Glutathione Depletion Exacerbates Methylenedianiline Toxicity to Biliary Epithelial Cells and Hepatocytes in Rats

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Methylenedianiline (DAPM) initially injures epithelial cells of major bile ducts, which is followed by cholestasis, cholangitis, and hepatocellular damage. This pattern of biliary injury resembles that produced by α-naphthylisothiocyanate (ANIT), a classic bile duct toxicant. Our goal was to determine whether prior depletion of hepatic glutathione (GSx), a condition reported to protect against biliary tract injury by ANIT, would also protect against DAPM-induced bile duct injury. A new protocol for extensive, sustained depletion of GSx was established. We found that administration of 1-bromohexane followed 1 h later by buthionine sulfoximine resulted in an ~96% depletion of hepatic GSx that persisted through 6 h without biochemical or morphological signs of hepatic injury. Treatment of rats with a minimally hepatotoxic dose of DAPM (without GSx depletion) produced at 6 h injury similar to previous studies: moderate oncosis of biliary epithelial cells (BEC), mild edema of portal triads, and increases in glutathione S-transferase (GST) activities without alterations in hepatic GSx/glutathione disulfide (GSSG), coenzyme A (CoASH)/coenzyme A-glutathione disulfide (CoASSG), or thiobarbituric acid-reactive substances (TBARS). In contrast, DAPM treatment of GSx-depleted rats produced severe oncosis of BEC, marked inflammatory and edematous alterations to portal tracts, and oncosis/apoptosis in scattered hepatocytes. The observed acceleration and enhancement of DAPM-induced liver injury by GSx depletion was associated with a concurrent sevenfold increase in hepatic CoASSG and a fourfold decrease in the ratio of CoASH to CoASSG, compounds presumably localized to mitochondria and a purported index of mitochondrial thiol/disulfide status. These results indicate that: (1) GSx depletion exacerbates BEC and hepatic cellular injury induced by DAPM, and (2) the mechanism by which DAPM causes liver injury is likely different from that of the classic bile duct toxicant, ANIT.

Key Words: methylenedianiline; glutathione depletion; bromoheptane; buthionine sulfoximine; GSH/GSSG; CoASH/CoASSG; oncosis; apoptosis.

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treatment leads to cholangitis and cholestasis comparable with that produced by ANIT (Kanz et al., 1992), and because the proximate toxicant of DAPM is excreted into bile (Kanz et al., 1995), modulation of DAPM injury by total glutathione (GSx) depletion seemed plausible. Thus, our aim was to investigate the effect of hepatic GSx depletion on the time course and degree of DAPM injury to BEC using a dosage (50 mg/kg) that produces moderate to marked BEC damage with minimal hepatocyte injury. The effect of GSx depletion on DAPM injury was assessed using endpoints for hepatotoxicity (serum parameters, histopathology, histochemistry), thiol/disulfide status (GSx/glutathione disulfide [GSSG], coenzyme A [CoASH]/coenzyme A-glutathione disulfide [CoASSG] ratios), lipid peroxidation (thiobarbituric acid-reactive substances [TBARS]), and conjugation capacity (phase II enzyme activities).

**MATERIALS AND METHODS**

**Animals.** Male Sprague-Dawley rats from Harlan (Indianapolis, IN) were kept in a 12-h light/dark cycle animal room with controlled temperature and humidity for 1 week prior to experiments. Rats were maintained in wire-floor cages over absorbent paper with chow and water ad libitum. Rats weighed from 290 to 350 g (mean ± SEM, 307 ± 2.5 g) in these experiments. The Institutional Animal Care and Use Committee of the University of Texas Medical Branch approved all animal care and experimental procedures.

**Chemicals.** 4,4′-Diaminodiphenylmethane (DAPM, 99% purity) and 1-bromohexane (BH, 99% purity) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Buthionine-[S,R]-sulfoximine (BSO), 1-chloro-2,4-dinitrobenzene (CDNB), CoASH (reduced form), CoASSG, N-ethylmaleimide (NEM), 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB), 3a-hydroxyxysteroid dehydrogenase, GSH (reduced form), GSSG, glutathione reductase, glycine, hydroxyl sulfate, β-nicotinamide adenine dinucleotide (β-NAD), β-nicotinamide adenine dinucleotide phosphate (reduced form, β-NADP+), tetra-butylammonium hydrogen sulfate (TBAH), 1,1,3,3-tetraethoxypropane, and taurocholate were obtained from Sigma Chemical Co. (St. Louis, MO).

