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Inducible nitric oxide has protective effect on fumonisin B₁ hepatotoxicity in mice via modulation of sphingosine kinase

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Abstract

Fumonisin B₁, a natural mycotoxin, is an inhibitor of ceramide synthase causing marked dysregulation of sphingolipid metabolism in cells. This mycotoxin causes accumulation of free sphingoid bases (sphingosine and dihydrosphingosine or sphinganine) and their metabolites, important messengers involved in signal transduction leading to either cell survival or death. Free sphingoid bases are known apoptotic molecules whereas their respective 1-phosphates are protective. We previously reported that fumonisin B₁ caused sphingosine kinase (SPHK) induction along with the increase of serine palmitoyltransferase (SPT). Fumonisin B₁ also increased inducible nitric oxide synthase (iNOS) expression. In the current study we employed mice strain with the targeted deletion of iNOS gene (Nos-KO) to evaluate the role of nitric oxide (NO) on fumonisin B₁-induced hepatotoxicity. The Nos-KO mice exhibited increased hepatotoxicity after subacute fumonisin B₁ exposure compared to their wild type counterparts, the liver regeneration was lower in Nos-KO compared to that in the WT mice. Increased hepatotoxicity in Nos-KO was not related to the extent of free sphingoid base accumulation after fumonisin B₁ treatment; however, it was accompanied by a lack of fumonisin B₁-induced SPHK induction. The fumonisin B₁-induced SPT was unaffected by lack of iNOS gene. Deletion of iNOS gene did not prevent fumonisin B₁-dependent induction of inflammatory cytokines, namely tumor necrosis factor α , interferon γ and interleukin-12. The lack of fumonisin B₁-induced SPHK induction in Nos-KO was supported by a similar effect on phosphorylated metabolites of sphingoid bases; the equilibrium between sphingoid bases and their phosphates is maintained by SPHK. We therefore conclude that iNOS induction produced by fumonisin B₁ modulates SPHK activity; the lack of iNOS prevents generation of sphingosine 1-phosphate and deprives cells from its protective effects.

1. Introduction

Fumonisin B₁, a mycotoxin produced by *Fusarium verticillioides* and related species commonly found in corn and other cereals, causes gender, age, dose-dependent toxic effects in farm and laboratory animals (Marasas. 2001). It was reported to be an etiological agent for equine leukoencephalomalacia and porcine pulmonary edema. Fumonisin B₁ was shown as hepatotoxic and nephrotoxic in mice and rats. The National Toxicology Program study reported

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renal carcinoma in male Fischer 344 rats and hepatic cancer in female B6C3F mice (Howard et al., 2001). In the Transkei region of South Africa a high incidence of esophageal cancer was associated with increased fumonisin B₁ consumption (Sydenham et al., 1990). International Agency for Research on Cancer (1992) classified fumonisin B₁ as possibly carcinogenic to humans or a Group 2B carcinogen.

Fumonisin B₁ is structurally similar to sphingoid bases and interferes with their incorporation into ceramide by inhibition of ceramide synthase (sphinganine or sphingosine *N*-acyltransferase, Wang et al., 1991). It is well established that the toxic effects of fumonisin B₁ are directly related to the accumulation of free sphingoid bases, sphingoid base metabolites, and depletion of more complex sphingolipids (Riley et al., 1996). The mechanism as how the disruption of sphingolipid metabolism leads to cellular death is not clear, the downstream toxic mechanisms involved with fumonisins may be complex and involve several molecular sites. Free sphingoid bases are apoptotic to cells whereas their respective 1-phosphates are protective (Le Stunff et al., 2002; Pyne and Pyne, 2000). Cells exposed to fumonisin B₁ *in vitro* and *in vivo* undergo a mixture of necrotic and apoptotic cell death (Tolleson et al., 1996). We have previously demonstrated increased apoptosis in liver and kidney of mice after a short-term treatment with fumonisin B₁ (Sharma et al., 1997); the hepatopathy and nephropathy were directly correlated with the accumulation of sphinganine in tissues (Tsunoda et al., 1998).

