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Deletion of IFN-γ Reduces Fumonisin-Induced Hepatotoxicity in Mice via Alterations in Inflammatory Cytokines and Apoptotic Factors

RAGHUBIR P. SHARMA,[1] QUANREN HE,[1] and VICTOR J. JOHNSON[1,2]

ABSTRACT

Fumonisin B_1 (FB_1) produces species-specific and organ-specific toxicity, including equine leukoencephalomalacia, porcine pulmonary edema, and hepatic or renal damage in other animals. FB_1 causes inhibition of ceramide synthase, leading to accumulation of free sphingoid bases. We previously reported that such cytokines as tumor necrosis factor-α (TNF-α) modify FB_1-induced hepatic apoptosis in male mice. FB_1 also caused induction of interferon-γ (IFN-γ) in mouse liver, and, therefore, it was worthwhile to determine the role IFN-γ plays in FB_1 toxicity. In the current study, male IFN-γ-knockout (GKO) mice and their wild-type (WT) counterparts, C57BL/6J, were treated subcutaneously (s.c.) with 2.25 mg/kg/day of FB_1 for 5 days and sampled 1 day after the last injection. The levels of circulating liver enzymes were increased in WT animals but considerably less in GKO mice. Reduced hepatotoxicity in GKO mice was evident by histologic evaluation and enumeration of apoptotic cells. The induction of TNF-α and interleukin-12 (IL-12) p40 by FB_1 in liver was less in GKO mice compared with WT animals. The GKO mice also had a reduced accumulation of liver sphinganine than did WT mice after FB_1 treatment. Results suggested the implication of IFN-γ in FB_1-induced hepatotoxicity, which can be explained by a lack of TNF-α and IL-12 amplification in the liver of the GKO mice. In addition, the GKO mice had altered expression of various apoptotic and antiapoptotic factors in liver. These changes were accompanied by a greater number of proliferating cells in the liver of GKO mice after FB_1 treatment, which may also contribute to the reduced hepatotoxicity of FB_1 in GKO mice. Whereas the GKO mice show reduced sensitivity to FB_1 and FB_1 treatment elevates IFN-γ expression, decreased hepatotoxicity to FB_1 could result from alterations in sphingolipid metabolism in the GKO strain.

INTRODUCTION

SINCE THE DISCOVERY OF FUMONISIN B_1 (FB_1) in 1988, much interest has been generated in evaluating the health effects of this chemical.[1,2] This mycotoxin, a member of a major class of fumonisins produced by *Fusarium verticillioides*, is a common contaminant of corn and corn products. Unique features of the biologic effects of this chemical are tissue and species specificity and even gender-dependent differences.[3–6] This compound produces leukoencephalomalacia in horses,[3] pulmonary edema in swine,[4] and liver and kidney damage in rodents.[5] It is a renal carcinogen primarily in male rats, whereas female mice exhibit primarily liver damage.[6]

Structurally, fumonisins are related to free sphingoid bases, including sphinganine and sphingosine. The primary molecular event in the biologic effects of FB_1 has been established as inhibition of sphinganine and sphingosine acyltransferase (ceramide synthase), thus interfering with the incorporation of these bases in ceramide or other complex sphingolipids, and an accumulation of sphinganine, synthesized *de novo* by serine palmitoyl transferase (SPT), and sphingosine, produced either by oxidation of sphinganine or via catalytic pathway from complex sphingolipids, including ceramide, in the cell.[7] Fumonisins inhibit ceramide synthase, leading to the accumulation of free sphingoid bases in all tissues and species, regardless of their sensitivity to toxic response against this mycotoxin. For example, mouse kidney shows high accumulation of sphinganine in response to FB_1 yet is less sensitive than liver to its toxic effects.[8]

We have shown previously the involvement of various cytokines in FB_1-induced hepatotoxicity in male and female

[1]Department of Physiology and Pharmacology, College of Veterinary Medicine, The University of Georgia, Athens, GA 30602.
[2]Present address: Toxicology and Molecular Biology Branch, Health Effects Laboratory Division, National Institute of Occupational Safety and Health, Morgantown, WV 26505.

mice.[9–13] In male BALB/c mice, the acute hematologic responses against FB_1 were reduced by pretreatment with antibodies against tumor necrosis factor-α (TNF-α), and macrophages obtained from FB_1-treated animals produced higher amounts of TNF-α.[9] Mice lacking either TNF-α receptor I (TNFRI) or TNFRII and treated with FB_1 had reduced hepatotoxicity compared with their wild-type (WT) counterparts.[10,11] A differential hepatotoxic response to FB_1 in male vs. female mice was related to a differential expression of various proinflammatory cytokines in their livers.[12] It was shown that a localized network of proinflammatory cytokines in liver, involving TNF-α, interferon-γ (IFN-γ), and interleukin-12 (IL-12), is involved in FB_1-induced hepatotoxicity in mice.[13]

We recently reported that a variety of cytokines and other apoptotic signaling molecules are modulated in FB_1 toxicity in male and female BALB/c mice.[14] In male mouse liver, a nearly 12-fold increase in IFN-γ was observed after FB_1 treatment. We, therefore, hypothesized that IFN-γ plays an important role in defining the hepatotoxic outcome of FB_1 in male mice. The purpose of the present study was to define the role of IFN-γ using an IFN-γ-knockout (GKO) strain of mice. Male GKO and their WT counterpart mice were treated with FB_1 employing a protocol used previously.[8–10] As hypothesized, the hepatotoxic response of FB_1 was reduced in GKO compared with WT mice. A lack of IFN-γ also reduced the FB_1-induced increase in TNF-α and IL-12. The FB_1-treated GKO animals had a higher cell proliferation in liver compared with FB_1-treated WT mice, suggesting a rapid regeneration process. The expression of a number of apoptotic signaling molecules was different in GKO than in WT animals, which may also account for the differential response to FB_1 in these strains.

MATERIALS AND METHODS

Animals and housing

Six-week-old male C57BL/6 mice with IFN-γ deleted (B6.129S7-*Ifng*^{tm1Ts}, stock no. 002287), henceforth referred as GKO, and their WT counterparts, C57BL/6J (stock no. 000664), referred to as WT, were procured from Jackson Laboratories (Bar Harbor, ME). The male mice of the GKO strain were homozygous/homozygous male offspring from animals with deleted IFN-γ produced by backcrossing of several generations of KO animals, originally developed by Dr. Timothy Stewart (Genentech Inc., San Francisco, CA).[15] The knockout status of GKO animals was confirmed by a reverse transcriptase polymerase chain reaction (RT-PCR) on their hepatic cDNA. On arrival, the animals were acclimated for 1 week before beginning treatment. Mice were group-housed in filter-barrier top cages under controlled environmental conditions targeted at 23°C and 50% relative humidity with a 12-h light/dark cycle. Animals were provided free access to food and water. Protocols for animal use followed the Public Health Service Policy on Humane Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee.

