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Deficiency of Glutathione Transferase Zeta Causes Oxidative Stress and Activation of Antioxidant Response Pathways

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ABSTRACT

Glutathione *S*-transferase (GST) zeta (GSTZ1-1) plays a significant role in the catabolism of phenylalanine and tyrosine, and a deficiency of GSTZ1-1 results in the accumulation of maleylacetoacetate and its derivatives maleylacetone (MA) and succinylacetone. Induction of GST subunits was detected in the liver of *Gstz1*$^{-/-}$ mice by Western blotting with specific antisera and high-performance liquid chromatography analysis of glutathione affinity column-purified proteins. The greatest induction was observed in members of the mu class. Induction of NAD(P)H:quinone oxidoreductase 1 and the catalytic and modifier subunits of glutamate-cysteine ligase was also observed. Many of the enzymes that are induced in *Gstz1*$^{-/-}$ mice are regulated by antioxidant response elements that respond to oxidative stress via the Keap1/Nrf2 pathway. It is significant that diminished glutathione concentrations were also observed in the liver of *Gstz1*$^{-/-}$ mice, which supports the conclusion that under normal dietary conditions, the accumulation of electrophilic intermediates such as maleylacetoacetate and MA results in a high level of oxidative stress. Elevated GST activities in the livers of *Gstz1*$^{-/-}$ mice suggest that GSTZ1-1 deficiency may alter the metabolism of some drugs and xenobiotics. *Gstz1*$^{-/-}$ mice given acetaminophen demonstrated increased hepatotoxicity compared with wild-type mice. This toxicity may be attributed to the increased GST activity or the decreased hepatic concentrations of glutathione, or both. Patients with acquired deficiency of GSTZ1-1 caused by therapeutic exposure to dichloroacetic acid for the clinical treatment of lactic acidosis may be at increased risk of drug- and chemical-induced toxicity.

The zeta-class glutathione *S*-transferases (GSTZs) are found in many eukaryotic species, including fungi, plants, insects, and mammals (Board et al., 1997). In mice and humans, a zeta-class GST is expressed in many tissues at a low level but is most abundant in the liver and kidney (P. G. Board, unpublished observations). The distribution and conservation of these enzymes over such a wide evolutionary range suggested that the zeta-class GSTs catalyze an important homeostatic reaction. The studies of Fernandez-Canon and Penalva (1998) subsequently demonstrated that GSTZ1-1 and maleylacetoacetate isomerase (MAAI) are identical. MAAI catalyzes the glutathione-dependent isomerization of maleylacetoacetate to fumarylacetoacetate, the penultimate step in the phenylalanine and tyrosine degradation pathway. Mutations of many enzymes in this pathway are associated with metabolic diseases in humans (Tanguay et al., 1996; Fernandez-Canon and Penalva, 1998; Mitchell et al., 2001).

Unlike many GSTs, the zeta-class isoenzymes do not catalyze the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with glutathione (Board et al., 1997). The zeta-class GSTs, however, do catalyze the biotransformation of a range of α-haloacids, including dichloroacetic acid, bromochloroacetic acid, chlorofluoroacetic acid, and dibromoacetic acid, but not difluoroacetic acid (Tong et al., 1998). Dichloroacetic acid is carcinogenic in rodents (Bull et al., 1990; DeAngelo et al., 1996), and human exposure to dichloroacetic acid occurs with

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ABBREVIATIONS: GSTZ, glutathione *S*-transferase zeta; MAAI, maleylacetoacetate isomerase; CDNB, 1-chloro-2,4-dinitrobenzene; DCNB, 1,2-dichloro-nitrobenzene; ARE, antioxidant response element; NQO1, NAD(P)H:quinone oxidoreductase 1; ALT, alanine aminotransferase; BHA, butylated hydroxyanisole; NAPQI, *N*-acetyl-*p*-benzoquinoneimine; GCL, glutamate-cysteine ligase; GSH, reduced glutathione; GSSG, glutathione disulfide; GST, glutathione *S*-transferase; m, mouse; r, rat; h, human.

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consumption of chlorinated drinking water (Krasner et al., 1989) and with its use in the treatment of metabolic lactic acidosis (Stacpoole et al., 1998).

The effects of GSTZ1/MAAI deficiency have never been fully investigated in humans or other mammals. An infant with symptoms consistent with MAAI deficiency died and was never fully characterized (Berger et al., 1988). Dichloroacetic acid is a mechanism-based inhibitor of GSTZ1-1, and treatment of rats with dichloroacetic acid causes a transient partial deficiency of activity that leads to the accumulation of maleylacetoacetate and maleylacetoacetone (Tzeng et al., 2000; Lantum et al., 2003). The therapeutic use of dichloroacetic acid for the treatment of lactic acidosis is also likely to cause a partial deficiency of GSTZ1-1/MAAI but seems to be well tolerated (Stacpoole et al., 1998).

Because it seemed likely that a complete deficiency of GSTZ1-1/MAAI may have severe metabolic consequences, *Gstz1* knockout mice have been developed in two different laboratories (Fernandez-Canon et al., 2002; Lim et al., 2004). Although these mice seem to survive and breed successfully, they show enlarged liver and kidneys as well as splenic atrophy (Lim et al., 2004). Light and electron microscopic examination revealed multifocal hepatitis and ultrastructural changes in the kidney (Lim et al., 2004). Tissue extracts from *Gstz1*$^{-/-}$ mice lacked MAAI activity, indicating that this reaction is not catalyzed by other GSTs or other enzymes (Lim et al., 2004). Despite the inability of other GSTs to catalyze the MAAI reaction, we noted the constitutive induction of several GSTs and NAD(P)H:quinone oxidoreductase 1 in the livers of *Gstz1*$^{-/-}$ mice (Lim et al., 2004). The induction of these enzymes is consistent with the activation of the Keap1/Nrf2 pathway that regulates the expression of genes that contain antioxidant response elements (AREs) in their promoter regions (Lim et al., 2004; Mathers et al., 2004). These observations suggest that even under normal dietary conditions, GSTZ1-1 deficiency results in the generation of a constant level of oxidative stress.

