Increased Sensitivity of Glutathione S-Transferase P-Null Mice to Cyclophosphamide-Induced Urinary Bladder Toxicity

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ABSTRACT

Hemorrhagic cystitis and diffuse inflammation of the bladder, common side effects of cyclophosphamide (CY) treatment, have been linked to the generation of acrolein derived from CY metabolism. Metabolic removal of acrolein involves multiple pathways, which include reduction, oxidation, and conjugation with glutathione. Herein, we tested the hypothesis that glutathione S-transferase P (GSTP), the GST isoform that displays high catalytic efficiency with acrolein, protects against CY-induced urotoxicity by detoxifying acrolein. Treatment of wild-type (WT) and *mGstP1/P2* null (GSTP-null) mice with CY caused hemorrhagic cystitis, edema, albumin extravasation, and sloughing of bladder epithelium; however, CY-induced bladder ulcerations of the lamina propria were more numerous and more severe in GSTP-null mice. CY treatment also led to greater accumulation of myeloperoxidase-positive cells and specific protein-acrolein adducts in the bladder of GSTP-null

than WT mice. There was no difference in hepatic microsomal production of acrolein from CY or urinary hydroxypropyl mercapturic acid output between WT and GSTP-null mice, but CY induced greater c-Jun NH₂-terminal kinase (JNK) and c-Jun, but not extracellular signal-regulated kinase or p38, activation in GSTP-null than in WT mice. Pretreatment with mesna (2-mercaptoethane sulfonate sodium) abolished CY toxicity and JNK activation in GSTP-null mice. Taken together, these data support the view that GSTP prevents CY-induced bladder toxicity, in part by detoxifying acrolein. Because polymorphisms in human *GSTP* gene code for protein variants differing significantly in their catalytic efficiency toward acrolein, it is likely that GSTP polymorphisms influence CY urotoxicity. In addition, pretreatment with dietary or nutrient inducers of GSTP may be of use in minimizing bladder injury in patients undergoing CY therapy.

Cyclophosphamide (CY) is a cytotoxic chemotherapeutic agent. Together with other chemotherapeutic drugs, it is used widely for the treatment of lymphomas, solid tumors, and autoimmune disorders such as rheumatoid arthritis and multiple sclerosis (Perini et al., 2007). It is a prodrug that is converted by mixed function oxidases in the liver to 4-hydroxycyclophosphamide and its tautomer aldophosphamide, which spontaneously generates phosphoramidate and acrolein (Low et al., 1982). Formation of acrolein from CY has been linked to the development of hemorrhagic cystitis or diffuse

inflammation of the bladder resulting in dysuria, hematuria, and hemorrhage. Between 2 and 40% of CY-treated patients develop hemorrhagic cystitis (Hader et al., 1993), which is thought to result from the generation of acrolein in the kidney or the bladder (Korkmaz et al., 2007). Evidence supporting a causal role of acrolein in the CY-induced hemorrhagic cystitis is derived from animal models showing that direct treatment with acrolein or aldophosphamide, but not with CY or phosphoramidate, induces bladder toxicity (Cox, 1979). In addition, treatment with thiols such as *N*-acetylcysteine (Cox, 1979; Chaviano et al., 1985) and glutathione (Batista et al., 2007), which readily form Michael adducts with unsaturated carbonyls, prevents CY toxicity in animals. Treatment with mesna (2-mercaptoethane sulfonate sodium) has also been shown to prevent or ameliorate hemorrhagic cystitis in CY-treated cancer patients (Shepherd et al., 1991). Despite the clinical use of mesna, a significant percentage of patients

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ABBREVIATIONS: CY, cyclophosphamide; GST, glutathione S-transferase; GSTP, glutathione S-transferase P; WT, wild type; CDNB, 1-chloro-2,4-dinitrobenzene; EA, ethacrynic acid; HPMA, hydroxypropyl mercapturic acid; NAC, *N*-acetylcysteine; HPLC, high-performance liquid chromatography; JNK, c-Jun NH₂-terminal kinase; ERK, extracellular signal-regulated kinase; H&E, hematoxylin and eosin; MPO, myeloperoxidase; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling.

treated with CY display severe hematuria and bleeding (Shepherd et al., 1991). Moreover, the mechanisms that determine individual susceptibility to CY and acrolein and those that mediate their bladder toxicity remain unclear.

Acrolein is a reactive α,β -unsaturated aldehyde, which reacts readily with nucleophilic cell constituents leading to widespread protein and DNA modification (Beauchamp et al., 1985). In high concentrations acrolein is cytotoxic, and unless removed or metabolized it leads to necrotic and apoptotic cell death (Beauchamp et al., 1985; Li et al., 1997). In most cells, acrolein is rapidly metabolized via several metabolic pathways. The major biochemical pathway for the metabolism of acrolein is conjugation with glutathione (Parent et al., 1998). Although because of its high reactivity acrolein reacts spontaneously with glutathione, the formation of Michael adducts between glutathione and acrolein is catalyzed by glutathione *S*-transferases (GSTs). Multiple GSTs catalyze the conjugation of glutathione with unsaturated aldehydes; however, glutathione *S*-transferase P (GSTP) displays the highest catalytic activity with small unsaturated aldehydes such as base propenals and acrolein (Berhane et al., 1994). Nonetheless, the role of GSTP in the *in vivo* metabolism of acrolein has not been studied, and it remains unclear whether GSTP regulates the urotoxic effects of acrolein generated during CY therapy. Accordingly, the present study was designed to study the role of GSTP in CY-induced bladder toxicity. Our data indicate that compared with wild-type (WT) mice, GSTP-null mice were more sensitive to bladder toxicity caused by treatment with CY. These results are consistent with the notion that acrolein is the key mediator of CY-induced bladder toxicity and that GSTP protects against CY-induced hemorrhagic cystitis. Hence the polymorphic forms of human GSTP, which differ in their catalytic efficiency with acrolein (Pal et al., 2000), and the level of expression of GSTP in the bladder may be important determinants of individual responses to CY therapy.

Materials and Methods

Mice. GSTP1/P2 null mice were generated on a 129 $\times$ MF1 background using homologous recombination as described previously (Henderson et al., 1998). GSTP-null mice were bred onto a B/6 background for six generations before phenotypic characterization. Mice heterozygous for the targeted locus (F1) were backcrossed, and GSTP^(-/-), GSTP^(+/-), and GSTP^(+/+) lines were established. GSTP-null and GSTP WT littermates were obtained from Drs. C. Henderson and R. Wolf (University of Dundee) and maintained as independent lines for use in this study. Mice were treated in accordance with the Declaration of Helsinki and with the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, 1996) as adopted and promulgated by the National Institutes of Health. Treatment protocols were approved by the University of Louisville Institutional Animal Care and Use Committee.

Polymerase Chain Reaction Protocol for GSTP1/P2 Screening. Polymerase chain reaction products were used to genotype WT and GSTP-null mice using primers that amplified a region between exons 5 and 6 of GSTP1 and a region in the *lacZ* gene to identify a null allele. Primers (5'-3') were WT (P1, ggccaccaactactgtgat; P2, agaaggccaggtcctaagc) and null (P3, ctgtagcggtgatgttgaa; P4, atggcattaccgttgatgt) (Henderson et al., 1998). All four primers were mixed with tail DNA, amplified using *Taq* polymerase (Promega, Madison, WI), and the products obtained were separated on 2% agarose gel with WT band at 200 base pair and null band at 300 base pair.