Treatment and sampling

Groups of 5 animals each were treated with 5 daily subcutaneous (s.c.) injections (1 ml/100 g body weight) of FB_1 (98% purity) (Promec, Tygerberg, South Africa) in phosphate-buffered saline (PBS), the dose per mouse being 0 (saline-treated controls) or 2.25 mg FB_1/kg/day. One day after the fifth and final injection, the animals were killed with halothane, and their organs were sampled.[10,11] Blood was collected in a heparinized syringe by cardiac puncture for blood counts and estimation of plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Liver, kidneys, spleen, and thymus were weighed. Aliquots of liver were quickly frozen on dry ice and stored at −85°C until analyzed.

Hematology and estimation of circulating liver enzymes

Total erythrocyte and leukocyte cell counts were determined using an electronic counter (Coulter Electronics, Hialeah, FL). Differential counts on blood smears were conducted by routine methods after Wright's stain and visual counting in a microscope. The numbers of lymphocytes and neutrophils in blood were calculated from the total leukocyte count and percentage of differential counts. Levels of plasma ALT and AST were determined using a Hitachi 912 Automatic Analyzer (Roche Diagnostics, Indianapolis, IN).

Histopathologic evaluation of liver

Liver tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned (4–5 μm), and stained with hematoxylin and eosin (H & E). Liver sections were screened to determine the type of effects present, in a random order and without knowledge of animal or group identity. Appearance was evaluated as described earlier.[16,17]

Determination of apoptosis in liver

Liver samples were fixed in 10% neutral buffered formalin overnight and routinely paraffinized. Sections (5 μm) were prepared and subjected to uridine triphosphate (UTP) nick-end labeling (TUNEL) of apoptotic cells with a peroxidase-based Apoptag® Plus kit (Oncor, Inc., Gaithersburg, MD).[17] Briefly, sections were deparaffinated with two changes of D-limolene:butylated hydroxyanisol mixture (Citrisolv™) (Fisher Scientific, Pittsburgh, PA), washed in ethanol, and hydrated with a series of descending concentrations of ethanol in water. After washing with PBS, the sections were treated with proteinase K (Fisher Scientific, Fair Lawn, NJ) for 15 min at ambient temperature. After further washing, the sections were quenched with 3% hydrogen peroxide in PBS to remove endogenous peroxidases and incubated first with equilibration buffer and then with a reaction mixture containing digoxigenin-UTP and terminal deoxynucleotidyltransferase (TdT) enzyme (Oncor). The sections were again washed with the buffer and treated with appropriately diluted antidigoxigenin antibody conjugated with peroxidase. The localized peroxidase enzyme was visualized with diaminobenzidene, which generates a brown product after catalysis. The sections were lightly counterstained with hematoxylin and observed under a light microscope. Positive (rat testis) and negative controls (sections without the reaction mixture) were simultaneously carried through the process. In each section, the apoptotic cells were counted, and the number was then normalized to a unit area.

Semiquantitative estimation of cytokine mRNA expression by RT-PCR

Total RNA was isolated from the tissue aliquots powdered in liquid nitrogen as described earlier.[10] The preparation of initial cDNA strand by reverse transcription and DNA amplification was similar to previously reported methods.[10] Primers used for various cytokines, TNF-α, IFN-γ, and IL-12p40, and the linearity of the PCR product formation have also been reported.[13] The conditions for annealing temperature, time, Mg^{2+} concentration, and number of cycles were optimized. The number of cycles was within the exponential increase of PCR products to avoid saturation.[13,18] The density of TNF-α, IFN-γ, and IL-12 bands was normalized to the β-actin band amplified from the same cDNA and by digitizing the gel photographs via densitometric imaging using UN-SCAN-IT software (Silk Scientific Inc., Orem, UT).

Analyses of sphingolipids

Free liver sphingosine and sphinganine concentrations were determined in base-treated lipid extracts by high performance liquid chromatography (HPLC) using a protocol reported previously.[8] The HPLC apparatus and procedures have also been described previously.[8] Briefly, the livers were homogenized in PBS and spiked with an internal standard C_{20}-sphinganine. Lipids were extracted in chloroform, followed by a base hydrolysis, the extracts were dried and residue was redissolved in aqueous methanol, and finally the free sphingolipids were derivatized with *o*-phthalaldehyde for fluorescence detection. The concentration of sphingoid bases was quantitated based on the recovery of the internal standard.

Enumeration of proliferating cells in liver

Paraffin-fixed liver tissues were sectioned at 5 μm, mounted on glass slides, and deparaffinated with several changes of Citrosolv and routinely hydrated with descending serial dilutions of ethanol. Antigens were retrieved by heat treatment of slides in a steamer containing citrate buffer (pH 6.5) for 25 min. Endogenous peroxidase activity was quenched by 3% hydrogen peroxide for 10 min, and sections were treated to block nonspecific binding with a solution containing goat serum and avidin-biotin blocking kit (Vector Laboratories, Burlingame, CA). The sections were incubated overnight with a 1:100 dilution of proliferating cell nuclear antigen (PCNA) antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The secondary biotinylated antibody was applied, and sections were stained with Vectastain ABC® kit following the manufacturer's protocol (Vector Laboratories). Tissues were visually examined under a light microscope, and the number of PCNA-positive cells was counted in the whole section and normalized to a square centimeter area.

RNA isolation and ribonuclease protection assay (RPA)

Total RNA was isolated from frozen livers (ca. 70–80 mg) with TRI® reagent (Molecular Research Center, Cincinnati, OH), using a protocol described earlier.[8] Frozen tissues were ground in liquid nitrogen in a mortar, TRI reagent was added, and the tissues were further homogenized. RPA was performed on samples using a RiboQuant™ RPA kit (PharMingen, San Diego, CA).[14,19] Briefly, three template sets, one for cytokines (mCK-2b), another for TNF-α and Fas signaling molecules (mAPO-3), and a third mouse template set containing TNF-α/Myc/Max/Mad/Bcl-2/Bax/Bad/caspases, were used. Synthesis of high specific-activity α^{32}P-UTP-labeled T7 RNA polymerase-directed antisense RNA probes was performed using the *in vitro* transcription kit according to the manufacturer's protocols (PharMingen). An aliquot of 50 μg sample RNA was hybridized with the probe overnight and digested with RNase A and RNase T1. The RNase-protected probes were purified and resolved on a denaturing polyacrylamide gel. The gels with α^{32}-labeled bands were exposed to an FX Imaging Screen K-HD® (Bio-Rad Laboratories, Hercules, CA) for 6–16 h and scanned by Bio-Rad Molecular Imager® Fx. The relative gene expression was digitized using Quantity One® software provided by the manufacturer (Bio-Rad) and normalized against the housekeeping gene, ribosomal protein L32.