In the present study, we have undertaken a more extensive investigation of the GSTs induced in *Gstz1*$^{-/-}$ mice and investigated the effect of GSTZ1/MAAI deficiency on tissue glutathione concentrations and redox balance. The constitutive induction of a range of enzymes involved in the disposition of drugs and xenobiotics that trigger oxidative stress suggests that GSTZ1-1 deficiency may have a significant impact on the metabolism and toxicity of some therapeutic agents. Hence, we have evaluated the response of *Gstz1*$^{-/-}$ mice to treatment with acetaminophen, which results in decreased hepatic glutathione concentrations.

Materials and Methods

***Gstz1*$^{-/-}$ Mice, Acetaminophen Treatment, and Plasma ALT Assays.** *Gstz1*$^{-/-}$ mice on a BALB/c background were generated as described previously (Lim et al., 2004). In some experiments, mice were given 3% (w/v) phenylalanine in the drinking water for 28 days, as described previously (Lim et al., 2004).

Mice were given 300 mg/kg i.p. acetaminophen in saline. Blood was collected into EDTA-containing tubes from the orbital vein at specified time points for preparation of plasma. Mice were killed 24 h after administering acetaminophen, and tissues were fixed in neutral buffered formalin. The least necrotic portions of liver were selected visually and frozen at −70°C. Plasma alanine aminotransferase (ALT) activities were determined by ACT Pathology (Canberra, Australia) with VITROS dry-slide technology (Ortho-Clinical Diagnostics, Rochester, NY). All studies were undertaken with the approval of the Australian National University Animal Ethics Experimentation Committee under the guidelines established by the Australian National Health and Medical Research Council.

GST Enzyme Activity. Enzymatic activity with CDNB, 1,2-dichloro-4-nitrobenzene (DCNB), and ethacrynic acid as substrates was determined spectrophotometrically (Habig et al., 1974; Mannervik and Widersten, 1995).

Western Blotting. Western blotting was performed as described previously (Lim et al., 2004); samples of 90 μg of protein from each sample were analyzed on 10% SDS-polyacrylamide gel electrophoresis gels and transferred to nitrocellulose membranes. Protein loading was checked by staining duplicate gels with Coomassie blue. Proteins were detected with rabbit antisera by enhanced chemiluminescence (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Antisera against mGSTA1/2, mGSTA3-3, rGSTA4-4, rGSTM1-1, hGSTM3-3, mGSTP1/2, and rat NAD(P)H:quinone oxidoreductase 1 (NQO1) were provided by Professor John Hayes (University of Dundee, Dundee, UK). Antisera against recombinant hGSTZ1-1, mGSTT1-1, hGSTT2-2, human glutathione synthetase, and γ-L-glutamate-L-cysteine ligase purified from sheep erythrocytes (Board et al., 1980) were raised in rabbits in the laboratory of Professor Philip Board (Australian National University, Canberra, Australia). The nomenclature used to describe mouse and human GST subunits is based the reports of Mannervik et al. (1992, 2005). In general, all of the GSTs referred to in this study are from mice. In those instances in which human enzymes are referred to or if there is a possibility of confusion, the human subunit name is preceded by an h (e.g., hGSTM3) and the mouse subunit name is preceded by an m (e.g., mGSTM5).

HPLC Analysis. The GST isoenzymes present in mouse liver were isolated by purification on glutathione-agarose followed by separation by reverse-phase HPLC according to Mitchell et al. (1997), with HPLC conditions adapted to a Shimadzu VP HPLC system (Shimadzu, Kyoto, Japan). The HPLC system consisted of two LC-8A pumps connected to a 2.6-ml mixing loop, an SCL-10A VP system controller, an SIL-10AD VP autoinjector, and an SPD-M10A VP diode-array detector. Purified GSTs were acid-precipitated with 1% trifluoroacetic acid and resuspended in 40% acetonitrile/0.1% trifluoroacetic acid. Individual subunits were separated on a Vydac 214TP54 C_4 reverse-phase column. The flow rate was 1.0 ml/min; all solutions contained 0.1% (v/v) trifluoroacetic acid, and the mobile phase was a gradient from acetonitrile/water (40:60, v/v) at 0 min to acetonitrile/water (54:46, v/v) at 25 min, followed by a 1-min gradient to acetonitrile/water (65:35, v/v) to remove remaining proteins from the column, a 1-min gradient to return to acetonitrile/water (40:60, v/v), and a 10-min re-equilibration time between injections. The absorbance of the eluate was monitored at 214 nm, and peaks were quantified with the Shimadzu Class-VP software. Subunits were quantified in preparations from three separate mice for each group. Fractions were collected, and Western blots were performed to confirm peak identities.