GST Expression and Enzymatic Activity. Western blots for tissue expression of specific GST isoforms (A, M, and P) were developed using commercially available standards and antibodies. Total glutathione-conjugating activity of GSTs with 1-chloro-2,4-dinitrobenzene (CDNB; 1 mM) and ethacrynic acid (EA; 200 μ M) was measured in fractions of kidney, liver, lung, small intestine, stomach, and urinary bladder homogenates (Habig et al., 1974).

CY Exposure. In a preliminary experiment, the dose dependence of CY-induced hemorrhagic cystitis (100–300 mg/kg, *i.p.*, 24 h) was measured in male C57BL/6 mice. The threshold for CY-induced dyslipidemia and cardiotoxicity was greater than 200 mg/kg, whereas increased bladder wet weight occurred with CY at the 200-mg/kg dose. Therefore, age- and strain-matched male WT and GSTP-null mice were exposed to sterile saline (control, 0.1 ml, *i.p.*) or to CY in saline (50 or 200 mg/kg, *i.p.*) and sacrificed at 4 or 24 h post-treatment to measure CY-induced effects. To assess the role of thiols in CY-induced toxicity, the mice were pretreated with mesna (2-mercaptoethanesulfonic acid; 80 mg/kg, *i.p.*; 1 h pre-CY) (Batista et al., 2007) and euthanized 4 h after treatment with CY.

For measurements of CY metabolism, isolated hepatic microsome fractions were incubated with CY, and acrolein (2-propenal) formation was monitored as a fluorescent product using *meta*-3-aminophenol (Alarcon, 1968). An acrolein standard curve (0–50 μ M; Sigma-Aldrich, St. Louis, MO) was prepared in 0.05 mM potassium phosphate buffer, 0.1 mM EDTA, pH 7.4, and mixed with a solution of 3-aminophenol (6 mg/ml) and hydroxylamine hydrochloride (6 mg/ml) in 1 M HCl. The mixture was heated at 90°C for 20 min and then cooled to room temperature. The fluorescence intensity of the product was measured at 350 nm excitation and 515 nm emission. Hepatic microsomal acrolein-generating activity was measured in microsomes pooled from three livers (~1 mg/ml) in two separate experiments.

Urine Collection. To measure whole-body CY and acrolein metabolism, the mice were treated with saline (0.1 ml, *i.p.*) or CY in saline (50 mg/kg) or acrolein (2 mg/kg) in water (0.1 ml, *i.p.*), and urine was collected over 15 h (~6 PM–9 AM). Because CY toxicity reduces urine flow (Wood et al., 2001), mice were acclimated to a glucose (3%) and saccharin (0.125%) solution substituted for drinking water overnight before treatment. This solution stimulates polydipsia and polyuria (~0.5–1 ml/h/mouse) without altering CY-induced urinary bladder toxicity in mice (Wood et al., 2001). Each mouse was housed singly in a metabolic cage overnight, and water and food consumption and urine production were measured. Urine was collected in a water-jacketed, chilled chamber (4°C); centrifuged (2000g, 5 min); urine protein, albumin, and creatinine content measured; and aliquots stored at –80°C until mercapturate analysis. Creatinine clearance was calculated as $V_U \times [\text{Creatinine}]_U / [\text{Creatinine}]_P$ in milliliters per hour.

Urine Hydroxypropyl Mercapturic Acid. Because acrolein is a substrate of GSTP and the reduced mercapturate of acrolein is the most abundant of acrolein-derived urinary metabolites in rat (Linhart et al., 1996), we measured the hydroxypropyl mercapturic acid (HPMA) in urine by gas chromatography/mass spectrometry. The internal standard, [¹³C₃]3-HPMA (10 nmol; in H₂O), was synthesized in our laboratory by incubating [¹³C₃]3-acrolein with a 10-fold excess of *N*-acetylcysteine (NAC) in 0.1 M K⁺-phosphate, pH 7.4, for 1 h at room temperature. NAC-propanal generated from this reaction was purified using reverse-phase high-performance liquid chromatography (HPLC) (C18 Microsorb-MV, 250 $\times$ 4.6 mm; Varian Inc., Palo Alto, CA). NAC-propanal was then reduced by incubating with aldehyde reductase (50 μ g of protein) and NADPH (15 mM) in 0.1 M K⁺-phosphate, pH 6.0, at 37°C for 3 h. Finally, [¹³C₃]3-HPMA was purified by HPLC and analyzed by electrospray ionization/mass spectrometry. For use as an internal standard [¹³C₃]3-HPMA was added to urine and submitted to solid-phase extraction (Carmella et al., 2007). For this, 500 mg of Oasis MAX (Waters, Milford, MA) solid-phase extraction cartridge was conditioned with MeOH (6 ml) and then with 2% NH₄OH (6 ml). The urine was applied to the

cartridge, and the cartridge was washed with 2% NH_4OH (6 ml) followed by methanol (6 ml). The cartridge was then dried with N_2 and washed with 2% formic acid (6 ml, aq.). The fraction containing 3-HPMA was collected with 30% MeOH/2% formic acid and dried under vacuum (SpeedVac; Thermo Fisher Scientific, Waltham, MA). HPLC separation was performed on a Waters HPLC (model 1525) with UV detection (Waters 2487 detector), and the HPMA fraction was collected. HPMA was detected after derivatization with bis(trimethylsilyl)trifluoroacetamide for 1 h at 70°C followed by injection (1 μl) into an Agilent Technologies (Santa Clara, CA) 6890 N gas chromatograph equipped with an HP-5 capillary column (50 m $\times$ 0.2 mm i.d. $\times$ 0.5- μm phase thickness) coupled to a 5973 detector operated in the positive chemical ionization mode with ammonia as the reagent gas. The ions m/z 366 and 369 were monitored for the analysis of HPMA and the internal standard [$^{13}\text{C}_3$]3-HPMA, respectively. Results were expressed as microgram of HPMA per total volume of urine excreted.

Plasma Lipids. Plasma total, high-density lipoprotein and low-density lipoprotein cholesterol, triglycerides, phospholipids, and free fatty acids were measured using Cholesterol E, L-Type HDL-L, L-Type LDL-L, Enzymatic Kits (Wako Bioproducts, Richmond, VA), L-Type TG-H Kit (Wako Bioproducts), phospholipids B Kit (Wako Bioproducts), and NEFA-C Free Fatty Acid Kit (Wako Bioproducts), respectively, using calibrated standards on a Cobas Mira Plus 5600 AutoAnalyzer (Roche, Basel, Switzerland).

Organ Analyses. Body and organ (i.e., gastrointestinal tract, heart, kidney, liver, lung, stomach, and urinary bladder) wet weights (nearest milligram) were measured, and individual organs were snap-frozen in liquid N_2 and stored at -80°C or formalin-fixed (10% neutral buffered formalin) for histological analyses.

