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Modulation of Hepatic and Renal Metabolism and Toxicity of Trichloroethylene and Perchloroethylene by Alterations in Status of Cytochrome P450 and Glutathione

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Abstract

The relative importance of metabolism of trichloroethylene (Tri) and perchloroethylene (Perc) by the cytochrome P450 (P450) and glutathione (GSH) conjugation pathways in their acute renal and hepatic toxicity was studied in isolated cells and microsomes from rat kidney and liver after various treatments to modulate P450 activity/expression or GSH status. Inhibitors of P450 stimulated GSH conjugation of Tri and, to a lesser extent, Perc, in both kidney cells and hepatocytes. Perc was a more potent, acute cytotoxic agent in isolated kidney cells than Tri but Perc-induced toxicity was less responsive than Tri-induced toxicity to modulation of P450 status. These observations are consistent with P450-dependent bioactivation being more important for Tri than for Perc. Incubation of isolated rat hepatocytes with Tri produced no acute cytotoxicity in isolated hepatocytes while Perc produced comparable cytotoxicity as in kidney cells. Modulation of P450 status in hepatocytes produced larger changes in Tri- and Perc-induced cytotoxicity than in kidney cells, with non-selective P450 inhibitors increasing toxicity. Induction of CYP2E1 with pyridine also markedly increased sensitivity of hepatocytes to Tri but had little effect on Perc-induced cytotoxicity. Increases in cellular GSH concentrations increased Tri- and Perc-induced cytotoxicity in kidney cells but not in hepatocytes, consistent with the role of GSH conjugation in Tri- and Perc-induced nephrotoxicity. In contrast, depletion of cellular GSH concentrations moderately decreased Tri- and Perc-induced cytotoxicity in kidney cells but increased cytotoxicity in hepatocytes, again pointing to the importance of different bioactivation pathways and modes of action in kidney and liver.

Keywords

Trichloroethylene; perchloroethylene; rat kidney cells; rat hepatocytes; cytochrome P450; glutathione conjugation; cytotoxicity

1. Introduction

Trichloroethylene (Tri)¹ and perchloroethylene (Perc) are colorless, odorless and lipid-soluble halogenated solvents that are used extensively in various industries. As a consequence of

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widespread use, Tri is the most commonly found chemical in groundwater at NPL sites in the U.S. and Tri and Perc are the most frequently found pair (Fay and Mumtaz, 1996). Both Tri and Perc are known animal carcinogens and limited evidence exists for their carcinogenicity in humans. The World Health Organization considers both chemicals to be “probably carcinogenic in humans” (IARC, 1995), and the U.S. Department of Health and Human Services lists each as “reasonably anticipated to be a human carcinogen” (NTP, 2002).

Tri has several potential tumor target organs, with the specific target varying between males and females, among species, and among different strains of a given species (Lash et al., 2006). Thus, whereas liver and lungs are the primary target organs in some strains of male mice, kidneys, Leydig cells, and the hematopoietic system are primary target organs in male rats. In humans, several potential target organs have been identified from epidemiology studies, and include kidneys, liver, cervix, and hematopoietic cells (i.e., non-Hodgkin's lymphoma) (Wartenberg et al., 2000). While there is compelling evidence that Tri exposure causes Leydig cell tumors in male rats (Cook et al., 1999; Forkert et al., 2002), the relevance of this to man is questionable.

As with Tri, Perc exhibits several potential tumor target organs that vary according to gender and species. Various cancer bioassays in laboratory animals identified liver tumors in male and female mice, kidney tumors and gliomas in male rats, and mononuclear cell leukemia in male and female rats from Perc exposure (Lash and Parker, 2001). Epidemiology studies in humans found significant increases in standardized incidence ratios for cervix, kidney, and non-Hodgkin's lymphoma, and significant increases in standardized mortality ratios for cervix, esophagus, urinary bladder, and non-Hodgkin's lymphoma (Lash and Parker, 2001), although not all studies found significance for all tissues. Based on the similarities in tumor target organs and chemical structure and properties, one might presume that both susceptibility and metabolism of Tri and Perc would be very similar. Such a conclusion, however, would be a serious mistake as significant differences are known in rates of metabolism and chemical reactivity of certain analogous metabolites (Lash and Parker, 2001). Additionally, intermediate precursors to these metabolites vary considerably.

In terms of metabolism, both Tri and Perc serve as substrates for cytochrome P450 (P450) and GSH *S*-transferases (GSTs). As illustrated in Figure 1, Tri and Perc yield many of the same metabolites from the P450 pathway (e.g., trichloroacetate (TCA), trichloroethanol and its glucuronide (TCOH and TCOG, respectively). Additionally, dichloroacetate (DCA) may be an important metabolite of Tri; its significance as a metabolite of Perc is unknown. Although P450 2E1 (CYP2E1) is the most active P450 enzyme for both compounds, Tri is a far better substrate than Perc (Lash and Parker, 2001; Lash et al., 2000a). Hence, the kinetics of formation of these common metabolites differ significantly between the two chemicals. For the GSH-dependent pathway, similar conjugate metabolites are generated, differing only by the presence of an additional chlorine in the Perc-derived metabolites. While fluxes of Tri and Perc through hepatic GST are similar, flux of Perc through renal GST is approximately 5-fold faster than that of Tri (Lash et al., 1998a). Perhaps the most critical difference between Tri and Perc with regards to the GSH-dependent pathway relates to the chemical reactivity of reactive intermediates derived from the two chemicals. Chemical reactivity of reactive intermediates