Glutathione Determinations. Total glutathione [reduced glutathione (GSH) + glutathione disulfide (GSSG)] and GSSG concentrations in mouse tissues were assayed with the glutathione reductase cycling assay of Baker et al. (1990). Where possible, tissues were homogenized fresh after being rinsed in ice-cold phosphate-buffered saline, but in some experiments in which total glutathione concentrations were measured, tissues were frozen on dry ice and stored at −70°C until analyzed. Portions (100–200 mg) of liver or kidney were homogenized with an Ultra-Turrax homogenizer in 20 volumes of ice-cold 5% sulfosalicylic acid. Tissues were allowed to stand for 15 min on ice and were then centrifuged at 14,000*g* for 10 min at 4°C. The acid supernatant was stored at −70°C until assayed. Assays were performed within 2 weeks of homogenization, with GSSG as a standard for both total glutathione and GSSG determinations.

Statistical analyses were performed with Student's *t* test; a level of

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$p < 0.05$ was selected for acceptance or rejection of the null hypothesis. Unless otherwise indicated, all errors shown are the standard deviation of the mean.

Results

The Effect of GSTZ1-1 Deficiency on GST Activities. CDNB is a substrate for most of the alpha-, mu-, and pi-class GSTs. To determine the general extent of GST induction in *Gstz1*$^{-/-}$ mice, we determined the GST activities of liver and kidney extracts from male and female wild-type and *Gstz1*$^{-/-}$ mice with CDNB as the substrate (Fig. 1). Some mice were given 3% phenylalanine in their drinking water to provide an increased burden on the phenylalanine/tyrosine catabolic pathway. As noted previously by others (Mitchell et al., 1997), the GST activity in the livers of male mice was considerably higher (×4) than in female mice. This gender difference was not observed in kidney tissue. GST activities in both the liver and kidney were significantly elevated in female *Gstz1*$^{-/-}$ mice compared with wild-type mice, with the levels in liver being increased off a low base to the level found in male mice. Significant induction was also found in kidneys of male *Gstz1*$^{-/-}$ mice compared with wild-type mice. The addition of 3% phenylalanine in the drinking water had no significant effect on GST activities in wild-type or *Gstz1*$^{-/-}$ mice, with the exception of the livers of female mice in which activities were significantly elevated compared with *Gstz1*$^{-/-}$ mice given water alone.

The activities of liver extracts with DCNB and ethacrynic acid as substrates were also measured (Table 1). Female *Gstz1*$^{-/-}$ mice showed a significant induction of GST activities with all three substrates tested, whereas in male *Gstz1*$^{-/-}$ mice, only GST activities with DCNB showed significant induction.

The Effect of GSTZ1-1 Deficiency on Specific GST Isoenzymes. We examined the specific GST isoenzymes that contributed to the observed changes in liver total GST activities with CDNB as the substrate by Western blotting with specific antisera and by HPLC fractionation of GSTs purified from liver by glutathione affinity chromatography. The relative induction of GSTA1/2, GSTM1, GSTM5, and GSTP1/2 in mice deficient in GSTZ1-1 as shown by Western blotting is shown in Fig. 2. The alpha-class GSTs were easily detected by Western blotting, and GSTA1 and -2 were strongly induced in *Gstz1*$^{-/-}$ mice. GSTA4 showed a modest induction in male mice, whereas GSTA3 showed no induction with GSTZ1-1 deficiency. The most striking increase occurs in the mu-class GSTs, in which a polypeptide that cross-reacted with anti-hGSTM3 was strongly expressed in *Gstz1*$^{-/-}$ mice but was barely detectable in the livers of male and female wild-type mice; this polypeptide is probably mGSTM5, which has the closest sequence similarity to hGSTM3 (Rowe et al., 1998). GSTM1 also showed considerable induction. The effect of sex on the induction of GSTP1/2 was also evident. Wild-type female mice have relatively low GSTP1/2 levels, and these enzymes were strongly induced in the livers of female *Gstz1*$^{-/-}$ mice. In contrast, Western blotting revealed little difference in GSTP1/2 levels in the livers of male wild-type and *Gstz1*$^{-/-}$ mice. These results are consistent with the

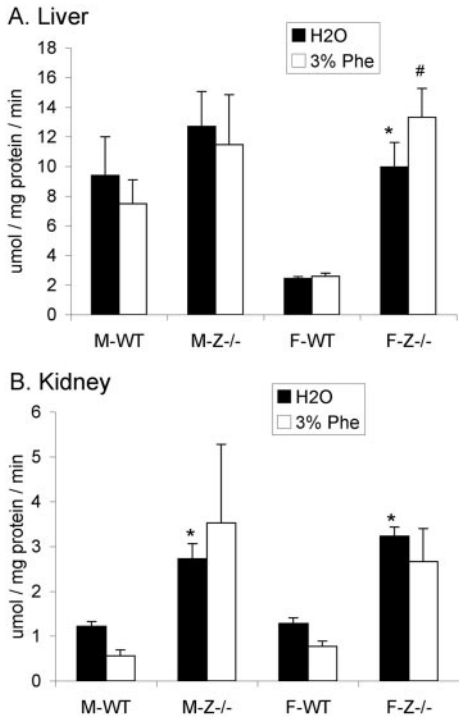

Fig. 1. GST conjugating activity with CDNB of liver (A) and kidney (B) from *Gstz1*$^{-/-}$ mice drinking normal water or water supplemented with 3% phenylalanine (Phe). M, male; F, female; *, $p < 0.001$ compared with wild-type tissue of the same gender; #, $p = 0.007$ compared with *Gstz*$^{-/-}$ mice receiving water.

TABLE 1
GST activities of mouse liver with CDNB, ethacrynic acid, and DCNB as substrates
Data are shown as mean ± S.D., $n = 4$ to 5.