Ceramide, an important sphingolipid involved in cell homeostasis, is produced by *N*-acylation of sphinganine and subsequent desaturation or by hydrolysis of sphingomyelin and is known to induce apoptosis in cells (Pettus et al., 2002). The toxic property of ceramide is likely shared by sphingosine or possibly even sphinganine (Le Stunff et al., 2002). However, the involvement of ceramide in the initiation or inhibition of the fumonisin B₁-induced apoptotic process is unclear (Tsunoda et al., 1998). Sphinganine, an intermediate in the *de novo* sphingolipid biosynthetic pathway, is present in healthy cells at very low levels unless downstream enzymes such as ceramide synthase are inhibited (see Fig. 1 for schematic presentation and He et al., 2006 for enzyme processes involved). While free sphinganine is clearly involved in the cellular effects of fumonisins, decreased ceramide biosynthesis, elevated levels of sphingoid base 1-phosphates; decreased levels of glucosyl ceramide and decreased lipid raft associated glycosylphosphatidylinositol anchored membrane proteins are all potential contributors to the cellular consequences of disrupted sphingolipid metabolism (He et al., 2001; Kim et al., 2001; Merrill et al., 2001; Riley et al., 2001).

The involvement of inflammatory cytokines in fumonisin B₁-induced murine hepatotoxicity has been shown from our laboratory. Cytokines, particularly tumor necrosis factor α (TNF α) and interferon γ (IFN γ) are involved in the untoward outcomes after fumonisin B₁ exposure (Dugyala et al., 1998; Sharma et al., 2001, 2003a). These cytokines are produced by the hepatic innate immune system; depletion of both the Kupffer cells (hepatic macrophages) and T helper cells prevents fumonisin B₁ hepatotoxicity in mice (He et al., 2005; Sharma et al., 2006). Fumonisin B₁ also induced other cytokine, namely interleukin (IL)-12 in mouse liver (Bhandari et al. 2002).

The influence of fumonisin B₁ on nitric oxide (NO) production has been reported. Murine macrophage cell line (J744A.1) pretreated with fumonisin B₁ exhibited increased lipopolysaccharide (LPS)-induced NO production (Meli et al., 2000); the effects were concentration-dependent both for LPS and fumonisin B₁. These effects were related with increased production of inducible nitric oxide synthase (iNOS); however, fumonisin B₁ without LPS was not studied in this report. Later Dresden-Osborne and Noblet (2002) reported a similar finding when murine RAW264.7 macrophage cultures were treated with fumonisin B₁ along with LPS or phorbol myristate acetate; the effects of LPS + fumonisin B₁ were antagonized by simultaneous presence of *N*-nitro-L-arginine methyl ester, an inhibitor of iNOS. In both of

these studies the levels of iNOS correlated with fumonisin B₁-induced cytotoxicity; however, if one was the cause of other was not identified. In our preliminary studies we observed induction of iNOS in liver when female mice were treated with fumonisin B₁ for 5 days (Suzuki et al., 2005). It was therefore of interest if NO production by iNOS was involved in fumonisin B₁-induced hepatotoxicity in mice. We hypothesized that induction of iNOS modulates fumonisin B₁ liver toxicity in mice. Since the role of iNOS derived NO is both toxic and protective in different tissues (James et al., 2003 Cals-Grierson and Ormerod, 2004); it was difficult to predict its role in fumonisin B₁ hepatotoxicity. In the current study we employed iNOS knockout strain of mice to evaluate the role of iNOS; since NO production is involved in function and production of inflammatory cytokines, the gene expression of selected cytokines was also evaluated.

2. Materials and methods

2.1. Animals

Six week old male mice (16-18 g body weight) were procured from Jackson Laboratories (Bar Harbor, ME). The B6.129P2-Nos2^{tm1Lau}/J mice (Stock # 002609) with NOS-2 gene deleted, henceforth referred as Nos-KO, and their wild-type counterparts, C57BL/6J (Stock # 000664), referred as WT, were randomly divided in four groups of 5 animals each. The Nos-KO mice were originally developed by Dr. Victor Laubach at the University of Virginia Health Sciences Center, Charlottesville (Laubach et al., 1998). Animals were acclimated for one week at the University of Georgia Animal Resources facility; all protocols complied with the Public Health Service Policy on Humane Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee.