¹Abbreviations: Acivicin, L-(α S,5S)- α -amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid; BSO, L-buthionine-S,R-sulfoximine; CGase, cysteinylglycinase; CH, chloral hydrate; CLO, clofibrate; CLX, chlorzoxazone; β -lyase, cysteine conjugate β -lyase; DBP, 1,3-dibromopropane; DCA, dichloroacetate; DCVC, S-(1,2-dichlorovinyl)-L-cysteine; DCVG, S-(1,2-dichlorovinyl)glutathione; DEM, diethylmaleate; DITC, diethyldithiocarbamate; F344, Fischer 344; FMO, flavin-containing monooxygenase; GC, gas chromatography; GGT, γ -glutamyltransferase; GSH, glutathione; GST, GSH *S*-transferase; HPLC, high-performance liquid chromatography; IARC, International Agency for Research on Cancer; LDH, lactate dehydrogenase; Metyr, metyrapone; NTP, National Toxicology Program; P450, cytochrome P450; Perc, perchloroethylene (also known as tetrachloroethylene or tetrachloroethene); PYR, pyridine; TCA, trichloroacetate; TCOG, trichloroethanol glucuronide; TCOH, trichloroethanol; TCVC, S-(1,2,2-trichlorovinyl)-L-cysteine; TCVG, S-(1,2,2-trichlorovinyl)glutathione; Tri, trichloroethylene (also known as trichloroethene).

derived from cysteine conjugate β -lyase (β -lyase)- or flavin-containing monooxygenase (FMO)-dependent metabolism of the respective cysteine conjugates (*S*-1,2-dichlorovinyl-L-cysteine [DCVC] and *S*-(1,2,2-trichlorovinyl)-L-cysteine [TCVC] from Tri and Perc, respectively) differs markedly; reactive intermediates derived from TCVC are more reactive than those derived from DCVC (Birner et al., 1997; Lash and Parker, 2001). Accordingly, isolated rat proximal tubular cells exhibit more cell injury when incubated with *S*-(1,2,2-trichlorovinyl)glutathione (TCVG) than with *S*-(1,2-dichlorovinyl)glutathione (DCVG) at the same concentrations (Lash et al., 2001,2002).

The differences in kinetics of Tri and Perc metabolism by P450 and GST suggest that the distribution of common metabolites among the two pathways will differ. Important pathway differences in precursors or downstream products to these common Tri and Perc metabolites occur. Because both acute cellular injury and chronic injury (carcinogenesis) are dependent on formation of specific metabolites, which varies among different target tissues, these facts imply two things: First, the dose or concentration dependence of cell injury will differ between Tri and Perc, as indicated above; second, alterations in expression or activities of enzymes of the P450 or GST pathways will affect metabolism and toxicity of Tri and Perc differently. As noted above, the first implication has been demonstrated in various biological systems. The second implication, however, has not been directly tested. In the present study, therefore, we hypothesized that modulation of P450 activity or expression or alteration of cellular GSH status will differentially alter both the metabolism and toxicity of Tri and Perc. This hypothesis was tested using isolated cells and subcellular fractions from two prominent target tissues in rats for Tri and Perc, the kidneys and liver.

2. Materials and Methods

2.1. Experimental design

Several experiments involved modulation of P450 enzyme activity or expression or cellular GSH status prior to measurements of Tri and Perc metabolism by either the P450/oxidative pathway or the GSH conjugation pathway. Acute cytotoxicity after the various pretreatments was then assessed to determine the degree of correlation between metabolism and toxicity and to assess the interplay between the two metabolic pathways in determining the cellular response.

P450 activity was altered by preincubation of isolated cells or microsomes for 15 min with various inhibitors of P450 metabolism [0.25 mM metyrapone (Metyr), 0.25 mM SKF-525A, 1 mM diethyldithiocarbamate (DITC), 1 mM chlorzoxazone (CLX)]. These concentrations of P450 inhibitors and incubation times have been previously established to produce maximal inhibition of P450 activity without significant cytotoxicity (Cummings et al., 1999; Lash and Tokarz, 1995; Lash et al., 1994). CYP2E1, the primary P450 enzyme that metabolizes Tri (Lash et al., 2000a) and Perc (Lash and Parker, 2001), was induced by pretreatment of rats with pyridine (PYR; ip injection; 0.1 g/kg body weight per day $\times$ 3 days). Previous work (Cummings et al., 2001) showed that this treatment induced protein expression of rat hepatic CYP2E1 approximately 5-fold and rat renal CYP2E1 approximately 2-fold. This induction was associated with a 2.6-fold and 2.0-fold increase in Tri metabolism to CH in rat liver and kidney microsomes, respectively. Rats were also treated with the peroxisome proliferator clofibrate (CLO; ip injection; 0.2 g/kg body weight per day $\times$ 3 days). Previous work showed that this induced both rat hepatic and renal expression of CYP2B1/2 by approximately 4-fold (Cummings et al., 1999) and induced rat renal, but not hepatic, expression of CYP2E1 and CYP2C11 by 2.6- and 2-fold, respectively (Cummings et al., 2001). Besides CYP2E1, both CYP2B1/2 and CYP2C11 can metabolize Tri to CH (Lash et al., 2000a; Nakajima et al., 1992).