**Experimental protocol.** DAPM was dissolved in absolute ethanol, then diluted to 25 mg/ml in 35% ethanol using ~40°C deionized water. The solution was kept warm until given by gavage at 2 ml/kg. Rats received a total ethanol dose of 0.56 g/kg, which was not expected to elicit major alterations in hepatic redox status because the reported threshold ethanol dose to induce GSH depletion is 3 g/kg (Videla et al., 1980). 1-Bromohexane (0.5 mmol/ml in corn oil) and buthionine-[S,R]-sulfoximine (0.555 mmol/ml in phosphate-buffered saline [PBS]) were administered by gavage at 2 ml/kg and ip at 4.5 ml/kg, respectively. Experiments began at 9:00 A.M. when rats (four per group) were pretreated with BH or corn oil, followed by pretreatment with BSO or PBS at 10:00 A.M. Treatment of rats with 50 mg DAPM/kg or 35% ethanol (vehicle) occurred 30 min later (at 10:30 A.M.). Rats were sacrificed at 0, 3, or 6 h after DAPM treatment. Rats were anesthetized with ether and opened along the ventral midline. Blood was collected from the inferior vena cava; blood samples were allowed to clot on ice, centrifuged, and the sera stored at ~20°C. Livers were rapidly removed, blotted, weighed, and chopped into pieces. Small pieces were immediately freeze-clamped using liquid nitrogen-cooled tongs and stored in liquid nitrogen until assayed for CoASH and CoASSG. Pieces of liver were washed with PBS, minced, and homogenized (1.5, wt/vol) in 5% sulfooxyacetic acid. After centrifugation, supernatants were aliquoted undiluted or mixed 1:1 (vol/vol) with 10 mM NEM and stored at ~80°C until total GSx and GSSG, respectively, were measured. Additional pieces of liver were rapidly homogenized in 250 mM phosphate-buffered sucrose (pH 7.4), then centrifuged at 9000 g, and the supernatants (S-9 fractions) were stored at ~80°C until assayed. Finally, liver slices from two major lobes were collected and fixed in 10% buffered formalin for histological assessment.

**Hepatotoxicity.** Serum alanine aminotransferase (ALT), alkaline phosphatase (ALP), bilirubin, and γ-glutamyltransferase (GGT) were determined using Sigma reagent kits 57, 20, 550, and 419, respectively. Serum bile salts were determined enzymatically according to the method of Koss et al. (1974) using 3a-hydroxyxysteroid dehydrogenase with taurocholate as a standard.

**Hepatic enzyme activities.** Glutathione S-transferase (GST) activity toward CDNB in liver S-9 fractions was assessed by monitoring the change in optical density at 340 nm, which represents the rate of formation of a conjugate between CDNB and GSH (Habig et al., 1974). Uridine diphosphate (UDP)-glucuronosyltransferase activity in S-9 fractions was determined using p-nitrophenol by the method of Mulder and Van Doorn (1975). Protein in S-9 fractions was measured using the bicinchoninic acid method (Smith et al., 1985).

**Liver TBARS.** Liver aliquots were homogenized in 10 mM phosphate-buffered (pH 7) saline (1:10, wt/vol) containing 50 μM butylated hydroxytoluene as an antioxidant (Esterbauer and Cheeseman, 1990; Pikul et al., 1983) and centrifuged. Supernatant aliquots were mixed with 10% trichloroacetic acid, incubated at 75°C for 2 min, mixed with 0.67% thiobarbituric acid, then incubated for another 10 min. After centrifugation of the samples, optical densities of supernatants were measured at 532 nm (Zhang et al., 1997). Concentrations of TBARS were calculated using a standard curve generated with 1,1,3,3-tetraethoxypropane.

**Liver GSx and GSSG.** GSx and GSSG were assayed by the enzymatic recycling method of Tietze (1969) using glutathione reductase, DTNB, and NADPH. GSx was measured at 405 nm in 96 well plates (BIO-RAD Microplate Reader, Bio-Rad Laboratories, Hercules, CA) according to Baker et al. (1990). GSH standards were prepared in the same buffer, and acid solutions as samples and were assayed at the same time. GSSG was determined in samples after conjugation of GSH with NEM and removal of excess NEM by C18 SepPak (Waters, Milford, MA) chromatography. GSSG standards and samples were assayed at 412 nm in a Beckman DU-8 spectrophotometer (Beckman Coulter, Inc., Fullerton, CA).