Each animal was treated with five daily subcutaneous injections of either phosphate-buffered saline (PBS) or 2.25 mg/kg/day of fumonisin B₁ (>98% purity, from Programme on Mycotoxins and Experimental Carcinogenesis, Tygerberg, South Africa) in PBS. The dose and duration of treatment for fumonisin B₁ was optimum for hepatotoxicity and sphinganine increase in mouse liver (Tsunoda et al., 1998), and was based on our previous studies in which it produced a consistent hepatotoxic damage in male mice (Sharma et al., 2001, 2003a,b). The employed dose of fumonisin B₁ corresponds to 14 mg/kg in rodent feed, well within the range of exposures encountered with toxicity episodes of natural exposures (Dutton, 1996). Animals were weighed daily. All treatments and samplings were performed at the same time of the day (mid-mornings).

2.2. Sampling and circulating liver enzymes

Twenty four hours after the fifth and final injection, mice were euthanized by halothane and the blood was drawn by heart puncture in heparinized tubes; plasma was isolated for determination of alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Livers were removed, weighed, and aliquots were fixed immediately in neutral 10% formalin or quickly frozen in liquid nitrogen and stored at -85°C until analysis. The time of sampling was based on our observations that effects of fumonisin B₁ decline rapidly after cessation of treatment (Enongene et al., 2002). Activities of plasma ALT and AST were determined using a Hitachi 912 Automatic Analyzer (Roche Diagnostics, Indianapolis, IN). The method used for determination of the activities of these enzymes was based on a kinetic reduction of β -nicotinamide adenine dinucleotide (reduced disodium salt hydrate) in the reaction measured photometrically; the assays conform to the standard procedures recommended by the International Federation of Clinical Chemistry (Bergmeyer et al., 1986).

2.3. Determination of apoptosis and PCNA positive cells in liver

Liver sections (5 μ m) were prepared from formalin-fixed paraffinized liver samples and subjected to terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) with a peroxidase-based *in situ* Cell Death Detection kit (Roche Diagnostics, Indianapolis, IN) as described previously (Sharma et al., 2003a). The stained apoptotic cells were counted in the whole section and normalized to the unit area. Hepatocyte proliferation was determined by analysis of proliferating cell nuclear antigen (PCNA). The primary anti-PCNA antibodies (Sigma Chemical Co., St. Louis, MO) were incubated with tissue sections at 4°C overnight in a humid chamber, secondary antibodies applied and sections were stained with Vectorstain ABC kit (Vector Laboratories, Inc., Burlingame, CA, USA). The numbers of PCNA positive cells were counted under a microscope and normalized to the unit area as described (Sharma et al., 1997).

2.4. Analyses of sphingolipids

Levels of free sphinganine and sphingosine in hepatic tissues were determined after base treatment of lipid extracts by high performance liquid chromatography (HPLC) according to the methods reported previously (Tsunoda et al. 1998). The concentration of sphingoid bases, sphinganine and sphingosine, was quantified after *o*-phthalaldehyde derivation based on the recovery of the C₂₀-sphinganine (D-erythro-C₂₀-dihydro-sphingosine; Matreya, Pleasant Gap, PA) as the internal standard.

2.5. RNA isolation and semi-quantitative estimation of cytokine expression

The RNA was isolated from liver samples, converted to cDNA by reverse transcriptase (RT) and amplified by polymerase chain reaction (PCR) using the protocol described earlier (Sharma et al., 2001), and using a thermal cycler (Eppendorf Scientific, Westbury, NY). The primer sequences used for *TNFA*, *IFN γ* and *IL-12* were similar to those reported earlier (Bhandari et al., 2002); ones for *iNOS* were as shown in Table 1. The number of cycles within the exponential increase of the product and annealing temperatures were optimized for each molecule. The amplified product was separated by electrophoresis on 2% agarose gels; gels were photographed using a back-lighted UV trans-illuminator (Ultra Lum, Inc., Carson, CA). The cytokine expression was normalized against β -actin and quantified via densitometric imaging using UN-SCAN-IT software (Silk Scientific Inc., Orem, UT).

2.6. Expression of serine palmitoyltransferase, sphingosine kinase 1 and iNOS

Gene expression for SPT subunits (LCB1 and LCB2), sphingosine kinase 1 (SPHK1) in liver was analyzed by RT-PCR as described above. The expression of *SPHK2* was not attempted as we have observed in other studies that fumonisin B₁ did not alter the expression of this isoform (He et al., 2006). The respective primers and PCR conditions are listed in Table 1.