Renal and hepatic GSH concentrations were depleted in isolated renal cells or hepatocytes by 30-min pretreatments with either L-buthionine-S,R-sulfoximine (BSO; 2 mM) to inhibit GSH synthesis or diethylmaleate (DEM; 1 mM) to conjugate GSH. Intracellular content of GSH was increased in isolated cells by a 30-min preincubation with either 5 mM GSH (renal cells) or 5 mM methionine (hepatocytes).

2.2. Materials

Tri and Perc (each reported to be 99.9% pure, as judged by electron ionization mass spectrometry), collagenase type I and IV, bovine serum albumin (fraction V), L-(α S,5S)- α -amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid (acivicin), and L- γ -glutamyl-L-glutamate were all purchased from Sigma Chemical Co. (St. Louis, MO). DCVG was synthesized as previously described by Elfarra et al. (1986), using Tri and GSH in sodium metal and liquid ammonia. TCVG was synthesized by a procedure modified from that used for DCVG synthesis, using a methanol/liquid sodium solvent system (Dekant et al., 1987), longer reaction times and acidification of the crude product to prevent base-catalyzed degradation (Lash et al., 1998a, 2002). Purity for both DCVG and TCVG (> 95%) was determined by high-performance liquid chromatography (HPLC) analysis. Identities were confirmed by proton nuclear magnetic resonance spectroscopy. All other chemicals were of the highest purity available and were obtained from commercial sources.

2.3. Animals

Male F344 rats (200-300 g; Charles River Laboratories, Wilmington, MA) were used in these studies. They were housed in a temperature- and humidity-controlled room on a 12-hr light/dark cycle, and were given commercial rat chow and water *ad libitum*. All procedures were conducted in accordance with state and NIH guidelines and were approved by the institutional Animal Investigation Committee.

2.4. Preparation of isolated kidney cells

Suspensions of renal cortical cells were prepared by the method of Jones et al. (1979) as modified by Lash (1989). Prior to surgery, animals were anesthetized by ip injection of sodium pentobarbital (0.11 mg/100 g body weight). The aorta was cannulated below the renal arteries with a 19-gauge steel cannula, and kidneys were perfused *in situ* at 8 ml/min with calcium-free, EGTA-containing Hanks' buffer. After 10 min, the kidneys were perfused in a recirculating manner with Hanks' buffer supplemented with 4 mM CaCl₂ and collagenase type I (0.15%, w/v, approximately 300 units/mg) at 5 ml/min for 15-18 min. All buffers were continuously bubbled with 95% O₂/5% CO₂ and were maintained at 37°C. Cells were released into Krebs-Henseleit buffer supplemented with 25 mM HEPES, pH 7.4, 0.2% (w/v) bovine serum albumin, 2.5 mM CaCl₂, 5 mM glucose, and 5 mM glutamine. Cell concentrations were estimated by counting on a hemacytometer. Cell viability was estimated by determining the fraction of cells that excluded trypan blue (0.2%, w/v, in 0.9%, w/v, saline) on a hemacytometer or by determining the fraction of cells that released lactate dehydrogenase (LDH; see below). By either method, cell viability was > 85%.

2.5. Preparation of isolated hepatocytes

Suspensions of hepatocytes were prepared by the method of Moldéus et al. (1978). Prior to surgery, animals were anesthetized by ip injection of sodium pentobarbital (0.11 mg/100 g body weight). Briefly, two sutures were placed around the portal vein as far apart as possible. A third ligature was placed around the vena cava close to the kidneys. A 16-gauge steel cannula was inserted into the portal vein between the two sutures. The liver was perfused *in situ* with Hanks' buffer supplemented with 25 mM NaHCO₃, 25 mM HEPES, pH 7.4, 0.5 mM EGTA, and 0.2% (w/v) bovine serum albumin (Hanks' I) at a flow rate of 10 ml/min for 5-7 min. All

buffers were continuously bubbled with 95% O₂/5% CO₂ and maintained at 37°C. The liver was then perfused in a recirculating manner with Hanks' buffer supplemented with 4 mM CaCl₂ and collagenase type IV (0.1%, w/v, approximately 300 units/mg) (Hanks' II) at 5 ml/min for 10-12 min. Cells were released by gentle rubbing of the liver surface with a glass rod and resuspended in Krebs-Henseleit buffer, supplemented with 25 mM HEPES, pH 7.4, 0.2% (w/v) bovine serum albumin, and 2.5 mM CaCl₂. Cell concentrations and viability (> 90%) were determined as above.

2.6. Preparation of microsomes from rat kidney and liver homogenates

Renal and hepatic microsomes were isolated from homogenates as described by Sharer et al. (1992). Prior to surgery, animals were anesthetized by ip injection of sodium pentobarbital (0.11 mg/100 g body weight). Livers or kidneys were removed, rinsed with buffer (250 mM sucrose, 10 mM triethanolamine, 1 mM EDTA·Na₂, pH 7.6), and homogenized in 3 ml of buffer/g tissue. Homogenates were initially centrifuged at 15,000 g for 5 min. The post-mitochondrial supernatant was then centrifuged for 60 min at 105,000 g. The resulting pellets were resuspended in buffer and centrifuged an additional 60 min at 105,000 g to produce the "washed" microsomal fraction. Microsomal pellets were resuspended in buffer containing 10% (v/v) glycerol and were stored at -80°C until used. Enzymatic activities were normalized to protein concentrations, which were determined by a Coomassie blue G dye-binding assay (Read and Northcote, 1981) with bovine serum albumin as the standard. Purity of the microsomal fraction was estimated by measurement of marker enzymes for several subcellular compartments as described previously (Lash et al., 1995). Results indicated that cross-contamination of the microsomal fraction with cytoplasm was < 5%, and contamination with mitochondria, lysosomes, and plasma membranes was < 2%.

