THE EFFECTS OF FUMONISIN B_1 TOXICITY UPON NEURONAL TISSUE

by

MARCIN FILIP OSUCHOWSKI

(Under the Direction of Raghubir P. Sharma)

ABSTRACT

Fumonisin B_1 (FB_1) is mycotoxin produced by *Fusarium verticillioides* (formerly *F. moniliforme*), a fungal contaminant of corn and other cereals found worldwide. Fumonisin B_1 causes number of diseases in farm animals including the species-specific disorder, equine leukoencephalomalacia (ELEM). FB_1 inhibits ceramide synthase and as a consequence disrupts metabolism of sphingolipids. Deregulation of sphingolipid metabolism results in accumulation of free sphingoid bases, activation of cytokine expression and depletion of complex sphingolipids. We hypothesized that lack of neurotoxic effects in species other than equine following systemic administration of FB_1 is due to the restricted availability of this mycotoxin in the brain.

The pre-exposure to the lipopolysaccharide (LPS) resulted in the disruption of blood brain barrier (BBB). When mice were treated concurrently with FB_1 an accumulation of sphinganine, a biomarker for FB_1 availability to the tissue, was observed in brain. The combined administration of LPS and FB_1 caused modulation of proinflammatory cytokines in brain and liver after repeated FB_1 treatment. These results indicate that sphingolipid metabolism in the adult brain is vulnerable to the exposure of FB_1 when accompanied by the endotoxin-related damage to the BBB.

The intracerebroventricular (icv) infusion of FB_1 led to the neuronal degeneration in cortex. The icv treatment caused regional accumulation of free sphingoid bases but sphingomyelin and complex sphingolipids were not depleted. Potent FB_1-induced activation of proinflammatory signaling was observed in cortex. These results show that adult murine brain is vulnerable to the direct exposure of FB_1 when the protective BBB barrier is bypassed.

FB_1 induced necrotic cell death in both BV-2 and primary astrocytes and exhibited potent antiproliferative properties toward the BV-2 cells. Neuronal cells were relatively resistant to the toxic effects of FB_1. In all cells the FB_1 treatment disrupted the sphingolipid metabolism, as indicated by the accumulation of free sphinganine and decrease of free sphingosine. These findings indicate that glial cells, namely microglia and astrocytes, may be a primary targets in the FB_1-induced neurotoxicity.

INDEX WORDS: Fumonisin B_1, ELEM, neurotoxicity, lipolysaccharide, sphinganine, sphingosine, intracerebroventricular infusion, BV-2, primary astrocytes, cortical neurons

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DEDICATION

To my dearest companion, friend and wife, Alicja. You are the Love and the Meaning of my life. Marrying You was the best thing that has ever happened to me. I pray to God that I will be able to repay adequately for all the goodness and love You bring into my existence.

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"This is my commandment, that you love one another as I have loved you."

John 5:10-Romans 8:39

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CHAPTER 1

INTRODUCTION

Humans have been exposed to fungi and their toxic products, mycotoxins, since ancient times. Writings by Hippocrates describe white patches of thrush in his patients, presumably of *Candida* origin and a book by Matossian (1989) captivatingly relates dietary intoxications by various mycotoxins to number of dramatic, health–related events noted in history of Europe and North America. Fumonisins are the mycotoxins isolated from *Fusarium verticillioides* (formerly *F. moniliforme*), a fungus that is a natural contaminant of corn on a worldwide scale (WHO 2000). Fumonisins have been isolated and characterized relatively recently in South Africa in 1988 with relation to the fatal neurodegnerative disorder occurring in horses, namely equine leukoencephalomalacia (Brownie and Cullen, 1987; Marasas, 2001). Fumonisin toxicity manifests also in the form of porcine pulmonary edema (PPE), liver cancer in rats and mice and general nephro- and liver toxicity in these and other species. Among numerous members of fumonisin family, fumonisin B_1 (FB_1) is the one of predominant importance from the health and toxicological standpoint. Considering its worldwide prevalence, fumonisin B_1-induced toxicity can pose a considerable threat to humans due to the popularity of corn-based products in the human diet within selected ethnic subpopulations. Cases of human esophageal cancer associated with the consumption of FB-containing foods diagnosed in Linxian county (China) and Transkei region (South Africa) support such exposure risk (Yoshizawa et al., 1994; Rheeder et al., 1992).

The exact mechanism of the fumonisin-related toxicity remains unclear. It has been established, however, that on the cellular level fumonisin potently disrupts the biosynthesis of sphingolipids that leads to accumulation of free sphingoid bases, their metabolites and depletion of more complex sphingolipids. The role of fumonisins in the stimulation of

proinflammatory cytokine signaling including TNFα in connection with liver toxicity has been also shown in our laboratory recently (Sharma et al., 2000; Bhandari et al., 2002).

With the liver and kidney as uniformly affected organs in majority of species the brain pathology in *equide* demonstrates a peculiar form of fumonisin-related neurotoxicity, the pathogenesis of which remains mostly unclear. Complex sphingolipids such as sphingomyelin or gangliosides, concentrated predominantly in the white matter, are vital components of the central nervous system (CNS). Thus, it is plausible that inhibition of *de novo* sphingolipid synthesis resulting in the further disruption of complex sphingolipids has the potential for adverse effects upon the brain. Such mode of fumonisin toxicity agrees with the observed histopathological changes of encephalomalacia, localized specifically within brain regions rich in myelin (Dutton, 1996). It is unclear whether the severe symptoms observed in ELEM are caused by the direct action of the lipid insoluble, hydrophilic fumonisin penetrating the blood-brain barrier (BBB) under certain circumstances (e.g. endotoxemia), or are the response to an adverse influence of other systems such as cardiovascular, compromised by the FB-dependent toxicity (Haschek et al., 2001). Horses are highly susceptible to various factors deregulating the balance in their gastrointestinal (GI) system and the GI disorders, regardless of species, compromise the integrity of intestinal lining. Colic events such as duodenitis-proximal jejunitis (DPJ) in horses, associated with *F. verticillioides*-containing feed (Goel et al., 1996) and FB_1 potential for endothelial barrier damage (Ramasamy et al., 1995; Bouhet et al., 2003), could promote an increase in systemic concentration of FB_1. These disorders will also cause increased absorption of endotoxins or endotoxin-producing bacteria from GI tract. The potential of proinflammatory cytokines, including TNFα, for disruption of blood-brain barrier

(BBB) integrity is known (Tsao et al., 2001) as well as the upregulation of proinflammatory signaling during colic-related endotoxemias (Morris, 1991). Damage to bowel mucosa related to FB_1 exposure, could further expose the equine immunological system to the residual, intestinal bacterial lipopolysaccharide (LPS), augmenting the adverse effects of an inflammatory process upon the BBB. Consequent elevated exposure of CNS to fumonisin could follow, leading to the interference with sphingolipid metabolism.

There is a limited data investigating the susceptibility of the CNS to fumonisin toxicity in species other than *equide*. Treatment with FB_1 decreased myelin depositions in aggregate rat brain cultures (Monnet-Tschudi et al., 1999) and hindered myelogenesis in postnatal rats (Kwon et al., 1997). Anecdotal cases of ELEM-like pathology were diagnosed in rabbits gavaged with purified form of FB_1 (Bucci et al., 1996) and in white tailed deer environmentally exposed to FB_1 (Howerth et al., 1989). Another, developmental aspect of FB_1-dependent toxicity, also pertaining to the nervous system, has emerged recently. Experiments on rodent embryos exposed to FB_1 *in vitro* demonstrated incidences of neural tube defects (NTD) (Sadler et al., 2002). In humans, occurrences of NTD cases were reported (often concurrently with esophageal cancer incidences) in populations whose diet is based on corn products, largely contaminated with fumonisins (Hendricks, 1999; Marasas et al., 2004).

At this point, however, the available data related to ELEM cannot definitely refute or confirm any of the existing hypotheses attempting to explain the pathogenesis of this disease. It is highly desirable to further investigate the mechanisms of FB_1-dependent toxicity of CNS. The development of a suitable laboratory model exhibiting the FB_1-induced brain toxicity would undeniably accelerate such pursuits.

Hypothesis and specific aims.

The exact pathophysiology of equine leukoencephalomalacia remains to be elucidated, including the question whether the FB_1-related neurotoxicity displayed in this disease is of primary or secondary nature. The laboratory *in vivo* models could be an useful alternative to study the pathogenesis of fumonisin-dependent disorder of equine central nervous system.

Therefore, the objective of the research encompassed in the dissertation was to test the hypothesis stating that: **"Lack of neurotoxic effects in species other than equine following systemic administration of FB_1 is due to the restricted availability of this mycotoxin in the brain"**. The following specific aims were attempted to accomplish the objective.

1. **To investigate the potential for FB_1-induced pathogenesis and modulation of sphingolipid biosynthesis and pro-inflammatory signaling in mouse brain during co-existing LPS-dependent disruption of blood-brain barrier.**

2. **To investigate the morphology, sphingolipid biosynthesis and proinflammatory signaling in mouse brain after direct intracerebroventricular infusion of FB_1.**

3. **To investigate the sensitivity of murine brain immunocompetent cells and neurons in response to various time- and dose-dependent FB_1 treatments.**

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CHAPTER 2

LITERATURE REVIEW

Fumonisin-background

Fumonisin B_1 was identified and characterized chemically for the first time in South Africa as a product of *Fusarium verticillioides*, a ubiquitous contaminant of local maize (Gelderblom et al., 1988). Infestation with *F. verticillioides* is not limited only to corn/maize but it was also observed in rice (Munimbazi and Bullerman, 1996), sorghum (Patel et al., 1996), navy beans (Tseng et al., 1995), oats (Wilson et al., 1985) and was even found in forage grass in New Zealand (Mirocha et al., 1992). Weather conditions favoring fungal growth lead to the development of so called *Fusarium* "ear rot" that usually correlates well with the fumonisin accumulation in the kernel (Pascale et al., 2002). The following types of fumonisins have been identified to date as a product of *F. verticillioides*: FA_1, FA_2, FB_1, FB_2, FB_3, FB_4 and FC_1 (Cawood et al., 1991). Structures of fumonisins are based on a long hydroxylated hydrocarbon chain containing methyl and either amino (FB_1 and FB_2 types) or acetyl amino groups (FA_1 and FA_2 types) (Dutton, 1996). Despite their relative variety, under natural conditions only FB_1, FB_2 and FB_3 are produced in quantities considered important from the toxicological standpoint. In the naturally contaminated maize FB_1 was determined to be the most abundant type with the ratio between FB_1 and FB_2 approximately 3:1 and between FB_1 and FB_3 approximately 12:1 (Sydenham et al., 1996).

Fumonisins are relatively resistant to high temperatures (Alberts et al., 1990) but after application of 220 C for 25 minutes to fumonisin-containing corn meal the toxin could not recovered with the available extraction methods. It remains to be established, however, whether it occurred due to disintegration of FB or only chemical transformation preserving its

toxic properties (Scott and Lawrence, 1994). Both FB_1 and FB_1 were highly resistant to prolonged gamma-irradiation of maize infested with these toxins: the process caused only about 20% decrease in fumonisin content (Visconti et al., 1996). Further stability studies by the same authors showed that fumonisins are stable in maize for at least 6 months at 25 degrees C or at least 4 weeks at 40 degrees C. The treatment with lime (nixtamalization) used in human food processing for corn-based products causes hydrolization of FB_1 and the formation of aminopentol (AP1; HFB_1) (Sydenham et al., 1996). In Mayan communities the nixtamalization of maize used in tortilla preparation, reduced total fumonisins by 50% (Palencia et al., 2003). Measurable levels of AP1 were detected in different corn-related products such as tortilla chips, masa or canned yellow corn (Hopmans and Murphy, 1993). Reports on mechanisms of AP1 toxicity are conflicting but its inhibitory effect upon ceramide biosynthesis was demonstrated in rat hepatocytes *in vitro* (Norred et al., 1992) and AP1 induced embryotoxicity in rat cultured fetuses (Flynn at al., 1997). In the latter report, a significant increase (29%) in NDT incidences was noted at the 100 μM AP1 dose. AP1-related embryotoxicity, however, was significantly lower when compared to the intact FB_1 as reported by Flynn et al., (1996).

FB is rather poorly absorbed from the GI tract, according to studies in rats, although limited assimilation was confirmed. The absorbed toxin persists in discrete tissues such as liver and kidney for at least 96 hours. After intragastric administration of FB_1 in dose of 5 mg/kg, its levels were calculated as follows: in liver 0.45 μg/g and in kidney 1.0 μg/g (Norred et al., 1993). The major route of FB_1 excretion is via feces (66%), urine (32%), liver (1%) and trace quantities in other tissues (e.g. kidney) as established by ^{14}C-labeled FB_1 circulation analysis in rat (Shephard et al., 1992).

Fumonisin and sphingolipid synthesis

The structural similarity of fumonisin to two of the intermediates in the *de novo* pathway of sphingolipid biosynthesis, namely sphingosine and sphinganine, indicates the possibility of FB_1-related interference with sphingolipid metabolism (Fig. 2.1). FB_1 is a potent and specific inhibitor of ceramide synthase (sphinganine and sphingosine-N-acyl tranferase), a key enzyme in the synthesis of ceramide (Fig. 2.2) (Wang et al., 1991; Merril et al., 1993b). Different inhibitors with high specificity toward the sphingolipid biosynthesis pathway, exist. For example myriocin/ISP-1, whose derivatives are successfully used in human transplantology as immunosuppresants, inhibits serine palmitoyltranferase (the first step in sphingolipid biosynthesis; Fig. 2.2).

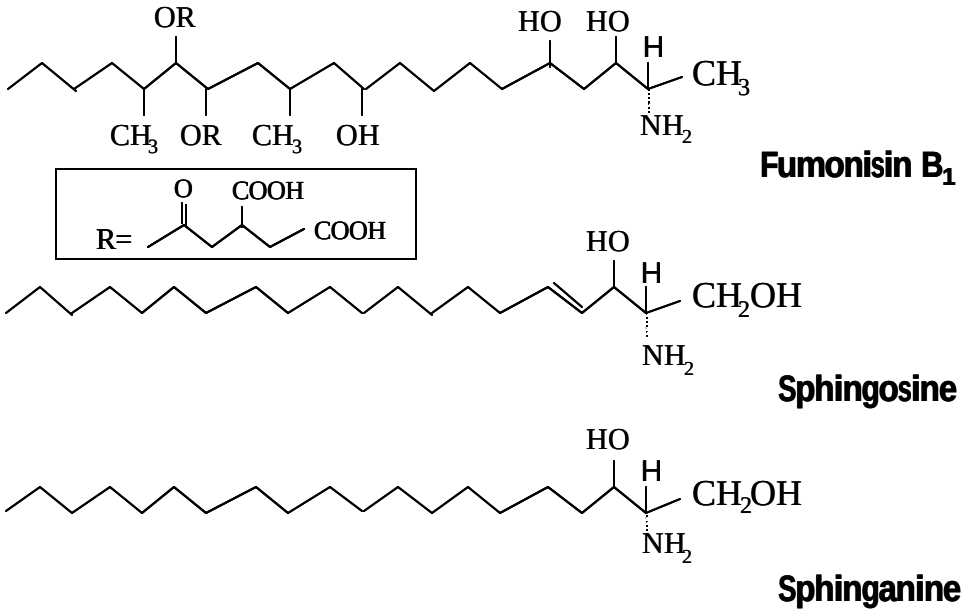

Fig. 2.1 Chemical structures of fumonisin B_1, sphingosine and sphinganine

Sphingolipids constitute a large family of various phospholipids and glycolipids containing a common core-sphinganine. Sphingolipids are integral part of all eukaryotic cell membranes, providing the highly resistant protective layer on the outer side of the lipid membrane and their crucial role in signal transduction pathways modulating cell growth, differentiation and death is known and expanding (Merrill et al., 2002).

The *de novo* sphingolipid biosynthesis begins with the merger of palmitoylCoA and L-serine catalyzed by serine palmitoyltransferase (Fig. 2.2). The 3-ketosphinganine is formed as a first product of this pathway. In consecutive steps ceramide is generated via three alternative routes, including the *de novo* route: synthesis from sphinganine (*de novo*), synthesis from sphingosine (both catalyzed by the same enzyme ceramide synthase) and by degradation of sphingomyelin featured by sphingomyelinases (Maceyka et al. 2002). Next, ceramide is deacylated by ceramidases, yielding a sphingosine ((2*S*,3*R*,4*E*)-2-amino-4-octadecen-1,3-diol) that is further catalized into the sphingosine-1-phosphate (S1P; phosphorylated by sphingosine kinase). The irreversible degradation of S1P to end metabolites catalyzed by S1P follows. Ceramide can be further incorporated into more complex final products such as sphingomyelin and number of glycosphingolipids including cerebrosides, gangliosides and globosides, which are vital components of the brain white matter (Riboni et al., 1997). Disruption of glycosphingolipid metabolism results in a number of debilitating and lethal disorders (e.g. Tay-Sachs disease), defined as lipid storage diseases (Aerts et al., 2003). The partial reverse process in the sphingolipid biosynthetic pathway is also possible, since S1P phosphatases and ceramide synthase allow S1P to be converted back to sphingosine and ceramide.

The events resulting in the deregulation of sphingolipid metabolism include inhibition of dihydroceramide synthesis, an increase in free sphinganine, a decrease in reacylation of sphingosine derived from complex sphingolipid turnover (and consequent increase in So), an increase in sphingoid base degradation products (i.e. sphingosine (sphinganine) 1-phosphate, ethanolamine phosphate and fatty acid aldehydes) and alterations in other lipids as a consequence of the carbon flux changes resulting from the elevation in the sphingoid base degradation products (Golderblom et al., 1992). The elevation in the levels of sphinganine and sphingosine in tissue and serum has been an accepted biomarker of exposure to FB_1, but these changes could not simultaneously explain their further involvement in FB_1-related toxicity (Merrill, 2002).

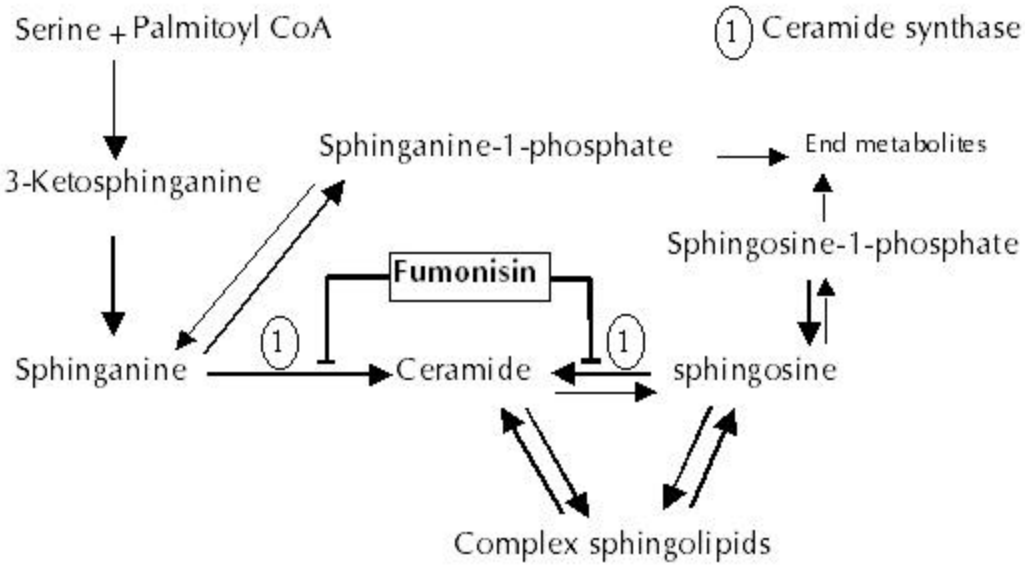

Fig. 2.2 Pathway of the *de novo* sphingolipid metabolism.

Initially, that ceramide levels do not decrease after FB_1-related inhibition of ceramide synthase is likely due to the breakdown of sphingomyelin. This process is catalyzed by

sphingomyelinase (mainly neutral in brain) what results in compensatory ceramide synthesis but also in depletion of complex sphingolipids (Buccoliero and Futerman, 2003). The sphingomyelinases are evidently activated by proinflammatory cytokines (mainly TNFα and IL-1β) (Maceyka et al. 2002; Buccoliero and Futerman, 2003) and FB_1-dependent upregulation of TNFα and IL-1β expression has been clearly demonstrated (Dugyala et al., 1998; Sharma et al., 2000; Bhandari et al., 2001; Bhandari et al., 2002). The FB_1-induced disruption of sphingolipid metabolism precedes and has been repeatedly correlated with the occurrence and severity of the *in vivo* kidney and liver toxicity in rodents (Riley et al., 1993; 1994b; 1996; Voss et al., 1996).

Overview of fumonisin toxicity

Toxicity of fumonisins manifests in various types of diseases in different animals affected, with the liver as a target organ in all tested species. Treatment with FB_1 in rats caused acute and chronic liver toxicity, bile duct proliferation, fibrosis further developing into cirrhosis, cholangiogenesis and hepatocellular carcinoma and/or cholangiocarcinoma (Gelderblom et al., 1991; Voss et al., 1993). Although the findings regarding the FB-related tumorgenesis in humans are inconclusive, FB_1 has been associated with the high incidences of esophageal cancer in Transkei, South Africa (Rheeder et al 1992; Dutton, 1996) and later reported in China (Yang, 1980; Li et al, 1980). A different form of tumor, the primary liver cancer, was also epidemilogically associated with FB_1 consumption in Haimen province, China (Ueno et al., 1997). Experiments with baboons (Kriek et al., 1981) and vervet monkeys (Jaskiewicz et al., 1987; Fincham et al., 1992; Gelderblom et al., 2001) demonstrated evident

hepatoxicity (hepatitis and/or cirrhosis) but carcinogenic development was not reported in these subjects. Carcinogenic properties of FB_1 observed in rodents are not associated with direct genotoxicity. As a potent promoter of apoptotic necrosis oncogenesis in liver FB_1 causes compensatory regeneration that is characterized by increased cell proliferation in affected tissues. Consequently, the constant elevation of DNA replication in exposed tissues has the potential for increasing the likelihood of cancer induction (Riley et al., 2001). Considering the available evidence in 1993 the International Agency for Research on Cancer has declared toxins of *F. moniliforme* as potential human carcinogens graded as class 2B (Vainio et al., 1993). Addressing human health concerns related to dietary fumonisin

Product	**Total Fumonisins (FB_1 + FB_2 + FB_3) parts per million (ppm)**
Degermed dry milled corn products (e.g., flaking grits, corn grits, corn meal, corn flour with fat content of < 2.25 %, dry weight basis)	2 ppm
Whole or partially degermed dry milled corn products (e.g., flaking grits, corn grits, corn meal, corn flour with fat content of ≥2.25 %, dry weight basis)	4 ppm
Dry milled corn bran	4 ppm
Cleaned corn intended for masa production	4 ppm
Cleaned corn intended for popcorn	3 ppm

Table 2.1. The recommended maximum levels for fumonisins in corn and corn products intended for human consumption (After FDA, 2002).

exposure, the Federal Drug Administration (FDA) recently issued a final guidance on fumonisin levels, that agency considers adequate to protect human and animal health, in human food and animal feeds (Table 2.1.).

Exposure of pigs to FB_1 results in porcine pulmonary edema (PPE) along with hepatotoxicity (Casteel et al., 1993). The animals fed feed with higher levels of FB_1 die of pulmonary edema and those exposed to lower levels exhibit subacute hepatotoxicosis. Chronic intoxication of piglets with FB_1 (10 mg/kg of feed) led to significant losses of productivity, as demonstrated by Dilkin et al., (2003). The PEE seems to be closely associated with FB_1-induced changes in cardiovascular system (Constable et al., 2003).

In poultry reduced body weight gain, hepatic necrosis, biliary hyperplasia, diarrhea and thymic cortical atrophy was shown, as in the case of day-old broiler chickens fed FB_1 in doses from 0 to 400 mg/kg (Brown et al., 1992). Ducklings exposed to *F. moniliforme* isolates, showed high mortality, loss in weight, low body fat and swollen and reddened livers (Dutton, 1996) and severe paralysis was observed in quail (Grimes et al., 1993). It has been suggested that the observed adverse effects of FB_1 toxicity in poultry are the result of general immunosuppression (Chatterrje et al., 1995; Martinova et al., 1995).

Fumonisin neurotoxicity

The major FB_1-related pathology associated with the CNS manifests in the form of disease called equine leukoencephalomalacia (ELEM). This CNS disorder is diagnosed solely in *equide* demonstrating the high species-specificity. An early description of ELEM is provided by Butler (1902), who linked the occurrence of the disease with the consumption of

moldy corn. Although FB_1 was established as a causative agent of ELEM only recently in 1988 in South Africa (Marasas et al., 1988), it has been associated with moldy feed for a long time. The same disease was also referred to by other names such as blind staggers, foraging disease, corn stalk disease, moldy corn poisoning, leucoencephalitis or cerebritis (Dutton, 1996). Progressing knowledge of the ELEM etiology determined that the mold infecting feed belongs to the genus *Fusarium* (Wilson and Maronpot, 1971). The disease shows a seasonal tendency with the highest number of incidences from late fall to early spring. This pattern agrees with the phases of fungus growth and toxin synthesis (Haliburton, 1979). The major outbreaks of the disease occurred in 1989/90 when numerous cases of ELEM were diagnosed in various regions of USA (Ross at al., 1991). Most recently, FB_1-related incidences of ELEM affected large number of donkeys and horses in Oaxaca, Mexico (Rosiles et al., 1998).

The clinical signs of ELEM demonstrate the similar progressive stages during its development as diagnosed in various cases. First, general symptoms feature lack of appetite after a period of contaminated feed consumption (stage 1) and lethargic behavior (stage 2). It is later followed (stage 3) by more specific neurotoxic effects (Dutton, 1996). There was a number of reported neurological symptoms that widely varied in their presentation and severity: depression, dullness, apathy, head bobbing, incoordination, staggers, ataxia combined with gait problems, confusion, impaired vision, general aggressiveness and paresis of the lower lip, which seems to occur relatively frequently. Complete paralysis and coma follow over the course of 2-10 days after the onset of clinical signs and most animals will die during convulsions at this stage in the disease. Animals surviving the disease demonstrate the evidence of persistent conscious proprioceptive deficits. The cases are reported, however,

where the death was not proceeded by any nervous symptoms or was accompanied by liver-related symptoms such as the nose and lips swelling, mild to severe icterus or cyanosis (Wilson and Maronpot, 1971; Kellerman et al., 1972; Marases et al., 1976; Pienaar et al., 1981; Wilson et al., 1990a, b; 1992; Dutton, 1996).

Consistent with the observed symptoms histopathological examinations reveal that brain and liver are two major target organs. Liver involvement is characterized as hepatic congestion and centrilobular necrosis. The central nervous system lesions include: focal necrosis of the cerebral and spinal white matter, enlarged and flattened cortical gyri, an enlarged cortex that is softer than normal, the white matter may feature a slight yellow discoloration, and there is evidence of vascular congestion and oedema and hemorrhaging. Brain lesions remain of relatively small magnitude (in mm), although cases were reported where they reached size of several cm in diameter forming large cavernae in the tissue. The lesions are unilateral and observed primarily in the frontal and temporal cortex and medulla oblongata but changes were located also in pons cerebri and basal ganglia. It was demonstrated by Brownie and Cullen (1987) that in the cerebro-spinal fluid (CSF) in horses affected with leukoencephalomalacia the concentration of myelin basic protein was elevated ~ 8-fold compared to control, suggesting the ongoing disruption of sphingomyelin.

Characteristically, all FB-related brain lesions diagnosed in equide are tightly associated with brain regions rich in white matter. Blockage of ceramide biosynthesis could affect the existing pool of complex sphingolipids such as sphingomyelin, gangliosides and/or cerebrosides, suggesting the potential for direct mode of FB_1 neurotoxicity. In support of such speculation, experiments showed that FB_1 treatment disrupted myelination in brain aggregate

cultures *in vitro* (Monnet-Tschudi et al., 1999) and decreased myelogenesis in developing rats *in vivo* (Kwon et al., 1997). In cultured hippocampal neurons FB-related inhibition of sphingolipid synthesis depleted ganglioside content and significantly affected axonal growth (Harel and Futerman, 1993). Thus, the hypothesized neurotoxicity of FB by direct mechanisms seems to agree, at least to some extent, with the existing evidence.

Interestingly, incidences of this equine-specific pathology are seldom observed in other animals. One case of experimentally-induced ELEM-like symptoms was reported in pregnant New Zealand White Rabbits (Bucci et al., 1996). Out of 4 dams gavaged for 20 days with 1.75 mg/kg/day of purified FB_1 two died exhibiting ELEM-like lesions in the hippocampus, midbrain and temporal cerebral cortex. There is no evidence regarding the embryotoxicity. The fetuses were either not examined or data was omitted from the publication. This finding was later challenged by the same author, after repeated, unsuccessful attempts to develop the similar neuropathology in rabbit (Bucci T.J., personal communication). A different group investigating embryotoxicity in the same species showed that FB treatment had no adverse effects on rabbit fetuses (LaBorde et al., 1997).

A single incident of ŀukoencephalomalacia similar to ELEM reported in nature was diagnosed in a white-tailed deer (Howerth et al., 1989). The affected animal was showing evident neurological symptoms (incoordination, stupor, constant twitching of the head and neck), which were later confirmed by pathological examination. Gross lesions consisted of malacia and cavitation in subcortical white matter accompanied by multiply petechial and ecchymotic hemorrhages. Corn was discovered in the rumen and within the area where animal was found, there were three cornfields containing corn-ears displaying high fungal infestation.

Subsequent examination identified 60% of molds as *Fusarium* species (i.e. F. *verticillioides* and *graminearum*). The presence of several fungal toxins was also detected, however, the corn was not specifically tested for FB_1 since the toxin has not been yet discovered and described.