Western Blot Analyses. Frozen bladder tissue was pulverized and suspended in lysis buffer (25 mM HEPES, pH 7.0, 1 mM EDTA, 1 mM EGTA, 1% Nonidet P40, 1% SDS, 1:100 protease inhibitor mixture, 1:100 phosphatase inhibitor mixture, and 50 mM *N*-ethylmaleimide), sonicated, and centrifuged (4000g, 15 min, 4°C), and heat-denatured (5 min, 95°C) protein samples in 5 $\times$ sample buffer (312.5 mM Tris base, pH 6.8, 10% glycerol, 11.5% SDS, 0.1% bromophenol, and 50 mM *N*-ethylmaleimide) were separated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA). Membranes were processed by standard immunodetection techniques using commercially available antibodies against phosphorylated or total stress-activated protein kinase/c-Jun NH_2 -terminal kinase (JNK), c-Jun, p42/44 [extracellular signal-regulated kinase (ERK)] and p38 (1:1000; Cell Signaling Technology Inc., Danvers, MA), actin (1:2000), albumin (goat anti-mouse horseradish peroxidase-conjugated; Bethyl Laboratories, Montgomery, TX), or IgG-purified rabbit polyclonal antibody against keyhole limpet hemocyanin acrolein antigen (gift from Dr. P. Burcham, University of Western Australia). Western blots were developed using enhanced chemiluminescence plus reagent (GE Healthcare, Little Chalfont, Buckinghamshire, UK) and detected with a Typhoon 9400 variable mode imager (GE Healthcare). Quantification of band intensities was performed using Image Quant TL software (GE Healthcare), and bands were normalized to unphosphorylated (total) or actin bands where appropriate.

Histology and Immunohistochemistry. Histopathology was performed on formalin-fixed, paraffin-embedded tissue sections (4 μm) stained with hematoxylin and eosin (H&E), rabbit polyclonal antibody against human GSTP1 (1:1500; Novocastra, Newcastle, UK) (Green et al., 2005), acidic toluidine blue for mast cells, rabbit polyclonal anti-myeloperoxidase (MPO) antibody (1:100; murine

MPO; Thermo Fisher Scientific Ab-1), IgG-purified rabbit polyclonal anti-protein-acrolein antibody (1:1000; IgG-purified preimmune rabbit serum served as negative control), or for apoptosis using terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) apoptosis kit (Millipore Bioscience Research Reagents, Temecula, CA) according to manufacturer's instructions. Secondary antibody for GSTP or protein-acrolein and MPO was anti-rabbit goat antibodies with a Vector Elite or Envision Plus staining kit, respectively, using diaminobenzidine (Dako North America, Inc., Carpinteria, CA) as chromagen. Tissue area in mid-bladder cross-sections was measured using Metamorph (Molecular Devices, Sunnyvale, CA). The H&E-stained sections were scored using published criteria (Batista et al., 2006). A grade of 0 was assigned to normal urothelium and no inflammatory infiltrate; 1 to mild flattening and/or sloughing of urothelium, limited hemorrhage and vascular congestion, limited expansion of lamina propria; and 2 to severe damage to urothelium, extensive hemorrhage, ulcerations in lamina propria, degraded connective tissue, and extensive edema. Intermediate scores were used when half the criteria were met. The number of mast cells (400 $\times$ magnification), MPO-positive cells (100 $\times$ magnification), or apoptosis-positive cells was counted in cross-section of the total lamina propria area excluding the smooth muscle (muscularis propria) and urothelium layers.

Chemicals. Unless otherwise indicated, all the chemicals were purchased from Sigma-Aldrich.

Statistics. Values are mean $\pm$ S.E.M. Group data were compared using *t* test or one-way analysis of variance with Bonferroni post-test where appropriate (SigmaStat; SPSS, Inc., Chicago, IL). Significance was accepted at $p < 0.05$.

Results

Tissue Distribution. GSTP protein abundance, localization, and activity were measured by Western blot, immunohistochemistry, and with two GST substrates, respectively, in several tissues (Fig. 1). In addition, to determine whether genetic deletion of the *GSTP1/P2* genes alters the level of other GST protein isoforms in the bladder, Western blots for GSTA and GSTM isoforms were performed. Young adult male WT and GSTP-null mice expressed similar levels of GSTA and GSTM proteins in urinary bladder and kidney. Immunohistochemical staining for GSTP protein was observed in WT but not GSTP-null tissues, including kidney (Fig. 1, A and B), liver (Fig. 1, C and D), lung (Fig. 1, E and F), small intestine (Fig. 1, G and H), stomach (Fig. 1, I and J), and urinary bladder (Fig. 1, K and L). Likewise, Western blot analyses confirmed GSTP in kidney and urinary bladder of WT but not in GSTP-null mice (Fig. 1O). GSTP was detected in high abundance in the epithelium of kidney proximal tubules, lung bronchioles, small intestine villi, stomach epithelium of distal (secretory portion) stomach with noticeably less in forestomach, and urinary bladder urothelium (Fig. 1). In addition, positive staining with anti-GSTP antibodies was observed in smooth muscle of small intestine, stomach, and urinary bladder, as well as in hepatocytes (Fig. 1). The GST activity with CDNB or EA in tissue homogenates of GSTP-null mice was significantly lower compared with WT mice, indicating that GSTP represents a significant fraction of

Fig. 1. Organ distribution of GSTP1/P2 protein and activity. Photomicrographs of sections obtained from kidney (A, WT; B, GSTP-null), liver (C, WT; D, GSTP-null), lung (E, WT; F, GSTP-null), small intestine (G, WT; H, GSTP-null), stomach (I, WT; J, GSTP-null), and urinary bladder (K, WT; L, GSTP-null) stained with anti-GSTP antibody. In M (WT) and N (GSTP-null), bladder cross-sections were stained in the absence of primary antibody. O, Western blots of GSTA (α), GSTM (μ), and GSTP (π) protein expression in urinary bladder and kidney of WT and GSTP-null mice. Total GST activity in tissue homogenates of WT and GSTP-null mice using CDNB (P) and EA (Q) as substrates. Values are mean $\pm$ S.E.M. *, $p < 0.05$ WT versus GSTP-null samples.

Fig. 2. Urinary bladder toxicity of CY in WT and GSTP-null mice. **A**, photomicrographs of sections from the urinary bladder of WT and GSTP-null mice treated with CY (200 mg/kg, i.p. for 24 h). Areas of lamina propria (LP), urothelium (UE), and muscularis propria (MP) are indicated. **B**, changes in urinary bladder wet weight/body weight ratio 4 and 24 h after CY treatment. **C**, H&E-stained sections showing changes in LP, UE, and MP 4 h after CY treatment. **D**, changes in different tissue layers of the urinary bladder 24 h after CY treatment. **E**, Western blots of albumin in bladder lysates of CY-treated WT and GSTP-null mice. Values are mean $\pm$ S.E.M. *, $p < 0.05$ between CY treatment and matched control; †, $p < 0.05$ WT versus GSTP-null; §, $0.10 > p > 0.05$ between CY-treated WT and GSTP-null mice.

TABLE 1

Histopathology of CY-induced bladder injury in WT and GSTP-null mice

H&E-stained cross-sections of urinary bladder at 4 and 24 h after saline (Control), mesna (2-mercaptoethanesulfonic acid; 80 mg/kg, i.p.), CY (200 mg/kg, i.p.), or mesna and CY treatment in 12- to 14-week-old male WT and GSTP-null mice were compared for level of histopathological damage. Median and individual scores (freq.) shown.