Statistics

Data from these studies were analyzed by a single-factor analysis of variance (ANOVA), followed by a *post hoc* Duncan's multiple-range test. Hepatic apoptosis and proliferating cell counts were compared using the Wilcoxon rank sum test (nonparametric comparison). All tests were performed using an SAS computer program (SAS Institute, Cary, NC). The level of $p \leq 0.05$ was considered statistically significant. Lower p values are shown with the respective results.

RESULTS

Treatment with FB_1 produced marginal clinical toxicity

Short-term treatment of WT and GKO mice with FB_1 produced no gross or behavioral changes when animals were observed every day. Treatment of WT mice with 2.25 mg/kg FB_1 for 5 days caused a marginal (<6%) but statistically significant decrease in body weight gain. Such difference was not observed in GKO mice (data not shown). Relative weights of liver, spleen, and thymus (normalized to final body weight) were unaltered in both WT and GKO strains. A slight decrease in relative kidney weights was observed in GKO mice only after the FB_1 treatment. No changes by FB_1 treatment were seen in total or differential blood cell counts (data not shown).

FB_1 hepatotoxicity was lower in GKO than in WT mice

Liver toxicity in response to FB_1 was reduced in the GKO strain compared with that in WT mice. Concentrations of circulating liver enzymes, ALT and AST, increased in WT mice after the FB_1 treatment (Fig. 1), indicative of liver toxicity. In the GKO mice, there was an increase in ALT concentration, but the increase was significantly lower than that observed for WT mice. Levels of AST were not altered by FB_1 treatment in GKO mice.

Changes in liver enzyme levels in plasma correlated with microscopic changes observed in the liver. In WT mice, the

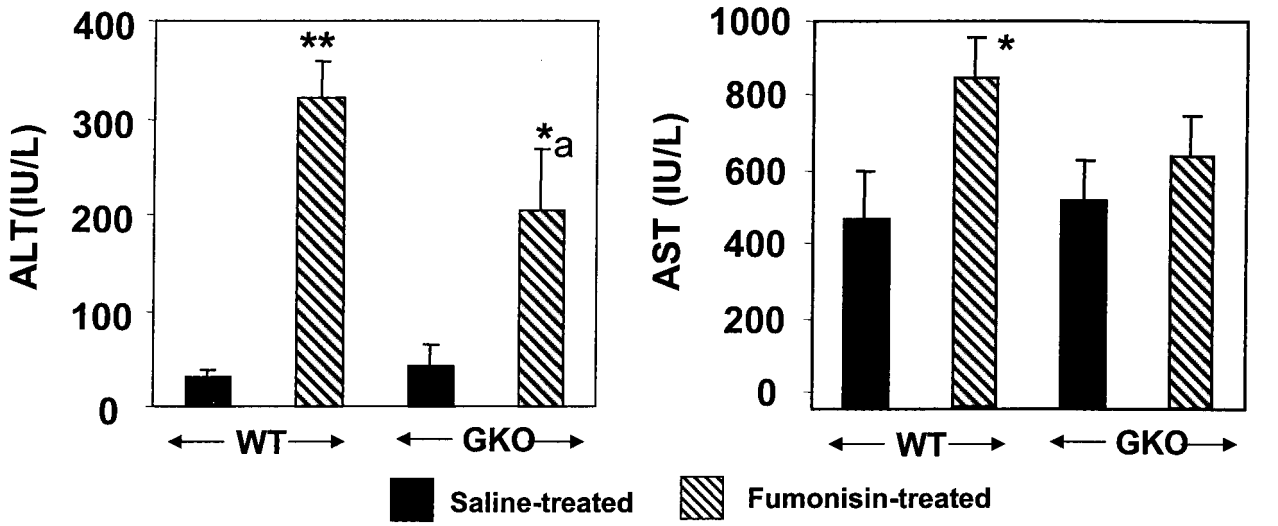

FIG. 1. Concentrations of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in the plasma of normal (WT) and GKO mice after treatment with FB_1 (mean ± SEM, $n = 5$). A significant difference between FB_1 and saline treatment of the same strain is indicated at $*p < 0.05$, $**p < 0.01$. The letter "a" on the bar indicates a significant difference ($p < 0.05$) between the FB_1-treated WT and GKO groups.

changes seen after FB_1 treatment were identical to those reported earlier for other strains of mice[16,17] and included primarily scattered apoptotic hepatocytes and occasional mitotic figures. Eosinophilic staining of cells in liver with lack of normal architecture was evident after FB_1 exposure. The incidence of apoptotic hepatocytes was less in FB_1-treated GKO mice than in FB_1-treated WT mice, as indicated by enumeration of apoptotic cells by the TUNEL assay. As illustrated in Figure 2, no apoptotic cells were seen in saline-treated WT or GKO animals. In FB_1-treated GKO mice, the number of apoptotic hepatocytes were 2.5-fold less than the number in respectively treated WT mice ($p < 0.05$). It is apparent, therefore, that GKO mice were relatively less susceptible to the hepatotoxic effects of FB_1 than were WT animals.

Hepatotoxic response correlated with accumulation of free sphinganine in liver

Figure 3 illustrates the increase in free sphingoid bases, sphinganine and sphingosine, after ceramide synthase inhibition by FB_1 in WT and GKO strains. Treatment of WT mice with FB_1 increased the free sphinganine concentration in liver by 30-fold. The increase was 30% less in GKO mice ($p < 0.05$) compared with similarly treated WT mice. Liver sphingosine levels were not altered by FB_1 treatment in either strain of mice used in this study.

Liver toxicity by FB_1 in both strains related to induction of inflammatory cytokines

The expression of TNF-α and IL-12 (determined by RT-PCR) is shown for both strains in Figure 4, along with the induction of IFN-γ in WT animals. As seen in our previous studies, the expression of three inflammatory cytokines (TNF-α, IFN-γ and IL-12p40) was induced by FB_1 treatment in WT mice. TNF-α and IL-12p40 were also increased in GKO mice, but the increase was significantly less than in WT mice. In the GKO mice, a lack of IFN-γ expression was confirmed by RT-PCR (Fig. 4). Results suggested that an increase in IFN-γ is necessary for optimal changes in TNF-α and IL-12p40 induced by FB_1, and the overall effect of reduced cytokine induction may be responsible for the decreased hepatotoxic response to this mycotoxin in GKO mice.

Lack of IFN-γ provided rapid regenerative response after FB_1 treatment

The occurrence of mitotic cells was measured by immunochemical staining for PCNA (Fig. 5). Very few PCNA-positive cells were observed in both WT and GKO mice without exposure to FB_1. Treatment of animals with FB_1 increased the number of PCNA-positive hepatocytes 60-fold in the liver of WT mice and nearly 200-fold in GKO mice. The number of PCNA-positive cells in GKO mice after FB_1 treatment was significantly higher than in the respective WT group ($p < 0.05$).

