

Genetic or Pharmacologic Activation of Nrf2 Signaling Fails to Protect Against Aflatoxin Genotoxicity in Hypersensitive GSTA3 Knockout Mice

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Mice are resistant to aflatoxin hepatotoxicity, primarily due to high expression of glutathione S-transferases (GSTs), and in particular the GSTA3 subunit. Nuclear factor erythroid 2 related factor 2 (Nrf2) signaling, which controls a broad-based cytoprotective response, was activated either genetically or pharmacologically in an attempt to rescue GSTA3 knockout mice from aflatoxin genotoxicity. Genetic activation of Nrf2 signaling was attained in a GSTA3: hepatocyte-specific Keap1 double knockout (DKO) mouse whereas pharmacologic activation of Nrf2 was achieved through pretreatment of mice with the triterpenoid 1-[2-cyano-3-,12-dioxoleana-1,9(11)-dien-28-oyl] imidazole (CDDO-Im) prior to aflatoxin B₁ exposure. Following oral treatment with aflatoxin, urine was collected from mice for 24 h and hepatic and urinary aflatoxin metabolites then quantified using isotope dilution-mass spectrometry. Although Nrf2 was successfully activated genetically and pharmacologically, neither means affected the response of GSTA3 knockout mice to chemical insult with aflatoxin. Hepatic aflatoxin B₁-N⁷-guanine levels were elevated 120-fold in GSTA3 knockout mice compared with wild-type and levels were not attenuated by the interventions. This lack of effect was mirrored in the urinary excretion of aflatoxin B₁-N⁷-guanine. By contrast, urinary excretion of aflatoxin B₁-N-acetylcysteine was >200-fold higher in wild-type mice compared with the single GSTA3 knockout or DKO mouse. The inability to rescue GSTA3 knockout mice from aflatoxin genotoxicity through the Nrf2 transcriptional program indicates that *Gsta3* is unilaterally responsible for the detoxication of aflatoxin in mice.

Key words: Nrf2; Keap1; aflatoxin; glutathione S-transferases; DNA adducts.

Aflatoxin B₁ (AFB₁) is a potent hepatocarcinogen produced by the mold *Aspergillus flavus*, which commonly grows on groundnuts and maize (Kensler *et al.*, 2011). AFB₁ is metabolized by cytochrome P450s to a reactive AFB₁-epoxide that can cause genotoxic damage by binding with DNA at the N – 7 atom of guanine (Eaton and Gallagher, 1994). This AFB₁-N⁷-guanine adduct is unstable and undergoes spontaneous depurination re-

sulting in an abasic site in DNA leading to the excretion of the N – 7 adduct in urine. The AFB₁-N⁷-guanine adduct can also rearrange to form a stable, ring-opened formamidopyrimidine adduct. Both forms of DNA lesions may lead to cytotoxicity and mutagenesis. The AFB₁-epoxide can be detoxified by conjugation with glutathione through the catalytic actions of glutathione S-transferases (GSTs). Upon further metabolism, these thiol conjugates are excreted in urine as water soluble aflatoxin mercapturic acids (AFB₁-NAC) (Busby and Wogan, 1984).

Significant disparities in sensitivity to AFB₁ carcinogenesis have been reported between species with humans, rats, ducks and trout exhibiting greater sensitivity, and adult mice greater resistance to the toxic effects of AFB₁ (Busby and Wogan, 1984). Interestingly, newborn mice are substantially more susceptible to hepatocarcinogenesis than adult mice (Veselinovitch *et al.*, 1972). In addition, newborn mice have very low hepatic GST levels compared with adult mice (Shupe and Sell, 2004). Such interspecies and age-related differences may reflect variations in the expression and catalytic activities of distinct GST isoforms toward conjugation of the aflatoxin epoxide. In particular, adult mice exhibit high constitutive levels of the GSTA3 subunit, which is very active with the AFB₁-epoxide as substrate (Buetler *et al.*, 1992). Mice in which the *Gsta3* gene is specifically knocked out exhibit accentuated acute cytotoxicity and genotoxicity following AFB₁ exposure (Ilic *et al.*, 2010). Thus, GSTA3 is an important determinant of resistance to AFB₁ in this species

Genetic intervention represents a means for alteration of nuclear factor erythroid 2 related factor 2 (Nrf2) signaling. The Nrf2-knockout mouse has been shown to be highly susceptible to many toxic compounds and carcinogens (reviewed in Slocum and Kensler, 2011). Correspondingly, hepatocyte-specific Keap1 (Kelch-like ECH associating protein 1) knockout mice, which exhibit constitutive up-regulation of Nrf2 signaling, demonstrate high hepatic expression of detoxication enzymes including GSTs and NAD(P)H:quinone acceptor oxi-

doreductase 1 (NQO1) (Okawa *et al.*, 2006). This mouse is resistant to the acute toxicities of many hepatotoxins (Liu *et al.*, 2013), although its relative sensitivity or resistance to AFB₁ is not known.

