Acetaminophen-induced Liver Injury Is Attenuated in Male Glutamate-cysteine Ligase Transgenic Mice*

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Acetaminophen overdose is a leading cause of drug-related acute liver failure in the United States. Glutathione, a tripeptide antioxidant protects cells against oxidative damage from reactive oxygen species and plays a crucial role in the detoxification of xenobiotics, including acetaminophen. Glutathione is synthesized in a two-step enzymatic reaction. Glutamate-cysteine ligase carries out the rate-limiting and first step in glutathione synthesis. We have generated C57Bl/6 mice that conditionally overexpress glutamate-cysteine ligase, and report here their resistance to acetaminophen-induced liver injury. Indices of liver injury included histopathology and serum alanine aminotransferase activity. Male transgenic mice induced to overexpress glutamate-cysteine ligase exhibited resistance to acetaminophen-induced liver injury when compared with acetaminophen-treated male mice carrying, but not expressing glutamate-cysteine ligase transgenes, or to female glutamate-cysteine ligase transgenic mice. We conclude that glutamate-cysteine ligase activity is an important factor in determining acetaminophen-induced liver injury. Male transgenic mice exhibited resistance to acetaminophen overdose for as much as 2 h post-APAP administration, whereas female transgenic mice exhibited increased levels of NAPQI-protein adducts and alanine aminotransferase activity. Male transgenic mice exhibited increased levels of NAPQI-protein adducts and alanine aminotransferase activity.

The tripeptide antioxidant glutathione (GSH; γ-glutamylcysteinylglycine) is one of the most abundant cellular thiols. It protects cells against oxidative damage from reactive oxygen species, maintains cellular redox status, promotes cell growth, and plays a crucial role in the detoxification of xenobiotics. GSH can directly scavenge free radicals, act as an antioxidant in GSH-mediated reduction of peroxides and act as a co-substrate for glutathione S-transferase-mediated detoxification of reactive intermediates formed during phase I metabolism (1).

GSH plays a major role in detoxifying many hepatotoxicants including acetaminophen (APAP), an over-the-counter analgesic and antipyretic (2–5). APAP overdose is responsible for nearly 50% of the acute liver failure cases in the United States (6) and is thus of high public health concern. APAP metabolism has been well defined, making it a good model for drug-induced liver toxicity. APAP is primarily metabolized through sulfation and glucuronidation pathways (7–9). However, a fraction of APAP is bioactivated by cytochrome P-450s to n-acetyl-p-benzoylguanidine (NAPQI), which can bind to cellular proteins (3, 7, 10–12). NAPQI also covalently binds to GSH and is either converted back to APAP, or forms the non-toxic APAP-GSH conjugate. APAP overdose results in depletion of hepatic GSH (by as much as 90%) (2). As GSH stores are depleted, increased levels of NAPQI-protein adducts form, and such adducts are thought to be an important contributor to APAP hepatotoxicity (3, 7, 8, 10, 11).

Pretreatment with N-acetylcysteine (7, 9, 10), a source of cysteine for GSH biosynthesis, attenuates APAP-induced hepatotoxicity. N-Acetylcysteine given soon after APAP (within 1–2 h) is highly protective against liver injury. However, this protection rapidly diminishes with time. Nonetheless, even when given as much as 2 h post-APAP administration, N-acetylcysteine affords some protection against hepatotoxicity (7).

In most tissues, the two-step biosynthesis of GSH is primarily limited by the activity of glutamate-cysteine ligase that carries out the first and rate-limiting step in GSH synthesis (13). Glutamate-cysteine ligase is a heterodimeric enzyme composed of catalytic (Gclc) and modifier (Gclm) subunits. All catalytic functions of the holoenzyme are carried out by Gclc, whereas Gclm influences the catalytic efficiency of Gclc by lowering the Km for glutamate and increasing the K for GSH feedback inhibition.