Induction of various signaling factors by FB_1 differed in WT and GKO animals

The expression of various signaling factors involved in apoptosis and cell cycling was evaluated by RPA, a quantitative technique that has been used previously in our laboratory.[14,19] Densitometric analyses of the bands (normalized to the housekeeping gene for ribosomal protein L32) indicated that many such factors were induced in liver after FB_1 treatment, a finding similar to those reported earlier for BALB/c mice.[14,19] In some cases, however, the extent of such increase caused by FB_1 was lower in GKO mice than in WT animals. As shown in Figure 6, FB_1-induced increases in IL-1β, b-*myc*, and c-*myc* were considerably less in GKO mice than in WT mice, whereas the expression of IL-1α and IL-1 receptor antagonist (IL-1Ra) was increased to the same extent in both strains after FB_1 treatment.

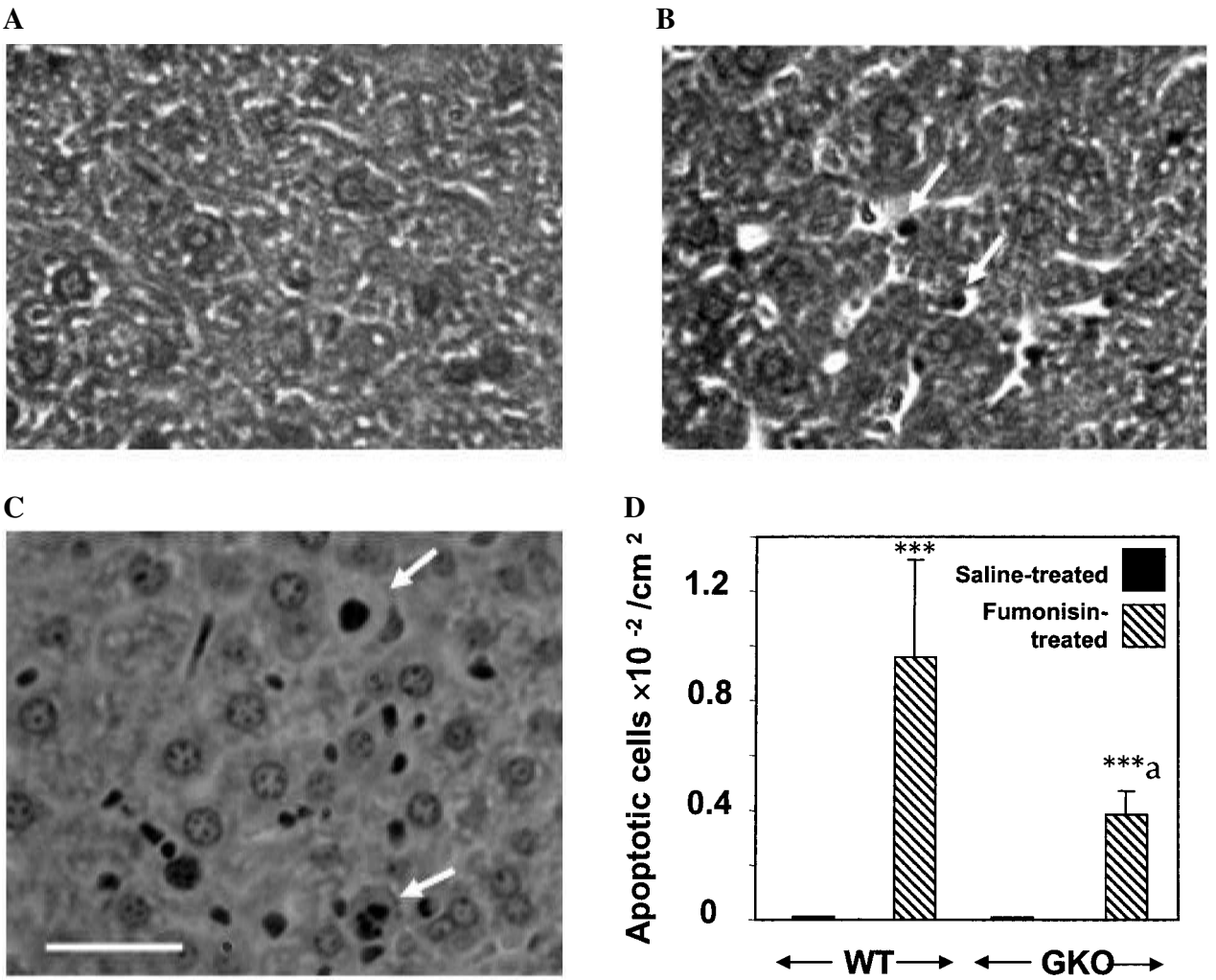

FIG. 2. Photomicrographs (H & E-stained) of liver showing (**A**) normal cytoarchitecture from a control (vehicle-treated) WT mouse and (**B**) liver from an FB_1-treated WT mouse, illustrating the hepatotoxic effects. Note the apoptotic cells (arrows), characterized by variation in cell (anisocytosis) and nucleus size (anisokaryosis) of hepatocytes. The condensed nuclei are separated from the cytoplasmic material. (**C**) Liver from FB_1-treated WT mice showing TUNEL-positive cells. The TUNEL-positive dark nuclei (arrows) are markedly different in texture and appearance from the normal ones, whereas TUNEL-positive nuclear fragments (dark spots) are scattered in the tissue. Bar = 40 μm. (**D**) Enumeration of TUNEL-positive cells in saline and FB_1-treated WT and GKO mice (mean ± SEM, $n = 5$). A significant difference between FB_1 and saline treatment of the same strain is indicated by $***p < 0.001$. The letter "a" on the bar indicates a significant difference ($p < 0.05$) between the FB_1-treated WT and the GKO groups.

FB_1 increased the expression of b-*myc* in WT mice only. No increase of b-*myc* was observed in GKO mice liver. Bcl-2 was unaffected in both strains after FB_1 treatment.

The expression of the proapoptotic factor Bax was increased by FB_1 in both WT and GKO mice (Fig. 6). No change by FB_1 treatment was seen for Max in GKO animals, whereas FB_1 induced Max in WT mice. Other factors, Bad and Mad, were unaltered by FB_1 treatment. However, Mad was expressed in greater amounts in control GKO animals than in control WT mice (40% higher). The basal expression of Max was also higher (by 30%) in GKO than in WT animals.

Densitometric analysis of the expression of TNF-α and related molecules measured by RPA is shown in Figure 7. The changes observed after FB_1 treatment in TNF-α expression confirmed the results obtained by RT-PCR (Fig. 3); that is, the FB_1-induced increase was lower in GKO mice than in WT mice. The expression of TNFRI and TNFR-related apoptotis-inducing ligand (TRAIL) was increased by FB_1 treatment in WT animals, whereas TRAIL was the only other TNF-α-related factor increased by FB_1 in the GKO strain. However, in saline-treated GKO mice, the expression of all TNF-α-related molecules (TNFRI, RIP, TRAIL, TRADD, and FADD) was 28%–60% higher than in the respective WT group. The expression of caspase-3 and caspase-6 was unaltered in both strains after FB_1 treatment. However, the basal expression of both caspase-3 and caspase-6 was again higher in the GKO group than in the respective WT mice by 55% and 30%, respectively.