In addition to genetic intervention, protection from the toxic and carcinogenic effects of aflatoxins can be conferred in sensitive species such as rats by many classes of compounds, including phenolic antioxidants, dithiolethiones, isothiocyanates, and synthetic oleanane triterpenoids (Yates and Kensler, 2007). These protective actions can be attributed largely to interactions with the Keap1-Nrf2 pathway. These compounds oxidize or alkylate cysteines in Keap1, thereby allowing the transcription factor Nrf2 to escape proteasomal degradation and translocate into the nucleus. Nrf2 in the nucleus drives the transcription of its target genes, including multiple isoforms of GSTs, through antioxidant response elements in their upstream regulatory domains (Chanas *et al.*, 2002). 1-[2-Cyano-3-,12-dioxoleano-1,9(11)-dien-28-oyl] imidazole (CDDO-Im) is among the most potent inducers of Nrf2 signaling *in vitro* and *in vivo* (Dinkova-Kostova *et al.*, 2005; Yates *et al.*, 2006). Cotreatment of rats with AFB₁ and CDDO-Im leads to a dramatic reduction of the hepatic burden of preneoplastic lesions compared with exposure to AFB₁ alone. Reductions of 40–90% of hepatic AFB₁-N⁷-guanine adducts coupled with declines of 85 to >99% for preneoplastic foci were reported over the range of 1–100 μmol CDDO-Im/kg body weight (Yates *et al.*, 2006). CDDO-Im is completely protective against AFB₁-induced hepatocarcinogenesis (Johnson *et al.*, forthcoming). Thus, in the rat, activation of Nrf2 signaling can affect profoundly the toxicity of aflatoxin.

To further probe the role of the Keap1-Nrf2 pathway as a modifier of chemical insult in mice, we examine whether genetic or pharmacologic activation of Nrf2 signaling could rescue the hyper-sensitive GSTA3 knockout mouse from the genotoxicity of AFB₁. Such a study could serve to delineate the unilateral role of a single, highly efficient detoxication enzyme versus a broad multigenic cytoprotective response as key effectors of murine resistance to aflatoxins.

MATERIALS AND METHODS

Caution. AFB₁ is a human carcinogen and dimethyldioxirane is an extremely volatile oxidant. Both should be used in properly vented areas exercising care to avoid personal exposure. Proper decontamination procedures should be followed.

Chemicals. AFB₁, potassium peroxy sulfate (Oxone; Dupont trademark), and *N*-acetylcysteine (NAC) were purchased from Sigma-Aldrich Co. (St Louis, MO). ¹³C¹⁵N-acetyl cysteine was purchased from Cambridge Isotope Laboratories (Tewksbury, MA). Dimethyldioxirane was prepared by the alkaline oxidation of acetone with the potassium peroxy sulfate and AFB₁-epoxide then prepared by oxidizing the dimethyldioxirane with

AFB₁. Subsequent synthesis of AFB₁-N⁷-guanine and AFB₁-N⁷⁻¹⁵N₅-guanine was conducted as described (Egner *et al.*, 2006). CDDO-Im was synthesized as described by Honda *et al.* (2000) and contributed by Dr. Michael Sporn, Department of Pharmacology and Toxicology, Dartmouth Medical School, Hanover, NH. All chromatographic solvents were HPLC grade and all chemicals used were of the highest purity grade possible.

Synthesis and purification of AFB₁-NAC. The disodium salt of NAC was prepared by adding a small piece of sodium metal to NAC dissolved in methanol (Bartels and Timchalk, 1990). A 10-fold excess of the reaction mixture was added to freshly prepared AFB₁-epoxide. After 1 min, the reaction was neutralized by the addition of 1M acetic acid. Solvents were evaporated and the crude AFB₁-NAC mixture purified by HPLC (Scholl *et al.*, 1997). A separate reaction was conducted substituting ¹³C¹⁵N-acetylcysteine was used to create the AFB₁-¹³C¹⁵N-NAC. Both the AFB₁-NAC and AFB₁-¹³C¹⁵N-NAC were over 99% isotopically pure.

