα; however, it prevented FB_1-induced expression of genes in the TNFα superfamily. This study suggests that silymarin protected against FB_1 liver damage through inhibition of free sphingoid bases signaling.

INDEX WORDS: Fumonisin B_1, Hepatotoxicity, Tumor necrosis factor α, sphinganine, signal transduction, Myriocin, Pentoxifylline, Silymarin, Kupffer cells

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DEDICATION

to my wife, Yinxiu

for her invaluable support, love and understanding

to my daughters, Chenxi and Rosy (Xizhi)

for their understanding when Dad had to work and not to play

to my father, Jianfa He, and to my mother, Jinhua Wu

for their enduring love, encouragement and inspiration

to my brothers, sisters and friends

who keep me focused on what is important to me

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CHAPTER 1

INTRODUCTION

Fumonisins are a group of mycotoxins produced by *Fusarium verticillioides* (=*F. moniliforme*) present on corn. Fumonisin contamination of animal feeds and human corn-based foods has been reported worldwide (WHO 2000). Fumonisin B_1 (FB_1), the most abundant fumonisin in *F. Verticillioides*, causes equine leukoencephalomalacia (Marasas 2001, Marasas *et al.*1988, Ross *et al.* 1991a, b), and porcine pulmonary edema (Colvin and Harrison 1992, Haschek *et al.* 2001, Osweiler *et al.* 1992). High incidences of human esophageal cancer in southern Africa and China have been epidemiologically associated with consumption of fumonisin-contaminated foods (Chu and Li 1994, Marasas 2001, Yoshizawa *et al.* 1994). Fumonisin B_1 is hepatocarcinogenic in male rats and female $B6C3F_1$ mice (Gelderblom *et al.* 1991, Lemmer *et al.* 2004, Howard *et al.* 2001) and nephrocarcinogenic in male F344 rats (Howard *et al.* 2001). Fumonisins are hepatotoxic and nephrotoxic in laboratory animals (Sharma *et al.* 1997, Voss *et al.* 1998, 2001). The biological effects of FB_1 at the cellular level consist of a mixture of necrosis and apoptosis (Lemmer *et al.* 1999, Sharma *et al*. 1997, Tolleson *et al.* 1996a, b).

Fumonisins structurally resemble free sphingoid bases (sphinganine and sphingosine), and inhibit ceramide synthase (Sphingosine *N*-acyltransferase) leading to inhibition of *de novo* sphingolipid synthesis (Merrill *et al.* 1993, Wang *et al.* 1991). By inhibiting ceramide synthase, fumonisins cause the accumulation of free sphinganine and sometimes of sphingosine in tissue, serum, and urine (Mathur *et al.* 2001, Riley *et al.* 1993, 1996, 1997, Wang *et al.* 1992, 1999). Fumonisins induce depletion of more complex sphingolipids (Wang *et al.* 1992, Yoo *et al.* 1996) and increase formation of other lipid metabolites such as sphingoid base-1-phosphates and downstream metabolites (Merrill *et al.* 2001, Smith and Merrill 1995). Toxicity of FB_1 is well correlated to tissue accumulation of free sphingoid bases (Riley *et al.* 2001, Tsunoda *et al.* 1998,

Yoo *et al.* 1992). Serine palmitoyltransferase (SPT), a key enzyme in *de novo* biosynthesis of sphingolipids, is responsible for the condensation of L-serine into palmitoyl-CoA to produce dihydrosphinganine (Hannun *et al.* 2001). Inhibition of SPT to reduce free sphinganine accumulation reversed FB_1 toxicity (He *et al.* 2002, Riley *et al.* 1999, Schmelz *et al.* 1998, Tolleson *et al.* 1999, Yoo *et al.* 1996). Myriocin, a specific inhibitor of SPT (Miyake *et al.* 1995), prevented accumulation of free sphinganine in kidney of mice exposed to FB_1, suggesting myriocin could be useful in protecting against FB_1 toxicity *in vivo* (Riley *et al.* 1999).

Tumor necrosis factor (TNF)α signaling pathways modulate FB_1 toxicity both *in vivo* and *in vitro*. Fumonisin B_1 treatment induced expression of various cytokines including TNFα and apoptotic signaling genes (Bhandari and Sharma 2002a, b). In response to lipopolysaccharide stimulation, peritoneal macrophages from FB_1-treated mice produced higher amount of TNFα than controls (Dugyala *et al*. 1998). Fumonisin B_1 hepatotoxicity was reduced in mice carrying human TNFα transgene or lack of either TNF receptor (TNFR) 1 (P55) or TNF receptor 2 (P75) (Sharma *et al.* 2000a, b, 2001); and TNFα knockout mice were more sensitive to FB_1 hepatotoxicity than their wild-type counterparts (Sharma *et al.* 2002). Transfection of a baculovirus gene, inhibitor of apoptosis (IAP), protected renal cells and fibroblasts from FB_1-induced apoptosis (Jones *et al.* 2001, Ciacci-Zanella and Jones 1999). Expression of TNFR-associated protein (TRAP) 2 was increased in FB_1-sensitive CV-1 cells but repressed in FB_1-resistant COS cells (Zhang *et al.* 2001), further suggesting roles of TNFα signaling pathways in FB_1 toxicity.

Fumonisin B_1 modulates a variety of cell signaling molecules other than TNFα, such as interferon (IFN) γ, interleukin (IL)-1, 6, 12, and c-my (Bhandari and Sharma 2002a, b, Bhandari

et al. 2002a). In response to FB_1, Kupffer cells, the resident macrophages in liver, express TNFα (Bhandari *et al.* 2002b). Kupffer cells play an important role in cell-cell interactions in liver and are involved in the signaling system of the liver by producing cytokines such as TNFα and IL-6. These cytokines act on hepatocytes and other cell types in the liver to produce the protective or deleterious effects on cell growth and survival (Bradham *et al.* 1998).

Free sphingoid bases, sphingoid base-1-phosphates, ceramide and complex sphingolipids are maintained a relative balance in cells and thereby regulate cell growth, proliferation, survival and death. Sphingolipids are important components of cellular structures and play important cell signaling roles (Maceyka *et al.* 2002, Ohanian and Ohanian 2001, Priechl and Baumruker 2000, Pyne and Pyne, 2000, Spiegel and Milstien, 2002). Fumonisins deregulate sphingolipid metabolism leading to accumulation of free sphingoid bases and reduction of ceramide and complex sphingolipids (Merrill *et al.* 2001, Riley *et al.* 1993, 1996, 1997, Wang *et al.* 1992, Yoo *et al.* 1992, 1996). Disruption of sphingolipid metabolism alters functions of various cell signaling pathways in response to FB_1. However, it remains controversial whether or not disruption of sphingolipid metabolism accounts for the induction of genes for cell signaling factors. Prevention of sphinganine accumulation by myriocin did not reduce FB_1-induced TNFα in LLC-PK_1 cells, a renal kidney epithelial cell line, suggesting that FB_1-induced expression of TNFα was unrelated to free sphinganine accumulation in these cells (He *et al.* 2001). Silymarin, a mixture extracted from seeds and fruits of *Silybum marianum*, is a very strong antioxidant, and can prevent apoptosis by inhibiting activation of caspases. Silymarin did not prevent free sphinganine accumulation but protected against FB_1 cytotoxicity in LLC-PK_1 cells (He *et al.* 2002).

Changes in the balance of different cell regulatory molecules are likely to play an important role in the pathogenesis of fumonisin-induced toxicity. The ability of FB_1 to alter cell signal transduction due to its disruption of sphingolipid metabolism and modulation of cell signaling cascades is involved in FB_1 toxicity and carcinogenicity (Gelderblom *et al.* 2001, Merrill *et al.* 2001, Riley *et al.* 2001). A better understanding of the mechanisms of fumonisin toxicity will dramatically improve risk assessment and provide evidence for investigating effective therapeutics for fumonisin intoxication in humans and animals.

The objective of the research comprising this dissertation is to test the hypothesis that **pharmacologic intervention of cell signaling molecules modifies FB_1-induced hepatotoxicity.** The following specific aims were attempted to achieve the objective.

1. **Investigate the role of free sphinganine in FB_1-induced liver damage and expression of hepatic cell signaling factors in mice.**
2. **Evaluate the effect of Kupffer cell depletion by gadolinium chloride on FB_1 hepaptotoxicity in mice.**
3. **Examine the effects of pentoxifylline and anti-TNFα antibodies on FB_1 hepatotoxicity.**
4. **Evaluate the effectiveness of silymarin in protecting against FB_1 toxicity *in vivo*.**

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CHAPTER 2

LITERATURE REVIEW

Fumonisins: mycotoxins

The study on moldy corn-related equine leukoencephalomalacia and possible role of fungal toxin in the etiology of human esophageal cancer in South Africa led to isolation and chemical characterization of a novel family of mycotoxins—fumonisins. Fumonisins were first isolated from the fungus *Fusarium verticillioides*, a common endophytic fungus on corn and corn-based product (Gelderblom *et al.* 1988), and structurally identified in 1988 (Bezuidenhout *et al.* 1988). Several types of fumonisins have been identified as products of *F. verticillioides*, fumonisin A_{1-3}, fumonisin B_{1-4}, fumonisin C_{1-4}, and fumonisin P_{1-3} (WHO 2000). In naturally contaminated corn and corn-based products, fumonisin B_1 (FB_1) is the most abundant and toxic of all fumonisin isomers investigated.

Occurrence of fumonisins

Significant fumonisin accumulation in corn occurs when weather conditions favor *Fusarium* kernel rot, and severity of ear infection is a good indicator of fumonisin accumulation in corn ears artificially inoculated with *F. verticillioides* (Pascale *et al.* 2002). *Fusarium. verticillioides* grow well at higher temperatures; ear rot and fumonisin production are related to drought and insect stress and growing hybrids outside their areas of adaptation (Miller 2001)

Because of the wide distribution of *F. verticillioides* in the world, it is not surprising to find global surveys reporting the presence of fungal strains capable of producing fumonisins. Dutton (1996) and WHO (2000) have summarized the results of fumonisin contamination in corn and corn-based products with a range of levels from 0.004 ppm – 334 ppm.

Structure of fumonisin B_1

Fumonisin B_1 is water-soluble polar molecule comprised of a long-chain aminopentol backbone (Bezuidenhout *et al.* 1988). The structure of FB_1 consists of a 20-carbon backbone; an amine group at C2; three hydroxyl groups at C3, C5, and C10; two methyl groups at C12 and C16; and two tricarballylic acid monoester groups at C14 and C15 (Fig 2.1). Fumonisin B_1 has a remarkable structural similarity to sphinganine and sphingosine, the long-chain sphingoid base backbone of sphingolipids (Fig. 2.1).

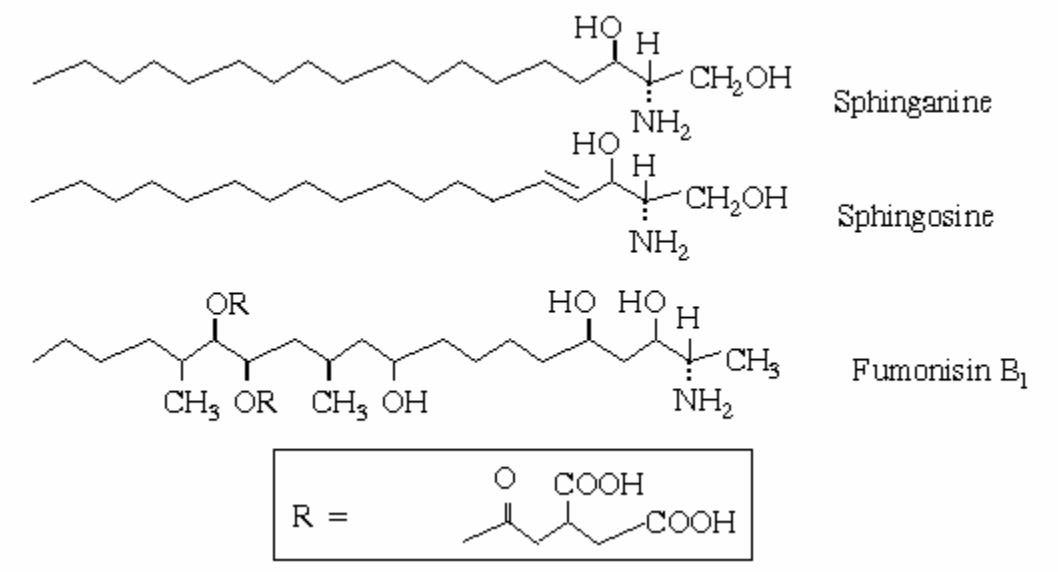

Fig. 2.1 Structures of sphinganine, sphingosine and fumonisin B_1

Toxicity of fumonisin B_1

Fumonisin B_1 induces species- and organ-specific toxicity in different species. This toxin causes equine leukoencephalomalacia (Kellerman *et al.* 1990, Marasas *et al.* 1988, Marasas 2001). Intravenous injection of pure FB_1 also causes porcine pulmonary edema (Colvin and Harrison 1992). Due to abnormal climate conditions during the fall of 1989 and spring of 1990, widespread, large-scale outbreaks of equine leukoencephalomalacia and porcine pulmonary edema occurred in the United States. Many horses and pigs died after ingesting fumonisin-contaminated corn (Osweiler *et al.* 1992, Ross *et al.* 1991a, b, 1992). Fumonisin B_1 is a cardiovascular toxin, which is assumed to be responsible for the porcine pulmonary edema (Haschek *et al.* 2001, Smith *et al.* 1999). Liver injury has been observed in all animals tested so far, and kidney is also a target of fumonisins in most animals (WHO 2000, Riley *et al.* 1994, Sharma *et al.* 1997, Voss *et al.* 1995, 1998, 2001). Fumonisin B_1 is immunotoxic in female mice (Johnson and Sharma 2001), and rats (Theumer *et al.* 2002). Decreased phagocytosis of macrophages exposed to FB_1 has been documented *in vitro* and *in vivo* (Haschek *et al.* 2001, Qureshi and Hagler 1992). Fumonisin B_1 caused a mixture of necrosis and apoptosis, accompanying mitogenesis (Lemmer *et al.* 1999, Sharma *et al.* 1997, Tolleson *et al.* 1996 a, b).

Studies have demonstrated that FB_1 is carcinogenic in liver and kidney of rodents (Geldeblom *et al.* 1991, Lemmer *et al.* 2004, Howard *et al.* 2001). Fumonisin B_1 promoted carcinogens such as aflatoxin B_1 and N-methyl-N'-nitro-nitrosoguanidine-initiated liver tumors in rainbow trout (Carlson *et al*. 2001). The high incidence of esophageal cancer in some areas of southern African and China has been associated with consumption of corn-based foods highly contaminated with *F. verticillioides* and fumonisins (Chu and Li 1994, Marasas 2001, Yoshizawa *et al.* 1994). Based on data available for carcinogenicity, the International Agency

for Research on Cancer evaluated the toxins produced by *F. verticillioides* as Group 2B carcinogens (possible human carcinogens) in 1993 (IARC, 1993). High levels of FB_1 in human food have been correlated with increased incidence of neural tube defects in parts of Texas, USA (Hendricks 1999). In mouse embryo cultures, FB_1 produces embryotoxicity and neural tube defects (Sadler *et al.* 2002). It has been documented or plausibly suggested that high incidences of neural tube defects occur in some regions of the world (Guatemala, South Africa, and China) where substantial consumption of fumonisins occurs (Marasas *et al.* 2004).

Sphingolipid metabolism

Sphingolipids are defined by their characteristic 1,3-dihydroxy, 2-aminoalkane (sphingoid bases) backbones; complex sphingolipids have a fatty acid linked to an amide linkage (forming ceramides) and a polar headgroup, such as sphingomyelin, and glycosphingolipids (Merrill *et al.* 2001).

The incorporation of L-serine into palmitoylCoA by serine palmitoyltransferase to form 3-ketosphinganine initiates the *de novo* biosynthesis of sphingolipids (Fig. 2.2). Ceramide is generated by (1) *de novo* synthesis from sphinganine and sphingosine catalyzed by ceramide synthase, and (2) catabolism of sphingomyelin by different forms of sphingomyelinase (Dbaibo *et al.* 2001, Maceyka *et al.* 2002). Ceramide is deacylated by ceramidases, yielding a sphingoid base, the most common of these in mammals is sphingosine. In order for the sphingoid base to be catabolized, it must be phosphorylated on the 1-OH by sphingosine kinases. The product of this reaction, sphingosine-1-phosphate, is irreversibly degraded in the endoplasmic reticulum by sphingosine-1-phosphate lyase to the end metabolites. Cells also contain sphingosine-1-phosphate phosphatase and cermide synthase activities, allowing sphingosine-1-phosphate to be

converted back to sphingosine and ceramide. Cells maintain a dynamic equilibrium in the levels of ceramide, sphingosine, sphingosine-1-phosphate, and other metabolites (Maceyka *et al.* 2002, Spiegel *et al.* 1998).

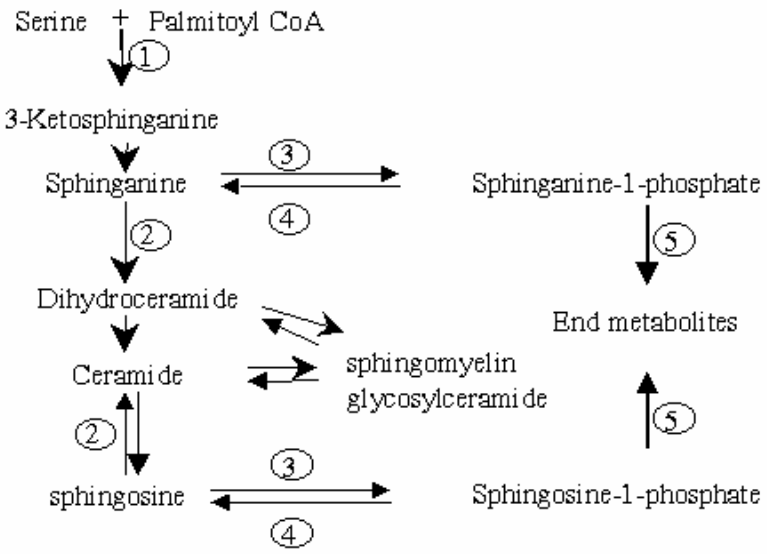

Fig. 2.2 Sphingolipid metabolism. → represents *de novo* biosynthesis pathway for sphingolipids. Important enzymes for sphingolipid metabolism: 1. Serine palmitoyltansferase, 2. Ceramide synthase, 3. Sphingosine kinase, 4. Sphingosine-1-phosphate phosphatase, 5. sphingosine-1-phosphate lyase. Modified from Merrill *et al.* 2001.

Roles of sphingolipids in cell signal transduction

Sphingoilipds are located in cellular membranes, lipoproteins, and other lipid-rich structures. Complex sphingolipids are critical for maintenance of membrane structure (Iwabuchi *et al.* 1998, Riboni *et al.* 1997), serve as binding sites for extracellular matrix proteins, and modulate the behavior of growth factor receptors. Complex sphingolipids also function as

precursors for second messengers that mediate cell responses to cytokines, differentiation factors, stress, and toxic insults (Dbaibo and Hannun, 1998, Merrill *et al.* 1997, Vit and Rosselli, 2003).

In addition to being structural constituents of biological membranes, sphingolipids play a role as signaling molecules. Some agonists, such as TNFα, and platelet-derived growth factor (PDGF), induce receptor-coupled activation of sphingomyelinase to generate ceramide, which can be converted into sphingosine and sphingosine-1-phosphate (Dbaibo *et al.* 2001, Spiegel *et al.* 1998). Each of these intermediates is a bioactive compound that can affect protein kinases, phosphoprotein phosphatase, and other cell regulatory pathways leading to growth inhibitory, pro-apoptotic, and anti-apoptotic effects (Merrill *et al.* 1997, 2001, Merrill 2002, Spiegel *et al.* 1998). In general, ceramide and sphingosine have been shown to initiate growth arrest and apoptosis (Hannun and Obeid, 2002, Merrill *et al.* 2001, Cuvillier 2002). Sphingosine-1-phosphate can act as an intracellular second messenger as well as an extracellular ligand for specific G-protein coupled receptors (GPCRs), sphingosine-1-phosphate receptors (S1PRs), to initiate signal transduction through G-proteins leading to affect activities of phospholipase (PL) C, D, adenylyl cyclase and mitogen-activated protein kinases (Maceyka *et al.* 2002, Pyne and Pyne, 2000, Spiegel and Milstien, 2002). Sphinosine-1-phosphate has an opposing role in cell growth. For example, sphingosine-1-phosphate stimulates proliferation or triggers apoptosis in mesangial cells, depending on cell density; however, it was believed that the apoptotic effect was not caused by sphingosine-1-phosphate itself, but by sphingosine produced upon removal of the phosphate group (Gennero *et al.* 2002). In human hepatic myofibroblasts, sphingosine-1-phosphate triggered pro-apoptotic effect through an S1PRs-independent but caspase 3-dependent mechanism, while it stimulated cell survival pathways via S1PRs–dependent mechanisms

(Davaille *et al.* 2002). Higher concentrations of sphingosine and ceramide are required for apoptosis induction than sphingosine-1-phosphate, and the increase in the activity of caspase 3 occurred earlier in human hepatic myofibroblasts exposed to sphingosine-1-phosphate than those treated with either sphingosine or ceramide ((Davaille *et al.* 2002). These results indicated that the apoptotic effect of sphingosine-1-phophate is unrelated to generation of sphingosine or ceramide in human hepatic myofibroblasts.

The intermediates of *de novo* sphingolipid biosynthesis are also highly bioactive. The most important intermediates in this pathway include free sphinganine, dihydroceramide, and ceramide. Under normal conditions, these compounds are kept at low levels in cells and maintain a dynamic balance (Merrill *et al.* 2001, Spiegel *et al.* 1998). Perturbation of sphingolipid metabolism causes imbalance of these bioactive intermediates and alterations of cell functions.

Fumonisins disrupt sphingolipid metabolism

Fumonisins potently inhibit activity of ceramide synthase, a critical enzyme for *de novo* synthesis of sphingolipid (Merrill *et al.* 1993, Wang *et al.* 1991). In primary hepatocytes, the IC_{50} (50% inhibitory concentration) of FB_1 for inhibition of serine incorporation into sphingosine is approximately 0.5 μM (Wang *et al.* 1991). In LLC-PK_1 cells, the IC_{50} for inhibition of *de novo* sphingosine synthesis is approximately 10 ~ 15 μM (Yoo *et al.* 1992). Fumonisin-induced disruption of sphingolipid metabolism also has been demonstrated in all animals that have been studied. The increase of free sphingoid bases have been consistently observed in tissues (Enongene *et al.* 2000, 2002, Riley *et al.* 1993, 1994), and serum (Mathur *et al.* 2001, Wang *et al.* 1992, 1999, Riley *et al.* 1993, 1994, 1996, 1997, 2001, Voss *et al.* 1995, 1998, 2001) from

animals exposed to fumonisins or given feed contaminated with *F. verticillioides*, the source of fumonisins.

Fumonisin B_1 appears to interact with the binding sites of sphingoid base and fatty-CoA competitively (Merrill *et al.* 2001). Structure-activity investigations suggest that the aminoalkyl backbone competes with sphingoid base binding site of ceramide synthase, and the anionic tricarballylic acid side chains interfere with utilization of the co-substrate fatty acyl-CoA (Merrill 2002, Merrill *et al.* 2001). Inhibition of ceramide synthase leads to accumulation of intracellular as well as extracellular free sphingoid bases and their metabolites or derivatives such as sphingoid base-1-phosphate (Merrill *et al.* 2001, Smith and Merrill 1995, Wang *et al.* 1992, Yoo *et al.* 1996). Furthermore, FB_1 blocks the production of sphingomyelin and diacyglycerol (DAG) from ceramide and phosphatidylcholine (PC), and depletes complex sphingolipids in long-term exposures as a consequence of ceramide synthase inhibition (Baron and Malhotra 2002, Merrill *et al.* 1993, 2001, Wang *et al.* 1992, Wu *et al.* 1995, Yoo *et al.* 1996).

Role of sphingolipid metabolism perturbation in FB_1 toxicity

The accumulation of free sphinganine in tissues is a very early event and precedes onset of cell death or tissue injury in FB_1 exposure both *in vitro* and *in vivo*. The increase in free sphinganine and decrease in the incorporation of ^{14}C-serine into sphingosine can be detected within a few hours of FB_1 exposure in primary cultured rat hepatocytes or LLC-PK_1 cells (Wang *et al.* 1991, Yoo *et al.* 1992, 1996). In LLC-PK_1 cells, the accumulation of free sphinganine at 7 hr preceded the decrease in complex sphingolipids at 24 hr, which was observed before the significant inhibition of cell growth at 48 hr (Yoo *et al.* 1992, 1996). Increase in serum sphinganine was detected before elevation of serum alanine aminotransferase activity, indicative

of liver damage, in horses and pigs exposed to fumonisins (Gumprecht *et al.* 1998, Wilson *et al.* 1992), suggesting that disruption of sphingolipid metabolism in tissues is responsible for the pathogenesis of fumonisins (Riley *et al.* 1994, 1997, Wang *et al.* 1992).

Accumulation of free sphingoid bases is positively correlated with the incidence and severity of apoptotic and necrotic cell death observed *in vivo* exposure of animals to FB_1 (Riley *et al.* 1997, 2001, Tsunoda *et al.* 1998). Pretreatment of cells with inhibitors of serine palmitoyltransferase, the first enzyme in the pathway of sphingolipid *de novo* biosynthesis, blocked the accumulation of free sphinganine, and consequently suppressed the toxicity of FB_1 in Swiss 3T3 cell (Schreder *et al.* 1994), human colonic cells (Schmelz *et al.* 1998), human keratinocytes (Tolleson *et al.* 1999), and LLC-PK_1 cells (He *et al.* 2002, Riley *et al.* 1999, Yoo *et al.* 1996). Depletion of cermide and other complex sphingolipids was believed to participate in FB_1-induced cell death (Tolleson *et al.* 1999, Yoo *et al.* 1996). These studies indicate that accumulation of free sphingoid bases and depletion of complex sphingolipids both are important contributors to FB_1 toxicity.