To investigate the influence of GSH biosynthesis capacity on the extent of hepatotoxicant-induced injury we developed transgenic mice that conditionally overexpress Gclc, Gclm, or both subunits. Our initial attempts to generate constitutively expressing glutamate-cysteine ligase transgenic mice using

3 The abbreviations used are: APAP, acetaminophen; NAPQI, n-acetyl-p-benzoylguanidine; Gclc, glutamate-cysteine ligase catalytic subunit; Gclm, glutamate-cysteine ligase modifier subunit; qRT, quantitative real time; HPLC, high performance liquid chromatography; tk, thymidine kinase; H&E, hematoxylin and eosin.
Glutamate-cysteine ligase is variably expressed in humans (15). Because GSH plays such an important role in detoxifying APAP, variation in glutamate-cysteine ligase expression may play a role in idiosyncratic reactions to APAP. This transgenic mouse model of inducible glutamate-cysteine ligase overexpression allowed us to directly test the hypothesis that glutamate-cysteine ligase expression influences susceptibility to APAP-induced liver injury.

MATERIALS AND METHODS

Plasmids—Mouse Gclc and Gclm cDNAs present in plasmid pCR3.1 (16, 17) were subcloned into plasmid p17 × 4-tk-CAT (18). The Gclc and Gclm amino acid coding regions plus polyadenylation fragments were amplified from Gclc/pCR3.1 or Gclm/pCR3.1 using primers having a 5′ BamHI (CCTATAGGGGATCCAGCTGCTAGCGTTTA-5′ BamHI/pCR3.1) and a 3′ KpnI (GCACTGTGACCTCCGCTGACGGCAT-3′ KpnI/pCR3.1) restriction site. The resulting amplicons were digested with BamHI and KpnI and used to replace the BglII/Kpn1 CAT fragment of p17 × 4-tk-CAT. The glutamate-cysteine ligase transgene plasmids comprise 4 copies of the Gal4 consensus binding sequence, a thymidine kinase (tk) minimal promoter, the amino acid coding sequence of either Gclc or Gclm cDNA, and a bovine growth hormone polyadenylation sequence. The p17 × 4-tk Gclc or Gclm plasmids were digested with AatII and KpnI to yield 2.8- and 1.7-kb fragments, respectively. These plasmid fragments were purified by gel electrophoresis and resuspended in Tris EDTA, pH 7.5, buffer.

Glutamate-cysteine Ligase Transgenic Mice—All animal procedures were carried out following protocols approved by the University of Washington Institutional Animal Care and Use Committee (IACUC). All mice were housed under specific pathogen-free conditions in microisolator caging. C57Bl/6 (75%) X C3H (25%) pronuclear embryos were collected on E0.5, following superovulation. E0.5 was taken as the morning of the day that copulation plugs were observed. Pregnant mare serum gonadotropin for superovulation was purchased from the National Hormone and Peptide Program (Torrance, CA). Constructs were injected into the pronuclei, and embryos were transferred into the oviducts of E0.5 pseudopregnant dams either on the day of injection or following overnight culture to the 2-cell stage and allowed to progress to term.

Genotyping of Gclc and Gclm Transgenic Mice—At 3–4 weeks of age tail biopsies were taken from pups and glutamate-cysteine ligase transgene status was determined by PCR using reverse primers designed to hybridize internally to either the Gclc (GAAGTAGCCCTCCTCCGCGG) or Gclm (CTGTGCAACTCAAGGACGGA) cDNA sequences, and a forward primer (GAACACCGACCGCCCTGCA) designed to hybridize to the thymidine kinase promoter region in the integrated plasmid fragment (Fig. 1).

Genotyping of GLVP Transgenic Mice—The same tail biopsies that were used to determine glutamate-cysteine ligase transgene status were used to identify the GLVP transgene status by PCR using GLVP forward (GACGCGCTAGACGATTTC) and reverse (AGCAAGAACTGCGAGGTG) primers (14).

Breeding of Gclm/Gclc Bigenic, Gclm/GLVP Bigenic, Gclc/GLVP Bigenic, and Gclm/GLVP Trigenic Mice—Progeny of the glutamate-cysteine ligase transgene positive founder lines were bred to GLVP transgenic mice, and the resulting progeny were backcrossed at least 6 generations with C57Bl/6 mice. All APAP experiments were conducted with mice that had been bred onto the C57Bl/6 genetic background.