**Liver CoASH and CoASSG.** CoASH and CoASSG in liver homogenates were determined by the method developed by Rogers et al. (2000), which utilizes conjugation of CoASH to NEM, followed by separation and quantitation using high-performance liquid chromatography (HPLC). Freeze-clamped liver aliquots (0.2 g) were powdered using a mortar and pestle filled with liquid nitrogen and immediately transferred into a chilled Dounce homogenizer containing 0.8 ml of 0.1-M phosphate buffer (pH 7.4) and 25 mM NEM. Aliquots of the homogenate were then added to 3.8% perchloric acid (1:1, vol/vol). Standards were prepared in the same buffer and acid solutions as samples and were assayed at the same time. Stock solutions of standards, prepared in 0.1-M phosphate buffer (pH 3.0), were diluted in 0.1-M phosphate buffer (pH 7.4) containing 40 μM NEM immediately before analysis. Final concentrations of the standards were 0–40 μM CoASH (now present as CoAS-NEM) and 0–5 μM CoASSG.

CoAS-NEM and CoASSG were analyzed by HPLC using a Waters 626 HPLC pump, a 600S gradient controller, and a 2487 dual-wavelength detector (Waters, Milford, MA). After injecting 0.1 ml of sample or standard, the separation was accomplished using a 15-cm, 4.6-mm I.D. Zorbax (MAC-MOD Analytical, Chadds Ford, PA) C8 reverse-phase column. Components of interest were eluted using a 30-min gradient from 88% A (25% methanol): 12% C (0.1 M tetrabutylammonium hydrogen sulfate, pH 5.2) to 12% A: 76% B (90% methanol): 12% C. Component elution was monitored by following absorbance at 260 nm. After sample elution, the column was washed for 15 min with 100% methanol, then reequilibrated with 88% A: 12% C for at least 10 min. For component identification, retention times of standards were compared with those in the samples. For quantitation, peak areas of standards were measured using Millennium 32 software, version 3.05 (Waters, Milford, MA), and standard curves were constructed.

**Histopathology.** Formalin fixed liver tissues were processed by standard histological techniques, sectioned, and stained with hematoxylin and eosin for evaluation. Also, liver sections from the 6-h groups treated with BH + BSO + DAPM (or vehicle) were histochemically stained for DNA fragmentation of dying cells using the KLENOV-Frag-EL detection kit (Onogene Research
Products, Cambridge, MA) according to the manufacturer’s instructions, except that sections were counterstained with Mayer’s hematoxylin (Polyscientific, Bayshore, NY).

Statistics. Data are expressed as mean ± SE of the mean (SEM). All data were analyzed by two-way ANOVA using SPSS (SAS Institute, Inc., Cary, NC), a statistical package for personal computers. Post hoc analyses compared treatment groups for the effects of time and for the effects of pretreatment/treatment at the same time point, using the Newman-Keuls multiple-comparisons test. A p value of < 0.05 was considered significant.

RESULTS

Effect of Pretreatment and DAPM on GSx and GSSG Levels

Hepatic GSx depletion is usually accomplished by administration of substrates that rapidly form GSH conjugates (e.g., diethylmaleate, phorone) or by inhibition of GSH synthesis via buthionine sulfoximine (BSO), an irreversible inhibitor of γ-glutamylcysteine synthetase (Mulder and Ouwerkerk-Mahadevan, 1997). However, GSH depletion via conjugation leads to rapid resynthesis of hepatic GSH within a few hours (Gerard-Monnier et al., 1992), whereas extensive GSH depletion by BSO treatment in rats requires high doses or repeated injections (Griffith, 1981). Our approach in this study was to produce a substantial depletion of liver GSx by administration of a GSH-transferase substrate, followed by BSO. 1-Bromoheptane was selected as the GSH-conjugate substrate because bromoalkanes rapidly deplete GSH in vivo and in vitro with minimal effects on phase I biotransformation and minimal cytotoxicity (Barnes et al., 1959; Khan and O’Brien, 1991).

Levels of GSx decreased by ~40% in vehicle-pretreated control animals through 6 h (Fig. 1A, open circles), analogous to previous reports of diurnal variation in hepatic GSx in rodents (Farooqui and Ahmed, 1984; Jaeschke and Wendel, 1985). GSSG levels declined more slowly (Fig. 1B, open circles) but no appreciable effect on the GSx/GSSG ratios was observed at either 3 or 6 h in control rats (Fig. 1C, open circles). The pretreatment protocol of BH followed 1 h later by BSO depleted hepatic GSx by ~60% within 90 min (i.e., 0 h), which then plummeted to ~96% depletion by 3 and 6 h (Fig. 1A, open squares). This pretreatment had no initial effect on GSSG levels at 0 h, but by 3 and 6 h, GSSG values fell by ~80% and ~90%, respectively (Fig. 1B, open squares). The faster depletion of GSx, compared with GSSG by BH + BSO, was sufficient to significantly decrease GSx/GSSG ratios at 3 but not 6 h (Fig. 1C, open squares).