Changes in the levels of cellular sphingolipids can disrupt cell membrane functions dependent on complex sphingolipids. For example, the folate receptor, a receptor responsible for folate uptake, is a glycosylphosphatidylinositol-anchored protein that is associated with membrane microdomains (rafts) enriched in cholesterol and sphingolipids (Brown and London, 1998). The folate receptor requires sphingolipids and cholesterol for normal functions. Treatment of intestinal cells (Caco-2 cells) with FB_1 in cultures disrupted folate receptor functions with subsequent reduced cellular folate uptake as a consequence of sphingolipid depletion (Stenvens and Tang, 1997). Depletion of complex sphingolipids may contribute to neural tube defects (NTD) as supplementation of folic acid and ganglioside G_{M1} to pregnant

dams reduced the incidence of NTD in FB_1 exposure (Sadler *et al.* 2002). FB_1 perturbation of sphingolipid metabolism is expected to deregulate sphingolipid-mediated cell signal transduction and subsequently cell survival and death.

Fumonisin-modulated expression of cell signaling genes

In addition to perturbations of cell signal transduction resulting from disrupted sphingolipid metabolism, FB_1 treatment causes increased expression of genes for cytokines, tumor necrosis factor (TNF) α, interleukin (IL)-1α, IL-1β, IL-1Ra, IL-12, and interferon (IFN) γ in mouse liver with the greatest increase in TNFα, IFNγ, and IL-6 (Bhandari and Sharma 2002a, b, Bhandari *et al.* 2002a). Alterations in gene expression occur very early following FB_1 treatment. For example, per os treatment of mice with FB_1 significantly increased expression of IFNγ and TNFα in 4 hr and 8 hr, respectively; increased expression of other genes can be detected within 4 hr – 8 hr FB_1 exposure (Bhandari *et al.* 2002a, Bhandari and Sharma 2002b).

Genes in TNFα signal pathways were increased during FB_1 exposure in mouse liver (Bhandari and Sharma 2002a, b). Expression of TNF receptor (TNFR) 1, TNFR-associated death domain (TRADD), and caspase 3 significantly increased after 5-daily FB_1 treatments (Bhandari and Sharma 2002a). No changes were observed in expression of receptor-interacting protein (RIP) and caspase 8 in a subacute FB_1 treatment (Bhandari and Sharma 2002a, b).

Exposure of mice to FB_1 for 5 days caused no alterations in Fas signaling molecules, namely Fas, Fas-associated death domain (FADD), Fas-associated protein factor (FAF), and Fas-associated protease (FAP, Bhandari and Sharma 2002a).

Expression of genes for c-Myc, B-Myc, Max and Mad were all activated after FB_1 treatment, without any gender differences with the highest increase in c-Myc, followed by B-

Myc, Mad and Max. Increased expression of pro-apoptotic Bcl-2 family genes, Bax and Bad was also observed following FB_1 treatment for 5 days (Bhandari and Sharma 2002a, b).

Increased expression of TNFα was consistently observed in peritoneal macrophages from mice treated with FB_1 (Dugyala *et al.* 1998). In LLC-PK_1 cells, FB_1 transiently increased expression of TNFα under different conditions (Gopee and Sharma, 2004, He *et al.* 2001). In response to FB_1, TNFR1-associated protein 2 was increased in FB_1-sensitive CV-1 kidney cells but decreased in FB_1-resistant COS-7 cells (Zhang *et al.* 2001). It remains controversial whether disrupted sphingolipid metabolism causes alterations of gene expression in FB_1 exposure. Previous studies have shown that sphingosine-1-phosphate and sphinganine-1-phosphate but not ceramide or sphingosine enhanced secretion of IL-2 and IFNγ in peritoneal blood T cells stimulated by anti-CD3 plus anti-CD28 (Jin *et al.* 2003). However, TNFα expression remained increased in FB_1-treated LLC-PK_1 cells treated with myriocin to completely prevent FB_1-induced sphinganine accumulation (He *et al.* 2001).

Kupffer cells express high level of TNFα in FB_1 exposure

The liver has its own innate immune system consisting of Kupffer cells, which are the largest population of resident macrophages in the body, and hepatic natural killer cells (Seki *et al.*, 2000). This innate immune system is responsible for the localized action involving the cytokine network in liver. Hyperplaisa of Kupffer cells occurs in response to FB_1 treatment (Haschek *et al.* 2001, Howard *et al.* 2002, Thumer *et al.* 2002). Mice treated with 2.25 mg/kg FB_1 daily for 5 days had increased expression of TNFα, IFNγ, and IL-12 p40 in liver without any changes in these cytokines in the kidney or spleen, suggesting the localized site of production of these cytokines. Kupffer cells express TNFα in response to FB_1, as detected by *in*

situ hybridization using digoxigenin-labeled antisense mouse TNFα probes (Bhandari *et al.* 2002b). The primary source of IL-12 production is also Kupffer cells in liver. Selective increase in T helper (Th1) cell cytokines (IFNγ and TNFα) with no change in Th2 cyokines (IL-4, IL-6, and IL-10) suggests that TNFα and IL-12 produced by Kupffer cells stimulate Th1 and natural killer (NK) T lymphocytes to produce IFNγ. The increased IFNγ further induces production of IL-12 and TNFα through a positive feedback loop (Bhandari *et al.* 2002b). Other cell types, biliary epithelial cells and venous endothelial cells, also produce TNFα in regenerating liver (Loffreda *et al.* 1997).

Biological roles of TNFα

TNFα is a pleitropic cytokine produced by many cell types in response to inflammation, infections, and many environmental challenges. The activities of TNFα are mediated through two distinct transmembrane receptors, TNFR1 and TNFR2 (Baud and Karin 2001, Meldrum 1998). TNFα produces a wide spectrum of cellular responses, including cell proliferation, differentiation, and apoptosis ((Baud and Karin 2001, Bradham *et al.* 1998, Heyninck and Beyaert, 2001, Heyninck *et al.* 2003, Locksley *et al.* 2001), and it has been implicated in the pathogenesis of a variety of human diseases, including sepsis, cancer, rheumatoid arthritis, Crohn's disease, and tissue injuries (Bradham *et al.* 1998, Heyninck *et al.* 2003, Luster *et al.* 1999, 2000, Meldrum, 1998, van Hogezand and Verspaget, 1998). In addition to its deleterious effects, TNFα plays a protective role in preventing tissue injury depending on its intracellular concentration and duration (Bruce-Keller *et al.* 1999, Kurrelmeyer *et al.* 2000, Nawashiro *et al.* 1997, Shohami *et al.* 1999).

TNFα-induced apoptosis primarily depends on the recruitment of a complex of adaptor proteins, including TRADD and FADD leading to the further recruitment and activation of various caspases, and subsequently, to programmed cell death (Baud and Karin 2001, Tartaglia *et al.* 1993). On the other hand, TNFR-associated factors (TRAF) mediate the cell activation, inflammatory reaction, and anti-apoptotic function of the TNF receptor superfamily (Arch et al. 2000, Dempsey *et al.* 2003, Locksley *et al.* 2001, Wajant *et al.* 2003). To date, six members of TRAF proteins have been identified in mammals from TRAF1 to TRAF6, with TRAF2 being the prototypical member of TRAF family. TRAF2 can interact directly or indirectly with various members of the TNF receptor superfamily to mediate the signal transduction via these receptors. TRAF2 can also interact with numerous intracellular proteins, such as RIP, MAPK kinase kinase, NFκB-inducing kinase (NIK), and cellular inhibitors of apoptosis proteins (cIAPs), and thereby transduces TNF signals required for the activation of the transcription factor NFκB, c-jun N-terminal kinase (JNK), and anti-apoptosis (Dempsey *et al.* 2003, Liu *et al.* 1996, Song *et al.* 1997, Wang *et al.* 1998).

Tumor necrosis factor α affects activities of several enzymes in sphingolipid metabolism pathways. For example, TNFα activates sphingomyelinase and inhibits sphingomyelin synthase as well as glucosylceramide synthase leading to generation of ceramide with subsequent induction of apoptosis (Adam-Klages *et al.* 1998, Bourteele *et al.* 1998, Garcia-Ruiz *et al.* 2003, Hannun *et al.* 2001). Previous studies have shown that TNFα stimulated activation of sphingosine kinase resulting in production of sphingosine-1-phosphate (Xia *et al.* 1999). A physical and functional interaction between TRAF2 and sphingosine kinase specifically transduces TNFα signal to activation of NFκB and anti-apoptosis (Xia *et al.* 1999, 2002).

Sphingosine-1-phosphate has emerged as an anti-apoptotic and mitogenic factor (Cuvillier *et al.* 1996, Maceyka *et al*. 2002). In addition to activation of NFκB, TNFα-induced generation of sphingosine-1-phosphate is able to activate phosphatidylinositol 3-kinase/Akt pathway, and subsequent protect cells from apoptosis (Osawa et al. 2001). Sphingosine kinase activation and subsequent sphingosine-1-phosphate production mediated or modulated TNFα effects (Osawa et al. 2001, Pettus *et al.* 2003, Xia *et al.* 1999, 2002). Taken together, all studies indicated that sphingolipid metabolites are important mediators in TNFα signal transduction.

TNFα is an important contributor to FB_1 hepatotoxicity

Expression of various cytokines and apoptotic signaling molecules were increased in response to FB_1 treatment (Bhandari and Sharma 2002a, b, Bhandari *et al.* 2002a, b). Cytokines and other cell signaling molecules play important roles in regulation of cell proliferation, differentiation, pro-apoptosis and anti-apoptosis. Each altered gene product could partially contribute to FB_1 toxicity. Of these genes, consistent induction of TNFα can modulate expression of other genes by affecting transcription factors. Previous studies have provided strong evidence that TNFα is an important mediator of FB_1-induced hepatotoxicity in mice. Treatment of mice with anti-TNFα antibodies reversed the acute hematologic effects of FB_1 and peritoneal macrophages treated with FB_1 produced increased TNFα in response to lipopolysaccharide *ex vivo* (Dugyala *et al.* 1998). Hepatotoxicity following FB_1 treatment was attenuated in transgenic mice carrying human TNFα gene (TG) (Sharma *et al.* 2000a). The observed less elevation of circulating liver enzymes in TG mice paralleled the accumulation of liver free sphinganine following FB_1 treatment, and the attenuated hepatotoxicity in TG liver could be explained by activation of nuclear factor (NF)κ B, which is essential for protection

against cell death (Beg and Baltimore 1996, Sharma *et al.* 2000a). In accordance with these findings, deletion of TNFα gene (TKO) increased susceptibility of liver to FB_1 toxicity (Sharma *et al.* 2002). Mice lacking either TNFR1 or TNFR2 showed reduced sensitivity to FB_1 hepatotoxicity demonstrated by histopathological examination and increases in activities of circulating liver enzymes, alanine aminotransferase (ALT) and aspartate aminotransferase (AST, Sharma *et al.* 2000b, 2001). The increased expression of hepatic TNFα in response to FB_1 treatment was not different between TNFR knockout mice and their wild-type counterparts (Sharma *et al.* 2000b, 2001). These studies indicated that both TNFRs are required for maximal FB_1 hepatotoxicity. In TKO mice, we found that there was an increased constitutive expression of hepatic Fas and Fas-related molecules, FAF, FADD, FAP, RIP, TNF-related apoptosis-inducing ligand (TRAIL) and caspase 8 compared to their wild-type mice; c-myc, bax and IL-1α was also increased; all these genes were further increased in response to FB_1, suggesting activation of Fas pathways and other pro-apoptotic molecules by FB_1 in TKO contributed to the increased sensitivity to FB_1 toxicity (Sharma *et al.* 2003). The available data suggest that TNFα play both protective and deleterious roles in FB_1-induced hepatotoxicity in mice.

Pigs fed fumonisin-containing *Fusarium moniliforme* culture material developed pulmonary edema, and TNFα-like activity was observed in serum of affected pigs (Guzman *et al.* 1997). Fumonisin B_1 induced apoptosis in CV-1 cells, primary human lung fibroblasts and neonatal kidney cells (Ciacci-Zanella and Jones 1999, Wang *et al.* 1996), and the apoptotic effects in these cells can be protected by transfection of a baculovirus gene, inhibitor of apoptosis (IAP), an important apoptosis inhibitor in TNF signal pathway Ciacci-Zanella and Jones 1999,

Jones *et al.* 2001). These studies supported that TNFα signal pathways are involved in FB_1 toxicity.

Summary and conclusion

Fumonisin inhibition of ceramide synthase leads to perturbation of the sphingolipid reheostat and subsequent interference with sphingolipid-mediated cell signaling events. Fumonisin also alters the cascade of cell signaling pathways as a consequence of modulating production of cell signaling molecules such as TNFα and other pro-apoptotic signaling factors. Both disruption of sphingolipid metabolism and alterations of cell signaling cascade like in TNFα pathways contribute to FB_1 toxicity. However, it remains unclear whether modulation of cell signaling factors is dependent on or independent of the disruption of sphingolipid metabolism in response to FB_1. Pharmacologic intervention of cell signaling is predicted to modulate FB_1 toxicity.

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CHAPTER 3

MYRIOCIN MODULATION OF FUMONISIN EFFECTS IN LIVER OF MICE: RELATION TO DISRUPTION OF SPHINGOLIPID METABOLISM[1]

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Abstract

Fumonisin B_1 (FB_1), a mycotoxin produced by *Fusarium verticillioides* present on corn and corn-based products, causes species- and organ- specific diseases. The hepatotoxic effects of FB_1 in Balbc mice have been closely correlated with the accumulation of free sphinganine, a marker for ceramide synthase inhibition, and reduced biosynthesis of more complex sphingolipids. It has been shown that FB_1 alters expression of many cell signaling factors. In the current study, we used myriocin, a specific inhibitor of serine palmitoyltransferase, to investigate the role of free sphinganine accumulation on FB_1-induced hepatotoxicity and increased expression of selected signaling genes in Balbc mice. The mice were pretreated daily with myriocin (intraperitoneal injection) at 1.0 mg/kg 30 min before injection of 2.25 mg/kg of FB_1 for 3 days. Results showed that this dose of myriocin alone was not hepatotoxic and the combination of myriocin plus FB_1 completely prevented the FB_1-induced elevation of hepatic free sphinganine and reversed the FB_1-induced overexpression of selected cell signaling genes to constitutive levels. However, although the combination slightly, but significantly, attenuated FB_1-increased activity of plasma aspartate aminotransferase, it did not reduce FB_1-induced hepatocyte apoptosis or increased PCNA staining. The hepatotoxic effects in mice seen in this study are most likely due to a combination of factors including accumulation of free sphinganine, depletion of more complex sphingolipids and sphingomyelin, and other unknown mechanisms. The accumulation of free sphinganine and /or its metabolites contributed to the FB_1-induction of the cell signaling factors including tumor necrosis factor (TNF)α, TNF related apoptosis-inducing ligand, TNF receptor 1, lymphotoxin β, interferon γ, and transforming growth factor β1.

Key words: fumonisin, myriocin, cytokine, hepatotoxicity, sphinganine, sphingolipid

1. Introduction

The study of moldy corn-related equine leukoencephalomalacia (ELEM) and the possible role of fungal toxins in the etiology of human esophageal cancer in South Africa led to isolation and chemical characterization of a novel family of mycotoxins—fumonisins. Fumonisins were first isolated in 1988 from the fungus *Fusarium verticillioides*, a common endophytic fungus in corn (Gelderblom et al., 1988). Several types of fumonisins have been identified so far as products of *F. verticillioides* in naturally contaminated corn and corn-based products. Fumonisin B_1 (FB_1) is the most abundant and most toxic of all fumonisin isomers investigated so far (WHO, 2000). Fumonisin B_1 induces species-specific toxicity in different species. This toxin is known to cause ELEM (Marasas, 2001) and in pigs pulmonary edema and cardiovascular damage (Haschek et al., 2001, Smith et al., 1999). The high incidence of esophageal cancer in some areas of southern African and China was correlated with *F. verticillioides* infection and fumonisin levels in home grown corn (Marasas, 2001, Yoshizawa et al., 1994). It has been demonstrated that FB_1 is hepato- and nephro-carcinogenic in male rats (Gelderblom et al., 1991, Howard et al., 2001), and hepatocarcinogenic in female mice (Howard et al., 2001). Fumonisins are hepatotoxic and nephrotoxic in rodents (Sharma et al., 1997, Voss et al., 1998, 2001). The cellular effects of fumonisins consist of a mixture of necrosis and apoptosis and regenerative proliferation (Lemmer et al., 1999, Howard et al., 2001, Sharma et al., 1997).

Fumonisins are structurally similar to free sphingoid bases (sphinganine and sphingosine), and inhibit ceramide synthase (sphingosine *N*-acyltransferase), a critical enzyme in the pathway of *de novo* sphingolipid synthesis (Merrill et al., 2001, Wang et al., 1991). By inhibiting ceramide synthase, FB_1 increases the level of free sphinganine in tissue, serum, and urine (Riley et al., 1993, 1996, 1997, Wang et al., 1992, 1999), decreases complex sphingolipids (Wang et al.,

1992, Yoo et al., 1996), and increases formation of other lipid metabolites such as sphingoid base-1-phosphates and downstream metabolites (Merrill et al., 2001, Smith and Merrill 1995). The hepatotoxicity of FB_1 is closely correlated with the accumulation of free sphinganine in male Balbc (Tsunoda et al., 1998,) and other mouse strains (Riley et al., 2001).

Serine palmitoyltransferase (SPT), the first enzyme in the pathway for *de novo* biosynthesis of sphingolipids, catalytically incorporates L-serine into palmitoyl-CoA to produce 3-keto-sphinganine, the immediate precursor to sphinganine (Hannun et al., 2001). Inhibition of SPT to reduce free sphinganine accumulation reversed FB_1 toxicity in mammalian cell cultures (He et al., 2002, Riley et al., 1999, Schmelz et al., 1998, Tolleson et al., 1999, Yoo et al., 1996). Myriocin, a selective inhibitor of SPT (Miyake et al., 1995), prevented accumulation of free sphinganine in kidney of mice exposed to FB_1, and therefore it has been proposed that myriocin might be useful in protecting against FB_1 toxicity *in vivo* (He et al., 2002, Riley et al., 1999a).

Numerous studies have shown that FB_1 can alter expression of cytokines. For example, FB_1 treatment induced expression of various cytokines including tumor necrosis factor (TNF) α and pro-apoptotic signaling genes in liver and kidney of mice (Bhandari and Sharma, 2002, Bhandari et al., 2002). Peritoneal macrophages from FB_1-treated mice produced more TNFα in response to lipopolysaccharide *ex vivo* (Dugyala et al., 1998). Treatment of LLC-PK_1 cells, a pig renal kidney epithelial cell line, with FB_1 increased expression of TNFα transiently, and the increased expression of TNFα by FB_1 was unaltered when free sphinganine accumulation was prevented in the cultures by myriocin (He et al., 2001). Expression of TNF receptor-associated protein (TRAP) 2 is induced in FB_1-sensitive CV-1 cells but repressed in FB_1-resistant COS cells (Zhang et al., 2001). It remains uncertain whether FB_1-induced alterations of gene expression in tissues are due solely to the disruption of sphingolipid metabolism.

Myriocin is able to inhibit the activity of hepatic SPT in mice (He et al., 2004a), and subsequently prevent the accumulation of free sphinganine in response to FB_1 both *in vivo* and *in vitro* (Enongene et al., 2002, Riley et al., 1999a, Schmelz et al., 1998). It has been widely used to study the role of sphinganine and *de novo* generated ceramide in regulation of cell functions under various conditions (He et al., 2001, 2002, Le Stunff et al., 2002, Riley et al., 1999a, Schmelz et al., 1998). In the present study, we investigated the effect of myriocin on FB_1 hepatotoxicity and gene expression of selected cytokines in mice. Myriocin effectively blocked the activity of SPT and prevented the FB_1-accumulation of free sphinganine in both liver and kidney; however, it did not reduce FB_1-induced hepatocyte apoptosis or increased PCNA staining, although the elevation of plasma aspartate aminotransferase (AST) was significantly reduced. In spite of minimal protection against FB_1-induced hepatotoxicity, myriocin reversed the FB_1-induced increase in expression of TNFα, TNF related apoptosis-inducing ligand (TRAIL), TNF receptor (TNFR) 1, lymphotoxin (LT)β, Interferon (IFN)γ, and transforming growth factor (TGF) β1. The results suggest that elevation in free sphinganine and/or its metabolites are involved in FB_1-induced alterations in expression of the tested cell signaling factors. However, with the dosing regime used in this study, myriocin would not provide significant protection from FB_1-induced hepatotoxicity and prolonged exposure to the combination of the two inhibitors of *de novo* sphingolipid biosynthesis could significantly potentiate the toxicity of either inhibitor alone.

2. **Materials and Methods**

2.1. Chemicals

Fumonisin B_1 (purity >98%) was obtained from Programme on Mycotoxins and Experimental Carcinogenesis (PROMEC, Tygerberg, South Africa). Myriocin, (2S, 3R, 4R, 6E-2- amino-3, 4-dihydroxy-2-hydroxymethyl-14-oxo-6-eicosenoic acid) was purchased from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA, USA). C_{20}-sphinganine standard (D-erythro-C_{20}-dihydro-sphingosine, purity 98%) was obtained from Matreya Inc. (Pleasant Gap, PA, USA). All other reagents were purchased from Sigma-Aldrich Chemical Company Inc. (St. Louis, MO, USA), unless otherwise stated in the text.

2.2. Animal treatments and sampling

Six-week-old female BALB/c mice weighing about 22 g were procured from Harlan Laboratories (Indianapolis, IN). They were adapted for 1 week before dosing under controlled environmental conditions at 23°C and 65% relative humidity with a 12 h light/dark cycle. Feed and water were available *ad libitum*. Animals were treated with humane care following the Public Health Service Policy on Humane Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee.

Mice were divided randomly into 4 groups with 5 animals each. Treatment with myriocin was by intraperitineal injection (1.0 mg/kg bw/day), whereas, FB_1 (2.25 mg/kg bw/day) was injected subcutaneously. Treatment groups were phosphate buffered saline (PBS) only, myriocin in PBS, FB_1 in PBS, or myriocin and then 30 min later FB_1. In a preliminary experiment mice were treated for 5 days and it was found that the myriocin alone (1 mg/kg bw/day) caused significant elevation of serum ALT and AST and the combination of myriocin and FB_1 was lethal (data not shown); no overt visual signs of toxicity were observed at 3 days. Therefore, in

subsequent experiments, the mice were given only 3 daily injections. The FB_1 treatment protocol has been proven to produce consistent liver damage in female mice exposed to FB_1 in our laboratory (He et al., 2004b).

One day following the final FB_1 treatment, mice were sacrificed by decapitation. Blood was collected in heparinized tubes, and plasma was subsequently isolated for analysis of ALT and AST. Livers and kidneys were collected from each animal and weighed; aliquots were fixed immediately in 10% neutral formaldehyde, or frozen in liquid nitrogen and stored at -85°C until analysis. Body weights were recorded during the treatment period.

2.3. Evaluation of liver damage

Activities of plasma ALT and AST were determined by using a Hitachi 912 Automatic Analyzer (Roche Diagnostics, Indianapolis, IN). The methods used for determination of the activities of these enzymes are based on a kinetic reduction of β-nicotinamide adenine dinucleotide (reduced disodium salt hydrate) in the reactions detected photometrically. The assays conform to the standard procedures recommended by the International Federation of Clinical Chemistry (Bergmeyer et al., 1986a, b).

Hepatocyte apoptosis was analyzed by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay as described previously (Sharma et al., 2003). Briefly, Liver tissue sections (5 μm) were prepared and subjected to dUTP nick-end labeling by TdT with a peroxidase-based *in Situ* Cell Death Detection kit (Roche Diagnostics, Indianapolis, IN). The TUNEL cells were counted and normalized to the unit area as described (Sharma et al., 1997).

2.4. Immunohistochemistry for proliferating cellular nuclear antigen (PCNA) assay

Hepatocyte proliferation was estimated by analysis of PCNA staining in liver tissue sections as described previously (Sharma et al., 2003). Briefly, the liver sections (5 μm) were hydrated followed by antigen retrieval in boiling citric acid buffer for 25 min. The primary anti-PCNA antibodies were incubated with the tissues at 4°C overnight in a humid chamber. Then the secondary antibodies were applied and the sections were stained with Vectorstain ABC kit (Vector Laboratories, Inc., Burlingame, CA, USA). The number of PCNA positive cells were counted and normalized to the unit area as described (Sharma et al., 1997).

2.5. Assay for the activity of serine palmitoyltransferase (SPT)

The activity of SPT in liver and kidney was analysed using the method described previously (Williams et al., 1984) with minor modification. Briefly, the frozen tissues were homogenized in homogenization buffer (50 mM N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid], 5 mM DL-dithiothreitol, 10 mM ethylenediaminetetraacetic acid, 0.25 M sucrose, pH 7.4), and the homogenate was centrifuged at 30,000 g for 30 min. Aliquots of 100 μg protein in the supernatant were used for analysis of SPT activity as previously described (He et al., 2004a, Williams et al., 1984). Bradford (Bio-Rad, Bio-Rad Laboratories, Hercules, CA, USA) reagent was used to determine the content of protein.