Mifepristone Administration—In initial experiments, a single intraperitoneal injection of mifepristone (5 mg/kg in sesame oil; Sigma) was administered to mice 8–9 h prior to sacrifice, to determine which founder strains were carrying responsive glutamate-cysteine ligase transgenes. In later experiments, animals from responsive strains were injected intraperitoneally 3 times with mifepristone (5 mg/kg each time at 24, 16, and 8 h prior to sacrifice). Control animals were administered vehicle only (sesame oil) at the same times.

Tissue Collection—Mice were sacrificed by CO2 narcosis/cervical dislocation. Livers were removed for biochemical, histological, and gene expression analyses.

Northern Blot Analysis—Total RNA was isolated from liver tissue using TRizol reagent (Invitrogen) and subjected to Northern blot analysis using standard methods (19). Blots were hybridized with either Gclc or Gclm 32P-labeled murine cDNA, corresponding to the amino acid coding regions (16, 17) or β-actin cDNA probes, and relative mRNA levels were determined by phosphorimager analysis (GelDoc, Bio-Rad). Glutamate-cysteine ligase transcripts originating from the transgenes were distinguished from the endogenous glutamate-cysteine ligase transcripts based on their differential migration in agarose gels (different 3′ non-coding region lengths).

Quantitative Real Time PCR (qRT-PCR)—Total liver RNA was extracted in TRizol reagent and used to generate cDNA by reverse transcription using Superscript reverse transcriptase (Invitrogen). The cDNA was then used to quantitate the mRNA levels of Gclc and Gclm endogenous and transgenes using an ABI 7700 Sequence Analyzer (Applied Biosystems, Foster City, CA). The technique involved the use of sequence-specific fluorogenic probes that border exon/exon boundaries in the processed mRNAs. PCR primers and probes were selected using Primer Express 1.5TM software (Applied Biosystems). A reference standard of normal mouse kidney RNA was serially diluted to derive a linear regression formula over 4 orders of magnitude that was then used to calculate and quantitate expression. Glyceraldehyde-phosphate dehydrogenase mRNA expression was used to normalize glutamate-cysteine ligase mRNA expression.
Endogenous transcripts were differentiated from transgenic sequences using primers specific for Gclc and Gclm and the bovine growth hormone polyadenylation region of the target gene (Table 1 and Fig. 1).

**GCL Transgenic Mice Are Resistant to Acetaminophen**

**TABLE 1**

| Description of oligonucleotide primers and probes used in quantitative real-time PCR analysis | |
|---|---|
| **Mouse primers and probe sequences** | **T_m (°C)** |
| Gclc forward, 5’-ATgTggACACCgATgCgTATT-3′ | 61.3 |
| Gclc reverse, 5’-TGtCTCTgTgATgTAAGgATgTT-3′ | 61.1 |
| Gclc probe, 5’-6FAM-TCTTCTgATgCTgTgTCTT-MGB-3′ | 72.2 |
| Gclm forward, 5’-gCACCgATgATTgATgCCTT-3′ | 61.3 |
| Gclm reverse, 5’-CgAggATgTTCTTgAggATgTCTT-3′ | 61.1 |
| Gclm probe, 5’-6FAM-CgAATgACCGgAAAGg-MGB-3′ | 73.4 |
| Transgenic reverse, 5’-gTgCTgATATgCTgAAgATT-3′ | 59.7 |
| GAPDH forward, 5’-TCTTgCAGGACCgATgCTT-3′ | 60.3 |
| GAPDH reverse, 5’-CgAggATgACCGgAAAGg-MGB-3′ | 59.9 |
| GAPDH probe, 5’-6FAM-CCTCgATgACCGgATgC-FAM-3′ | 70.0 |