Animals and characterization. All procedures were approved by the Johns Hopkins University Animal Care and Use Committee. Wild-type C57BL/6J mice and B6.Cg-Tg(Alb-Cre)21Mgn/J (AlbCre) mice were obtained from The Jackson Laboratories (Bar Harbor, ME). AlbCre:Keap1^{flox/flox} (Okawa *et al.*, 2006) and GSTA3 knockout mice (Ilic *et al.*, 2010) were crossed to produce compound GSTA3:Alb:Cre:Keap1^{flox/flox} double knockout mice (DKO). Mouse genotypes were verified through analysis of tail genomic DNA using previously published PCR assays (Jowsey *et al.*, 2003b; Okawa *et al.*, 2006). Male mice were used in all studies.

Quantitative real-time PCR. Total RNA was extracted from frozen liver samples from untreated mice using the Trizol reagent (Life Technologies, Carlsbad, CA). RNA samples were treated with TURBO DNase (Life Technologies) to remove any possible genomic DNA contamination and were further purified using the RNeasy Mini Kit (Qiagen, Germantown, MD). RNA was quantified spectrophotometrically at 260 nm. RNA purity was estimated by the optical absorbance ratio $A_{260\text{ nm}}/A_{280\text{ nm}}$ and RNA integrity was assessed by agarose gel electrophoresis. cDNA was prepared from 1 μg of each RNA sample using the qScript cDNA Synthesis Kit (Quanta Biosciences, Gaithersburg, MD). The cDNA samples were used as a template for qRT-PCR analysis using the iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) and specific primers for each gene synthesized by IDT (Coralville, IA). The primer sequences were obtained from the PrimerBank (Wang *et al.*, 2012) and are shown in Table 1. The qRT-PCR reactions were run in technical tetraplicates for each biological replicate in a MyiQ Single Color real-time PCR system (Bio-Rad) using the following conditions; 95°C for 3 min and then 95°C for 10 s (step 1), 61°C for 30 s (step 2), go to step 1, 39 times. The correct size of the PCR products was confirmed by agarose gel electrophoresis and their

purity was assessed by melt curve analysis using the MyiQ optical system software version 1.0410 (Bio-Rad). PCR efficiency was calculated from a standard curve using serial dilutions of pooled liver samples and relative mRNA levels were calculated by the comparative threshold cycle method using GAPDH as the housekeeping gene and the Pfaffl method to calculate fold changes (Pfaffl, 2001). The mean ratio was then calculated by dividing the relative mRNA levels of each target gene in the GSTA3 knockout mice by the relative mRNA levels of the same gene in the wild-type mice taking into account the error propagation that may arise by this method (Holmes and Buhr, 2007).

Treatment protocol. Approximately 9-week-old male mice were treated by gavage with three doses of vehicle or 30 $\mu\text{mol/kg}$ of CDDO-Im every other day (Monday, Wednesday, and Friday). This dose and schedule provides maximal induction of Nrf2 target genes *in vivo* in multiple murine tissues (Yates *et al.*, 2007) and completely protects against AFB₁-induced hepatocarcinogenesis in rats (Johnson *et al.*, forthcoming). The vehicle used for CDDO-Im was 10% DMSO (dimethyl sulfoxide), 10% Cremophor-EL and PBS. Twenty-four hours after receiving the last dose, a single oral 0.8 mg/kg dose of AFB₁ dissolved in DMSO was administered. Urine was collected from these mice by placing four mice in a glass metabolic cage for 24 h between administration of AFB₁ and euthanasia. Urine volumes were recorded and aliquots were frozen for analysis. Identical protocols were followed for experiments using DKO mice; however, without pretreatment with CDDO-Im or vehicle. Livers were removed, snap frozen in liquid nitrogen, and stored at -80°C prior to analysis. Hepatic AFB₁-N⁷-guanine, urinary AFB₁-N⁷-guanine, and AFB₁-NAC levels were measured by isotope-dilution mass spectrometry. A separate cohort of mice, treated identically but sacrificed 2 h after treatment with AFB₁ was used for NQO1 enzyme activity and immunoblot analyses.