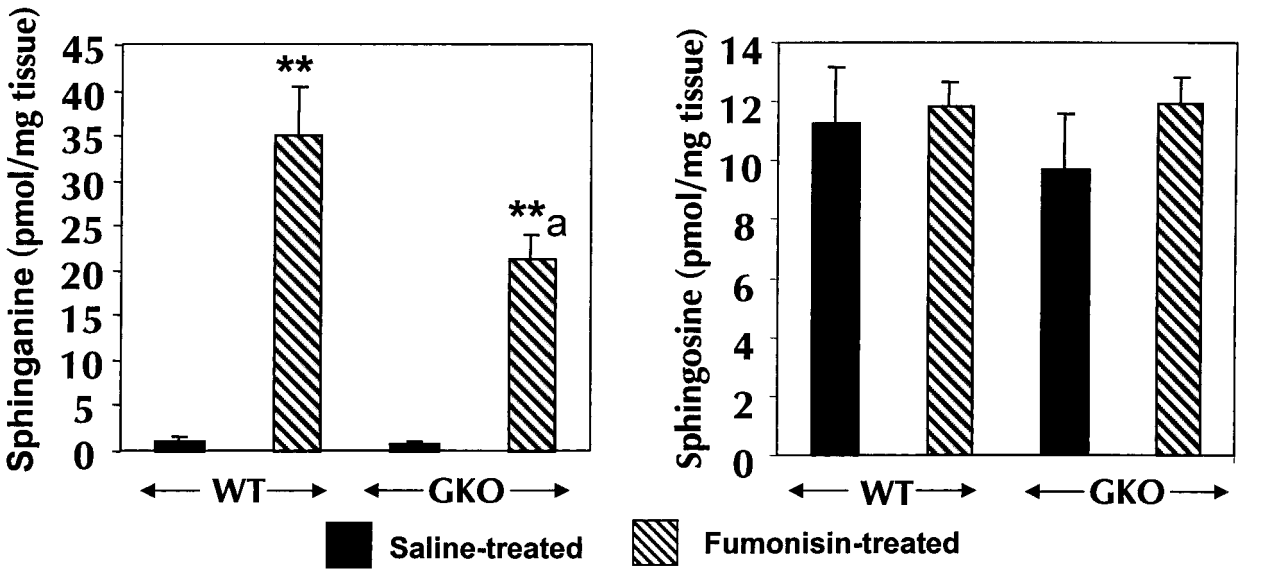

FIG. 3. Free sphinganine and sphingosine levels in the liver of WT and GKO mice after FB_1 treatment (mean ± SEM, $n = 5$). **Significant difference between FB_1 and saline treatment of the same strain, $p < 0.01$. The letter "a" on the bar indicates significant ($p < 0.05$) difference compared with FB_1-treated groups of the two strains.

The expression of Fas was similar in both WT and GKO mice and was unaltered by FB_1 treatment in both strains (results not shown). The expression of Fas-related factors, Fas-associated protease (FAP) and Fas-associated protein factor (FAF), was higher by 74% and 25%, respectively, in GKO animals than in WT mice, although no increase with FB_1 treatment was observed in either strain (Fig. 7).

Deletion of IFN-γ modulated a variety of apoptotic and antiapoptotic factors in GKO mice

As is evident from the results shown in Figures 6 and 7, the basal expression of a variety of signaling genes was higher in GKO than in WT animals. It is interesting to note that the GKO mice had higher basal levels of nearly all apoptotic factors than did the WT mice. No differences were observed for Fas or Fas ligand (FasL) in the two strains, and these factors were unaltered by FB_1 treatment (data not shown). The same was also true for IL-18 (not shown).

DISCUSSION

Results of the present study suggest that the presence of IFN-γ is necessary for optimal hepatotoxicity in mice following FB_1 exposure. The GKO animals were consistently less susceptible than their WT counterparts to various biologic effects of FB_1. There was a decrease in body weights observed after FB_1 treatment in WT but not in GKO mice. The decrease in body weight

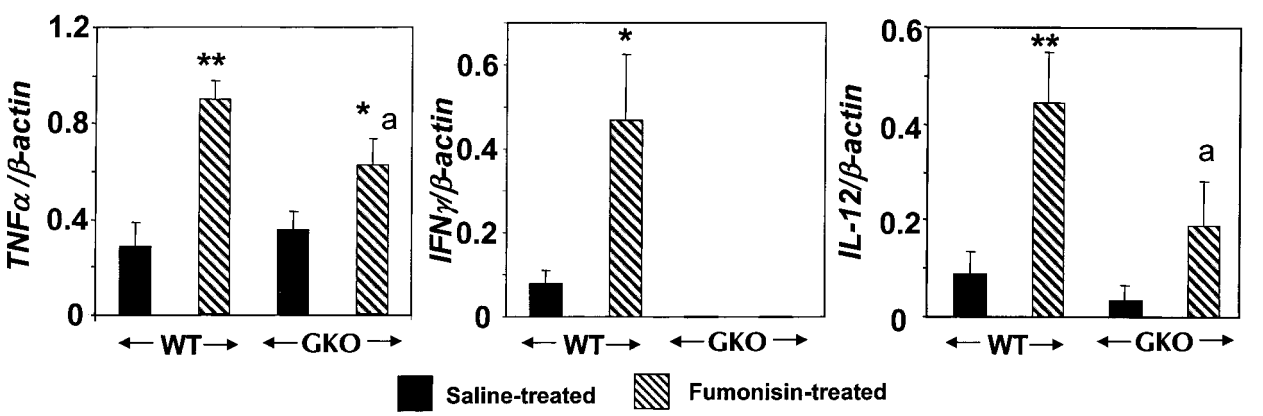

FIG. 4. Expression of TNF-α, IFN-γ, and IL-12p40 in liver (normalized against β-actin) in WT and GKO mice after FB_1 treatment, measured by RT-PCR (mean ± SEM, $n = 5$). A lack of IFN-γ expression is evident in GKO mice. Significant differences between FB_1 and saline treatment of the same strain are indicated at $*p < 0.05$, $**p < 0.01$. The letter "a" on the bars indicates a significant ($p < 0.05$) difference between the FB_1-treated groups of the two strains.