2.6. Sphingolipid analysis by high performance liquid chromatography (HPLC)

Free sphingoid bases or complex sphingolipids were determined in base-treated or acid-treated lipid extracts by HPLC utilizing the extraction methods described previously (Riley et al., 1999b). Sphingoid bases were quantified based on the recovery of a C_{20}-sphinganine standard. The HPLC apparatus and derivation procedure were similar to those described before (He et al., 2001, 2004b).

2.7. Sphingomyelin assay in the liver by enzyme catalysis fluorescence-based method

Sphingomyelin was analyzed by a fluorescence-based method following enzyme catalysis (He et al., 2002). Briefly, the livers were homogenized in 0.25% Triton X-100 in PBS (at a ratio of 1:20 g/ml) and centrifuged (10,000g for 5 min). Aliquots of 20 μl supernatant were mixed with equal volumes of homogenizing buffer followed by heating at 70°C for 5 min; then the heated samples were cooled to room temperature, and centrifuged briefly. Ten microliters of the supernatant was used for sphingomyelin determination. The reaction contains an enzyme cocktail consisting of 12.5 mU of *Bacillus cereus* sphingomyelinase, 400 mU of alkaline phosphatase, 120 mU choline oxidase, 200 mU of horseradish peroxidase, and 20 nmol of 10-acetyl-3-dihydrophenoxazine, a sensitive fluorogenic probe for hydrogen peroxide (Amplex® red reagent, Molecular Probes, Inc., Eugene, OR, USA) in reaction buffer. For each sample, a negative control well contained 10 μl of the sample and the same reaction mixture without sphingomyelinase. After 20 min incubation at 37°C, the microtiter plate was read using a fluorescence microplate reader (Molecular Devices Corp., Sunnyvale, CA, USA). The excitation and emission wavelengths were set to 560 and 590 nm, respectively. Sphingomyelin amounts were calculated from the difference in fluorescence between the test and the negative control samples and compared with sphingomyelin standard curve.

2.8. RNase protection assay (RPA) for selected gene expression

Total RNA from liver tissue was extracted with TRI® reagents (Molecular Research Center, Cincinnati, OH). An aliquot part of 50 μg RNA was used for RPA using RiboQuant™ RPA starter kit (B.D Biosciences, San Diego, CA, USA) as recently described (Sharma et al., 2003). Briefly, high specific-activity α-^{32}P-UTP-labeled anti-sense RNA probes were synthesized using

T7 RNA polymerase *in vitro* transcription kit according to the manufacturer's protocol (BD Biosciences, San Diego, CA, USA). The synthesized probes were hybridized with RNA overnight at 65°C followed by treatment with RNase A and T1, and then protease K. The RNase-protected products were extracted and resolved on gels containing 5% polyacrylamide/7 M urea. The α-^{32}P-labeled bands were exposed to an FX Imaging Screen K-HD® (Bio-Rad Laboratories, Hercules, CA, USA) for 6-24 hr and scanned by Bio-Rad Molecular Imager® FX. The relative gene expression was digitized using the Quantity one® software (Bio-Rad Laboratories) and normalized against ribosomal protein L32, the housekeeping gene.

2.9. Statistical Analysis

Results are presented as mean ± standard error (SE). Data were analyzed by two-way analysis of variance (ANOVA) followed by Duncan's multiple range tests unless otherwise stated in the text. All statistical analyses were done using the SAS program (SAS, Cary, NC, USA). The level of $p < 0.05$ was considered significant.

3. **Results**

3.1. Toxicity of FB_1 after pretreatment of mice with myriocin

During 3 daily treatments, no obvious behavioral changes were observed in any of the treatment groups. Myriocin treatment alone did not change body weights compared to saline control. Treatment with either FB_1 or myriocin + FB_1 significantly reduced body weights to a similar content. Both absolute and relative liver weights were not different from each other among these treatments (data not shown). Treatment of mice with myriocin alone for 3 days did

not increase activities of plasma ALT and AST (Fig. 3.1), whereas treatment with FB_1 alone or FB_1 plus myriocin caused a significant increase in both plasma ALT and AST. The increase in plasma AST was significantly decreased in the FB_1 plus myriocin group compared to FB_1 alone (Fig. 3.1). Plasma ALT was slightly, but not significantly reduced in the FB_1 plus myriocin group compared to FB_1 alone (Fig. 3.1).

As expected, the FB_1 alone treatment caused a significant increase in apoptotic cells in liver (Table 3.1). There was no evidence of ongoing apoptosis in either the control-treated or myriocin alone-treated mice, and the combination of myriocin plus FB_1 did not significantly reduce the number of apoptotic cells compared to FB_1 alone (Table 3.1). The number of PCNA-positive cells in liver of mice treated with FB_1 alone or myriocin alone was similar although markedly greater than in the controls, the increase was not statistically significant (Table 3.1). The combination of FB_1 plus myriocin appeared to have an additive effect on the number of PCNA-positive cells compared to FB_1 did, however, once again, because of the large amount of variability in staining, the differences were not statistically significant. No attempt was made to determine if the increased PCNA staining was due to cells being arrested in specific stage of the cell cycle.

3.2. Myriocin blocked activity of serine palmitoyltransferase (SPT)

The activity of SPT was significantly increased following FB_1 treatment compared to that of controls in liver but not in kidney (Fig. 3.2). Consistent with our recent studies (He et al., 2004a), myriocin reduced SPT activity to 30% of control levels in liver, and efficiently blocked FB_1-induced activation of SPT (Fig. 3.2).

3.3. Myriocin prevented FB_1-induced accumulation of free sphinganine and inhibited the biosynthesis of more complex sphingolipids and sphingomyelin

Treatment with FB_1 significantly increased the concentrations of free sphinganine in both liver and kidney (Fig. 3.3). Myriocin alone significantly reduced the level of free sphingosine in both liver and kidney and the FB_1-induced accumulation of free sphinganine in liver and kidney was effectively blocked by co-treatment with myriocin (Fig. 3.3). The levels of more complex sphingolipids were significantly reduced by FB_1, myriocin, and the combination of myriocin plus FB_1 (Table 3.2). However, the greatest reduction was seen in the FB_1 plus myriocin treatment group (Table 3.2).

The level of hepatic sphingomyelin was significantly decreased by FB_1, myriocin, and myriocin plus FB_1, with the greatest reduction by myriocin plus FB_1 (Table 3.2).

3.4. Myriocin reversed FB_1-induced expression of selected cell signaling factors

FB_1 significantly increased expression of TNFα, TNFR1, TRAIL, LTβ, IFNγ and TGFβ1 (Fig. 3.4-3.5). Myriocin alone did not alter the constitutive levels of selected gene expression in the liver; however, it reduced the FB_1-induced overexpression of all the above genes to constitutive levels (Fig. 3.4 – 3.5).

4. **Discussion**

The most well studied biochemical effect of FB_1 is inhibition of ceramide biosynthesis with resultant accumulation of free sphinganine (Wang et al., 1991). Many downstream and concurrent effects resulting from exposure to FB_1 have been demonstrated including increases in

production of various cell signaling factors (Bhandari et al., 2002; Bhandari and Sharma, 2002). In the present study, we found that myriocin significantly prevented the accumulation of free sphinganine and reversed the induction of selected cell signal factors in the TNFα signal pathway and other cytokines in response to FB_1, but had minimal or no protective effect on FB_1-induced increase in activities of plasma ALT and AST and the number of apoptotic hepatocytes.

Myriocin prevents FB_1-induced cell death *in vitro* in LLC-PK_1 cells, a porcine kidney epithelial cell line (He et al., 2002, Riley et al, 1999), and HT29 cells, a human colonic cell line (Schmelz et al., 1998). However, our findings in the current *in vivo* study showed that myriocin did not significantly prevent FB_1-induced liver damage even though it has been shown that there is a close correlation between the extent and severity of fumonisin-induced apoptosis/hepatotoxicity and the degree of elevation in free sphinganine, a marker for FB_1 inhibition of ceramide synthase in BALBc mice (Tsunoda et al., 1998; Riley et al., 2001). While there is a clear protective effect of myriocin on FB_1-induced cell death in short term *in vitro* studies, demonstration of a protective effect *in vivo* is not as easily accomplished for several reasons. For example, there is no information on the specifics of the toxicity of myriocin, the kinetics of its distribution, or its peripheral effects. While it is known to be a potent inhibitor of serine palmitoyltransferase, the first and rate limiting step in *de novo* sphingolipid biosynthesis, it is also known to be a potent immunosuppressant (Miyake et al., 1995) and its immunosuppressive effects are independent of its ability to inhibit SPT (Fujita et al., 1996)

The use of myriocin to prevent the sphinganine accumulation and subsequent FB_1 toxicity, as done in this study, was not successful. In fact, the combination of myriocin and FB_1 was highly toxic after 5 daily treatments for the survival of female BALB/c mice. There was 100% mortality after 5 daily treatments of myriocin plus FB_1 and yet after three days of exposure to the

combination there was no evidence of increased hepatotoxicity. It is possible that the combined effects of myriocin on the biosynthesis of complex sphingolipids and sphingomyelin could have been the cause of the increased toxicity at 5 days or the lack of a protective effect at 3 days. Present data show that levels of complex sphingolipids and sphingomyelin were reduced by FB_1 and myriocin, and the reduction was greater by myriocin plus FB_1 than by either agent alone. Inhibition of ceramide synthase in response to FB_1 results not only in the accumulation of free sphingoid bases but also in the reduction of ceramide and complex sphingolipids (Tolleson et al., 1999, Wang et al., 1992, Yoo et al., 1996). Myriocin potently inhibits activity of SPT, and thereby blocks biosynthesis of free sphinganine, the precursor of *de novo* synthesized ceramide (Hannun et al., 2001). The reduction of complex sphingolipids by the combination of FB_1 and β-chloroalanine, a nonspecific inhibitor of SPT, was greater than that mediated by either compound alone in LLC-PK_1 cells (Yoo et al., 1996). Thus, under our present *in vivo* study conditions, myriocin probably had an additive effect on decreased complex sphingolipid and sphingomyelin biosynthesis, and thereby depletion of complex sphingolipids and sphingomyelin occurred more rapidly in mice treated with combination of myriocin and FB_1 than that in either FB_1 or myriocin-treated mice. Sphingolipids and their metabolites mediate a variety of cell signaling processes involved in cell growth, apoptosis, and proliferation (Ohanian and Ohanian, 2001). Depletion of sphingolipids disrupts cell functions (Hidari et al., 1996). Previous studies have shown that elevated free sphingoid bases as well as depletion of ceramide and complex sphingolipid contributed to FB_1 cytotoxicity in cell cultures (Tolleson et al., 1999, Yoo et al., 1996). It is possible that myriocin facilitated the decrease in ceramide and complex sphingolipid as well as sphingomyelin biosynthesis and thus potentiated disruption of downstream effects that are dependent on more complex sphingolipids and sphingomyelin. .

Free sphinganine and sphingosine can be metabolized to sphinganine/sphingosine-1-phosphate by sphingosine kinase (Maceyka et al., 2002, Merrill et al. 2001). Accumulation of free sphingoid bases following FB_1 exposure results in accumulation of intracellular sphingoid base-1-phosphates (Merrill et al., 2001). It is possible that sphinganine-1-phosphate accumulation in tissues of animals exposed to FB_1 could promote cell survival. While sphingosine-1-phosphate is clearly an important cell survival and anti-apoptotic signaling factor (Maceyka et al., 2002), the possible role of sphinganine-1-phosphate in cell survival *in vivo* after FB_1 exposure is unknown. It is likely that myriocin reduced the formation of sphingoid base-1-phosphates as a consequence of blocking sphinganine synthesis. As a result, any possible protective effects of sphinganine-1-phosphate in FB_1-treated liver would be prevented.

It has been demonstrated that FB_1 increases expression of many types of genes in different cell signal transduction pathways. For example, treatment of mice with FB_1 increased expression of TNFα, IL12, IFNγ, c-myc and many other genes in liver and kidney (Bhandari and Sharma, 2002, Bhandari et al., 2002). It is becoming apparent that expression of cell signaling genes is related to the disruption of sphingolipid metabolism following FB_1 exposure. In LLC-PK_1 cells, FB_1 induced a transient increase in TNFα expression, while the induction of TNFα was not reduced by myriocin, suggesting accumulation of free sphingoid bases was not the cause of TNFα induction (He et al., 2001). However, it is possible that sphinganine/sphingosine-1-phosphates resulting from accumulated free sphinganine and sphingosine induce expression of these genes following FB_1 exposure. It has been shown that sphingosine-1-phosphate as well as sphinganine-1-phosphate, but not sphingosine or sphinganine, enhanced the secretion of IL-2 and IFNγ via pertussis toxin sensitive sphingosine-1-phosphate receptors in peripheral blood T cells stimulated with anti-CD3 plus anti-CD28 (Jin *et al.* 2003). In the current study, myriocin

treatment did not alter constitutive expression of TNFα, TNFR1, TRAIL, LTβ, IFNγ and TGFβ1, FB_1 increased expression of all these genes in liver, while myriocin prevented FB_1-induced increases in expression of these genes. The reversion of FB_1-induced expression of the above genes was concomitant with the reduced accumulation of free sphingoid bases by myriocin. These results suggest that accumulation of free sphinganine or its metabolites as a result of FB_1 exposure could mediate overexpression of the above hepatic genes.

In conclusion, the current study demonstrated that five day exposure of mice to myriocin plus FB_1 resulted in overall death, indicating that myriocin plus FB_1 is highly toxic and therefore myriocin used under the conditions described in this study would have no therapeutic potential for preventing FB_1 intoxication. However, myriocin efficiently prevented FB_1-induced accumulation of free sphinganine as a consequence of SPT inhibition and it reversed FB_1-induced expression of TNFα superfamily signal molecules and other cytokines to the constitutive level. Results suggest that accumulation of free sphinganine contributed to FB_1-modulated expression of these cell signal factors in liver and depletion of complex sphingolipids as well as sphingomyelin produced liver damage in FB_1 exposure. Dosing regimes of myriocin that minimize its potential toxic effects may be possible. For example, because FB_1 is quickly eliminated, exposure to myriocin for a brief period after cessation of FB_1 exposure could prevent the harmful effects of free sphinganine and signaling factors induced by free sphinganine while avoiding the additive effects of co-exposure to two potent inhibitors of sphingolipid biosynthesis.

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TABLE 3.1.

Effects of myriocin on FB_1-induced cell apoptosis and proliferation#

Treatment	Animal number	Apoptosis incidence	Apoptotic cells/cm^2	Proliferation incidence	Proliferating cells/cm^2
Control	5	0/5	0	3/5	3.8 ± 2.6
FB_1	5	5/5	49.1 ± 20.0*	2/5	69.0 ± 51.4
Myriocin	5	0/5	0	5/5	68.1 ± 29.7
Myriocin+FB_1	5	5/5	41.4 ± 14.3*	5/5	189.6 ± 94.4

[#] Cell apoptosis and proliferation were analyzed by TUNEL assay and PCNA immunohistochemistry, respectively. The positive stained cells were counted under a light microscope. Mean ± SE. * $p < 0.05$ vs. control.

Table 3.2

Concentrations of complex sphingolipids and sphingomyelin after myriocin and FB_1 treatment[a]

Treatment	Complex sphingolipid containing sphingosine[b]	Complex sphingolipid containing sphinganine[b]	Complex sphingolipid containing sphingosine and sphinganine[b]	Sphingomyelin[c]
Control	297.8 ± 12.3^{a}	18.4 ± 3.1^{a}	312.6 ± 12.2^{a}	252.8 ± 14.0^{a}
FB_1	67.9 ± 10.5^{bc}	20.2 ± 2.2^{a}	90.1 ± 9.0^{b}	47.9 ± 12.8^{b}
Myriocin	70.0 ± 2.3^{b}	5.2 ± 3.4^{b}	74.2 ± 3.4^{b}	128.8 ± 9.5^{c}
Myriocin+FB_1	56.1 ± 2.0^{c}	6.1 ± 1.4^{b}	60.9 ± 2.9^{c}	30.1 ± 9.6^{b}

a. The values of sphingolipids and sphingomyelin are all expressed as mean ± SE (pmol/mg wet tissue, $n=5$). Different letters besides the values indicate significant difference at $p<0.05$.

b. Sphingolipids were extracted by acid hydrolysis and determined by HPLC. Complex sphingolipids are the differences between total complex sphingolipids and free sphingoid bases.

c. Determined by enzyme-coupled Amplex® Red assay.

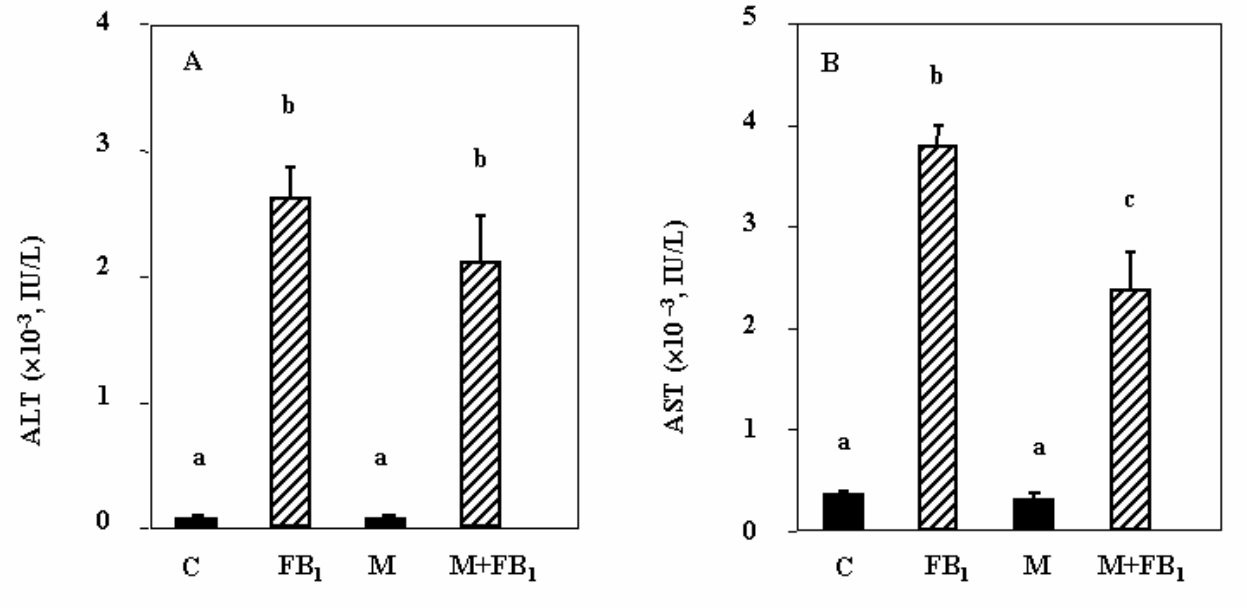

Fig. 3.1. Effects of myriocin (M) on fumonisin B_1 (FB_1)-induced increase of plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities. Female BALB/c mice were daily treated with 1.0 mg/kg myriocin intraperitoneally, and/or 2.25 mg/kg FB_1 subcutaneously for 3 days. One day after the last FB_1 treatment, the animals were sacrificed and plasma was used for analysis of ALT and AST. Mean ± SE (n=5). Different letters indicate statistical difference at $p<0.05$.

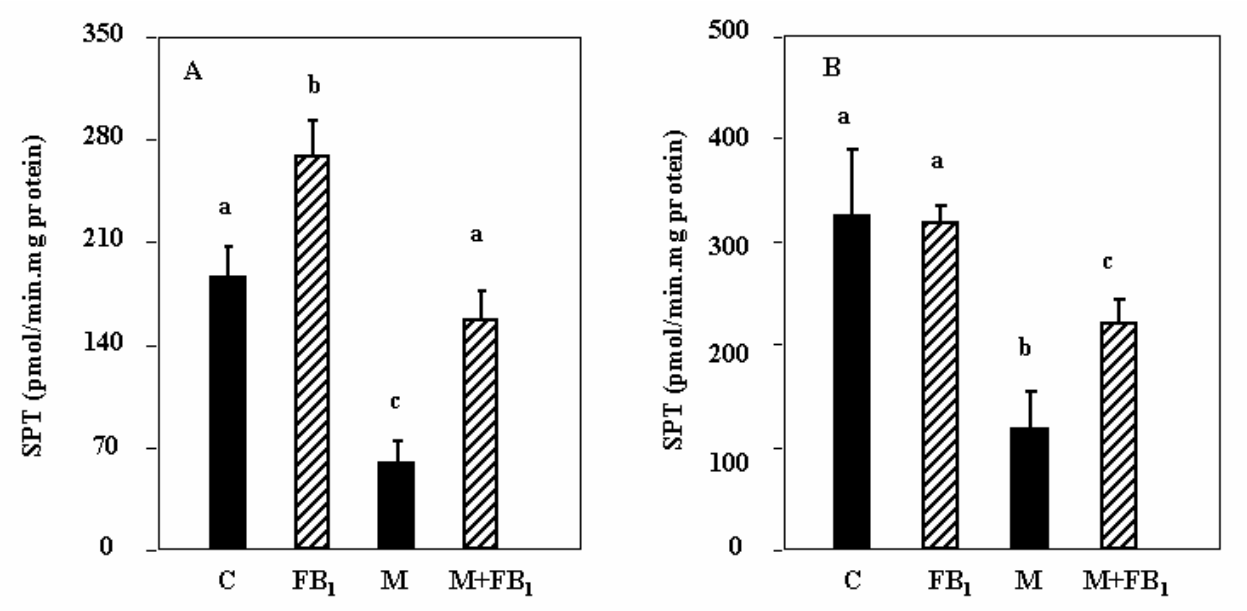

Fig. 3.2. Myriocin inhibition of serine palmitoyltransferase (SPT) activity in liver (A) and kidney (B). Mean ± SE (n=5). Different letters indicate statistical difference at $p<0.05$.

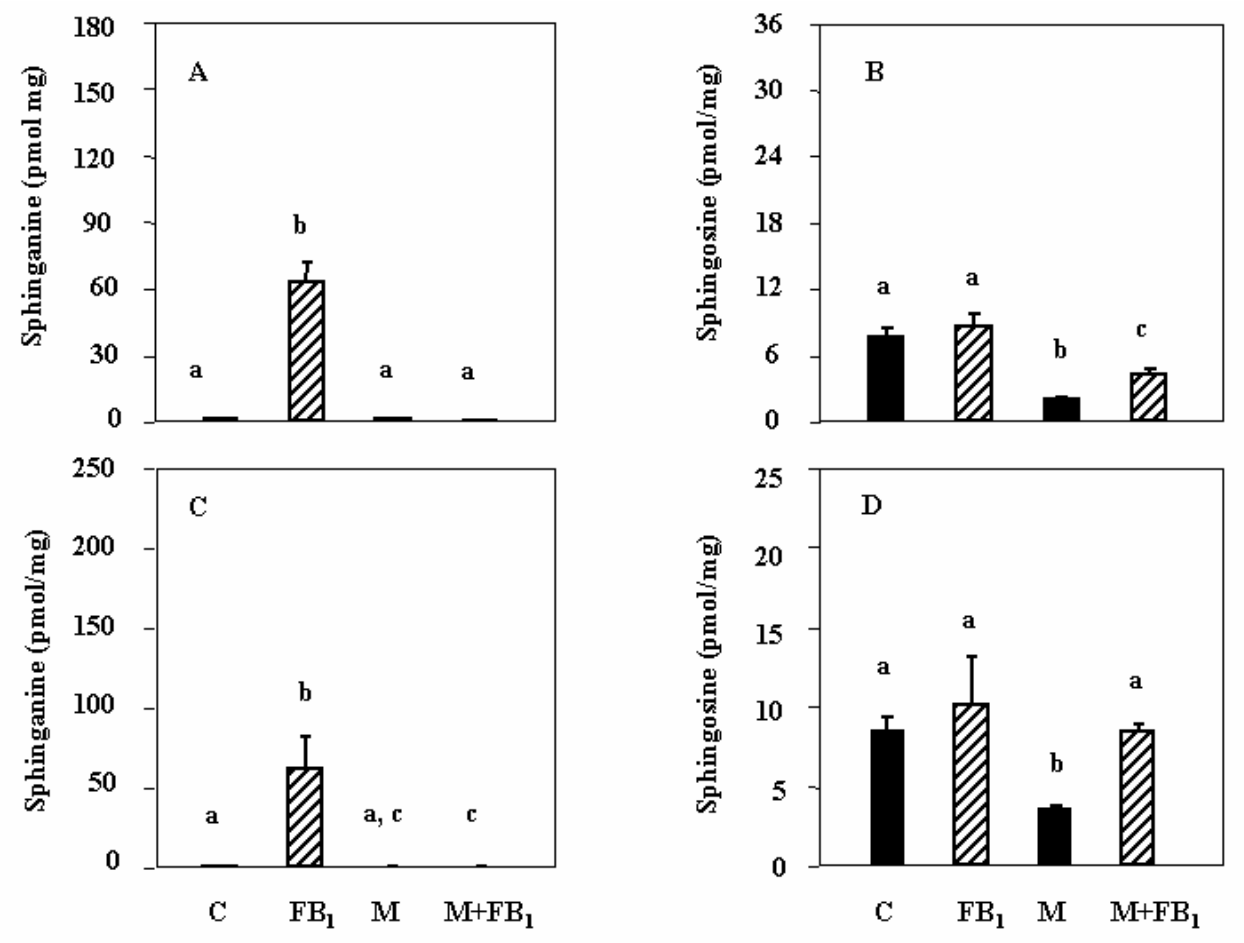

Fig. 3.3 Effects of myriocin (M) on fumonisin B_1 (FB_1)-induced accumulation of free sphingoid bases, sphinganine (Sa) and sphingosine (So), in liver (A, B) and kidney (C, D). Mean ± SE (n=5). Different letters indicate statistical difference at $p<0.05$.