Protein preparation and immunoblot analyses. Tissue was homogenized in radioimmunoprecipitation assay buffer (RIPA-I), which contained protease inhibitor cocktail (Roche, Indianapolis, IN). An equal volume of 2 \times SDS sample buffer was added, followed by denaturation via boiling for 5 min. Samples were run through a SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to an Amersham Hybond-C Extra nitrocellulose membrane (GE Life Sciences, Piscataway, NJ). Membranes were blocked with tris-buffered saline containing 0.1% Tween 20 and 5% skim milk followed by treatment with primary antibody. NQO1 antibody (ab80588) was obtained from Abcam (Cambridge, MA), GAPDH (NB300-221) antibody was sourced from Novus Biologicals (Littleton, CO) and anti-GSTA3 antibody was kindly provided by Dr John D. Hayes, University of Dundee, UK. Membranes were then reacted with the appropriate secondary antibodies conjugated to horseradish peroxidase (Bio-Rad, 170-6515, 170-5047). The

immunocomplexes were visualized with enhanced chemiluminescence.

Quantification of NQO1 enzyme activity. NQO1 enzyme activity was measured according to the method of Prochaska and Santamaria (1988) in homogenates prepared from livers of mice pretreated with three doses of CDDO-Im or vehicle and then sacrificed 2 h after dosing with AFB₁. NQO1 levels were normalized to protein concentration (Pierce Protein BCA Kit no. 23225, ThermoFisher Scientific, Rockford, IL).

Analysis of AFB₁ adducts and conjugates. Liver DNA was isolated 24 h after AFB₁ treatment and the AFB₁-N⁷-guanine adducts isolated as previously described (Kensler *et al.*, 1985). AFB₁-¹⁵N₅-guanine was used as an internal standard for both DNA adduct analyses as well as the urinary AFB₁-N⁷-guanine determinations. All mass spectrometric conditions have been previously reported (Egner *et al.*, 2006). AFB₁-NAC excreted into the urine was measured under the same chromatographic conditions using AFB₁-¹³C¹⁵N-NAC as an internal standard. Retention time for the AFB₁-N⁷-guanine was ~ 4.0 min while the AFB₁-NAC eluted at 6.2 min. Limits of detection for AFB₁-N⁷-guanine and AFB₁-NAC were 0.2 and 2 pmol/mg creatinine, respectively.

RESULTS

Generation and Characterization of Knockout and Transgenic Mice

The GST isoforms shown to be expressed in the liver of mice by Knight *et al.* (2008) were measured using real-time PCR methods to determine the impact, if any, of disruption of *Gsta3* on either the compensatory expression or off-target disruption of other hepatic GST isoforms. As shown in Figure 1A, ratios (*Gsta3* knockout/wild-type) of transcript levels of *Gsta1*, *Gsta4*, *Gstm1*, *Gstm2*, *Gstm3*, *Gstm4*, *Gstm5*, *Gstm6*, *Gstp1*, *Gstt1*, and *Gstt2* demonstrated only modest differences in expression by genotype. By comparison, transcripts for *Gsta3* were undetectable in the knockout mice. Collectively, these results indicate that the primary genotype and phenotype of these mice include normal expression of all hepatic GSTs except for *Gsta3* in the GSTA3 knockout mice.

GSTA3 knockout mice and hepatocyte-specific Keap1 knockout mice were mated to generate a AlbCre:Keap1^{flox/flox}::GSTA3^{-/-} double knockout mouse. PCR assays were utilized to verify this DKO genotype (Fig. 1B). Both male and female DKO mice appear healthy and demonstrate no phenotypic abnormalities. Immunoblot analyses substantiated that neither GSTA3 knockout nor DKO mice expressed the GSTA3 protein (Fig. 1C). Upon treatment with the triterpenoid CDDO-Im, hepatic protein and activity levels for NQO1, an enzyme not involved in AFB₁ metabolism (Eaton and Gallagher, 1994), increased in both wild-type and