Another incident demonstrating ELEM-like symptoms was reported in turkeys (Ficken et al., 1993). Sudden, high mortality, preceded by various nervous symptoms, was concurrent with histopathology reminiscent of encephalomalacia. Conversely, analysis of suspected feed did not show any contamination, specifically lack of fumonisin presence that could have been linked to the reported outbreak. It is interesting that the given incident occurred in the same time period, during which number of severe ELEM outbreaks were diagnosed in horses. Moreover, since fumonisin was identified only two years earlier the detection methods were not entirely established and the sensitivity of those available was relatively poor.

Fumonisin and gastrointestinal tract

The information regarding the FB-related impact on equine gastrointestinal (GI) tract is very limited. It can be explained by the lack of interest in organs not regarded as major targets of FB_1 toxicity, and because occurring GI-related changes may be considered as of secondary importance being more colloquial compared to those present in brain and liver. However, existing evidence implicates the exposure to FB_1 in occurrences of pathological events (in a form of duodenitis-proximal jejunitis (DPJ; proximal enteritis)) in the equine GI system. During an experimental trial horses were fed *F. moniliforme* isolates cultured from corn in South Africa, MRC 826, which were shown to cause natural outbreaks and experimentally

induced ELEM incidences (Marasas et al., 1988). Animals exposed to MRC 826 developed evident intestinal lesions consistent with DPJ and moderate ELEM lesions in most brain tissues examined. Horses exposed to AU 2/3 isolates, associated previously with clinical signs of DPJ, have developed even more severe brain damage ranging from perivascular and perineuronal edema to necrosis in all tissues except gray matter of the frontal lobe and hypothalamus-dentate gyrus with concurrent elevation of free sphinganine in selected brain regions (Goel et al., 1996). Indirect support comes from the cases of poultry and human exposures to fumonisin. Chickens fed with FB_1-contaminated feed in Brasil developed diarrhea (Sydenham et al., 1992). The same symptoms along with histopathological changes in GI tract were observed in cockerels and hens in India (Prathapkumar et al., 1997). Human patients in India consuming moldy maize and sorghum showed acute GI disorder (abdominal pain and diarrhea) with the onset of symptoms within ½ to 1 h following the ingestion (Bhat et al., 1997). Moreover, FB_1 was the most abundant mycotoxin detected. Although there were no further attempts to investigate the reported relationship, the association between F. *verticillioides*-infested feed (containing FB_1) and concurrent GI injury should be carefully studied.

It is common knowledge that horses are highly susceptible to various factors disrupting the balance in their gastrointestinal (GI) system. Dietary exposure to corn infested with *F. verticillioides* containing FB_1 could compromise the physiological functionality of gut lining in *aquide* leading to GI disturbances followed by colic. Such a notion agrees with the *in vitro* studies reporting the vulnerability of endothelial cells to FB_1 treatment. The exposure to FB_1 disrupted barrier function of cultured porcine endothelium derived either from arteries (as

measured by the rate of albumin transfer; Ramasamy et al., 1995) or intestine (as assayed by transepithelial electrical resistance; Bouhet et al., 2003**)** and is important to potential pathogenic mechanisms**.** Thus, the disruption of GI homeostasis by FB containing infested feed could be jointly accomplished by number of partially understood, indirect and direct effects. Damage of the endothelial layer usually results in its increased permeability exposing the residual, intestinal bacterial lipopolysaccharide (LPS) to the immune system. Bacterial LPS is a potent stimulator of proinflammatory cytokine signaling (e.g. TNFα, IL-1β, IL-6) and the consequent increase in BBB permeability in LPS-induced endotoxemias is evident (Morris, 1991; Tsao et al., 2001). Studies performed on horses clearly demonstrate that TNFα is a central factor in the pathogenesis of endotoxemia: TNFα was highly elevated in colic events attributable to GI diseases (Morris et al., 1991) and in the number of experimentally induced endotoxemias (Morris et al., 1990). FB_1 could also contribute to the subsequent, TNFα-dependent effects, since the key role of this cytokine in the FB-induced hepato-and nephrotoxicity has been demonstrated in numerous studies (Dugyala et al., 1998; Sharma et al., 2000; Bhandari et al., 2001; Bhandari et al., 2002).

Thus, it could be speculated that events causing release of TNF and other related pro-inflammatory factors, regardless of their origin, could result in BBB damage. This could increase FB_1 penetration to the CNS to the point where the FB_1 could exert its toxic effects directly upon the brain contributing, along with other still unclear mechanisms, to the development of neurotoxicity as seen in ELEM. Penetration of FB_1 to the brain during development of ELEM cannot be ruled out, since increases of sphinganine concentration were reported in brain of horses afflicted with this disorder (Goel et al., 1996). Considering the

suggested bowel-damaging potential of *Fusarium*-infested corn, much lower GI tolerance coupled with the unique endotoxemic-type of response to the insults targeting equine digestive system, and the proven role of LPS in the BBB disruption, the suggested cascade of events could be considered.

Fumonisin and developmental neurotoxicity

Another aspect of FB-related neurotoxicity may be described as developmental. A number of studies have linked this mycotoxin to different forms of embryotoxicity including developmental alteration in neural tissue. First, exposure of chicken embryos to FB_1 resulted in elevated mortality and developmental anomalies of the CNS were observed such as exencephaly and head enlargements (Javed et al., 1993). Pregnant hamsters when gavaged with FB_1-containing extracts of *F. moniliforme* also demonstrated increased embryotoxicity, however relatively high doses (over 6 mg/kg) were employed (Floss et al., 1994). These initial findings were later contrasted by Voss et al., (1996), who showed no adverse effects on mating, fertility, reproductive performance and lack of developmental anomalies after FB_1 in the fetuses of pregnant rats. Some embryotoxic effects were associated with maternal toxicity rather than with direct impact of FB_1 treatment upon fetuses, especially while the employed doses were high (Collins et al., 1998a,b).

Later study in pregnant rats, however, showed a significant decrease of myelin deposits in the brain concurrent with elevated levels of brain sphinganine and sphinganine/sphingosine ratio (Kwon et al., 1997). Although the demyelination was also observed in nutritionally deficient controls the effect of FB_1 upon white matter depletion in FB_1-treated rats was

significantly greater. The same group suggested that postnatal rats with undeveloped BBB may be vulnerable to the FB_1 exposure. After a single 8.0 (and also 0.8) mg/kg FB_1 dose was given sc free sphingoid bases in brain were elevated and simultaneously the presence of FB_1 in this organ was detected (Kwon et al., 1997b). Pharmacokinetic analyses executed in the same study indicated that the sphinganine increase in brain was caused by the FB_1-dependent inhibition of ceramide synthesis and not due to the accumulation of the systemic sphinganine. Infiltration of FB_1 across the leaky, due to the immaturity, BBB implies that a similar penetration could occur when the integrity of BBB is compromised by pathological stressors.

One issue, whether the placenta is permeable to funonisins, is of the utmost importance, since developing organisms including humans are exceptionally vulnerable to the adverse effects of various chemicals *in utero*. It has been recently shown for the first time in inbred (as which are known to be generally more susceptible to various stimuli and insults) pregnant mice that exposure to FB_1 resulted in detectable levels of this mycotoxin found in uterus (Maddox et al., 2001). This FB_1 concentration was similar to the doses later employed in experimentation with *ex vivo* fetuses, in which FB_1 caused neural tube defects (NTD) (Maddox et al., 2001). In the connection with these studies, a novel hypothesis has surfaced regarding the mechanism of FB toxicity: that depletion of sphingolipids clustered in lipid membrane rafts causes folate receptor damage and consequent inhibition of vitamin uptake. *In vitro* treatment of Caco-2 cells (cells derived from intestine) with FB_1 disrupted functions of folate receptor what was subsequently followed by reduced uptake of cellular folate (Stenvens and Tang, 1997). The association between FB toxicity and development of NTDs has been supported by the recent study by Sadler et al., (2002) also implicating the importance of folate vitamins in this FB_1-

induced defect. The study employing neurulating mouse embryo cultures demonstrated that the FB-induced inhibition of sphingolipid biosynthesis, confirmed by elevation of sphinganine/sphingosine levels, is coincidental with increased cases of NTD both at short-(50 μM of FB_1) and long-term (2, 50 and 100 μM of FB_1) exposure. Moreover, supplementation of folate vitamins to embryos exposed to FB_1 partially reduced the NTD incidences and improved embryo growth without affecting the elevated levels of free sphingoid bases.

The reported evidence regarding a relationship between NTD incidences in mice and exposure to FB_1 puts in new context epidemiological data describing clusters of the same pathology diagnosed in Mexican-American women in 1990, in Texas (Hendricks, 1999). Females from this region had a substantially higher NTD rate during this particular year compared to earlier time periods. Maize products constitute a major diet component for certain populations, especially of Hispanic origin, and despite food processing applied to corn products the significant amounts of FB_1 and its hydrolysis products can be detected: e.g. tortilla (FB_1-187 ng/g) and masa (FB_1-262 ng/g) (Stack, 1998). Given these facts, it has been recently suggested that consumption of fumonisin constitutes a risk factor for human birth defects such as neural tube malformation and craniofacial abnormalities (Marasas et al., 2004).

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CHAPTER 3

ENDOTOXIN EXPOSURE ALTERS BRAIN AND LIVER EFFECTS OF FUMONISIN B_1 IN BALB/C MICE: IMPLICATION OF BLOOD BRAIN BARRIER[1]

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[1] Osuchowski, Marcin F., He, Quanren, Sharma, Raghubir P. Submitted to Food and Chemical Toxicology 06/05/2004

Abstract

Fumonisin B_1 (FB_1), a mycotoxin produced by *Fusarium verticillioides*, causes equine leukoencephalomalacia and hepatoxicity. We studied the modulation of FB_1 toxicity in brain and liver of female BALB/c mice after endotoxin administration to compromise the blood-brain barrier (BBB) integrity. Mice were injected saline or 3 mg/kg of lipopolysaccharide (LPS) followed 2 h later by either a single or three daily doses of 2.25 mg/kg of FB_1. After 4 h of a single FB_1 injection free sphingoid bases increased in liver. Circulating alanine aminotransferase increased by LPS alone at this time. The expression of inflammatory cytokines, tumor necrosis factor (TNF) α, interferon (IFN) γ, interleukin (IL)-1β, IL-6, and IL-12, increased in brain by LPS; no effect of FB_1 was observed. In liver LPS + FB_1 decreased the expression of TNFα and IFNγ compared to LPS alone. One day after 3-day FB_1 treatment sphinganine increased in brain and liver; it was further elevated when LPS was given before FB_1. Circulating liver enzymes increased in FB_1-treated mice but were lower after LPS + FB_1 compared to FB_1 alone. FB_1 decreased the LPS-induced brain expression of IFNγ and IL-1β, whereas it increased expression of IL-6 and IL-12. In liver FB_1 decreased the expression of IL-1β IFNγ compared to LPS alone. Results indicated that endotoxemia concurrent with FB_1 intoxication facilitated the permeability of fumonisin in brain indicated by increased accumulation of sphinganine and endotoxin modifies the effect of FB_1 in both brain and liver.

Keywords: lipopolysaccharide, fumonisin, blood-brain barrier, sphingolipids, cytokines, brain

1. Introduction

Fumonisins are a group of mycotoxins produced by the *Fusarium verticillioides* (formerly *F. moniliforme*), a common fungal contaminant of corn and other cereals (Dutton, 1996). Fumonisin B_1 (FB_1) is the most abundant and noxious of this class and has been linked to various diseases in humans and animals. The role of FB_1 as an etiologic agent in diseases such as equine leukoencephalomalacia (ELEM, Brownie et al., 1987) and porcine pulmonary edema (PPE) has been established (Dutton, 1996). An association between dietary exposure to FB_1 and occurrences of human esophageal cancer in Southern Africa and primary liver cancer in China was reported (Dutton, 1996). Cancerous hyperplasia after chronic FB_1 treatment is also shown in laboratory animals with high species/gender specificity (Howard et al., 2001). Oral exposure to FB_1-containig feed compromised the integrity of gastrointestinal (GI) system in horses (Brownie et al., 1987) and poultry (Dutton, 1996). Incidences of acute GI disorders in humans concurrent with the consumption of fumonisin-containing food products were reported in India (Bhat et al., 1997). Among all studied species, however, liver is an organ displaying uniform susceptibility to the FB_1 exposure (Voss et al., 2001). Epidemiological importance of FB_1 triggered further studies revealing the magnitude of cytokine-related signaling. Key role of proinflammatory cytokines such as tumor necrosis factor α (TNFα) in FB_1-related toxicity has been shown in mouse liver (Sharma et al., 2000; Bhandari et al., 2002) and porcine kidney (He et al., 2001).

The initial mechanism of fumonisin toxicity on the cellular level involves the inhibition of ceramide synthase deregulating the de novo sphingolipid biosynthesis. The inhibition leads to accumulation of sphinganine, sphingosine and their metabolites, all of which are implicated

in cellular signals regulating cell growth, differentiation, survival and apoptosis (Merrill, Jr. et al., 2001). Inhibition of ceramide synthase is followed by depletion of more complex sphingolipids such as sphingomyelin and gangliosides crucial for function and stability of neuronal tissue.

The brain damage induced by fumonisins is consistent in *equide* and specific only for this species. The exact pathogenesis of ELEM is not clear but direct impact of FB_1 on neuronal tissue has been shown in number of reports. Fumonisins were implicated in the possible etiology of neural tube defects (NTD) in children (Hendricks, 1999). FB_1 treatment disrupted myelination in rat brain aggregate cultures in vitro (Monnet-Tschudi et al., 1999), interrupted axonal growth in cultured rat hippocampal neurons (Harel and Futerman, 1993), and altered the sphinganine concentration and oligodentrocyte activity in the rat primary glial/oligodenrocyte culture (Kwon et al., 2000). FB_1 was toxic during the developmental period; it hindered myelogenesis in postnatal rats (Kwon et al., 1997b) and caused the folate-dependent cranial NTD in neurulating mouse embryos (Sadler et al., 2002). FB_1-dependent changes in electrophysiological activity in rat neocortex were also reported (Banczerowski-Pelyhe et al., 2001). There are no laboratory in vivo models for ELEM.

Endotoxins are common dietary contaminants and are also produced by gut microflora. Lipopolysaccharide (LPS) is a component of gram-negative bacteria and a potent proinflammatory factor affecting central nervous system (Lee et al., 2002) and liver (Van Amersfoort et al., 2003). The systemic exposure to LPS occurs frequently via several routes and may be concurrent with intake of mycotoxins (Ganey et al., 2001). LPS applied systemically in nontoxic concentration upregulates the expression of brain cytokines (Pitossi et

al., 1997) and their receptors (Utsuyama and Hirokawa, 2002). Higher LPS doses compromise the blood-brain barrier (BBB) facilitating the passage of typically non-permeable molecules (Minami et al., 1998). A single dose of LPS was shown to modify the liver FB_1 toxicity when given concurrently (Dugyala et al., 1999). Non-injurious systemic doses of LPS can exacerbate existing mycotoxin-related hepatoxicity (Barton et al., 2001).

The FB_1-related CNS effects are poorly understood and the existing evidence largely focuses on developmental and in vitro aspects of neurotoxicity lacking the studies in adult laboratory models. Similarly, the mechanisms leading to the FB_1-related toxicity in liver are only partially explained. The incidence of concurrent mycotoxin intake or gram-negative bacterial infection combined with potential to modulate their systemic toxicity and evidence of LPS-dependent BBB damage (Minami et al., 1998) encourage further studies of CNS-related FB_1 effects. The present study investigated the modulation of FB_1 toxicity in the brain and liver after pretreatment of mice with endotoxin at a dose known to cause an increase in the permeability of the blood-brain barrier.

2. Materials and methods

2.1. Animals

Female 7-8 week old BALB/c mice were purchased from Harlan Inc. (Indianapolis, IN) and acclimated for one week prior to treatment at 21°C and 50% humidity with a 12-h light/dark cycle at the University of Georgia Animal Resources facility. Mice were maintained on mycotoxin-free commercial rodent chow (Harlan-Teklad, Madison, WI) and deionized water *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.

2.2. Chemical

Lipopolysaccharide from *E. coli*, serotype 026:B6, was purchased from Sigma-Aldrich Chemical Company (St. Louis, MO). Fumonisin B_1 (98% purity) was purchased from Promec (Tygerberg, South Africa).

2.3. Blood-brain barrier permeability assay

The BBB integrity was evaluated 2, 24 and 48 h after a single intraperitoneal (ip) dose of 3 mg/kg LPS. The employed dose, as shown in previous studies investigating drug delivery to the CNS in sepsis models, allows for a relatively long period of increased BBB permeability (Xaio et al., 2001, Minami et al., 1998). Sodium fluoresceine (NaFl, 200 µl of 0.5%) or a similar volume of physiologically buffered saline (PBS) was injected intravenously 1 h before sampling the animals. The brains were dissected after euthanizing mice and fluorescence determined following a method previously described (Minami et al., 1998). The NaFl was employed since its molecular weight of 376 daltons is within the same order of magnitude as that of FB_1 (721 Da).

2.4. Treatments

Mice (5 per group) were injected ip with LPS (3 mg/kg) or PBS at the beginning of treatment and FB_1 (2.25 mg/kg) administered by subcutaneous route either as in a single injection (2 h after the LPS, referred hereafter as a single FB_1 injection) or in 3 daily doses (6, 24 and 48 h after LPS, referred henceforth as repeated FB_1 treatment). The employed dose of

FB_1 was optimal in affecting cytokine expression and sphingolipid synthesis without apparent clinical signs of toxicity, as reported in number of previous studies (Sharma et al., 2000; Bhandari et al., 2001; Bhandari et al., 2002). Control animals received PBS via the respective route. Weights were recorded daily in repeated FB_1 treatment.

2.5. Sampling

Mice were decapitated 4 h after the single FB_1 injection or 24 h following the last FB_1 injection for repeated FB_1 treatment. Blood was collected in heparinized tubes and plasma was subsequently isolated for analysis of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), using a Hitachi 912 Automatic Analyzer (Roche Diagnostics, Indianapolis, IN). Livers were removed and brains were immediately dissected on ice into cortex, cerebellum, midbrain, medulla oblongata. Hypothalamus and thalamic regions were included in the midbrain section, whereas the hippocampus was dissected together with cortex. Collected tissue samples were quickly frozen and stored at -85°C until analysis.

2.6. Sphingolipid analysis

Free sphingosine and sphinganine in brain and liver extracts were determined by high performance liquid chromatography using extraction methods and equipment identical to described previously by He et al., (2001). The sphingoid bases were analyzed in cortex, cerebellum, midbrain and medulla oblongata. The concentration of sphingoid bases was quantitated based on the recovery of the C_{20}-sphinganine internal standard.

2.7. Semiquantitative RNA expression analysis

The RNA was isolated from the homogenized brain cortex and liver aliquots; initial cDNA strand prepared by reverse transcription and DNA amplified as described previously (Sharma et al., 2000). Primers used for thermal amplification, along with optimized conditions (annealing temperature, time, Mg^{+} concentration, and number of cycles), are shown in Table 1. Saturation of the product was avoided by maintaining the number of cycles within the exponential increase of the product. The density of the cytokine bands was normalized to the -actin band of the same cDNA product by digitizing gel photographs.

2.8. Liver cells proliferation assay

Frozen liver tissues were embedded in paraffin and proliferating cell nuclear antigen (PCNA) assay was performed as described recently (Sharma et al., 2003). The method employed the PCNA primary antibody (1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) coupled with the secondary biotinylated antibody and stained with Vectastain ABC® (Vision Biosystems, Mount Waverley, Victoria, Australia) kit. The PCNA-positive cells were counted in the sections and normalized to the unit area as described previously (Sharma et al., 1997).

2.9. Statistics and data presentation

All data are expressed as mean ± standard error (SE). Data were analyzed by two-way analysis of variance (ANOVA) followed by the Duncan's multiple comparison test. Proliferating cell counts were compared by means of the nonparametric Wilcoxon Rank Sum

Test. All tests were performed using SAS software (SAS Institute, Cary, NC). The level of $p < 0.05$ was considered significant.

3. Results

3.1. BBB permeability after LPS treatment

Intraperitoneal treatment with LPS altered the BBB permeability as measured by the NaFl uptake in the whole brain (Fig.1). An increase in the NaFl concentration in brain was observed at all time points as 500% after 2 h, 750% at 24 h, and 400% at 48 h.

3.2. Body and organ weights

Exposure to FB_1 and/or LPS in repeated FB_1 treatment decreased the body weights significantly (Table 2). In the FB_1-treated animals the body weight declined by 9% whereas the LPS treatment decreased it by 17%. The greatest weight loss (by 26%) was observed in animals treated both with FB_1 and LPS. The decrease after FB_1 + LPS treatment was significantly greater than in mice treated with either FB_1 or LPS alone. Liver weights were significantly increased only after combined FB_1 + LPS treatment; no effect was noticed after administration of either FB_1 or LPS alone. Brain weights remained unaffected in all treatment groups (data not shown).

3.3. Sphingolipid concentration in brain

No changes in free sphinganine (Fig. 2) or sphingosine were observed in the cortex 4 h after the single FB_1 injection for either group treated with FB_1. Other regions are not shown, as there were no FB_1-related changes. In the repeated FB_1 treatment animals administered FB_1

alone showed an increase in the regional brain concentrations of sphinganine; 51% in cortex, 57% in cerebellum and 48% in medulla oblongata, compared to the saline treated mice (Fig.2). No increase of sphinganine was noted in the midbrain in the FB_1-treated mice. The LPS treatment did not cause a change in sphinganine concentration in any brain region. When mice received repeated FB_1 treatment after LPS, the sphinganine levels in all brain regions further increased compared to the animals treated with FB_1 alone. FB_1 and/or LPS did not alter sphingosine in any brain region regardless of the treatment duration (data not shown).

3.4. Cytokine gene expression in brain

The LPS treatment upregulated the expression of all cytokines in both experiments, except TNFα in repeated FB_1 treatment, compared to control or FB_1-treated animals (Fig. 3). Fumonisin B_1 given as a single injection after LPS did not influence the LPS-induced cytokines. However, the repeated FB_1 treatment lowered the expression of IFNγ and IL-1β but increased the expression of IL-6, compared to mice receiving only LPS. The IL-12 expression with repeated FB_1 treatment was elevated with FB_1 alone. After repeated FB_1 treatment the observed level of cytokine expression in combined FB_1 + LPS treatment surpassed the levels noted in FB_1 only-treated mice, except for TNFα.

3.5. Concentration of liver enzymes in plasma

The animals treated with FB_1 alone showed no change in the concentration of ALT and AST after a single FB_1 injection; however, displayed a dramatic increase in the level of both enzymes after repeated FB_1 treatment (Fig.4). The LPS alone treatment in the short term increased the activities of circulating ALT but did not modify ALT or AST in the repeated-

treatment experiment. When both FB_1 and LPS were administered in the repeated FB_1 treatment, the mice showed a significantly lower activity of circulating ALT and AST compared to animals given FB_1 alone.

3.6. Sphingolipid concentration in liver

In animals treated with FB_1 only the sphinganine concentration in liver increased in both after single and repeated FB_1 treatment, whereas the sphingosine level increased only after a single FB_1 injection (Fig. 5). The LPS treatment did not cause any alteration in the liver sphinganine levels but elevated the sphingosine concentrations in both experiments (after 6 h and 3 days). When mice were pretreated with LPS and received FB_1 for 3 days the sphinganine concentration increased further compared to FB_1 only-treated animals. The LPS pretreatment did not change FB_1-induced sphingosine accumulation after the single FB_1 injection.

3.7. Cytokine gene expression in liver

In mice treated with FB_1 increased the expression of TNFα, IFNγ and IL-1β after a single injection; the cytokine expression was unaltered after repeated FB_1 treatment (Fig 6). At both time points the LPS alone increased the expression of TNFα and IL-1β but not the expression of IFNγ. The animals treated with FB_1 after LPS showed decreased expression of TNFα and IFNγ after a single FB_1 injection; however, that of IFNγ and IL-1β after repeated FB_1 injection compared to the mice administered LPS only.

3.8. Liver cells proliferation assay

This assay was conducted after repeated FB_1 treatment only. In livers from control animals very few PCNA-positive cells were evident (Fig. 7). Both FB_1 and LPS treatment increased the number of PCNA-positive cells indicating the augmented regeneration in this organ. When mice were treated with FB_1 after LPS pretreatment, the number of PCNA-positive cells was higher than those in LPS-treated animals but similar to those treated with FB_1 alone.

4. **Discussion**

The results presented here support the hypothesis that the endotoxin modifies the FB_1-effects in mouse brain and liver. The pre-exposure to the LPS resulted in the disruption of BBB indicated by increased accumulation of sphinganine, a biomarker for FB_1 availability to the tissue. Consequently FB_1-induced increase in sphinganine in brain occurred along with modulation of proinflammatory cytokines after repeated FB_1 treatment.

Periods of increased BBB permeability, regardless of whether of physiological or pathogenic origin, are likely to promote the CNS exposure to the FB_1. In postnatal rats, which may have incomplete BBB, FB_1 treatment increased the sphinganine concentration and sphinganine/phingosine ratio in brain. This effect was due to direct FB_1-dependent inhibition of ceramide synthase in this organ, since FB_1 was detected in brain after both (0.8 and 8 mg/kg) doses (Kwon et al., 1997a). In developing rats (*in utero*) the exposure to FB_1 was concomitant with the CNS hypomyelination (Kwon et al., 1997b). Sphingolipid biosynthesis in

the adult CNS may be vulnerable to the FB_1 exposure after LPS-related endotoxemia by compromising BBB integrity (Xaio et al., 2001, Minami et al., 1998).

Due to the high environmental prevalence of both fumonisin and endotoxin a concurrent exposure to these agents is likely to be frequent. Infestation of cereals by FB_1-producing *Fusarium* is widespread (Dutton, 1996) and the contact with bacterial endotoxin is commonplace whether via infection or residual microflora. Stressing stimuli such as liver disease or dietary modification may increase the plasma concentration of GI-derived endotoxin in various species (Ganey et al., 2001). Equines seem to be particularly exposed due to the gram-negative flora residing in the gut and frequency of colic disorders (Morris, 1991). Endotoxemia secondary to GI diseases is a primary cause of fatality in horses (Morris, 1991). The fumonisin intoxication may potentially promote the systemic exposure to LPS since the FB_1 compromised the barrier function of intestinal (Bouhet et al., 2003), as well as endothelium of pulmonary arteries in vitro (Ramasamy et al., 1995), and was responsible for GI damage reported in horses (Goel et al., 1996), humans (Bhat et al., 1997) and poultry (Dutton, 1996).

The observation showing the increases of regional brain sphinganine after co-administration of FB_1 and LPS compared to mice treated with FB_1 only indicated that the de novo biosynthesis of sphingolipids in brain was further affected by FB_1 after LPS treatment. An elevation of sphinganine was also observed in three brain regions after FB_1 treatment alone. In an early report a insignificant increase of free sphinganine was reported in whole brain after systemic administration of FB_1 (Tsunoda et al., 1998a), whereas FB_1-induced regional imbalance of some brain neurotransmitters and their metabolites was observed (Tsunoda et al.,

1998b). Free sphingoid bases may play a role in FB_1-related neurotoxicity since their elevation was demonstrated in brain of ELEM-affected horses (Goel et al., 1996), and in rat brain displaying hypomyelination after FB_1 treatment (Kwon et al., 1997b). The increase of sphingoid bases paralleled FB_1-induced hepato-and nephrotoxicity in rodents in numerous studies (Sharma et al., 2000; Voss et al., 2001; Bhandari et al., 2002). The relationship between sphingoid bases and CNS effects, however, remains controversial partly due to a general lack of brain sphinganine/sphingosine analyses in studies performed on horses and partly due to the absence of appropriate experimental animal model confirming such association.

All cytokines analyzed in our study are known to be CNS-borne and are expressed on-site by neurons (IL-1β, IL-6), astrocytes (IL-1β, IL-6, TNFα, IFNγ) and microglia (IL-1β, IL-6, IL-12, TNFα) (Szelenyi, 2001). The expression of all cytokines in brain after the short-term treatment was upregulated in LPS-treated groups. The longer exposure to LPS and/or FB_1 revealed a significant modulation of expression of various cytokines except TNFα when mice received both toxicants. The insensitivity of TNFα in the repeated FB_1 treatment is not surprising, since it has been previously shown that the expression of this cytokine predominantly occurs in the first phase of LPS-related insult but returns to control levels after several h (Brochu et al., 1999). The transition in expression of brain IFNγ, IL-1β, IL-6 and IL-12 observed after repeated treatment only is likely due to the activation nature of these cytokines, secondary to TNFα, as well as delayed availability of this organ for signaling molecules (LPS or FB_1), thus deterred initiation of the inflammatory cascade. In liver the changes in the LPS + FB_1 were already present at the early time point. The modulatory

changes in cytokine expression in brain by co-exposure to FB_1 and LPS were not uniform showing an augmentation for IL-6 and IL-12 but antagonism in the case of IL-1β and IFNγ. Cytokine effects exerted upon the brain are consequence of a complex interplay involving cytokines from peripheral sources coupled with autonomic neuroregulation (Szelenyi, 2001). The IL-1β, IL-6, TNFα and IFNγ are primarily associated with neuronal injury although their role in neuroprotection is also known (Stoll et al., 2002). Based on the presented data it cannot be interpreted whether such disparity in modulatory effect after combined LPS + FB_1 treatment would or would not promote neurotoxic effects.

