Fumonisin B1-Induced Toxicity Was Not Exacerbated in Glutathione Peroxidase-1/Catalase Double Knock Out Mice

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Abstract
Fumonisin B1 (FB1) structurally resembles sphingolipids and interferes with their metabolism leading to sphingolipid dysregulation. We questioned if FB1 could exacerbate liver or kidney toxicities in glutathione peroxidase 1 (Gpx1) and catalase (Cat) knockout mice. While higher serum levels of thiobarbituric acid reactive substances (TBARS) and sphinganine (Sa) were measured in Gpx1/Cat knockout mice (Gpx1/Cat KO) than wild type mice after 5 days of FB1 treatment, serum levels of alanine aminotransferase (ALT), sphingosine-1 phosphate (So-1-P), and sphinganine-1 phosphate (Sa-1-P) were found to be relatively low. Although Sa was highly elevated in Gpx1/Cat KO mice and wild mice, lower levels of So and Sa were found in both the kidney and liver tissues of Gpx/Cat KO mice than wild type mice after FB1 treatment. Paradoxically, FB1-induced cellular apoptosis and necrosis were hastened under oxidative stress in Gpx1/Cat KO mice.

Key Words: Fumonisin B1, Catalase, Glutathione peroxidase 1, Sphingosine, Sphinganine

INTRODUCTION
Sphingolipids are composed of sphingosine, sphinganine (dihydrosphingosine), or a phytosphingosine backbone covalently linked to fatty acid molecules (Ebenezer et al., 2016). Although mammalian cells are unable to synthesize sphingosine from dihydrosphingosine (Chalfant and Spiegel, 2005), ceramidases act on ceramide to generate sphingosine (So), which can be converted into So-1-P by sphingosine kinases (Takabe et al., 2008). Sphingolipid dysregulation has been tied with obesity, insulin resistance, hyperglycemia, dyslipidemia and hypertension involving various extracellular and intracellular signals (Aburasayn et al., 2016; Chen et al., 2016; Kuzmenko and Klimentyeva, 2016). However, emerging evidence is unraveling the crucial role of sphingolipid (Cer and So-1-P) metabolism in regulating liver injury, repair, and regeneration (Nojima et al., 2015).

Food containing fusarium mycotoxins poses considerable public health hazard worldwide (Lin and Guo, 2016), particularly in areas where regulatory measures are either lacking or poorly enforced (Alberts et al., 2016). Various fusarium mycotoxins (fumonisins) are produced from Fusarium fujikuroi, Fusarium proliferatum, and Fusarium verticillioides (Matic et al., 2013). Out of all the fumonisins, FB1 is the most prevalent in both toxicity and incidence (Raiola et al., 2015). Chemically, FB1 consists of different stereoisomers with an unsubstituted primary amine group that interferes with sphingolipid metabolism (Escriva et al., 2015) and speeds up sphingoid base accumulation which depletes complex sphingolipids (Bouhet and Oswald, 2007). While the liver and kidneys are the primary target organs for FB1 toxicity in mice, further studies unveiled that FB1 toxicity extends to aggravate necrotic enteritis in broiler chickens (Antonissen et al., 2015) and facilitate seizures in mice (Poersch et al., 2015). The molecular mechanisms behind FB1 toxicity in liver and kidneys are supposed to be linked with early events associated with altering DNA methylation (Demirel et al., 2015) and oxidative stress (Wang et al., 2016). Catalase (Cat) and glutathione peroxidase 1 (Gpx1) are antioxidant enzymes responsible for controlling intracellular hydrogen peroxide levels. However, their
genetic polymorphism and gene deficiency could hasten liver and kidney injury (Kang and Alexander, 1996; Kim et al., 2009; Sousa et al., 2016). Therefore, we posed that Cat and Gpx1 gene depletion could exacerbate FB1 intoxication in mice via dysregulated sphingolipid metabolism.