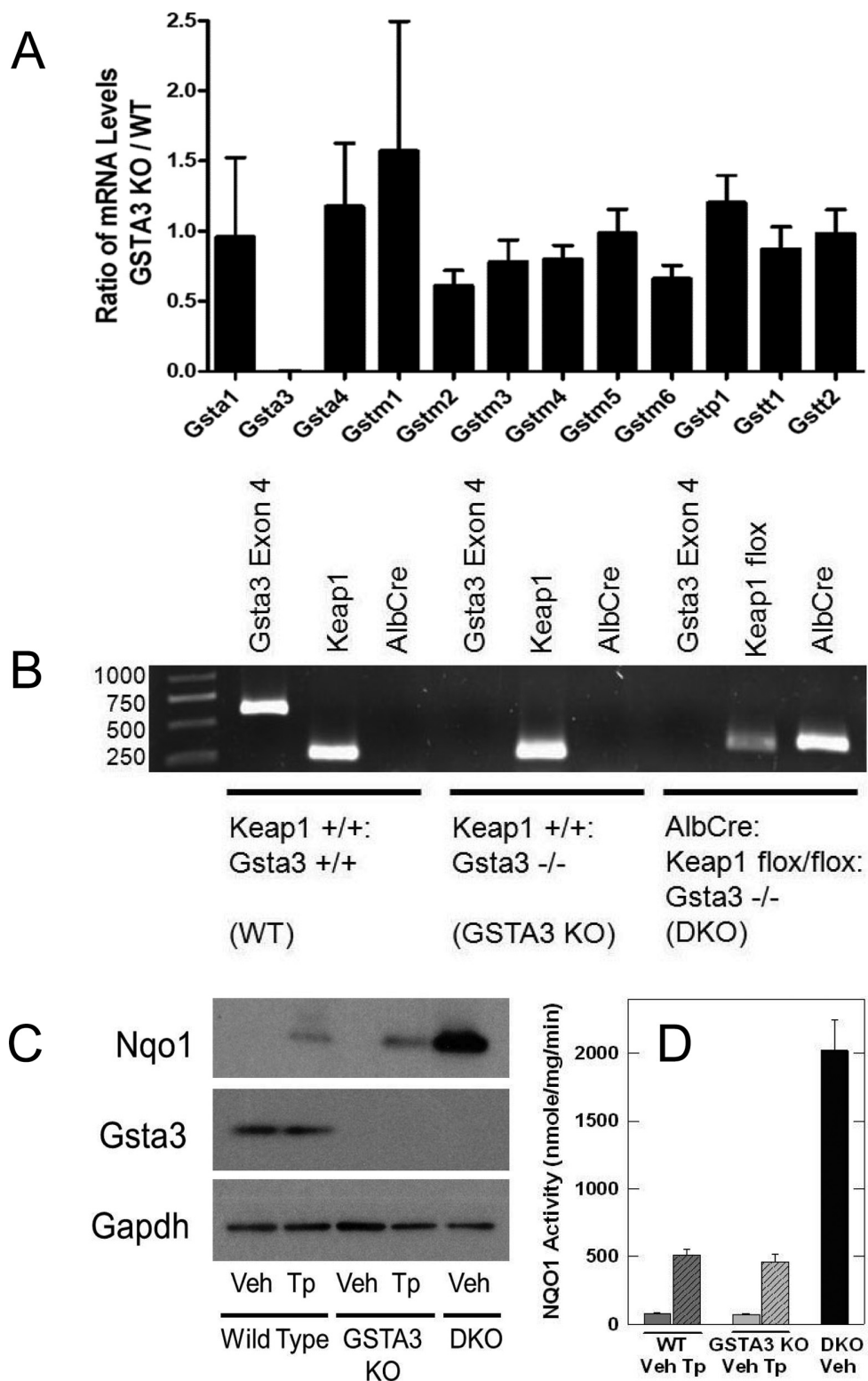


FIG. 1. Genotypic and phenotypic characterization of wild-type (WT), GSTA3 knockout (GSTA3 KO), and GSTA3:AlbCre:Keap1^{flox/flox} double knockout (DKO) mice. (A) Ratio of mRNA levels of hepatic GST isoforms between GSTA3KO and WT mice. Bars show mean \pm SD. (B) Genotypic characterization of mice by PCR. *Gsta3* was only present in the WT mice (576 bp). WT and GSTA3 KO mice demonstrated WT *Keap1* (250 bp) while DKO mice demonstrated floxed *Keap1* (350 bp). Albumin Cre recombinase was present only in the DKO mice (356 bp). (C) NQO1 and GSTA3 protein expression by Western blot. Veh, vehicle; Tp, triterpenoid (CDDO-Im). (D) Hepatic NQO1 activity. Values are the mean \pm SE, $n = 4$.