	CDNB	Ethacrynic Acid	DCNB
	μmol/mg protein/min	*nmol/mg protein/min*	
Male			
Wild type	9.39 ± 2.61	99 ± 15	61 ± 23
Gstz1$^{-/-}$	12.69 ± 2.37	84 ± 16	197 ± 45
p	0.07	0.14	0.0003
Female			
Wild type	2.44 ± 0.12	13 ± 1.9	17 ± 1.4
Gstz1$^{-/-}$	9.96 ± 1.66	42 ± 4.1	70 ± 11
p	<0.0001	<0.0001	<0.0001

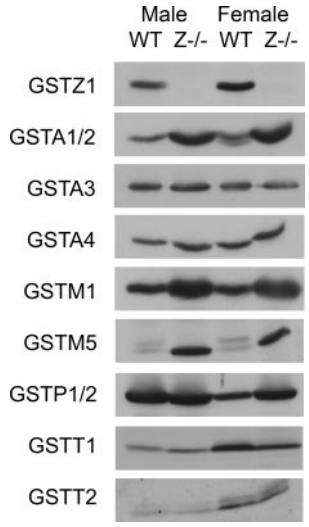

Fig. 2. Induction of GST isoenzymes in *Gstz1*$^{-/-}$ mouse liver detected by Western blot.

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changes in activity observed toward the different GST substrates shown in Table 1. Pi-class GSTs show particularly strong activity with ethacrynic acid as the substrate (Mannervik and Widersten, 1995), and no change was seen in this activity in livers from male wild-type mice, whereas increased activities were observed in female *Gstz1*$^{-/-}$ mice. Conversely, DCNB is a poor substrate for pi-class GSTs but is a good substrate for mu- and alpha-class GSTs (Mannervik and Widersten, 1995), and activity with this substrate was significantly induced in livers from both male and female *Gstz1*$^{-/-}$ mice (Table 1). The theta-class GSTs, like GSTP1, also show a gender difference, with higher levels of GSTT1 and GSTT2 being present in female liver; no induction of these isoenzymes was seen in *Gstz1*$^{-/-}$ mice.

To gain a quantitative understanding of the levels of each isoenzyme, GSTs from mouse liver were purified by glutathione affinity chromatography and fractionated by HPLC. Individual peaks were identified by their elution time (compared with Mitchell et al., 1997) and by cross-reaction with specific antisera. Typical chromatograms are shown in Fig. 3, and quantitative analysis of data obtained from multiple animals is shown in Fig. 4. Whereas alpha-class GSTs were easily detected by Western blotting, these isoenzymes appeared as minor peaks by HPLC. Mitchell et al. (1997) only identified a peak for GSTA3, a relatively abundant alpha-class GST, which was unchanged in *Gstz1*$^{-/-}$ mice. The combination of concentrating proteins by acid precipitation before injection and the induction of GSTA1/2 and GSTA4 in *Gstz1*$^{-/-}$ mice allowed the positive identification of these peaks in the HPLC eluates (Fig. 3, inset) by Western blotting of collected fractions. GSTA1 and GSTA2 were assigned to separate peaks on the basis of their elution under similar conditions in the previous experiments reported by Andorfer et al. (2004). Both the GSTA1/2 and GSTA4 antisera showed some cross-reactivity with the GSTA3 peak at the relative abundances present in these samples, which may account for their apparent detection in the wild-type livers by Western blotting. On the other hand, significant amounts of these isoenzymes may have been lost during the affinity purification step. Nevertheless, the pattern of changes in GSTA1/2 and GSTA3 seen by Western blotting was confirmed by HPLC and, although GSTA4 cannot be detected in wild-type livers, HPLC analysis clearly showed that it was induced in both male and female *Gstz1*$^{-/-}$ livers.

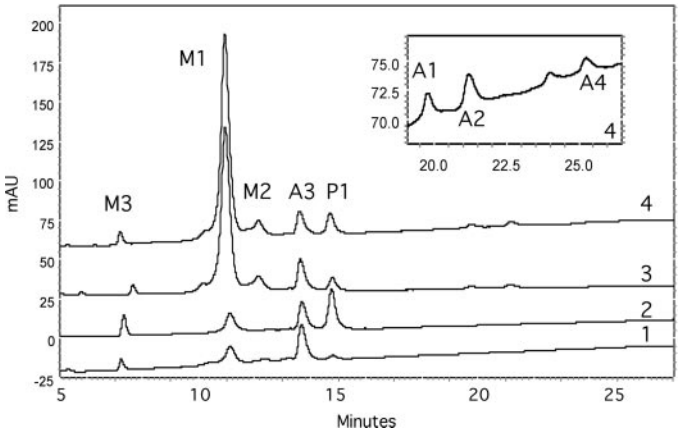

Fig. 3. Separation of GST isoenzymes by HPLC. Representative traces from wild-type female (1), wild-type male (2), *Gstz1*$^{-/-}$ female (3), and *Gstz1*$^{-/-}$ male (4) mouse liver. Peaks were identified by the time of elution and comparison with the results of Mitchell et al. (1997) and by Western blotting of collected fractions. Inset, enlargement of a section of trace 4 showing the detection of alpha class GSTs GSTA1, GSTA2, and GSTA4.