For protein analyses liver tissues were homogenized in lysis buffer [20 mM Tris-HCl pH 8.0, 137 mM NaCl, 2 mM ethylenediaminetetraacetate (EDTA), 10% glycerol, 1% Triton X-100, 100 μ M dithiothreitol, 1 mM sodium orthovanadate, 20 μ M phenylmethylsulfonyl fluoride, 10 μ g/ml of each leupeptin and aprotinin]. Lysates were centrifuged for 30 min at 42,000 $\times$ g, and supernatants subjected to western blot analysis after quantifying protein concentrations. An aliquot of 10 μ g protein from each sample was subjected to electrophoresis on polyacrylamide gels containing 10% sodium dodecylsulfate, and then transferred to polyvinylidene difluoride membranes. Equal loading and transfer of protein was confirmed by staining the membranes with reversible Ponceau S before incubation with respective primary antibodies. Proteins were detected with polyclonal antibodies against LCB1, LCB2 (BD Biosciences, Rockville, MD), SPHK1 (Oncogene, San Diego, CA), and monoclonal antibody against iNOS of mouse origin (Santa Cruz Biotechnology, Santa Cruz, CA), respectively, using enhanced

chemiluminescence detection kit (Amersham Pharmacia, Piscataway, NJ, USA). The exposure time of the film was optimized to be in the linear range before the saturation occurs.

2.7. Determination of sphingoid base 1-phosphate

Concentrations of sphingosine- and sphinganine 1-phosphates were determined by HPLC after enzymatic dephosphorylation of sphingoid base 1-phosphates as described elsewhere (Min et al., 2002). Briefly, liver aliquot was homogenized and free sphingoid bases and sphingoid base 1-phosphates were extracted and isolated in the organic (chloroform/methanol) phase and alkaline aqueous phases, respectively; recoveries of sphingoid base 1-phosphates were monitored using C₁₇-sphingosine 1-phosphate as an internal standard (Avanti Polar Lipids, Inc., Alabaster, AL). The aqueous phase containing the sphingoid base 1-phosphates was then subjected to treatment with calf intestine alkaline phosphatase (M. P. Biomedicals, Irvine CA) to release the sphingoid base backbones which were extracted into chloroform and analyzed by HPLC. It has been shown previously (Min et al., 2002) that the amount of free sphingosine in the aqueous phase is less than 1% of the free sphingosine recovered in the organic phase following the chloroform/methanol extraction; thus the sphingosine and sphinganine recovered following alkaline phosphatase treatment is most likely derived from the release of the sphingoid base backbones of the sphingoid base 1-phosphates. Since there was a likelihood that the phosphatase employed here could yield sphingoid bases from lysosphingomyelin in the cell membranes, specificity of enzymatic hydrolysis was verified using graded amounts of D-erythro-sphingosyl-phosphorylcholine (Matreya Inc., Pleasant Gap, PA), which yielded undetectable free sphingosine suggesting that lysosphingomyelin did not interfere with the assay.

2.8. Statistics

Data from these experiments were analyzed by two-way analysis of variance (ANOVA) followed by Duncan's multiple range test. Results are presented as mean $\pm$ standard error (SE). All tests were performed using an SAS computer program (SAS Institute, Cary, NC). The level of $p \leq 0.05$ was considered statistically significant.

3. Results

3.1. Deletion of iNOS increased fumonisin B₁ toxicity in mice

Fumonisin B₁ caused a slight but statistically significant decrease in body weights on day 5 of the treatment and at the time of sampling in Nos-KO strain only compared to other three groups (Fig. 2); there were no effects on relative liver, kidney, spleen or thymus weights in either strain by FB treatment (data not depicted). The decline in body weight of all groups on the day of sampling was due to taking the animals off-feed overnight after the last treatment, desirable to evaluate hepatic lesions with less glycogen contents. There were no gross or behavioral changes observed in any group during this treatment protocol. The concentrations of circulating ALT and AST were significantly higher in fumonisin B₁-treated Nos-KO mice than the similarly treated WT strain (Fig. 3). The relatively increased hepatotoxicity of fumonisin B₁ in Nos-KO was confirmed by microscopic evaluation of liver sections and the incidence of TUNEL-positive nuclei in liver. The effects of fumonisin B₁ in murine liver included apoptotic hepatocytes and occasional mitotic figures scattered throughout the liver tissue with inflammatory changes characterized by anisocytosis and cytoplasmic vacuolation (Sharma et al., 1997; Voss et al., 1995); the effects were relatively prominent in the Nos-KO strain than the WT. Relative incidence of TUNEL-positive cells in liver of the two strains is illustrated in figure 4.