In liver, after repeated FB_1 treatment the co-administration of FB_1 and LPS resulted in partial attenuation of hepatotoxicity, which was paralleled by generally decreased cytokine expression compared to LPS alone. It was however contrasted by enhanced accumulation of free sphingoid bases by co-exposure to FB_1 and LPS. In all species studied FB_1 caused liver damage associated with increases of cytokines including TNFα (Sharma et al., 2000), IL-1β (Bhandari et al., 2001; Bhandari et al., 2002) and IFNγ (Bhandari et al., 2002; Sharma et al., 2003). There is gender-specific high constitutive liver expression of TNFα, IFNγ, IL-1β and IL-12 in liver of female mice, related to higher hepatoxicity (Bhandari et al., 2001). The modulation of cytokine expression by FB_1 + LPS in both brain (after repeated FB_1 treatment) and liver (in both experiments) was correlated with the accumulation of free sphinganine in these organs. Since food deprivation and weight loss-related stress may influence the expression of inflammatory cytokines, the use of simultaneous food-restricted controls would be desirable in future studies. We hypothesize, however, that the observed cytokine modulation by FB_1 + LPS after repeated treatment was minimally influenced by body weight

loss, since similar effects were noted in liver at the early time point when the food/water deprivation and/or weight loss were not a factor.

The toxic potential of a number of xenobiotics including fungal toxins can be augmented by endotoxin (Ganey et al., 2001); the non-injurious doses of LPS enhanced aflatoxin B_1-induced hepatoxicity in rat (Barton et al., 2001). A single dose of FB_1 + LPS treatment showed similar liver damage elevating the activity of circulating ALT and AST in mice reported earlier where the injury was partially prevented by pretreatment with anti-TNF antibodies (Dugyala et al., 1999). The evident hepatic injury caused by FB_1 in current study was partially ameliorated after pre-exposure to LPS without concurrent increase in liver regeneration. Both FB_1 and FB_1 + LPS-treated animals showed an equal level of tissue restoration that agrees with the reported lack of the TNFα-dependent effect upon PCNA expression in rat liver (Barton et al., 2001).

The accumulation of sphinganine is tightly correlated with the occurrence and severity of the in vivo toxicity, particularly in the liver (Voss et al., 2001). The observed increase of liver sphingosine after FB_1 + LPS treatment may be unrelated to FB_1, rather it is LPS-dependent since sphingosine increased in the LPS but not FB_1-treated mice. Sphingosine metabolite, sphingosine-1-phosphate, is a potent promoter of cell survival (Merrill, Jr. et al., 2001) and the sphingosine increase may be associated with the observed reduction in hepatotoxicity. Endotoxin stimulated the sphingolipid biosynthesis (Memon et al., 1999) and an increase of liver sphingolipids after FB_1 + LPS treatment may be a consequence of an earlier LPS-dependent upregulation.

In the FB_1 + LPS-dependent interaction observed both in brain and liver the cross-tolerance between the two toxicants may be considered. TNFα and IL-1β induced such phenomenon in brain (Zimmermann et al., 2001) and liver (Henricson et al., 1991) when preconditioned with endotoxin. The expression of these mediators was induced by LPS (Van Amersfoort et al., 2003) and also by FB_1 (Bhandari et al., 2002). Interestingly, a cross-tolerance to TNFα in neuronal culture was associated with increased level of ceramide in vivo, and the addition of FB_1 abolished the protective effect (Liu et al., 2000), suggesting the FB_1 treatment promoted neurotoxicity. The combined FB_1 + LPS treatment modulated the expression of cytokines in both brain and liver; the antagonistic effect of FB_1 + LPS hepatotoxicity was in discord to the enhanced accumulation of sphinganine in liver.

The present study demonstrated that sphingolipid metabolism in the adult brain is vulnerable to the exposure of FB_1 when accompanied by the endotoxin-related damage to the BBB. The inhibition of the sphingolipid metabolism in brain after FB_1 treatment alone suggests that limited transfer of the mycotoxin across the BBB exists. Considering the often prolonged and subclinical specificity of the gram-negative infection and mycotoxin exposure further long-term studies of the interaction of endotoxin and FB_1 are warranted.

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TABLE 3.1.

Primer sequences and selected assay conditions

	Primers	Annealing temperature (°C)	PCR cycles
IL-1b	S-5' ATC TGC GAC GAG GAA GAG AA 3' AS-5' ATC GCA GAT GAA GCT CTG GT 3'	54	38
IL-6	S-5' TTC CAT CCA GTT GCC TTC TT 3' AS-5' CAG AAT TGC CAT TGC ACA AC 3'	54	40
IL-12	S-5' AGG TGC GTT CCT CGT AGA GA 3' AS-5' AAA GCC AAC CAA GCA GAA GA 3'	54	39
TNFa	S-5' GTT CTA TGG CCC AGA CCC TCA CA 3' AS-5' TCC CAG GTA TAT GGG TTC ATA CC 3'	54	33
IFNã	S-5' AAG TTC TGG GCT TCT CCT C 3' AS-5' CCT GTG GGT TGT TGA CCT 3'	54	35
b-actin	S-5' TAT GAC TCC ACT CAC GGC AA 3' AS-5' GTG GTT CAC ACC CAT CAC AA 3'	54	25

*Other PCR conditions: hot start: 95 °C 5 min, 1 cycle, followed by: denaturation, 94 °C 15 sec, annealing, at indicated temperature 15 sec; elongation, 72 °C 30 min for indicated cycles, and finally elongation at 72 °C 1 min for 1 cycle.

TABLE 3.2.

Body and liver weights after indicated (FB_1 repeated) treatments in BALB/c mice

Weight (g)	Control	FB_1	LPS	FB_1 + LPS
liver	$0.863 \pm 0.04^{*a}$	0.843 ± 0.05^{a}	$0.886 \pm 0.02^{a,b}$	$0.935 \pm 0.01^{b,c}$
body	20.26 ± 0.72^{a}	18.64 ± 0.45^{b}	17.28 ± 0.29^{c}	16.00 ± 0.25^{d}
liver/body %	4.61 ± 0.16^{a}	5.01 ± 0.12^{a}	5.39 ± 0.09^{b}	5.84 ± 0.10^{c}

* Data presented as mean ± SE (n = 5). Different letters as superscripts denote a statistical significance of at least $P < 0.05$.

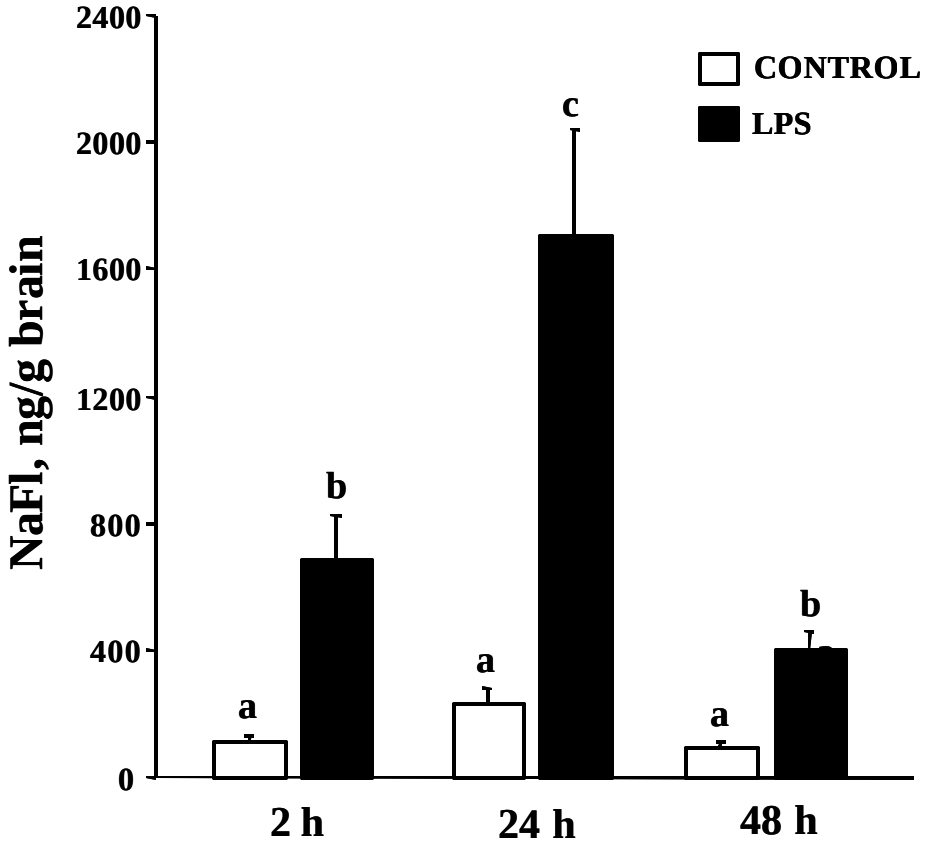

Fig. 3.1. Concentrations of sodium fluoresceine (NaFl) in brain of BALB/c mice after single lipopolysaccharide (LPS) injection at indicated time points. Mean ± SE (n = 4). Different letters on top of bars denote a statistical significance at $p < 0.05$.

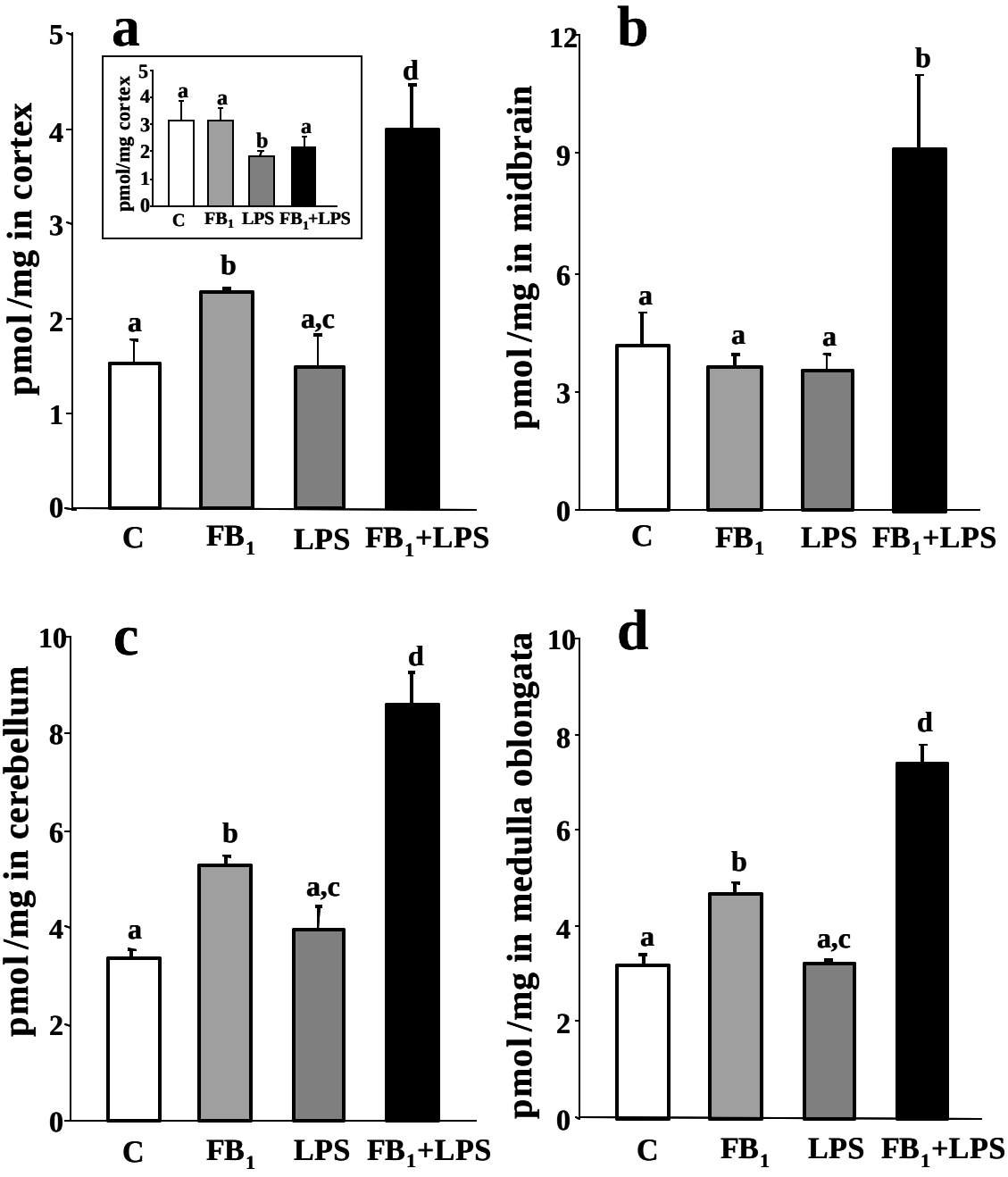

Fig. 3.2. Concentration of sphinganine in different brain regions of BALB/c mice from the repeated FB_1 treatment in (a) cortex, (b) midbrain, (c), cerebellum, and (d) medulla oblongata. The concentration of sphinganine after a single FB_1 injection in cortex as inset in (a). Mean ± SE (n = 5). Different letters on the top of bars denote a statistical significance at $p < 0.05$.

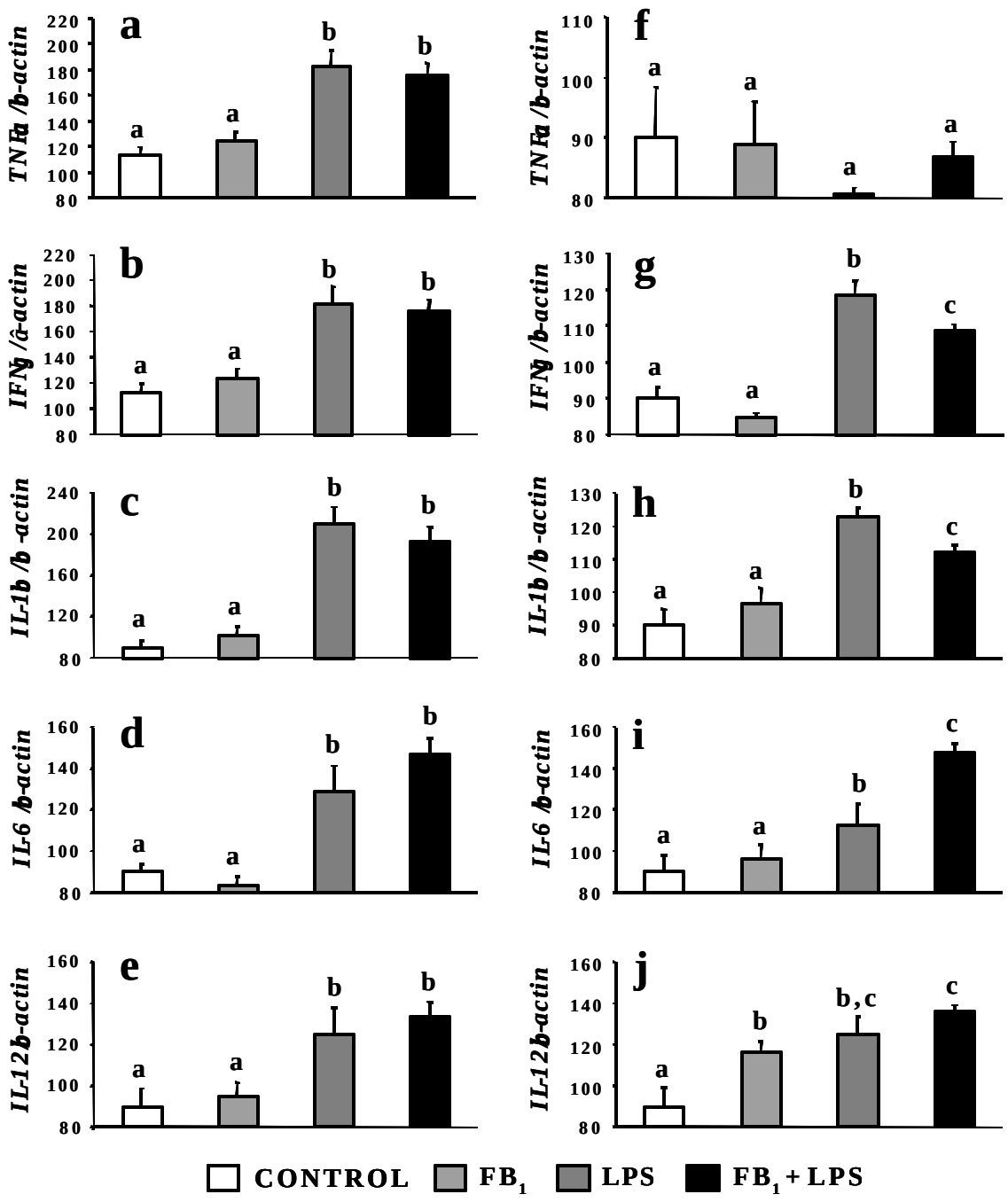

Fig. 3.3. Relative expression of *TNFα*, *IFNγ*, *IL-1β*, *IL-6* and *IL-12* in the cortex of BALB/c mice after a single FB_1 injection (a, b, c, d and e), or after repeated FB_1 treatment (f, g, h, i and j). Mean ± SE (n = 5). Different letters denote a statistical significance at $p < 0.05$.

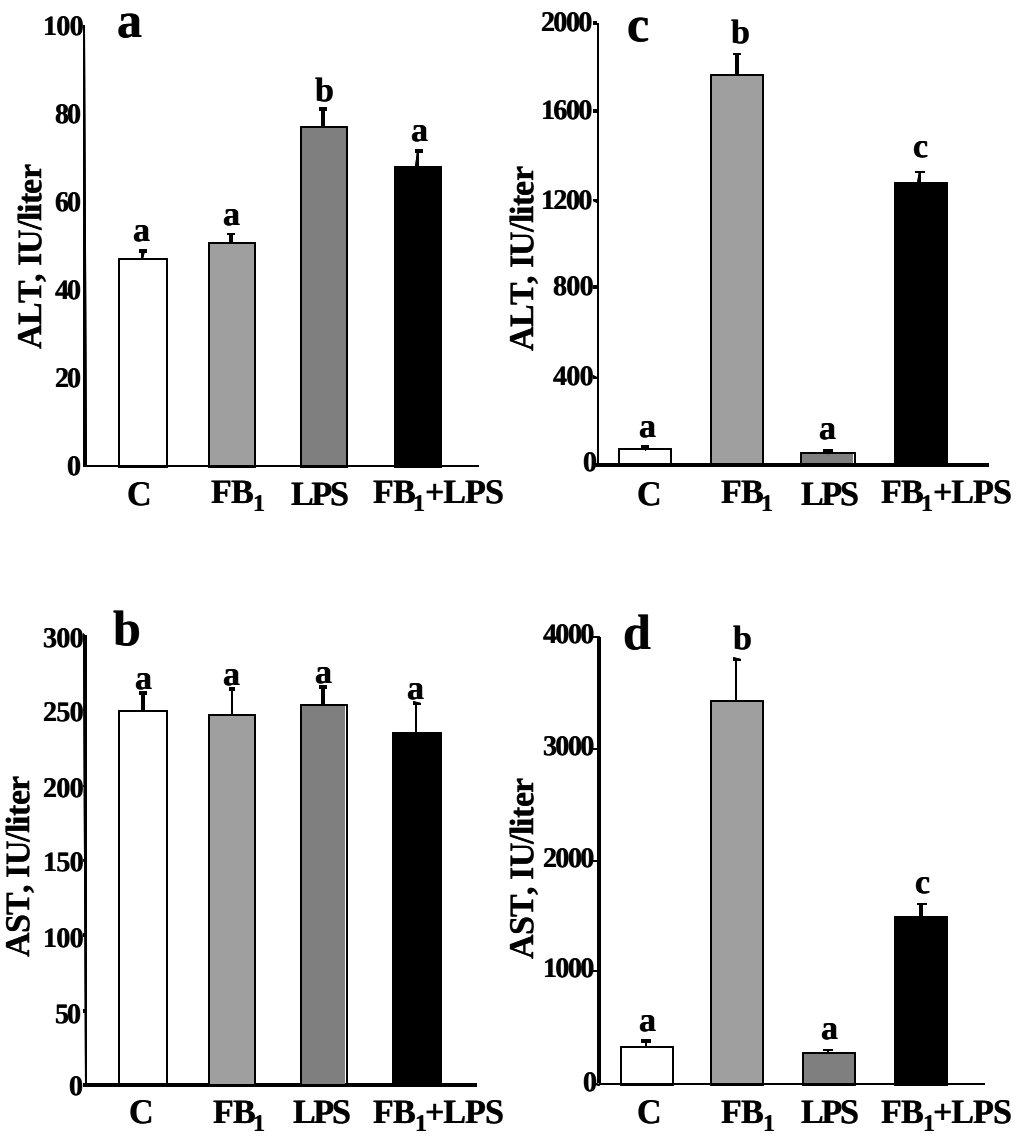

Fig. 3.4. Activity of circulating alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in BALB/c mice after a single FB_1 injection (a, b) or after repeated FB_1 treatment (c, d). Mean ± SE (n = 5). Different letters on top of bars denote a statistical significance at $p < 0.05$.

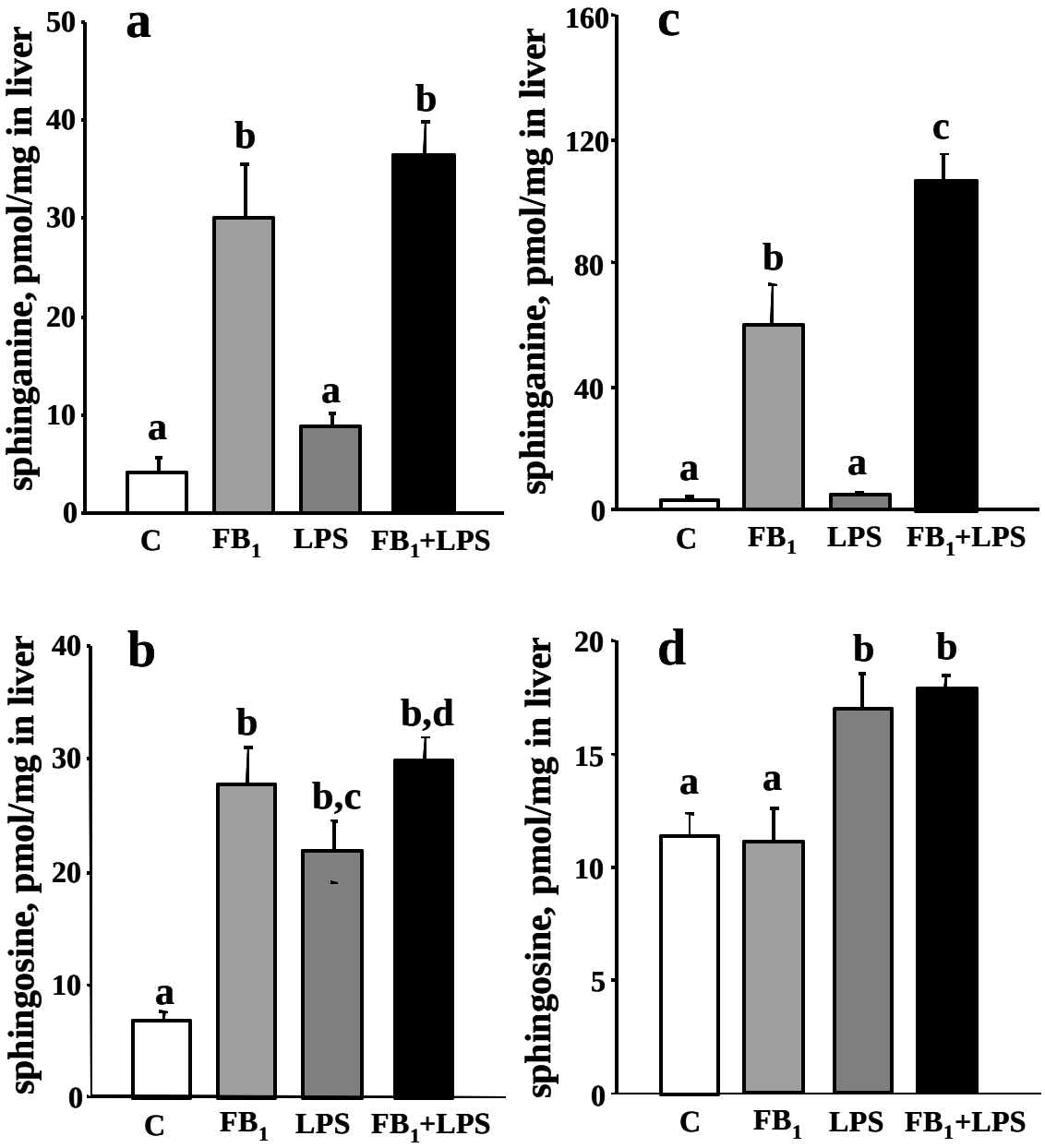

Fig. 3.5. Concentrations of sphinganine and sphingosine in the liver of BALB/c mice after a single FB_1 injection (a, b), or after repeated FB_1 treatment (c, d). Mean ± SE (n = 5). Different letters on top of bars denote a statistical significance at $p < 0.05$.

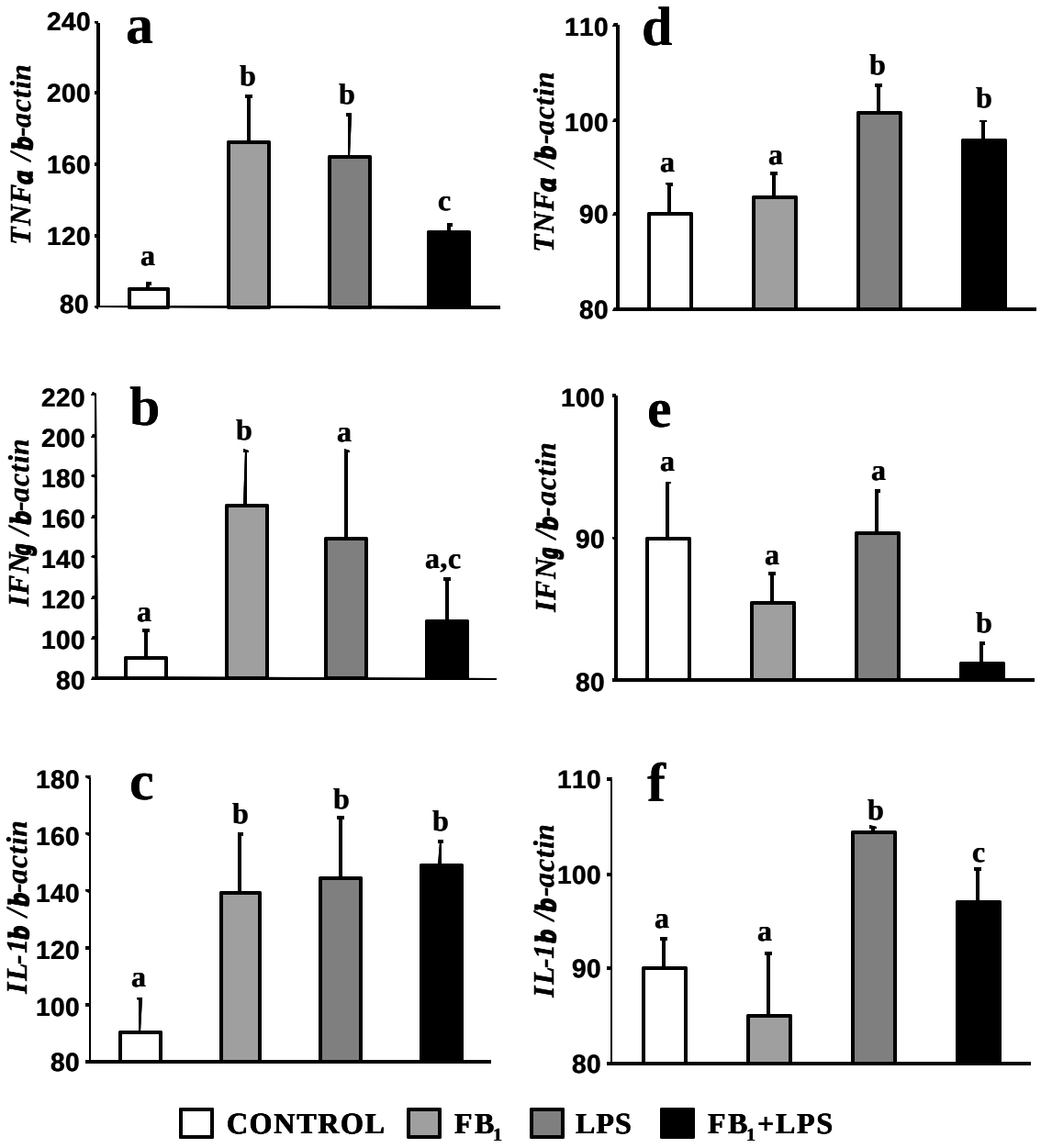

Fig. 3.6. Relative RNA expression of *TNFa*, *IL-1b* and *IFNg* in the liver of BALB/c mice after single FB_1 injection (a, b), or after repeated FB_1 treatment (c, d). Mean ± SE (n = 5). Different letters on top of bars denote a statistical significance at $p < 0.05$.