TABLE 1
Primers Used for Measuring GST Transcript Levels in Wild-type and GSTA3 Knockout (KO) Mouse Liver

Gene symbol	Forward primer	Reverse primer
GAPDH	AGGTCGGTGTGAACGGATTG	TGTAGACCATGTAGTTGAGGTCA
GST α 1	AAGCCCGTGCTTCACTACTTC	GGGCACTTGGTCAAACATCAAA
GST α 3	AGATCGACGGATGAACTGG	CAGATCCGCCACTCCTTCT
GST α 4	TGATTGCCGTGGCTCCATTTA	CAACGAGAAAAGCCTCTCCGT
GSTM1	ATACTGGGATACTGGAACGTCC	AGTCAGGGTTGTAACAGAGCAT
GSTM2	ACACCCGCATACAGTTGGC	TGCTTGCCAGAAAATCAGAG
GSTM3	GCGGACTGACTCACTCCATC	CCCCATGACATATCTCTTCTCCT
GSTM4	CTGAAGGTGGAATACTTGAGC	GCCAGGAAGTGTGAGAAGA
GSTM5	TCATCCAAGTCTATGGTTCTGGG	CCACAGATGTACCGTTTCTCCT
GSTM6	ACAGGTCATGGACACTCGAAT	TGGCTTCCGTTTCTCAAAGTC
GSTP1	ATGCCACCATACACCATTTGTC	GGGAGCTGCCCATACAGAC
GSTT1	AGGCTCGTGCTCGTGTAGA	CAGGGAACATCACCTTATGCC
GSTT2	TGCCAAAGTCCACGAATACC	CCATTCTATCTGTTCGGTCC

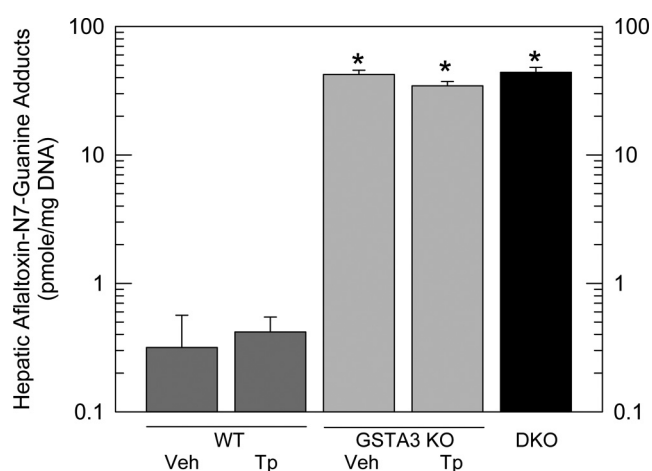


FIG. 2. Quantification of hepatic AFB₁-N⁷-guanine adducts (pmol/mg DNA) by isotope-dilution mass spectrometry for wild-type (WT) and GSTA3 KO mice receiving CDDO-Im (Tp) or vehicle (Veh) and in DKO mice 24 h after treatment with AFB₁. Values are the mean \pm SE, $n = 8$. (*) Differs from WT vehicle, $p < 0.05$.

GSTA3 knockout mice (Figs. 1C and D). In addition, NQO1 activity was approximately fourfold greater in livers of DKO mice as compared to wild-type or GSTA3 knockout mice receiving CDDO-Im (Figs. 1C and D). As *Nqo1* is regulated by Nrf2 (Nioi *et al.*, 2003), these analyses confirm that efforts to genetically and pharmacologically activate Nrf2 signaling were successful.

Effect of Nrf2 Activation on Hepatic Aflatoxin B₁-N⁷-Guanine Levels

Comparison of AFB₁-N⁷-guanine adduct levels in livers collected 24 h after administration of AFB₁ across genotypes and treatments indicates that neither genetic nor pharmacologic activation of Nrf2 signaling significantly altered the hepatic adduct burden in GSTA3 knockout mice (Fig. 2). GSTA3 knockout mice receiving vehicle pretreatment had a mean 42.3 ± 3.1

pmol/mg DNA adducts as compared with 0.3 ± 0.0 pmol/mg DNA among wild-type mice receiving the vehicle pretreatment. Pretreatment with the triterpenoid CDDO-Im had insignificant effects on both GSTA3 knockout and wild-type mice, with a mean adduct burden of 34.5 ± 2.7 pmol AFB₁-N⁷-guanine adducts/mg DNA among GSTA3 knockout mice and 0.4 ± 0.1 pmol/mg DNA among wild-type mice receiving CDDO-Im. Hepatocyte-specific disruption of Keap1 to enhance Nrf2 signaling likewise did not alter the adduct burden of the GSTA3 knockout mice as there was a mean 44.0 ± 4.0 pmol AFB₁-N⁷-guanine adducts/mg DNA among DKO mice. The great disparity in hepatic aflatoxin genotoxicity between GSTA3 knockout and wild-type mice (Ilic *et al.*, 2010) was not reduced through pharmacologic or genetic activation of Nrf2 signaling.