Time	Control	Mesna	CY	Mesna + CY
WT				
4 h	0.25	0.25	1.5	0
Scores	0 (3); 0.5 (2); 1 (1)	0 (2); 0.25 (1); 0.5 (1)	1 (1); 1.5 (4); 2 (1)	0 (3); 0.5 (1); 1.5(1)
24 h	0.5	—	1.5	—
Scores	0 (2); 0.5 (4)	—	1.5 (4); 2 (2)	—
GSTP-null				
4 h	0	0.5	2	0.5
Scores	0 (3); 0.5 (2)	0 (2); 0.5 (3)	1 (1); 2 (5)	0 (3); 0.5 (1); 1.5(1)
24 h	0.5	—	2	—
Scores	0 (2); 0.5 (2); 1 (1)	—	2 (6)	—

—, not measured.

TABLE 2

Neutrophil infiltration after CY treatment in WT and GSTP-null mice

Numbers of MPO⁺-stained cells in cross-section of urinary bladder lamina propria at 4 h after saline (Control), mesna (2-mercaptoethanesulfonic acid; 80 mg/kg, i.p.), CY (200 mg/kg, i.p.), or mesna + CY treatment in 12- to 14-week old male WT and GSTP-null mice were counted. Values are mean $\pm$ S.E.M. (range; *n* = number of mice).

Time	Control	Mesna	CY	Mesna + CY
WT				
4 h	1.4 $\pm$ 0.6	1.8 $\pm$ 0.3	13.3 $\pm$ 5.7*	7.8 $\pm$ 3.3
(range; <i>n</i>)	(0–4; 6)	(1–2; 4)	(1–34; 4)	(0–18; <i>n</i>)
GSTP-null				
4 h	0.5 $\pm$ 0.3	0.6 $\pm$ 0.4	35.8 $\pm$ 6.3*†	6.6 $\pm$ 1.7
(range; <i>n</i>)	(0–1; 4)	(0–2; 5)	(18–61; 6)	(2–12; 5)

* *p* < 0.05 from matched control.† *p* < 0.05 from matched mesna + CY group.

total GST activity in these tissues (Fig. 1, P and Q). Immunological staining intensity for GSTP was predictive of the tissue EA activity and thus supported the specific relationship between total immunoreactive GSTP protein and EA activity (Fig. 1). For example, the most intense staining with anti-GSTP antibody was in the small intestine and urinary bladder, and these organs also had the highest specific EA activity, whereas the limited, focal GSTP staining in kidney and lung was associated with the lowest tissue EA activity (Fig. 1Q). In contrast to previous work (Henderson et al., 1998), both CDNB and EA activities were significantly lower in the liver of GSTP-null mice compared with WT mice (Fig. 1P). Moreover, there was no evidence of a compensatory increase in GSTA or GSTM expression in urinary bladder of GSTP-null mice (Fig. 1O). It is interesting to note that GSTM protein was noticeably abundant in the urinary bladder. It is likely that GSTM is a major contributor of total CDNB activity in the bladder, which was equivalent of total hepatic GST activity (Fig. 1P).

Hemorrhagic Cystitis of CY. Treatment of WT or GSTP-null mice with CY led to a significant increase in the wet weight/body weight ratio of the bladder 4 and 24 h post-treatment. However, twenty-four hours after treatment, the increase in urinary bladder wet weight/body weight ratio was significantly greater in GSTP-null than in WT mice (Fig. 2A). H&E-stained cross-sections showed exfoliation of urothelium and edematous expansion and hemorrhage of the lamina propria layer consistent with increased wet weight of the bladder in both WT and null mice at 4 and 24 h after CY treatment (Fig. 2B). Four hours after CY treatment, epithelial exfoliation, hemorrhage, and disintegration of lamina propria appeared more severe in GSTP-null mice than in

Fig. 3. CY-induced apoptosis in the urinary bladder. WT and GSTP-null mice were treated with CY (200 mg/kg, i.p.), and apoptosis was measured in the urinary bladder 24 h after treatment. Positive apoptosis (TUNEL) staining in the lamina propria of CY-treated WT and GSTP-null (B and D) mice but not in saline-treated control mice (A and C). Insets, photomicrographs (400 $\times$) of TUNEL-positive cells of the lamina propria in CY-treated WT (top) and GSTP-null mice (bottom). Bar = 100 μ m.

treated WT mice (Fig. 2C; Table 1). Treatment with CY significantly enhanced lamina propria area from <25% of cross-sectional area in untreated mice to >50% in both WT and GSTP-null mice (Fig. 2D). Likewise, treatment with CY significantly decreased urothelium area; however, this decrease was much greater in GSTP-null than in WT mice, indicating additional sloughing of the urothelium in GSTP-null mice at 24 h after treatment (Fig. 2, C and D). After 4 h of CY treatment, albumin extravasation was significantly increased in GSTP-null and WT mice over untreated controls, and albumin was slightly more in GSTP-null compared with WT mice (Fig. 2E), although this did not reach statistical significance ($0.10 > p > 0.05$).

Because histamine release from mast cells could contribute to CY-induced increase in vascular permeability and urinary bladder edema (Bjorling et al., 1999), the number of mast cells in bladder lamina propria was measured by acidified toluidine blue staining. There was no change in mast cell number or granulation status at 4 or 24 h after treatment in either WT or GSTP-null mice (data not shown). In contrast, a significant increase in MPO-positive (MPO⁺) cells was observed at 4 h post-treatment (Table 2). The number of MPO⁺ cells was significantly greater (by $269 \pm 47\%$; *n* = 5) in

Fig. 4. Measurement of HPMA. Gas chromatogram of native and [¹³C]-HPMA recovered from the urine of mice. Inset, mass spectra of selected ions (*m/z* 366 and 369) corresponding to native HPMA and [¹³C]-HPMA standard, respectively. Ratio of native to standard was used to calculate absolute amount of excreted HPMA after saline, CY, or acrolein treatment.

CY-treated GSTP-null mice, indicating a greater level of inflammation in the GSTP-null mice, than in WT mice. Moreover, there was an ~10-times increase in the number of cells stained positive for apoptosis (TUNEL⁺ stain) in the lamina propria layer in WT (WT + saline, 1.3 ± 0.6 ; WT + CY, 13.5 ± 2.9 ; $n = 6, 6$) and GSTP-null (null + saline, 1.6 ± 0.7 ; null + CY, 14.6 ± 3.6 ; $n = 5, 5$) bladders at 24 h after CY treatment. However, there was no difference in the number of or distribution of TUNEL⁺ cells in the urinary bladder of WT and GSTP-null mice (Fig. 3).