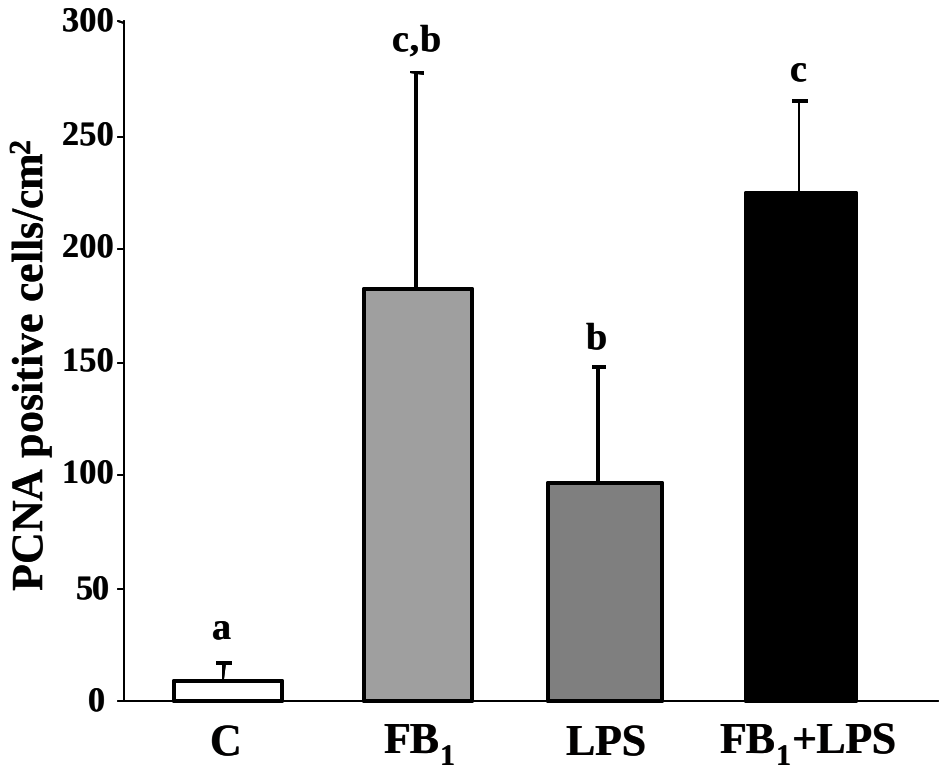

Fig. 3.7. The incidence of proliferating cell nuclear antigen (PCNA)-positive cells in the liver of BALB/c mice after repeated FB_1 treatment. Mean ± SE (n = 5). Different letters on the top of bars denote a statistical significance at $p < 0.05$.

CHAPTER 4

FUMONISIN B_1-INDUCED NEURODEGENERATION IN MICE AFTER INTRACEREBROVENTRICULAR INFUSION IS CONCURRENT WITH DISRUPTION OF SPHINGOLIPID METABOLISM AND ACTIVATION OF PROINFLAMMATORY SIGNALING[2]

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Abstract

Fumonisin B_1 (FB_1), a mycotoxin produced by *Fusarium verticillioides*, causes equine leukoencephalomalacia. This study compared neurotoxicity of FB_1 in mouse after its intracerebroventricular (icv) or subcutaneous (sc) infusion. Female BALB/c mice (5/group) were infused (0.5 μl/hr) with total doses of 0, 10 or 100 μg FB_1 in saline over 7 days via osmotic pumps implanted either via icv cannulation of the brain bypassing the blood-brain barrier or subcutaneously. One day after the treatment, brains were dissected either fresh or after intracardiac paraformaldehyde fixation. In mice given 100 μg of icv FB_1 FluoroJade B revealed neurodegeneration in cortex and anti-glial fibrillary acidic protein staining detected activated astrocytes in hippocampus. High performance liquid chromatography indicated accumulation of free sphinganine after FB_1 given icv in all brain regions and increased free sphingosine after the 100 μg dose in cortex. The concentration of cortical sphingomyelin and complex sphingolipids remained unchanged. Icv administration of FB_1 induced expression of tumor necrosis factor α, interleukin-1β, interleukin-6 and interferon γ after both doses, as assayed by the real-time polymerase chain reaction. The sc administration of 100 μg FB_1 caused slight sphinganine accumulation and increased IL-1β expression in cortex only. Results indicate the icv treatment with FB_1 caused neurodegeneration simultaneous with inhibition of *de novo* ceramide synthesis, stimulation of astrocytes and upregulation of pro-inflammatory cytokines in the murine brain. A lack of fumonisin B_1 permeability into brain could be responsible for the absence of its neurotoxicity in mouse.

Keywords: Fumonisin; Brain, Neurotoxicity; Sphingolipids; Cytokines; Intracerebroventricular infusion

1. Introduction

Fumonisins belong to the relatively recently discovered group of mycotoxins produced by the fungus *Fusarium verticillioides* (formerly *F. moniliforme*), a widespread fungal contaminant of various cereals, predominantly corn (Dutton, 1996). In this class, fumonisin B_1 (FB_1) is proven to be the most abundant and toxic and has been linked to number of diseases in humans and animals. The FB_1 is a causative agent of porcine pulmonary edema (Dutton, 1996) and chronic exposures to this mycotoxin caused cancerous hyperplasia in laboratory animals with high species/gender specificity (Howard *et al.*, 2001). In humans, reported cases of esophageal cancer in Southern Africa and primary liver cancer in China were associated to dietary exposure to FB_1 (Dutton, 1996). Liver is an organ displaying susceptibility to the FB_1 exposure among all studied species (Voss *et al.*, 2001).

The inhibition of ceramide synthase resulting in deregulation of the *de novo* sphingolipid biosynthesis pathway is considered as a primary mechanism of fumonisin toxicity both *in vivo* and *in vitro*. The FB_1-induced accumulation of sphinganine and sphingosine is an established biomarker of exposure to this mycotoxin (Dutton, 1996). Free sphingoid bases and their metabolites are crucial regulatory elements of cellular signaling regarding cell growth, differentiation, survival and death (Merrill, Jr. et al., 2001). Prolonged inhibition of ceramide synthase leads to the depletion of more complex, sphingosine-and sphinganine-based molecules such as sphingomyelin, cerebrosides and gangliosides crucial in maintaining the functional and structural balance of neuronal tissue.

To date, the only recognized neurological disorder caused by exposure to fumonisins occurs in a form of equine leukoencephalomalacia (Brownie and Cullen, 1987) and fumonisins show high specificity for equide. The disease is associated primarily with FB_1 and is characterized by high mortality as noted during ELEM outbreaks in the US and elsewhere (Wilkins et al., 1994; Wilson et al., 1990). The reported FB_1 content in feed associated with natural ELEM cases varied widely from 1 to 126 ppm with the estimated exposure time ranging between 7 and over 35 days before the onset of clinical symptoms (Ross et al., 1991b; Ross et al., 1991a; Ross et al., 1992; Wilson et al., 1990). In histopathological examinations the pathognomonic focal necrotic lesions, located primarily in the subcortical white matter, are apparent (Dutton, 1996). The exact pathophysiology of the disease remains to be elucidated, including the question whether the FB_1-related neurotoxicity displayed in ELEM-affected animals is of primary or secondary nature. The laboratory *in vivo* models could be an useful alternative to study the pathogenesis of fumonisin-dependent equine central nervous system (CNS) disorders (Lee and Lee, 2002). Another, emerging neurodevelopmental aspect of FB_1 toxicity was recently suggested implicating the consumption of fumonisins in the etiology of neuronal tube defects (NTD) in children (Marasas et al., 2004). Treatment with FB_1 caused NTDs in *ex vivo* neurulating mouse embryos (Sadler et al., 2002) and this effect could be related to the folic acid receptor deficiency as a result of the FB_1-dependent lipid rafts depletion (Stevens and Tang, 1997).

The detrimental effects of FB_1 on neuronal tissue have been shown in number of reports indicating its potential for direct neurotoxicity. FB_1 treatment inhibited development of cultured neurons (Harel and Futerman, 1993), altered the sphinganine concentration and oligodentrocyte activity of primary glial/oligodenrocytes (Kwon et al., 2000) in rat and disrupted myelination *in vitro* (Monnet-Tschudi et al., 1999), and *in vivo* (Kwon *et al.*, 1997b). FB_1-dependent changes in neurotransmitter metabolite levels in murine (Tsunoda et al., 1998a) and rat (Porter et al., 1993) brain and alteration of electrophysiological activity in rat neocortex (Banczerowski-Pelyhe *et al.*, 2001) were also reported.

The CNS disorders induced by fumonisins are poorly understood and the existing evidence primarily investigates developmental and *in vitro* aspects of neurotoxicity. The studies in adult laboratory models featuring the direct exposure of the CNS to these mycotoxins are lacking. We hypothesized that the lack of FB_1-dependent neurotoxicity in murine CNS is due to the restricted permeability of this mycotoxin into brain. The present study investigated the CNS homeostasis after the direct sub-acute administration of FB_1 to the brain via the intracerebroventricular infusion bypassing the protective blood-brain barrier (BBB).

2. Materials and methods

2.1 Animals

Female 7-8 week old BALB/c mice were purchased from Harlan Inc. (Indianapolis, IN) and acclimated for one week at 21°C and 50% humidity with a 12-h light/dark cycle prior to treatment. Females were used because they have been shown

to be more sensitive to FB_1 effects than males (Bhandari et al., 2001). Mice were maintained on mycotoxin-free commercial rodent chow (Harlan-Teklad, Madison, WI) and deionized water *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 University of Georgia Institutional Animal Care and Use Committee.

2.2 Surgery and treatments

Animals were treated for 7 days with saline or 10 and 100 μg (total dose per animal) of fumonisin B_1 (98% purity; Promec, Tygerberg, South Africa) via either intracerebral cannulation of the lateral ventricle (ivc) or subcutaneously (sc). The continuous (0.5 μl/h) infusion of fumonisin B_1 was facilitated via Alzet® osmotic pumps (model 1007D; 100 μl nominal reservoir volume; Durect, Copertino, CA) implanted sc (dorsal region). All pumps were primed overnight before implantation according to manufacturer's protocol. During surgery mice were anesthetized with a mixture of ketamine (1.8 mg/kg) and xylazine (12.2 mg/kg) at 10 ml/kg body weight intraperitoneally (ip). For intracerebroventricular application, a 22-gauge stainless steel connector cannula (model 328OPM/SPC; Plastics One®, Roanoke, VA) was inserted stereotaxically over the lateral cerebral ventricle. Coordinates for the guide cannula were 0.4 mm posterior and 1.6 mm lateral to bregma and 2.2 mm below the skull surface at the point of entry according to stereotaxical atlas of Paxinos and Watson (2004). Control dye injection validated the stereotaxic coordinates as appropriate. The cannula was connected to pump via vinyl tubing (Plastics One®, Roanoke, VA). For

subcutaneous application pumps were dorsally inserted through a small incision into the scapular region. Mice were weighed and observed daily.

2.3 Sampling

Mice were euthanized 1 day after the last day of treatment and brains were dissected either fresh for sphingolipid and gene expression assays or after transcardiac fixation with 4% paraformaldehyde for histochemical studies. Fresh brains were immediately dissected on ice into cortex, cerebellum, midbrain, medulla oblongata. Hypothalamus and thalamic regions were included in the midbrain section, whereas the hippocampus was dissected together with cortex. Fresh tissue samples were quickly frozen and stored at -85°C and fixed brains were cut into 20 μm sections, collected on slides and frozen at -20°C until analysis.

2.4 FluoroJade B labeling

The neuronal degeneration was detected by the histofluorescent labeling with FluoroJade B following the previously described method (Schmued and Hopkins, 2000). Briefly, slide-mounted 20 μm thick sections were immersed in a solution of basic alcohol (1% potassium hydroxide in 80% alcohol) for 5 min, followed by incubation in 70% alcohol (2-min) and a rinse in distilled water (2-min). Sections were then incubated in potassium permanganate (0.06%; 10 min) to reduce background staining. Next, slides were rinsed briefly in distilled water and stained for 20 min in a 0.0004% solution of Fluoro-Jade B (Histo-Chem, Jefferson, AR) dissolved in a vehicle of 0.1% acetic acid. Slides were then rinsed in distilled water, air dried, cleared in Citrosolv™ (Fisher Scientific, Pittsburgh, PA) and coverslipped with DPX non-

fluorescent mounting media (Fluka/Sigma). Slides were visualized using a filter cube designed for fluorescein (FITC).

2.5 Immunohistochemistry and histofluoresccence

The primary antibody directed against murine glial fibrillary acidic protein (GFAP) conjugated to Cy3 dye was obtained from Sigma-Aldrich (St. Louis, MO). Slide-mounted 20 μm thick sections were incubated first in 0.1 M neutral phosphate buffered saline (PBS) containing 0.3% Triton-X detergent and 2% normal goat serum (vehicle) for 30 min and then in the primary antibody solution (diluted 1:250 in vehicle) overnight at 4 °C. After the incubation sections were rinsed with two 5 min changes in distilled water, air dried and were stained with 0.01% 4, 6-diamidino-2-phenylindole (DAPI, Sigma) alone (to visualized nuclei of viable cells) or combined with FluoroJade B (as described earlier) for 20 minutes (Schmued and Hopkins, 2000). Next, slides were cleared in Citrosolv™ and coverslipped with DPX mounting media and visualized by using a filter cube designed for the tetramethylrhodamine isothiocyanate (for GFAP) and/or ultraviolet illumination (for DAPI).

2.6 Sphingolipid analyses

Sphinganine and sphingosine levels were determined by high performance liquid chromatography using extraction methods by (Riley et al., 1999) and equipment identical to described previously (He et al., 2001). The free sphingoid bases, in fresh base-treated brain extracts, were analyzed in cortex, cerebellum, midbrain and medulla oblongata, whereas the fresh acid-treated cortex was assayed for complex sphinganine

and sphingosine concentration. The concentration of sphingoid bases was quantitated based on the recovery of the C_{20}-sphinganine internal standard.

2.7 Sphingomyelin assay

The relative sphingomyelin concentration difference in cortex between control and treated animals was analyzed based on the generation of resorufin from the reaction of Amplex Red (AR) with hydrogen peroxide as recently described (He et al., 2002). Briefly, cortical tissue was weighed and homogenized in 0.25% Triton X-100 and centrifuged (10,000*g* for 5 min). The lipid extraction followed, utilizing the detergent/heating method: 20 µl of the cortex homogenate was mixed with equal volumes of 0.25% Triton X-100 and then heated at 70°C for 5 min, cooled to room temperature, and briefly centrifuged. Next, 5 µl of the supernantant was added to individual wells of a 96-well microtiter plate containing an enzyme mixture that consisted 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 AR in 100 µl of reaction buffer (50 mM Tris–HCl, 5 mM $MgCl_2$, pH 7.4; Molecular Probes, Eugene. OR). After 20 min incubation at 37°C the plate was read using a fluorescence microplate reader (Molecular Devices Corp., Sunnyvale, CA) at 560 (excitation) and 587 (emission) nm. Relative sphingomyelin amounts were calculated from the difference in fluorescence between the test and the control samples.

2.8 Quantitative real-time polymerase chain reaction

The total RNA was isolated from the homogenized brain cortex, quantified spectrophotometrically at 260/280 nm and initial cDNA strand prepared using the

SuperScript™ II transcriptase system with oligo $(dT)_{12\text{-}18}$ primer (Invitrogen, Carlsbad, CA) as previously described in detail (Sharma et al., 2000). Quantitative determination of gene expression levels was performed on the ABI PRISM® 7900HT sequence detection system using the TaqMan® Universal Master Mix (Applied Biosystems, Foster City, CA). TaqMan® MGB probes (5'-FAM™ dye-labeled) combined with Assay-on-Demand™ (Applied Biosystems) primers (sequences are proprietary–manufacturer's catalog number listed) for following murine genes were used: β-glucoronidase-Mm00446953_m1, tumor necrosis α-Mm00443258_m1, interferon γ-3339850T, interleukin (IL)-1β-Mm00446190_m1 and IL-6-Mm00434228_m1. All primers are designed to bind to separate exons or exon/intron boundaries to avoid the amplification of genomic DNA. After the amplification efficiency of each target and reference was validated the relative gene expression levels were determined using the C_T method described previously by (Livak and Schmittgen, 2001) following the manufacturer's protocol (Applied Biosystems; user bulletin#2-P/N 4303859). β-glucoronidase was used as a reference gene in a singleplex reaction.

2.9 Statistics and data presentation

All data are expressed as mean ± standard error (SE) except where noted otherwise. Data were analyzed by two-way analysis of variance (ANOVA) followed by the Duncan's multiple comparison test. All tests were performed using SAS software (SAS Institute, Cary, NC). The level of $p < 0.05$ was considered significant.

3. Results

3.1 Gross observations

Intracerebroventricular exposure to FB_1 resulted in reduced weight gain only in mice treated with the higher icv (100 μg) infusion of FB_1 (Table 1). No effect on body weight was noticed after sc administration of FB_1. Brain weight remained unaffected in all treatment groups (data not shown).

Condition of animals infused via the icv route with 100 μg of FB_1 deteriorated progressively during the treatment. Mice developed locomotoric problems such as shaky gait and tremors and spasticity of extremities occurred toward the end of the treatment period. Mice treated via sc (both doses) and with the lower icv dose (10 μg) of FB_1 did not display apparent neurotoxic symptoms.

3.2 Neurodegeneration

Mice treated with the 100 μg FB_1 via icv displayed apparent neurodegenerative changes as assessed by the FluoroJade B fluorescence (Fig. 1). The damage was confined to the cortical region (frontal and temporal lobe) and both nonpyramidal and pyramidal neurons were affected. The FluoroJade B-positive neurons were located in superficial supragranular layers (Fig. 1b) as well as in deeper, internal pyramidal/granular layers (Fig. 1c). Both perikarya and fibers were distinctly visualized (Fig. 1 d) suggesting rather advanced stages of neurodegeneration. None of the other groups displayed such alterations.

3.3 Astrocyte activation

In the hippocampus of mice treated with 100 μg of icv FB_1 the FluoroJade B labeled cells morphology was characteristic of astrocytes (Fig. 2b). This observation was consistent with recent evidence showing, that activated astrocytes are stained by FluoroJade B after acute injury in primate and in Alzheimer's diagnosed human brain (Colombo and Puissant, 2002). The staining of astrocyte-like cells was much weaker compared to the FluoroJade B-positive cortical neurons and labeled cells were present throughout the molecular and granular layers of the entire hippocampus (including the dentate gyrus and subiculum) but were not apparent in other brain regions. To validate the identity of hippocampal FluoroJade B-positive cells the specific anti-GFAP staining followed (coupled with DAPI or FluoroJade B/DAPI). Large numbers of high-intensity GFAP-positive astrocytes were visualized in hippocampus (Fig. 2d) mimicking the distribution of cells stained by FluoroJade B but no concurrent neurodegeneration was present. Similar to the FluoroJade B staining, the activated astrocytes were predominantly located within the hippocampus and not in other brain regions. We did not detect activated GFAP-positive astrocytes adjacent to FluoroJade B-positive degenerating cortical neurons during double-label staining, which is likely due to the oxidation of tissue after $KMnO_4$ pretreatment that weakens the GFAP staining as shown previously (Schmued and Hopkins, 2000). When the single labeling was employed it revealed GFAP-positive cells in cortical layers where degeneration was observed as well as in unaffected regions (data not shown) but only in hippocampus was the difference apparent.

3.4 Concentration of free sphingoid bases

The animals treated icv with 10 μg of FB_1 had an increased sphinganine concentration in all regions except medulla oblongata (Fig.3). The 100 μg dose further elevated sphinganine levels in all brain regions, especially in cerebellum where the potent accumulation of sphinganine was nearly 20-fold higher compared to the control group. The sphingosine concentration was elevated after the high dose icv (100 μg) treatment in cortex and showed an increasing trend for medulla oblongata, but it was statistically not significant.

The mice given FB_1 via the sc route had a significantly elevated level of sphinganine in cortex after the higher (100 μg) FB_1 dose (Fig.4), whereas the level of sphingosine remained unaltered in all regions (data not shown).

3.5 Concentration of complex sphingolipids

The concentration of sphinganine and sphingosine in complex sphingolipids measured after acid hydrolysis of cortex tissue extracts from mice treated with FB_1 via icv infusion remained unaltered after either dose (Fig. 5). Similarly, the analysis of cortical sphingomyelin concentration did not reveal any changes in any of the dose groups (Fig. 5).

3.6 The relative cytokine gene expression

Expression of selected inflammatory cytokines in cortex increased markedly after animals were treated with 10 μg of FB_1 via icv infusion (Fig.6). The 100 μg dose further induced proinflammatory gene expression for TNFα, IL-1β and IL-6 but not for

IFNγ. The increase of IL-1β expression after the high FB_1 dose was especially potent increasing nearly 30-fold over control.

In mice given FB_1 via sc route a marginal elevation of IL-1β expression in cortex after both doses was observed (Fig. 7). Expression of cortical TNFα, IL-6 and IFNγ in mice treated via the sc route remained unchanged after both FB_1 doses.

4. Discussion

The present results support the hypothesis that FB_1 disrupts CNS homeostasis when brain tissue is directly exposed to this toxin. The icv infusion of FB_1 led to the neuronal degeneration in cortex concurrent with disruption of sphingolipid metabolism, as indicated by regional accumulation of free sphingoid bases, and activation of proinflammatory signaling.

The species specificity of equines for FB_1-dependent ELEM is not clear. Anecdotal reports described cases of FB_1-related ELEM-like signs in rabbit (Bucci et al., 1996), white-tailed deer (Howerth et al., 1989) and turkeys (Ficken et al., 1993). However, the exposure to FB_1 only in equines triggers ELEM pathology. Equines are highly prone to gastrointestinal (GI) disorders coupled with frequent colic/gram negative bacteria-related endotoxoemias (Morris, 1991) that may impair BBB integrity (Burroughs et al., 1992). *F. verticillioides*-infested feed induced GI disorders in equines (Goel et al., 1996). Also, FB_1 compromised the endothelial barrier function (Bouhet et al., 2003; Ramasamy et al., 1995) and there is possibly a limited BBB transfer of FB_1 (Chapter 3). From a safety and therapeutic standpoint, it is important to

establish whether the primary and/or secondary mechanisms of FB_1-induced toxicity lead to the development of equine neurotoxicity.

It has been shown that FB_1 interrupted axonal growth in cultured rat hippocampal neurons *in vitro* (Harel and Futerman, 1993) and disrupted myelination in rat brain aggregate culture (Monnet-Tschudi et al., 1999) without a concurrent change in neuronal viability. We noted the distinct neurodegeneration in cortex of mice treated with the higher dose of FB_1 via icv infusion. Such a discrepancy is not surprising since the *in vitro* models only partially emulate the structural characteristics of brain cellularity that may contribute to susceptibility to toxic insults. The location of degenerating neurons varied between mice from the supragranular to the internal cortical layers. Within the area of cortical neurodegeneration the activation of adjacent astrocytes was no different from control. In contrast, in hippocampus the intact neurons were in the vicinity of numerous highly active astrocytes.

Free sphingoid bases may be important in FB_1-related neurotoxicity since their elevation after FB_1 treatment was demonstrated in brain of ELEM-diagnosed horses (Goel et al., 1996). Furthermore, in numerous studies it paralleled FB_1-induced hepato-and nephrotoxicity in rodents (Sharma *et al.*, 2000; Voss *et al.*, 2001; Bhandari *et al.*, 2002). This relationship however, is controversial first due to a general lack of free sphingoid base analyses in brain in experiments performed on equines, and secondly due to the absence of a suitable experimental animal model validating such association.

In mice icv infusion of FB_1 caused an accumulation of sphinganine in all brain regions analyzed indicating that the *de novo* ceramide biosynthesis was inhibited and this effect was especially potent in cerebellum. The highest accumulation of sphinganine in cerebellum could be explained by the structural specificity of this region featuring large surface contact area with CSF leading to larger FB_1 penetration into tissue; thereby greater inhibition of cerebellar sphingolipid synthesis. In cortex of mice treated with FB_1 via sc route sphinganine accumulation was observed. Partial inhibition of sphingolipid metabolism after systemic FB_1 treatment observed in our current and recent experiments (Chapter 3), suggests that there may be a limited transfer of FB_1 across the BBB.

Although the sphinganine accumulation was dose-dependent and correlated with FB_1-induced cell death in cultured cells (Kim et al., 2001; Yu et al., 2001), in our study its potent accumulation in cerebellum was not concurrent with pathological damage. In cortex, where abundant degenerating neurons were detected sphinganine accumulation was much lower compared to cerebellum and did not exceed levels detected in midbrain and medulla oblongata. The sphingosine concentration, however, increased by 2-fold in mice treated icv with 100 ug of FB_1 and this accumulation was specific only for cortex.

Sphingosine is an intermediate in the sphingolipid biosynthetic pathway between ceramide, a promoter of neuronal death (Buccoliero and Futerman, 2003) and sphingosine-1-phosphate (S1P), an enhancer of cell survival (Merrill, Jr. et al., 2001). Sphingosine itself is a bioactive molecule inducing cell growth inhibition and apoptosis

(Maceyka et al., 2002). Ceramide, sphingosine and S1P maintain a dynamic life/death equilibrium and dysregulation of such a fine balance may shift eukaryotic systems in either direction. Because FB_1 inhibits ceramide synthesis both from dihydroceramide (*de novo* pathway) and sphingosine, the only alternative for ceramide production is sphingomyelinase (nSMase)-dependent compensatory depletion of sphingomyelin. The observed accumulation of sphingosine could be incurred via the sphingomyelin/ceramide pathway. The icv infusion of 10 and 100 μg FB_1 did not alter the concentration of cortical sphingomyelin or complex sphingosine and sphinganine. The duration of our treatment may be insufficient to induce a detectable depletion of structural sphingolipids *in vivo*. FB_1 at high doses not only failed to reduce complex sphingolipids, but paradoxically increased concentration of complex sphinganine in liver and kidney of BALB/c mice after acute FB_1 treatments 2.0-2.5 fold (Sharma et al., 2000; Tsunoda et al., 1998). The observed lack of elevation in cortical complex sphinganine after icv treatment compared to its previously reported increase in other organs may suggest that the disruption of structural sphingolipids in brain was in fact initiated. The correlation between neurodegeneration and the elevation of sphingosine in cortex suggests that sphingosine rather then sphinganine could be associated with the observed neurotoxicity.

All cytokines analyzed in our study are CNS-borne and are expressed on-site by neurons (IL-1β, IL-6), astrocytes (IL-1β, IL-6, TNFα, IFNγ) and microglia (IL-1β, IL-6, TNFα) (Szelenyi, 2001). The IL-1β, IL-6, and TNFα are primarily associated with neuronal injury, thus the neuronal damage will be accompanied by their elevated

expression. We previously demonstrated that in target organs such as liver and kidney FB_1-induced toxicity depends on activation of proinflammatory-cytokines (Bhandari et al., 2002; Sharma et al., 2000). There are no reports investigating the cytokine changes following the FB_1 exposure in brain. We observed that the expression of all assayed cytokines in cortex increased, occurring concurrent with the neuronal damage. The increased expression of TNFα and IL-1β could also indirectly enhance the neuronal damage via ceramide-mediated signaling since both cytokines activate the brain nMSase and the role of ceramide-dependent neurodegeneration mediated by nSMase is reported (Buccoliero and Futerman, 2003; Pettus et al., 2002). The relatively high upregulation of IL-1β expression suggests that in murine brain IL-1β may be especially responsive to FB_1 either directly or more likely via secondary TNFα induction. This observation agrees with studies describing the pivotal role of IL-1β in the development and progression of neuroinflammation associated with neurodegeneration (Oprica et al., 2003). Although not analyzed, microglial cells likely contribute to FB_1-dependent upregulation of inflammatory signaling in cortex.

The present study demonstrates that homeostasis in the adult murine brain is vulnerable to the direct exposure of FB_1 when the protective BBB barrier is bypassed. The potential for CNS damage after intracerebroventricular infusion of FB_1 suggests that the mycotoxin could partly contribute to FB_1-related neurological diseases via primary mechanisms. Further studies are needed to gain more insight into the role played by fumonisins during the development of brain disorders observed in equide and in scenarios of increased BBB permeability.

Acknowledgements

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TABLE 4.1.

Body weight change between day 1 and day 8 of the treatment with FB_1.

Body weight (g)	Saline	FB_1, 10 μg	FB_1, 100 μg
After ICV	$+0.94 \pm 0.3$[a]	$+0.34 \pm 0.4$	-2.51 ± 0.6[*]
After SC	$+1.3 \pm 0.3$	$+0.75 \pm 0.5$	-0.64 ± 0.2

[a] Data presented as mean ± SE ($n = 5$). [*] $P < 0.05$ compared to the saline treated group.

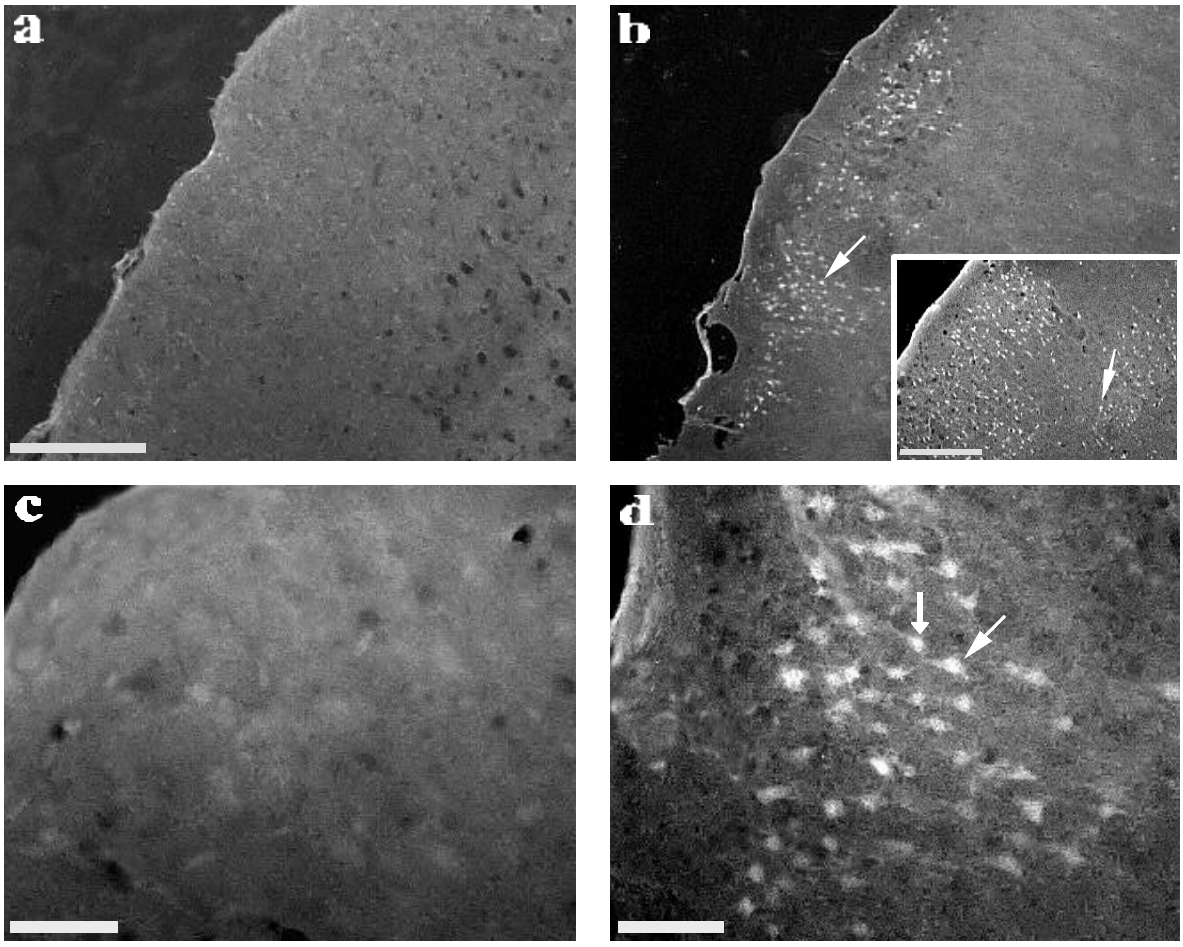

Fig. 4.1. FluoroJade B staining in the mouse cortex after the icv infusion of saline (a and c) and 100 μg of FB_1 (b and d). FluoroJade B labeling revealing degenerated neurons in supragranular (b) and internal (b-inset) layers of the temporal cortex. Bar = 100 μm. (d) Same region as in b-higher magnification. FluoroJade B revealing degenerating pyramidal (arrow) and granular cells (block arrow) with visible terminals in supragranular layers. Bar = 10 μm.