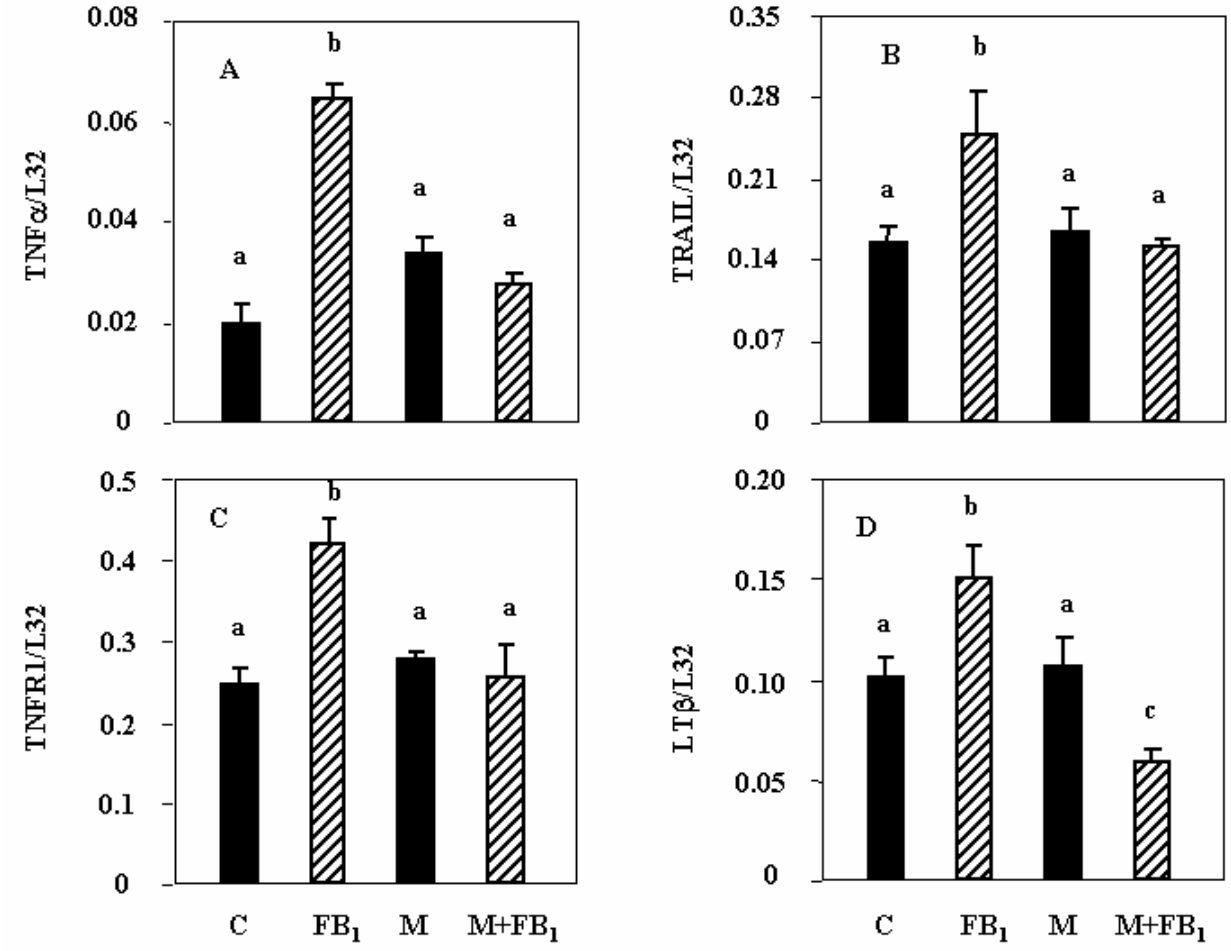

Fig. 3.4. Effects of myriocin (M) on fumonisin B_1 (FB_1)-induced expression of tumor necrosis factor (TNF) α, TNF receptor (TNFR) 1, TNF-related apoptosis-inducing ligand (TRAIL), and lymphotoxin (LT) β in mouse liver. Mean ± SE (n=5). Different letters indicate statistical difference at $p<0.05$.

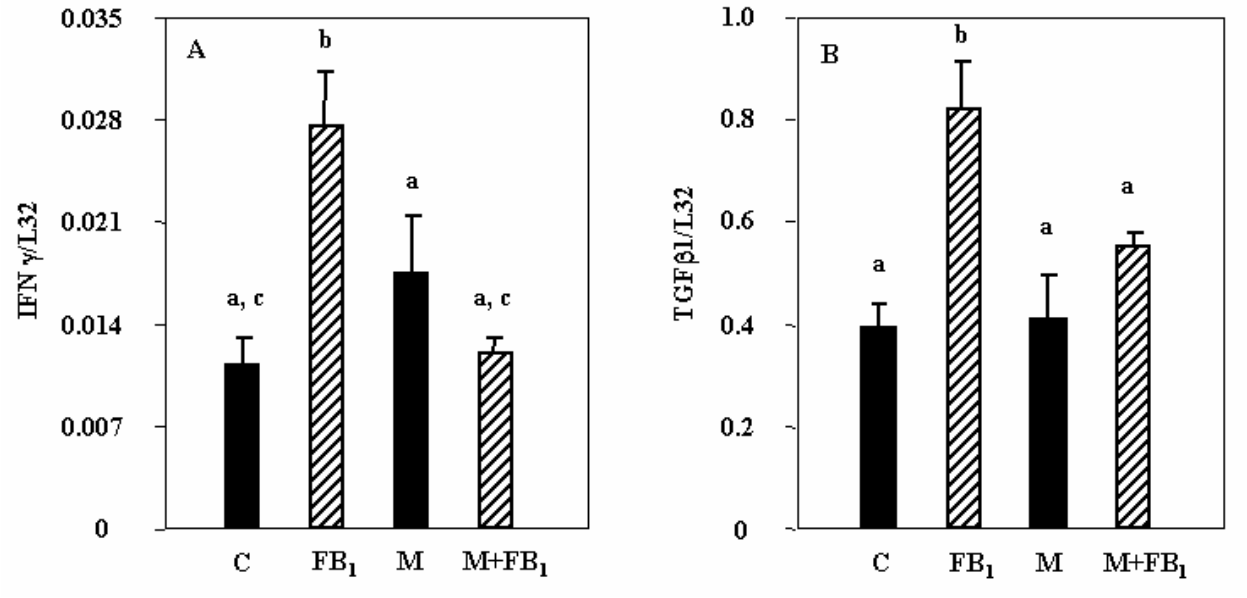

Fig. 3.5. Effects of myriocin (M) on fumonisin B_1 (FB_1)-induced expression of interferon (IFN) γ and transforming growth factor (TGF) β1 in mouse liver. Mean ± SE (n=5). Different letters indicate statistical difference at $p<0.05$.

CHAPTER 4

FUMONISIN B_1 HEPATOTOXICITY IN MICE IS ATTENUATED BY DEPLETION OF KUPFFER CELLS BY GADOLINIUM CHLORIDE[2]

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[2]Q. He, Kim J., Sharma R.P. To be submitted to *Toxicology*.

Abstract

Fumonisin B_1 (FB_1) is a toxic and carcinogenic mycotoxin produced by *Fusarium verticillioides* found on corn worldwide. The biological effects of FB_1 are attributed to sphingolipid metabolism disruption as a result of ceramide synthase inhibition. Tumor necrosis factor α (TNFα) is an important modulator of FB_1 hepatotoxicity. Kupffer cells are major source of cytokine production in liver. In the present study we investigated the effects of Kupffer cell depletion by gadolinium on FB_1 hepatotoxicity in female BALB/c mice. Mice were given saline or 50 mg/kg of gadolinium chloride once via the tail vein; 16 h later they were treated with subcutaneous injections of vehicle or 2.25 mg/kg/day FB_1 in saline for 3 successive days. Gadolinium significantly attenuated FB_1-induced increases in the activities of circulating alanine aminotransferase and aspartate aminotransferase and reduced the FB_1-induced hepatocyte apoptosis and free sphinganine accumulation in liver. Both gadolinium and FB_1 treatments individually increased the expression of selected cell signal factors; e.g., TNFα, TNF receptor 1, TNF-related apoptosis-inducing ligand, lymphotoxin β, interferon γ, and transforming growth factor β1, gadolinium chloride did not alter FB_1-induced the expression of the above genes. Results indicated that Kupffer cells play a role in FB_1 hepatotoxicity; Decreased FB_1-induced sphinganine accumulation and increased protective TNFα signaling by gadolinium chloride may in part account for its ameliorating effect on FB_1 liver damage.

Key words: Fumonisin; hepatotoxicity; Tumor necrosis factor α; Sphinganine; Kupffer cells

1. Introduction

Fumonisins, including fumonisin B_1 (FB_1), belong to a group of structurally related mycotoxins produced by *Fusarium verticillioides*, a common endophytic fungus on corn. Fumonisin contamination of animal feeds and human corn-based food has been reported worldwide (WHO, 2000). Fumonisin B_1 is the most abundant and toxic among various types of fumonisins. Fumonisins caused field outbreaks of equine leukoencephalomaracia and porcine pulmonary edema in the Unites States in 1989-1990 (Ross et al., 1992). Areas with high incidence of human esophageal cancer in southern Africa and China have been correlated with high level of FB_1 contamination of human food (Chu and Li, 1994; Marasas, 2001). Feeding studies demonstrated that FB_1 is hepatocarcinogenic in BD IX rats (Gelderblom et al., 1991), in female B6C3F1 mice (Howard et al., 2001), and nephrocarcinogenic in F344 rats (Howard et al., 2001). Fumonisins produce liver injury in rodents (Sharma et al., 1997; Voss et al., 2001), horses (Ross et al., 1993), milk-fed caves (Mathur et al., 2001), and pigs (Haschek et al., 2001).

Fumonisin B_1 induces intracellular accumulation of free sphinganine, and usually of free sphingosine, due to inhibition of ceramide synthase (sphinganine-*N*-acyltransferase), a critical enzyme responsible for conversion of sphinganine to ceramide (Wang et al., 1991). Cell death in response to FB_1 exposure has been related to disruption of sphingolipid metabolism both *in vivo* and *in vitro* (Riley et al., 2001; Tolleson et al., 1999; Tsunoda et al., 1998; Yoo et al., 1996).

Tumor necrosis factor α (TNFα) signaling pathways are important in modulating FB_1 toxicity. In response to FB_1 exposure, TNFα was increased in mouse liver along with other inflammatory cytokines (Bhandari and Sharma, 2002) and FB_1 hepatotoxcity was reduced in mice lacking either TNFα receptor (TNFR) 1 or TNFR 2 (Sharma et al, 2000a; 2001). On the

other hand, mice carrying a human TNFα transgene exhibited less sensitivity to FB_1 hepatotoxicity (Sharma et al., 2000b).

Kupffer cells, usually referred to as fixed hepatic macrophages, have diverse functions including phagocytosis, endocytosis, immunomodulation and synthesis and secretion of numerous biological active mediators (Laskin et al., 2001). Several reports indicate that Kupffer cells are principal source of inducible nitric oxide (NO) synthase (iNOS), NO production, and of cytokines, e.g., TNFα, interleukin (IL)-1β, IL-6, IL-12 (Laskin et al., 2001; Ishiyama et al., 2000). Kupffer cells have been linked in the pathogenesis of liver injury induced by various hepatotoxicants such as carbon tetrachloride (Edwards et al., 1993), acetaminophen (Laskin et al., 1995; Ju et al., 2002), ethanol (Wheeler et al., 2001), and cadmium (Yamano et al., 2000). It is believed that the regulatory role of Kupffer cells in chemical-induced liver damage is mediated through their production of superoxides and cytokines (Ju et al., 2002; Michael et al., 1999; Wheeler et al., 2001; Yamano et al., 2000).

In addition to induction of hepatocellular apoptosis, necrosis and mitosis (Howard et al., 2001; Sharma et al., 1997; Voss et al., 2001), Histopathological examination by light microscopy revealed that FB_1 exposure increased the number of Kupffer cells in rats, and Kupffer cell hyperplasia in pigs and mice (Haschek et al., 2001; Howard et al.; 2002; Theumer et al., 2002). We previously demonstrated that cytokines such as TNFα and interferon γ (IFNγ) are important contributors in murine hepatotoxicity by FB_1 (Sharma et al., 2000b, 2002, 2003). Kupffer cells were suggested as a source of TNFα in response to FB_1 treatment (Bhandari et al., 2002). It was proposed that TNFα and IL-12 secreted by Kupffer cells after FB_1 stimulation activated T lymphocytes and natural killer cells in liver to produce IFNγ, which further amplified the

production of TNFα through a positive feedback loop (Bhandari et al., 2002). These findings suggested an important role of Kupffer cell in FB_1-induced liver toxicity and that the modulation of Kupffer cell function would alter production of cytokines to modulate FB_1 hepatotoxicity. In the present study, we studied FB_1 hepatotoxicity in mice after eliminating Kupffer cells by gadolinium chloride, a selective Kupffer cell toxicant in liver (Hardonk et al., 1992). Results demonstrated that gadolinium chloride at the dose used in the current study eliminated Kupffer cells in liver. Pretreatment of mice with gadolinium chloride reduced FB_1-induced hepatotoxicity in response to three daily FB_1 treatments, suggesting that Kupffer cells are involved in FB_1 hepatotoxicity.

2. Materials and Methods

2.1. Chemicals

Fumonisin B_1 (purity >98%) was purchased from Programme on Mycotoxins and Experimental Carcinogenesis (PROMEC, Tygerberg, South Africa). Purified rat anti-mouse Mac-3 monoclonal antibody (clone M3/84) was obtained from BD Biosciences (San Diego, CA, USA). Gadolinium chloride ($GdCl_3.6H_2O$) and all other reagents were purchased from Sigma-Aldrich Chemical Company Inc. (St. Louis, MO, USA), unless stated otherwise.

2.2. Animals and treatment

Female BALB/c mice weighing 20-22 g were obtained from Harlan Laboratories (Indianapolis, IN). They were acclimated for 1 week before treatment under controlled environmental conditions at 23°C and 65% relative humidity with a 12 h light/dark cycle. Feed

and water were provided *ad libitum*. Mice were treated with humane care following the Public Health Service Policy on Humane Care and Use of Laboratory Animals; the protocol was approved by the Institutional Animal Care and Use Committee.

The mice were divided randomly into 4 groups with 5 animals each, and injected with 0.9% sterilized sodium chloride solution or 50 mg/kg gadolinium chloride in saline solution once through tail vein 16 h before beginning treatments with FB_1. The mice were given 3 daily subcutaneous injection of either physiological buffered saline (PBS) or 2.25 mg/kg/day of FB_1 in PBS. The protocol has been used to produce consistent liver damage in female mice exposed to FB_1 in our laboratory (He et al., 2004).

Body weights were recorded daily in the course of experiments. One day after the last FB_1 treatment, mice were sacrificed by decapitation. Blood was collected in heparinized tubes, and plasma subsequently isolated for determination of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities. Livers were collected and weighted from each animal; aliquots were fixed immediately in neutral 10% formalin, or quickly frozen in liquid nitrogen and stored at -85°C until analyses.

2.3. Analysis of liver enzymes in plasma

Activities of plasma ALT and AST were determined using a Hitachi 912 Automatic Analyzer (Roche Diagnostics, Indianapolis, IN) and expressed as international units per liter (IU/L). The methods used for determination of the activities of these enzymes are based on a kinetic reduction of β-nicotinamide adenine dinucleotide (reduced disodium salt hydrate) in the reactions photometrically. These assays conform to the standard procedures recommended by the International Federation of Clinical Chemistry (Bergmeyer et al., 1986a, b).

2.4. Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay for DNA fragmentation

Liver tissue sections (5 μm) were prepared and subjected to dUTP nick-end labeling by TdT with a peroxidase-based *In Situ* Cell Death Detection kit (Roche Diagnostics, Indianapolis, IN) as described previously (Sharma et al., 2003). The positively stained hepatocytes were counted under a light microscope and normalized to the unit area as described (Sharma et al., 1997). A few endothelial cells were labeled with TUNEL, but only hepatocytes were counted as TUNEL positive cells.

2.5. Immunohistochemical assay for Kupffer cells and hepatocyte proliferating cellular nuclear antigen (PCNA) assay

Formalin fixed, paraffin-embedded liver sections (5 μm) were subjected to standard immunohistochemical staining with Kupffer cell specific mac-3 antibodies according to the manufacturer's protocol. Briefly, the tissues were incubated with rat anti-mouse mac-3 antibodies (1:1,000 dilution) at 4°C overnight, and visualized after a three-step staining procedure involving biotinylated anti-rat IgG as the secondary antibody, streptravidin-horseradish peroxidase (Vectorstain® ABC kit, Vector laboratories, Inc., Burlingame, CA, USA) and diaminobenzidine tetrahydrochloride detection system.

Hepatocyte proliferation was determined by analysis of PCNA in formalin-fixed, paraffin-embedded liver tissues as described recently (Sharma et al., 2003). Briefly, the liver sections (5 μm) were hydrated followed by antigen retrieval in boiling citric acid buffer for 25 min. The primary anti-PCNA antibodies were incubated with the tissues at 4°C overnight in a humid chamber. Then the secondary antibodies were applied and the sections were stained with

Vectorstain ABC kit (Vector Laboratories, Inc., Burlingame, CA, USA). The numbers of PCNA positive cells were counted under a microscope and normalized to the unit area as described (Sharma et al., 1997).

2.6. Free sphinganine and sphingosine analysis

Hepatic levels of free sphinganine and sphingosine in base-treated lipid extracts were determined by high-performance liquid chromatography (HPLC) utilizing a modification of the extraction methods described earlier (Merrill et al., 1988). Sphingoid bases were quantitated based on the recovery of a C_{20}-sphinganine standard (D-erythro-C_{20}-dihydro-sphingosine, Matreya Inc. Pleasant Gap, PA, USA). The HPLC apparatus and derivation procedure were similar to those described before (He et al., 2001).

2.7. RNase protection assay (RPA) for selected gene expression

Total RNA from liver tissue was extracted using TRI® reagent (Molecular Research Center, Cincinnati, OH). An aliquot of 50 μg RNA was used for RPA using RiboQuant™ RPA starter kit (BD Biosciences, San Diego, CA, USA) as described previously (Sharma et al., 2003). Briefly, high specific-activity α-^{32}P-UTP-labeled anti-sense RNA probes were synthesized using T7 RNA polymerase *in vitro* transcription kit according to the manufacturer's protocol (BD Biosciences, San Diego, CA, USA). The synthesized probes were hybridized with RNA overnight at 65°C followed by treatment with RNase A and T1, and then protease K. The RNase-protected products were extracted and resolved on gels containing 5% polyacrylamide/7 M urea. The α-^{32}P-labeled bands were exposed to an FX Imaging Screen K-HD® (Bio-Rad Laboratories, Hercules, CA, USA) for 6-24 hr and scanned by Bio-Rad Molecular Imager® FX.

The relative gene expression was digitized using the Quantity one® software (Bio-Rad Laboratories) and normalized against ribosomal protein L32, the housekeeping gene.

2.8. Statistical analysis

Results are presented as mean ± standard error (SE). Data were analyzed by two-way analysis of variance (ANOVA) followed by Duncan's Multiple Range test. In selected cases where unequal variances of different groups were present the Wilcoxon Rank Sum test was employed. All analyses were done with the SAS software (SAS Institute Inc., Cary, NC, USA). The level of $p < 0.05$ was considered significant.

3. Results

3.1. Lack of general effects in response to gadolinium and fumonisin B_1

Treatment of mice with either gadolinium or FB_1 produced no gross behavioral effects. Consumption of feed and water was similar for all groups. Body weights or relative organ weights were not affected by gadolinium and/or FB_1 treatment (data not shown).

3.2. Gadolinium chloride eliminated Kupffer cells

Treatment of mice with gadolinium chloride effectively eliminated Kupffer cells demonstrated by immunohistochemical staining with antibody against mac-3 antigen, a specific glycoprotein in macrophages. Abundant mac-3 positive Kupffer cells were observed in livers from mice pretreated with saline (Fig. 4.1A); no Kupffer cells were detected in liver after gadolinium treatment (Fig. 4.1B).

3.3. Hepatotoxicity of FB_1 after gadolinium chloride pretreatment in mice

The activities of ALT and AST were not different between saline controls and gadolinium-treated mice. Consistent with our previous findings, FB_1 caused increases in activities of plasma ALT and AST, indicative of liver damage. Gadolinium pretreatment significantly reduced the increase of ALT and AST activities in response to FB_1 (Fig. 4.2).

The histopathological effects of FB_1 on liver were limited to the presence of apoptotic hepatocytes with no evident oncotic changes; and such histopathological changes in liver of mice treated with FB_1 have been consistently reported in our previous studies (He et al., 2004, Sharma et al., 1997, 2000a, b 2002). The morphologic changes in response to FB_1 in haemtoxylin-eosin stained livers of the same strain mice have been consistently reported previously (He et al., 2004, Sharma et al., 1997). Thus, the change in hepatotoxic response was analyzed by immunochemical detection for apoptotic cells in liver using TUNEL stain reagents. No TUNEL positive cells were observed in livers from either saline- or gadolinium-treated mice. Consistent with alterations in plasma ALT and AST activities, FB_1 induced hepatocyte apoptosis; gadolinium pretreatment significantly decreased the number of FB_1-induced apoptotic hepatocytes (Fig. 4.3A).

Treatment of animals with FB_1 increased the number of PCNA positive cells (Fig. 4.3B). Gadolinium chloride did not increase PCNA positive cells compared to the saline treatment. The numbers of PCNA positive cells in liver were similar between treatments with FB_1 alone or FB_1+gadolinium (Fig. 4.3B).

3.4. Hepatic free sphingoid bases after gadolinium and FB_1 administration

The levels of free sphinganine and sphingosine in liver in response to gadolinium and FB_1 treatment are presented in Fig. 4.4. Gadolinium caused no change in liver sphinganine, but

marginally increased sphingosine compared to saline treatment. Treatment with FB_1 increased hepatic levels of free sphinganine and sphingosine. Pretreatment with gadolinium chloride significantly prevented FB_1-induced accumulation of hepatic free sphinagnine (Fig. 4.4); no FB_1-induced increase was observed for free sphingosine in gadolinium-treated mice.

3.5. Expression of selected genes for signaling factors after gadolinium and FB_1 treatment

An illustration of RNase protection assay for selected genes involved in cellular signaling is presented in Fig. 4.5. Neither gadolinium nor FB_1 caused any change in expression of CD95 (Fas) signal factors including Fas, Fas associated death domain, Fas associated protease, and Fas associated factor (Fig. 4.5). The expression of caspase 8, TNF receptor associated death domain, and receptor-interacting protein also remained unaltered after gadolinium or FB_1 treatment.

Treatment with gadolinium chloride or FB_1 increased expression of signaling factors of TNFα superfamily, and of IFNγ and TGFβ1 (Fig. 4.5). A quantitative estimation for the expression of selected gene is shown in Fig. 6. Both gadolinium and FB_1 significantly increased expression of TNFα, TNF receptor 1, TNF-related apoptosis-inducing ligand (TRAIL), lymphotoxin β (LTβ), IFNγ and TGFβ1. Gadolinium chloride had no effect on FB_1-induced expression of all the above signal factors.

4. Discussion

Gadolinium chloride is a selective Kupffer cell toxicant that completely eliminates Kupffer cells from liver, and the repopulation of immature Kupffer cells does not occur until 4 days after a single injection of gadolinium chloride (Hardonk et al., 1992; Lieber et al., 1997). It has been

used *in vivo* to investigate the role of Kupffer cells in a variety of hepatotoxic processes and has been shown to protect animals from chemical-induced liver damage (Edwards et al., 1993; Laskin et al., 1995; Wheeler et al., 2001; Yamano et al., 2000).

In our current study, immunohistochemical technique indicated no mac-3 positive Kupffer cells in liver 4 days after a single treatment with 50 mg/kg of gadolinium chloride. Gadolinium is specifically toxic to Kupffer cells and exerts no direct toxicity to other cell types of liver in mice in doses up to 60 mg/kg (Harstad and Klaassen, 2002). Removal of Kupffer cells by gadolinium in the present study alleviated FB_1 liver injury, indicating Kupffer cells play a role in fumonisin hepatotoxicity. Kupffer cells produce and release superoxide anions, hydrogen peroxide, nitric oxide, hydrolytic enzymes (Laskin et al., 2001). Gadolinium chloride-induced depletion of Kupffer cells in liver presumably led to reducing lysosomal enzymes and superoxides; therefore, it provided to some extent protective effects against FB_1 toxicity.

In the current study mice pretreated with gadolinium had less hepatic accumulation of free sphinganine in response to FB_1 than those pretreated with saline. The reduced accumulation of free sphinganine also could at least in part account for the attenuating liver damage by FB_1. It has been established that free sphinganine causes cell death after FB_1 exposure. Inhibition of free sphinganine accumulation reversed FB_1 cytotoxicity in numerous *in vitro* studies (He et al., 2002, Tolleson et al., 1999; Yoo et al., 1996). Hepatotoxicity was correlated with the hepatic free sphinganine content in FB_1-treated animals (Riley et al., 2001; Tsunoda et al., 1998).

The Mechanisms by which gadolinium attenuates FB_1-induced accumulation of free sphinganine are not totally clear. Previous studies showed that TNFα activated sphingosine kinase, leading to conversion of sphingosine to sphingosine-1-phosphate (Osawa et al., 2001; Xia et al., 1999). We observed in the present study that gadolinium pretreatment increased

production of TNFα signaling factors such as TNFα and TNF receptor 1. It is plausible that activation of sphingosine kinase by the increased TNFα signaling could promote sphinganine metabolism, thereby reducing accumulation of free sphinganine. Sphinganine is produced through *de novo* biosynthesis by serine palmitoyltransferase (SPT, Merrill, 2002). We recently have shown that hepatic SPT was activated upon exposure to FB_1 (He et al., 2004). It is unknown whether or not gadolinium pretreatment influenced SPT activity; however, since both FB_1 and TNFα increase SPT activity (He et al., 2004; Memon et al., 1998), the decrease in sphinganine accumulation is unlikely related to the change of this enzyme activity.