2.7. Incubations for assay of GSH conjugation of Tri and Perc in isolated kidney cells and hepatocytes

Incubations were performed in 25-ml polypropylene Erlenmeyer flasks on a Dubnoff metabolic shaking incubator (60 cycles/min) at 37°C. Flasks were sealed with rubber serum bottle stoppers to minimize evaporation of Tri or Perc, which are volatile halogenated solvents. Additions to and sampling from sealed flasks were made with glass syringes to maintain the sealed environment of the incubations. Isolated kidney cells were preincubated for 15 min with 0.25 mM acivicin to inhibit GGT activity before performing incubations to measure GSH conjugate formation. This preincubation irreversibly inhibits GSH or GSH *S*-conjugate degradation by > 95% (Lash, 1989; Lash and Jones, 1985). A similar preincubation of hepatocytes was unnecessary because they contain insignificant levels of GGT activity (Lash et al., 1998b). For some samples, cells were also preincubated with P450 inhibitors for 15 min. Cells were then incubated for 10 or 30 min with 5 mM GSH, 2 mM Tri or Perc (stock solutions made in acetone; final concentration < 1%, v/v), and 0.1% (v/v) Triton X-100 to solubilize the plasma membranes. This concentration of GSH was chosen, because it is the approximate normal, physiological concentration of GSH in renal and hepatic cells. Reactions were terminated by addition of 10% (w/v) perchloric acid, and samples were processed for analysis of DCVG or TCVG as described previously (Lash et al., 1995) using the HPLC method described below.

2.8. HPLC assay for GSH, DCVG, and TCVG

Perchloric acid extracts of kidney cells or hepatocytes, for either analysis of GSH content or for GSH conjugation of Tri or Perc, were derivatized with iodoacetic acid and 1-fluoro-2,4-dinitrobenzene as described previously (Lash et al., 1995). The *S*-carboxymethyl-*N*-dinitrophenyl derivative of GSH and the *N*-dinitrophenyl derivatives of either DCVG or TCVG were analyzed by the ion-exchange HPLC method of Fariss and Reed (1987). Analyte content

in samples was quantitated with respect to authentic standards and was adjusted according to the content of added L- γ -glutamyl-L-glutamate as an HPLC internal standard. The limit of detection for GSH, DCVG, and TCVG derivatives was 50 pmol, and efficiency of derivatization was estimated to be 85 to 110%, with a correction made for this variation by use of the internal standard. A typical set of retention times (R_f values) was 17.5 min for *N*-dinitrophenyl-DCVG, 19.0 min for *N*-dinitrophenyl-TCVG, 27.5 min for *N*-dinitrophenyl-L- γ -glutamyl-L-glutamate (internal standard), 36.0 min for *S*-carboxymethyl-*N*-nitrophenyl-GSH, and 42.5 min for *N,N*-bisdinitrophenyl-glutathione disulfide. Actual R_f values and resolution varied according to the age of the amine column.

Besides comparison of retention times with those of the derivatives of authentic DCVG or TCVG, identity of peaks was also confirmed as done previously (Lash and Jones, 1985). This involved a 15-min treatment of selected samples with 1 unit of a partially purified preparation of GGT that also contains dipeptidase activity; as a consequence of such treatment, GSH conjugates were converted to the corresponding cysteine conjugates, whose *N*-dinitrophenyl derivatives are also detected by this HPLC method (Lash and Anders, 1989).

2.9. GC assay for oxidative metabolites of Tri and Perc

P450- and other oxidatively-derived metabolites of Tri and Perc were measured by a gas chromatography (GC) assay essentially as described previously (Cummings et al., 2001;Elfarra et al., 1998). All incubations were carried out in 1-ml glass vials at 37°C for 30 min. Microsomes from kidney or liver homogenates (1 mg protein/ml) were resuspended in 0.5-1.0 ml of 50 mM Tris-HCl buffer, pH 7.4. For experiments testing effects of P450 inhibitors, reaction mixtures were preincubated for 15 min with the inhibitor. After incubating with Tri or Perc for 3 min at 37°C, 1 mM NADPH was added to initiate the reaction. Reactions were allowed to proceed for 15 min, stopped by flash-freezing in a dry ice-acetone bath and thawed. One μ l of 1,3-dibromopropane (DBP; Aldrich Chemicals, Milwaukee, WI; diluted 1000-fold in 0.5% (v/v) aqueous acetonitrile) was added as an internal standard. Samples were then extracted with 0.25 ml ethyl acetate. Sample analysis was carried out with a Perkin-Elmer Autosystem XL gas chromatograph fitted with a PE-210 30 m $\times$ 0.25 mm ID, 0.5 μ m thickness column (Perkin-Elmer, Norwalk, CT) and an electron capture detector. Metabolites were analyzed by injection of ethyl acetate extracts into a split injector set at 200°C, with a detector temperature of 300°C and a He flow rate of 24.8 cm/sec. The initial oven temperature of 35°C was maintained for 11 min. Temperature was then increased at 10°/min to 120°C, which was held for 19 min. R_f values for DCA, Tri, CH, TCOH, DBP, TCA, and Perc were approximately 2.5, 3.9, 6, 14, 16, 19, and 22 min, respectively. Assay limits of detection for DCA, Tri, CH, TCOH, TCA, and Perc were 3, 0.4, 0.1, 0.1, 3, and 0.4 fmol/mg protein, respectively. Control incubations, performed in the absence of NADPH or substrate, exhibited no detectable formation of P450-derived metabolites, as shown previously (Cummings et al., 2001;Lash et al., 1995,1998a,1998b,1999).