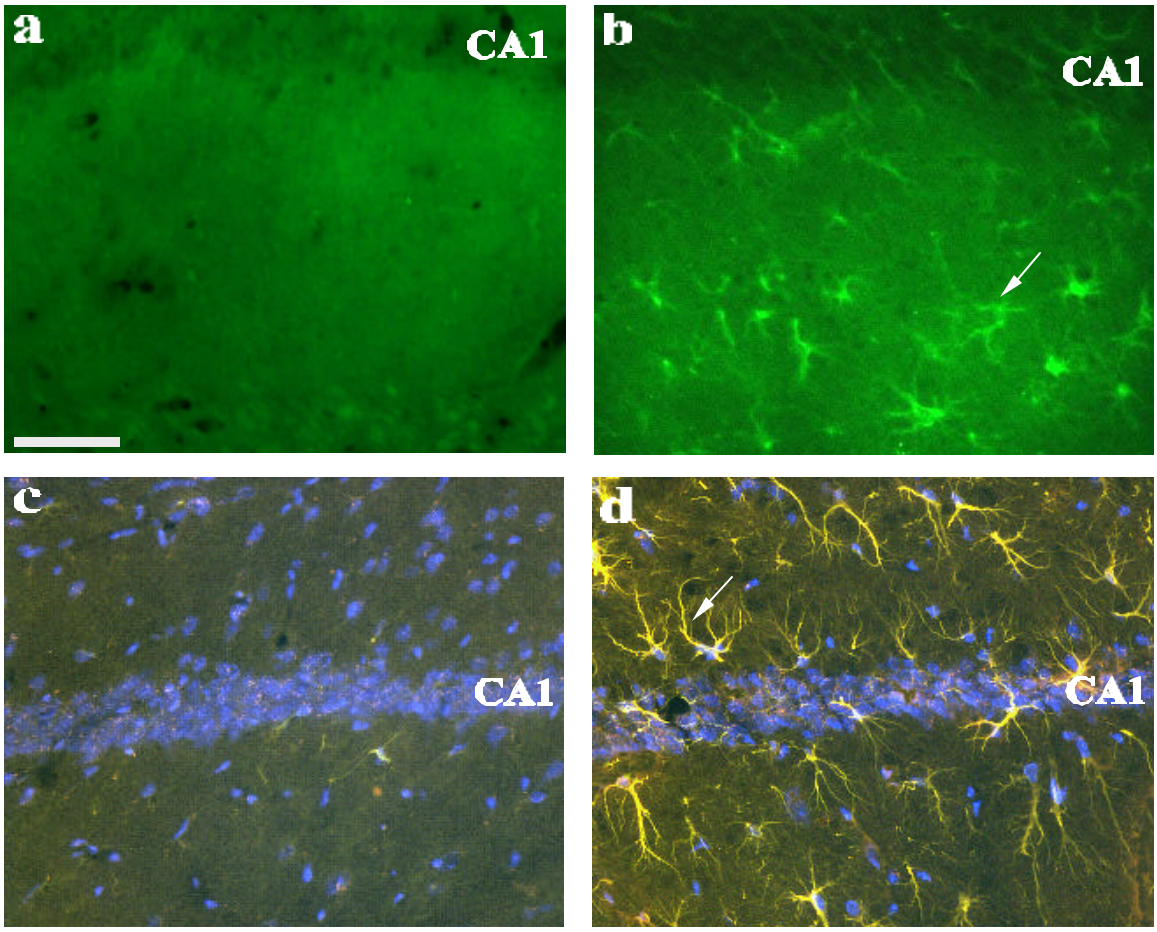

Fig. 4.2. Combination of stainings in mouse hippocampus following the exposure to saline (a and c) and 100 μg of FB_1 (b and d) administered via icv infusion. FluoroJade B labeling revealing activated astrocyte-like cells in CA1 region of hippocampus (b). FluoroJade B/GFAP/DAPI revealing activated astrocytes (arrow) throughout the striatum ariens, pyramidale and radiatum of the hippocampal CA1 region (d). Merged pictures from ultraviolet and fluorescein filters. Yellow GFAP positive astrocytes and blue DAPI stained nuclei of viable cells (CA 1 pyramidal layer). Bar = 10 μm.

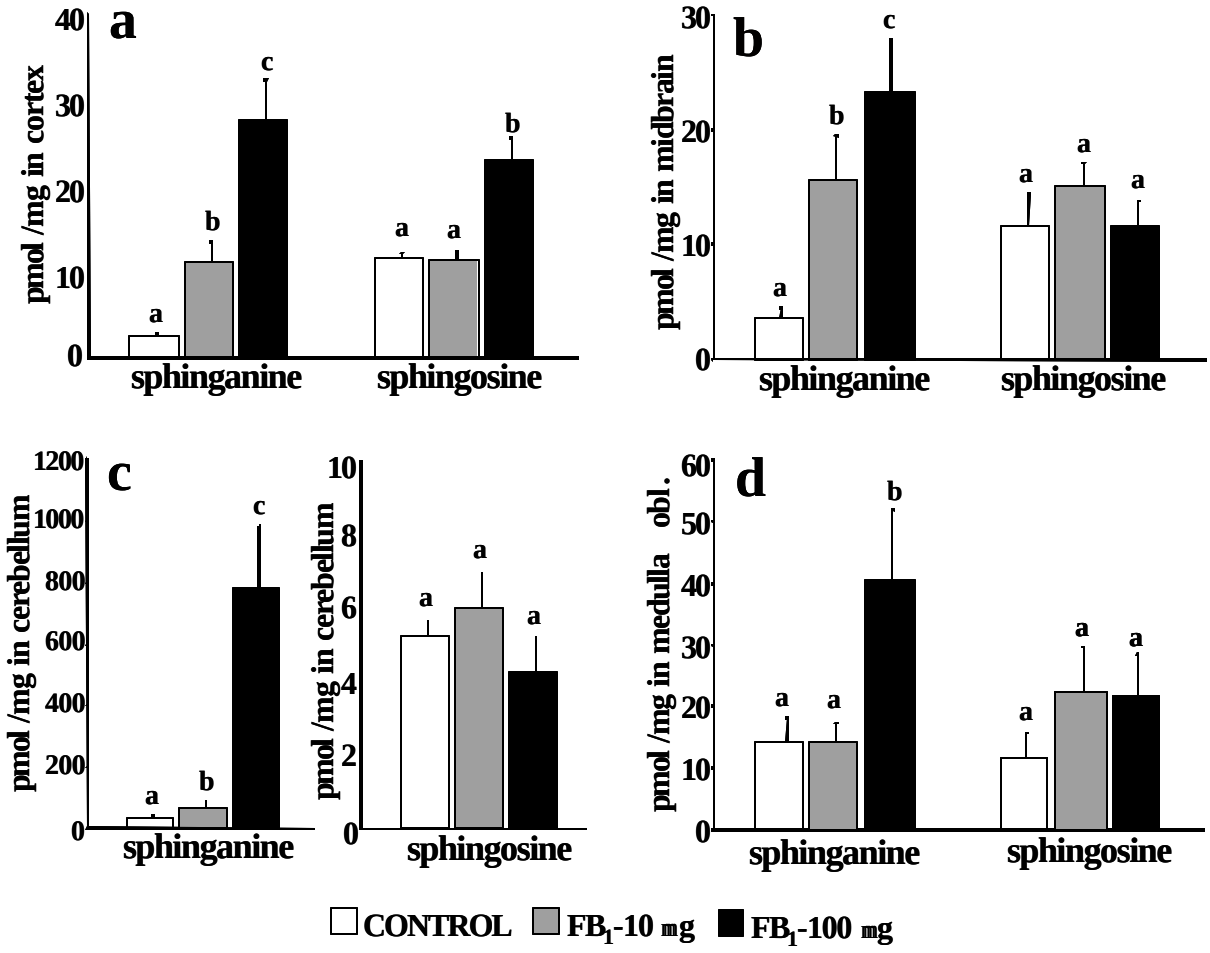

Fig. 4.3. Concentration of sphinganine and sphingosine in (a) cortex, (b) midbrain, (c), cerebellum, and (d) medulla oblongata of BALB/c mice after icv infusion of FB_1. Mean ± SE (n = 5). Different letters on the top of bars denote a statistical significance at $p < 0.05$.

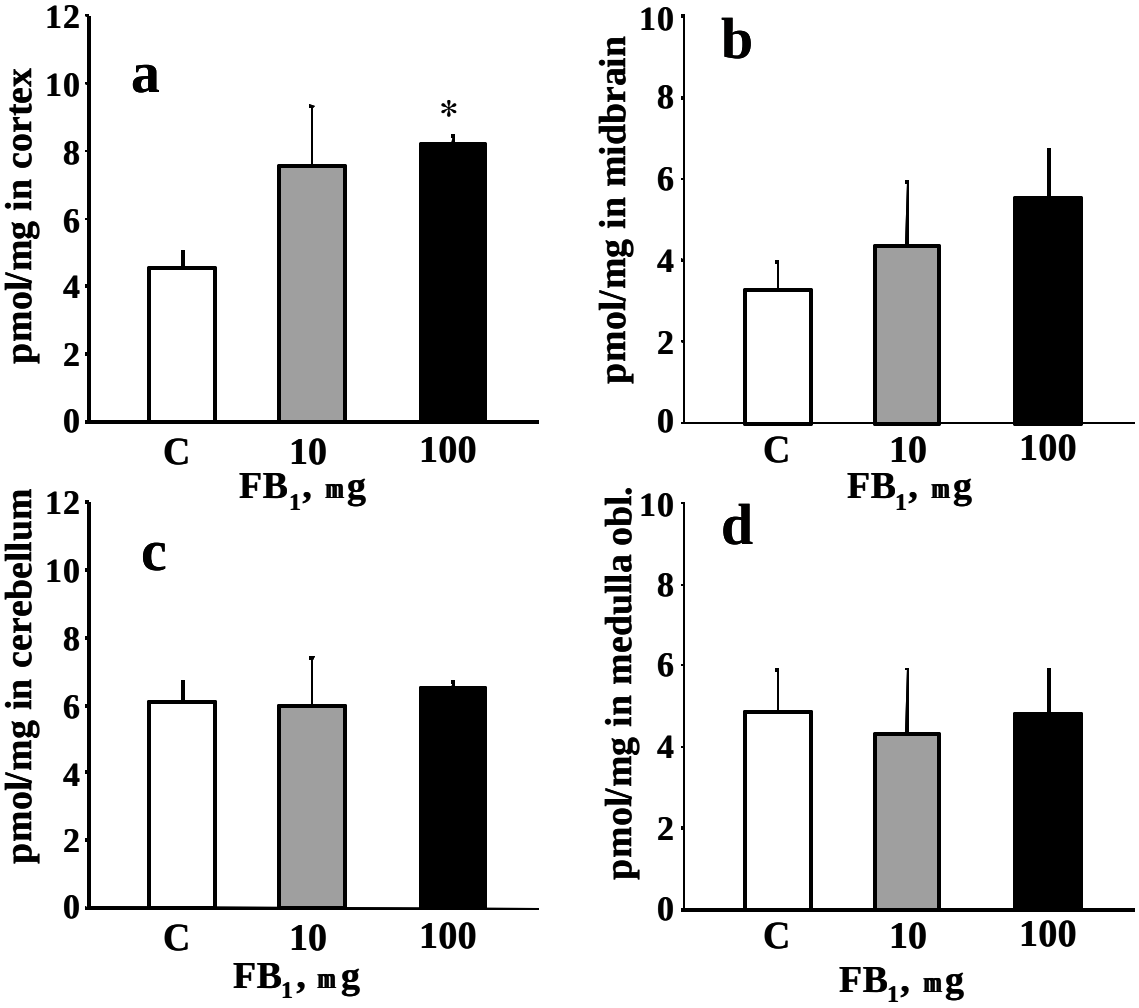

Fig. 4.4. Concentration of sphinganine in (a) cortex and (b) midbrain (c) cerebellum and (d) medulla oblongata of BALB/c mice after sc treatment with FB_1. Mean ± SE (n = 5). * $p < 0.05$.

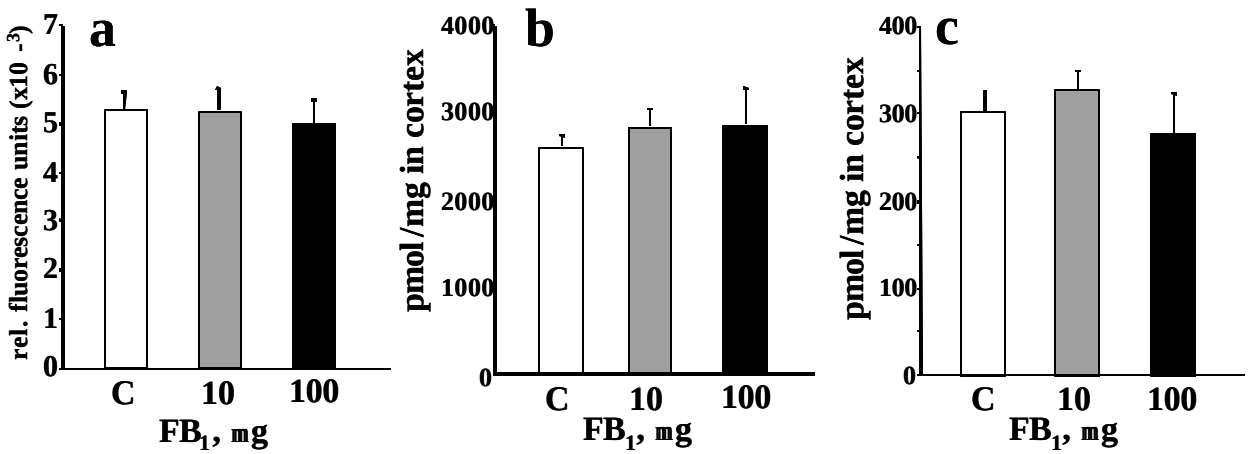

Fig. 4.5. Concentration of sphingomyelin (a), complex sphingosine (b) and sphinganine (c) in cortex of BALB/c mice after icv treatment with FB_1. Mean ± SE (n = 5). No significant differences.

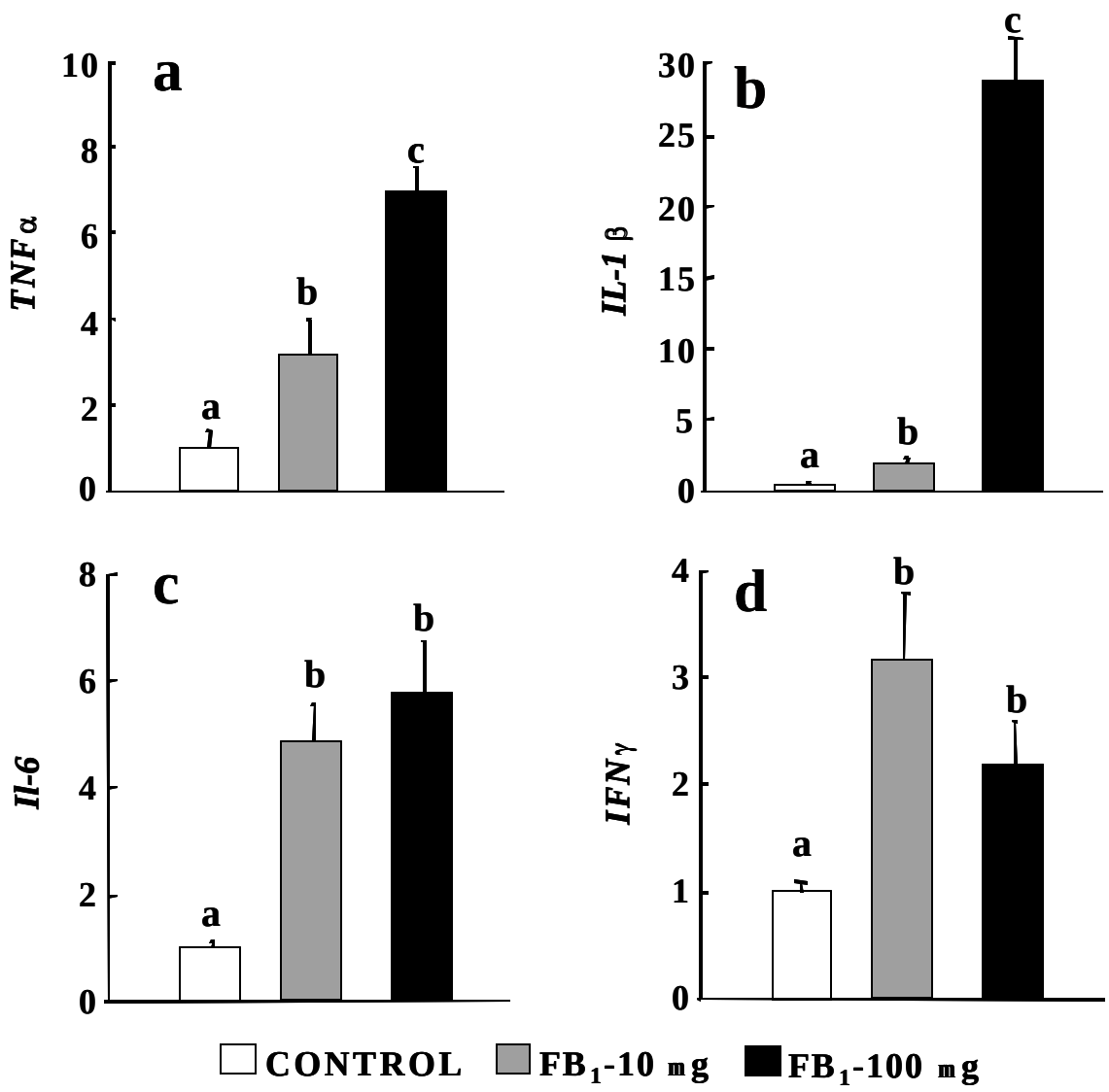

Fig. 4.6. Relative expression levels of TNFα (a), IL-1β (b), IL-6 (c) and IFNγ (d) in the cortex of BALB/c mice after icv treatment with FB_1, determined by real-time PCR. Presented as fold-change compared to control. Mean ± SD (n = 5). Different letters on top of bars denote a statistical significance at $p < 0.05$.

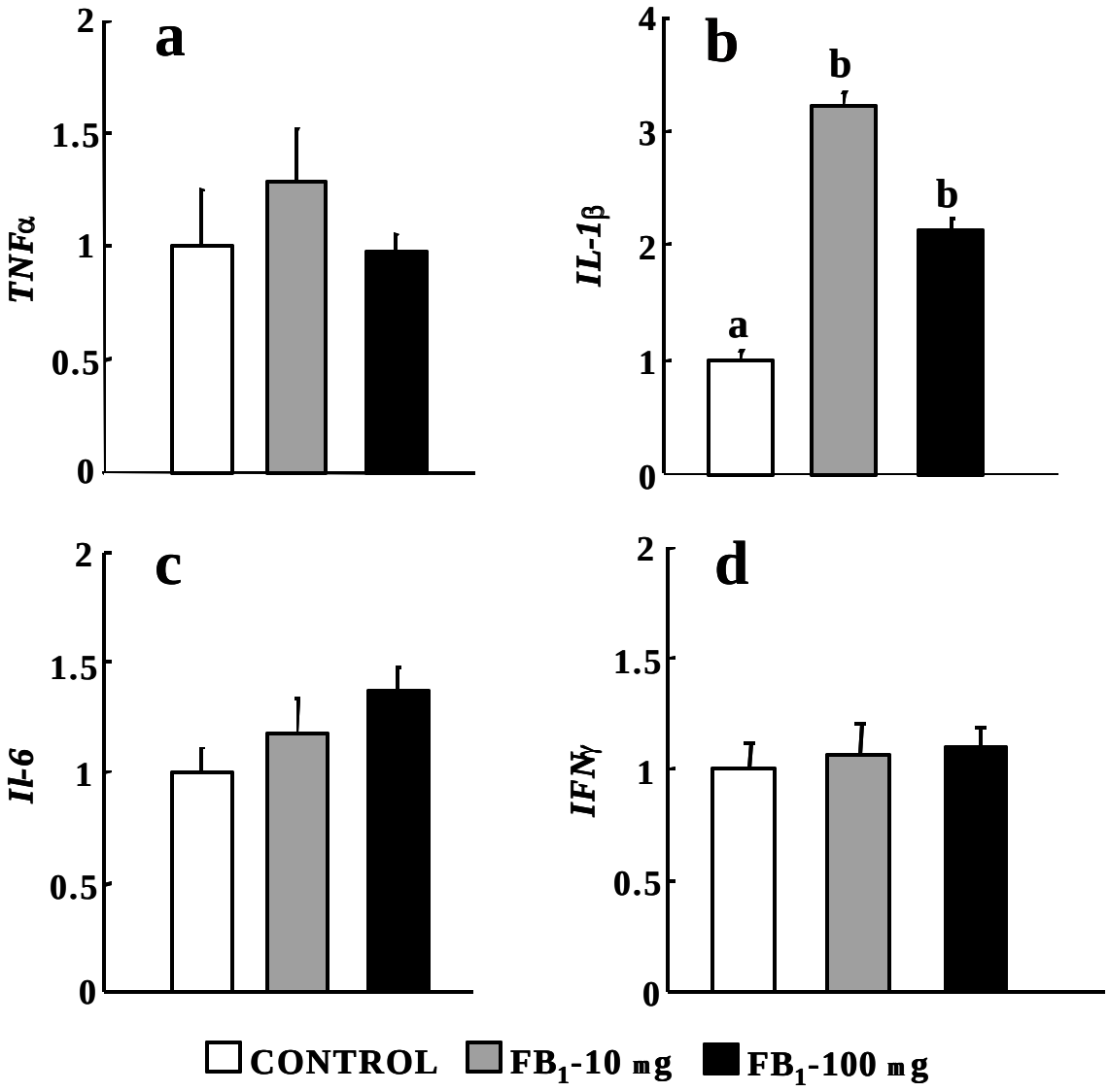

Fig. 4.7. Relative expression levels of TNFα (a), IL-1β (b), IL-6 (c) and IFNγ (d) in the cortex of BALB/c mice after sc treatment with FB_1, determined by real-time PCR. Presented as fold-change compared to control. Mean ± SD (n = 5). Different letters on top of bars denote a statistical significance at $p < 0.05$.

CHAPTER 5

THE EFFECTS OF SUBCUTANEOUS AND INTRACEREBROVENTRICULAR APPLICATION OF FUMONISIN B_1 UPON LIVER HOMEOSTASIS IN BALB/C MICE[3]

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[3] Osuchowski, Marcin F., Edwards, Gaylen L., Sharma, Raghubir P. To be submitted to Toxicology Letters

Abstract

Fumonisin B_1 (FB_1), a ceramide biosynthesis inhibitor, is a mycotoxin produced by *Fusarium verticillioides*. Free sphingoid bases and proinflammatory cytokines play a key role in FB_1-induced hepatotoxicity. In this experiment, a continuation of the study investigating the FB_1-induced neurotoxicity, liver homeostasis was assessed after continuous subcutaneous (sc) FB_1 application and compared to the intracerebroventricular (icv) treatment. Female BALB/c mice (5/group) were infused (0.5 μl/hr) with 10 or 100 μg FB_1 or saline over 7 days via osmotic pumps implanted either sc or via icv cannulation. The high 100 μg (5 mg/kg) dose constitutes ~ 50% of the total FB_1 bolus doses we previously employed *in vivo*. As indicated by a similar increase of free sphinganine and sphingosine, *de novo* ceramide biosynthesis was inhibited equally by both routes in liver. After 100 μg FB_1 icv the elevation of circulating liver enzymes, a hepatoxicity biomarker, surpassed the increase after sc treatment. Real-time quantitative PCR showed that the expression of tumor necrosis factor α and interleukin (IL)-1β was much higher and the IL-6 expression was similar after the sc compared to icv treatment. The 100 μg FB_1 dose given continuously via sc infusion effectively induced liver cytokine signaling and the disruption of sphingolipid biosynthesis, followed by a relatively low hepatoxicity. It is suggestive that the continuous FB_1 treatment may be relevant to study mechanisms of FB_1-induced hepatotoxicity.

Keywords: fumonisin, sphingolipids, cytokines, liver, osmotic pump, subcutaneous

1. Introduction

Fumonisins are mycotoxins synthesized by a number of *Fusarium* species, predominantly *F. verticillioides* (formerly *F. moniliforme*), which commonly contaminates various cereals, mainly corn (Dutton, 1996). Out of at least 15 members of its class, fumonisin B_1 (FB_1) has been established as the most prevalent and toxic. FB_1 has been detected in corn-based products worldwide (mg/kg levels) (Dutton, 1996), is relatively resistant to food processing (Voss et al., 2003), and its derivatives (e.g. AP_1) are also toxic (Howard et al., 2002). The exposure to this toxicant *per os* is not uncommon either in agriculture animals or in human subpopulations, which rely on maize as a primary source of their diet (Dutton, 1996). Consumption of fumonisin-contaminated foods was concurrent with gastrointestinal disturbances in humans, horses and poultry (Dutton, 1996). The FB_1 is causative agent in diseases such as equine leukoencephalomalacia and porcine pulmonary edema. In countries with high dietary exposure to fumonisins (South Africa, China) occurrences of human malignancies (esophageal and liver cancers) have been associated with this toxin (Dutton, 1996). Recently, based on the epidemiological and laboratory evidence, the consumption of fumonisin has been considered as a risk factor for neural tube and related birth defects in humans (Marasas et al., 2004)

Due to the structural similarity to free sphingoid bases sphinganine and sphingosine, FB_1 potently inhibits ceramide synthase, an enzyme involved in the ceramide biosynthesis pathway. The consequent accumulation of sphinganine, sphingosine and their metabolites in tissues is an established biomarker of FB_1 exposure.

Since ceramide, free sphingoid bases and their derivatives modulate cell growth, survival and apoptosis (Merrill, Jr. et al., 2001) the FB_1-dependent disruption of sphingolipid metabolism deregulates the life/death equilibrium in the cell. The depletion of complex sphingolipids (e.g. sphingomyelin, cerebrosides) after the FB_1 treatment reported both *in vitro* and *in vivo* is another effect of FB_1 toxicity (Dutton, 1996).

Liver is a affected target organ among all species diagnosed with different FB_1-related disorders (Voss et al., 2001). Chronic FB_1 feeding trials induced hepatic and renal tumors in laboratory animals with gender/species specificity (Howard et al., 2001). It was proposed that the non-genotoxic mode of cancer incidences after exposure to FB_1 is due to the enhanced tissue regeneration triggered by the FB_1-induced apoptotic/nectrotic cell death (Dragan et al., 2001). In rodents, treatment with FB_1 caused acute and chronic hepatotoxicity including hepato-and cholangiofibrosis, cirrhosis, apoptotic/oncotic necrosis and proliferation of hepatocytes (Dutton, 1996). In short and long-term studies the correlation between tissue accumulation of free sphingoid bases and FB_1-induced pathologies was shown in liver and other organs (Voss et al., 2001). The key role of cytokines such as tumor necrosis factor α (TNFα) and proinflammatory signaling cascades in FB_1-related hepatotoxicity in mice has been widely established in previous studies (Sharma et al., 2000; Bhandari et al., 2002a).

Causative role of fumonisin B_1 in established and emerging health hazards threatening humans and livestock has increased the interest in investigations of known and suspected FB_1-induced disorders. Rodents are the frequent choice for toxicological

assessments *in vivo*. Since the mechanisms leading to the FB_1-related toxicity in liver and other organs are only partially understood, effective *in vivo* models are vital in future experiments investigating the pathophysiology of diseases caused by this toxin. In a previous experiment we investigated the effects of FB_1 administered either via intracerebroventricular (icv) or subcutaneous (sc) routes on the central nervous system (CNS). In the present study the continuous subcutaneous infusion of FB_1 via osmotic pump implants was used to assess the disruption of liver homeostasis and compare effects of sc infusion with effects of infusion via the intracerebroventricular route and to previously reported effects of repeated bolus doses.