It has been reported that gadolinium chloride activated nuclear transcriptional factor NFκB leading to production of TNFα in liver (Rose et al., 2001). Consistent with these findings, we observed increased production of TNFα signaling factors after gadolinium treatment in the present study. However, the source of TNFα and other cytokines following gadolinium depletion of Kupffer cells remains unclear. Immunohistochemical studies earlier demonstrated that biliary epithelial cells and portal vein endothelial cells, which are resistant to gadolinium, produced TNFα in regenerating rat liver (Loffreda et al., 1997).

In the current study, the increased TNFα signal production in liver of mice pretreated with gadolinium chloride could partly explain the observed reduction of FB_1 hepatotoxicity. Tumor necrosis factor itself could activate NFκB, an essential factor for protection from TNFα-induced cell death (Beg and Baldwin, 1994; Beg and Baltimore, 1996). Beneficial effects of TNFα in protecting against cell death have been reported in a variety of previous studies (Bruce-Keller et al., 1999; Cheng et al., 1994; Kurrelmeyer et al., 2000; Nagwashiro et al., 1997). It is apparent that TNFα has both cell death signaling and cell survival roles (Kurrelmeyer et al., 2000,

Shohami et al., 1999). Tumor necrosis factor α therefore may play roles in inducing apoptosis as well as preventing cell damage after FB_1 treatment in mouse liver. We previously demonstrated that transgenic mice carrying human TNFα gene reduced susceptibility to FB_1 hepatotoxicity (Sharma et al., 2000b). Lack of TNFα expression increased sensitivity of mice to FB_1-induced liver damage (Sharma et al., 2002). These studies suggest a protective role of TNFα in FB_1 hepatotoxicity in mice.

It has been suggested that gadolinium chloride exerts hepatoprotective effects on cadmium liver damage in addition to depleting Kupffer cells (Harstad and Klaassen, 2002). The effects of gadolinium chloride on liver are not limited to Kupffer cell depletion. For example, gadolinium chloride reduces expression of glutathione-S-transferase (Kim and Choi, 1997), and induces metallothionein in liver (Harstad and Klaassen, 2002). It is unknown currently whether or not these effects of gadolinium chloride contribute to reducing FB_1 hepatotoxicity in mice. In the present study, Kupffer cells are effectively depleted by gadolinium chloride. This does not exclude the role of Kupffer cells in FB_1-induced liver damage. In conclusion, present study demonstrated that gadolinium chloride depletion of Kupffer cells attenuated FB_1 liver injury, indicating Kupffer cells are involved in FB_1 pathogenesis. Gadolinium chloride-alleviated FB_1 liver injury may partly be attributed to decreasing FB_1-hepatic accumulation of free sphinganine as well as increasing protective TNFα signaling.

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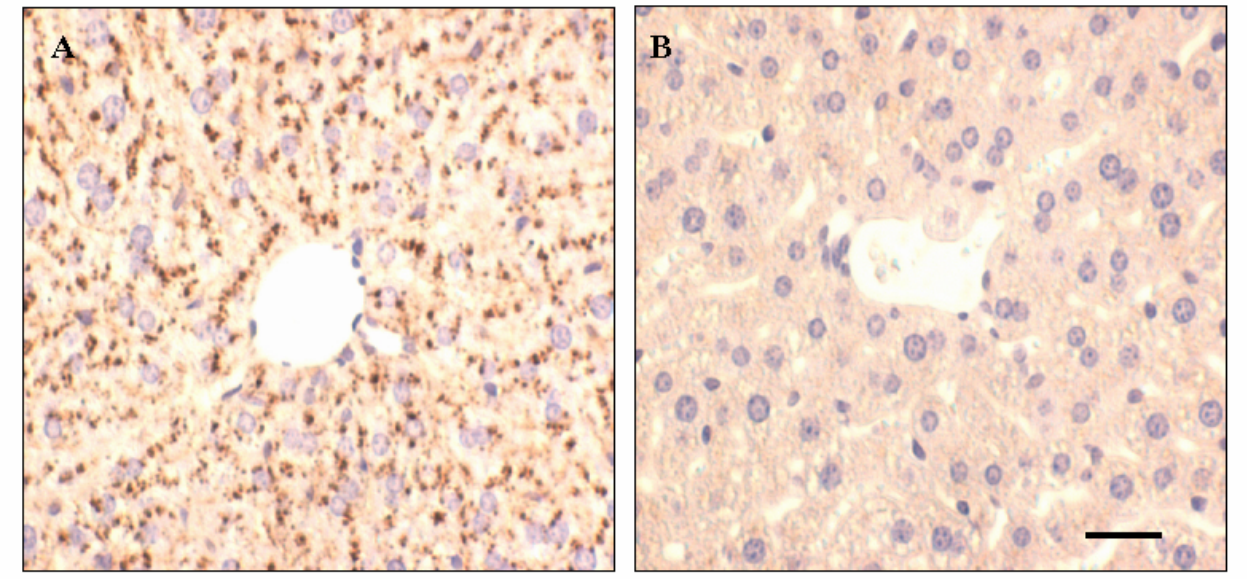

Fig. 4.1. Immunological localization of Kupffer cells in liver after gadolinium chloride. Kupffer cells in liver sections were stained with mac-3 antibodies (M3/84). A, normal control liver showing abundant Kupffer cells; B, gadolinium-treated liver showing no Kupffer cells. Bar on the lower right indicates 20 μm.

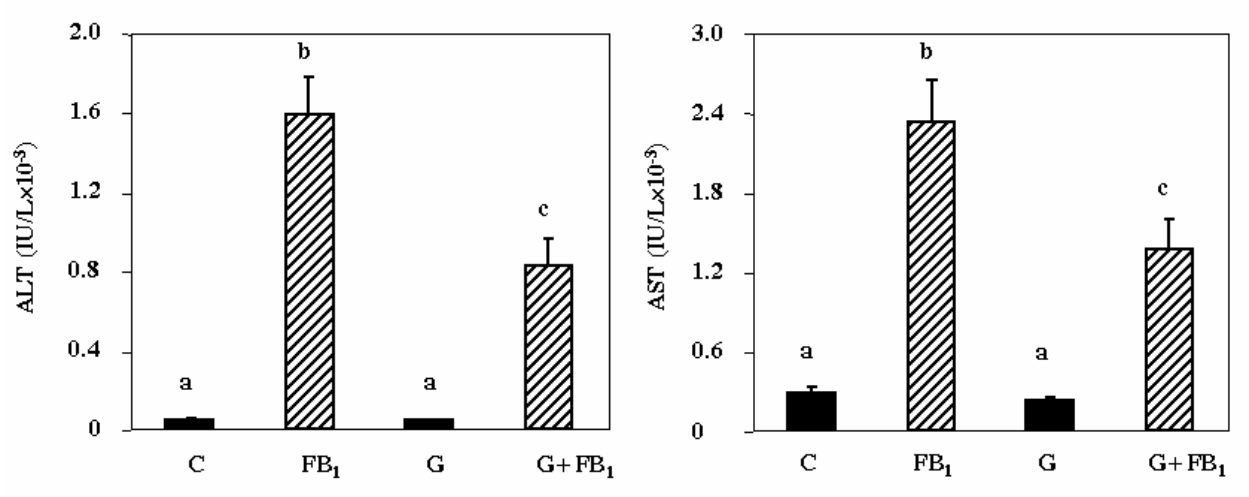

Fig. 4.2. Effect of gadolinium (G) on FB_1-induced increase in plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities. Female BALB/c mice were treated once with 50 mg/kg gadolinium chloride via tail vein before treatment with 2.25 mg/kg FB_1 subcutaneously for 3 days. Mean ± SE (n=5). Different letters on bars indicate statistical difference at $p<0.05$.

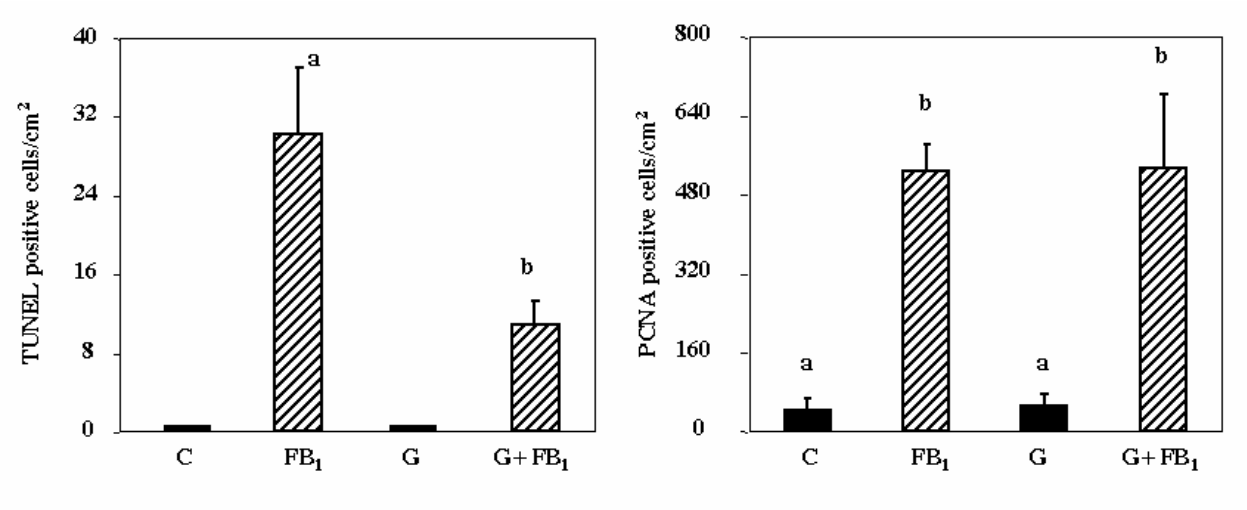

Fig. 4.3. Effect of gadolinium (G) on FB_1-induced hepatocyte apoptosis and proliferation. Cell apoptosis was analyzed by TUNEL; proliferation was detected by proliferating cellular nuclear antigen (PCNA). Mean ± SE (n=5). Different letters indicate statistical difference at $p<0.05$ by the Wilcoxon Rank Sum test.

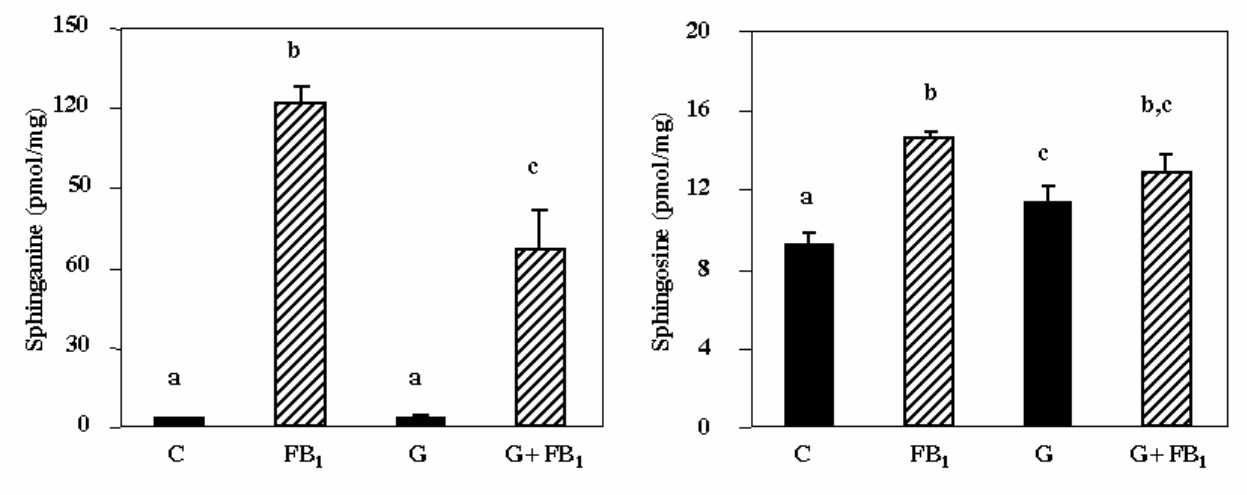

Fig. 4.4. Effect of gadolinium (G) on FB_1-induced accumulation of free sphingoid bases sphinganine and sphingosine in liver. Mean ± SE (n=5). Different letters denote statistical difference at $p<0.05$.

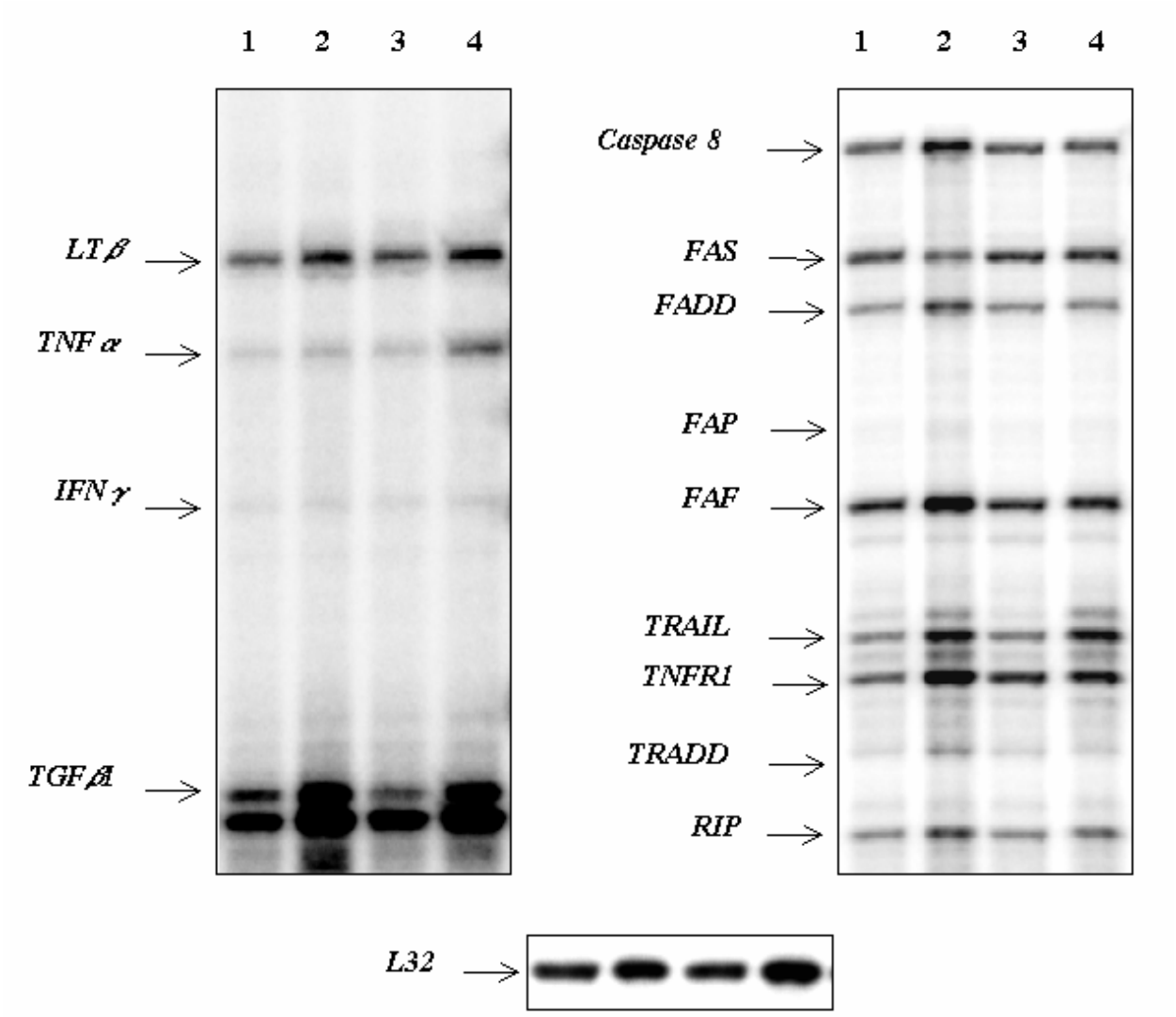

Fig. 4.5. Representative gel from RNase protection assay. The lanes indicate samples from mice treated with 1, saline; 2, FB_1; 3, gadolinium; and 4, gadolinium+FB_1. Bands shown are LTβ, lymphotoxin β; TNFα, tumor necrosis factor α; IFNγ, interferon γ; TGF β1, transforming growth factor β1; FAS, CD95; FADD, Fas associated death domain; FAP, Fas associated protease; FAF, Fas associated factor; TRAIL, TNF-related apoptosis-inducing ligand; TNFR1, TNF receptor 1; TRADD, TNF receptor associated death domain; and RIP, receptor-interacting protein. The ribosomal L-32 was used as a housekeeping gene to normalize the expression for various genes.

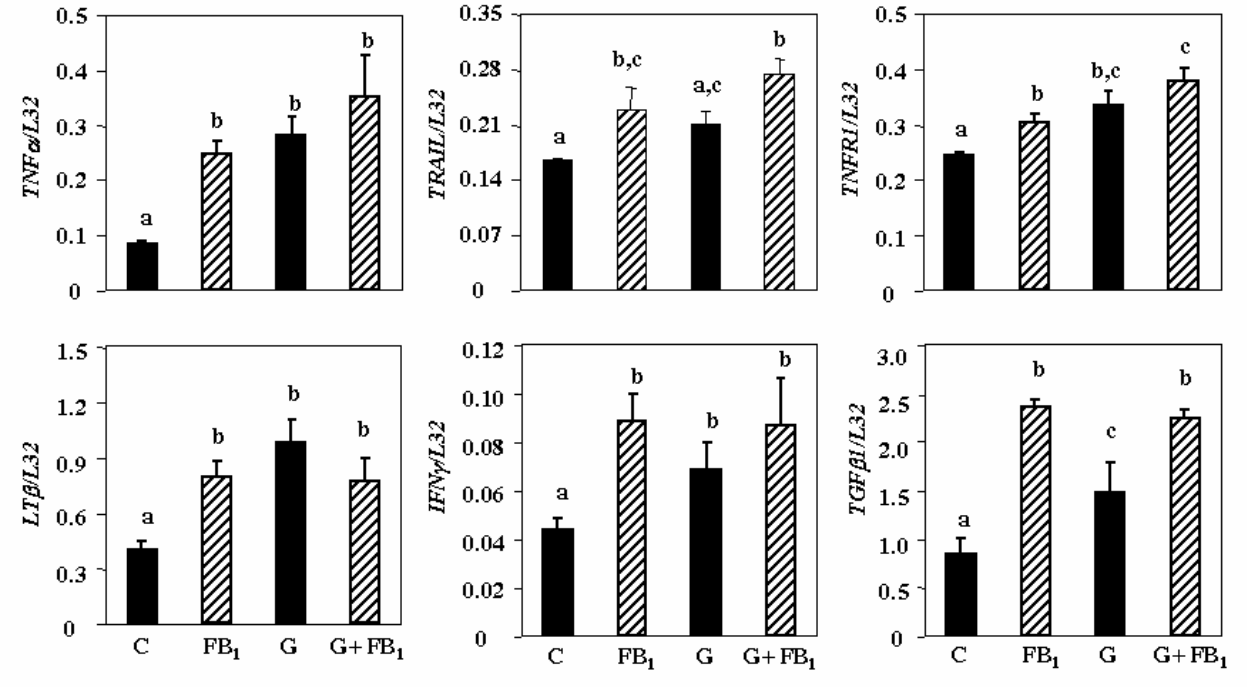

Fig. 4.6. Effects of gadolinium (G) on FB_1-induced expression of tumor necrosis factor α (TNFα), TNF-related apoptosis-inducing ligand (TRAIL), TNF receptor (TNFR) 1, lymphotoxin (LT) β, interferon γ (IFNγ) and transforming growth factor β1 (TGFβ1) in liver. Mean ± SE (n=5). Different letters indicate statistical difference at $p<0.05$.

CHAPTER 5

EXACERBATION OF FUMONISIN HEPATOTOXICITY IN MICE BY ANTI-TUMOR NECROSIS FACTOR α ANTIBODIES AND PENTOXIFYLLINE[3]

[3]Q. He, Sharma R.P. To be submitted to *Toxicological Sciences*

Fumonisin B_1 (FB_1), the most abundant and toxic fumonisin produced by *Fusarium verticillioides* detected in corn and corn-based foods, causes species- and organ-specific toxicity such as equine leukoencephalomalacia, porcine pulmonary edema, and liver and kidney damage in most animal species. Fumonisin B_1 disrupts sphingolipid metabolism by inhibiting ceramide synthase and induces expression of many cytokines including tumor necrosis factor (TNF) α. In the current study, male C57BL/6N mice were injected with anti-TNFα antibodies to block TNFα signal transduction at 150 μg each through the tail vein once 16 h before 5 daily FB_1 treatment, or pentoxifylline at 150 mg/kg twice a day for 5 days to inhibit TNFα production; FB_1 was given by subcutaneous injection at 2.25 mg/kg daily for 5 days. One day after the last FB_1 injection, the mice were euthanized and blood and tissues were sampled for analyses. Results showed that both anti-TNFα antibodies and pentoxifylline augmented FB_1-induced increases in activities of plasma alanine aminotransferase and aspartate aminotransferase and number of apoptotic hepatocytes. Anti-TNFα antibodies did not alter FB_1-induced accumulation of free sphingoid bases and expression of TNFα, interleukin (IL)-12, and interferon (IFN) γ; pentoxifylline significantly reduced accumulation of free sphinganine and expression of TNFα without altering IL-12 and IFNγ expression in response to FB_1. These findings suggest a partially protective role of TNFα signaling activation in FB_1 hepatotoxicity.

Key words: Fumonisin B_1, pentoxifylline, tumor necrosis factor α, sphingolipid, hepatotoxicity

INTRODUCTION

Fumonisins, mycotoxins produced by *Fusarium verticillioides* (=*F. moniliforme*), has been reported globally as a contaminant of animal feeds and human corn-based foods (WHO, 2000). Fumonisin B_1 (FB_1), the most abundant fumonisin found in *F. verticillioides*, causes species- and organ-specific toxicity, such as equine leukoencephalomalacia (Marasas 2001), and porcine pulmonary edema (Haschek *et al.*,, 2001). Fumonisin B_1 is hepatocarcinogenic in male rats and female $B6C3F_1$ mice (Gelderblom *et al.*, 1991, Howard *et al.*, 2001) and nephrocarcinogenic in male F344 rats (Howard *et al.*, 2001). High incidences of human esophageal cancer in southern Africa and China have been epidemiologically linked to consumption of fumonisin-contaminated foods (Chu and Li, 1994, Marasas, 2001). Fumonisins produce toxic damage in liver and kidney of rodents (Sharma *et al.*, 1997, Voss *et al.*, 2001).

Fumonisins structurally resemble free sphingoid bases (sphinganine and sphingosine), and inhibit ceramide synthase (Sphingosine *N*-acyltransferase), a critical enzyme in sphingolipid biosynthetic pathway, leading to blocking *de novo* sphingolipid biosynthesis and subsequent accumulation of free sphinganine (Merrill *et al.*, 1993, Wang *et al.*, 1991). Toxicity of FB_1 is correlated to intracellular accumulation of free sphingoid bases (Riley *et al.*, 2001, Tsunoda *et al.*, 1998). Inhibition of serine palmitoyltransferase (SPT), the first enzyme in the *de novo* biosynthetic pathway of sphingolipids, reduced free sphinganine accumulation and reversed FB_1 toxicity (He *et al.*, 2002, Riley *et al.*, 1999, Tolleson *et al.*, 1999, Yoo *et al.*, 1996), further supporting a role of free sphinganine accumulation in FB_1 toxicity.

Tumor necrosis factor (TNF)α signaling pathways modulate FB_1 toxicity both *in vivo* and *in vitro*. Fumonisin B_1 treatment induced expression of various cytokines including TNFα and apoptotic signaling genes (Bhandari and Sharma, 2002a, b, He *et al.*, 2001). In response to

lipopolysaccharide stimulation, peritoneal macrophages from FB_1-treated mice produced higher amounts of TNFα than controls (Dugyala *et al.,* 1998). Hepatotoxicity in response to FB_1 was reduced in mice lacking TNF receptor (TNFR) 1 or TNFR 2 (Sharma *et al.,* 2000a, 2001). On the other hand, FB_1 hepatotoxicity was reduced in mice carrying human TNFα transgene (Sharma *et al.,* 2000b), and TNFα knockout mice were more sensitive to FB_1 hepatotoxicity than their wild-type counterparts (Sharma *et al.,* 2002). It is therefore apparent that pharmacologic modulation of TNFα signal transduction would alter FB_1 toxicity.

Binding of TNFα to its receptors initiates transducation of TNFα signaling, leading to cell death or survival (Beg and Baltimore, 1996, Shohami *et al.,* 1999, Kurrelmeyer *et al.,* 2000). Anti-TNFα antibodies can inhibit TNF signal transduction by preventing binding of TNFα to its receptors, thereby inhibiting TNFα-mediated responses. These antibodies have been used to block TNFα biological effects experimentally and clinically (Iimuro *et al.,* 1997).

Pentoxifylline, a non-specific inhibitor of phosphodiesterase (PDE), has been shown to be an effective agent for inhibiting the production of TNFα (Bernard *et al.,* 1995, Zabel *et al.,* 1993). It has been used to investigate the role of TNFα in ischemic injury of liver (Rüdiger and Clavien, 2002), and chemical hepatotoxicity (Barton *et al.,* 2001, Sneed *et al.,* 2000).