As previously reported from our laboratory (Kanz et al., 1998), DAPM treatment did not decrease GSx, GSSG, or the GSx/GSSG ratio at 3 or 6 h (Figs. 1A,B,C, closed circles), compared with the vehicle-treated control group. Similarly, DAPM treatment did not alter the decreases in GSx, GSSG, or GSx/GSSG induced by BH + BSO pretreatment (Figs. 1A,B,C, closed squares).

FIG. 1. Influence of BH + BSO pretreatment on time course of changes in hepatic levels of total GSx (A), GSSG (B), and GSx/GSSG ratio (C) at 0, 3, and 6 h after DAPM treatment. Symbols represent means ± SEM of four rats per group; \( p < 0.05 \), compared with 0-h vehicle treatment control (CO + PBS + Vehicle) group; \( p < 0.05 \), compared with 0-h pretreatment control (BH + BSO + Vehicle) group.

Effect of Pretreatment and DAPM on Coenzyme A Thiol Status

Severe depletion of the cytosolic GSx pool can lead to depletion of the mitochondrial GSx pool, which enhances the
vulnerability of cells to mitochondrial dysfunction and cell death (Meredith and Reed, 1983). Isolation of mitochondria for measurements of GSx/GSSG can lead to artifactual increases/decreases in disulfides/thiols due to lengthy procedures and numerous manipulations. Recently, measurement of the CoASH/CoASSG ratio has been proposed as a potential biomarker of the thiol/disulfide status of mitochondria (O’Donovan et al., 2002; Rogers et al., 2000) because 80–95% of liver CoASH is present in mitochondria (Siess et al., 1978; Soboll et al., 1976) and because CoASSG is believed to form via nonenzymatic oxidation of CoASH and GSH or via sulf-hydryl exchange between GSSG and CoASH (Crane et al., 1982; Dyer and Wilken, 1972).

The GSx depletion protocol was found to produce only a modest effect on CoASH, e.g., a ~25% increase at 6 h (Fig. 2A, open squares) without an effect on CoASSG (Fig. 2B, open squares). This differential effect of GSx depletion on coenzyme A thiol status increased the CoASH/CoASSG ratio by ~60% at 6 h (Fig. 2C, open squares). In contrast, treatment of GSx-depleted rats with DAPM produced a striking approximately sevenfold rise in the CoASSG level between 3 and 6 h (Fig. 2B, closed squares). This striking rise in the coenzyme A-mixed disulfide at 6 h was associated with a ~80% decline in the CoASH/CoASSG ratio (Fig. 2C, closed squares).

**Effect of Pretreatment and DAPM on TBARS**

Neither DAPM treatment alone nor DAPM treatment following GSx depletion increased TBARS levels at 3 or 6 h (data not shown).

**Effect of Pretreatment and DAPM on Serum Indices**

Potential effects on hepatic integrity were assessed by monitoring serum levels of ALT, an index of leaky hepatocytes, as well as serum levels of bilirubin and bile salts, compounds efficiently removed from blood by hepatic uptake and export into bile. BH + BSO pretreatment unexpectedly elevated serum bile acids approximately fourfold at 0 h (Table 1); this elevation in bile acids was transient because bile acid values returned to baseline by 3 h. In contrast, a later elevation of two- to threefold in serum bile acids was evident at 6 h after treatment with DAPM alone or BH + BSO + DAPM. No appreciable changes in serum bilirubin or ALT (Table 1), or in serum ALP or GGT (data not shown) were found, and no pretreatment or treatment effects were observed on liver/body weight ratios (data not shown).

**Effect of Pretreatment and DAPM on Histopathology**

The GSx depletion protocol produced no morphological alterations in liver at any time point (Fig. 3A). At 6 h after DAPM treatment, BEC of the major bile ducts in the medium- to large-size portal triads were flattened with pyknotic nuclei or were becoming denuded from the basement membrane (Fig. 3B). In some animals, BEC injury extended throughout the intrahepatic biliary tree to bile ducts in the smallest size portal triads. Portal triads also displayed mild to moderate edema and fibrous networks within the transudate (Fig. 3B). Hepatocellular injury was apparent as occasional bile infarcts adjacent to the largest portal triads.
In contrast, pretreatment of rats with BH + BSO prior to DAPM accelerated and enhanced BEC injury because bile duct injury was evident within the larger portal triads at the earlier time of 3 h (not shown) and became substantial by 6 h (Fig. 3C). At 3 h, BEC were swollen, occasionally pyknotic, or partially denuded from the basement membrane. By 6 h, oncotic necrosis of BEC in major bile ducts was severe, with many ducts denuded of cells (Fig. 3C). Edema, transudate, inflammatory infiltrate, and connective tissue disarray were frequently observed in portal triads, whereas periportal hepatocytes surrounding some portal triads were eosinophilic with condensed nuclei (Fig. 3C). In addition, an unusual pattern of hepatocyte death was observed to varying degrees among the BH + BSO + DAPM animals at 6 h. This pattern consisted of individual, scattered hepatocytes across the zones displaying characteristics of either apoptosis (specifically, rounding up and engulfment within vacuoles of adjacent parenchymal cells [Fig. 3D]), or oncosis (specifically, pyknotic nuclei and eosinophilia [Fig. 3D]). Cells displaying the characteristics of oncocytic cell death were frequently surrounded by inflammatory cells (Fig. 3D).