The marked induction of mu-class GSTs in *Gstz1*$^{-/-}$ mice was also confirmed by HPLC (Figs. 3 and 4). GSTM1 is induced 7- to 10-fold in the *Gstz1*$^{-/-}$ livers, making it by far the most abundant GST detected by this method. A peak corresponding with the elution time of mGSTM3 reported by Mitchell et al. (1997) was also induced in both male and female mice (increases of 21 and 72%, respectively). Whereas mGSTM5 induction seems most dramatic by Western blotting, mGSTM5 was not detected in liver by HPLC. This is consistent with the reports of Mitchell et al. (1997) and Andorfer et al. (2004) and suggests that mGSTM5 is of relatively low abundance in the liver. The data shown in Figs. 3 and 4 confirm the significant effect of gender on the level of expression of GSTP1/2, with a 4-fold induction observed in female *Gstz1*$^{-/-}$ mice but no similar induction observed in male liver.

We examined the expression of other enzymes by Western blotting and confirmed the induction of NQO1 in *Gstz1*$^{-/-}$ mice (Fig. 5). In addition, glutamate-cysteine ligase catalytic subunit and modifier subunit were also induced in *Gstz1*$^{-/-}$ mice. Like NQO1, GCL subunits are regulated by an ARE. In contrast, glutathione synthetase was not up-regulated in *Gstz1*$^{-/-}$ mice.

Effect of GSTZ1-1 Deficiency on Glutathione Concentrations. Because GCL is believed to be the rate-limiting step in glutathione synthesis (Richman and Meister, 1975), we examined the total glutathione (primarily GSH) and

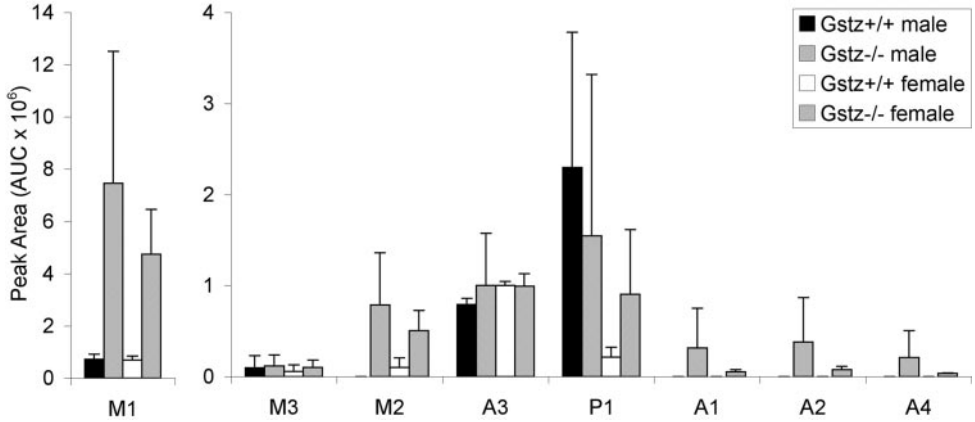

Fig. 4. Quantitation of GST isoenzymes by HPLC (n = 3 mice per group). Note the different scale of the y-axis for GSTM1 data.

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GSSG concentrations in the liver and kidneys of wild-type and *Gstz1*$^{-/-}$ mice (Fig. 6).

In the livers of male *Gstz1*$^{-/-}$ mice, total glutathione and GSSG concentrations were significantly diminished. Total glutathione concentrations were only 25% of wild-type liver, whereas the GSSG concentration was also significantly decreased, but only to 72% of wild-type levels, which resulted in a 3-fold increase in the GSSG/GSH ratio. In contrast, kidney total glutathione levels were significantly elevated in *Gstz1*$^{-/-}$ mice, but the changes in GSSG concentrations and in the GSSG/GSH ratio were not significant. A similar pattern was observed in female *Gstz1*$^{-/-}$ mice, but the changes in glutathione concentrations were smaller (liver total glutathione decreased to 58% of wild-type levels, and kidney increased by 26%; data not shown), and no other changes were significant. In a separate experiment with frozen male liver tissues, the effect of phenylalanine loading on total glutathione levels was examined. Glutathione depletion in *Gstz1*$^{-/-}$ mice was further exacerbated by the administration of 3% phenylalanine in the drinking water to levels at the limit of detection in most mice, but levels were not significantly changed in wild-type mice (Fig. 6D).

The depletion of liver glutathione concentrations in BALB/c *Gstz1*$^{-/-}$ mice contrasts with the results of Fernandez-Canon et al. (2002) for C57BL/6 *Gstz1*$^{-/-}$ mice, in which no significant change in liver glutathione concentrations was reported. To examine whether this was a strain-specific phenomenon, liver and kidney tissues from BALB/c *Gstz1*$^{-/-}$ mice backcrossed for 10 generations to the C57BL/6 strain were analyzed for total glutathione and GSSG concentrations. A pattern of changes identical with that occurring in the BALB/c *Gstz1*$^{-/-}$ mice was observed (data not shown), indicating that there was no strain-specific effect. The previous report conducted the analysis on tissues from fasted mice, which may remove the physiological stress to *Gstz1*$^{-/-}$ mice by removing dietary phenylalanine and tyrosine. We examined this possibility in female mice (Fig. 7) and found that fasting resulted in a significant decrease in total glutathione levels in wild-type mice and a further small decrease in *Gstz1*$^{-/-}$ livers, such that there was no longer a significant difference between the total glutathione concentration of the livers of the two groups of mice (fasted wild-type mice compared with fasted *Gstz1*$^{-/-}$ mice). Thus, the difference between this and the previous report is attributable to fasting of animals rather than to a strain-specific effect.