2.10. LDH release assay for cytotoxicity

Isolated renal cortical cells or hepatocytes (2×10^6 cells/ml) were incubated in 25-ml polypropylene Erlenmeyer flasks with the indicated additions and concentrations of Tri or Perc for up to 3 hr at 37°C in a Dubnoff metabolic shaking incubator (60 cycles/min). Flasks were sealed with a rubber serum bottle stopper, and the atmosphere in each flask was equilibrated with 95% O₂/5% CO₂. Cell viability was determined by measurement of lactate dehydrogenase (LDH) release (Lash, 1989). Solvent vehicle for Tri and Perc (acetone, 1%, v/v) was added to control cells, and those cells exhibited no differences in LDH release from cells incubated only with Krebs-Henseleit buffer.

2.11. Data analysis

All values are means $\pm$ SE of measurements made of the indicated number of cell or microsomal preparations. Significant differences between means were first assessed by oneway analysis of variance. When significant *F* values were obtained with the analysis of variance, Fisher's protected least-significance *t*-test was performed to determine which means were significantly different from one another, with two-tail probabilities < 0.05 considered statistically significant.

3. Results

3.1. Modulation of renal and hepatic P450- and GSH-dependent metabolism of Tri and Perc

To investigate the influence of alterations in P450 activity on metabolism and toxicity of Tri and Perc, we first induced P450s by pretreatment of rats with either CLO or PYR, and then isolated microsomes from both kidneys and liver to measure P450-dependent metabolism of Tri or Perc (Table 1). Additionally, kidney and liver microsomes from either untreated rats or CLO-treated rats were incubated with either SKF-525A, a non-selective P450 inhibitor, or CLX, a CYP2E1 substrate and competitive inhibitor.

P450-dependent metabolism of Tri was 26.2-fold greater in liver microsomes than in kidney microsomes (Table 1; **left two data columns**). Neither SKF-525A nor CLX significantly affected renal Tri metabolism. Both compounds, however, significantly inhibited P450-dependent metabolism of Tri in liver microsomes by $> 50\%$. CLO induction of P450s increased both renal and hepatic metabolism of Tri by 2.1- and 1.6-fold, respectively. Pretreatment of microsomes isolated from CLO-treated rats with SKF-525A inhibited the increase in both renal and hepatic metabolism of Tri. In contrast, pretreatment of microsomes isolated from CLO-treated rats with CLX only inhibited the increase in hepatic metabolism of Tri. P450-dependent metabolism of Tri in microsomes from PYR-induced rats was increased 4.1-fold in kidney, but was not altered in the liver.

As with Tri, P450-dependent metabolism of Perc was 31.9-fold greater in liver microsomes than in kidney microsomes (Table 1; **right two data columns**). P450-dependent metabolism of Perc was 52.9% and 64.6% of that of Tri in kidney and liver microsomes, respectively. Unlike results with Tri, pretreatment of microsomes with SKF-525A had no effect on Perc metabolism in either tissue, whereas CLX only slightly inhibited Perc metabolism in liver microsomes. CLO induction of P450s increased renal and hepatic Perc metabolism by 2.4- and 1.9-fold, respectively, similar to its effects on Tri metabolism. SKF-525A slightly inhibited the CLO-dependent increase in renal Perc metabolism but had no effect on hepatic Perc metabolism. CLX treatment of microsomes from CLO-induced rats had the opposite effect on P450-dependent metabolism of Perc, moderately decreasing hepatic and having no effect on renal metabolism. PYR pretreatment of rats produced similar effects on P450-dependent metabolism of Perc as it had on Tri; renal metabolism of Perc was increased 2.6-fold, and hepatic metabolism of Perc was unaffected.

We next explored the effects of modulation of P450 activity on GSH conjugation of Tri and Perc (Figure 2). In isolated kidney cells and hepatocytes from untreated rats, DCVG formation from Tri and TCVG formation from Perc were approximately 8-fold greater at both time points in hepatocytes than in kidney cells. As shown previously (Lash et al., 1998a, 1998b), GSH conjugation of Perc was modestly higher than GSH conjugation of Tri in isolated cells from both tissues. Isolated rat kidney cells and hepatocytes were then preincubated with either of two non-specific P450 inhibitors (Metyr, SKF-525A) or a selective CYP2E1 inhibitor (DITC). In kidney cells, DCVG formation was significantly stimulated by all three inhibitors by 25% to 50%, although stimulation by the two non-specific inhibitors was greater than that by DITC

(Figure 2A). In kidney cells, Perc metabolism was less responsive to the P450 inhibitors; it was significantly but slightly stimulated by the two non-specific inhibitors but was not affected by DITC. In hepatocytes, DCVG formation was only stimulated by the two non-specific inhibitors, and TCVG formation was only stimulated by SKF-525A (Figure 2B).