To characterize this pattern of cell death further, liver sections from animals given either DAPM or its vehicle following BH + BSO pretreatment were stained for nuclear DNA fragmentation by the KLENOW-Frag-EL procedure. Through in situ nick translation, this procedure is reported to detect DNA single-strand breaks or double-strand breaks with protruding 5’ termini (Jin et al., 1999). Staining that apparently identified apoptotic nuclei (brown staining, Fig. 4B) was rarely observed in livers at 6 h after the GSx depletion protocol. However, staining for DNA fragmentation was regularly observed at 6 h in livers of animals treated with DAPM after GSx depletion (Figs. 4B,C). The extent of this staining in apoptotic hepatocytes that were apparently engulfed within other hepatocytes was highly variable, ranging from dark clumps in cells where nuclear material was evident to no staining in cell fragments where substructure was absent (Fig. 4B). Staining of engulfed cells was also observed more frequently in the livers with the largest number of cells exhibiting characteristics of apoptosis (Fig. 4B). In contrast, no nuclear staining for DNA fragmentation was observed in hepatocytes exhibiting characteristics of oncosis that were surrounded by inflammatory cells (Fig. 4C).

### Effect of Pretreatment and DAPM on Hepatic Phase II Enzyme Activities

A variety of conjugated metabolites of DAPM have been tentatively identified in urine (Cocker et al., 1986) and bile (T. R. Dugas, 2000, unpublished observations). Therefore, potential confounding effects of the GSx-depletion protocol on hepatocellular UDP-glucuronosyltransferase and GST activities were assessed. The GSx depletion protocol had no apparent effect on the activities of the phase II enzymes at 3 or 6 h (Table 2). DAPM alone increased GST activity (toward the universal substrate CDNB) at both time points, whereas GSx depletion plus DAPM further elevated GST activity by ~45% (Table 2).

### DISCUSSION

#### A New GSH/GSSG Depletion Protocol

Investigations about the role of GSx in the detoxification or intoxication of drugs and chemicals have utilized agents that deplete glutathione, either by serving as substrates for glutathione S-transferases (GST) or by inhibiting GSH synthesis. Care must be taken in the choice of agents used to deplete GSx because their administration will also alter other cellular processes. For example, the two most commonly used GST substrates, diethyl maleate (DEM) and phorone, are known to alter multiple processes, including high-energy phosphate metabo-
lism (Kobayashi et al., 1993), microsomal heme oxygenase and cytochrome P450 activities (Anders, 1978; Burk and Correia, 1979), cytochrome P450 content (Yoshida et al., 1987), and lipid peroxidation (Miccadei et al., 1988). An alternative GST substrate, BH, was found to deplete GSx in isolated hepatocytes more rapidly and more completely than either DEM or phorone with less lipid peroxidation and effects on cytochrome P450 activity (Khan and O’Brien, 1991). Furthermore, other bromoalkanes efficiently deplete hepatic GSx by ~50% within 4 h (Barnes et al., 1959) at dosages (1.5 mmol/kg) half that required for comparable GSx depletion by DEM (3 mmol/kg) (Gerard-Monnier et al., 1992). A pronounced degree of hepatic glutathione depletion can be produced by repeated administration of the GSH synthesis inhibitor BSO at 8 mmol/kg (Griffith, 1981). However, a 4-mmol/kg dosage of BSO inhibits S-adenosyl-L-methionine synthetase activity in liver (Corrales et al., 1991) and reduces bile flow (Dahm and Roth, 1991).