The Toxicity of Acetaminophen in *Gstz1*$^{-/-}$ Mice. Acetaminophen metabolism in the liver leads to decreased GSH concentrations. Because *Gstz1*$^{-/-}$ mice have a partial deficiency of GSH and elevated GST activities, we were interested in determining whether *Gstz1*$^{-/-}$ mice showed altered susceptibility to acetaminophen toxicity. Untreated *Gstz1*$^{-/-}$ mice showed no significant evidence of liver damage, as measured by plasma ALT activities (48.9 ± 9.2 U/l in female wild-type mice compared with 48.2 ± 7.3 U/l in *Gstz1*$^{-/-}$ mice, $n = 10$ per group), although ALT activities were significantly different between wild-type male mice (48.7 ± 9.9 U/l) and *Gstz1*$^{-/-}$ mice (65.2 ± 20.0 U/l, $p = 0.03$, $n = 10$ per group). Acetaminophen treatment (300 mg/kg) caused liver necrosis after 24 h in both wild-type and

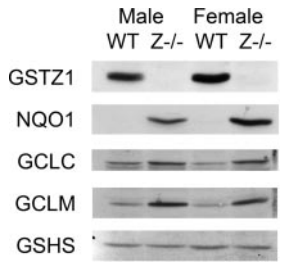

Fig. 5. Induction of oxidative stress genes in the liver of *Gstz1*$^{-/-}$ mice.

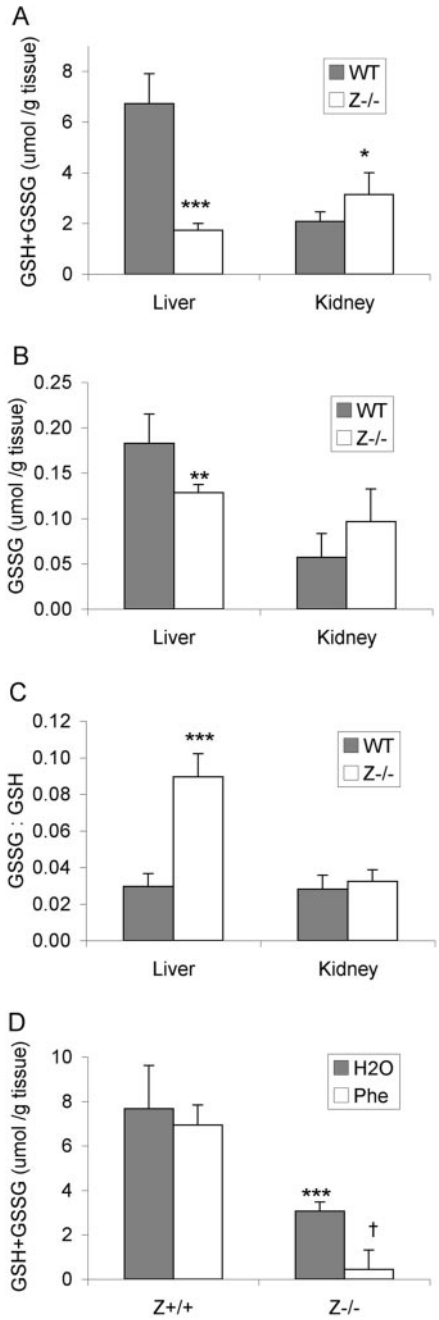

Fig. 6. Glutathione content of mouse liver and kidney in male wild-type ($n = 5$) and *Gstz1*$^{-/-}$ ($n = 4$) mice. A, total glutathione (GSH + GSSG). B, glutathione disulfide (GSSG). C, GSSG/GSH ratio. D, effect of 3% phenylalanine in drinking water for 28 days on total glutathione concentrations (GSH + GSSG) in male mouse livers ($n = 4$–5 per group). Assays for panels A–C were performed on freshly collected tissues, whereas results shown in D were obtained with frozen tissues. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$ compared with wild-type mice. †, $p < 0.001$ compared with *Gstz1*$^{-/-}$ given water alone.

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Gstz1$^{-/-}$ male mice: plasma ALT activities were elevated 7-fold in *Gstz1*$^{-/-}$ mice compared with treated wild-type mice 3 and 6 h after treatment (Fig. 8). Enhanced acetaminophen toxicity was also observed in female *Gstz1*$^{-/-}$ mice (data not shown).

Discussion

The present studies confirmed and extended our previous observation that the expression of several antioxidant enzymes is elevated in *Gstz1*$^{-/-}$ mice. We followed the method of Mitchell et al. (1997) for the purification and HPLC fractionation of mouse liver GSTs and obtained similar chromatographic elution profiles. We used specific antisera to confirm the previous assignment of individual peaks to specific GST isoenzymes. In addition, we were able to assign GSTA1, GSTA2, and GSTA4-4 to previously unidentified minor peaks. The changes in levels of GST isoenzymes detected in *Gstz1*$^{-/-}$ mice by these methods corresponded to changes in GST activity with CDNB, ethacrynic acid, and DCNB as substrates. In wild-type mice, the gender difference in GST profiles was notable. Constitutively higher levels of GSTP1 in male mice have been noted before (Sharma et al., 1993), but we also found constitutively higher levels of the theta-class isoenzymes in female liver. The difference in basal levels of GSTP1 expression between male and female mice shows that the induction of GSTP1 we reported previously in female *Gstz1*$^{-/-}$ mice (Lim et al., 2004) was not observed in male mice.