3.2. Presence of iNOS is involved in hepatic regeneration

The prevalence of proliferating cells in liver after fumonisin B₁ was evaluated by PCNA-positive cells; results are presented in Figure. 4. The fumonisin B₁-treated Nos-KO mice had only 40% as much proliferating cells as the fumonisin B₁-treated WT, suggesting that iNOS is important for liver regeneration. The increased hepatotoxicity of fumonisin B₁ in Nos-KO is very likely related to decreased hepatocyte regeneration; however, there is also a possibility that increased hepatotoxicity in Nos-KO mice could be independent of hepatocyte regeneration.

3.3. Increased hepatotoxicity was not due to increased free sphingoid bases in liver

Since the fumonisin B₁ toxicity is related to the levels of free sphingoid bases particularly that of sphinganine, the sphingoid base levels were measured in liver; results are presented in Figure 5. Both sphingosine and sphinganine levels were increased after fumonisin B₁ treatment of mice in the two strains; however, the increase was much lower in the Nos-KO mice compared to that in WT. In fact, the fumonisin B₁-induced increase of sphingosine in Nos-KO was not statistically significant and that of sphinganine was nearly 4 times lower in the Nos-KO than in the WT mice. It is therefore apparent that increased hepatotoxicity of fumonisin B₁ in Nos-KO was not due to accumulated sphingoid bases alone.

3.4. Expression of iNOS did not influence fumonisin B₁-induced inflammatory cytokines

Figure 6 presents the mRNA expression of *TNFα*, *IFNγ* and *IL-12* in liver of mice after treatment with fumonisin B₁. The fumonisin B₁-mediated induction of these cytokines was comparable in the two strains. This figure also presents the mRNA and protein expression of iNOS; fumonisin B₁ produced nearly 8-fold increase in the iNOS mRNA in WT mice. This increase was somewhat related to the protein expression for iNOS in this strain. No iNOS mRNA was detectable in Nos-KO mice, confirming the deletion of iNOS gene; no protein was also observed in this strain (not depicted).

3.5. Sphingosine kinase induction after fumonisin B₁ was dependent on the presence of iNOS

Treatment of WT mice with fumonisin B₁ caused an induction of SPHK1 expression as reported earlier (Suzuki et al., 2005). A significant increases in both mRNA and protein levels for SPHK1 were obvious in WT after fumonisin B₁ (Fig. 7); the effects were not noticed in Nos-KO animals. No effect of fumonisin B₁ on SPHK2 was observed (He et al., 2006). The involvement of iNOS on enzymes of sphingolipid metabolism appears to be selective on SPHK1 as neither the mRNA expression nor the protein expression for the two subunits of SPT (LCB1 and LCB2) was different in WT or Nos-KO after fumonisin B₁ treatment (Fig. 8); indeed in both strains a comparable induction of these proteins was observed.

3.6. Levels of sphingosine 1-phosphate were related to sphingosine kinase

Since the balance of free sphingoid bases and their respective phosphates is important for the toxicity outcome and the expression of SPHK1 was lower in fumonisin B₁-treated Nos-KO than the WT, the concentrations of sphingosine and sphinganine 1-phosphates were estimated in mice livers. Fumonisin B₁ increased the levels of both phosphates in liver of WT mice (Fig. 9); comparable increases in phosphate concentrations were not observed in the liver of Nos-KO mice. The sphingoid phosphate levels therefore appear to be in agreement with the expression of SPHK1 indicated above.

4. Discussion

Results of current study suggest that NO production by iNOS had a protective effect in fumonisin B₁ hepatotoxicity in mice, the species where liver is the main target of this

mycotoxin. The mice deficient in iNOS provided a greater extent of hepatotoxicity than the corresponding wild type strain. Not only liver toxicity was increased but fumonisin B₁-treated mice in the Nos-KO group lost body weight compared to all other groups suggesting a generalized increase in toxicity. The protective role of iNOS in liver has been demonstrated in other models of hepatotoxicity; when Nos-KO mice were treated with the hepatotoxic drug acetaminophen, it increased lipid peroxidation only in animals lacking the iNOS gene (James et al., 2003). These authors suggested that NO played a role in controlling lipid peroxidation and reactive oxygen/nitrogen species were involved in mediating the toxic responses. A protective role of iNOS was also described against carbon tetrachloride liver toxicity in mice; TNF α was a putative mediator of hepatotoxicity in this case (Morio et al., 2001). Nitric oxide provided protection against TNF α -induced apoptosis in osteoblasts (Chae et al., 2006).