2. Materials and methods

2.1 Animals

Female 7-8 week old BALB/c mice were purchased from Harlan Inc. (Indianapolis, IN) and acclimated for one week prior to treatment at 21°C and 50% humidity with a 12-h light/dark cycle at the University of Georgia Animal Resources facility. Females were used because they have been shown to be more sensitive to FB_1 effects then males (Bhandari et al., 2001). Mice were maintained on mycotoxin-free commercial rodent chow (Harlan-Teklad, Madison, WI) and deionized water *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 University of Georgia Institutional Animal Care and Use Committee.

2.2 Treatments and Sampling

The current experiment extends a previous study (Chapter 4), which investigated the FB_1-dependent neurotoxicity. Animals were treated for 7 days with saline,10 or 100 µg (total dose per animal) of fumonisin B_1 (98% purity; Promec, Tygerberg, South Africa) via either an icv or sc route. The continuous (0.5 µl/h) infusion of fumonisin B_1 was facilitated via Alzet® osmotic pumps (model 1007D Durect, Copertino, CA; 100 µl nominal reservoir volume) implanted sc. Anesthesia and the surgical procedures including intracerebroventricular implantation of connector cannula were detailed previously (Chapter 4). All pumps were primed overnight before implantation according to manufacturer's protocol. The connector cannula was connected to the pump via vinyl tubing (Plastics One®, Roanoke, VA). For subcutaneous application pumps were inserted through a small incision into the scapular region. Mice were weighed and observed for behavioral effects daily.

One day after the last day of treatment mice were euthanized and blood was collected into heparinized tubes and plasma was subsequently isolated for analysis of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), using an Hitachi 912 Automatic Analyzer (Roche Diagnostics, Indianapolis, IN). Fresh livers were dissected immediately on ice for sphingolipid and gene expression assays. Fresh tissue samples were quickly frozen and stored at -85°C until analysis.

2.3 Sphingolipid analyses

Free sphinganine and sphingosine from fresh tissue were determined by high performance liquid chromatography in fresh base-treated liver extracts using methods

(Riley et al., 1999) and equipment identical to the described previously (He et al., 2001). The concentration of sphingoid bases was quantitated based on the recovery of the C_{20}-sphinganine internal standard.

2.4 Quantitative real-time polymerase chain reaction

The total RNA was isolated from the liver homogenates, quantified spectrophotometrically at 260/280 nm and initial cDNA strand prepared using the SuperScript™ II transcriptase system with oligo $(dT)_{12-18}$ primer (Invitrogen, Carlsbad, CA) as previously described in detail (Sharma et al., 2000). Quantitative determination of gene expression levels was performed on the ABI PRISM® 7900HT sequence detection system using the TaqMan® Universal Master Mix (Applied Biosystems, Foster City, CA). TaqMan® MGB probes and primers were listed elsewhere (Chapter 4). After the amplification efficiency of each target and reference was validated the relative gene expression levels were determined using the C_T method described previously by (Livak and Schmittgen, 2001) following the manufacturer's protocol (Applied Biosystems; user bulletin#2-P/N 4303859). β-glucorinidase (β-GD) was used as a reference gene in the singleplex reaction.

2.5 Terminal deoxynucleotidyl transferase–(TdT-) mediated dUTP nick-end labeling (TUNEL) assay for apoptosis.

Liver tissue sections (5 mm) were prepared and subjected to dUTP nick-end labeling by TdT with a peroxidase-based, *in situ* Cell Death Detection kit (Roche Diagnostics), 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 previously (Sharma *et al.*, 1997).

2.5 Statistics and data presentation

All data are expressed as mean ± standard error (SE) except where noted otherwise. Data were analyzed by two-way analysis of variance (ANOVA) followed by the Duncan's multiple comparison test. All tests were performed using SAS software (SAS Institute, Cary, NC). The level of $p < 0.05$ was considered significant.

3. Results

Exposure to FB_1 via the icv route resulted in a reduced weight gain and increased liver and liver/body weight ratios in mice treated with the high (100 μg) icv infusion of FB_1 (Table 5.1). No effect on body weight was noted after sc administration of FB_1.

Condition of mice infused via the icv route with 100 μg of FB_1 was behaviorally compromised as previously described (Chapter 4), whereas animals treated via sc (both doses) and with the lower icv dose (10 μg) of FB_1 did not display abnormal behavioral symptoms.

Circulating concentrations of ALT increased dose-dependently in both icv and sc-infused animals treated with 10 and 100 μg dose of FB_1 (Fig. 5.1a). Concentration of AST increased dose-dependently only in animals treated via icv route (Fig. 5.1b). The AST increase in sc-treated mice was not dose-related (Fig. 5.1b). The increase of both ALT and AST in animals exposed to 100 μg FB_1 via the icv route was much

greater compared to mice treated via sc infusion. In 100 μg FB_1 icv-treated mice ALT level was nearly 2.5 fold higher than similarly treated controls, whereas the AST increased 3-fold compared to the respective sc-treated group.

Free sphinganine (Fig. 5.2a) and sphingosine (Fig. 5.2b) in liver of animals treated with 10 and 100 μg of FB_1 increased dose-dependently in icv and sc-treated mice. The accumulation of sphinganine was nearly identical compared between groups. After the low (10 μg) dose of FB_1, accumulation of liver sphinganine in icv and sc-treated mice was ~10-fold higher and after 100 μg dose ~30-fold higher compared to respective control animals. The accumulation of sphingosine was slightly higher in the sc-treated mice compared to icv-infused animals. After 10 μg dose of FB_1, sphingosine increased 1.6-fold in icv and 2-fold in sc-treated group, whereas the 100 μg caused ~2-fold increase in icv compared to 2.7-fold elevation in sc mice compared to control, respectively.

The expression of selected proinflammatory cytokines increased in liver of mice treated via both exposure routes. After the 10 μg FB_1 dose for TNFα (Fig. 5.3a) and IL-6 (Fig. 5.3b) the relative fold-increase of mRNA in animals treated via sc infusion was similar to the icv group. After the high FB_1 dose (100 μg), the relative expression of TNFα in the icv-treated mice was not significantly higher, whereas in the sc group it increased greatly compared to respective controls. The relative expression of IL-6 increased similarly regardless of the exposure route compared to respective controls.

For IL-1β (Fig. 5.4a), mRNA expression after 10 μg FB_1 was significantly higher compared to control in mice treated via the sc route and remained unchanged in

icv-infused animals. After the 100 μg dose, the expression of IL-1β in icv-treated mice increased significantly over the control but was only half as much as in mice treated via sc-infusion. No changes were observed for IFNγ expression in either of the groups (Fig. 5.4b).

Number of apoptotic cells showed a dose-dependent increase in liver of mice treated with FB_1 both via icv and sc route (Fig. 5.5). In mice infused with FB_1 via sc the number of apoptotic hepatocytes was higher in all groups compared to respective groups in icv-treated mice. Because of a high variability among animals of the same group the differences between sc and icv FB_1-treated mice at the 10 μg dose-group were not statistically significant, although a greater incidence of TUNEL-positive cells in livers from sc mice was apparent.

4. Discussion

The results presented indicate that low doses of FB_1 (10 and 100 μg) administered via continuous sc infusion inhibited *de novo* ceramide synthesis and induced the expression of selected proinflammatory cytokines in liver. These effects were followed by a relatively low hepatotoxicity compared to FB_1 administered via icv infusion, as indicated by the activities of circulating ALT and AST and apoptotic hepatocytes. The FB_1 treatment in sc-or icv-infused mice caused similar disruption of sphingolipid metabolism. After the icv treatment with FB_1 there was less upregulation of cytokine expression but enhanced hepatotoxicity compared to the sc-treated animals.

In the majority of studies investigating FB_1-induced toxicity daily injections of FB_1 via subcutaneous (Bhandari et al., 2001; Kwon et al., 1997; Sharma et al., 2000)

or infrequently intraperitoneal (Bondy et al., 2000) route have been employed. The repeated animal handling is a considerable stressor. Such stimuli could likely modulate death/survival signaling pathways and alter the expression/transcription rate in number of proinflammatory factors, especially during acute studies, in females and/or in genetically modified animals. The key role of proinflammatory cytokines such as TNFα, IL-1β, I-6, IFNγ and related cascades in FB_1-induced hepatoxicity, including the oncotic/apoptotic death is widely demonstrated in mice (Bhandari et al., 2001; Bhandari et al., 2002a; Sharma et al., 2002; Sharma et al., 2003; Sharma et al., 2000; Theumer et al., 2002). In subchronic and chronic trials the total food-intake variability between animals is rather negligible and unrestricted gavage of FB_1-containing feed (Howard et al., 2002; Voss et al., 1996) is the most reasonable choice.

The considerably lower upregulation of TNFα, IL-1β and IL-6 expression noted in liver of mice treated icv with FB_1 compared to the sc group, is likely related to the antinflammatory events mediated by hypothalamic-pituitary-adrenal (HPA) axis. The activation of the HPA axis will result in production and systemic release of glucocorticoids (Eskandari and Sternberg, 2002). Adrenal hormones are potent immunosuppressive and antiinflammatory regulators, and as such, they inhibit the expression of proinflammatory mediators such as arachidonic acid derivatives, cytokines (primarily) and cytokine receptors, shifting the balance in favor of anti-inflammatory signaling (Elenkov et al., 1999; Eskandari and Sternberg, 2002). In earlier studies (Chapter 4) we found the icv application of FB_1 potently induced the expression of TNFα, IL-1β and IL-6 in brain cortex. Proinflammatory cytokines

trigger the release of glucocorticoids and their releasing factors (Eskandari and Sternberg, 2002). In rat, the intracerebroventricular infusion of IL-1β at doses as low as 0.1 μg /day caused a significant increase in plasma adrenocorticotropin (ACTH, 10-fold) and corticosterone (CS, 20-fold) (van der Meer et al., 1996). IL-1β, TNFα and IL-6 induced a similar elevation of circulating ACTH and CS when administered systemically (Wang and Dunn, 1998).

The decreased apoptosis in liver of icv-treated mice compared to the sc-treated group agrees with the observed profile of proinflammatory signaling in liver. In sc-treated mice the concentration of circulating liver enzymes was lower compared to icv-infused animals. This effect was not related to the inhibition of ceramide synthesis, since the bioavailability of FB_1 in liver, as measured by the concentration of free sphingoid bases, seemed to be identical after both routes. Based on current data it is difficult to explain the disparity between the upregulation of proinflammatory signaling and attenuated hepatoxicity. The liver toxicity is a net result of interplay between number of dynamic processes involving various cytokines and only selected factors were analyzed in our study. Cytokine stimulated, death effector domain-containing proteins also exert antiapoptotic functions (Barnhart et al., 2003) and paradoxical relationships between FB_1-related hepatotoxicity and proinflammatory cytokine signaling was reported previously (Sharma et al., 2002; Sharma et al., 2003). The continuous infusion of FB_1 effects could also activate mechanisms promoting cell survival and regeneration despite obvious toxicity. At the same time, the lower hepatotoxicity (as assessed by ALT and AST) in sc animals when compared to icv-

treated mice may be misleading. Glucocorticoids have been shown to stimulate the release of ALT and AST enzymes (O'Brien et al., 2002), thus defining the sc treatment as relatively less hepatotoxic when compared to icv route, with regard to the level circulating liver enzymes. The comparison of apoptosis incidence in liver of mice treated via sc and icv infusion, seem to support the latter notion.

The 100 μg (5 mg/kg) of FB_1 per animal constitutes less then 50% of the total dose of 11.25 mg/kg (2.25 mg/kg of FB_1 injected daily for 5 days) employed in a number of recent studies investigating the cytokine-mediated FB_1 toxicity (Bhandari et al., 2001; Bhandari et al., 2002a; Sharma et al., 1997; Sharma et al., 2000; Tsunoda et al., 1998) and is much lower than doses used elsewhere (Banczerowski-Pelyhe et al., 2002; Tryphonas et al., 1997). The ALT and AST levels in BALB/c females we observed after sc FB_1 infusion were lower compared to both males and females of the same strain treated with the 11.25 mg/kg of FB_1 via 5 daily sc injections (Bhandari et al., 2001). The reduced hepatoxicity was not concurrent with the decrease of free sphingoid bases in liver. Sphinganine accumulation (136 pmol/mg tissue) in sc infused mice exceeded the sphinganine concentration after 2.25 mg/kg/day of FB_1 for 3 days in females in our previous experiment (chapter 3). He et al., (2004) reported a sphinganine increase after 65 pmol/mg and unchanged sphingosine concentration in females after the same treatment (6.75 mg/kg FB_1 total over 3 days). When female mice were treated sc with 11.25 mg/kg FB_1 total dose (2.25 mg/kg/day daily for 5 days) sphinganine levels in liver reached 110 pmol/mg and sphingosine increased 2-fold (Bhandari et al., 2001).

The upregulation of expression for three liver cytokines after a cumulative 11.25 mg/kg dose of FB_1 via 5 daily sc injections was much greater compared to the TNFα, IL-1β and IL-6 expression as measured by semiquantitative PCR in both genders (Bhandari et al., 2002a; Bhandari et al., 2002b), and similar when compared to their expression by the RNase protection assay in females (Brann et al., 2002). In the current study the expression of IFNγ remained unchanged regardless of the dose and/or route compared to its previously reported decreased (Bhandari et al., 2001) or increased (Bhandari et al., 2002b) expression after 11.25 mg/kg of FB_1 in female.

In this experiment we demonstrated that sphingolipid metabolism and proinflammatory signaling was very sensitive to the low but continuously administered doses of FB_1 and this effect was concurrent with low hepatotoxicity. The intracerebroventricular infusion of FB_1 caused similar inhibition of sphingolipid metabolism but less pronounced hepatoxicity compared to its application via subcutaneous route. The osmotic pumps could become a useful alternative to bolus injections since they feature an uniform and animal-friendly delivery of FB_1, simplicity of implantation and the time range allowing for subacute (up to 2 weeks in mice) and subchronic (up to 4 weeks in rats) exposures.

Acknowledgments

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TABLE 5.1.

Liver weight change between day 1 and day 8 of the treatment with FB_1.

Route	liver weight (g)	liver weight (g)	liver wt. (g/100 g of bw)
SC	Saline	1.06 ± 0.05[a]	5.33 ± 0.27
	FB_1 10 μg	1.13 ± 0.06	5.76 ± 0.22
	FB_1 100 μg	1.15 ± 0.07	5.46 ± 0.25
ICV	Saline	0.98 ± 0.03	4.57 ± 0.15
	FB_1 10 μg	0.91 ± 0.05	4.29 ± 0.23
	FB_1 100 μg	1.10 ± 0.04[*]	5.61 ± 0.40[*]

[a] Mean ± SE ($n = 5$). [*] $P < 0.05$ compared to the corresponding saline group.

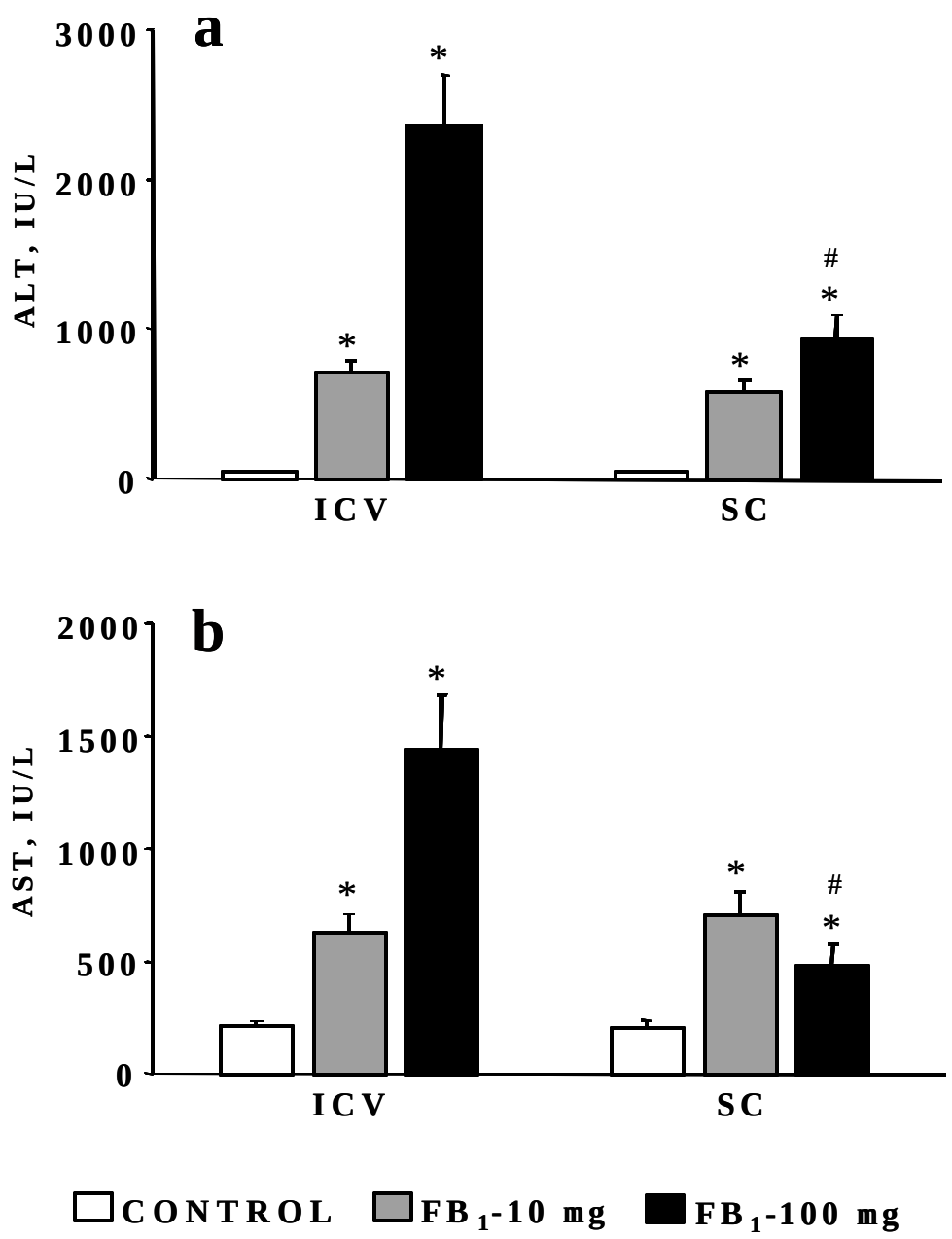

Fig. 5.1. Activity of circulating alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in BALB/c mice treated with two doses (10 and 100 μg) of FB_1 via icv (a) and sc (b) infusion. Mean ± SE (n = 5). * $p < 0.05$. # $p < 0.05$ compared to icv route.

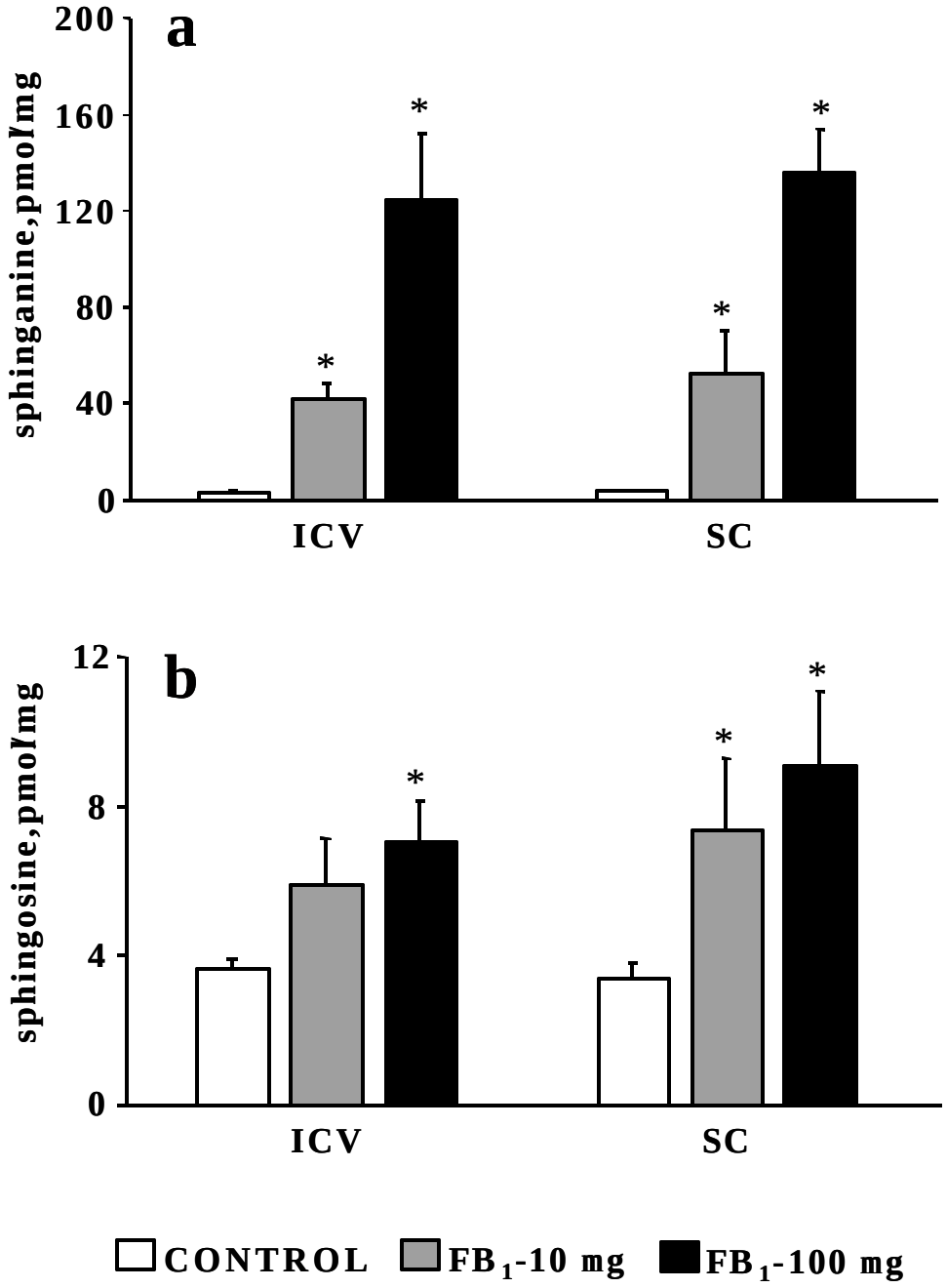

Fig. 5.2. Concentrations of sphinganine and sphingosine in the liver of BALB/c mice treated with two doses (10 and 100 μg) of FB_1 via icv (a) and sc (b) infusion. Mean ± SE (n = 5). * $p < 0.05$.

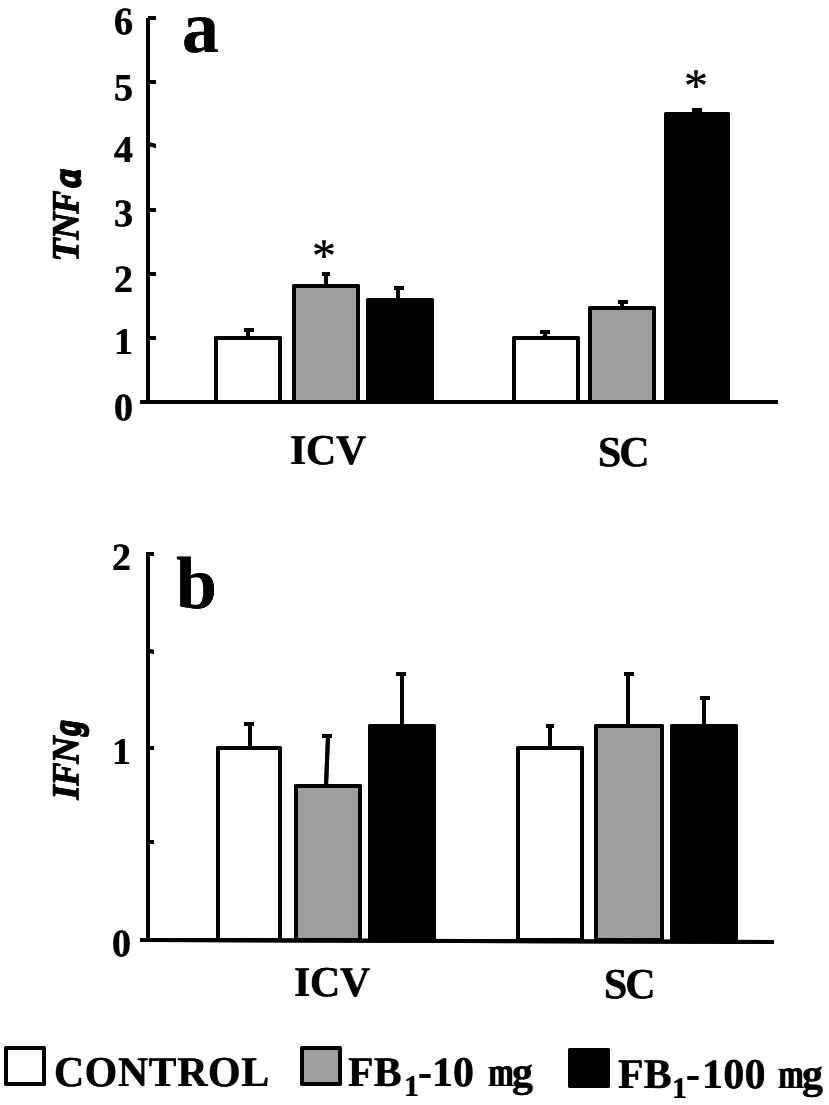

Fig. 5.3. Relative RNA expression levels of TNFα (a) and IFNγ (b) in the liver of BALB/c mice treated with two doses (10 and 100 μg) of FB_1 via icv and sc infusion. Presented as fold-change compared to control. Mean ± SD (n = 5). * $p < 0.05$.

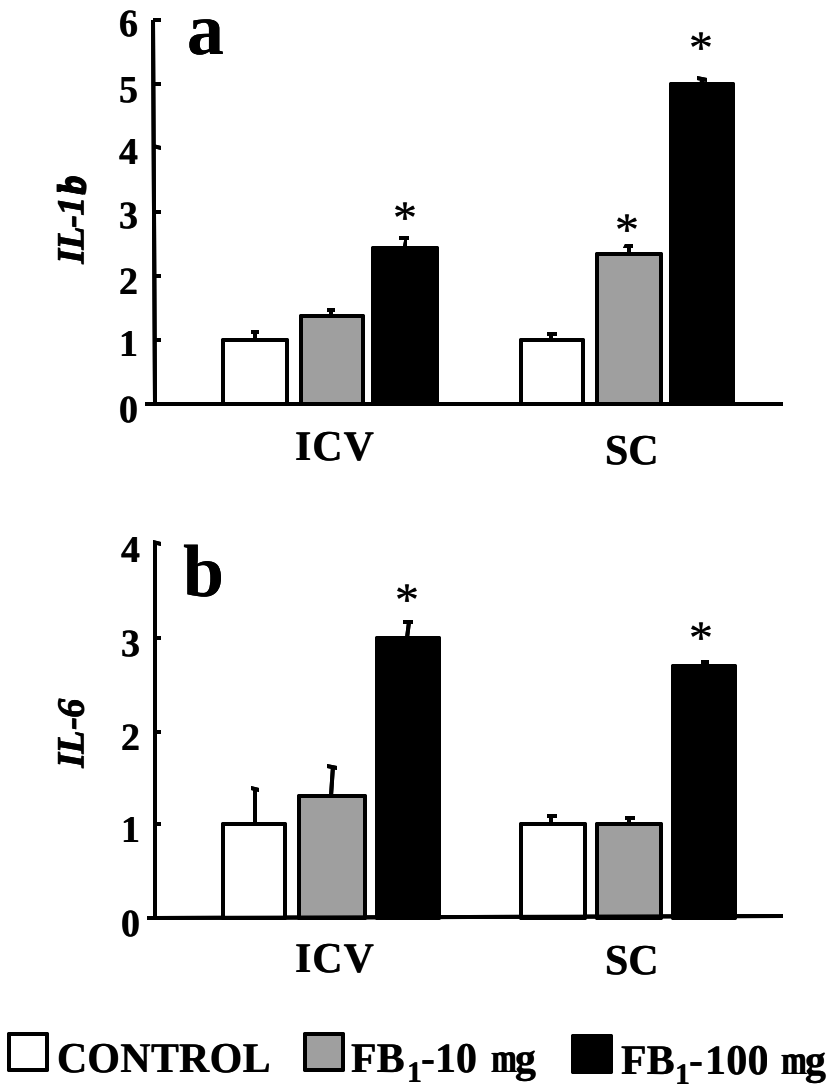

Fig. 5.4. Relative RNA expression levels of IL-1β (a) and IL-6 (b) in the liver of BALB/c mice treated with two doses (10 and 100 μg) of FB_1 via icv and sc infusion. Presented as fold-change compared to control. Mean ± SD (n = 5). * $p < 0.05$.