In the present study, we investigated the role of TNFα in the pathogenesis of FB_1 liver damage. It was hypothezed that interefering with TNFα signaling will modify FB_1-induced hepatotoxicity. The signal transduction or production of TNFα was inhibited using specific antibodies to TNFα and pentoxifylline. Results showed that FB_1 hepatotoxicity was augmented in mice pre-treated with either anti-TNFα antibodies or pentoxifylline. The accumulation of free sphinganine in the liver following FB_1 treatment remained unchanged after treatment with anti-

TNFα antibodies or pentoxifylline. Anti-TNFα antibodies did not change FB_1-induced expression of TNFα, interleukin (IL)-12, and interferon (IFN)γ; pentoxifylline significantly prevented the FB_1-induced increase in the expression of TNFα, but did not alter the expression of IL-12 and IFNγ in response to FB_1. Data indicate that, in addition to free sphingoid base accumulation, TNFα signaling can modify FB_1 hepatotoxicity.

MATERIALS AND METHODS

Chemicals. Fumonisin B_1 (purity >98%) was obtained from Programme on Mycotoxins and Experimental Carcinogenesis (PROMEC, Tygerberg, South Africa). Rabbit anti-mouse TNFα antibodies was purchased from Pierce Biotechnology, Inc. (Rockford, IL, USA). Pentoxifylline (3,7-dimethyl-[5-oxohexyl]xanthin) and all other reagents were purchased from Sigma-Aldrich Chemical Company Inc. (St. Louis, MO, USA), unless stated otherwise.

Animals. Six-week-old male C57BL/6N mice weighing about 22-24 g were obtained from Harlan Laboratories (Indianapolis, IN, USA). They were acclimated for 1 week before dosing under controlled environmental conditions at 23°C and 65% relative humidity with a 12 h light/dark cycle. Feed and water were available *ad libitum*. Protocols for animal use followed the Public Health Service Policy on Humane Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee.

Treatment. Each animal receiving antibodies was treated once with 150 μg of anti-TNFα antibodies by tail vein injection 16 h before the first FB_1 treatment; pentoxifylline was injected intraperitoneally at 150 mg/kg every 12 h for 5 days. The mice were given 5 daily

subcutaneous injections of either phosphate buffered saline (PBS) or 2.25 mg/kg of FB_1 in PBS. The protocol has been proved to produce consistent liver damage in mice exposed to FB_1 in our laboratory (Sharma *et al.*, 2000a, b, 2002).

One day after the final FB_1 treatment, mice were sacrificed by decapitation. Blood was collected in heparinized tubes, and plasma was subsequently isolated for analysis of alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Livers were collected from each animal, and aliquots were fixed immediately in neutral 10% formalin, or quickly frozen in liquid nitrogen and stored at -85°C until analysis.

Analysis of liver enzymes in plasma. Activities of plasma ALT and AST were determined using a Hitachi 912 Automatic Analyzer (Roche Diagnostics, Indianapolis, IN).

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay for DNA fragmentation. Liver tissue sections (5 μm) were prepared and subjected to dUTP nick-end labeling by TdT with a peroxidase-based *In Situ* Cell Death Detection kit (Roche Diagnostics, Indianapolis, IN) as described previously (Sharma *et al.*, 2003). The stained apoptotic cells were counted under a light microscope and normalized to the unit area as described (Sharma *et al.*, 1997).

Sphingolipid analysis. Free sphingosine and sphinganine of liver in base-treated lipid extracts were determined by HPLC utilizing a modification of the extraction methods described earlier (Merrill *et al.*, 1988). Sphingoid bases were quantitated based on the recovery of a C_{20}-sphinganine standard (D-erythro-C_{20}-dihydro-sphingosine, Matreya Inc. Pleasant Gap, PA, USA). The HPLC apparatus and derivation procedure were similar to those described before (He *et al.*, 2001).

Reverse transcriptase-polymerase chain reaction for semiquantitative analyses of TNFα, IL-12 and IFNγ expression. Total RNA from liver tissue was extracted with TRI® reagents (Molecular Research Center, Cincinnati, OH, USA). An aliquot of 2.5 μg RNA was subject to cDNA synthesis using Superscript™III reverse transcriptase and Oligo(dT)$_{12-18}$ primer (Inveitrogen® Life Technologies, Carsbad, CA, USA). The abundance of mRNA for TNFα, IL-12 and IFNγ in liver tissues was analyzed by polymerase chain reaction (PCR) using Taq DNA polymerase and 0.2 μM of each primer in 1X PCR buffer containing 2 mM $MgCl_2$. The PCR reactions were performed in an Eppendorf Mastercycler® gradient (Eppendorf Scientific Inc., Westbury, NY, USA). The respective primers (chosen by Primer3 program, Whithead Institute, Cambridge, MA, USA) are shown in Table 1. The annealing temperatures for each reaction were optimized, and the number of cycles was optimized to yield the product in exponential range to avoid saturation. The PCR products were separated on 2% agarose gel containing ethidium bromide and detected by UV transilluminator (Ultra Lum Inc., Carson, CA, USA). Images were captured using a Kodac DC290 camera followed by digitization using UN-SCAN-IT software (Silk Scientific Inc., Orem, UT, USA). Density of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the same sample was used to normalize the expression of each gene. The quantative validity of RT-PCR was confirmed by either northern blot in lipopolysaccharide-treated J774.A macrophages (He *et al.*, 2001) or RNase protection assay in mouse liver (Bhandari and Sharma 2002a)

Statistical analysis. Results are presented as mean ± standard error (SE). Data were analyzed by two-way analysis of variance (ANOVA) followed by Duncan's multiple range test, unless otherwise stated. In selected cases where unequal variances of different groups were

obvious the Wilcoxon rank sum test was employed. All statistical analyses were performed using SAS software programs (SAS Institute Inc., Cary, NC, USA). The level of $p < 0.05$ was considered significant.

RESULTS

Treatment with anti-TNFα antibodies or pentoxifylline increased liver damage following FB_1 exposure

No obvious behavioral abnormality was observed in all animals during the 5 daily treatment period. Body weight gain and food intake were not different from each other among treatments (data not shown).

Anti-TNFα antibodies treatment did not increase plasma ALT and AST activities. In response to FB_1, mice pretreated with anti-TNFα antibodies had higher activities of plasma ALT and AST than those receiving FB_1 only (Fig. 5.1). The increase of plasma ALT and AST activities was consistent with the increased hepatocyte apoptosis (Fig. 5.1).

Pentoxifylline alone caused no effects on plasma ALT and AST activities, and cell apoptosis. Similar to anti-TNFα antibodies, pentoxifylline significantly enhanced the FB_1-induced increase in plasma enzyme activities and number of apoptotic hepatocytes (Fig. 5.2).

The histopathological effects of FB_1 on liver were limited to the presence of apoptotic hepatocytes with no evident oncotic changes; and such histopathological changes in liver of mice treated with FB_1 have been consistently reported in our previous studies (He *et al.*, 2004, Sharma *et al.*, 2000a, b, 2002). The apoptotic changes in response to FB_1 have been reported recently (He *et al.*, 2004, Sharma *et al.*, 1997).

Alterations of free sphingoid bases following anti-TNFα antibodies or pentoxifylline and FB_1 exposure

Treatment with anti-TNFα antibodies did not change levels of free sphinganine and sphingosine in liver (Fig. 5.3). Fumonisin B_1 significantly increased the levels of hepatic free sphinganine and sphingosine, and the increases in free sphingoid bases resulting from FB_1 were not changed by anti-TNFα treatment (Fig. 5.3).

There were significantly higher levels of free sphinganine and sphingosine in liver of mice treated with pentoxifylline compared to saline controls (Fig. 5.4). The accumulation of hepatic free sphinganine in the pentoxifyline + FB_1 group was lower than that in the FB_1 alone group, while free sphingosine contents were not different between these two groups (Fig. 5.4).

Expression of TNFα, IL-12 and IFNγ in liver after treatment with anti-TNFα antibodies or pentoxifylline and FB_1

Neither anti-TNFα antibodies nor pentoxifylline altered the constitutive expression of TNFα, IL-12 and IFNγ (Fig. 5.5 - 5.6). Consistently FB_1 increased the expression of the above genes in liver. Anti-TNFα antibodies pretreatment did not change FB_1-induced expression of hepatic TNFα, IL-12 and IFNγ (Fig. 5.5).

Pentoxifylline treatment significantly reduced FB_1-induced hepatic TNFα expression to its constitutive level, while it did not alter the induction of liver IL-12 and IFNγ as a result of FB_1 treatment (Fig. 5.6).

DISCUSSION

The current study demonstrated that inhibition of TNFα signal transduction by anti-TNFα antibodies or TNFα production by pentoxifylline enhanced FB_1 hepatotoxicity. Treatment of animals with either anti-TNFα antibodies or pentoxifylline did not increase the accumulation of hepatic free sphingoid bases in response to FB_1. The expression of TNFα in response to FB_1 was reduced by pentoxifylline, while IL-12 and IFNγ remained unaffected by either anti-TNFα or pentoxifylline.

The primary biochemical effect of FB_1 is ceramide synthase inhibition leading to accumulation of free sphinganine, the precursor of ceramide in *de novo* biosynthetic pathway of sphingolipid (Merrill *et al.,* 1993, Wang *et al.,* 1991). Inhibition of ceramide synthase by FB_1 ultimately results in depletion of ceramide and more complex sphingolipids (Merrill *et al.,* 2001). Signaling by free sphingoid bases involves cell growth inhibition and apoptosis induction (Merrill *et al.,* 2001). Previous studies have shown that hepatotoxicity of FB_1 was correlated with hepatic free sphinganine content (Riley *et al.,* 2001, Tsunoda *et al.,* 1998); inhibition of SPT by myriocin or β-chloroalanine prevents formation of free sphinganine, an earlier step in sphingolipid biosynthesis at least temporarily protects cells in cultures from FB_1-induced cytotyoxicity. These findings indicate that free sphinganine is an important mediator in FB_1 toxicity (He *et al.,* 2002, Riley *et al.,* 1999, Tolleson *et al.,* 1999, Yoo *et al.,* 1996). The present study showed that liver content of free sphinganine in all FB_1-treated groups was as 4-6 fold higher than respective saline controls, further supporting a role of free sphinganine in FB_1 toxicity. However, both anti-TNFα antibodies and pentoxifylline exacerbated FB_1-induced liver damage, though neither enhanced accumulation of free sphinganine in response to FB_1. Results

suggest that TNFα signal pathways, in addition to sphinganine accumulation, are involved in FB_1 hepatotoxicity in mice.

In response to FB_1, expression of hepatic TNFα signaling factors including TNFα and TNF receptor 1 was increased (Bhandari and Sharma, 2002a, b). Peritoneal macrophages from FB_1-treated mice produced more TNFα than those from saline controls following lipopolysaccharide stimulation *ex vivo* (Dugyala *et al.,* 1998). In contrast, the hepatoxicity of FB_1 in mice carrying human TNFα transgene was attenuated compared to their wild-type controls (Sharma *et al.,* 2000b). In accordance with studies in the TNFα transgenic mice, TNFα knockout mice also exhibited greater sensitivity to FB_1 liver toxicity (Sharma *et al.,* 2002). All these studies indicated that TNFα is an important regulatory molecule in FB_1 hepatotoxicity.

Although TNFα is generally associated with inflammation and cell death, it also protects cells from death induced by different types of stimuli including TNFα (Shohami *et al.,* 1999). The reduced hepatotoxicity in TNFα transgenic mice exposed to FB_1 could be explained by the activation of nuclear factor (NF) κB (Sharma *et al.,* 2000b), which is essential for protection from TNFα cell death (Beg and Baltimore, 1996). Beneficial effects of TNFα in protecting against cell death have been reported in a variety of previous studies (Bruce-Keller *et al.,* 1999, Kurrelmeyer *et al.,* 2000, Nagwashiro *et al.,* 1997, Shohami *et al.,* 1999). In the present study, it seemed that TNFα played a protective role in FB_1-induced liver damage. Anti-TNFα antibodies could inhibit TNFα-induced cell death as well as survival signal transduction by blocking interaction of TNFα with its receptors and subsequent recruitment of multiple intracellular adapter proteins. Inhibition of TNFα signal cascade by anti-TNFα antibodies could prevent activation of NFκB and therefore sensitize the liver of mice to FB_1 toxicity. Reduced FB_1

hepatotoxicity in TNFα transgenic mice was correlated with an increase in nuclear NFκB (Sharma *et al,* 2000b).

Pentoxifylline has been shown to inhibit the production of TNFα in different types of cells e.g., vascular cells and macrophages (Bernard *et al.,* 1995, Poulakis *et al.,* 1999). The current study showed that pentoxifylline decreased FB_1-induced expression of hepatic TNFα with no effects on IL-12 and IFNγ expression. Results suggest a selective inhibitory effect of pentoxifyline on TNFα production in liver. It has been shown that pentoxifylline did not alter IL-12 stimulated IFNγ production in mitogen-activated spleenic T cells (Coon *et al.,* 1999). Previous studies have shown that lack of TNFα production in mice increased susceptibility of liver to FB_1 toxicity (Sharma *et al.,* 2002), which could be explained by increased expression of pro-apoptotic factors such as those in Fas signal pathways (Sharma *et al.,* 2003). It is unknown currently whether or not prevention of FB_1-stimulated TNFα production by pentoxifylline alters expression of pro-apoptotic factors. Since TNFα itself could be anti-apoptotic (Bruce-Keller *et al.,* 1999, Kurrelmeyer *et al.,* 2000, Nawashiro *et al.,* 1997, Shohami *et al.,* 1999), prevention of TNFα production would be predicted to reduce the anti-cell death properties of TNFα. It is plausible that pentoxifylline inhibition of TNFα alone was sufficient to increase vulnerability of hepatocytes to FB_1-induced cell death.

In summary, prevention of TNFα signaling transduction by anti-TNFα antibodies or inhibition of TNFα production by pentoxifylline both augmented FB_1 hepatotoxicity. Results suggest that activation of TNFα signal cascade would play a protective role, to some extent, in response to FB_1-induced liver injury.

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Table 5.1.

Primers and PCR conditions for hepatic genes expression analysis*

Gene name	Primers	Annealing temperature (°C)	PCR cycles
TNFα	sense 5' GTT CTA TGG CCC AGA CCC TCA CA 3' anti-sense 5' TCC CAG GTA TAT GGG TTC ATA CC 3'	55	32
IL-12	sense 5' CTG GTG CAA AGA AAC ATG GA 3' anti-sense 5' GTC CCT GAT GAA GCT GG 3'	55	40
IFNγ	sense 5' TTC TGC CCT TTT TGG ATG AG 3' anti-sense 5' AGT CGC TGG AAC TGA GGT GT 3'	55	32
GAPDH	sense 5' TAT GAC TCC ACT CAC GGC AA 3' anti-sense 5' GTG GTT CAC ACC CAT CAC AA 3'	55	22

* Other PCR conditions include: hot start: 95 °C 5min, 1 cycle, followed by: denaturation, 94 °C 30 sec, annealing, indicated temperature 30 sec; elongation, 72 °C 1 min for indicated cycles, and finally elongation at 72 °C 1 min for 1 cycle. For all experiments, the conditions were optimized to keep the number of cycles within the range of exponential product increase.

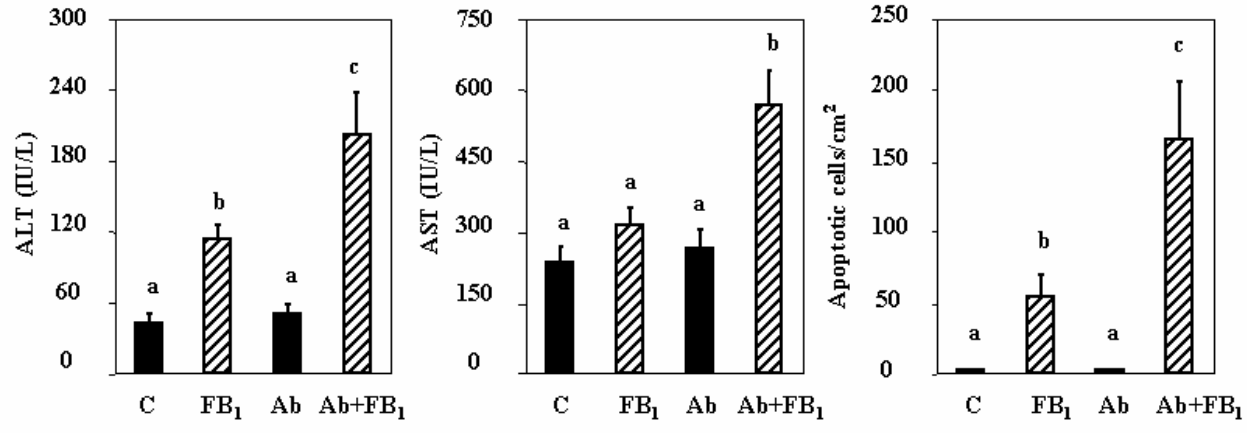

Fig. 5.1. Anti-TNFα antibodies (Ab) enhanced fumonisin B_1 (FB_1)-induced increase of plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities and number of apoptotic hepatocytes. C57BL/6N mice were injected with polyclonal anti-TNFα antibodies at 150 μg/each only once before daily treatment with 2.25 mg/kg FB_1 subcutaneously for 5 days. One day after the last FB_1 treatment, the animals were sacrificed and plasma was used for analysis of ALT and AST. Apoptotic hepatocytes in formalin fixed, paraffin-embedded liver sections were analyzed by TUNEL. Mean ± SE (n=5). Different letters indicate statistical difference at $p<0.05$.

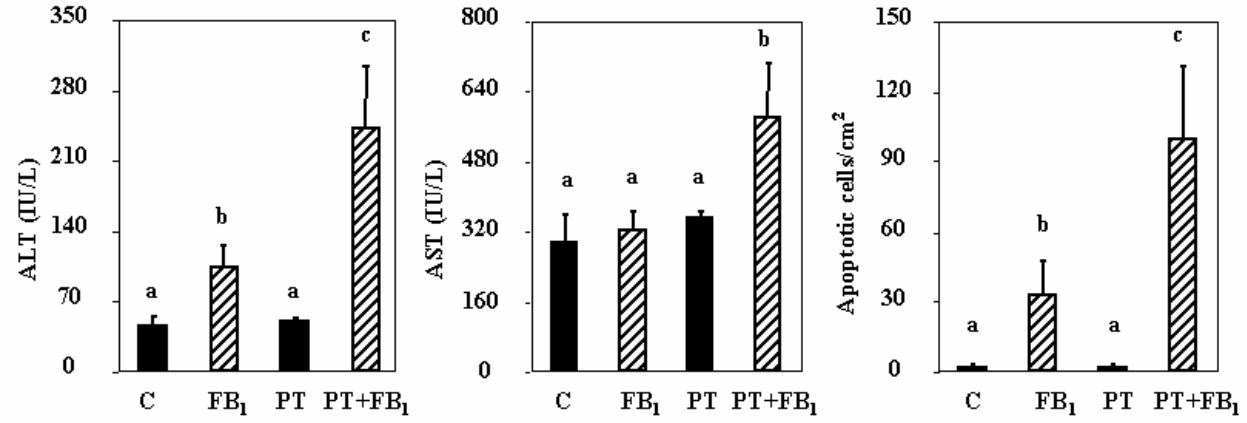

Fig. 5.2. Pentoxifylline (PT) increased fumonisin B_1 (FB_1)-induced increase of plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities and number of apoptotic hepatocytes. C57BL/6N mice were injected intraperitoneally with 150 mg/kg of pentoxifylline twice a day and 2.25 mg/kg FB_1 subcutaneously for 5 days. One day after the last FB_1 treatment, the animals were sacrificed and plasma was used for analysis of ALT and AST. Apoptotic hepatocytes in formalin fixed, paraffin-embedded liver sections were analyzed by TUNEL. Mean ± SE (n=5). Different letters indicate statistical difference at $p<0.05$.

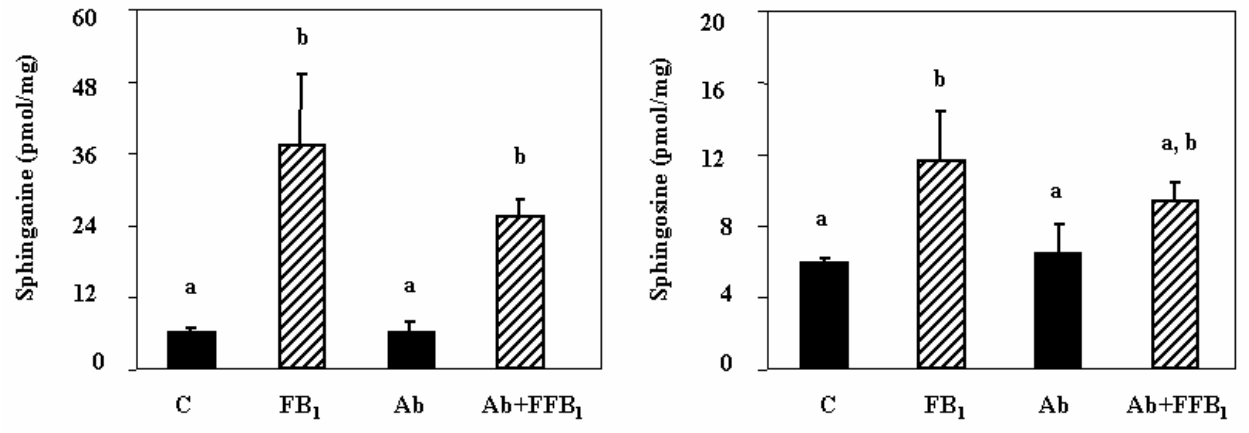

Fig. 5.3. Effects of Anti-TNFα antibodies (Ab) on fumonisin B_1 (FB_1)-induced accumulation of free sphingoid bases, sphinganine and sphingosine, in liver. Mean ± SE (n=5). Different letters indicate statistical difference at $p<0.05$.

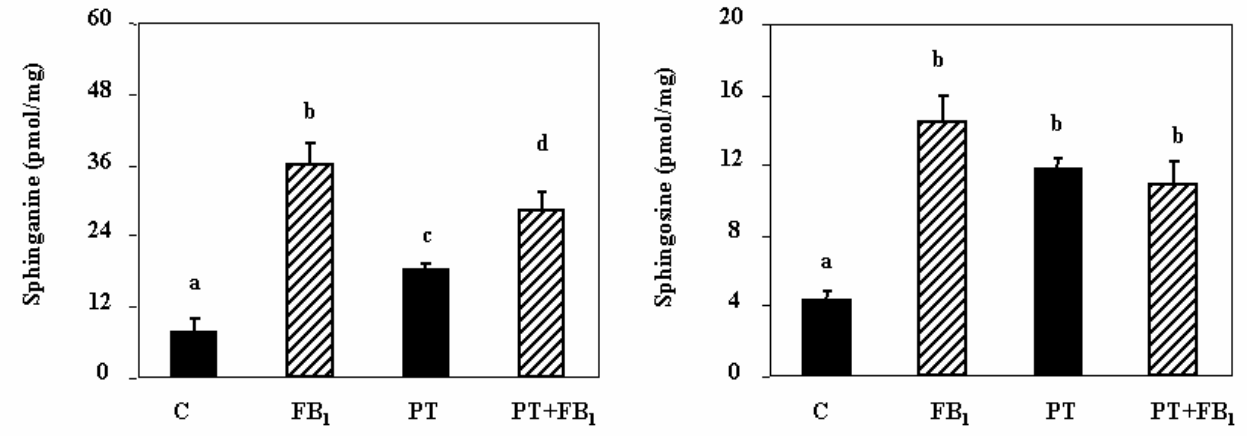

Fig. 5.4. Effects of pentoxifylline (PT) on fumonisin B_1 (FB_1)-induced accumulation of free sphingoid bases, sphinganine and sphingosine, in liver. Mean ± SE (n=5). Different letters indicate statistical difference at $p<0.05$.

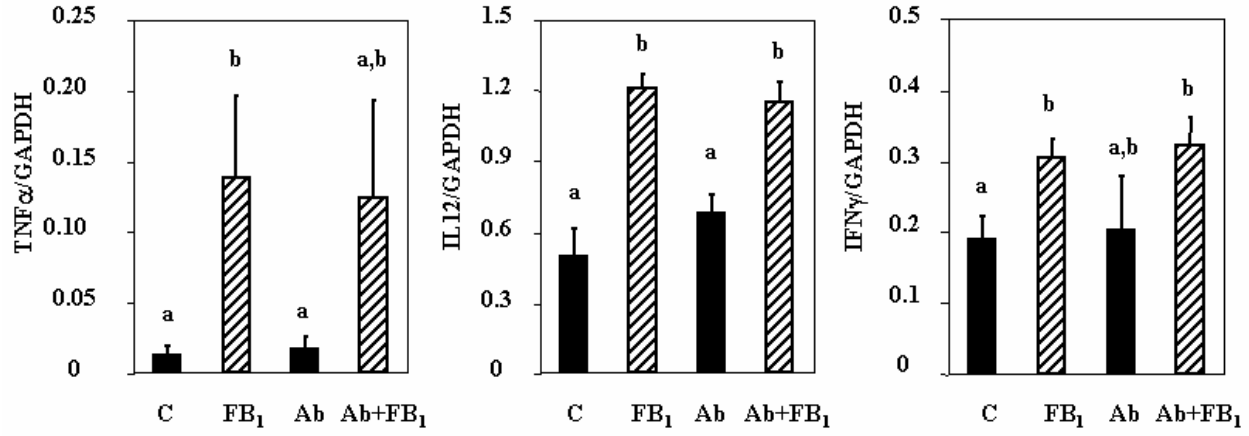

Fig. 5.5. Effects of Anti-TNF antibodies (Ab) on fumonisin B_1 (FB_1)-induced expression of tumor necrosis factor (TNF) α, interleukin (IL)-12, and interferon (IFN) γ in liver. Mean ± SE (n=5). Different letters indicate statistical difference at $p<0.05$.