*GAPDH, glyceraldehyde-3-phosphate dehydrogenase.*

**Acetaminophen Protein and Glutathione Adducts**—We modified the method of Muldrew et al. (26) to measure the levels of APAP protein and APAP glutathione adducts using HPLC with electrochemical detection. For glutathione APAP adducts, liver tissue was homogenized (1:5, w/v) in 10 mM sodium acetate, pH 6.5, and then centrifuged at 16,000 × g for 20 min at 4 °C. One hundred μl of supernatant was combined with 100 μl of 20% ice-cold trichloroacetic acid for 15 min, and then centrifuged for 5 min at 16,000 × g. Fifty μl of supernatant was then added to 950 μl of 10 mM sodium acetate, pH 6.5, and aliquots were then assayed using reverse-phase HPLC with electrochemical detection. For protein APAP adducts, livers were homogenized as above in 10 mM sodium acetate and centrifuged at 16,000 × g. Five hundred μl of supernatant was then mixed 1:1 with 10 mM sodium acetate and subjected to dialysis (Slide-A-Lyser, Pierce; 3,500 MWCO) against 10 mM sodium acetate, pH 6.5, for 19–22 h at 4 °C. Samples were then diluted 1:3 in 10 mM sodium acetate buffer. Ten μl of protease solution (200 mg/ml, Sigma) was then added to 1 ml of dialyzed sample and incubated at 50 °C for 20–24 h. One-hundred fifty μl of digested sample was then mixed with 150 μl of 20% trichloroacetic acid, placed on ice for 15 min, and then centrifuged at 16,000 × g for 5 min at 4 °C. Fifty μl of supernatant was then added to 450 μl of 10 mM sodium acetate buffer, and samples were analyzed by HPLC with electrochemical detection as above. APAP-glutathione adduct standards and APAP-cysteine adduct standards were generated by incubating 250 μM GSH or 250 μM cysteine with 250 μM APAP for 4 h in the presence of NADPH (2 mM) and microsomes (400 μg of protein/ml) con-
containing recombinant human cytochrome P450 2E1 (BD Biosciences).

**Statistical Analyses**—The results for GSH levels, glutamate-cysteine ligase activity, glutathione and protein APAP adducts, alanine aminotransferase activity, and histopathology scores were analyzed by analysis of variance and Student’s t test. Regression analyses and F tests were also performed on selected comparisons of these data. Differences yielding a p value of less than 0.05 were considered statistically significant.

**RESULTS**

**Generation of Glutamate-cysteine Ligase Transgenic Mice**—Using standard techniques, we generated mice possessing Gclc or Gclm cDNA transgenes flanked by the 17×4 Gal4 recognition elements and the minimal tk promoter (Fig. 1). Four Gclc and 7 Gclm transgenic founder lines were identified by PCR using reverse primers specific for the glutamate-cysteine ligase subunit cDNA portion of each plasmid construct, as well as a forward primer homologous to the tk promoter (present in both glutamate-cysteine ligase subunit plasmid constructs; Fig. 1). These transgenes were inherited in a simple Mendelian manner, and there were neither gender bias of heredity in the progeny, nor fertility problems associated with the presence of any of the transgenes (data not shown).

Gclc and Gclm transgenic mice were crossed with mice carrying the liver-specific and mifepristone responsive transactivator GLVP. Subsequently, these two lines were intercrossed (Gclc/GLVP X Gclm/GLVP) to generate trigenic mice (Gclc/Gclm/GLVP). All mice were backcrossed onto a C57Bl/6 genetic background for at least 6 generations prior to the initiation of the APAP exposures.

**Characterization of Glutamate-cysteine Ligase Overexpressing Transgenic Mice**—The 4 Gclc/GLVP and the 7 Gclm/GLVP founder lines were analyzed for inducible RNA transgene expression by administration of mifepristone. Initially, both Northern blot and qRT-PCR analyses were applied to detect both the endogenous and the transgenic Gclc and Gclm transcripts. After several concurring experiments qRT-PCR became the preferred method of detection and quantification of these transcripts (Fig. 2). Utilizing a primer sequence from the bovine growth hormone poly(A) 3′ end of the target gene construct (Fig. 1), we were able to distinguish the transgenic transcripts from the endogenous glutamate-cysteine ligase transcripts. Several of our founder lines showed no detectable transgene mRNA expression.

Protein expression and glutamate-cysteine ligase activity were next evaluated in Gclc/GLVP bigenic, Gclm/GLVP bigenic, and Gclc/Gclm/GLVP trigenic mice that showed inducible transgene mRNA expression. There was an increase in Gclm and Gclc protein expression that was predicted by the presence of Gclc and Gclm transgene mRNA expression. Several of our founder lines showed increased levels of Gclc protein 9 h after mifepristone treatment (even though Gclc transgene mRNA was not increased in these mice; Fig. 2). This may be
due to a transient stabilizing effect of Gclm on Gclc protein in this strain of transgenic mice. Importantly, there was an increase in glutamate-cysteine ligase activity associated with mifepristone-induced increases in glutamate-cysteine ligase protein expression (Fig. 3D).