The role of inducible NO has been shown both toxic and protective in different models. It was shown that burn trauma related upregulation of iNOS leading to peripheral vasodilatation; activation of nuclear factor κ B (NF- κ B) and prompting upregulation of transcription and translation of numerous inflammatory cytokines (Horton, 2003). Nitric oxide may also interact with superoxide radical to yield peroxynitrite, which is highly reactive and mediates tissue injury. The ultraviolet light mediated iNOS induction was also implicated in skin melanogenesis and immune suppression (Cals-Grierson and Ormerod, 2004). Nitric oxide is a known mediator of nitrosative stress (Kroncke, 2003) and it increased cellular levels of ceramide in keratinocytes and apoptosis in human fibroblasts (Gallala et al., 2004). On the other hand, NO protected myocardial tissue from ischemic damage (Dawn and Bolli, 2002). The wound healing process triggered by NO is supposedly diverse; it supports inflammation while increasing angiogenesis and cellular proliferation (Schwentker et al., 2002).

The paradoxical effects of induced NO are possibly dependent on the involvement of the transcription factor, NF- κ B in iNOS induction or the role of NO in cytokine production. It is generally believed that activation of NF- κ B by LPS or cytokines is an important mediator of iNOS induction (Kleinert et al., 2003). Immunomodulatory compounds that increase cytokine levels generally inhibit iNOS induction at the transcriptional or posttranscriptional levels (Kim et al., 2002). On the other hand, NO is an important mediator of NF- κ B activation; reactive oxygen and nitrogen species are both known for the activation of redox-sensitive transcription factor, NF- κ B (Janssen-Heininger et al., 2000).

Regulation of iNOS expression depends on various cellular signals, in particular some of the inflammatory cytokine pathways (Kleinert et al., 2003). In addition to the NF- κ B, the interferon regulatory factor-1 (IRF-1) and signal transducer and activator of transcription 1 α (STAT-1 α) were reported to activate iNOS. The TNF α is a known activator of NF- κ B and would in turn induce iNOS. TNF α -dependent production of iNOS was protective against acute liver injury (Muntane et al., 2000). The inflammatory cytokine IL-1 is also a potent inducer of iNOS (Dayer, 2002). We have earlier reported that fumonisin B₁ activated NF- κ B and TNF α in the porcine kidney epithelial cell line (Gopee and Sharma, 2004). Since fumonisin B₁ has been reported to increase expression of all inflammatory cytokines (Bhandari and Sharma, 2002; Sharma et al., 2003b), it was not unexpected that this mycotoxin will induce iNOS. In the current study both gene expression and translated product for iNOS were increased after a five-day treatment of mice with fumonisin B₁. Induction of iNOS by fumonisin B₁ in murine macrophages has been reported earlier (Meli et al., 2000; Dresden-Osborne and Noblet, 2002) and since it is well known that macrophages are the rich source for iNOS (Chien et al., 2005) it may be contemplated that Kupffer cells in liver were producing iNOS. Kupffer cells are involved in fumonisin B₁-induced hepatotoxicity in mice; depletion of Kupffer cells by gadolinium chloride significantly attenuated fumonisin B₁ hepatotoxicity (He et al., 2005).

It has also been suggested that NO produced by iNOS stimulated production of various cytokines (Schwentker et al., 2002). Since nitrosative stress is a known inducer of transcription, especially via activation of NF- κ B, it will be expected that NO was a cytokine inducer (Kroncke, 2003). However, in the case of fumonisin B₁ hepatotoxicity in mice it appears that iNOS-induced NO was not involved in the regulation of cytokine induction; in the current study fumonisin B₁ treatment had comparable increased expression of *TNF α* , *IFN γ* and *IL-12*, with or without the presence of iNOS.