MATERIALS AND METHODS

Animals
C57/BL6 male mice (21 g body weight and 7-8 weeks old) obtained from Daehan Biolink (Eumsung, Korea), and Gpx1/Cat KO mice were generously donated by Dr. Goo Taeg Oh (Ewha Womans University, Seoul, Korea). Gpx1/Cat KO mice and their congenic background strain (C57BL/6) were acclimated for 1 week in the animal facility (22°C, 12 h light/dark cycle). All procedures were conducted according to the guidelines approved by the Animal Care and Use Committee, Ewha Womans University. Mice were housed in groups (n=8) supplied with chow diet and water ad libitum. Wild and Gpx1/Cat KO mice were intraperitoneally (i.p.) administered with FB1 (5 mg/kg) for 5 days. Control groups of mice received saline instead of FB1 in a similar manner. After blood collection from mice 7 h following the final injection, serum was separated and used for enzymatic analysis and lipid peroxidation assays. Liver and kidneys were isolated, and the tissues were sectioned and homogenized to determine sphingolipid levels.

Enzymatic and lipid peroxidation assays
Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in serum were assayed to assess for liver function using an automated chemistry analyzer as described earlier (Jung et al., 2016). The release of thiobarbituric acid reactive substances (TBARS) was assayed for lipid peroxidation via Cayman’s TBARS Assay Kit (Cayman, Ann Arbor, MI, USA).

Sphingolipid determination
Levels of sphingolipids such as So, Sa, Cer, sphingomyelin (SM), dihydroceramide (DH Cer), Sa-1-P, and Sa-1-P from serum and tissue lysates of liver and kidney were determined by high-performance liquid chromatography (HPLC) as described previously (Min et al., 2002; Kim et al., 2006). Briefly, liver and kidney tissue homogenates were lysed in 0.2 N NaOH. Next, chloroform/methanol was added to 200 µg protein and 100 µL serum. 5 min incubation was performed for lipid extraction. After centrifugation (15,000×g, 15 min), the alkaline aqueous phase containing Sa-1-P was transferred to fresh tubes whereas the chloroform phase was mixed with 400 µL of 0.15 M methanolic KOH for sphingoid bases analysis.

Histological examination
Mice were anesthetized with ether and perfused with 0.05 M phosphate-buffered saline (PBS) through the heart. Liver and kidneys were isolated and fixed using 4% paraformaldehyde in 0.1 M phosphate buffer. Then, tissues were embedded in paraffin, sectioned at 5 µm, stained with hematoxylin and eosin (H&E), and examined under a LEICA DM750 microscope (Leica Microsystems, Seoul, Korea).

Statistical analysis
All values were expressed as mean ± SEM and comparisons between groups were performed using ANOVA followed by Newman-Keuls multiple comparison test. p<0.05 was considered as significant.

RESULTS

FB1-treatment in Gpx1/Cat KO mice was associated with elevated level of TBARS in the serum
Acute liver damage is associated with increased levels of ALT and AST in the serum. We determined the levels of these enzymes at the beginning to examine if acute liver damage occurred after administration of FB1. We found that mice treated with FB1 showed an increased level of AST and ALT enzymes in both wild and Gpx1/Cat KO mice. However, both AST and ALT levels were relatively lower in the serum of Gpx1/Cat KO than their wild-type counter parts although significance was shown only in the ALT levels. Similarly, levels of TBARS (indicator of lipid peroxidation) were increased in FB1-treated wild and Gpx1/Cat KO mice compared to saline-treated groups. Unlike ALT/AST, levels of TBARS in the serum were relatively higher in Gpx1/Cat KO groups of mice than wild type mice treated with FB1 (Fig. 1).