CY and Acrolein Metabolism. To assess the effect of GSTP deficiency on clearance of CY-derived acrolein, the concentration of HPMA, the primary metabolite of acrolein, was measured in the urine of mice treated with either a nontoxic dose of CY (50 mg/kg) or acrolein (2 mg/kg, i.p.). HPMA concentration was measured by gas chromatography/mass spectrometry using ¹³C-HPMA as an internal standard (Fig. 4). Treatment with either CY or acrolein significantly increased urinary HPMA; however, there were no differences between WT and GSTP-null mice in basal, CY-, or acrolein-induced HPMA levels (Table 3). Moreover, CY treatment had no effect on urine flow, urine albumin, urine creatinine, or urine total protein in WT and GSTP-null mice (data not shown). In contrast, acrolein treatment had no effect on urine flow in WT mice but significantly decreased urine flow (ml/15 h) in GSTP-null mice by >50% (pre, 19.5 ± 3.5 ml; post, 7.3 ± 1.4 ml; $n = 5$). However, creatinine clearance, a measure of renal function, was unaffected by acrolein. Acrolein induced a significant decrease (~50%) in fluid intake (preacrolein, 28.5 ± 3.4 ml; postacrolein, 15.7 ± 1.9 ml; $n = 5$) in GSTP-null mice, which probably accounted for the decreased urine

flow. Nevertheless, total HPMA excretion was not different between WT and null mice after acrolein treatment (Table 3). Taken together, these data indicate that GSTP deletion does not affect systemic excretion of HPMA in CY-treated mice, suggesting that exaggerated bladder toxicity in GSTP-null mice was not the result of decreased systemic metabolism of acrolein.

Protein-Acrolein Adducts. Because GSTP has the highest catalytic efficiency of GSTs with acrolein (Berhane et al., 1994), we hypothesized that GSTP deficiency would lead to a decrease in acrolein metabolism and, consequently, to a greater accumulation of protein-acrolein adducts in the bladders of CY-treated mice. Although overall HPMA excretion was not different between WT and GSTP-null mice treated with CY, we localized tissue protein-acrolein adducts in bladder cross-sections by immunohistochemistry and measured adducts in lysates by slot blot and Western blot. As shown in Fig. 5, A through F, CY-induced protein-acrolein adducts colocalized in the lamina propria with dilated blood vessels and degraded connective tissue—the area of greatest hemorrhagic and edematous damage. The basal level of protein-acrolein adducts was significantly increased in GSTP-null mice bladder lysates compared with WT mice as detected by an increase in the band intensities by slot blot (Fig. 5G). Both WT and null mice had significantly more protein-acrolein adducts 4 h after CY treatment than in saline-treated control mice, indicating bladder injury was associated with increased protein-acrolein adducts in WT and null mice. Moreover, CY-treated GSTP-null mice had significantly more adducts than CY-treated WT mice by slot blot (Fig. 5G). Likewise, CY treatment significantly enhanced the quantity

TABLE 3

Measurement of 3-HPMA in urine of WT and GSTP-null mice

HPMA calculated as total micrograms in urine volume collected over 15 h pre- and post-treatment with CY (50 mg/kg, i.p., $n = 5$, 5 mice, respectively) or acrolein (2 mg/kg, i.p.; $n = 4$, 5 mice, respectively). Values are mean $\pm$ S.E.M.

	WT ($n = 5, 4$)	GSTP-Null ($n = 5, 5$)
Pre-CY treatment	4.13 $\pm$ 0.26	4.06 $\pm$ 0.20
Post-CY treatment	22.06 $\pm$ 1.66	18.11 $\pm$ 1.9
Ratio (Post/Pre: CY)	5.42 $\pm$ 0.49	4.46 $\pm$ 0.42
Pre-acrolein treatment	4.07 $\pm$ 0.44	3.16 $\pm$ 0.42
Post-acrolein treatment	21.41 $\pm$ 1.93	24.34 $\pm$ 2.37
Ratio (Post/Pre: acrolein)	5.38 $\pm$ 0.60	7.93 $\pm$ 0.58*

* $p < 0.05$ between WT and GSTP-null mice.

of specific protein-acrolein adducts observed by Western blotting at molecular masses of >250 , ~ 150 , ~ 40 , ~ 35 , ~ 25 , and ~ 20 kDa in WT mice compared with saline-treated WT mice, whereas an ~ 25 -kDa band was significantly increased by CY in GSTP-null mice compared with control null mice when normalized to actin (Fig. 5, H and I).

JNK/Mitogen-Activated Protein Kinase Activation.

Genomic deletion of GSTP in mice has been shown to result in constitutive activation of JNK in liver, lung, and bone marrow (Elsby et al., 2003; Gate et al., 2004); however, we found no evidence of constitutive JNK activation in the bladder of GSTP-null mice. Nevertheless, to determine whether GSTP regulates mitogen-activated protein kinase activation, we measured changes in the phosphorylation of JNK, ERK, and p38 in the bladders of CY-treated mice. We found that treatment with CY led to a significant increase in phospho-JNK in both WT and GSTP-null mice; however, the increase in JNK phosphorylation was greater in GSTP-null than in WT mice (Fig. 6A). In addition, c-Jun phosphorylation was significantly greater in GSTP-null mice treated with CY than in CY-treated WT mice (Fig. 6B). Phosphorylation of ERK was also stimulated by CY treatment, but the increase was similar in both WT and GSTP-null mice (Fig. 6C). Phospho-p38 status was unchanged by CY treatment (Fig. 6D). We conclude, based on these observations, that even though the deletion of GSTP increased CY-induced JNK and c-Jun phosphorylation, it did not alter phosphorylation status of ERK or p38 after CY treatment.

Mesna Prevented CY-Induced Bladder Toxicity. To assess the role of electrophilic injury in CY treatment, mice were pretreated with mesna (80 mg/kg, i.p.). We found that mesna pretreatment prevented CY-induced increase in urinary bladder wet weight (WT, $96 \pm 8\%$ of control; $n = 5$; null, $86 \pm 6\%$ of control; $n = 5$) and the associated changes in histopathology (Fig. 7A; Table 3). Mesna pretreatment significantly attenuated CY-induced increase in the number of MPO⁺ cells (Fig. 7B; Table 3) and decreased protein-acrolein adduct staining in the lamina propria of urinary bladder of CY-treated GSTP-null mice (Fig. 7C). Mesna pretreatment prevented JNK phosphorylation in WT mice and similarly prevented the hyperphosphorylation of JNK in GSTP-null mice (Fig. 7D). Because excessive bladder injury, inflammation, protein-acrolein adduct formation, and JNK activation in GSTP-null mice were all prevented by mesna, these observations indicate that the lack of GSTP exacerbates bladder injury, inflammation, and stress by mechanisms consistent with excessive electrophilic injury (i.e., increased acrolein accumulation) and was not a result of constitutive, irre-

versible changes in the bladder caused by deletion of the *mGstp1/p2* genes.