The results of early experiments with this transgenic model indicated that maximal glutamate-cysteine ligase protein expression in the transgenic mice was between 8 and 9 h after a single mifepristone injection. To achieve more consistency in glutamate-cysteine ligase induction and expression, we administered 3 consecutive 5 mg/kg doses of mifepristone given 8 h apart. With repeated mifepristone dosing there was again a notable increase in Gclm protein expression than controls (GLVP only; *, p < 0.05). The number of mice in genotype groups is indicated within each bar. 

**FIGURE 3.** Mifepristone induction of liver glutamate-cysteine ligase protein expression. Mice were treated with mifepristone and liver tissue was harvested as for Fig. 2. A, chemiluminescent image of Gclc and Gclm protein detected with anti-glutamate-cysteine ligase antisera 9 h after a single injection of 5 mg/kg mifepristone. B, mifepristone-mediated induction of Gclc protein expression. Gclc/Gclm/GLPV trigenic mice had significantly higher protein expression than controls (GLVP only; *, p < 0.05). The number of mice in genotype groups is indicated within each bar. C, mifepristone-mediated induction of Gclm protein expression. Both Gclm/GLVP bigenic and Gclc/Gclm/GLPV trigenic mice had significantly higher Gclm protein expression than controls (GLVP only; *, p < 0.05). The number of mice in genotype groups is indicated within each bar. D, mifepristone-mediated induction of liver glutamate-cysteine ligase activity. With a single mifepristone treatment of 5 mg/kg, Gclc/Gclm/GLPV trigenic mice show significantly higher levels of liver glutamate-cysteine ligase activity than control (GLVP only) mice 9 h after treatment (*, p < 0.05). The number of mice in genotype groups is indicated within each bar.
Repeated doses of mifepristone (RU486) in female control mice. Gclc protein was not changed at this time point after mifepristone treatment in either genotype. Gclc protein remained elevated for at least 8 h after the last mifepristone injection (RU486) in Gclc/Gclm/GLVP trigenic mice but not in Gclc/Gclm control mice. Gclm protein was not changed at this time point after mifepristone treatment (Fig. 5).

**FIGURE 4. Persistent elevation in glutamate-cysteine ligase modifier subunit protein expression in trigenic mice.** Mice were injected 3 times at 8-h intervals with 5 mg/kg mifepristone (+) or vehicle (−), then assessed 8 h after the last injection for Gclc and Gclm protein expression by Western immunoblot. Gclm protein remained elevated for at least 8 h after the last mifepristone injection (RU486) in Gclc/Gclm/GLVP trigenic mice but not in Gclc/Gclm control mice. Gclm protein was not changed at this time point after mifepristone treatment in either genotype.

**FIGURE 5. Mifepristone-mediated induction of liver glutamate-cysteine ligase activity in trigenic mice.** A, mice were treated with multiple doses of mifepristone or vehicle as described in the legend to Fig. 4. Female Gclc/Gclm/GLVP trigenic mice show significantly higher levels of glutamate-cysteine ligase activity than Gclc/Gclm/GLVP female trigenic mice not treated with mifepristone or Gclc/Gclm female mice (irrespective of mifepristone treatment) 8 h after the last injection (**, p < 0.01). The number of mice in genotype groups is indicated within each bar. Liver glutathione content in Gclc/Gclm and Gclc/Gclm/GLVP trigenic mice treated with mifepristone. Mice were treated with mifepristone or vehicle (sesame oil) as described in the legend to Fig. 4. The number of mice in genotype groups is indicated within each bar.