The low level of free sphinganine in the Nos-KO mice was surprising. In the presence of fumonisins, the most likely route for elimination of accumulated free sphinganine is via phosphorylation to sphinganine 1-phosphate and subsequent decomposition via sphinganine kinase lyase. The fact that serine palmitoyltransferase was induced and SPHK1 was not induced in the Nos-KO mice suggests that either the ceramide synthase in Nos-KO mouse liver is insensitive to fumonisins or that there is an alternative pathway for elimination of accumulated sphingoid bases. Sphinganine *N*-acyl transferases that are insensitive to fumonisin inhibition have been described (Riebeling et al., 2003) but alternative pathways for the metabolism of free sphinganine have not been described.

A major contribution of the present study is to explain at least one of the mechanisms in the partial protection of liver injury afforded by iNOS after fumonisin B₁ treatment. Nos-KO mice had decreased levels of sphingosine 1-phosphate after fumonisin B₁ treatment compared to their wild type counterparts; this was accomplished by a lack of SPHK1 induction in the strain lacking iNOS gene. Sphingosine 1-phosphate provides survival signals in cells (Pyne and Pyne, 2000), that will counteract the apoptotic signaling initiated by accumulated free sphingoid bases in cells after fumonisin B₁ treatment. Both SPHK and sphingosine 1-phosphate are important in providing pro-growth and anti-apoptotic signaling (Maceyka et al., 2002). Our laboratory has reported earlier that the activity of SPHK was responsible for relative resistance to fumonisin B₁ in human embryonic kidney (HEK-293) cells (Sharma et al., 2004). It has been reported that TNF α activates both NF- κ B and SPHK, thereby providing protective mechanism; and also activates sphingomyelinase that will increase cellular ceramide contents (Spiegel and Milstein, 2000). In addition TNF α can stimulate death-domain containing TNF α receptors or initiates recruitment of death-domain molecules (Darnay and Aggarwal, 1997). Fumonisin B₁ is a known inducer of TNF α and NF- κ B (Gopee and Sharma, 2004) and it appears that NF- κ B is a mediator of SPHK1 induction but not of SPT, another important enzyme in sphingolipid metabolism. The fumonisin B₁-induced SPHK1 was not observed in Nos-KO, while the effect on SPT was similar to that in the WT strain in the current study. The pro-growth effects of increased sphingosine 1-phosphate were supported by relatively less frequency of apoptosis and higher regeneration of hepatocytes in mice with intact iNOS gene after fumonisin B₁ treatment.

In summary, the present study suggests a partial protective role of iNOS in fumonisin B₁-induced hepatotoxicity in mice. Fumonisin B₁ induced both SPT and SPHK1, the former will provide higher accumulation of sphinganine while the latter will increase sphingosine 1-phosphate levels in cells, the two molecules with opposite outcomes on cell survival. The mechanism involved in fumonisin B₁ hepatotoxicity are indeed complex and depend on opposing signals, NO production favoring the survival signals. The TNF α stimulated SPHK1 production (Xia et al., 1999) may involve signaling via NO as this effect was lost without the presence of iNOS.

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Figure 1.

Schematic representation of sphingoid bases and their biotransformation. Only major factors relevant in this study are indicated; the original synthesis of sphinganine and ultimate metabolites of their phosphates are not shown. Sphingosine kinase converts both sphinganine and sphingosine to their respective phosphates; very little sphinganine 1-phosphate is formed because of the low levels of sphinganine in cells as this base is rapidly incorporated in ceramide or sphingomyelin. The sphingoid 1-phosphates are hydrolyzed by phosphatases to original bases or may be degraded by respective lyase. Ceramide, sphingosine and sphinganine are putative apoptotic agents whereas the sphingoid 1-phosphates impart survival properties in cells.

Figure 2. Body weights of mice after treatment with fumonisin B₁. On days 5 and 6 the body weights in FB-treated Nos-KO were lower than saline-treated WT or Nos-KO and also fumonisin B₁-treated WT groups. Mean±SEM of 5 animals each. * $p < 0.05$ compared to other groups.

Figure 3.

Five-day treatment of mice with fumonisin B₁ produced an increase in alanine aminotransferase (ALT) levels of both WT and Nos-KO strains; however, the increase of ALT was significantly higher in fumonisin B₁-treated Nos-KO than in similarly treated WT mice. The level of circulating aspartate aminotransferase (AST) increased only in Nos-KO mice after fumonisin B₁ treatment; AST was not increased after fumonisin B₁ treatment in WT. Mean $\pm$ SEM of 5 animals each. * $p < 0.05$ to respective saline-treated group, # $p < 0.05$ compared to fumonisin B₁-treated WT.