CY-Induced Hepatic and Systemic Toxicity. In addition to changes in bladder, we also measured systemic markers of general toxicity to assess whether GSTP-induced protection was restricted to the bladder. Treatment with CY (200 mg/kg, i.p.) significantly increased plasma total and low-density lipoprotein cholesterol. CY increased plasma aspartate aminotransferase and decreased plasma total protein in GSTP-null mice but not in WT mice (Table 4). Because these data indicated a hepatic locus of CY toxicity, we measured hepatic metabolism of CY. Our results show that acrolein formation from CY was similar in hepatic microsomes isolated from WT and GSTP-null mice (WT, 2.28 ± 0.15 ; GSTP-null, 2.10 ± 0.06 nmol product formed/min/mg protein). These observations show that there was no difference in the microsomal activation of CY in the livers of CY-treated WT and GSTP-null mice. Likewise, we did not observe any obvious alterations in hepatic histology in H&E-, MPO⁺-, acidified toluidine blue-, and apoptosis-stained sections in CY-treated WT and GSTP-null mice (4 and 24 h post-CY),

Fig. 5. Protein-acrolein adducts in the urinary bladder of CY-treated GSTP-null mice. A and B, photomicrographs (40 $\times$) of H&E-stained cross-sections of urinary bladder from saline (A) or CY-treated (B) GSTP-null mice. C through F, bladder cross-sections stained with IgG-purified rabbit polyclonal anti-protein-acrolein antibody (1:1000) using diaminobenzidine as chromagen and photographed at 100 $\times$ (C and E) or 400 $\times$ (D and F) magnification, respectively. Anti-protein-acrolein antibody staining in urinary bladder of GSTP-null mice treated with saline (C and D) or CY (200 mg/kg, i.p.; 4 h) (E and F). Outlined box in E is expanded in F to show greater detail of protein-acrolein adduct staining. Slot blot (SB; G) and Western blot (WB; H) of protein-acrolein adducts in urinary bladder lysates of individual mice. I, densitometric analysis of discrete protein-acrolein adduct bands ($n = 5$ mice/group). BV, blood vessel; MP, muscularis propria; LP, lamina propria; UE, urothelium. Values are mean $\pm$ S.E.M. *, $p < 0.05$ treated versus matched control; †, $p < 0.05$ GSTP-null versus matched WT; §, $0.10 > p > 0.05$ treated (or GSTP-null control) versus matched control.

G SB: protein-acrolein adducts

H WB: protein-acrolein adducts

Fig. 5. (Continued)

Fig. 6. JNK/mitogen-activated protein kinase activation in urinary bladder of WT and GSTP-null mice. Western blots of bladder lysates from WT and GSTP-null mice treated with saline (C, control) or CY (200 mg/kg, i.p.) for 4 h and developed with anti-phospho-JNK and JNK (A), anti-phospho-c-Jun and c-Jun (B), anti-phospho-ERK and ERK (C), and anti-phospho-p38 and p38 antibodies (D). Values are mean $\pm$ S.E.M. *, $p < 0.05$ CY-treated versus matched control; †, $p < 0.05$ WT versus GSTP-null ($n = 5$ /group).

Fig. 7. Effects of mesna (2-mercaptoethanesulfonic acid) on CY-induced bladder toxicity. WT and GSTP-null mice pretreated with mesna (80 mg/kg, i.p.) before CY (200 mg/kg, i.p.) had bladders removed 4 h after treatment. Changes in urinary bladder wet weight/body weight ratio (A), MPO⁺ cells (B), protein-acrolein adduct staining (C), and Western blot analysis (D) of bladder lysates developed with anti-phospho-JNK and anti-JNK antibodies. Values are mean $\pm$ S.E.M. *, $p < 0.05$ CY-treated versus matched control (n indicated in circle for A and B and $n = 3/\text{group}$ for D); †, $p < 0.05$ treated-null and mesna-treated-null; §, $0.10 > p > 0.05$ treated versus matched control.

indicating no obvious hepatic damage. No significant differences in the relative weight of major organs were noted (Table 5). Mesna pretreatment significantly decreased the gain in stomach weight gain in treated GSTP-null mice ($+138 \pm 12\%$ of control; $n = 5$) to a greater degree than in WT mice ($+214 \pm 15\%$ of control; $n = 5$). Overall, these observations suggest that deletion of GSTP neither exaggerated systemic CY toxicity nor affected hepatic activation of the prodrug.

Discussion

The major finding of this study is that GSTP protects against CY-induced bladder toxicity, in part by promoting bladder-specific metabolism and detoxification of acrolein, the major urotoxic CY metabolite. This conclusion is based on the observations that CY-treated GSTP-null mice displayed

greater bladder injury, disintegration of lamina propria, and additional sloughing (exfoliation) of the urothelium than in the WT mice. The GSTP-null mice also displayed a greater increase in vascular permeability (edema, albumin leakage) and inflammation. These changes were accompanied by a greater accumulation of protein-acrolein adducts and JNK/c-Jun hyperactivation in the bladder of CY-treated GSTP-null mice. Nevertheless, pretreatment with mesna prevented increased bladder injury and inflammation in CY-treated GSTP-null mice to the same extent as in WT mice, indicating that these changes in GSTP-null mice were caused by exaggerated electrophilic stress and not the result of nonspecific genetic changes caused by constitutive deletion of the *GSTP* gene. Taken together, this evidence supports the notion that GSTP activity in the bladder is a critical determinant of urotoxicity caused by acrolein generated on CY treatment.

Although an effective chemotherapeutic strategy, CY

TABLE 4

Blood and plasma parameters in WT and GSTP-null mice

Effects of saline (control) or CY (mg/kg, i.p.) treatment in unfasted 12- to 14-week-old male WT and GSTP-null mice 24 h after exposure to CY. Values are mean $\pm$ S.E.M.; (n) = number of mice.