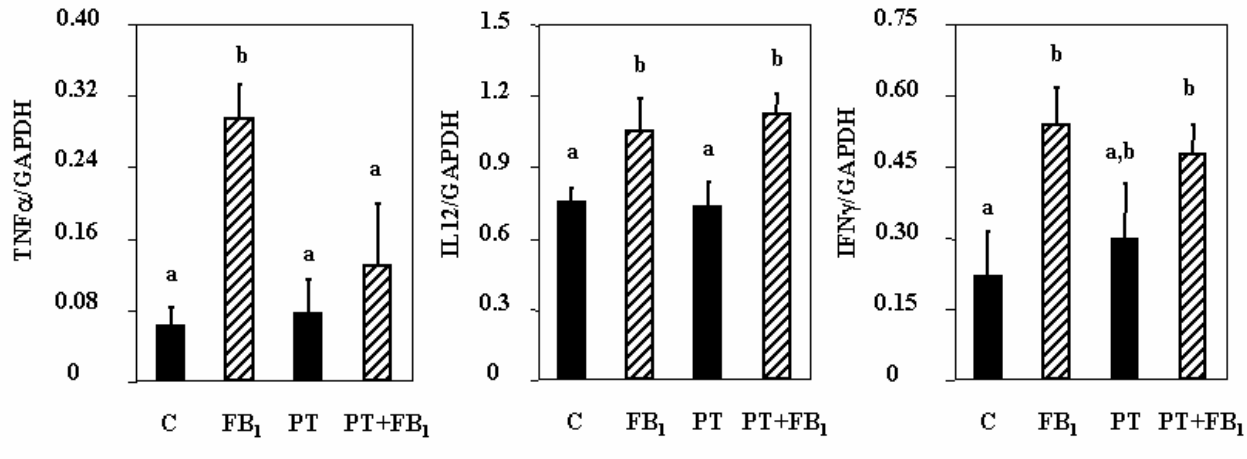

Fig. 5.6. Effects of pentoxifylline (PT) on fumonisin B_1 (FB_1)-induced expression of tumor necrosis factor (TNF) α, interleukin (IL)-12, and interferon (IFN) γ in liver. Mean ± SE (n=5). Different letters indicate statistical difference at $p<0.05$.

CHAPTER 6

SILYMARIN PROTECTS AGAINST LIVER DAMAGE IN BALB/C MICE EXPOSED TO FUMONISIN B_1 DESPITE INCREASING ACCUMULATION OF FREE SPHINGOID BASES[4]

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[4]Q. He, Kim J., Sharma R.P. *Toxicological Sciences* 2004, 80, 335-342

Fumonisin B_1 (FB_1) is a mycotoxin produced by *Fusarium verticillioides* present on corn and corn-based foods. It causes equine leukoencephalomalacia, porcine pulmonary edema, and liver and kidney damage in most animal species. Fumonisin B_1 perturbs sphingolipid metabolism by inhibiting ceramide synthase activity leading to production of cell signaling factors including tumor necrosis factor (TNF) α. The signal pathways of TNFα are important factors in the pathogenesis of FB_1 hepatotoxicity. In the present study, female BALB/c mice were treated daily with 750 mg/kg silymarin by gavage and 2.25 mg/kg FB_1 subcutaneously for 3 days. One day after the last FB_1 injection, the mice were euthanized and blood and tissues were sampled for analyses. Silymarin significantly diminished FB_1-induced elevation of plasma alanine aminotransferase and aspartate aminotransferase activities and the number of apoptotic hepatocytes, while it augmented hepatocyte proliferation indicated by an increase in proliferating cells. Silymarin dramatically potentiated FB_1-induced accumulation of free sphinganine and sphingosine in both liver and kidney. Silymarin itself slightly increased expression of hepatic TNFα; however, it prevented the FB_1-induced increases in TNFα, TNF receptor 1, TNF receptor-associated apoptosis-inducing ligand, lymphotoxin β, and interferon γ. The induction of transforming growth factor β1 expression in liver following FB_1 treatment was not affected by silymarin. These findings suggest that silymarin protected against FB_1 liver damage through inhibiting biological functions of free sphingoid bases and increasing cellular regeneration.

Key words: Fumonisin B_1, silymarin, tumor necrosis factor α, sphingolipid, hepatotoxicity

INTRODUCTION

Fumonisins are a group of mycotoxins produced by *Fusarium verticillioides* (=*F. moniliforme*) found on corn worldwide (WHO, 2000). Fumonisin B_1 (FB_1), the most abundant fumonisin, causes equine leukoencephalomalacia (Marasas, 2001) and porcine pulmonary edema (Marasas, 2001, Haschek *et al.*, 2001). A high incidence of human esophageal cancer in southern Africa and China was epidemiologically associated with consumption of fumonisin-contaminated foods (Marasas, 2001). Fumonisin B_1 is a hepatic and renal carcinogen in rats and hepatic carcinogen in mice (Gelderblom *et al.*, 1991, Howard *et al.*, 2001). Fumonisins are hepatotoxic and nephrotoxic in laboratory animals (Sharma *et al.*, 1997, Voss *et al.*, 2001). The toxic effects of FB_1 at the cellular level consist of a mixture of both necrosis and apoptosis (Howard *et al.*, 2001, Lemmer *et al.*, 1999).

Fumonisins are structurally similar to free sphingoid bases and inhibit ceramide synthase (Merrill *et al.*, 1993, Wang *et al.*, 1991) resulting in accumulation of free sphinganine and subsequently sphingosine, leading to depletion of ceramide and complex sphingolipids (Merrill *et al.*, 1993, Riley *et al.*, 1993, 1997, Wang *et al.*, 1991, Yoo *et al.*, 1996). Accumulation of free sphingoid bases promotes formation of other sphingolipid metabolites such as sphingoid base-1-phosphates and downstream metabolites (Merrill *et al.*, 2001). Toxicity of FB_1 is well correlated with the accumulation of free sphinganine (Riley *et al.*, 2001, Tsunoda *et al.*, 1998, Yoo *et al.*, 1996) and depletion of complex sphingolipids (Tsunoda *et al.*, 1998, Yoo *et al.*, 1996).

Fumonisin B_1 induces expression of various cytokines including tumor necrosis factor (TNF)α in mice (Bhandari and Sharma, 2002). It has been established that TNFα signaling pathways modulate FB_1 toxicity both *in vivo* and *in vitro*. Fumonisin B_1 hepatotoxicity was reduced in mice lacking either TNF receptor (TNFR) 1 (P55) or TNFR 2 (P75) (Sharma *et al.*,

2000a, 2001). Transfection of a baculovirus gene, inhibitor of apoptosis (IAP), an inhibitor of TNFα-induced cell death, protected renal cells and fibroblasts from FB_1-induced apoptosis (Ciacci-Zanella and Jones 1999, Jones *et al.,* 2001). Expression of TNFR-associated protein (TRAP) 2 was induced in FB_1-sensitive CV-1 cells but repressed in FB_1-resistant COS cells following FB_1 treatment (Zhang *et al.,* 2001), supporting the concept that TNFα signaling pathways are involved in FB_1 toxicity. The signaling cascade in TNFα pathways results in apoptosis upon activation of different downstream signaling molecules after binding of TNFα to TNFRs (Bradham *et al.,* 1998).

Silymarin, an extract from seeds and fruits of *Silybum marianum*, is a mixture of flavonoid isomers such as silibinin, isosilibinin, silidianin, and silichristin. Silymarin suppresses activation of caspases and nuclear factor (NF) κB in various cell types following TNFα treatment (Manna *et al.,* 1999). Silymarin or silibinin, a major active component of silymarin, inhibited production of cytokines, e.g. TNFα, interferon (IFN)γ, interleukin (IL)-2, IL-4, IL-6, and IL-8, in mouse liver in response to concanavalin A-induced, T-cell dependent liver injury (Schümann *et al.,* 2003). Suppression of NFκB activation by silymarin probably accounts for its inhibitory effects on cytokine production (Schümann *et al.,* 2003, Manna *et al.,* 1999). We recently reported that in contrast to increasing TNFα expression by itself in LLC-PK_1 cells, silymarin prevented FB_1-induced overexpression of this cytokine and effectively protected LLC-PK_1 cells from FB_1 cytotoxicity (He *et al.,* 2002). Silymarin has been recommended in prevention of alcoholic liver disease (Saller *et al.,* 2001). It protected liver injury from various other hepatotoxicants such as carbon tetrachloride, paracetamol (Saller *et al.,* 2001), and concanavalin A (Schümann *et al.,* 2003).

In the current study, the protective effect of silymarin on FB_1 hepatotoxicity was investigated in female mice. Results demonstrated that silymarin protected mice from FB_1-induced liver injury as indicated by reduced activities of circulating alanine aminotransferase (ALT) and aspartate aminotransferase (AST) and number of apoptotic hepatocytes. Silymarin caused liver regeneration indicated by an increased number of proliferating cells in liver. Fumonisin-induced expression of proinflammatory cytokines was effectively prevented by pretreatment with silymarin.

MATERIALS AND METHODS

Chemicals. Fumonisin B_1 (purity >98%) was obtained from Programme on Mycotoxins and Experimental Carcinogenesis (PROMEC, Tygerberg, South Africa). Silymarin (product number: 254924), a mixture of toxifolin (4%), silichristin (27.9%), silidianin (2.9%), silybin A (19.3%), silybin B (31.3%), isosilybin A (8.2%) and isosilybin B (2.3%), determined by high performance liquid chromatography (HPLC)/277 nm detection, and all other reagents were purchased from Sigma-Aldrich Chemical Company Inc. (St. Louis, MO, U.S.A), unless stated in the text otherwise.

Animals. Six-week-old female BALB/c mice weighing about 22 g were obtained from Harlan Laboratories (Indianapolis, IN). They were acclimated for 1 week before dosing under controlled environmental conditions at 23°C and 65% relative humidity with a 12 h light/dark cycle. Feed and water were available *ad libitum*. Mice were treated with humane care following the Public Health Service Policy on Humane Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee.

Treatment. Animals were divided randomly into 4 groups with 5 mice each, and treated orally with water or silymarin by gavage once daily at 750 mg/kg 16 h before FB_1 dosing. The mice were given 3 daily subcutaneous injection of either physiological buffered saline (PBS) or 2.25 mg/kg of FB_1 in PBS. The protocol has been proved to produce consistent liver damage in female mice exposed to FB_1 in our laboratory.

One day after the final FB_1 treatment, mice were sacrificed by decapitation. Blood was collected in heparinzed tubes, and plasma was subsequently isolated for analysis of alanine aminotransferase (ALT) and aspartate aminotransferase (AST). The livers and kidneys were collected from each animal, aliquots were fixed immediately in neutral 10% formalin, or quickly were frozen in liquid nitrogen and stored at -85°C until analysis.

Analysis of liver enzymes in plasma. Activities of plasma ALT and AST were determined using a Hitachi 912 Automatic Analyzer (Roche Diagnostics, Indianapolis, IN).

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay for apoptosis. Liver tissue sections (5 μm) were prepared and subjected to dUTP nick-end labeling by TdT with a peroxidase-based *In Situ* Cell Death Detection kit (Roche Diagnostics, Indianapolis, IN) as described previously (Sharma *et al.*, 2003). The stained apoptotic cells were counted under a light microscope and normalized to the unit area as described (Sharma *et al.*, 1997).

Immunohistochemistry for proliferating cellular nuclear antigen (PCNA) assay. Hepatocyte proliferation was determined by analysis of PCNA in formalin-fixed, paraffin-embedded liver tissues as described recently (Sharma *et al.*, 2003a, b). The number of PCNA positive cells were counted under a microscope and normalized to the unit area as described

(Sharma *et al.,* 1997). A few other cells beside hepatocytes such as endothelial cells were labeled with PCNA, but only PCNA positive hepatocytes were counted.

***Histology*.** Liver specimens were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned (4-5 μm), and stained with hematoxylin and eosin (H&E). The tissues were examined under a microscope in a random order and without knowledge of animal or group.

Sphingolipid analysis. Free sphingosine and sphinganine of liver and kidney in base-treated lipid extracts were determined by HPLC utilizing a modification of the extraction methods described earlier (Merrill *et al.,* 1988). Sphingoid bases were quantitated based on the recovery of a C_{20}-sphinganine standard (D-erythro-C_{20}-dihydro-sphingosine, Matreya Inc. Pleasant Gap, PA, USA). The HPLC apparatus and derivation procedure were similar to those described before (Merrill *et al.,* 1988) except the fluorescence detector used in this study was Luminescence Spectrometer LS30 (Perkin-Elmer Inc., Norwalk, CT, USA).

Assay for the activity of serine palmitoyltransferase (SPT). The activity of SPT in liver and kidney was analysed using the method described by Williams *et al.,* (1984) with minor modification. Briefly, the frozen tissues were homogenized in homogenization buffer (50 mM N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid), 5 mM DL-dithiothreitol, 10 mM ethylenediaminetetraacetic acid, 0.25 M sucrose, pH 7.4), and the homogenate was centrifuged at 30,000 g for 30 min. Aliquots of 100 μg protein in the supernatant were used for analysis of SPT activity as previously described (He *et al.,* 2004, Williams *et al.,* 1984). The content of protein was determined by Bio-Rad Bradford reagent according to the manufacturer's protocol (Bio-Rad Laboratories, Hercules, CA, USA).

RNase protection assay (RPA) for selected gene expression. Total RNA from liver tissue was extracted with TRI® reagents (Molecular Research Center, Cincinnati, OH). An aliquot part

of 50 μg RNA was used for RPA using RiboQuant^{TM} RPA starter kit (B.D Biosciences, San Diego, CA, USA) as recently described by Sharma *et al.,* (2003). The relative gene expression is normalized against ribosomal protein L32.

Statistical analysis. Results are presented as mean ± standard error (SE). Data were analyzed by two-way analysis of variance (ANOVA) followed by Duncan's multiple range tests, unless otherwise stated in the text. In selected cases where unequal variances of different groups were obvious, the Wilcoxon rank sum test was employed. The level of $p < 0.05$ was considered significant.

RESULTS

Silymarin reduced FB_1-induced increases in plasma ALT and AST activities

Following FB_1 treatment, the plasma activity of ALT increased by 33-fold over that of control. Silymarin significantly reduced the FB_1-induced elevation of ALT by 70% (Fig. 6.1A). Silymarin treatment decreased FB_1-induced plasma AST elevation to the control level (Fig. 6.1B). Silymarin itself did not alter plasma activities of ALT and AST.

Silymarin diminished FB_1-induced hepatocyte apoptosis and stimulated cell proliferation

No apoptotic cells were observed in the control or silymarin alone treatment groups. Similar to the changes in ALT and AST, the number of apoptotic hepatocytes as well as the incidence of apoptosis in response to FB_1 treatment was significantly reduced by silymarin (Table 6.1).

In response to the injury caused by FB_1, the liver underwent a compensatory regeneration indicated by increase in the number of PCNA-positive hepatocytes. The number of PCNA-positive cells was increased by 5-fold in silymarin plus FB_1-treated mice compared to that in FB_1-alone treated ones (Table 6.1).

The effect of three-day treatment with FB_1 on mouse liver was limited to the presence of scattered apoptotic hepatocytes, characterized by the presence of small round to ovoid cells, and occasional mitotic figures. No swollen cells indicative of oncotic changes were observed. The cells undergoing apoptosis were usually present as single cells with no leukocytic infiltration and were surrounded by normal cells. The architecture of liver tissue was not influenced by either treatment, and treatment with silymarin provided no distinguishable differences from livers of control animals. Figure 6.2 illustrates an H&E stained section of liver and also a TUNEL stained liver section from an FB_1-treated mouse. The extent of damage was corroborated with ALT and AST activities and enumeration of apoptotic cells in tissue sections by TUNEL assay. The appearance of PCNA-positive cells in FB_1-treated mouse liver was identical to ones reported earlier (Sharma *et al*., 2003a).

Effect of silymarin on FB_1-induced accumulation of free sphingoid bases

By inhibiting the activity of ceramide synthase, FB_1 causes accumulation of free sphinganine, a precursor of dihydroceramide and ceramide (Merrill *et al.,* 1993, Wang *et al.,* 1991). Consistent with these studies, FB_1 significantly increased in the level of hepatic free sphinganine but not sphingosine (Fig. 6.3 A, B). Unexpectedly, the levels of free sphinganine and sphingosine in liver were significantly higher in silymarin plus FB_1-treated mice than those in FB_1 alone-treated ones, whereas silymarin alone did not alter concentrations of free sphingoid bases (Fig. 6.2 A, B).

To investigate whether or not the potentiation of free sphingoid bases accumulation by silymarin in FB_1 exposure was specific for liver, we measured concentrations of renal free sphinganine and sphingosine. The results demonstrated, as observed in liver, that the content of kidney free sphingoid bases was significantly higher in mice treated with combination of silymarin and FB_1 than that in FB_1 alone-treated one (Fig. 6.3 C, D).

Silymarin prevented FB_1-induced activation of SPT in liver

As the overall content of free sphingoid bases was much higher in silymarin-pretreated mice in response to FB_1, we measured the activity of SPT, the first enzyme in the pathway of *de novo* biosynthesis of sphingolipid (Hannun *et al.*, 2001). Compared to the controls, a significant increase in the activity of hepatic SPT was observed in FB_1 alone-treated mice (Table 6.2). Silymarin significantly decreased the activity of liver SPT to a similar content in mice treated with silymarin or silymarin plus FB_1.

The activity of SPT in kidney was not altered upon FB_1 treatment (Table 6.2). Silymarin decreased kidney SPT activity compared to the controls, but it did not significantly change the SPT activity in kidney following FB_1 exposure (Table 6.2).

Effects of silymarin on expression of selected genes in response to FB_1 treatment

Fumonisin B_1 treatment increased expression of hepatic genes for selected TNFα superfamily, namely TNFα, TNFR1, TNF-receptor-associated apoptosis-inducing ligand (TRAIL) and lymphotoxin (LT) β (Fig. 6.3). Silymarin moderately increased expression of TNFα in liver; however, it completely prevented FB_1-induced increases of these genes (Fig. 6.4).

The expression of IFNγ, and transforming growth factor (TGF) β1 in liver was significantly increased in response to FB_1 treatment (Fig. 6.5). The induction of IFNγ expression

following FB_1 exposure was completely reversed by silymarin. Silymarin partially diminished FB_1-induced overexpression of TGFβ1 by 17%. The increased expression of hepatic TGFβ1 mRNA in silymarin plus FB_1-treated mice was not significantly different than that in control mice (Fig. 6.5). The increased expression of hepatic TGFβ1 mRNA in silymarin plus FB_1-treated mice was also not significantly different from that in mice treated with FB_1 only.

Neither FB_1 nor silymarin altered expression of Fas signaling factors such as Fas ligand, Fas, Fas-associated death domain (FADD), and Fas-associated phosphatase (FAP) (data not shown).

DISCUSSION

This study demonstrated that silymarin prevented FB_1-induced liver injury and overexpression of selected genes for TNFα superfamily and IFNγ. Fumonisin B_1 increases free sphingoid bases in tissues via inhibition of ceramide synthase (Merrill *et al.*, 1993, Wang *et al.*, 1991). Free sphingoid bases could mediate cell death following FB_1 treatment (Schmelz *et al.*, 1998, Tolleson *et al.,* 1999). In contrast to its inhibitory effects on liver damage and selected gene induction, silymarin dramatically increased FB_1-induced accumulation of free sphingoid bases.

The fumonisin-induced alterations in mouse liver were similar to those reported earlier employing similar protocols (Sharma *et al.*, 1997; 2003a, b). The only difference in treatments was the duration (3 days vs. 5 days in former reports) and gender (females in the current experiments). Exposure of mice to fumonisin caused the appearance of apoptotic cells in liver with no other noticeable alterations. The PCNA positive cells were also increased in fumonisin-

treated mice. In the group treated with both silymarin and fumonisin the number of apoptotic (TUNEL positive) cells was decreased and those of proliferating (PCNA positive) cells was increased; changes implying that silymarin both decreased the cellular damage and increased the regeneration of liver when coadministered with FB_1.

Silymarin has been reported to stimulate enzymatic activity of DNA-dependent RNA polymerase 1, and subsequent biosynthesis of RNA and protein resulting in DNA biosynthesis and cell proliferation (Sonnenbichler and Zetl, 1986). The stimulatory effect of silymarin on liver regeneration was observed only in damaged but not normal livers (Sonnenbichler and Zetl, 1986). These findings indicate that silymarin increases regeneration potency of damaged liver tissues. Consistent with these studies, the current investigation demonstrated that silymarin increased proliferating hepatocytes in response to FB_1-induced cell death without modulation of cell proliferation in normal livers (Table 1). The capability of silymarin to stimulate regeneration activity of liver tissue in FB_1 intoxication could partly account for the observed hepatoprotective actions.

It has been appreciated that silymarin has clinical applications in the treatment of cirrhosis, ischemic injury, and toxic hepatitis induced by various toxins such as ethanol, carbon tetrachloride, acetaminophen, organic solvents, and toxic mushroom (Saller *et al.,* 2001). The pharmacological properties of silymarin involve regulation of cell membrane permeability and integrity, inhibition of leukotriene, reactive oxygen species scavenging, suppression of NFκB activity, depression of protein kinases and collagen production (Saller *et al.,* 2001). Silymarin is able to reduce the cellular uptake of xenobiotics including mushroom poisons (Saller *et al.,* 2001); it has been recently shown that silymarin can potentiate doxorubicin cytotoxicity by inhibiting P-glycoprotein-mediated drug efflux (Zhang and Morris, 2003). In response to FB_1,

mice co-treated with silymarin had greater accumulation of free sphingoid bases in both liver and kidney than those only exposed to FB_1 (Fig. 6.3). It has been suggested that the hydrophilic FB_1 entered cells in an LLC-PK_1 cell model through passive diffusion (Enongene *et al.,* 2002). Moreover, we previously reported that mice lacking p-glycoprotein genes exhibited a similar response to FB_1 hepato- and nephro-toxicity to their wild-type counterparts, suggesting little role of multi-drug transport system in FB_1 toxicity (Sharma *et al.,* 2000b). Therefore, the effect of silymarin on P-glycoprotein would not account for its protection against FB_1 toxicity. The mechanisms by which silymarin potentiates FB_1-induced accumulation of free sphingoid bases are currently unknown.

The activity of SPT was not increased in response to silymarin or silymarin plus FB_1, suggesting that silymarin potentiation of FB_1-induced free sphingoid bases accumulation in liver and kidney tissues is not due to increased *de novo* biosynthesis. Sphingosine kinase is responsible for the conversion of free sphingoid bases to their 1-phosphate metabolites (Hannun *et al.,* 2001). It is unclear whether or not silymarin affects the activity of sphingosine kinase or efflux of intracellular free sphingoid bases from cells. Silymarin is able to stabilize cellular membrane (Saller *et al.,* 2001). The higher levels of free sphinganine and sphingosine in silymarin plus FB_1-treated mouse livers and kidneys might result from reduced efflux of intracellular free sphingoid bases as a consequence of inhibiting cellular membrane damage.

Free sphingoid bases are pro-apoptotic and cell growth inhibitory (Merrill *et al.,* 2001). The disruption of sphingolipid metabolism resulting from inhibition of ceramide synthase by FB_1 is believed responsible for FB_1 toxicity. Accumulation of free sphinganine and depletion of complex sphingolipid correlated with FB_1 toxicity *in vitro* and *in vivo* (Riley *et al.,* 2001, Tsunoda *et al.,* 1998, Yoo *et al.,* 1996). Inhibition of SPT prevented the accumulation of free

sphinganine, and reversed FB_1 cytotoxicity in various cell types (He *et al.,* 2002, Schmelz *et al.,* 1998, Tolleson *et al.,* 1999, Yoo *et al.,* 1996). In the present study, the FB_1-induced elevation of serum ALT and AST and apoptotic hepatocytes was dramatically decreased by silymarin, though greater accumulation of hepatic free sphingoid bases was observed in co-treatment with silymarin and FB_1. These results suggest that silymarin protects FB_1 toxicity through blocking the actions of free sphingoid bases. The ability of silymarin to preserve the integrity of cellular and mitochodrial membrane could in part explain its protective effects on FB_1 hepatotoxicity.

Fumonisin increases expression of many cytokines and apoptotic signaling factors (Bhandari and Sharma, 2002). Silymarin has been shown to protect liver from hepatotoxin injury through inhibiting production of TNFα, IFNγ, IL-2, and IL-4 as a consequence of blocking hepatic NFκB activation (Schümann *et al.,* 2003). Consistent with these findings, we observed in the present study that silymarin reversed FB_1 induction of TNFα, TNFR1, TRAIL, LTβ, and IFNγ (Fig. 6.4 and Fig. 6.5). Silymarin also has been shown to repress TNFα-induced activation of NFκB and apoptosis in various cell types (Manna *et al.,* 1999). Either TNFR1 or TNFR2 knockout mice exhibited less sensitivity to FB_1 liver injury compared with their wild-type counterparts (Sharma *et al.,* 2000a, 2001). It has been shown that transfection of a baculovirus gene, an important inhibitor of apoptosis (IAP) in TNFα-induced apoptosis pathway, protected CV-1 cells from FB_1-induced activation of caspase 8 and apoptosis (Ciacci-Zanella and Jones 1999, Jones *et al.,* 2001). These studies support that the signal pathways of TNFα play an important role in the pathogenesis of FB_1. Mice lacking IFNγ have also been shown to be less responsive to FB_1 treatment (Sharma *et al.,* 2003b). Silymarin-reversed FB_1 overexpression of

signal factors in TNFα superfamily and IFNγ could in part account for its protective role in FB_1 hepatotoxicity.