As a prelude to examining the influence of modulation of cellular GSH status on Tri- and Perc-induced cytotoxicity, the influence of three compounds on GSH concentrations in isolated kidney cells and hepatocytes was determined (Figure 3). To decrease cellular GSH concentrations, isolated kidney cells and hepatocytes were incubated with either BSO or DEM, which decreased GSH concentrations by 60% to 80% in both cell types. To increase cellular GSH concentrations, isolated kidney cells were incubated with 5 mM GSH and hepatocytes were incubated with 5 mM Met. These incubations resulted in > 100% increase in GSH concentration in isolated kidney cells and approximately a 50% increase in that of hepatocytes.

3.2. Effect of modulation of P450 status on Tri- and Perc-induced cytotoxicity in isolated kidney cells and hepatocytes

Incubation of isolated kidney cells for 3 hr with either 2 mM or 10 mM Tri produced a moderate degree of acute cytotoxicity, increasing LDH release to approximately 35% and 50%, respectively (Figure 4A and 4B). In contrast, Perc was a more potent, acute cytotoxic agent in isolated kidney cells, increasing LDH release to approximately 50% and 75% after 3-hr incubations with 2 mM and 10 mM Perc, respectively (Figure 4C and 4D). Pretreatment of isolated kidney cells with Metyr or SKF-525A had no effects on Tri-induced LDH release, whereas the CYP2E1-selective inhibitor DITC slightly increased LDH release (Figure 4A and 4B). This contrasts with the metabolism data, which showed DITC to be a less potent stimulator of DCVG formation than the non-selective P450 inhibitors. Pretreatment of isolated kidney cells with any of the three P450 inhibitors had no effect on Perc-induced LDH release (Figure 4C and 4D), consistent with the relatively low rate of renal P450-dependent metabolism of Perc. Examination of the influence of PYR induction of P450 on Tri- and Perc-induced cytotoxicity in isolated kidney cells also highlights the differences between the two chemicals (Figure 4E). Tri-induced LDH release in isolated kidney cells was moderately increased from PYR-induced as compared to control rats, whereas Perc-induced LDH release in isolated kidney cells was either unchanged (2 mM) or slightly reduced (10 mM).

In contrast with its modest cytotoxicity in isolated kidney cells, incubation of isolated hepatocytes for 3 hr with 2 mM or 10 mM Tri had no effect on LDH release (Figure 5A and 5B). Incubation of isolated hepatocytes for 3 hr with 2 mM or 10 mM Perc produced a moderate to high degree of cytotoxicity (Figure 5C and 5D), similar to the extent of LDH release produced in kidney cells. Also in contrast with the results in isolated kidney cells, pretreatment of hepatocytes with either Metyr or SKF-525A significantly increased Tri-induced LDH release at both 2 mM and 10 mM concentrations of Tri and significantly, albeit slightly, increased Perc-induced LDH release only at the 2 mM concentration of Perc. Pretreatment with DITC had no effect on cytotoxicity of Tri but slightly decreased Perc-induced LDH release at the 10 mM concentration of Perc. In general, however, P450 inhibitors exhibited little effect on the cytotoxicity of either concentration of Perc in isolated hepatocytes. Incubation of isolated hepatocytes from PYR-pretreated rats exhibited increased cytotoxicity from Tri but slightly decreased cytotoxicity from Perc (Figure 5E). The results with Tri are consistent with a significant role for CYP2E1 in Tri metabolism. The results with Perc, however, are somewhat surprising, although they are consistent with the lower rates of CYP2E1-dependent, and overall P450-dependent metabolism of Perc as compared to those with Tri as substrate.

3.3. Effect of modulation of GSH status on Tri- and Perc-induced cytotoxicity in isolated kidney cells and hepatocytes

Pretreatment of isolated rat kidney cells with 5 mM GSH to increase renal cellular levels of GSH markedly increased LDH release induced by 2 mM Tri (Figure 6A) but had no effect on that induced by 10 mM Tri (Figure 6B). Conversely, the only effect on Tri-induced LDH release of pretreatment of rat kidney cells with either BSO or DEM, to deplete renal cellular levels of GSH, was to slightly decrease LDH release caused by 10 mM Tri. Perc-induced LDH release in isolated kidney cells was similarly increased at the 2 mM concentration of Perc by preincubation with GSH but was similarly not affected by either BSO or DEM pretreatment (Figure 6C and 6D).

Results of effects of modulation of the GSH concentration in isolated hepatocytes differed markedly from the patterns observed in isolated kidney cells (Figure 7). Increase of hepatocyte GSH concentrations by preincubation with 5 mM Met had no effect on Tri-induced LDH release (Figure 7A and 7B) but markedly decreased Perc-induced LDH release at both 2 mM and 10 mM Perc (Figure 7C and 7D). Also in contrast to results in isolated kidney cells, decreases in hepatocyte GSH concentrations by preincubation with either BSO or DEM significantly increased LDH release induced by both Tri and Perc.