FB1 treatment in Gpx1/Cat KO mice was closely linked with excessive accumulation of sphingoid bases in the serum
Inside the body, FB1 interferes with sphingolipid metabolism at early stage by obliterating the formation of complex sphingolipids while enhancing the accumulation of sphingoid bases such as So and Sa. To determine if this imbalance was

![Fig. 1](http://www.biomolther.org/image)

**Fig. 1.** The levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and thiobarbituric acid reactive substances (TBARS) in serum of FB1-treated mice. Mice were treated with FB1 (5 mg/kg, i.p.) daily for 5 days. The levels of ALT and AST (A, B) were assayed from collected serum at 7 h after final injection of FB1 via Automated Chemistry Analyzer. The release of TBARS (C) was assayed via Cayman’s TBARS Assay Kit (Cayman, Ann Arbor, MI, USA). *Significant between wild and Gpx/Cat KO groups after treatment with FB1 (p<0.05), (n=8).
exacerbated under oxidative stress, we evaluated the concentration of So, Sa, and their phosphorylation, i.e., So-1-P and Sa-1-P in the serum. While concentrations of So and Sa were relatively higher in FB1-treated Gpx1/Cat KO mice than wild-type mice without statistical significance, the levels of So-1-P and Sa-1-P were lower in Gpx1/Cat KO mice compared to wild-type mice (Fig. 2A, 2B). Like So and Sa levels, serum DH Cer level was relatively higher in Gpx1/Cat KO mice than wild-type after treating with FB1. However, serum Cer level was lower in Gpx1/Cat KO mice and wild mice (Fig. 2C).

Low levels of So and Sa were determined in liver and kidney tissues of Gpx1/Cat KO mice with exposure of FB1

The liver and kidneys are the primary organs targeted by FB1 toxicity in mice. Therefore, we determined the concentration of sphingoid bases in these organs. Moreover, histopathological preparations were carried out to examine any damage after FB1 intoxication. When we looked into the histopathological sections in both liver and kidney tissues, leukocyte infiltrations were excess in wild type mice than Gpx1/Cat KO mice (Fig. 3). Lower levels of So and Sa were found both in liver and kidney tissues after FB1-treatment in Gpx1/Cat KO mice.
mice than wild type mice (Fig. 4). Although Cer and SM levels were found to be decreased or same level in FB1 treated mice compared to saline administered groups in both Gpx1/Cat KO mice and wild mice, concentration differences were not seen between wild type and Gpx1/Cat KO mice after FB1 treatment (Fig. 5).

FIGURE 4. The elevation of the sphingolipid in tissues of FB1-treated mice. Mice were treated with FB1 (5 mg/kg, i.p.) daily for 5 days. *Significant between wild and Gpx/Cat KO groups after treatment with FB1 (p<0.05), (n=8).

FIGURE 5. The levels of ceramide and sphingomyelin in tissues of FB1-treated mice. Mice were treated with FB1 (5 mg/kg, i.p.) daily for 5 days. *Significant between wild and Gpx/Cat KO groups (p<0.05), (n=8).

DISCUSSION

Considering the rampant nature of FB1 toxicities (Lin and Guo, 2016), in vivo studies involving sphingolipid metabolism and the anti-oxidant system could have paramount importance. Fusarium species of fungi produce nearly 15 different fumonisins (Escriva et al., 2015) with FB1 being predominant. FB1 is highly toxic by inhibiting the enzyme ceramide synthase which metabolizes intracellular sphingosine (So) and sphinganine (Sa), which produces deleterious effects to cells (Raiola et al., 2015). We studied FB1 toxicity in wild type mice and Gpx1/Cat KO mice. Since the liver is one of the primarily targeted organs in acute FB1 toxicity in mice, we measured the levels of AST and ALT in the serum. We found that serum levels of AST and ALT were increased in FB1 administered mice in a similar manner to acute liver injury during lipopolysaccharide induced inflammation in mice (Tang et al., 2016). Serum levels of TBARS were also increased in FB1-treated mice. However, Gpx1/Cat KO mice showed relatively higher level of TBARS than wild type. In contrast, ALT and AST serum levels were relatively lower in knocked down groups of mice than wild type. Increased levels of tissue reactive substances in the serum may be partially explained by oxidative tissue damage either through genetic depletion of major anti-oxidant enzymes or malfunction that could aggravate tissue injury by apoptotic cell death (Dunning et al., 2013) thereby increasing serum levels of TBARS as biomarkers of oxidative stress (Tsai and Huang, 2015). In addition, the roles of densely populated peroxisomes in the liver and kidneys may possess unequivo-

cal merit compared to oxidative injury to these tissues (Vasko, 2016). Therefore, it is plausible to speculate that higher serum levels of TBARS in FB1 treated Gpx1/Cat KO mice in this study could be partially explained by sustained cellular apoptosis in the liver and kidney tissues rather than acute tissue necrosis associated with elevation of liver enzymes in the serum. However, further research may be warranted to justify discrepancies between liver enzymes and TBARS levels in the serum during FB1 intoxication.