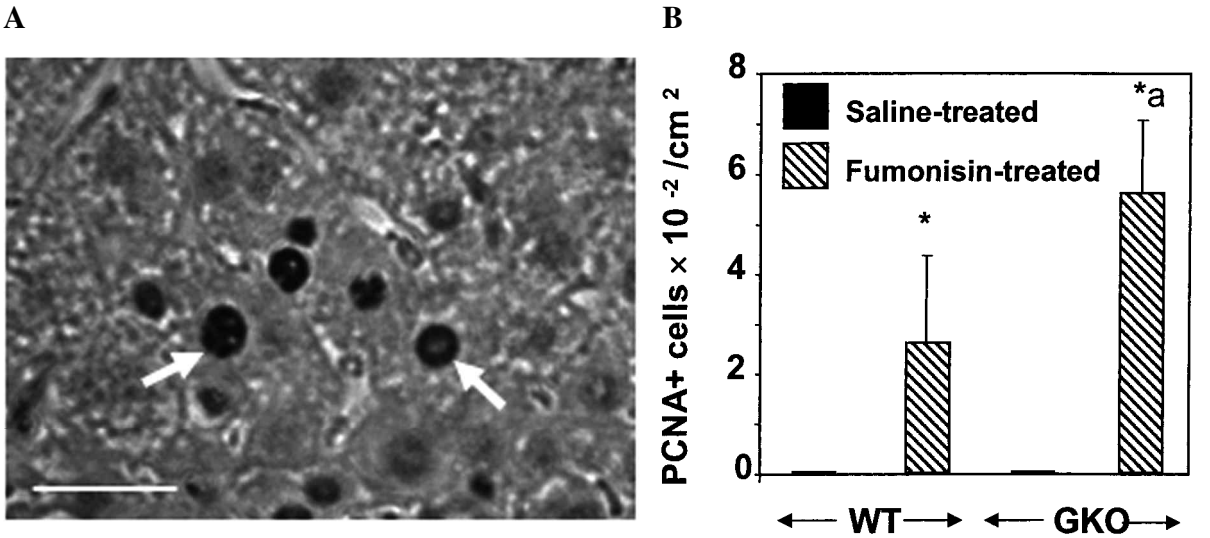

FIG. 5. Evaluation of proliferating cells using PCNA antibody. (**A**) PCNA-positive cells (dark nuclei) in the liver of an FB_1-treated GKO mouse are shown (two of these are marked with arrows). Bar = 40 μm. (**B**) Cells were counted in liver sections and normalized to the unit area (mean $\pm$ SEM, $n = 5$). Significant difference between FB_1 and saline treatment of the same strain is indicated by $*p < 0.05$. The letter "a" on the bar indicates a significant ($p < 0.05$) difference compared with FB_1-treated groups of the two strains.

gain after FB_1 treatment was similar to that observed in our previous experiment involving male BALB/c mice.[12]

In mice, the target organ for FB_1 toxicity is the liver, and females are more sensitive to the hepatotoxic effects of FB_1 than are their male counterparts.[6,12] A reduced hepatotoxic response to the same dose of FB_1 in GKO animals compared with that in WT mice was clearly apparent in the current study. This reduced hepatotoxic response to FB_1 in GKO mice was evident by levels of circulating liver enzymes, histopathologic evaluation of the liver, and enumeration of TUNEL-positive hepatocytes. In our previous reports, we have shown that IFN-γ was induced in intact male mice after a similar FB_1 treatment.[13,14] It could not be ascertained, however, if such increase was responsible for the resulting hepatotoxicity or was in response to liver damage by other mechanisms. The current study emphasizes that IFN-γ is a partial mediator of hepatotoxicity in mice.

It should be noted, however, that IFN-γ may not be essential for the FB_1 hepatotoxicity in mice. All parameters of hepatotoxicity observed here, namely, circulating ALT levels and the presence of apoptotic cells in liver, were reduced in GKO animals compared with those in WT mice but were not totally abolished. In FB_1-treated GKO mice, the increase in ALT was 36% lower that that in WT mice, and the increase in AST was totally abolished. The TUNEL-positive cells in FB_1-treated GKO mice were reduced by 60% from that observed in the FB_1-treated WT strain. It should also be noted that the accumulation of free sphinganine in FB_1-treated GKO mice was 32% less than that in the similarly treated WT group. The cause of diminished sphinganine accumulation in the liver of FB_1-treated GKO animals is not understood at present and may be related to reduced activity of SPT in this strain. We have observed recently that similar treatment of mice with FB_1 induced SPT activity by nearly 5-fold, and there was a good correlation of FB_1-induced sphinganine increase with SPT activity in male vs. female mice (unpublished data). A relationship between SPT activity and TNF-α was documented earlier,[20] and the induction of TNF-α in GKO animals by FB_1 treatment was less than that for the WT mice.

It is, therefore, likely that the reduced hepatotoxicity by FB_1 in GKO mice was due to the decreased activity of SPT. Although cytokines modulate SPT activity,[20] the role of IFN-γ in such modulation is yet to be defined. It could either be a direct effect on SPT or via modulation of TNF-α expression. We are in the process of investigating if the GKO mice had a low basal SPT activity or if it was induced to a lesser extent after FB_1 treatment in this strain. Our recent studies with porcine renal cells have indicated that FB_1-induced accumulation of free sphingoid bases caused a transient activation of protein kinase C (PKC) α, followed by the induction of TNF-α, and resulting apoptosis and nuclear localization of NF-κB. The latter responses (TNF-α induction, apoptotis, and NF-κB activation) were effectively abrogated by the PKC inhibitor, calphostin C (unpublished observations). These observations imply that the resulting cytokine alterations after sphingoid base accumulation are responsible for FB_1-induced toxic responses.

The cause of partial protection from FB_1 hepatotoxicity in GKO mice may, therefore, involve decreased accumulation of sphingoid bases, followed by altered modulation of the cytokine network. It has been well known that the inflammatory cytokine TNF-α is a contributor to FB_1 hepatotoxicity.[9–11] Fumonisin-induced apoptosis in African green monkey kidney cells (CV-1) involved activation of caspase-8, an important mediator of the TNF-α signaling pathway, and the apoptosis in these cells was abolished by inhibitor of apoptosis protein (IAP) produced by a baculovirus gene.[21] The reduced hepatotoxicity in GKO mice after FB_1 treatment was comparable in extent to the reduced induction of TNF-α in liver of this strain compared with FB_1-treated WT mice, as confirmed by both RT-