Mice were injected 3 times at 8-h intervals with 5 mg/kg mifepristone (RU486) or vehicle (sesame oil) as described in the legend to Fig. 4. Female Gclc/Gclm/GLVP trigenic mice received 3 doses of mifepristone (5 mg/kg) or vehicle (sesame oil) at 8-h intervals. APAP (300 mg/kg intraperitoneal) was administered 8 h after the final mifepristone dose. Mice were then sacrificed 6 h after APAP administration. *+, significantly different from control (trigenic vehicle-treated) mice within the same gender (p < 0.05). #, significantly different from all other groups within the same gender (p < 0.01).

**FIGURE 6. Glutamate-cysteine ligase activity in APAP-treated mice.** Mice received 3 doses of mifepristone (5 mg/kg) or vehicle (sesame oil) at 8-h intervals. APAP (300 mg/kg intraperitoneal) was administered 8 h after the final mifepristone dose. Mice were then sacrificed 6 h after APAP administration. *+, significantly different from control (trigenic vehicle-treated) mice within the same gender (p < 0.05). #, significantly different from all other groups within the same gender (p < 0.01).

We next evaluated the effects of mifepristone induction of glutamate-cysteine ligase on the extent of APAP-induced liver injury. Because livers from the APAP-treated mice were not harvested until 14 h after the 3rd dose of mifepristone, we wanted to determine whether glutamate-cysteine ligase activity was still elevated at this time. In fact, it was significantly higher in both male and female trigenic mice receiving mifepristone and APAP than in all the other groups, including Gclc/Gclm mice receiving both mifepristone and APAP (Fig. 6).
male trigenic mice receiving APAP without mifepristone pretreatment, or in male Gclc/Gclm mice receiving APAP, irrespective of mifepristone pretreatment. Interestingly, APAP-treated female mice experienced only a moderate increase in alanine aminotransferase (relative to male mice, despite their relatively high glutamate-cysteine ligase activity). This gender-dependent sensitivity to APAP-induced liver injury has been recently noted by others (27). Moreover, female Gclc/Gclm/GLVP trigenic mice pretreated with mifepristone were not protected from APAP-induced liver injury (Fig. 7B). We saw no effect of mifepristone treatment alone on serum alanine aminotransferase activity in either Gclc/Gclm mice or Gclc/Gclm/GLVP trigenic mice (data not shown).

Further support for the protective effect of high glutamate-cysteine ligase expression in male mice comes from a direct comparison of serum alanine aminotransferase activity with glutamate-cysteine ligase activity in APAP-exposed trigenic mice pretreated with mifepristone. There is a clear inverse relationship between glutamate-cysteine ligase activity and serum alanine aminotransferase activity in APAP-treated male mice, and those mice with the highest glutamate-cysteine ligase activity showed the best protection from APAP-induced increases in serum alanine aminotransferase activity (Fig. 7C). No such relationship was observed in female trigenic mice (Fig. 7D). In addition, there was no correlation between glutamate-cysteine ligase and serum alanine aminotransferase activities in APAP-treated male or female trigenic mice that had not been pretreated with mifepristone, or in APAP-treated male or female Gclc/Gclm mice irrespective of mifepristone pretreatment (data not shown).

A spectrum of histopathological patterns of centrilobular injury was observed in the livers of mice treated with 300 mg/kg APAP, including hepatocellular glycogen depletion.