Effect of Nrf2 Activation on Urinary Aflatoxin Metabolite Levels

Analyses of aflatoxin metabolites in urine collected for 24 h after administration of 0.8 mg/kg of AFB₁ corroborated the failure of activation of Nrf2 signaling to enhance aflatoxin detoxication in GSTA3 knockout mice (Table 2). Pretreatment with CDDO-Im failed to significantly reduce the urinary levels of excreted AFB₁-N⁷-guanine adducts among GSTA3 knockout mice, which exhibited greater than 100-fold higher urinary concentrations of adducts than the wild-type mice. Correspondingly, the wild-type mice excreted several hundred-fold greater amounts of the mercapturic acid, AFB₁-NAC, suggesting a greater aflatoxin detoxication capacity than for the GSTA3 knockout mice. Hepatocyte-specific Keap1 disruption similarly did not reduce the level of urinary AFB₁-N⁷-guanine adducts or increase excretion of AFB₁-NAC among GSTA3 knockout mice. Indeed, the disparity in aflatoxin detoxication capacity between GSTA3 knockout and wild-type mice is highlighted by nondetectable levels of AFB₁-N⁷-guanine in the urine of wild-type mice and nondetectable levels of AFB₁-NAC in the urine of GSTA3 knockout mice.

TABLE 2
Urinary Excretion of AFB₁-N⁷-Guanine and AFB₁-NAC

Genotype	Pretreatment	AFB ₁ -N ⁷ -guanine (pmol/mg creatinine)	AFB ₁ -NAC (pmol/mg creatinine)
Wild-type	Vehicle	<0.2 ^a	471 ± 81
	Triterpenoid	<0.2	655 ± 83
GSTA3 KO	Vehicle	71.2 ± 14.8	<2
	Triterpenoid	60.3 ± 2.4	<2
DKO	None	69.2 ± 12.0	<2

^aFour pools of four mice each were used for the urinary analyses. Values are mean ± S.E.

DISCUSSION

Aflatoxin B₁ is metabolized by cytochrome P450s to AFB₁-epoxides, which can bind with DNA or undergo detoxication. In mice, the principal pathway for detoxication is through conjugation of the epoxide with glutathione mediated by GSTs. Murine GSTs exhibit substantially greater conjugative activity with the AFB₁-epoxide than those in rats or other species with higher susceptibility to aflatoxin toxicity (Eaton and Gallagher, 1994). Alpha class GSTs are believed to play the primary role in aflatoxin detoxication and constitutive expression of the *Gsta3* subunit accounts for 35% of all GSTs in the livers of male mice (Mitchell *et al.*, 1997). Upon transfection into hamster V79 cells, murine *Gsta3* reduced AFB₁ genotoxicity by 70–80% and increased resistance to cytotoxicity 4.6-fold (Fields *et al.*, 1999).

There is robust evidence that many murine GSTs, are regulated through the Keap1-Nrf2 signaling pathway, including *Gsta1*, *Gsta2*, *Gsta4*, *Gstm1*, *Gstm2*, *Gstm3*, *Gstm4*, and *Gstm5* (Chanas *et al.*, 2002; Hayes *et al.*, 2000; Knight *et al.*, 2008; McWalter *et al.*, 2004). However, evidence for the regulation of *Gsta3* by an antioxidant response element in the Keap1-Nrf2 pathway in mice is ambiguous. Heightened levels of GSTA3 were observed when mouse Hepa-1c1c7 cells were treated with the isothiocyanate, sulforaphane, a potent Nrf2 inducer. (Jowsey *et al.*, 2003a; McWalter *et al.*, 2004). Additionally, these studies reported that *Nrf2*^{-/-} mice demonstrated lower constitutive expression of GSTA3 than wild-type, and that GSTA3 was not inducible in these transgenic mice. Other studies have indicated that there was no reduction of basal GSTA3 in *Nrf2*^{-/-} mice, and likewise Nrf2 inducers such as butylated hydroxyanisole, oltipraz, and ethoxyquin had no effect on hepatic GSTA3 expression (Chanas *et al.*, 2002; Hayes *et al.*, 2000; Knight *et al.*, 2008). In this study, treatment with CDDO-Im had no effect on hepatic GSTA3 expression in wild-type C57BL/6J mice, lending credence to the hypothesis that *Gsta3* is not regulated by the Keap1-Nrf2 pathway in the mouse. Nevertheless, given the arsenal of phase II enzymes, including other GSTs, which are reg-