There are striking similarities between the induction of GSTs in *Gstz1*$^{-/-}$ mice and the induction of GSTs by butylated hydroxyanisole (BHA) (Sharma et al., 1993). Treatment of mice with BHA results in a 3- to 4-fold increase in liver GST activity in male mice compared with a 10-fold increase in female mice with CDNB as the substrate, and BHA induced a greater increase in GSTP expression in female mice than in male mice. Comparison of the induction of GSTs by BHA with the response of *Gstz1*$^{-/-}$ mice suggests that the mechanism of GST induction in *Gstz1*$^{-/-}$ mice may occur by a similar pathway. BHA induces the expression of genes modulated by AREs (Rushmore et al., 1991). The elevated expression of genes encoding enzymes, such as NQO1, glutamate-cysteine ligase catalytic subunit, and glutamate-cysteine ligase modifier subunit, that are also regulated by AREs (Nioi et al., 2003; Mathers et al., 2004) supports the view that deficiency of GSTZ1-1 causes constitutive oxidative stress. Regulation of genes with AREs occurs via the Keap1/Nrf2 pathway (Hayes et al., 2005). The importance of the Nrf2 pathway in induction of GSTs has been demonstrated in Nrf2 knockout mice (Ishii et al., 2000; Chan et al., 2001; Chanas et al., 2002). Keap1 contains reactive cysteine residues that act as sensors of oxidative stress. The formation of intersubunit disulfide bonds or the alkylation of these thiols results in the release of bound Nrf2, which activates ARE sequences (Wakabayashi et al., 2004). Maleylacetoacetate, the substrate for GSTZ1-1, and its metabolite maleylacetone are electrophiles and can potentially alkylate a range of macromolecules. Their formation and accumulation may play a role in the toxicities associated with GSTZ1 deficiency (Lantum et al., 2003). Thus, the constitutive accumulation of maleylacetoacetate or maleylacetone could also be responsible for the alkylation of Keap1 and the subsequent Nrf2-mediated up-regulation of genes with ARE sequences.

The Keap1/Nrf2 pathway may also be induced by the oxidative stress associated with depletion of GSH. Total glutathione concentrations were decreased to 25 and 58% of the wild-type value in the livers of male and female *Gstz1*$^{-/-}$ mice, respectively. Treatment of *Gstz1*$^{-/-}$ mice with phenylalanine exacerbated both the depletion of liver glutathione concentrations (Fig. 6D) and the accumulation of plasma succinylacetone (the reduction product of the metabolic intermediates maleylacetoacetate, maleylacetone, and succinylacetoacetate) (Lim et al., 2004); it did not, however, result in further changes in GST activities (Fig. 1) or in the further induction of NQO1 (data not shown). This suggests that stimulation of the Keap/Nrf2 signaling pathway, whether it be through GSH depletion or by alkylation of Keap1, is already functioning at maximal levels in the livers of *Gstz1*$^{-/-}$ mice with a normal phenylalanine/tyrosine intake.

The increased expression of GCL subunits in the livers of *Gstz1*$^{-/-}$ mice suggests that their capacity to synthesize GSH is higher than in wild-type mice; hence, the low concentrations of GSH and GSSG infer a dramatically increased consumption of glutathione. GSH can catalyze the isomerization of maleylacetoacetate in the absence of GSTZ1-1, but at a much lower rate than the enzyme-catalyzed reaction, and higher concentrations of GSH are required (Edwards and Knox, 1956). Fumarylacetoacetate produced in the liver in this way will be rapidly hydrolyzed by fumarylacetoacetate isomerase. Maleylacetoacetate and fumarylacetoacetate may

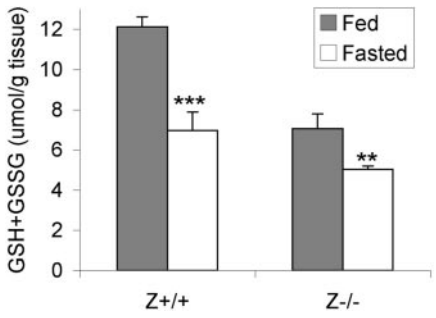

Fig. 7. Effect of fasting on total glutathione concentrations in female mouse liver from wild-type and *Gstz1*$^{-/-}$ mice (n = 4–5 fed groups, n = 2 fasted groups). ***, $p < 0.001$; **, $p = 0.02$ fed compared with fasted mice of the same genotype.

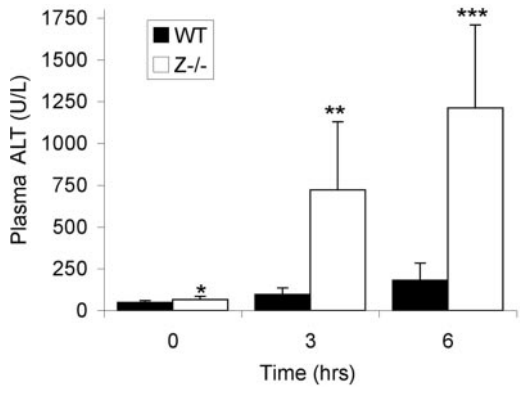

Fig. 8. Liver damage after giving acetaminophen (300 mg/kg i.p.) to male wild-type and *Gstz1*$^{-/-}$ mice, as measured by plasma ALT activities. Data from two independent experiments have been combined. Wild-type, n = 5; *Gstz1*$^{-/-}$, n = 6. *, $p = 0.03$; **, $p = 0.01$; ***, $p = 0.003$ compared with acetaminophen-treated wild-type mice at the same times.