In prior studies that used more than one agent to deplete GSH, simultaneous injection of DEM + BSO (Baggett and Berndt, 1986) or DEM given 0.5 h after BSO (Monroe and Eaton, 1988) were reported to decrease GSx by 85–95% within 2.5 h, but the sustained effects of administering these agents were not described. Our goal was to develop a pretreatment protocol that produced a significant yet persistent depletion of GSH using low concentrations of drug. Thus, we first rapidly depleted GSx by administration of a GST substrate (BH, 1 mmol/kg, an expected mercapturic acid excretion rate of ~40

FIG. 3. Photomicrographs of representative H&E-stained liver sections of experimental groups at 3 or 6 h. (A) Pretreatment control at 3 h (BH + BSO + Vehicle). Livers appeared morphologically normal after GSx depletion; no alterations were observed in the architecture of the portal triads at any time point. (B) DAPM at 6 h (CO + PBS + DAPM). BEC of major bile ducts were oncotic and partially denuded (arrows) from the basement membrane. Portal triads were edematous, and fibrous exudates were observed in lymph vessels (asterisks). (C) GSx depletion plus DAPM at 6 h (BH + BSO + DAPM). Epithelial cells lining the bile ducts (asterisks) were absent. Portal triads were edematous and contained scattered inflammatory cells (arrowheads); connective tissue appeared loosened in this portal triad. Periportal hepatocytes immediately adjacent to the portal triad were eosinophilic with condensed nuclei (arrows). (D) GSx depletion plus DAPM at 6 h (BH + BSO + DAPM). Scattered individual hepatocytes were either oncotic (asterisks) and surrounded by inflammatory cells or shrunken, apoptotic (arrows), and engulfed within other hepatocytes. BD, bile duct; HA, hepatic artery; PV, portal vein. Magnifications: (A) ×185; (B) ×175; (C) ×165; (D) ×340.
then prevented resynthesis of GSH by administration of BSO (2.5 mmol/kg) 1 h later. This protocol depleted GSx by ~60% at 0 h (i.e., 30 min after BSO), then to ~96% by 3 h (i.e., 3.5 h after BSO), which was maintained for another 3 h (Fig. 1A). Depletion of GSSG occurred more slowly but eventually reached ~90% (Fig. 1B). Furthermore, minimal levels of GSx were attained without an apparent increase in lipid peroxidation (as measured by TBARS) or effects on hepatic morphology (Fig. 3A), UDP-glucuronosyltransferase or GST activities (Table 2), or serum indicators of hepatotoxicity (Table 1). The only indicator of impaired hepatic function was a transient, approximately three-fold elevation of serum bile acids. Thus, pretreatment with BH + BSO is a promising new protocol for investigating the effects of GSx depletion on chemical and drug hepatotoxicity.

One curious finding of our study was that severe GSx depletion was associated with an ~25% increase in hepatic CoASH by 6 h (Fig. 2A) without an apparent effect on CoASSG levels (Fig. 2B). We are currently unable to explain the increase in hepatic CoASH induced by GSx depletion. More extensive studies measuring numerous forms of CoA (e.g., CoASH, acetyl-CoA, acyl-CoA, fatty acyl-CoA; CoASS-CoA, CoASSG) may be necessary to clarify this influence of GSx depletion on CoASH status.

Exacerbation of DAPM Toxicity following GSx Depletion

The hepatotoxic effects of DAPM observed in this study are consistent in direction and magnitude with our previous findings (Kanz et al., 1998): (1) morphological injury was confined to BEC in portal triads (Fig. 3B), (2) alterations in serum markers of hepatocellular injury were minimal (Table 1), and (3) liver levels of GSx and GSSG were not appreciably affected (Figs. 1A and 1B). Results from this study also indicate that DAPM does not alter hepatic CoASH or CoASSG levels (Figs. 2A and 2B), nor does it elevate TBARS in liver, suggesting that DAPM does not produce significant effects on the redox status of a majority of the cells in the liver (e.g., hepatocytes). However, our observations do not provide information regarding injury markers or thiol/disulfide ratios in BEC, the target cell of DAPM. Detection of specific constitutive changes induced by DAPM within BEC will require in vitro investigations with cultured cells.

Unlike the protection against biliary toxicity of ANIT observed by others in GSx-depleted animals (Dahm and Roth, 1991), we found that depletion of GSx enhanced DAPM toxicity. Depletion of hepatic GSx accelerated DAPM-induced DNA fragmentation in BEC (Fig. 4). Brown staining in presumably apoptotic bodies (arrow) was infrequently observed in the livers of control, GSx-depleted rats. Brown staining in cells engulfed by other hepatocytes ranged from minimal/moderate (arrows) to none (arrowheads). Inflammatory cells typically surrounded hepatocytes undergoing oncosis (asterisk). An oncotic hepatocyte (asterisk) displays vacuolation and pyknosis, and is associated with inflammatory cells; a condensed hepatocyte with circumscribed brown staining is observed within another hepatocyte whose uninterrupted cell membrane can be identified (arrows). Magnification: ×750.
BEC injury by at least 3 h, as well as increased the severity of BEC injury at 6 h (Fig. 3C). Constitutively, the level of GSH in BEC is about one-third that of hepatocytes (Parola et al., 1990a). These low levels of GSH make BEC particularly vulnerable to toxicants that oxidize GSH and produce glutathione-protein mixed disulfides (Parola et al., 1990b). Thus, if the pretreatment protocol produced only a 50% depletion of GSx levels in BEC, GSx concentrations would have dropped below 5 nmol/mg protein (Parola et al., 1990a,b). At such minimal levels of GSx, BEC would have little protection against potential reactive intermediates of DAPM secreted into bile. In addition, we found that depletion of hepatic GSx altered the response to DAPM with noticeable hepatocyte damage, as evidenced by the scattered apoptotic or oncotic dying cells (Figs. 3D, 4B,C) and by hepatocyte dysfunction, as evidenced by increases in serum bile acid (Table 1) and increases in hepatic CoASSG levels (Fig. 2B) and GST activity (Table 2).