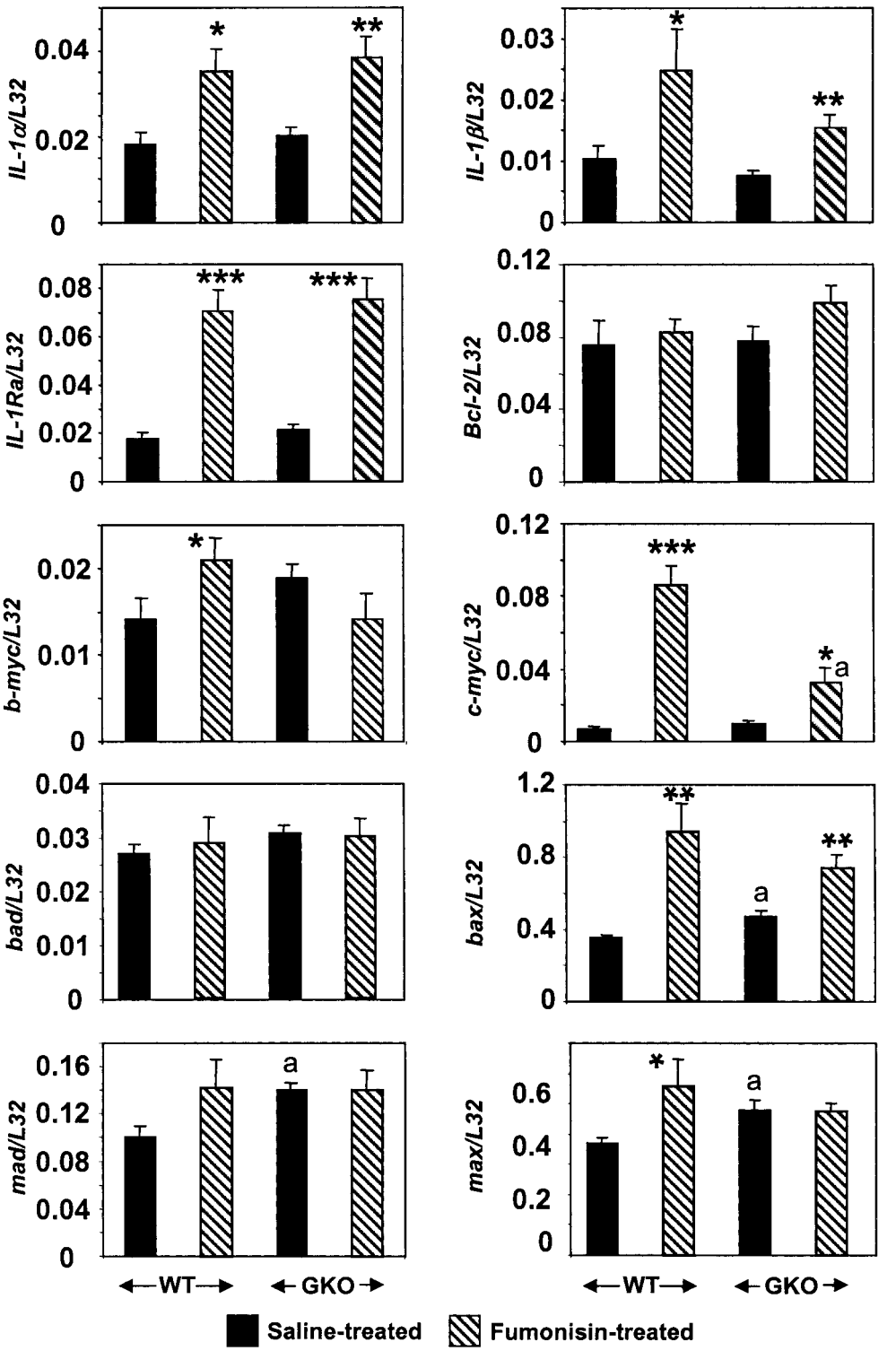

FIG. 6. Alterations in the expression of selected cytokines and apoptosis-related factors in the livers of saline-treated and FB_1-treated WT and GKO mice, measured by RPA. The relative mRNA expression was normalized against a housekeeping gene for ribosomal protein L32 (mean ± SEM, $n = 5$). Significant differences between the saline-treated group and FB_1-treated groups of the same strain are indicated at $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. A significant difference between the similar treatment groups of the two strains is marked by the letter "a."

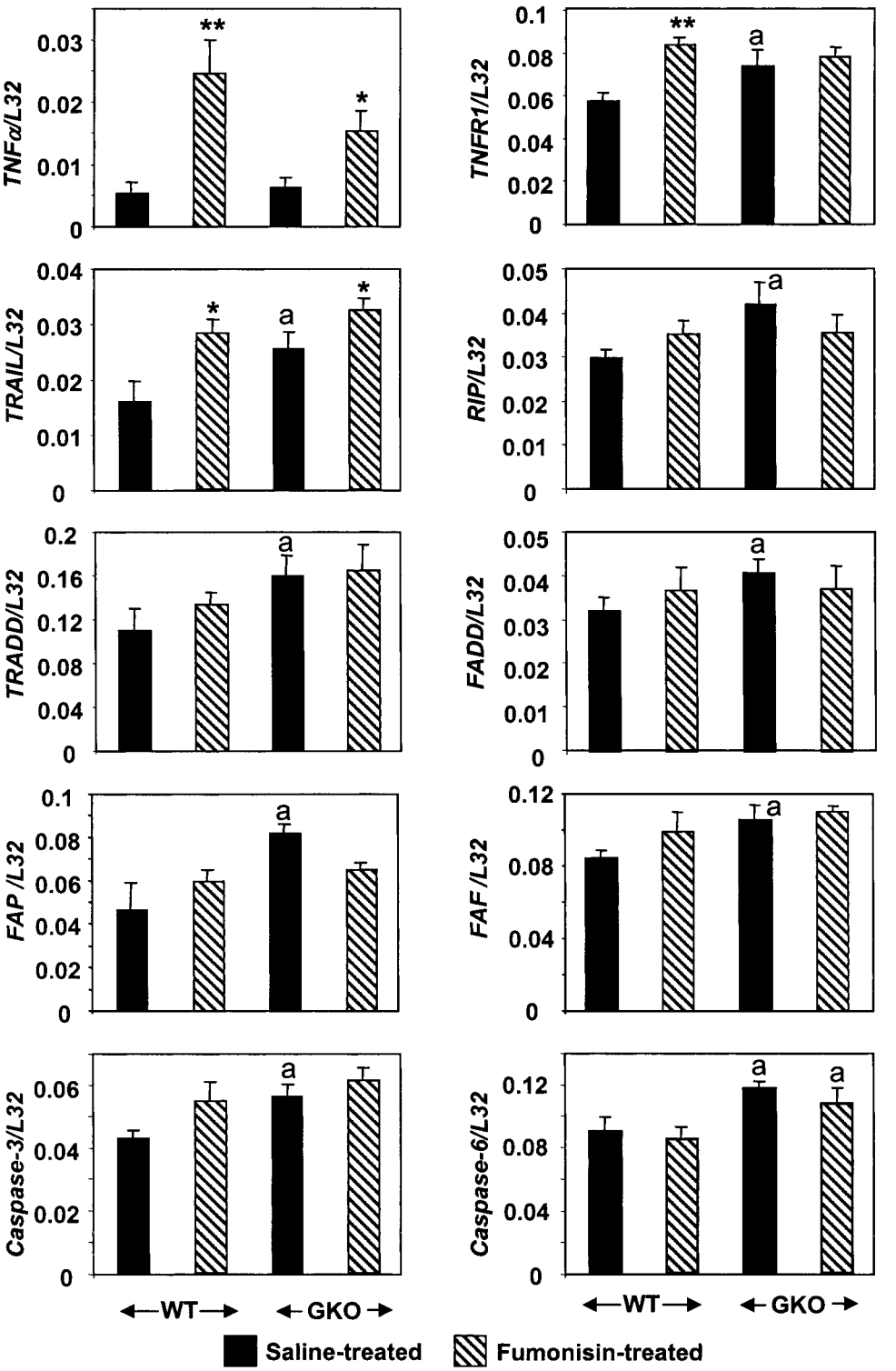

FIG. 7. Alterations in the expression of TNF-α and TNF-α-related factors in the livers of saline-treated and FB_1-treated WT and GKO mice, measured by RPA. The relative mRNA expression was normalized against L32 (mean ± SEM, $n = 5$). Significant differences between the saline-treated group and FB_1-treated groups of the same strain are indicated at $^*p < 0.05$ and $^{**}p < 0.01$. A significant difference between the saline-treated groups of the two strains is marked by the letter "a."