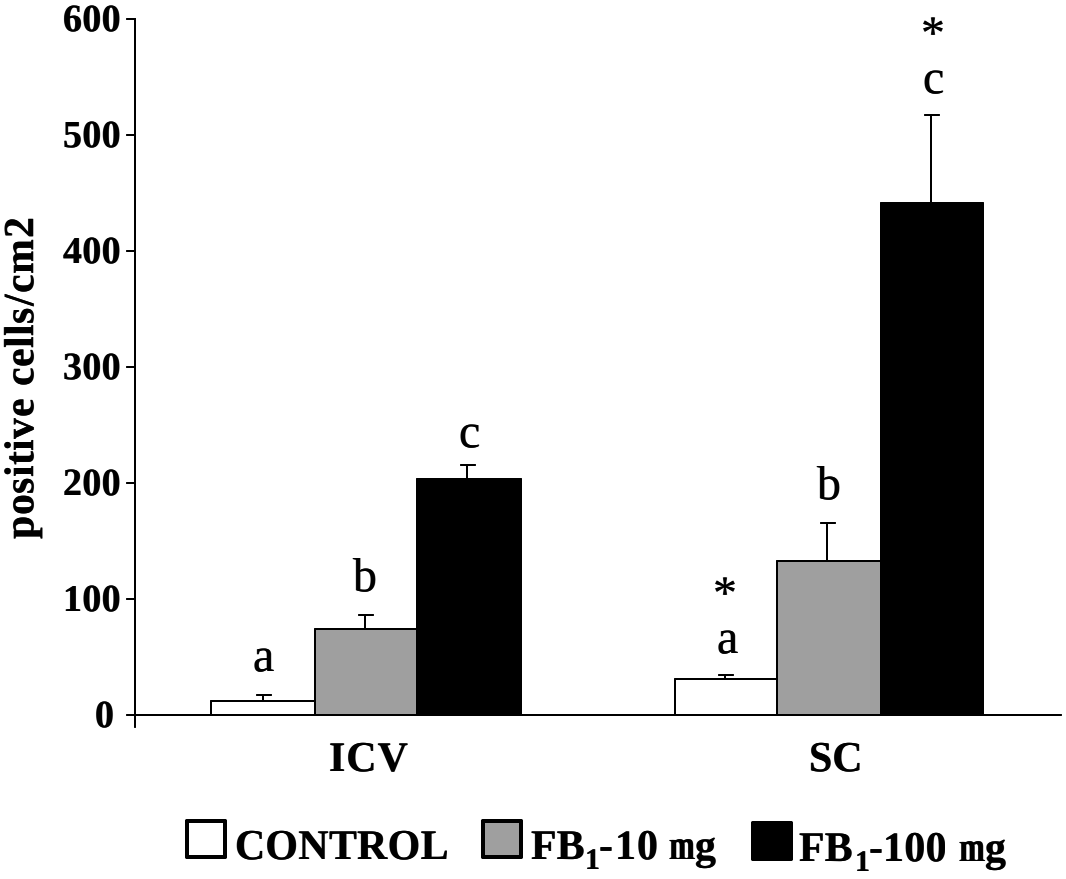

Fig. 5.5. The incidence of TUNEL-positive cells in the liver of BALB/c mice after icv and sc infusion of FB_1. Mean ± SE (n = 5). Different letters on the top of bars denote a statistical significance at $p < 0.05$. * $p < 0.05$ compared to icv route.

CHAPTER 6

FUMONISIN B_1 INDUCES NECROTIC CELL DEATH IN BV-2 AND MURINE CULTURED ASTROCYTES AND IS ANTIPROLIFERATIVE IN BV-2 CELLS BUT NOT N2A AND PRIMARY CORTICAL NEURONS ARE RESISTANT[4]

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[4]Osuchowski, Marcin F., Sharma, Raghubir P. To be submitted to NeuroToxicology

Abstract

Fumonisin B_1 (FB_1), a mycotoxin produced by *Fusarium verticillioides*, causes equine leukoencephalomalacia, impairs myelination and inhibits neuronal growth *in vitro*. This study investigated toxicity of FB_1 upon murine microglial (BV-2), neuroblastoma (N2A) lines and primary astrocytes and cortical neurons. BV-2 and N2A lines and cells prepared from neonatal and postnatal brains of BALB/c mice were exposed to various doses of FB_1 for 4 (BV-2 and N2A) or 4 and 8 days (astrocytes and cortical neurons). FB_1 at 25 μM decreased viability in BV-2, whereas at 50 μM caused necrotic but not apoptotic cell death in both BV-2 and primary astrocytes (day 8 only) as assessed by lactic dehydrogenase release, pripidium iodide and Annexin V staining. Thymidine incorporation indicated FB_1-induced decrease in proliferation only in BV-2 cells at 2.5 μM concentration. DNA analysis by flow cytometry showed that the inhibition was not caused by cell cycle arrest. The mitochodrial activity decreased dose-dependently in BV-2 cells only and was significantly elevated after 25 μM (but not 50 μM) FB_1 at day 4 and 8 in astrocytes. In BV-2 and astrocytes the expression of TNFα and IL-1β analyzed by real-time polymerase chain reaction was downregulated at 6 and 24 h. In all cell types tested the FB_1 treatment caused accumulation of free sphinganine and decrease in free sphingosine level at selected time points. Results indicate that the murine brain immunocompetent cells but not neurons are vulnerable to the FB_1-dependent cytotoxicity *in vitro*.

Keywords: Fumonisin; Brain, Fumonisin; BV-2, Astrocytes; N2A; Cortical neurons; Sphingolipids, Proliferation inhibition; Necrosis

1. Introduction

Fumonisins are toxic metabolites synthesized by the fungus *Fusarium verticillioides* (formerly *F. moniliforme*). The occurrence of fumonisin infestation is worldwide and affects various cereals, predominantly maize (Dutton, 1996). The most abundant and metabolically active member of fumonisin family is fumonisin B_1 (FB_1). FB_1 causes number of disorders in farm and laboratory animals such as porcine pulmonary edema, nephrosis in sheep, hepatotoxicity and nephrotoxicity in rodents and equine leukoencephalomalacia (ELEM) (Dutton, 1996). Chronic feeding with FB_1 caused liver and kidney cancer in mice and rats with high species/gender specificity (Howard et al., 2001). Incidences of esophageal cancer diagnosed in humans were associated to dietary exposure to FB_1 in regions of Southern Africa and China where high levels of this mycotoxin are present in foods (Dutton, 1996).

The specific biochemical effect of exposure to FB_1 both *in vivo* and *in vitro* is an inhibition of ceramide synthase, an enzyme of the sphingolipid biosynthetic pathway. The inhibition of ceramide synthesis leads to the accumulation of free sphingoid bases, namely sphinganine and sphingosine. The FB_1-dependent deregulation of the *de novo* sphingolipid biosynthesis is considered as a primary mechanism of fumonisin toxicity. The accumulation of free sphingoid bases, especially sphinganine, after FB_1 treatment is an established biomarker of exposure to this mycotoxin (Dutton, 1996). Dynamic balance between free sphingoid bases and their metabolites controls several cellular processes including growth, differentiation, survival and death (Merrill et al., 2001). Long-term inhibition of ceramide synthase results in the depletion of complex

sphingolipids such as sphingomyelin, cerebrosides and gangliosides, which maintain the function and structure of neuronal tissue. Number of reports demonstrated the key role of proinflammatory cytokines such as tumor necrosis (TNF) α in FB_1-induced toxicity in target organs such as liver (Bhandari et al., 2002; Sharma et al., 2000) and kidney (Bhandari and Sharma, 2002).

The equine leukoencephalomalacia (Brownie and Cullen, 1987) is the only known naturally occurring disorder affecting brain caused by the environmental exposure to FB_1. The disease is specific for *equidae*, shows high mortality (Wilkins et al., 1994; Wilson et al., 1990) and is characterized by focal necrosis in brain, localized primarily in the subcortical white matter. Recently, the consumption of fumonisins was implicated in the etiology of neuronal tube defects (NTD) in children (Marasas et al., 2004). NTDs were induced experimentally in neurulating murine embryos (Sadler et al., 2002) and this effect was related to decreased uptake of folic acid caused by exposure to FB_1 (Stevens and Tang, 1997).

In vitro and *in vivo* experiments demonstrated potential for toxic effects upon neuronal tissue by FB_1. FB_1 treatment altered neurotransmitter metabolite levels in murine (Tsunoda et al., 1998) and rat (Porter et al., 1993) brain and modulated the electrophysiological activity of rat neocortex (Banczerowski-Pelyhe *et al.*, 2001). Harel and Futerman (1993) reported the FB_1-dependent inhibition of neuronal development in culture, whereas Kwon et al., (2000) showed that FB_1 treatment decreased the activity of rat primary glia/oligodenrocytes concurrently with the accumulation of sphinganine. The FB_1-induced disruption of myelination was

demonstrated *in vitro* (Monnet-Tschudi et al., 1999), and *in vivo* (Kwon *et al.*, 1997b). In cultured rat astrocytes FB_1 caused DNA damage but did not alter viability (Galvano et al., 2002). FB_1 treatment in J774A.1 macrophage deregulated their membrane homeostasis (Ferrante et al., 2002) and increased the LPS-dependent production of nitric oxide and prostaglandin E2 (Meli et al., 2000). In a number of cell lines FB_1 displayed strong antiproliferative properties, which were often concurrent with cell cycle arrest (Ciacci-Zanella et al., 1998; Johnson et al., 2003; Mobio et al., 2000; Tolleson et al., 1996b). Apoptosis seems to be an important mode of FB_1-induced death in non-neuronal cells *in vitro* (Tolleson et al., 1996a; Tolleson et al., 1996b; Yu et al., 2001).

Abundance of studies investigating carcinogenetic properties of FB_1 is contrasted by relatively low interest in its neurotoxic effects. Scarce experiments studying the susceptibility of brain neuronal and immunocompetent cells to FB_1 warrant further insight into this aspect of FB_1-related toxicity. It is not known what cells in the neuronal tissue are sensitive to FB_1 and likely target for the degenerative effects in equines. In the present study we investigated the effect of FB_1 treatment upon viability and proliferation of murine neuronal and glial cells and correlation of these effects to the sphingolipid metabolism and proinflammatory signaling.

2. Materials and methods

2.1.1. BV-2 and N2A cell lines

The cultured murine microglial cells were immortalized after infection with a v-raf/v-myc recombinant retrovirus forming a cell line that features the morphological and functional characteristics of microglia and is a suitable model for *in vitro* studies. (Bocchini et al., 1992). The cells were maintained at 37 °C and 5% CO_2 in RPMI 1640 (Gibco Laboratories, USA), supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 0.5% penicillin–streptomycin (Pen-Strep, Gibco Laboratories). For the experiments, BV-2 cells growing in 75 cm^3 flasks were plated before first passage in various concentrations (10-150 th/ml) in either 96 or 6-well culture plates. BV-2 cells were allowed to grow for 24 h, and then exposed to FB_1. In the majority of experiments, the FB_1 (98% purity; Promec, Tygerberg, South Africa) was at 20 and 50 µM concentration for the duration of 4 days. To develop a dose–response curve for the viability and proliferation study, BV-2 were treated with FB_1 concentrations of 0.625-50 µM for 4 days. In a short-term proliferation experiment BV-2 were treated with 25 µM FB_1 for 24 h. For cytokine analysis the BV-2 cells growing in RPMI media with or without FBS were treated with 50 µM FB_1 for 6 and 24 h.

Neuro-2a (N2A; CCL-131, American Type Culture Collection, Manassas, VA) murine neuroblastoma cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS) and 1% penicillin–streptomycin (Pen-Strep, Gibco Laboratories). Cells were used before first passage and plated in various concentrations (40-300 th/ml) in either 96 or 6-well

culture plates. The treatment concentrations and duration is identical with the described above in BV-2 cultures.

2.1.2. Neocortical neuron culture

Primary cultures of neocortical neurons were obtained from embryonic day 16-17 BALB/c mice as described previously (Dravid et al., 2004). Briefly, pregnant mice were killed by CO_2 asphyxiation and embryos were removed under sterile conditions. Neocortices were collected, stripped of meninges, minced by trituration with a Pasteur pipette and treated with trypsin for 25 min at 37°C. The cells were then dissociated by two successive pippeting and sedimentation steps in soybean trypsin inhibitor and DNase containing isolation buffer, centrifuged, and resuspended in Eagle's minimal essential medium with Earle's salt and supplemented with 2 mM L-glutamine, 10% fetal bovine serum, 10% horse serum, 100 IU/mL penicillin and 0.10 mg/mL streptomycin, pH 7.4. For majority of experiments cells were plated onto poly-L-lysine coated 96-well (9 mm) clear-bottomed black-well culture plates (Corning Costar, Acton, MA, USA) at a density of 1.5 x 10^6 cells/ml. For free sphingoid base analysis cells were plated on 12-well plates at a density of 2.0 x 10^6 cells/ml. Cells were incubated at 37°C in a 5% CO_2 and 95% humidity atmosphere. Cytosine arabinoside (10 µM) was added to the culture medium on day 2 after plating to prevent proliferation of non-neuronal cells. The culture media was replenished with minimal essential medium supplemented with 2 mM L-glutamine, 5% horse serum, 100 IU/mL penicillin and 0.10 mg/mL streptomycin, pH 7.4. The cultures were treated with 25 and 50 µM FB_1 for 4 and 8 days starting between 3 and 5 day *in vitro*.

2.1.3. Glial cell culture

The whole brains from postnatal day 1-2 mice were aseptically removed, and then cortices were dissociated and mechanically triturated as described in the previous section. Cells (1.0×10^6 cells/ml) were plated into 96-well cell culture plates (Costar), and maintained at 37°C and 5% CO2 in DMEM containing 10% FBS, 2 mM l-glutamine, 1 mM sodium pyruvate, 50 U/ml penicillin, and 50 mg/ml streptomycin. The culture medium was replenished 1 and 3 days after plating. The FB_1 was introduced on the 3 day *in vitro*. In cultures treated with 25 and 50 μM FB_1 for 4 days media was not changed, whereas in 8 day-treated cells it was replenished once on day 4. Cell preparations yielded at least 95% purity as confirmed by staining with antibodies specific for an astrocyte marker, glial fibrillary acidic protein (GFAP) conjugated to Cy3 dye (Sigma-Aldrich, St. Louis, MO). Henceforth, the cells will be referred to as primary astrocytes.

2.2. Cytotoxicity Assays

Cell death induced by FB_1 was determined using the lactate dehydrogenase (LDH) release assay as previously described (He et al., 2002). Briefly, 40 μl of culture media was added to a 96-well plate and then 100 μl of 4.6 mM pyruvic acid in 0.1 M potassium phosphate buffer (pH 7.5) was added using a repeater pipette. The total LDH was released from the cells by the addition of 0.1% Triton X-100 followed by brief incubation at 37 C. 100 μl of 0.4 mg/ml reduced nicotinamide adenine dinucleotide in 0.1 M potassium phosphate buffer (pH 7.5) was added to medium and/or cell lysate and the kinetic change in absorbance at 340 nm was read for a

duration of 1 min using a PowerWaveX™ absorbance microplate reader (Bio-Tek Instruments Inc., Winooski, VT). Data are presented as percentage of control (no treatment) LDH release.

The fluorescent probes propidium iodide (PI) and annexin V were used to detect the apoptotic and necrotic cells, respectively. Cells were grown and treated in 96 and 6 well plates. At the end of treatment, cells were incubated with PI (20 µg/ml) and/or annexin V (5 µL/100 µL in annexin binding buffer). Fluorescence intensity was recorded using Spectramax Gemini fluorescent plate reader (Molecular Devices, Irvine, CA). PI fluorescence was detected with excitation at 535 nm and emission at 617 nm; and the fluorescence of annexin V with excitation at 495 nm and emission at 520 nm. Visual documentation of cellular effects was performed using Olympus IX71 inverted microscope (Olympus America, Melville, NY) and digital images were acquired using Magnafire SP digital camera.

2.3. Mitochondrial activity

Mitochondrial enzyme activity was determined using the 3(4,5-dimethylthiazolyl-2)2,5-diphenyl tetrazolium bromide (MTT) assay. 4 h before the end of FB_1 exposure the MTT was added to each well (96-well plate) at a final concentration of 0.5 mg/ml. Media was removed and the purple formazan crystals were dissolved in 100% dimethyl sulfoxide. Absorbance at 570 nm was determined using a Spectra SLT absorbance reader (Tecan, Durham, NC, USA).

2.4. Proliferation analysis

DNA synthesis measured by the [methyl-^{3}H] thymidine incorporation as an index of cell proliferation was described previously (Johnson et al., 2003). After cells were seeded in 96-well plates FB_1 at selected concentration and 20 µl of [methyl-^{3}H] thymidine (25 µgCi/ml, 6.7 Ci/mmol, DuPont NEN Products, Boston, MA) was added. After 4 (BV-2 and N2A) and 8 days (primary astrocytes) of treatment cells were collected with the cell harvester (PHD, Cambridge Technology, Cambridge, MA) and the uptake of [methyl-^{3}H] thymidine in harvested cells was counted in liquid scintillation counter (Pharmacia, Turku, Finland) and expressed as of control proliferation.

2.5. Cell cycle analysis

BV-2 cells treated with FB_1 for 24 h and 4 days were scrapped and re-suspended in nuclear isolation medium (NIM, 50 µg/ml PI, 1 mg/ml RNase A, 0.1% Triton X-100). Nuclear fluorescence is proportional to DNA content. Analysis of DNA ploidy and discrimination of cells in G0/G1 versus S versus G2/M phases of the cell cycle was done by measuring cellular DNA content using fluorescent automated cell sorting with a FAXL-MCL four-color flow cytometer (Coulter Electronics, Hileah, FL). DNA histograms were analyzed using Coulter's System II software, version 3.0.

2.6. Quantitative polymerase chain reaction for inflammatory cytokines

BV-2 cells and primary astrocytes treated with FB_1 for duration of 6 and 24 h were harvested from 6 well plates, the total RNA was isolated and the initial cDNA

strand prepared using the SuperScript™ II transcriptase system with oligo $(dT)_{12-18}$ primer (Invitrogen, Carlsbad, CA) as previously described (Sharma et al., 2000). The expression of cytokines was measured only in cells that exhibited cytoxicity to FB_1 (i.e. BV-2 and primary astrocytes). In addition, neuronal cells express only receptors for TNFα but not the cytokine itself and the expression of interleukin (IL)-1β in neurons is substantially lower compared to cells of glial origin (Szelenyi, 2001). The procedure of quantitative determination of gene expression was described in detail elsewhere (Chapter 4). Briefly, the ABI PRISM® 7900HT sequence detection system using the TaqMan® Universal Master Mix (Applied Biosystems, Foster City, CA), TaqMan® MGB probes combined with Assay-on-Demand™ (Applied Biosystems) primers for murine β-glucoronidase, TNFα and IL-1β genes were used. After the amplification efficiency was validated the relative gene expression levels were determined using the C_T method described by (Livak and Schmittgen, 2001) following the manufacturer's protocol (Applied Biosystems). β-glucoronidase was used as a reference gene in a singleplex reaction.

2.7. Sphingolipid analyses

Determination of intracellular free sphingoid bases was performed as previously described (He et al., 2001). Briefly, at the end of 4 or 8 day treatment with FB_1, the medium was aspirated, and cells were washed once with ice-cold PBS and then scraped into 1 ml ice-cold PBS. An aliquot (0.1 ml) of cell suspension in PBS was transferred to another tube, spun at 2,000g, 4°C for 5 min. The cell pellet was lysed and stored at -85°C until analysis of total protein by Bio-Rad Bradford reagent (Bio-Rad

Laboratories, Hercules, CA) using 96-well plate according to the manufacturer's protocol. Free sphingoid bases were extracted from the remainder of cells by base hydrolysis. The relative amounts of free sphinganine and sphingosine in base-treated cell extracts were determined by high performance liquid chromatography (HPLC) as described earlier (He et al., 2002). Sphingoid bases were quantified based on the recovery of a C_{20} sphinganine internal standard. Concentrations of analyzed free sphingoid bases in pmol/mg of protein are expressed as % change compared to control.

2.8. Statistics and data presentation

Experiments were repeated at least 3 times yielding consistent results. All data are expressed as mean ± standard error (SE) except where noted otherwise. Data were analyzed by two-way analysis of variance (ANOVA) followed by the Duncan's multiple comparison test. All tests were performed using SAS software (SAS Institute, Cary, NC). The level of $p < 0.05$ was considered significant.

3. Results

3.1. Determination of cytoxicity

In BV-2 cells treated for 4 days with FB_1, 20 μM was the lowest concentration at which the significant release of LDH was observed (Fig. 6.1a). The LDH concentration increased further compared to control after the BV-2 cells were treated with 20 or 50 μM of FB_1. In primary astrocytes after 8 days of treatment the significant release of LDH was observed at the 50 μM FB_1, whereas an increasing trend was noted at the lower (20 μM) FB_1 concentration (Fig. 6.1b). The shorter treatment

period (4 days) with the same FB_1 concentration (25 and 50 μM) caused no effect upon the LDH release (data not shown). The FB_1-induced toxicity in primary astrocytes at 50 μM was further confirmed by the PI fluorescence intensity (Fig. 6.1b-inset). None of the selected FB_1 concentrations affected the LDH release in N2A (Fig. 6.1c) and primary cortical neurons (Fig. 6.1d).

The FB_1-dependent loss of BV-2 cells and primary astrocytes was confirmed by visual inspection under the fluorescence microscope revealing the increase in number of PI-positive cells treated with FB_1 compared to control. After the 50 μM FB_1 dying BV-2 cells formed numerous and widespread cell clusters (Fig. 6.2a). Smaller and less frequent but similar conglomerates of necrotic BV-2 cells were observed after the lower (20 μM) FB_1 concentration (data not shown). No evidence of apoptosis (stained by annexin V) was apparent after FB_1 treatment in this cell line. In primary astrocytes the increase in number of PI-positive cells was evident only after 8 days of treatment with FB_1 at the high (50 μM) concentration compared to control (Fig. 6.2b). The necrotic astrocytes were evenly scattered throughout the entire well surface. The simultaneous staining with Annexin V dye (apoptotic cell death) at day 4 (BV-2) and day 8 (astrocytes) revealed cells undergoing apoptosis in both BV-2 and primary astrocytes but treatment with the selected FB_1 concentrations did not modify this process compared to control (data not shown). No FB_1-related increase was observed in number of PI-positive cells in either N2A or cortical neurons at any dose or time point (data not shown).

3.2. Determination of mitochondrial activity

The MTT assay is helpful to assess changes in cell viability but does not directly detect dead cells, since the generated signal depends on the degree of cellular mitochondrial activity. In BV-2 the activity of succinate dehydrogenase after FB_1 treatment decreased in a concentration-dependent fashion (Fig. 6.3a). It was significantly lower compared to control at the 8 μM FB_1, decreased further after 20 μM and plateaued at the 50 μM FB_1. The MTT concentration-response curve of BV-2 cells treated with FB_1 closely mimicked the curve shapes of LDH release (Fig. 6.1a). It is likely, that the decrease in the formazan concentration is due to the decline in total cell number, apart from being indicative of ongoing cell death in BV-2 line.

The marginal decrease of succinate dehydrogenase activity in N2A cells was recorded at the 50 μM FB_1 dose (Fig. 6.3b).

In primary astrocytes the succinate dehydrogenase activity was elevated after the 25 μM of FB_1 at both 4 and 8 day of treatment compared to control (Fig. 6.3c). After the 50 μM FB_1 dose, succinate dehydrogenase activity was unaltered at day 4 and showed a slightly decreasing trend (statistically insignificant) at the 8 day of treatment. In primary astrocytes, the cell death was not observed after the 25 μM FB_1 dose but occurred at the concentration of 50 μM. It is suggestive, that the elevated succinate dehydrogenase activity after the 25 μM FB_1 indicated that at this concentration FB_1 was toxic to primary astrocytes even though without immediate lethal effect.

The succinate dehydrogenase activity in cortical neurons remained unchanged regardless of the dose and time of treatment (Fig. 3d).

3.3. Determination of proliferation and cell cycle analysis

In BV-2 cells FB_1 treatment (4 days) caused the concentration-dependent decrease in [^{3}H] thymidine incorporation (Fig. 6.4a). The 2.5 μM of FB_1 was the lowest concentration at which the decrease of [^{3}H] thymidine incorporation was significant. When BV-2 cells were treated with higher FB_1 concentrations, the thymidine incorporation declined further reaching plateau at the 20 μM concentration. BV-2 cells treated only for 24 h with 25 μM FB_1 showed identical decrease in [^{3}H] thymidine incorporation compared to the 4-day treatment (Fig. 6.4a-inset). The DNA analysis by flow cytometry was employed to analyze the cell cycle profile in BV-2 cells. Only BV-2 cells were assayed since in no other cell types the evident FB_1-dependent decline in proliferation was observed. The FB_1 dose of 25 μM for 4 days did not modulate the BV-2 cell ratio in different phases of the cell cycle as compared to untreated cells (Fig. 6.4b).

Proliferation of primary astrocytes exposed to 50 μM FB_1 was not significantly changed at either 4 and/or 8 day of the treatment (Fig. 6.4c). In N2A the thymidine incorporation also remained unaltered after 8 days (Fig. 6.4d).

3.4. The relative cytokine gene expression

In BV-2 cells and primary astrocytes treated with 50 μM FB_1, a uniform decline in the expression of TNFα and IL-1β was observed at selected time points (6 and 24 h). In BV-2 cells the TNFα expression was downregulated equally after both 6 and 24-h FB_1 treatment compared to control (Fig. 6.5a). The IL-1β expression remained unchanged at the 6-h time point and decreased when BV-2 cells were treated with FB_1

for 24 h (Fig. 6.5b). In primary astrocytes treated with FB_1 the expression of TNFα and IL-1β decreased in a similar fashion both after 6 and 24-hour exposure (Fig. 6.5c,d). When cells were grown in media deprived of FBS prior to the PCR assay to eliminate the presence of factors that could possibly modulate the FB_1-dependent impact on cytokine expression, similar results were obtained (data not shown).

3.5. Concentration of free sphingoid bases

In all cell types treated for 4 or 8 days with 50 μM of FB_1 the sphinganine concentration dramatically increased, whereas the level of sphingosine declined compared to their respective controls. After BV-2 cells were treated with FB_1 for 4 days the accumulation of sphinganine was 20-fold higher and of sphingosine 14-fold lower compared to the control (Fig. 6.6a). After 8-day FB_1 treatment of primary astrocytes resulted in 14-fold increase of sphinganine level and 8-fold decline of sphingosine concentration over respective controls (Fig. 6.6b). In N2A cells exposed to FB_1 for 4 days sphinganine increased by 26-fold and sphingosine was lower by 8-fold compared to control (Fig. 6.6c). The greatest sphinganine accumulation was observed in primary cortical neurons, where sphinganine was 50-fold higher, while sphingosine decreased only by 40% compared to control, respectively (Fig. 6.6d).

4. Discussion

The present results show that FB_1 induced necrotic cell death in both BV-2 and primary astrocytes and exhibited potent antiproliferative properties toward the BV-2 cells only. These effects were simultaneous with the downregulation of proinflammatory signaling in both primary astrocytes and BV-2 cells. The N2A cell

line and cortical neurons were resistant to the toxic effects of FB_1 in the employed doses. In all cell types the FB_1 treatment disrupted the sphingolipid metabolism, as indicated by the accumulation of free sphinganine and decreased level of free sphingosine.

The sensitivity of astrocytes *in vitro* to FB_1 was reported in handful of experiments, whereas the FB_1 effects upon microglial cells have not been studied. In the aggregating cell culture from fetal rat telencephalon treated with FB_1 (1-40 μM) a significant decrease in astrocyte cytoskeletal marker (GFAP) was observed at the dose as low as 1 μM between *in vitro* days 25 and 35, suggesting the FB_1-induced decrease in astrocytes activity and/or maturation (Monnet-Tschudi et al., 1999). We did not observe any FB_1-related alterations in primary astrocytes after GFAP staining (data not shown) but the cells in our study were exposed to FB_1 for much shorter period. Kwon et al., (2000) reported that FB_1 (5-75 μM) stimulated population of oligodendrocytes and astrocytes in a mixed primary culture after 10 days of treatment. In mixed cultures the effects of brain glia-dependent inflammatory signaling are amplified with respect to cells of the same lineage (Sola et al., 2002) as well as to neurons (Ahlemeyer et al., 2002; He et al., 2002) resulting in increased cytoxicity. We have observed similar activation of murine hippocampal astrocytes (as assessed by anti-GFAP labeling) *in vivo* after intracerebroventricular application of FB_1 (Chapter 4). In primary rat astrocytes culture the FB_1 treatment (48, 72 h and 6 days) caused DNA damage but this effect was not followed by the reduction in GFAP-positive staining or cell death (Galvano et al., 2002).

The studies reporting unaltered viability of glial cells *in vitro* (Kwon et al., 2000; Monnet-Tschudi et al., 1999; Galvano et al., 2002) even after very high FB_1 doses (up to 100 μM) contrast here the observed loss of BV-2 cells and primary astrocytes. The reason for such disparity is not clear but the treatment timing employed in our study could at least partially explain the observed cell death. To include early developmental phase of primary astrocytes we begun FB_1 treatment at the 3 day *in vitro*, which is significantly earlier compared to previous studies (Galvano et al., 2002; Kwon et al., 2000; Monnet-Tschudi et al., 1999). Such early FB_1 insult to immature cells of relatively low density could promote changes that would afterward render some cells more vulnerable to the treatment at later time points. BV-2 cells seeded in high density and when confluent and matured did not exhibit cytotoxic response after FB_1 (data not presented). A similar phenomenon was noted previously in HT29 cells (Schmelz et al., 1998).

The relationship between proliferation stages and the antimitotic properties of FB_1 treatment was reported before (Riley et al., 1998). Although the mitotic effect of FB_1 was reported in Swiss 3T3 fibroblasts (Schroeder et al., 1994) and rat lymphocytes (Dombrink-Kurtzman et al., 2000) fumonisin is mainly antiproliferative (Schmelz et al., 1998; Tolleson et al., 1996a; Tolleson et al., 1996b). The noted FB_1-dependent inhibition of proliferation in BV2 cells that did not rely on cell cycle arrest can be compared to previous reports on HT29 cell line (Schmelz et al., 1998) and normal human keratinocytes (Tolleson et al., 1996b), whose growth was similarly inhibited but without differences in cell cycle progression.

In majority of mammalian cell lines responsive to FB_1 exposure *in vitro* (e.g. LLC-PK_1, CV-1, HET-1A and Hep-G2) the FB_1-induced necrosis was accompanied by the programmed cell death (Ciacci-Zanella et al., 1998; Schmelz et al., 1998; Tolleson et al., 1996a; Tolleson et al., 1996b). We observed mainly necrotic mode of cell death in BV-2 and primary astrocytes after FB_1 treatment. In Chinese hamster ovary (CHO) cells, despite their relative resistance when compared to the highly susceptible LLC-PK_1 line, the FB_1 treatment produced significant cytoxicity that did not produce apoptotic cell death and even suppressed this process (Yu et al., 2001). It could be possible that in certain cell types (e.g. less sensitive to FB_1) the pro-apoptotic signaling is not initiated and the FB_1-induced cell loss is caused primarily by necrosis.