**FIGURE 7. Influence of glutamate-cysteine ligase transgene induction on APAP-induced increases in serum alanine aminotransferase activity.** Mice were treated with mifepristone (or sesame oil vehicle) and APAP (or saline) as described in the legend to Fig. 6. A, APAP treatment resulted in an increase in serum alanine aminotransferase in Gclc/Gclm bigenic male mice (irrespective of mifepristone pretreatment) and in Gclc/Gclm/GLVP trigenic male mice not pretreated with mifepristone. However, in APAP-treated Gclc/Gclm/GLVP trigenic male mice, pre-treatment with mifepristone resulted in significantly lower serum alanine aminotransferase activity. B, female mice exhibited less APAP-induced alanine aminotransferase activity than males, and mifepristone pretreatment did not result in a lowering of alanine aminotransferase activity in female Gclc/Gclm/GLVP trigenic mice. C, there exists an inverse relationship between glutamate-cysteine ligase activity and serum alanine aminotransferase activity in APAP-treated Gclc/Gclm/GLVP trigenic male mice, indicating more protection in mice with high glutamate-cysteine ligase activity. D, there was no significant correlation between glutamate-cysteine ligase activity and serum alanine aminotransferase activity in APAP-treated trigenic female mice. *, significantly different from control (saline only) mice (p < 0.01); #, significantly different from APAP-treated trigenic mice not pretreated with mifepristone, and from APAP-treated Gclc/Gclm bigenic mice, irrespective of mifepristone treatment (p < 0.01). The number of mice in each genotype is indicated above (controls) or within each bar.
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FIGURE 8. Patterns of APAP-induced liver injury. Control mice were treated with mifepristone (or sesame oil vehicle) and APAP (or saline) as described in the legend to Fig. 7. Mice were sacrificed 6 h later and liver tissue was excised, fixed in 4% paraformaldehyde, paraffin imbedded, sectioned, and stained with hematoxylin and eosin, using standard methods. A, control (saline) treated liver. B, glycogen depletion. C, glycogen depletion with microvascular injury/hemorrhage. D, steatosis. Panel E, steatosis (magnified). F, centrilobular necrosis. Magnification: panels A–D and F, ×100; panel E, ×200.

The level of APAP protein adducts after APAP overdose has been correlated with liver injury (8). Thus, we determined the level of residual GSH and APAP protein adducts in the livers of mifepristone-pretreated Gclc/Gclm/GLVP male trigenic and Gclc/Gclm male bigenic mice, 2 h after intraperitoneal injection of 300 mg/kg APAP (Fig. 10). There was a modest correlation between glutamate-cysteine ligase activity and residual GSH in the liver, and there were significantly fewer APAP protein adducts in the trigenic mice than in the Gclc/Gclm mice, which indicates a more efficient scavenging of NAPQI in mice overexpressing glutamate-cysteine ligase. There was no correlation between glutamate-cysteine ligase activity and glutathione-APAP adducts, which may reflect the rapid disposal of these adducts at 2 h after treatment (data not shown).

DISCUSSION

The role of GSH as a cellular chemoprotectant and antioxidant is well established (1, 7, 30, 31). Treatment with many xenobiotics results in GSH depletion due either to direct binding of toxic metabolites to GSH or through glutathione S-transferase-mediated pathways (1, 31). When GSH is depleted, cells become more susceptible to damage from oxidative and nitrosative species sometimes leading to cell death (10, 32). Moreover, various cytokines, environmental chemicals, and drugs have been shown to influence the levels of glutamate-cysteine ligase mRNA and protein expression, or to have direct effects on its activity (5, 33–37).

In this study, we report that high expression of glutamate-cysteine ligase is protective against APAP-induced liver injury in male mice. Presumably this is due to the increased ability of these glutamate-cysteine ligase transgenic mice to resynthesize GSH subsequent to or during APAP-induced GSH depletion. Male glutamate-cysteine ligase transgenic mice showed diminished serum ALT activity, and less evidence of liver injury (as indicated by histopathological evaluation) after APAP treatment. Because GSH can limit glutamate-cysteine ligase activity via non-allosteric feedback inhibition (38, 39), GSH is usually restored via increased glutamate-cysteine ligase activity released from the suppressive effects of high levels of GSH (13). In addition, GSH resynthesis can be enhanced through increased glutamate-cysteine ligase gene transcription, which results in increased mRNA and protein expression, and increased activity. We have previously shown in cultured mouse hepatocytes that increasing glutamate-cysteine ligase expression can increase GSH synthesis and attenuate the apoptotic effects of tumor necrosis factor-α (24). Using the inducible glutamate-cysteine ligase transgenic mouse model...
reported here, we were able to investigate protection afforded by increasing glutamate-cysteine ligase levels \textit{in vivo}.