PCR and RPA (30% and 35%, respectively). The increases in TNF-α in liver after FB_1 treatment were 4.8-fold vs. 2.5-fold in WT and GKO mice, respectively, using quantitative RPA.

TNF-α, IL-12, and IFN-γ reportedly are involved in a positive feedback loop that may lead to hepatotoxicity.[22] TNF-α and IL-12 are produced by the Kupffer cells of the liver and can act on resident natural killer (NK) cells or Th1 cells in liver to produce IFN-γ. The IFN-γ can then activate Kupffer cells to produce more TNF-α and IL-12, eventually producing more IFN-γ from the effector cells. Elevated levels of TNF-α and IFN-γ play a major role in other models of hepatotoxicity.[23] The lack of IFN-γ in GKO mice will likely interrupt this loop, leading to a limited induction of TNF-α and IL-12, as observed in the current study. The two inflammatory cytokines, TNF-α and IFN-γ, also may have synergistic effects in causing liver damage.[24] All three cytokines, IL-12, IFN-γ, and TNF-α, have been shown to be important in the generalized Shwartzman reaction.[24] IFN-γ may thus determine the extent of liver damage as it modulates the level of TNF-α expression.

Genetically modified animals serve as good models to investigate the role of individual gene products. The GKO mice are viable and fertile and appear to be normal in a clean environment. In response to pathogens, however, these animals show reduced macrophage function. Specifically, the macrophages have impaired production of antimicrobial products and reduced expression of MHC II antigens.[15] The GKO mice have uncontrolled proliferation of splenocytes in response to mitogens and alloantigens and reduced resting NK cell activity. A lack of impact of FB_1 on spleen size in both WT and GKO mice indicated that FB_1 did not act as an antigenic stimulus in these animals. Using macrophages from GKO mice, Xing et al.[25] showed that IL-12 and nitric oxide release in response to bacterial infection was mediated through endogenous IFN-γ. Both mycobacteria and IFN-γ released TNF-α synergistically.[25] Concanavalin A (ConA)-induced hepatitis was suppressed in GKO mice but not in homogygous TNF-α-knockout mice.[26] On the other hand, overexpression of IFN-γ, as in IFN-γ transgenic mice, provides a suitable model for chronic hepatitis from the age of 6–10 weeks.[27] Taken together, these findings corroborate the role IFN-γ plays in the development of liver injury, including that caused by FB_1.

Fumonisins produce hepatotoxicity in most animal species. However, liver is the principal target organ in mice. Both sphinganine and sphingosine are important cell signal mediators. It is not fully understood how accumulation of free sphingoid bases causes altered cell signaling, for both death and proliferation (depending on the cell type), but preventing sphingoid base accumulation by inhibitors of SPT effectively counteracts the FB_1-induced toxic effects.[28,29] Other effects of FB_1 are perhaps secondary to impairment of sphingolipid metabolism and include modulation of cytokine expression, activation and suppression of PKC and mitogen-activated protein kinases, and regulation of a variety of apoptotic and cell-cyling intermediates.[30]

The current report suggests that the expression of a variety of signaling factors is modulated by FB_1, and these modulations can be important in the hepatotoxic response of FB_1 in mice. The changes observed here in WT (C57BL/6J) mice were similar to those reported earlier for male BALB/c mice.[14] It is noteworthy, however, that not only are the GKO mice just lacking IFN-γ, but a whole series of other signaling factors are also affected by knocking out the *IFN-γ* gene. The GKO mice had a relatively higher basal expression level of a number of cell cycling and apoptotic factors compared with WT animals. High expression of Mad and Myc, which repress transcription and growth, may account for part of the refractory nature of GKO mice in response to FB_1 by reducing the transcription of other death-signaling factors. Max and Mad may have both apoptotic and antiapoptotic effects.[31] Our previous studies suggested that higher basal levels of proinflammatory cytokines, including TNF-α, IFN-γ, and IL-12p40, in female BALB/c mice liver than those observed in their male counterparts may account for the higher hepatotoxicity in females after FB_1 treatment. The basal levels of other apoptosis and signaling genes were not different in the livers of two sexes in that strain.[14]

The FB_1-induced increase in several signaling factors was less in GKO than in WT mice. These included the inflammatory cytokines TNF-α and IL-1β and other proapoptotic factors, including c-*myc* and *bax*. Various factors that have antiapoptotic influence, for example, b-*myc* and *bcl-2*, were also lower in FB_1-treated GKO mice than in similarly treated WT animals. These may have a lesser influence in the total hepatotoxic outcome of FB_1 in GKO mice. It is likely that the hepatotoxic response of FB_1 in GKO animals is influenced by a balance of various signaling factors in the cell, including those that have opposite effects.

Results of this study suggest that a network of signaling factors rather than a single cytokine may be responsible in the pathologic outcome of FB_1 hepatotoxicity in mice. Although the GKO mice show reduced hepatotoxicity to FB_1 and treatment of normal mice with FB_1 elevates IFN-γ expression, the overall data do not establish a role for elevated IFN-γ production in the pathogenesis of hepatotoxicity. Evidence from prior work implicates sphingolipid metabolism in the hepatotoxicity in FB_1-treated mice, and this is modulated by cytokines, including TNF-α. IFN-γ may play a limited role in the final expression of the hepatotoxic response to FB_1, and the absence of this cytokine may influence FB_1 effects in these animals by modulating TNF-α induction, enhancing the regeneration process, or by causing an imbalance in various apoptotic and antiapoptotic signals. The reduced hepatotoxicity in GKO mice may also result from altered sphingolipid metabolism. This is a topic of our current studies.

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Address reprint requests or correspondence to:
Dr. Raghubir P. Sharma
Department of Physiology and Pharmacology
College of Veterinary Medicine
The University of Georgia
Athens, GA 30602-7389

Tel: (706) 542-2788
Fax: (706) 542-3015
E-mail: rpsharma@vet.uga.edu

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