Figure 4.

TUNEL and PCNA positive cells in liver after FB treatment. Incidence of TUNEL positive cells was higher in fumonisin B₁-treated Nos-KO than in similarly treated WT. The increase in PCNA positive cells in Nos-KO was lower than in WT after fumonisin B₁ treatment. Mean $\pm$ SEM of 5 animals each. * p < 0.05 to respective saline-treated group, # p < 0.05 compared to fumonisin B₁-treated WT.

Figure 5.

Concentration of sphingoid bases sphinganine and sphingosine in the liver of WT and Nos-KO mice after saline or fumonisin B₁ exposure. The treatment of Nos-KO with fumonisin B₁ did not cause an increase of sphingosine in liver, the fumonisin B₁-induced increase in liver sphinganine was statistically different in Nos-KO compared to similarly treated WT mice. Mean±SEM of 5 animals each. **p* < 0.05 compared to respective saline-treated group, #*p* < 0.05 compared to FB-treated WT.

Figure 6.

Treatment of mice with fumonisin B₁ modulated the expression of inflammatory cytokines. Normalized mRNA expression of cytokines to that of β -actin, a housekeeping protein, is depicted. The increases of fumonisin B₁-induced $TNF\alpha$, $IL-12$ and $IFN\gamma$ were similar in both WT and Nos-KO. The expression of iNOS is also shown in lower right panel, the inset represents iNOS protein expression analyzed by a western blot. Mean $\pm$ SEM (n=5). * $p < 0.05$ to respective saline treated group.

Figure 7.

Expression of SPHK1 in mouse liver after fumonisin B₁ treatment measured by RT-PCR (left) and western blot (right, also showing representative gels). Fumonisin B₁ induced SPHK1 in WT but not in the Nos-KO strain. Mean±SE (n=5). **p*< 0.05 to respective saline treated group, # *p*< 0.05 compared to FB-treated WT.

Figure 8.

Treatment of mice with fumonisin B₁ induced expression of SPT subunits in both WT and Nos-KO. Upper panels: Expression of mRNA was determined by RT-PCR and normalized against β-actin. Lower panels: Treatment of mice with FB induced protein levels of SPT subunits. Enzyme levels were determined by western blotting, representative gels are presented. Mean±SE (n=5). **p*< 0.05 to respective saline treated group.

Figure 9.

Levels of sphingosine 1-phosphate and sphinganine 1-phosphate in liver with or without fumonisins B₁ treatment of the two strains. Mean±SE (n=5). **p* < 0.05 to respective saline treated group, # *p* < 0.05 compared to FB-treated WT.

Table 1

Primers and PCR conditions for genes*

Genes	Primers	Annealing temperature (°C)	Number of cycles
<i>iNOS</i>	sense 5' CAC CTT GGA GTT CAC CCA GT 3' anti-sense 5' ACC ACT CGT ACT TGG GAT GC 3'	54	28
<i>LCB1</i>	sense 5' AAG AAC CAC CCT GCT CTC AA 3' anti-sense 5' CAG GCG CTC TTC TAA ATC CA 3'	54	30
<i>LCB2</i>	sense 5' CAG ACT GTC AGG AGC AAC CA 3' anti-sense 5' ATC ACT TCA GGA AGG CGA AC 3'	54	30
<i>SPHK1</i>	sense 5' ACC GAT AAG AAG CTG AAC GCA GGA 3' anti-sense 5' ACC ACC TCA TGC ATC AGA CCA TCA 3'	55	42
β -actin	sense 5' AGC CAT GTA CGT AGC CAT CC 3' anti-sense 5' CTC TCA GCT GTG GTG GTG AA 3'	54	28

* Primers are based on Primer3 program (Whitehead Institute, Cambridge, MA). The amplification conditions include: hot start: 95 °C 5min, 1 cycle, followed by: denaturation, 94 °C 30 sec, annealing, indicated temperature 30 sec; elongation, 72 °C 1 min for indicated cycles, and finally elongation at 72 °C 1 min for 1 cycle. For all experiments, the conditions were optimized to keep the number of cycles within the range of exponential product increase.