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also react nonenzymatically with GSH, but fumarylacetoacetate reacts faster than maleylacetoacetate or maleylacetone to give a glutathione conjugate of succinylacetoacetate, which is not metabolized by fumarylacetoacetate isomerase (Edwards and Knox, 1956). Fernandez-Canon et al. (2002) proposed a model for the nonenzymatic bypass of MAAI deficiency that is dependent on GSH to nonenzymatically isomerize maleylacetoacetate to fumarylacetoacetate. In cases of phenylalanine overload, it was predicted that maleylacetoacetate would accumulate and be exported from the liver, resulting in the appearance of fumarylacetoacetate and succinylacetoacetate in the urine of mice. We propose a variant on this mechanism whereby in situations of overload of the nonenzymatic bypass pathway, a glutathione conjugate of maleylacetoacetate is exported from the liver, which results in the massive depletion of total liver glutathione concentrations. Although an accumulation of metabolites, such as maleylacetoacetate or succinylacetoacetate, may contribute to the phenotype of GSTZ1-1 deficiency, many of the acute symptoms of phenylalanine challenge may be attributed to the dramatic depletion of GSH.

The severe depletion of GSH in the livers of *Gstz1*$^{-/-}$ mice contrasted with the increased concentration found in their kidneys. GST activity with CDNB as a substrate was elevated in both the liver and kidney of *Gstz1*$^{-/-}$ mice (Fig. 1), suggesting that a similar spectrum of enzymes is induced in each tissue in response to the accumulation of maleylacetoacetate and its metabolites. Indeed, Western blotting of female kidney extracts indicated strong induction of both pi- and mu-class GSTs in *Gstz1*$^{-/-}$ mice (data not shown). Thus, it is probable that as occurs in the liver, GCL subunits are also induced in the kidney of *Gstz1*$^{-/-}$ mice, allowing increased synthesis of GSH. However, because the vast majority of tyrosine catabolism occurs in the liver (Mitchell et al., 2001), it seems likely that the accumulation of maleylacetoacetate results in a far greater consumption of glutathione in the liver than in the kidney of *Gstz1*$^{-/-}$ mice. Thus, the kidneys have responded to the chemical stress and increased GSH synthesis to levels that are beyond the level of consumption.

We originally observed an increase in GSTP1 expression in female *Gstz1*$^{-/-}$ mouse liver (Lim et al., 2004). *Gstp*1/2-null mice are resistant to acetaminophen-induced hepatotoxicity, and Henderson et al. (2000) suggest that GSTP may mediate the toxicity of acetaminophen by the inhibition of Jun-N-terminal kinase. We therefore hypothesized that *Gstz1*$^{-/-}$ mice would be more sensitive to acetaminophen because of the induction of GSTP1. In the present experiments, we found that *Gstz1*$^{-/-}$ mice did, indeed, show increased acetaminophen-induced hepatotoxicity, but this could not be attributed to elevated levels of GSTP1, because both male and female mice showed similar increases in hepatotoxicity, but male mice did not show increased GSTP1 levels. The toxicity of high doses of acetaminophen is attributable to the formation of reactive intermediate *N*-acetyl-*p*-benzoquinoneimine (NAPQI) by several cytochromes P450. NAPQI is normally detoxified by a GST-catalyzed enzymatic reaction and also by nonenzymatic reaction with GSH. When acetaminophen is given in high doses, GSH concentrations are depleted, and unconjugated NAPQI causes oxidative stress and covalently modifies cellular proteins. Alternative mechanisms for the increased toxicity of acetaminophen in *Gstz1*$^{-/-}$ mice could include the induction of cytochromes P450 responsible for catalyzing the formation of NAPQI or an increased rate of consumption of glutathione for the detoxication of NAPQI caused by the increased levels of other GST isoenzymes, such as alpha-class GSTs, which have activity with NAPQI (Coles et al., 1988). The simplest mechanism for which we have good evidence is that the low hepatic GSH concentrations in *Gstz1*$^{-/-}$ mice is responsible for their increased sensitivity to acetaminophen-induced hepatotoxicity. Induction of the Keap1/Nrf2 pathway is critical for responses to oxidative stress and for the regulation of glutathione synthesis. Although *Gstz1*$^{-/-}$ mice have a highly active Keap1/Nrf2 pathway, their inability to increase further the Keap1/Nrf2 pathway in response to additional stresses, such as phenylalanine loading or acetaminophen toxicity, is consistent with the increased sensitivity of Nrf2 knockout mice to acetaminophen (Chan et al., 2001; Enomoto et al., 2001).

The available data suggest, therefore, that GSTZ1-1 negatively modulates the induction of ARE-regulated phase II enzymes by the endogenous metabolites of tyrosine and phenylalanine (Hayes et al., 2005). The constitutive induction of a range of enzymes involved in drug and xenobiotic metabolism and the depletion of hepatic GSH concentrations in GSTZ1-1 deficiency, arising from either a genetic mutation or from exposure to dichloroacetic acid, have the potential to alter the pharmacokinetics and toxicity of a range of drugs. No humans with confirmed GSTZ1-1 /MAAI deficiency have been identified, despite the severity of deficiencies in other enzymes in the tyrosine catabolic pathway. The lack of a phenotype in *Gstz1*$^{-/-}$ mice and the apparent nonenzymatic bypass of the MAAI reaction under normal dietary conditions suggest that cases of human deficiency may go undetected. The elevated susceptibility of *Gstz1*$^{-/-}$ mice to acetaminophen-induced hepatotoxicity suggests that humans with GSTZ1-1 deficiency may suffer liver damage when exposed to therapeutic doses of acetaminophen. Furthermore, because dichloroacetic acid is an irreversible inhibitor of GSTZ1-1 (Tzeng et al., 2000), patients given dichloroacetic acid for the treatment of chronic lactic acidosis (Stacpoole et al., 1998) may have a therapeutically induced deficiency of GSTZ1-1 and may be at risk of hepatotoxicity when given acetaminophen or drugs that induce an oxidative stress.

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