In conclusion, we have clearly demonstrated that silymarin plays a protective role in FB_1 hepatotoxicity in a mouse model. These findings suggest a therapeutic potential of silymarin in fumonisin liver injury in humans or animals exposed to fumonisin-producing fungus contaminated feeds. Efficacy of silymarin in protection of liver damage after long-term exposure to the mycotoxin needs to be investigated.

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TABLE 6.1

Effects of silyamrin on FB_1-induced cell apoptosis and proliferation[a]

Treatment	Animal number	Apoptosis incidence	Apoptotic cells/cm^2	Proliferation incidence	Proliferating cells/cm^2
Control	5	0/5	0	3/5	3.8 ± 2.6
FB_1	5	5/5	49.1 ± 20.0*	2/5	69.0 ± 51.4
Silymarin	5	0/5	0	0	0
Silymarin+FB_1	5	3/5	7.9 ± 3.9*#	5/5	433.2 ± 222.1*#

[a] Cell apoptosis and cell proliferation were analyzed by TUNEL assay and PCNA immunohistochemistry, respectively. The positive stained cells were counted under a light microscope. Data are presented as mean ± SE. * $p< 0.05$ vs. control, # $p<0.05$ vs. FB_1.

Table 6.2

Activity of serine palmitoyltransferase (SPT) in liver and kidney of mice following FB_1 exposure[a]

Treatment	Hepatic SPT activity	Renal SPT activity
Control	186.2 ± 21.4	325.4 ± 63.5
FB_1	267.5 ± 24.6*	319.5 ± 14.8
Silymarin	80.0 ± 12.9 *#	220.3 ± 19.4*#
Silymarin+FB_1	71.0 ± 15.6 *#	251.1 ± 13.6

[a] Activity of SPT is expressed as pmol product/min.mg protein. Data are presented as mean ± SE. * $p< 0.05$ vs control, # $p<0.05$ vs. FB_1 treatment.

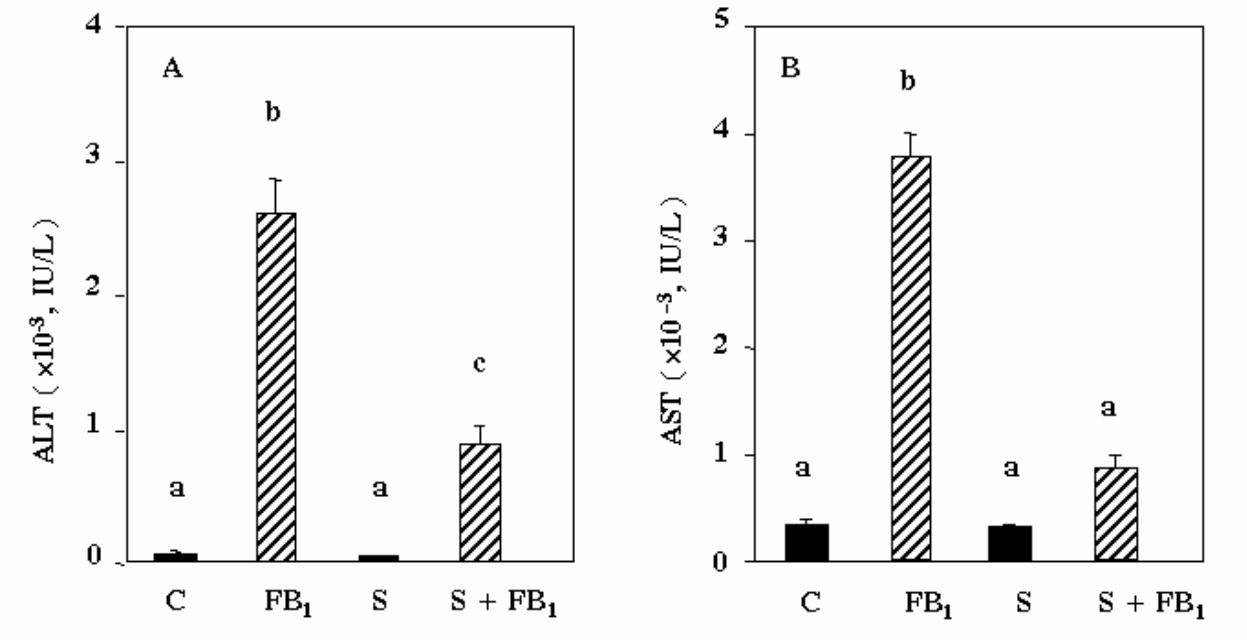

Fig. 6.1. Effects of silymarin (S) on fumonisin B_1 (FB_1)-induced increase of plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities. Female BALB/c mice were daily treated with 750 mg/kg silymarin by gavage, and 2.25 mg/kg FB_1 subcutaneously for 3 days. One day after the last FB_1 treatment, the animals were sacrificed and plasma was used for analysis of ALT and AST. Data are presented as mean ± SE (n=5). Different letters indicate statistical difference at $p<0.05$.

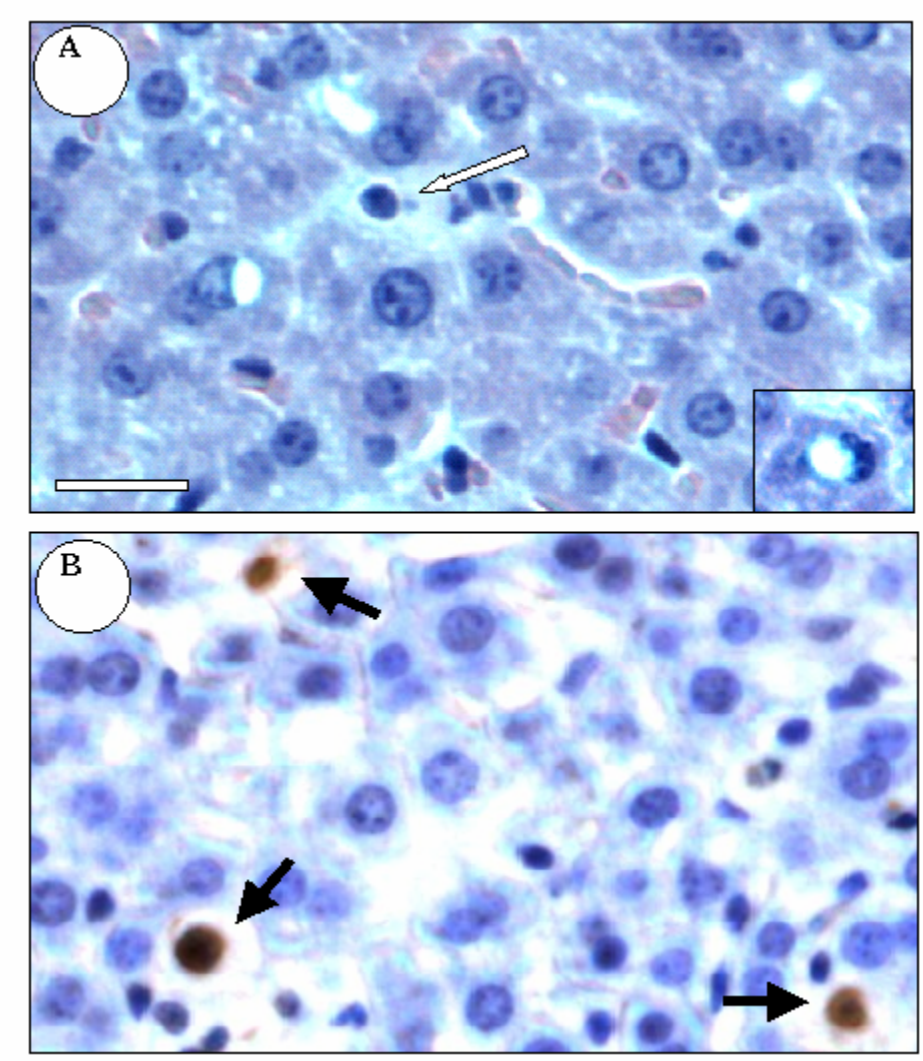

Fig. 6.2. Microscopic structure of liver after treatment of mice with fumonisin B_1. A, H&E stained section of a mouse liver. A condensed nucleus, separated from the surrounding cytoplasm, is indicated by a white arrow. The surrounding cells are normal in appearance. Bar in the lower left indicates 25 μm. The inset in lower left illustrates a cell with crescent-shaped nucleus, characteristic of apoptotic changes. B, Liver from FB_1-treated mice indicating TUNEL positive cells. The brown-darkened nuclei are pointed by dark arrows.

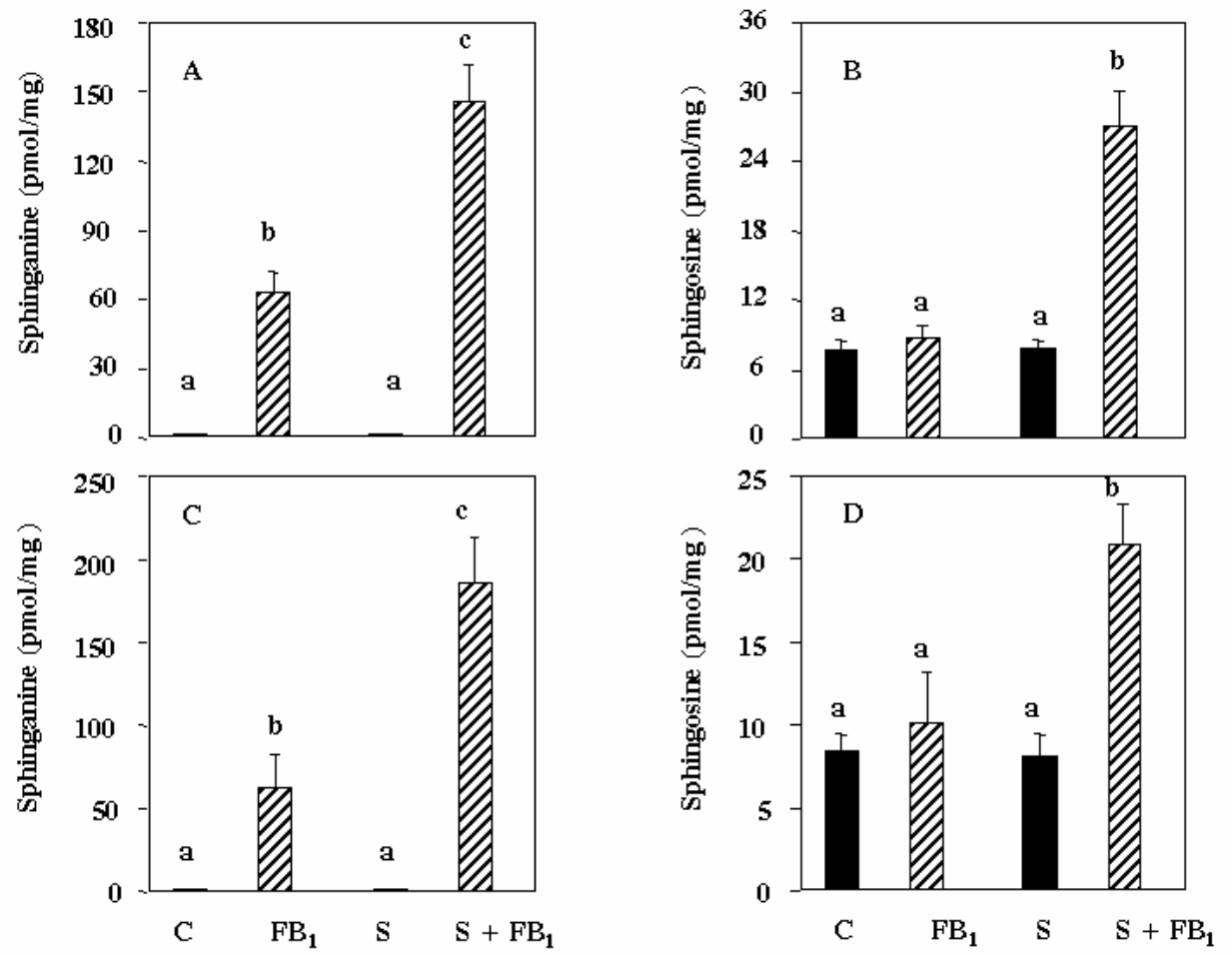

Fig. 6.3. Effects of silymarin (S) on fumonisin B_1 (FB_1)-induced accumulation of free sphingoid bases, sphinganine (Sa) and sphingosine (So), in liver (A, B) and kidney (C, D). Data are presented as mean ± SE (n=5). Different letters indicate statistical difference at $p<0.05$.

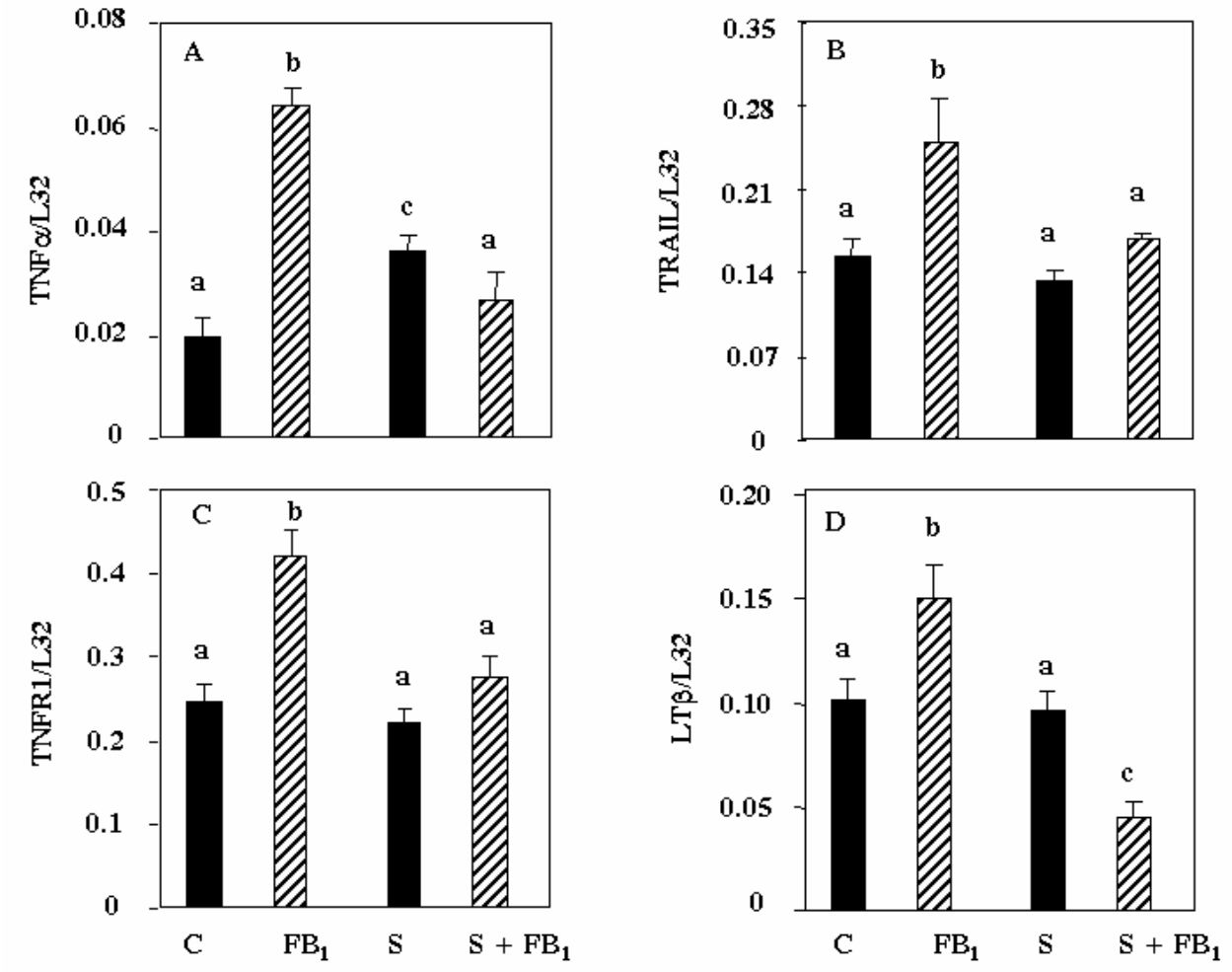

Fig. 6.4. Effects of silymarin (S) on fumonisin B_1 (FB_1)-induced expression of tumor necrosis factor (TNF) α, TNF-related apoptosis-inducing ligand (TRAIL), TNF receptor (TNFR) 1, and lymphotoxin (LT) β in liver. Data are presented as mean ± SE (n=5). Different letters indicate statistical difference at $p<0.05$.

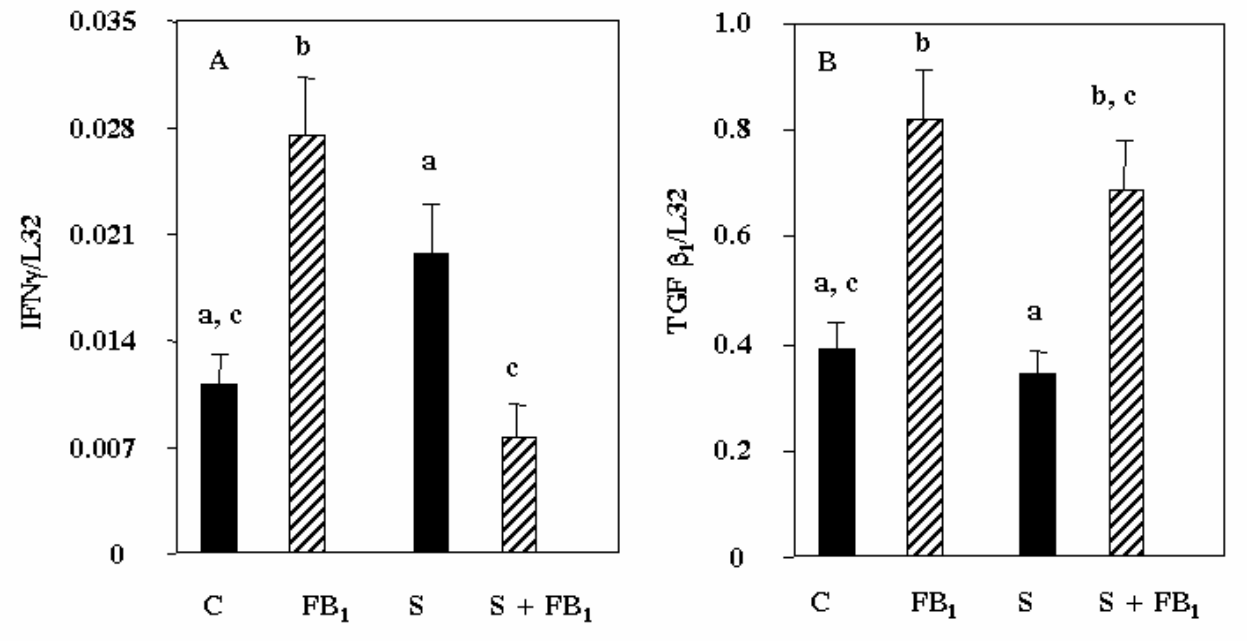

Fig. 6.5. Effects of silymarin (S) on fumonisin B_1 (FB_1)-induced expression of interferon (IFN) γ and transforming growth factor (TGF) β1 in liver. Data are presented as mean ± SE (n=5). Different letters indicate statistical difference at $p<0.05$.

CHAPTER 7

SUMMARY AND CONCLUSIONS

Fumonisin B_1 (FB_1), produced by *Fusarium verticillioides*, is a common mycotoxin present on corn and corn-based foods. It produces species-, organ- and gender-specific toxicity in animals and is carcinogenic. The primary biochemical effect of FB_1 is its inhibition of ceramide synthase leading to disruption of sphingolipid metabolism including accumulation of free sphinganine and sphingosine, formation of free sphingoid base-1-phosphates, and depletion of complex sphingolipids. Fumonisin B_1 induces expression of tumor necrosis factor (TNF)α and other cytokines *in vivo* and *in vitro*. Various sphingolipids and cytokines are important cell signaling factors. Pharmacologic intervention to modifying these cell signaling pathways may modulate FB_1 hepatotoxicity.

The objectives of the present study were to (1) investigate the role of free sphinganine and complex sphingolipids in FB_1-induced liver damage and expression of hepatic cell signaling factors in mice, (2) evaluate the effect of Kupffer cell depletion by gadolinium chloride on FB_1 hepatotoxicity in mice, (3) examine the effects of anti-TNFα antibodies and pentoxifylline on FB_1 hepatotoxicity, and (4) evaluate the effectiveness of silymarin in protecting against FB_1 toxicity *in vivo*.

In the first study we observed 100% mortality within 12 hr after 5 daily treatments of female BALB/c mice with FB_1 in the presence of myriocin, a specific and potent inhibitor of serine palmitoyltransferase. In subsequent experiments, the mice were given only 3 daily treatments. We demonstrated that treatment with myriocin did not prevent FB_1-induced liver damage. Daily myriocin alone treatment for 3 days did not detected any increases in activities of plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) as well as the number of apoptotic hepatocytes. Myriocin prevented FB_1-induced free sphinganine accumulation in mouse liver as a result of inhibiting serine palmitoyltransferase, the first critical

enzyme in sphingolipid *de novo* biosynthetic pathway. The hepatic levels of more complex sphingolipids and sphingomyelin were reduced by either FB_1 or myriocin, and further decreased by combination of myriocin plus FB_1. Treatment with myriocin daily for 3 days reversed FB_1-induced overexpression of selected cell signaling genes to constitutive levels including TNFα, TNF related apoptosis-inducing ligand (TRAIL), TNF receptor (TNFR) 1, lymphotoxin (LT)β, interferon (IFN)γ, and transforming growth factor (TGF)β1. These findings suggest that myriocin may facilitate FB_1 toxicity by inhibiting *de novo* biosynthesis of complex sphingolipids and sphingomyelin; accumulation of free sphinganine and /or its metabolites contribute to the induction of the above cell signaling factors in response to FB_1.

The second study evaluated the effect of gadolinium depletion of Kupffer cells on FB_1 hepatotoxicity and cytokine expression in female BALB/c mice. Gadolinium completely depleted Kupffer cells in the experimental period. Depletion of Kupffer cells significantly attenuated circulating ALT and AST, and reduced apoptotic hepatocytes and free sphinganine following FB_1 treatment. Gadolinium and FB_1 increased expression of TNFα, TNFR1, LTβ, IFNγ and TGFβ1 in liver to similar levels, whereas FB_1 further increased TRAIL expression in gadolinium-pretreated mouse liver. Gadolinium chloride did not alter FB_1-induced expression of the above genes in liver. Results indicated that Kupffer cells play a role in FB_1 liver injury; the reduced sphinganine accumulation and increased TNFα signaling may in part account for the observed ameliorating effect of gadolinium on FB_1 hepatotoxicity.

In the third study we investigated FB_1 hepatotoxicity in male C57BL/6N mice in the presence of anti-TNFα antibodies or pentoxifylline. Results showed that both anti-TNFα antibodies and pentoxifylline augmented FB_1-induced increases in activities of plasma ALT and

AST, and number of apoptotic hepatocytes. Anti-TNFα antibodies did not alter FB_1-induced accumulation of free sphingoid bases and expression of TNFα, interleukin (IL)-12, IFNγ; pentoxifylline significantly reduced accumulation of free sphinganine and expression of TNFα without altering IL-12 and IFNγ expression induced by FB_1. These findings suggest a partially protective role of TNFα signaling activation in FB_1 hepatotoxicity.

The final study determined the effectiveness of silymarin, an extract from *Silybum marianum*, in protecting against FB_1 hepatotoxicity in BALB/c mice. Silymarin significantly decreased FB_1-induced elevation of plasma ALT and AST activities and number of apoptotic hepatocytes, while it augmented hepatocyte proliferation indicated by an increase in proliferating cells. Silymarin markedly augmented FB_1-induced accumulation of free sphinganine and sphingosine in both liver and kidney. Silymarin alone slightly but significantly increased expression of hepatic TNFα; however, it prevented FB_1-induced increases in TNFα, TNFR 1, TRAIL, LTβ, and IFNγ. The induction of TGFβ1 expression in liver following FB_1 treatment was not affected by silymarin. These findings suggest that silymarin protected against FB_1 liver damage through inhibiting pro-apoptotic effects of free sphingoid bases and increasing cellular regeneration (repair).

All together, the data presented in this dissertation indicate that perturbation of cell signaling by FB_1 as a result of disrupted sphingolipid metabolism and cytokine expression plays an important role in FB_1 hepatotoxicity in mice. Myriocin prevented accumulation of free sphingoid bases and simultaneously reversed expression of cytokines induced by FB_1, suggesting a role of accumulated free sphingoid bases in FB_1-induced cytokine production. Gadolinium depletion of Kupffer cells induced TNFα expression and ameliorated FB_1 liver damage; blocking

TNFα signaling or TNFα production exacerbated FB_1 hepatotoxicity. These findings suggest a protective role of endogenous TNFα in FB_1 hepatotoxicity. Myriocin treatment in the presence of FB_1 is highly toxic and therefore myriocin had no therapeutic potential for FB_1 intoxication under the conditions used in these studies, while silymarin protected against FB_1 liver damage in mice, suggesting a therapeutic potential of silymarin in FB_1 liver injury in humans and animals exposed to fumonisin contaminated foods. The molecular mechanisms and efficacy of silymarin in protection of hepatotoxicity after long-term exposure to the mycotoxin needs to be investigated.