We found that serum levels of So and Sa were elevated compared to their phosphorylation types, So-1-P and Sa-1-P, in mice treated with FB1. The levels of So and Sa were significantly increased in Gpx1/Cat KO mice treated with FB1 compared to their wild type counter parts whereas So-1-P and Sa-1-P tend to be decreased. We suggest that elevated levels of So and Sa might have occurred most likely either due to the dephosphorylation processes of So-1-P and Sa-1-P by lipid phosphate phosphatases (Tang et al., 2015) or created as a metabolite of ceramide during oxidative stress (Hoehn et al., 2016). Nevertheless, Gpx1/Cat KO mice serum having depleted So-1-P levels may work against its cardio-protective effect (Knapp et al., 2013). In addition, ceramide levels were decreased in Gpx1/Cat KO mice and wild type mice after FB1 treatment. However, dihydroceramide level was elevated in both groups. We presumed that higher Cer and DHcer level in FB1-treated Gpx1/Cat KO mice could be due to the accumulation of reactive oxygen species in the body (Sordillo et al., 2015). Due to the fact that the liver and kidneys are primarily targeted organs by FB1 toxicity in mice, we measured the sphingolipid levels from these tissues. Furthermore, we made histological preparations to determine if any pathological conditions were present. When we see the pathway of de novo synthesis of sphingolipid and its metabolism, we can understand why the level of So (So) was elevated but the level of Cer was decreased after FB1 treatment in mice. FB1 inhibits ceramide synthase and results in the accumulation of So and Sa. Blockade of ceramide synthase by FB1 resulted in
Fig. 6. De novo synthesis of sphingolipid and metabolism. Fumonisin B1 (FB1) inhibits ceramide synthase and results in the accumulation of sphinganine and sphingosine. Blockade of ceramide synthase by FB1 results in the decrease of the level of ceramide.

The decreased level of Cer (Fig. 6). FB1 toxicity in mice was accompanied by marked elevation of Sa levels in liver and kidney tissues. While in Gpx1/Cat KO mice with their wild type counter parts, we found So and Sa concentrations were less in tissue homogenates obtained from Gpx1/Cat KO mice than wild type mice. We speculated that this could be due to the accumulation of reactive oxygen species in the organs. This notion could be supported by a previous study that showed variations in sphingoid bases in HL-60 cells incubated with H2O2 (Son et al., 2007), and strengthened by linking the reactive oxygen species with inflammation accompanied by leucocyte infiltration (Voltan et al., 2016), as it could be observed from our histological sections infiltrated with leucocytes in liver and kidney tissues.

We conclude that FB1-induced intoxication is associated with escalating levels of Cer, So, and Sa in the serum collected from Gpx1/Cat KO mice compared to their wild counter parts. Although So and Sa levels were found to be diminished within liver and kidney tissues, concentration of So-1-P in the serum was relatively lower in FB1-treated mice of the knocked down group than the wild type. We speculated that FB1 intoxication in Gpx1/Cat KO mice may hasten cellular apoptosis via sphingoid bases during oxidative stress. Considering that FB1-induced tissue injury primarily targets the liver and kidneys, rising serum levels of AST, ALT, and TBARS in Gpx1/Cat KO mice could be an indication for severe necrotic and apoptotic effects of FB1 toxicity in stressed mice. Therefore, enhancing the oxidative system in the body could be an alternative option to reduce the rampant FB1-associated intoxication worldwide.

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