Utilizing the conditional transactivator GLVP, Wang \textit{et al.} (14) developed a system in which expression of target genes in transgenic mice can be controlled via the administration of mifepristone. We generated a plasmid vector with either the \textit{Gclc} or \textit{Gclm} cDNA as targets for GLVP transactivation. We crossed mice carrying these target glutamate-cysteine ligase transgenes to GLVP transgenic mice in which the \textit{GLVP} gene was driven by a liver-specific (transthyretin) promoter. We then determined which founder lines had increased levels of glutamate-cysteine ligase mRNA, protein, and activity following mifepristone pretreatment. Administration of a single dose of mifepristone resulted in highly variable glutamate-cysteine ligase transgene mRNA expression (Fig. 2). Importantly, endogenous \textit{GlcC or GclM} mRNA expression was apparently unaffected by mifepristone administration (data not shown). \textit{Gclc} protein levels were only slightly increased in \textit{Gclc/GLVP} bigenic mice, whereas \textit{Gclm} protein was significantly induced in \textit{Gclm/GLVP} bigenic mice after receiving mifepristone. This stronger effect of the \textit{Gclm} transgene remained when these bigenic mice were intercrossed to generate \textit{Gclc/Gclm/GLVP} trigenic mice (Fig. 3).

Kitteringham \textit{et al.} (5) showed that APAP (530 mg/kg) decreased glutamate-cysteine ligase activity in the liver of CD-1 mice at 1 and 24 h even though both the mRNA and protein levels had increased. In our studies, livers were harvested 6 h after APAP administration, and at this time we found glutamate-cysteine ligase activity to be significantly higher in trigenic mice when compared with mice carrying just the \textit{GLVP} transactivator gene (Fig. 3).
High glutamate-cysteine ligase activity in male trigenic mice was protective against APAP-induced changes in serum alanine aminotransferase (Fig. 7), and centrilobular necrosis (Fig. 9).

When mice are administered high doses of APAP, GSH is rapidly depleted due to covalent binding to the APAP metabolite NAPQI (2, 7, 11, 12). Subsequently, NAPQI is able to bind to cysteine on proteins. We found that residual GSH levels were correlated with glutamate-cysteine ligase activity, and an inverse relationship between glutamate-cysteine ligase activity and residual GSH level after APAP treatment (Fig. 10C). Because most adduct formation occurs by 2 h after APAP administration (44, 46, 47), glutamate-cysteine ligase likely protects against NAPQI protein adduct formation via an increased supply of GSH resulting in less steatosis and necrosis.

The mechanisms by which APAP induces hepatocellular necrosis is still uncertain, but many causative factors have been rejected including NADPH oxidase (48), lipid peroxidation (49), and Kupffer cell activation (50). In addition, NAPQI protein adduct levels per se do not necessarily lead to hepatotoxicity (10). Recent studies support the view that oxidative stress may not be the only factor responsible for APAP toxicity. For example, several studies support the hypotheses that peroxynitrite formation (50–53) and mitochondrial permeability transition (10, 43) are critical for APAP-induced hepatocellular necrosis.

The reasons for the increased APAP sensitivity of male mice relative to female mice are not clear. Recently, Dai and colleagues (27) also reported that female mice were relatively resistant to APAP-induced liver injury, despite the fact that APAP-protein adducts were similar in male and female mice. Differences in the amount of GST per liver in their mice might be one attributing factor in the differential sensitivity of male and female mice. Our data suggests that GSH synthesis by glutamate-cysteine ligase may also be a gender-dependent protective factor, whereby males are much more dependent upon GSH synthesis for protection from APAP than female mice.

Because glutamate-cysteine ligase activity can be limiting for APAP metabolism (44, 46, 47), glutamate-cysteine ligase likely protects against NAPQI protein adduct formation via an increased supply of GSH resulting in less steatosis and necrosis.
increased risk of cardiovascular disease (54–56). These polymorphisms may also be important determinants of APAP-induced liver injury. If so, this suggests that administration of GSH ethyl ester, or other GSH pro-drugs that by-pass the need for de novo GSH synthesis may be a more effective therapy in APAP overdose than N-acetylcysteine, especially for those people carrying glutamate-cysteine ligase alleles associated with lower GSH synthetic capacity.

Acknowledgments—We thank Monica Hendsch for excellent technical assistance, and Dr. Edward Kelly, Department of Pharmaceutics, and Dr. Haley Neff-LaFord, Department of Environmental and Occupational Health Sciences, University of Washington, for helpful discussions. We also thank Drs. Sophia Tsai and Bert W. O'Malley, Department of Molecular and Cellular Biology, Baylor College of Medicine, for providing GLVP mice.

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