4. Discussion

The overall hypothesis that was tested in the present study was that alterations in cellular GSH status or expression or activities of enzymes of the P450 pathway will affect metabolism and toxicity of Tri and Perc differently. The foundation for this hypothesis is based on both metabolism and toxicity data, showing that while many identities and similarities exist between the two chemicals, significant differences are also present. A key question is whether these differences are of pharmacological or toxicological importance. To address this question, P450 and GSH status were modulated and influences on P450- or GSH-dependent metabolism and acute cytotoxicity were determined. P450 activity was modulated by the use of non-selective P450 inhibitors (SKF-525A, Metyr), CYP2E1-selective inhibitors (DITC, CLX), or P450 inducers (PYR, CLO). Cellular GSH concentrations were decreased by use of either a GSH-synthesis inhibitor (BSO) or an electrophilic GST substrate (DEM), and were increased by preincubation of kidney or liver cells with GSH or Met, respectively. The concept of two alternate bioactivation pathways interacting is not a novel concept, and has been considered before for both Tri (Lash et al., 1999) and Perc (Dekant et al., 1987; Green et al., 1990).

For both Tri and Perc, P450-dependent metabolism was markedly higher in isolated hepatocytes than in isolated kidney cells, and the rate of metabolism was faster with Tri than with Perc as substrate (cf. Table 1), which are as expected. While many of the responses to pretreatments or inducers were similar between the two chemicals and between the two tissues for a given chemical, some responses differed. For example, SKF-525A only inhibited hepatic Tri metabolism but had no effects on either renal Tri or hepatic or renal Perc metabolism. CLX also only inhibited hepatic and not renal Tri metabolism but did significantly inhibit hepatic Perc metabolism, although the extent of inhibition was much less than that for Tri. The various effects on P450-dependent metabolism are consistent with Tri and Perc both being metabolized by CYP2E1 (Guengerich et al., 1991; Halmes et al., 1997; Lipscomb et al., 1997), although other P450 enzymes, in particular the CYP2B family and possibly the CYP3A family, play important roles for Perc (Costa and Invanetich, 1980; Hanioka et al., 1995). The lack of an effect of PYR pretreatment on hepatic metabolism of Tri suggests that either other P450 enzymes besides CYP2E1 are important in Tri metabolism and/or that PYR influences other P450 enzymes that may result in decreased hepatic metabolism of Tri, thereby balancing the increase in CYP2E1 expression.

When considering toxic effects of Tri and Perc, human health risk assessment is typically concerned with the chronic, low-dose exposures that can occur in the environment. These types of exposures are difficult to model in an *in vitro* system. Hence, we have used acute exposures at relatively high toxicant concentrations to derive information about relative susceptibility and selected mechanistic aspects. Although the studies presented in this paper were conducted in cells and subcellular fractions from rat liver and kidney, they have some relevance for humans in terms of basic mechanisms and principles. Additionally, the concentrations of Tri and Perc used in the present study, while needed to enable quantitation of metabolism and to elicit acute cytotoxicity responses, are much higher (three- to five-orders of magnitude or more) than those to which humans are exposed either occupationally or environmentally. There are significant species-dependent differences that are both quantitative and qualitative (Lash et al., 1995,1998a,1998b,1999). Hence, the current studies should be considered as a model system and any direct extrapolation to humans needs to be done carefully.

We have consistently found Perc to be more potent than Tri as an acute cytotoxicant in both isolated kidney cells (cf. Figure 4) and isolated hepatocytes (cf. Figure 5) (also see Lash et al., 1995,2001,2002). Depending on what parameter is being measured, however, other patterns may be observed. For example, Toraason et al. (1999) exposed F344 rats to Tri and Perc by gavage and observed various indices of oxidative stress, including those for lipid peroxidation and DNA damage. They found that Tri, but not Perc, caused an increase in oxidative DNA damage in rat liver. Taking into account that Tri is a significantly better substrate for P450s than Perc and that Perc is a better substrate for GSTs than Tri, the overall higher susceptibility of renal cells and hepatocytes to acute cytotoxicity from Perc is consistent with a GST-derived metabolite of Perc playing a significant role in cytotoxicity. A more detailed understanding of the basis for the differences in susceptibility to these two chemicals will require additional studies.

Schemes summarizing the experimental design and results of this study (Figures 8 and 9) highlight both the similarities and differences between Tri and Perc and between their handling in liver and kidney cells. An important factor in the tissue-specificity of responses to these solvents is that it is the P450-derived metabolites that are associated with hepatic injury and the GST-derived metabolites that are associated with renal injury. Even though most of the GSH conjugation reaction for Tri and Perc occurs in the liver when these pathways are studied *in vivo* (Lash et al., 2000a) and a significant amount of GSH conjugate will be formed in the isolated hepatocytes or other *in vitro* models (Lash et al., 1995,1998a,1998b,1999,2006), this pathway is not toxicologically relevant for the liver. Rather, GSH conjugate is efficiently exported to the blood or bile and either goes to the kidneys as the GSH conjugate (hepato-renal circulation) or is degraded in the bile to the cysteine conjugate, returned to the liver and then exported to the kidneys via the blood (entero-hepatic-renal circulation).

As noted above, one factor in the different responses to Tri and Perc is the greater activity of Tri as compared with Perc as a P450 substrate and the moderately higher activity of Perc as compared with Tri as a GST substrate. CYP2E1 plays a greater role in Tri metabolism than in Perc metabolism. Accordingly, DITC, a selective CYP2E1 inhibitor, decreased Tri-induced hepatotoxicity but actually slightly increased Perc-induced hepatotoxicity. Whereas several of the agents used to modulate P450 activity influenced GST-dependent metabolism and nephrotoxicity of Tri, most of the agents were without effect on GST-dependent metabolism or nephrotoxicity of Perc. The markedly different responses of PYR-pretreated renal cells (cf. Figure 4E) and hepatocytes (cf. Figure 5E) to Tri and Perc also highlight the different roles of P450-dependent metabolism in their cytotoxicity.