The approximately sevenfold increase in CoASSG at 6 h after DAPM may represent a late oxidant stress response in the liver, compartmentalized in the mitochondria (O’Donovan et al., 2002; Rogers et al., 2000), because 80–95% of liver CoASH is found in the mitochondria (Siess et al., 1978; Soboll et al., 1976). Previously, Crane et al. (1982) had shown that perfusion of isolated rat livers with t-butyl hydroperoxide for 10 min elevated hepatic CoASSG levels approximately fourfold while decreasing CoASH and acetyl-CoA levels by ~80% and 75%, respectively, without substantial changes in total CoA species. These authors concluded that elevated levels of GSSG produced by hydroperoxide metabolism via glutathione peroxidase were responsible for increases in hepatic CoASSG. In our experiments, because increases in CoASSG were observed only at 6 h, a time point considerably after evident injury to BEC, it is unlikely that this late alteration in the presumed mitochondrial thiol/disulfide ratio is mechanistically important in BEC injury but it may be relevant to the unusual pattern of hepatocyte injury observed at 6 h (Figs. 3D, 4B,C).

DAPM treatment was found to increase liver GST activity mildly, which was further elevated in GSx-depleted rats treated with DAPM (Table 2). The increase in GST activity induced by DAPM is comparable in extent and timing with that previously reported for DEM and sodium valproate in rat liver (Seçkin et al., 1999; Younes et al., 1980). Known inducers of GSTs include electrophilic substrates for GSTs (Eaton and Bammler, 1999) and specific transcription factors that bind to an antioxidant response element (ARE) in response to oxidative stress (Dhakshinamoorthy et al., 2000). DAPM administration had no effect on TBARS and GSSG levels, which suggests that DAPM does not induce extensive lipid peroxidation or GSH oxidation. Therefore, the observed increases in GST activity are more likely due to the metabolism of DAPM to electrophilic intermediates. Support for DAPM metabolism to electrophiles is our preliminary identification of DAPM-glutathione conjugates in rat bile (Dugas, 2000, unpublished observation). One additional noteworthy fact regarding our results is that the pretreatment protocol for depleting ~96% of liver GSx does not appear to alter cell homeostasis sufficiently to upregulate GSTs (Table 2).

### Apoptosis/Oncosis Pattern of DAPM Hepatocellular Injury

Numerous studies have shown that mitochondrial perturbations (respiratory uncoupling, calcium changes, activation of the mitochondrial permeability transition pore [MTP], loss of membrane potential, enhanced reactive oxygen species formation, release of cytochrome C) are critical events in the early, shared pathways that terminate in either oncosis or apoptosis (for review, see Kroemer et al., 1998). In fact, Lemasters (1999) has proposed that cell death is a continuum ranging from apoptosis to oncosis and that tissue responses to insult are a mixture of events associated with both types. At 6 h after DAPM treatment of GSx-depleted animals, we observed hepatocytes with the swollen, leaky appearance of death by oncosis.

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**Table 2: Effect of Bromoheptane (BH) plus Buthionine Sulfoximine (BSO) Pretreatment on DAPM-Induced Changes in Hepatic Phase II Enzyme Activities**