Variable	WT			GSTP-Null		
	Control	CY (50 ^a)	CY (200)	Control	CY (50 ^a)	CY (200)
Blood glucose ^b	243 $\pm$ 9 (9)	201 $\pm$ 7 (6)	239 $\pm$ 6 (18)	248 $\pm$ 3 (12)	175 $\pm$ 8 (5)	257 $\pm$ 4 (26)
Hct (%)	41.2 $\pm$ 0.5 (9)	40.2 $\pm$ 1.4 (6)	42.2 $\pm$ 0.6 (12)	42.3 $\pm$ 0.6 (12)	39.8 $\pm$ 2.1 (5)	41.5 $\pm$ 0.6 (20)
Buffy coat (%)	1.1 $\pm$ 0.1 (3)	1.2 $\pm$ 0.2 (6)	1.6 $\pm$ 0.2 (12)	1.3 $\pm$ 0.2 (7)	1.5 $\pm$ 0.3 (5)	1.1 $\pm$ 0.1 (20)
Cholesterol ^b	94 $\pm$ 6 (8)	94 $\pm$ 2 (6)	104 $\pm$ 7 (18)	85 $\pm$ 4 (12)	85 $\pm$ 4 (5)	100 $\pm$ 3 (26)*
HDL ^b	75 $\pm$ 4 (9)	68 $\pm$ 3 (6)	70 $\pm$ 5 (15)	64 $\pm$ 3 (12)	61 $\pm$ 3 (5)	68 $\pm$ 3 (23)
LDL ^b	18 $\pm$ 1 (9)	19 $\pm$ 1 (6)	24 $\pm$ 3 (15)	15 $\pm$ 1 (11)	17 $\pm$ 1 (5)	21 $\pm$ 1 (23)*
Phospholipids ^b	155 $\pm$ 7 (9)	152 $\pm$ 9 (5)	137 $\pm$ 9 (18)	158 $\pm$ 5 (12)	153 $\pm$ 10 (5)	141 $\pm$ 8 (26)
Triglycerides ^b	28 $\pm$ 4 (9)	12 $\pm$ 1 (5)	24 $\pm$ 4 (18)	38 $\pm$ 6 (12)	12 $\pm$ 2 (5)	32 $\pm$ 3 (26)
TP ^c	4.60 $\pm$ 0.06 (9)	4.53 $\pm$ 0.15 (6)	4.34 $\pm$ 0.10 (15)	4.50 $\pm$ 0.08 (12)	4.48 $\pm$ 0.21 (5)	4.24 $\pm$ 0.05 (25)*
ALB ^c	2.96 $\pm$ 0.04 (9)	3.11 $\pm$ 0.10 (6)	2.86 $\pm$ 0.05 (18)	2.95 $\pm$ 0.18 (12)	3.04 $\pm$ 0.11 (5)	2.80 $\pm$ 0.03 (24)*
LDH ^d	156 $\pm$ 16 (3)	121 $\pm$ 6 (6)	108 $\pm$ 12 (3)	246 $\pm$ 31 (6)	146 $\pm$ 29 (5)	272 $\pm$ 102 (7)
CK ^d	218 $\pm$ 53 (3)	201 $\pm$ 17 (6)	113 $\pm$ 20 (12)	169 $\pm$ 8 (6)	301 $\pm$ 110 (5)	253 $\pm$ 49 (19)
ALT ^d	33 $\pm$ 3 (3)	23 $\pm$ 3 (6)	30 $\pm$ 2 (12)	25 $\pm$ 2 (7)	19 $\pm$ 2 (5)	49 $\pm$ 8 (20)
AST ^d	65 $\pm$ 8 (3)	72 $\pm$ 4 (6)	75 $\pm$ 6 (12)	66 $\pm$ 5 (6)	87 $\pm$ 8 (5)	123 $\pm$ 12 (20)*
ALP ^d	22 $\pm$ 8 (3)	8 $\pm$ 2 (6)	27 $\pm$ 4 (3)	25 $\pm$ 7 (7)	9 $\pm$ 3 (5)	32 $\pm$ 11 (7)
Creatinine ^b	0.26 $\pm$ 0.01 (3)	0.29 $\pm$ 0.02 (6)	0.25 $\pm$ 0.01 (3)	0.25 $\pm$ 0.01 (7)	0.27 $\pm$ 0.01 (5)	0.25 $\pm$ 0.01 (7)
sICAM-1 ^e	27.0 $\pm$ 1.8 (6)	—	25.0 $\pm$ 2.0 (6)	19.3 $\pm$ 3.3 (6)	—	17.4 $\pm$ 1.0 (6)

Hct, hematocrit; HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol; TP, total protein; ALB, albumin; LDH, lactate dehydrogenase; CK, creatine kinase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; —, not measured.

* $p < 0.05$ from control by one-way analysis of variance and Bonferroni post-test.^a CY treatment in mice acclimated to 3% glucose and 0.125% saccharin drinking solution to stimulate diuresis.^b mg/dl.^c g/l.^d U/l.^e ng/ml.

TABLE 5

Body weight (BWT) and organ wet weight/BWT ratios in WT and GSTP-null mice

Effects of saline (Control) or CY (mg/kg, i.p.) treatment in unfasted, 12- to 14-week-old male WT and GSTP-null mice 24 h after exposure to CY. Values are mean $\pm$ S.E.M.; (n) = number of mice.

Variable	WT			GSTP-Null		
	Control	CY (50 ^a)	CY (200)	Control	CY (50 ^a)	CY (200)
BWT	27.7 $\pm$ 0.8 (9)	23.1 $\pm$ 0.5 (5)	25.5 $\pm$ 0.4 (18)	25.6 $\pm$ 0.6 (12)	24.7 $\pm$ 0.5 (5)	23.8 $\pm$ 0.4 (26)
BWT change ^b	-0.9 $\pm$ 0.2 (9)	-0.5 $\pm$ 0.2 (6)	-1.9 $\pm$ 0.2 (18)*	-1.0 $\pm$ 0.3 (12)	-0.6 $\pm$ 0.2 (5)	-1.7 $\pm$ 0.2 (26)*
Heart ^c	5.1 $\pm$ 0.1 (9)	4.8 $\pm$ 0.1 (5)	5.2 $\pm$ 0.1 (18)	4.8 $\pm$ 0.1 (12)	5.1 $\pm$ 0.1 (5)	5.0 $\pm$ 0.1 (26)
LV ^c	3.4 $\pm$ 0.03 (3)	—	3.2 $\pm$ 0.1 (12)	3.4 $\pm$ 0.1 (7)	—	3.1 $\pm$ 0.1 (20)
Liver ^c	45.7 $\pm$ 1.2 (9)	42.7 $\pm$ 1.3 (5)	44.2 $\pm$ 0.9 (18)	48.3 $\pm$ 1.7 (12)	40.1 $\pm$ 1.2 (5)*	46.3 $\pm$ 0.5 (26)
Lungs ^c	5.9 $\pm$ 0.5 (3)	6.5 $\pm$ 0.2 (5)*	5.7 $\pm$ 0.1 (12)	5.9 $\pm$ 0.2 (7)	6.7 $\pm$ 0.04 (5)*	6.3 $\pm$ 0.1 (20)
Kidney ^c	11.7 $\pm$ 0.3 (9)	11.7 $\pm$ 0.2 (5)	12.5 $\pm$ 0.3 (16)	12.7 $\pm$ 0.4 (12)	13.0 $\pm$ 0.3 (5)	12.6 $\pm$ 0.2 (26)
GI tract ^c	105.1 $\pm$ 3.0 (3)	—	121.3 $\pm$ 2.8 (12)*	115.3 $\pm$ 2.2 (7)	—	129.2 $\pm$ 2.1 (20)*
Stomach ^c	16.3 $\pm$ 1.6 (3)	—	29.9 $\pm$ 1.7 (12)*	14.2 $\pm$ 0.5 (7)	—	32.8 $\pm$ 1.3 (20)*
Intestine ^d	88.8 $\pm$ 4.5 (3)	—	91.4 $\pm$ 2.1 (12)	101.1 $\pm$ 2.2 (7)	—	96.4 $\pm$ 1.6 (20)
Urinary bladder ^c	1.2 $\pm$ 0.2 (7)	1.3 $\pm$ 0.2 (5)	2.0 $\pm$ 0.1 (18)*	1.1 $\pm$ 0.1 (12)	1.3 $\pm$ 0.1 (5)	2.3 $\pm$ 0.2 (26)*

LV, left ventricle; GI tract, intact stomach and intestine; —, not measured.

* $p < 0.05$ from control by one-way analysis of variance and Bonferroni post-test.^a CY treatment in mice acclimated to 3% glucose and 0.125% saccharin drinking solution to stimulate diuresis.^b Grams.^c Milligram of tissue wet weight/gram BWT.^d Intestine mass was difference between total GI tract wet weight and stomach wet weight.

treatment is associated with multiple side effects, particularly urinary bladder hemorrhagic cystitis (Cox, 1979; Bon et al., 2003; Morandi et al., 2005). The high urotoxicity of CY has been linked to the generation of acrolein (Cox, 1979); hence we examined whether GSTP, which catalyzes the conjugation of acrolein with glutathione (Berhane et al., 1994), prevents CY toxicity by promoting acrolein metabolism. To test the role of GSTP, we used GSTP-null mice. Because mouse *GSTP* genes are orthologous to human *GSTP1*, sharing 85% sequence identity at the genetic level (Board, 2007), deletion of *GSTP* genes in mice represents a true null genetic model of human GSTP1 deficiency. The GSTP-null mice develop, grow, and reproduce normally and have normal urinary bladder histology as in WT mice. There are no obvious anatomical or physiological defects caused by GSTP deletion;

however, it is known that the GSTP-null mice are more sensitive to TPA-induced skin tumorigenesis (Henderson et al., 1998), as well as cigarette smoke- and acrolein-induced endothelial dysfunction (Conklin et al., 2009). In contrast, GSTP-null mice are more resistant to acetaminophen-induced hepatotoxicity (Henderson et al., 2000), indicating the need to assess the effects of GSTP deletion on the sensitivity of a specific target tissue.