ulated through the Nrf2 signaling pathway, it would be expected that they could compensate for the loss of GSTA3 in mitigating AFB₁ genotoxicity. However, no reduction in AFB₁ genotoxicity was attained in the GSTA3 knockout mice despite successful activation of the Keap1-Nrf2 signaling pathway achieved either pharmacologically or genetically. These data indicate that *Gsta3* is unilaterally responsible for the detoxication of AFB₁ in the mouse. Such a single deterministic outcome defining natural resistance to an environmental carcinogen in mammals is unusual, but not unprecedented. Pi class GST knockout mice are more sensitive to skin, lung and colon carcinogenesis (Henderson *et al.*, 1998; Ritchie *et al.*, 2007, 2009). However, whether such mice can be rescued by members of the Keap1-Nrf2 gene battery is not known.

Other attempts to define unilateral determinants of resistance to AFB₁ genotoxicity have failed. The epoxide-derived dialdehyde of AFB₁ has been hypothesized to contribute indirectly to the carcinogenic effects of AFB₁ via protein adduction and subsequent hepatotoxicity. This aldehyde is reduced through the activity of aflatoxin aldehyde reductases (AKR7A1), which is the most highly inducible gene yet described in rat liver by CDDO-Im and other inducers known to affect Nrf2 signaling (Knight *et al.*, 1999; Yates *et al.*, 2006). Rats are a species with greater vulnerability to the effects of AFB₁ than mice. However, AKR7A1 transgenic rats still maintained congruent susceptibility to hepatocarcinogenesis as wild-type rats despite robust expression of the transgene and demonstrable alterations in the disposition of the aflatoxin dialdehyde *in vivo* (Roebuck *et al.*, 2009). The mechanism of protection in the rat likely lies in the induction of GSTA5, which is the orthologous GST to murine GSTA3 and has a very high catalytic activity towards the AFB₁ epoxide (Buetler *et al.*, 1995).

Disruption of *Nrf2* has been shown to enhance the sensitivity of mice to chemical carcinogenesis in models targeting the forestomach with benzo[a]pyrene (Ramos-Gomez *et al.*, 2001), the bladder with N-nitrosobutyl(4-hydroxybutyl)amine (Iida *et al.*, 2007), the skin with dimethyl-

benz[a]anthracene (Xu *et al.*, 2006), and the liver with 2-amino-3-methylimidazo[4,5]quinoline (Kitamura *et al.*, 2007). Correspondingly, *Keap1*^{flox/flox} mice are more resistant to tongue and esophageal carcinogenesis following treatment with 4-nitroquinoline-1-oxide than wild-type (Okhoshi *et al.*, 2013). The cytoprotective transcriptional program regulated through Nrf2 consists of several hundred genes, many but not all of which encode enzymes engaged in the detoxication of chemicals. Thus, there is a presumption that multiple gene products contribute to this broad sweep of protection. In this context, it is remarkable that a single gene, *Gsta3*, has evolved to determine sensitivity of mice to AFB₁. The extent to which GSTs determine human sensitivity to AFB₁, and their inducibility in either Nrf2-dependent or -independent manners is not resolved. Human alpha class GST proteins that are constitutively expressed in the liver (hGSTA1 and hGSTA2) have little, if any activity toward the AFB₁-epoxide, although mu class GSTs may afford some protection (Eaton *et al.*, 2001). Early chemoprevention trials with the Nrf2 inducer oltipraz provoked enhanced rates of urinary excretion of the AFB₁-NAC conjugate compared to placebo treated participants (Wang *et al.*, 1999). Collectively, studies across species highlight the importance of GSTs in the detoxication of aflatoxin and as possible targets for induction by chemopreventive agents.

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