<table>
<thead>
<tr>
<th>Pretreatments</th>
<th>Treatment</th>
<th>Sacrifice time</th>
<th>UDP-Glucuronosyltransferase (nmol/min per mg protein)</th>
<th>Glutathione S transferase (nmol/min per mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO PBS</td>
<td>Vehicle</td>
<td>0 h</td>
<td>2.23 ± 0.28</td>
<td>1246 ± 103</td>
</tr>
<tr>
<td>CO PBS</td>
<td>Vehicle</td>
<td>3 h</td>
<td>2.72 ± 0.22</td>
<td>935 ± 66</td>
</tr>
<tr>
<td>CO PBS</td>
<td>DAPM</td>
<td>3 h</td>
<td>3.68 ± 0.54</td>
<td>1722 ± 199*</td>
</tr>
<tr>
<td>CO PBS</td>
<td>Vehicle</td>
<td>6 h</td>
<td>3.27 ± 0.64</td>
<td>1008 ± 92</td>
</tr>
<tr>
<td>CO PBS</td>
<td>DAPM</td>
<td>6 h</td>
<td>2.82 ± 0.42</td>
<td>1402 ± 73*</td>
</tr>
<tr>
<td>BH BSO</td>
<td>Vehicle</td>
<td>0 h</td>
<td>2.33 ± 0.27</td>
<td>1251 ± 127</td>
</tr>
<tr>
<td>BH BSO</td>
<td>Vehicle</td>
<td>3 h</td>
<td>2.49 ± 0.48</td>
<td>1234 ± 64</td>
</tr>
<tr>
<td>BH BSO</td>
<td>DAPM</td>
<td>3 h</td>
<td>2.66 ± 0.31</td>
<td>2060 ± 360*</td>
</tr>
<tr>
<td>BH BSO</td>
<td>Vehicle</td>
<td>6 h</td>
<td>2.81 ± 0.38</td>
<td>1352 ± 79</td>
</tr>
<tr>
<td>BH BSO</td>
<td>DAPM</td>
<td>6 h</td>
<td>2.78 ± 0.32</td>
<td>2043 ± 166*</td>
</tr>
</tbody>
</table>

*Note.* Values are the means ± SEM of four rats per group. Abbreviations for pretreatments and treatments are as indicated in Table 1.

*p < 0.05, compared with the respective 3-h or 6-h vehicle treatment control groups.

**p < 0.05, compared with the respective 6-h group given CO + PBS + DAPM.
and other hepatocytes with the shrunken, compacted appearance of death by apoptosis (Fig. 3D). Histchemical staining with the KLENOF-Frag-EL technique for DNA strand breaks was equivocal because only some of the hepatocytes engulfed by other cells showed KLENOF-Frag-EL-positive staining, and that was of highly variable staining intensity (Fig. 4B). DNA strand breaks (as detected by TUNEL staining) have been reported in both apoptotic and oncocytic cells (Grasl-Kraupp et al., 1995; Ohno et al., 1998); thus, the presence of DNA strand breaks is not a definitive marker of apoptosis. A pattern of mixed oncocytic and apoptotic cell death has been reported previously in rat liver following toxicant administration (Levin et al., 1999) and following tissue injury by other kinds of insults, such as ischemia/reperfusion and viral infection (Lemasters, 1999). Based on the observed morphology, we conclude that DAPM induces both apoptosis and oncosis in hepatocytes depleted of GSx. This mixed oncocytic and apoptotic cell death could be related to the DAPM-induced alterations in thioldisulfide ratios in the presumed mitochondrial compartment. Alternatively, such severe GSx depletion may have modulated the sensitivity of hepatocytes to apoptotic cell death by other factors, such as inflammatory cytokines. Nagai et al. (2002) recently showed that GSx depletion of isolated hepatocytes by DEM or phorone treatment produced dose-dependent necrosis, whereas GSx depletion in the presence of tumor necrosis factor (2002) recently showed that GSx depletion of isolated hepatocytes depleted of GSx. This mixed oncocytic and apoptotic cell death could be related to the DAPM-induced alterations in thioldisulfide ratios in the presumed mitochondrial compartment. Alternatively, such severe GSx depletion may have modulated the sensitivity of hepatocytes to apoptotic cell death by other factors, such as inflammatory cytokines. Nagai et al. (2002) recently showed that GSx depletion of isolated hepatocytes by DEM or phorone treatment produced dose-dependent necrosis, whereas GSx depletion in the presence of tumor necrosis factor α induced similar levels of necrosis with increased levels of apoptosis. These authors suggested that GSx depletion could be altering redox-sensitive steps in the apoptosis cascade, which could make cells more susceptible to activation of this pathway.

In summary, pretreatment of rats with BH + BSO produced a substantial and sustained depletion of hepatic glutathione without significant alterations in markers of cell injury or thiol/disulfide status. Administration of DAPM to rats with minimal amounts of hepatic glutathione accelerated injury to BEC and produced an unusual pattern of cell death, with dying hepatocytes exhibiting characteristics of either apoptosis or oncosis. Because glutathione depletion exacerbated BEC and hepatocyte injury induced by DAPM, unlike its protective influence on ANIT injury, our observations suggest that the mechanisms of BEC injury caused by the bile duct toxicants DAPM and ANIT are dissimilar. Further studies comparing DAPM and ANIT injury in rats depleted of GSx by the same protocol will be required to clarify differences in the mechanisms of these two bile duct toxicants.

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