The FB_1-induced inhibition of TNFα and IL-1β expression in BV-2 and astrocytes was surprising, given the potent upregulation of these cytokines (also IL-6 and IFNγ) after FB_1 was administered directly to the murine brain (Chapter 4). *In vivo*, the FB_1-induced apoptosis in liver, correlated with the activation of proinflammatory cytokines including TNFα (Bhandari et al., 2002; Bhandari and Sharma, 2002; Sharma et al., 2000). In LLC-PK_1 line the TNFα-induced apoptosis increased after cells were pretreated with FB_1 (Johnson et al., 2003). He et al., (2001) demonstrated that FB_1-related apoptosis in the same cell line was simultaneous with the expression of TNFα, substantiating key role of this cytokine in the FB_1-dependent toxicity. In BV-2 cells aluminium caused an identical decline of TNFα expression within the same time frame as observed in our study (Johnson, 2003). Based on an evident correlation between TNFα signaling and programmed cell death, the observed decrease in TNFα and IL-1β

expression could be related to a lack of apoptosis in BV-2 and primary astrocytes. One possible explanation could be that such downregulation follows induction shortly after the beginning of treatment. This can be measured by investigating the kinetics of expression of inflammatory cytokines at early time points. This was not the objective of the present study but will be investigated in future.

The FB_1-induced deregulation of sphingolipid metabolism has been proposed to be a preliminary step in cytotoxicity and carcinogenicity of FB_1 (Merrill, Jr. et al., 1996; Merrill, Jr. et al., 2001). The accumulation of sphinganine after exposure to FB_1 was correlated with the occurrence and severity of the *in vivo* toxicity (Voss et al., 2001). In number of cell lines, the FB_1-induced elevation of sphinganine concentration-dependently correlated with the cell death (Kim et al., 2001; Yu et al., 2001). In contrast, neurons seemed to be resistant to FB_1. The sphinganine accumulation in neuronal cells was not related to cytotoxicity in our present work. Others have reported similar findings: the disruption of sphingolipid biosynthesis did not affect viability of rat hippocampal neurons *in vitro* (Harel and Futerman, 1993; Schwarz et al., 1995; Schwarz and Futerman, 1998) and the accumulation of sphinganine in murine granular neurons was not followed by cell death (Merrill, Jr. et al., 1993). In all cell types tested, FB_1 treatment caused the decrease in free sphingosine. This effect was previously reported in cultured brain cells (Kwon et al., 2000) and in cell lines of non-CNS origin (Wang et al., 1991; Sharma et al., 2004), and is likely related to the FB_1-induced depletion of sphingosine-based complex sphingolipids.

The present study demonstrates that in culture, immunocompetent cells of murine brain but not of neuronal origin are vulnerable to the cytotoxic effects of FB_1. This observation indicates that glial cells, namely microglia and astrocytes, may be a primary targets in the FB_1-induced neurotoxicity. The mechanism by which FB_1 caused the suppression of TNFα and IL-1β signaling in BV-2 and astrocytes remains to be elucidated. The relative resistance of isolated neurons in culture to FB_1 treatment suggests that neuronal death observed *in vivo* could be a secondary effect mediated in part via CNS-borne immunocompetent cells. *In vitro* studies employing the mixed glial/neuronal cultures, which more closely emulate the *in vivo* environment of the CNS, could further clarify nature of this relationship.

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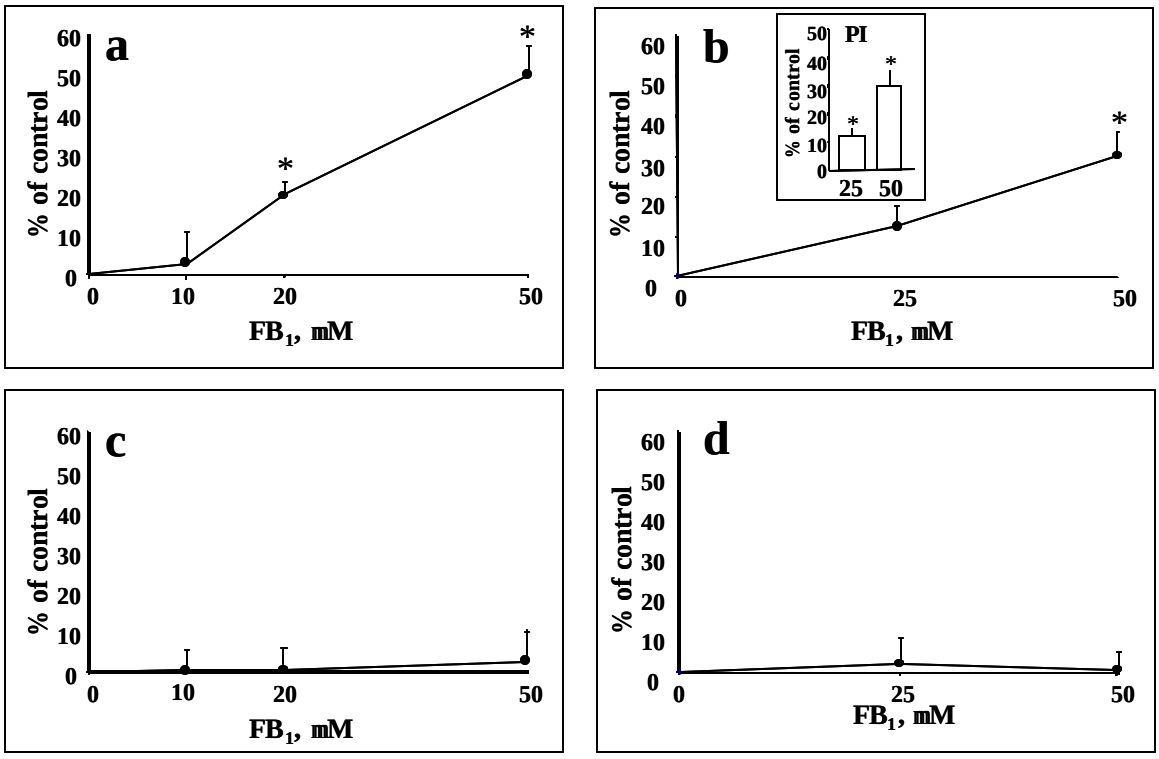

Fig. 6.1. Release of lactate dehydrogenate after treatment with FB_1 at selected concentrations in (a) BV-2 (after 4 days), (b) primary astrocytes (after 8 days), (c) N2A (after 4 days) and (d) cortical neurons (after 8 days). Increase of propidium iodide (PI) fluorescence intensity after primary astrocytes (b-inset) were treated with 25 and 50 μM FB_1 for 8 days. Mean ± SE (n = 5) from a representative experiment repeated independently three times. * $p < 0.05$.

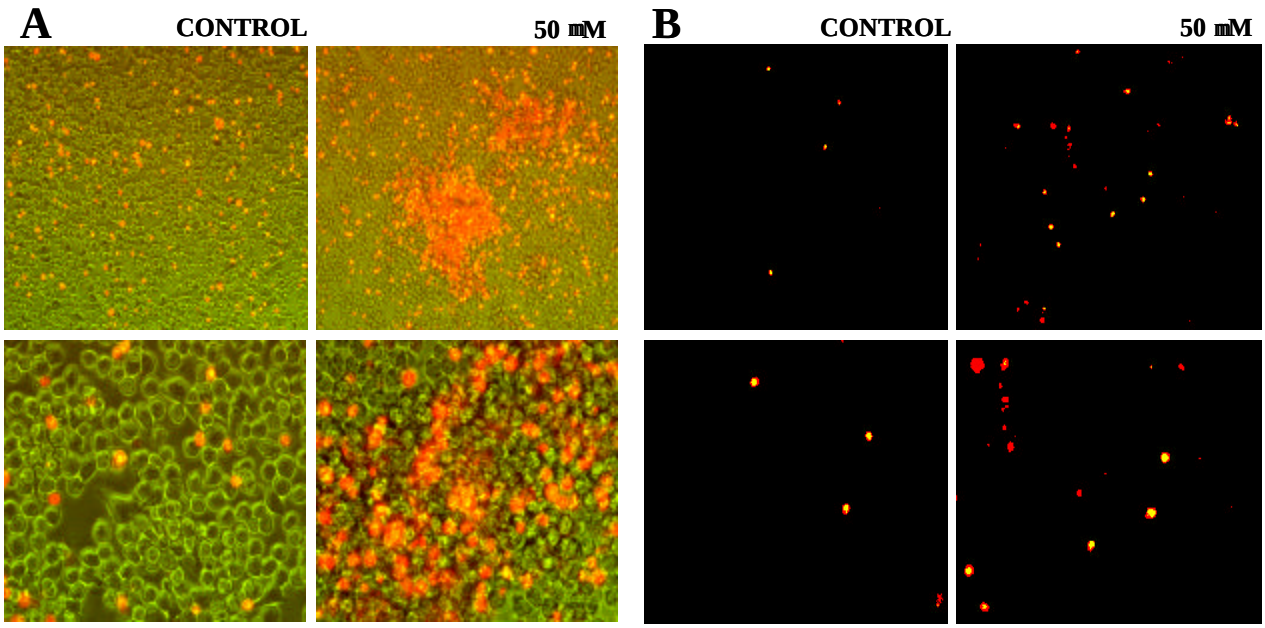

Fig. 6.2. Nuclear staining with propidium iodide (PI) revealing necrotic death in BV-2 cells (a) and primary astrocytes (b) after treatment with FB_1 at 50 μM concentration for 4 (BV-2) or 8 days (prim. astrocytes). Characteristical clusters formed by necrotic BV-2 are presented (a). Experiment repeated independently three times. Representative micrographs are shown.

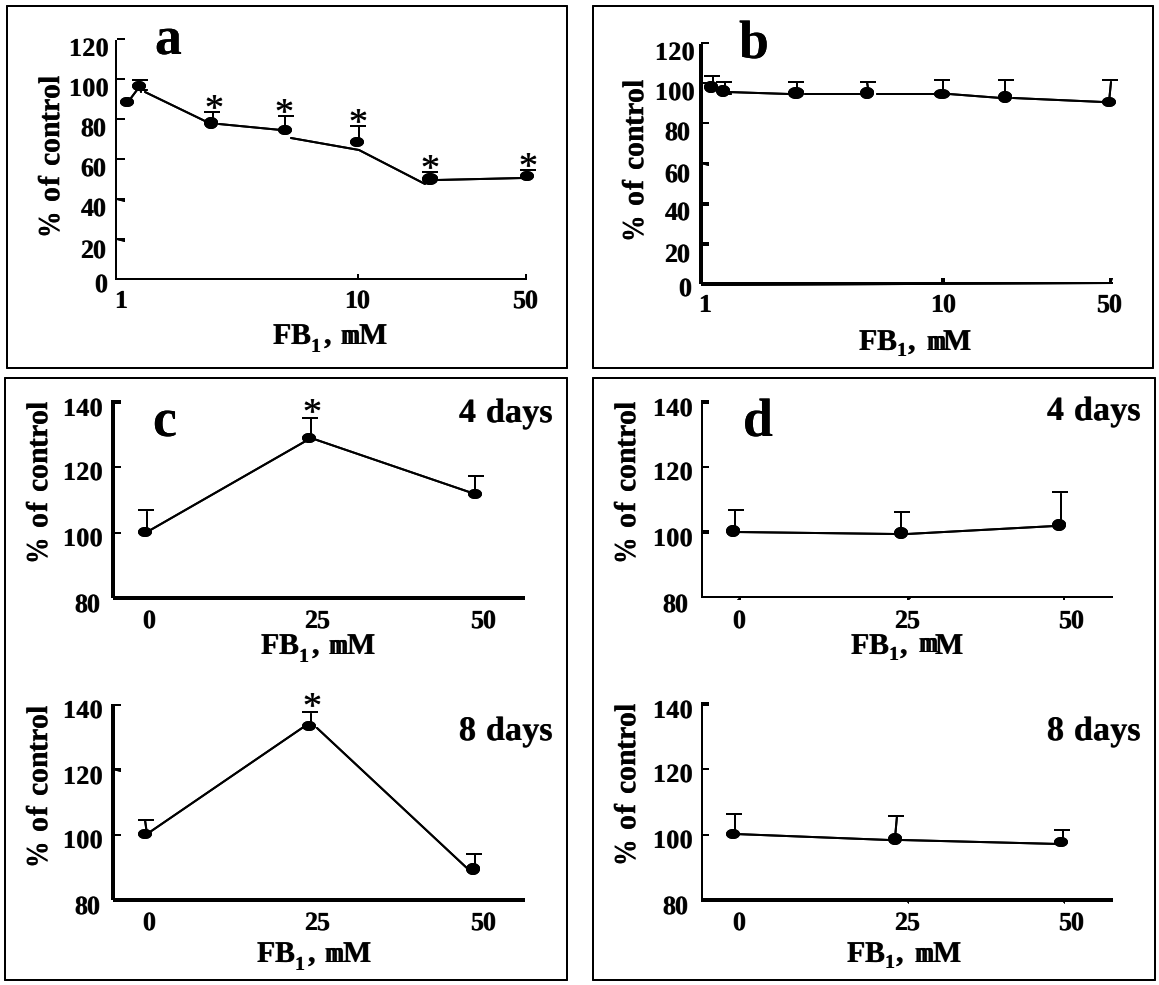

Fig. 6.3. Activity of succinate dehydrogenase after treatment with FB_1 at selected concentrations in (a) BV-2, (b) N2A (both after 4 days), (c) primary astrocytes and (d) cortical neurons. Mean ± SE (n = 5) from a representative experiment repeated independently three times. * $p < 0.05$.

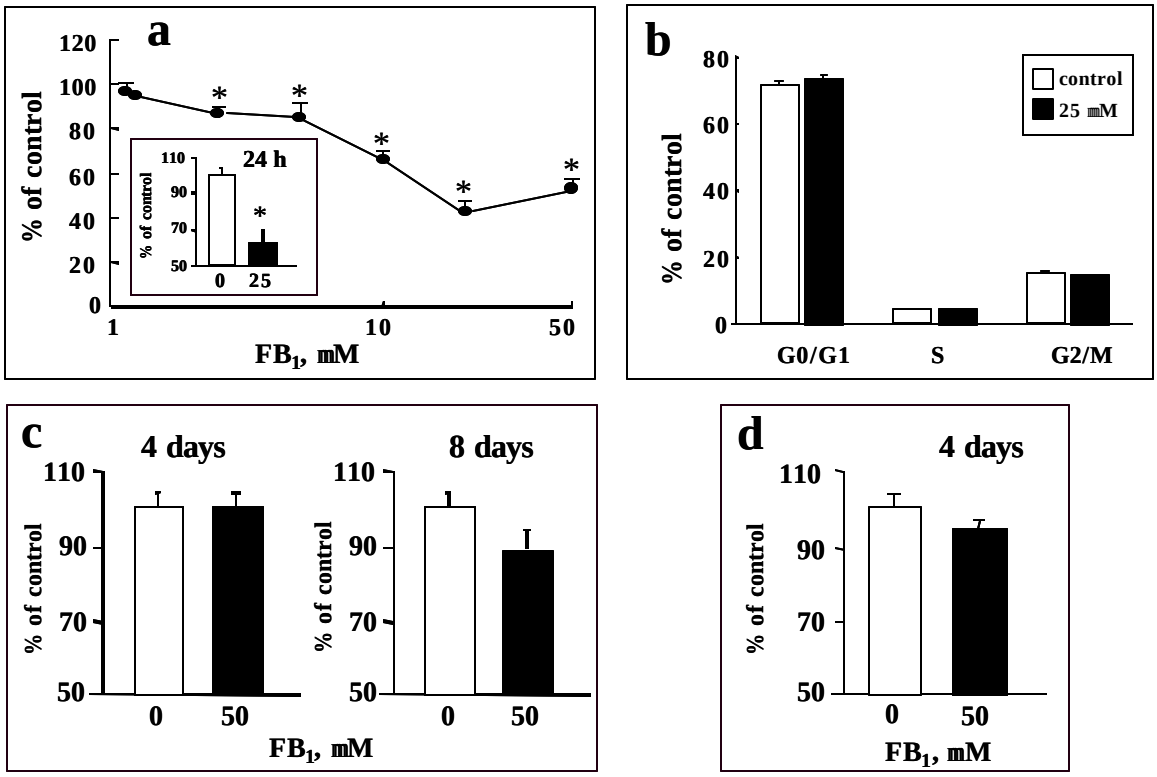

Fig. 6.4. Incorporation of [^{3}H] thymidine (4 days) after BV-2 cells were treated with FB_1 at selected concentrations for 4 days (a) and 24 h (a-inset). Phase profiles of the cell cycle in BV-2 (b) cells after 4-day treatment with FB_1 at 25 µM concentration (b). Incorporation of [^{3}H] thymidine after primary astrocytes (c) and N2A (d) cells were treated with FB_1 at 50 µM concentration for 4 and/or 8 days. Mean ± SE (n = 5) from a representative experiment repeated independently three times. * $p < 0.05$.

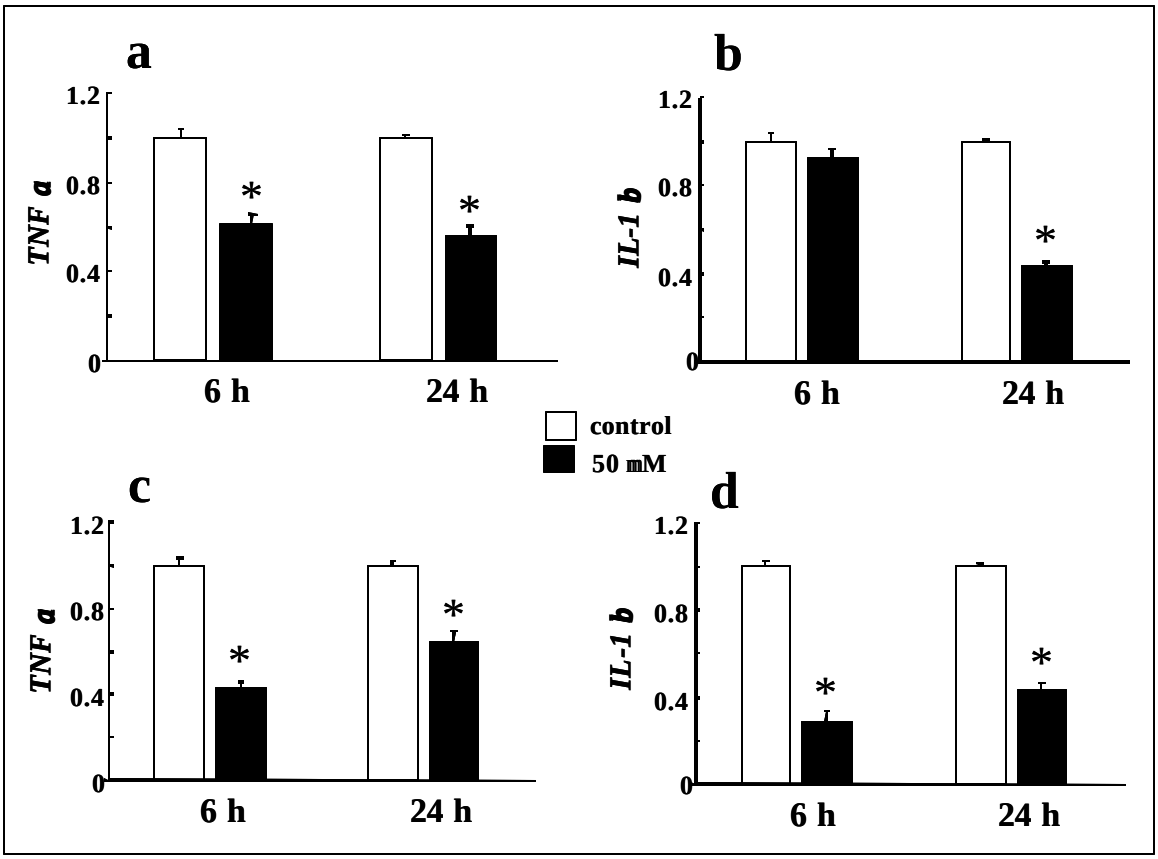

Fig. 6.5. Expression profile of selected proinflammatory cytokines determined by real-time PCR in BV-2 (a and b) cells and primary astrocytes (c and d) treated with FB_1 at 50 μM concentration for 6 and 24 h. BV-2 cells: relative expression levels of TNFα (a) and IL-1β (b) after 6 and 24 h, respectively. Primary astrocytes: relative expression levels of TNFα (c) and IL-1β (d) after 6 and 24 h, respectively. Presented as fold-change compared to control. Mean ± SE (n = 5) from a representative experiment repeated independently three times.* $p < 0.05$.

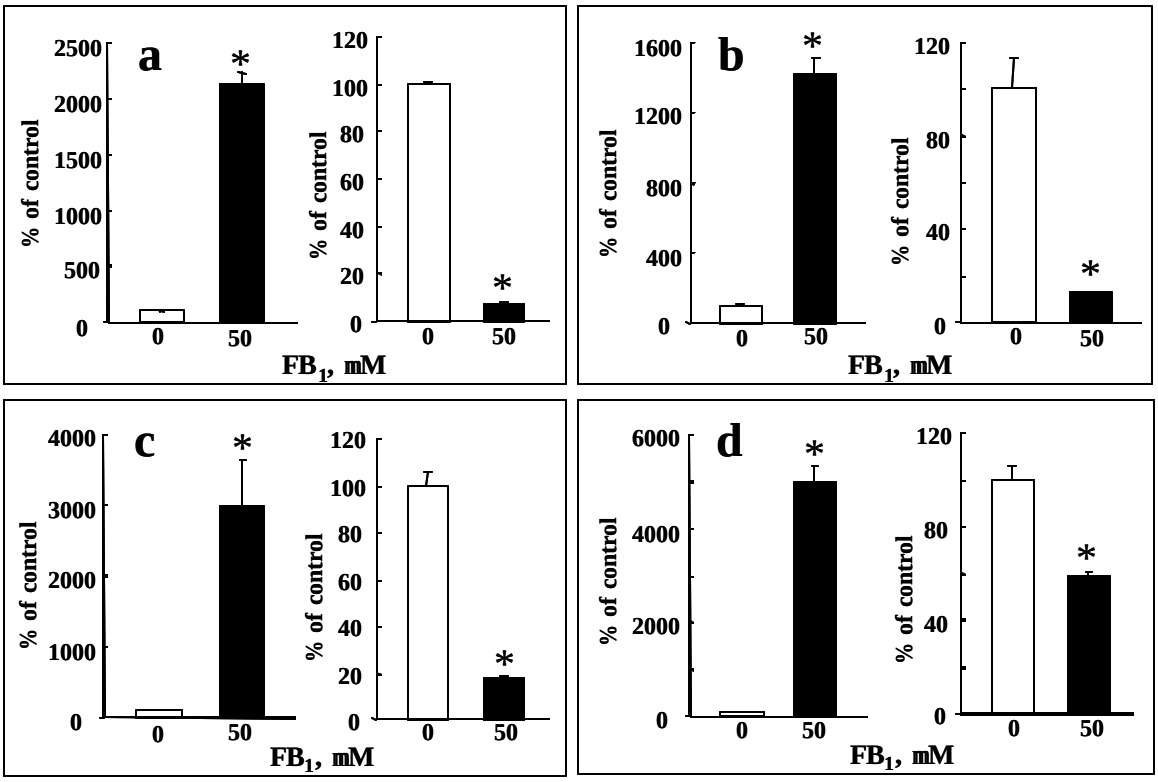

Fig. 6.6. Concentration of sphinganine and sphingosine in BV-2 (a), primary astrocytes (b), N2A (c) cells and cortical neurons (d) after treatment with FB_1 at 50 μM concentration for 4 (BV-2 and N2A) and 8 (primary astrocytes and cortical neurons) days. Mean ± SE (n = 5). Different letters on the top of bars denote a statistical significance at $p < 0.05$.

CHAPTER 7

SUMMARY AND CONCLUSIONS

Fumonisin B_1 (FB_1), produced by *Fusarium verticillioides*, is a widespread mycotoxin contaminating various cereals, predominantly corn. Ingestion of FB_1 causes number of species-, organ- and gender-specific disorders in farm animals. In humans, exposure to FB_1 is associated with cancer incidences and it was recently proposed that the consumption of fumonisin constitutes a risk factor for human neural tube defects. Wide use of corn-based foods destined for both human and animal consumption poses a potential health threat for all species that may be potentially exposed to fumonisin. The equine leukoencephalomalacia (ELEM) is the most frequently diagnosed FB_1-induced disorder affecting animals. Inhibition of ceramide synthase resulting in disruption of sphingolipid metabolism and followed by the accumulation of free sphingoid bases, formation of free sphingoid base-1-phosphates, and depletion of complex sphingolipids is a key biochemical effect of FB_1 in tissue. The pathophysiological mechanisms leading to ELEM are unclear including the question as to what extent, if at all, the direct effects of FB_1 upon brain tissue contribute to this disorder.

The objectives of the present study were to (1) investigate the modulation of sphingolipid biosynthesis and pro-inflammatory signaling in mouse brain during co-existing LPS-dependent disruption of blood-brain barrier, to (2) evaluate the morphology, sphingolipid biosynthesis and proinflammatory signaling in mouse brain after direct intracerebroventricular infusion of FB_1 and to (3) examine the sensitivity of murine brain immunocompetent cells and neurons in response to various time- and dose-dependent FB_1 treatments.

In the first study it was established that the pre-exposure to the LPS resulted in the disruption of blood brain barrier (BBB) as indicated by the increased accumulation of sodium fluorescein in mouse brain. After the FB_1 was administered, the accumulation of sphinganine,

a biomarker for FB_1 availability to the tissue, and the modulation of proinflammatory cytokines after repeated FB_1 treatment followed in brain. Accumulation of sphinganine in selected brain regions also occurred when mice were treated with FB_1 alone. These findings demonstrate that in the adult mouse brain sphingolipid metabolism is vulnerable to the exposure of FB_1 when accompanied by the endotoxin-related damage to the BBB and that the LPS modifies the FB_1-effects upon the inflammatory signaling. Whether the modulation of proinflammatory cytokines in brain after combined LPS + FB_1 treatment would or would not promote neurotoxicity cannot be inferred based on the presented data. The inhibition of the sphingolipid metabolism in brain after FB_1 treatment alone suggests that limited transfer of the mycotoxin across the BBB exists.

The following study investigated homeostasis of the central nervous system (CNS) after the direct sub-acute administration of FB_1 to the brain via the intracerebroventricular (icv) infusion bypassing the protective blood-brain barrier (BBB). The icv infusion of FB_1 led to the neuronal degeneration in cortex concurrent with the accumulation of free sphinganine in all analyzed brain regions and increase of free sphingosine in cortex. Also, the icv treatment with FB_1upregulated the expression of all proinflammatory cytokines assayed in cortex. These effects were not followed by the depletion of cortical complex sphingolipids. In mice infused FB_1 via sc route there was a significant accumulation of sphinganine and upregulation of IL-1β expression in cortex. Results indicate that the adult murine brain is vulnerable to the direct exposure of FB_1 when the protective BBB barrier is bypassed. The observed inhibition of the sphingolipid metabolism in cortex after systemic sc infusion of FB_1, can be compared to the similar findings demonstrated in the first study.

The third study evaluated the sensitivity of immunocompetent and neuronal cells (immortalized and primary cultures) from murine brain in response to various modes of time- and dose-dependent treatment with FB_1. Results show that FB_1 caused necrotic cell death in both BV-2 and primary astrocytes. The FB_1 treatment inhibited the proliferation of BV-2 cells but did not alter the progression of cell cycle phases. These effects were simultaneous with the downregulation of proinflammatory signaling in both primary astrocytes and BV-2 cells. The N2A cell line and cortical neurons were resistant to the employed doses of FB_1. In all cells the FB_1 treatment disrupted the sphingolipid metabolism, as indicated by the accumulation of free sphinganine and decrease of free sphingosine. It can be inferred from this study that in culture, immunocompetent cells of murine brain but not of neuronal origin are vulnerable to the FB_1-dependent disruption of sphingolipid metabolism. This observation indicates that glial cells, namely microglia and astrocytes, may be a primary targets in the FB_1-induced neurotoxicity.

The FB_1-induced disruption of sphingolipid metabolism in brain preceded by the LPS treatment indicates that stressors known to compromise the BBB integrity, namely endotoxemia caused by gram-negative organisms, may facilitate the penetration of fumonisin to the CNS. The evident neuronal degeneration induced by FB_1 when the protective BBB barrier was bypassed, shows that the observed neurotoxicity was due to a direct effect of FB_1 on brain tissue and that the adult murine brain is vulnerable to the direct exposure of FB_1. The simultaneous lack of complex sphingolipid disruption after FB_1 treatment indicates that the FB_1-induced damage of white matter, characteristic of the ELEM disease, may be preceded by the FB_1-induced neuronal death. The relative resistance of isolated neurons in culture to FB_1

treatment coupled with the evident sensitivity of glial cells exposed to FB_1 both *in vivo* and *in vitro* suggests that neuronal death observed *in vivo* could be a secondary effect mediated in part via CNS-borne immunocompetent cells.

Summarizing, the data presented in this dissertation indicates that the fumonisin B_1 displays evident potential for neurotoxicity in brain when the availability of the toxin is not restricted to this organ. It is suggestive that fumonisin B_1 via direct toxic effects upon the CNS could at least partly contribute to the ELEM disorder diagnosed in *equide*. The further studies investigating the mechanisms underlying the development of ELEM are warranted and could aid the prevention and treatment of this disease in the future.