Our results show that deletion of GSTP does not affect overall CY metabolism or systemic CY toxicity. We found no difference in the CY metabolism in WT and GSTP-null mice hepatic microsomes and no difference in the excretion of HPMA, which is the main urinary metabolite of acrolein in mice. These findings suggest that deletion of GSTP does not alter the overall production of acrolein from CY and that it

does not affect systemic removal of acrolein. In addition to GSTP, other GST isoforms can also participate in conjugating acrolein. Thus, even though GST isoforms other than GSTP display relatively weak catalytic activity with acrolein *in vitro*, their high abundance may compensate for the loss of GSTP *in vivo*, particularly in tissues such as liver and kidney. However, such compensation is unlikely to be effective in tissues or specific cell types where GSTP is expressed at higher levels. Hence, it appears that greater bladder toxicity in GSTP-null mice may relate to the quantitatively higher contribution of this isoform to acrolein detoxification in the bladder (or in parts of the bladder) than in other tissues.

The protection of the bladder by GSTP appears likely to be a function of its metabolic activity. GSTP deletion significantly decreases GST activity in all the organs of GSTP-null mice tested; thus, GSTP contributes to a significant part, albeit to varying degrees, of the total organ GST activity. Although GSTP contributes only a fraction of the total GST activity in the urinary bladder, the specific GSTP activity toward ethacrynic acid is absent in GSTP-null bladders. Significantly, GSTM protein is expressed in high abundance in the bladder and likely accounts for the relatively high total CDNB activity in the bladder, which was comparable with that of liver. Nevertheless, GSTM did not compensate for the loss of GSTP protein. This further emphasizes the specific contribution of GSTP activity in the bladder; thus, despite being relatively enriched in total GST activity (i.e., GSTM), bladders of GSTP-null mice were highly sensitive to CY toxicity. GST isoforms other than GSTP may be involved in acrolein removal; for example, it has been reported that when *hGSTP* polymorphism (*hGSTP105V*) is combined with *hGSTM1*-null and *hGSTT1*-null genotypes, it increases steroid-dependent remission of idiopathic nephritic syndrome in children receiving intravenous CY (Sharda et al., 2008), suggesting that GST isoforms other than GSTP may also be involved in the detoxification of acrolein, although this remains to be quantitatively established.

Our findings provide further support of the role of acrolein as a causative agent in CY-induced hemorrhagic cystitis (Batista et al., 2006, 2007; Bjorling et al., 2007). Our results show, for the first time, that intense protein-acrolein adduct staining is localized in the lamina propria (i.e., suburothelium), which is the site of the earliest and most intense focal damage, including expansive edema, hemorrhage, schistocyte formation, albumin extravasation (leakage), ulcerative dissolution of connective tissue, and inflammatory cell infiltration, and apoptosis. Protein-acrolein adducts were present in large aggregates ("clumps") of filamentous protein near blood vessels in the lamina propria, which is composed mostly of fibroblasts, connective tissue, and vasculature underlying the urothelium and is largely devoid of GSTP protein. In contrast, relatively intense GSTP staining is present in the urothelium and outer smooth muscle layers (muscularis externa) of WT mice (see Fig. 1). Thus, our histological data support the view that high concentrations of acrolein are present in the bladder of CY-treated mice and that increased levels of protein-acrolein adducts accumulate in areas of GSTP deficiency in WT and GSTP-null mice. Moreover, we found that CY-induced damage is increased in the urothelium and to a lesser degree in the muscularis externa of GSTP-null mice; this region is enriched in GSTP staining of wild-type mice. Slot and Western blotting analyses confirmed

that basal levels of protein-acrolein adduction were higher in GSTP-null mice compared with WT mice (see Fig. 5) and that CY substantially increases adducts in both WT and null mice; however, the relative increase was similar, perhaps in part because of saturation of available sites, i.e., the susceptible proteins were already modified at baseline and their bulk modification could not be increased even if acrolein concentration was increased. Although speculative, this view is supported by a significant increase in specific adducts (in GSTP-null and WT) as exemplified by the increase in the 25-kDa band (there may be others). These data indicate that although the relative extent of adduct generation on CY treatment may appear similar, modification of proteins is enhanced by GSTP deletion.

A causative role of acrolein as a mediator of CY-induced cystitis is further supported by our observation that pretreatment with mesna afforded protection against CY-induced bladder injury. This finding is consistent with previous studies showing that thiol pretreatment protects humans and experimental animals from CY-induced bladder injury (Gurtoo et al., 1983; Roberts et al., 1994). In addition, our study shows that mesna pretreatment significantly decreased protein-acrolein adduct staining in bladder and thus provides evidence for a functional link between protein-acrolein adducts and CY-induced urinary bladder injury. It is noteworthy that complete prevention of exaggerated toxicity in GSTP-null mice by mesna also supports a metabolic role of GSTP in protection against CY-induced urotoxicity, indicating that deletion of GSTP creates a metabolic deficit and not a chronic susceptible state or a structural defect (e.g., changes in protein-protein interaction), which could not be overcome by a direct nucleophilic intervention. It follows then that these results are consistent with the idea that GSTP protects by detoxifying acrolein by conjugating it with glutathione. Thus, even though it has been reported that acrolein-glutathione conjugates also are toxic in bladder and kidney (Horvath et al., 1992; Ramu et al., 1996), our data support the general hypothesis that free acrolein is causative in CY-induced bladder cystitis and that toxicity of exogenously delivered acrolein-glutathione may be derived from their ability to dissociate and liberate free acrolein or transfer free acrolein to other cellular nucleophiles.

In summary, the results of this study show that GSTP protects against CY-induced urotoxicity, perhaps in part by detoxifying acrolein in the bladder. These findings suggest that humans with different polymorphisms of GSTP (which display different catalytic efficiencies with acrolein) (Pal et al., 2000) may differ in their sensitivity to CY-induced urotoxicity. Further identification of GSTP genotype-dependent CY sensitivity in humans could lead to a more personalized therapy, avoiding high-dose CY in susceptible individuals or taking additional preventive measures before therapy to avoid bladder hemorrhage and cystitis. In addition, because GSTP is a highly inducible enzyme, its expression level could be enhanced to minimize CY toxicity. Several environmental and dietary supplements such as garlic organosulfur compounds (Tsai et al., 2005), chemopreventive selenocysteine conjugates (t Hoen et al., 2002), and coffee induce GSTP (Steinkellner et al., 2005) and may be of use in preventing CY-induced urotoxicity, which continues to plague a significant portion of the CY-treated population (Ekhardt et al., 2008).

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