The influence of modulating cellular GSH status illustrates the tissue-dependent differences even more clearly. As far as tissue specificity of responses, alterations in GSH status have

complex effects on potential nephrotoxicity. On the one hand, increases in renal GSH concentrations provide more co-substrate for GST, which enhances formation of DCVG and subsequent nephrotoxicity. Although the same mechanism should occur for Perc metabolism to TCVG, no significant effects were observed in either hepatocytes or kidney cells. On the other hand, reactive metabolites generated from the subsequent renal metabolism of these conjugates can lead to oxidative stress (Lash and Parker, 2001; Lash et al., 2000b). With oxidative stress, in contrast, decreases in renal cellular concentrations of GSH may exacerbate cytotoxicity whereas increases in renal cellular concentrations of GSH may protect. In the kidneys, therefore, there must exist some balance between utilization of GSH for Tri or Perc bioactivation and for detoxication of reactive electrophiles or oxygen species.

In contrast to this scenario in the kidney cells, the available data from the present and other studies suggest that in isolated hepatocytes, GSH functions to protect the cells from any oxidative stress induced by metabolites of Tri or Perc. Moreover, although GST activity is quite prominent in the isolated hepatocytes, alterations in GSH content do not seem to significantly alter the proportion of Tri or Perc undergoing P450-dependent metabolism (Lash et al., 1999). A likely explanation is that the affinity of P450s for Tri and Perc is much higher than that of GSTs, and there is considerably higher P450 activity in hepatocytes than in kidney cells.

In summary, the present study has demonstrated that modulation of P450 or GSH status in either rat kidney or liver cells significantly affects metabolism and acute cytotoxicity of Tri and Perc. Differential effects are based, at least in part, on kinetic differences in P450s and GSTs for Tri and Perc as substrates, and on chemical reactivities of metabolites derived from the two chemicals. One important implication of these findings is that individuals with differences in P450 or GSH status (possibly as a consequence of genetic polymorphisms or exposures to other drugs or chemicals that are P450 or GST substrates), may handle Tri and Perc differently and exhibit distinct sensitivities to injury from these compounds. Such differences are likely to be important primarily at higher doses of Tri and Perc; their influence at low, environmental doses, however, is uncertain and requires further study.

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Figure 1. Scheme for oxidative and conjugative metabolism of Tri and Perc

Summary scheme for key metabolites formed during renal and hepatic metabolism of Tri and Perc by P450 and GSH conjugation. Tri or Perc can be metabolized by either P450s or GSTs. The former initially yields either chloral or epoxides, which are subsequently converted to yield TCA, TCOH, and TCOG as major metabolites. Although this oxidative pathway is much more active in liver, it can occur in kidney as well. GSH conjugation occurs primarily in the liver, but can occur to a significant extent in kidney too. Subsequent processing of the GSH conjugate occurs primarily in the kidneys and generates reactive, nephrotoxic species. Abbreviations: β -lyase; cysteine conjugate β -lyase; CGase, cysteinylglycinase; DCVC, *S*-(1,2-dichlorovinyl)-L-cysteine; DCVG, *S*-(1,2-dichlorovinyl)glutathione; FMO, flavin-containing

monooxygenase; GGT, γ -glutamyltransferase; GST, GSH *S*-transferase; Perc, perchloroethylene; P450, cytochrome P450; TCA, trichloroacetate; TCOH, trichloroethanol; TCOG, trichloroethanol glucuronide; TCVC, *S*-(1,2,2-trichlorovinyl)-L-cysteine; TCVG, *S*-(1,2,2-trichlorovinyl)glutathione; Tri, trichloroethylene.

Figure 2. Effect of P450 inhibitors on DCVG and TCVG formation by GSH conjugation of Tri and Perc, respectively, in isolated rat kidney cells (A) and isolated rat hepatocytes (B) from male F344 rats

Isolated kidney cells or hepatocytes (1×10^6 cells/ml) were preincubated for 15 min with either buffer (= Control), 1 mM DITC, 0.25 mM Metyr, or 0.25 mM SKF-525A. Kidney cells were also preincubated for 15 min with 0.25 mM acivicin to inhibit further metabolism of GSH *S*-conjugates. Cells were then incubated with 5 mM GSH and either 2 mM Tri or 2 mM Perc in the presence of 0.1% (v/v) Triton X-100. After 10 or 30 min of incubation, aliquots of samples were processed for analysis of DCVG or TCVG formation by HPLC. Results are means $\pm$ SE of measurements from 4-6 separate cell preparations. *Significantly different ($P < 0.05$) from the corresponding control sample.

Figure 3. Modulation of GSH concentrations in isolated rat kidney cells and hepatocytes

Total cellular GSH concentrations in isolated rat kidney cells and hepatocytes were modulated by preincubating cells (1×10^6 cells/ml) for 30 min at 37°C with either buffer (= Control), 2 mM BSO, 1 mM DEM, or either 5 mM GSH (kidney cells) or 5 mM methionine (hepatocytes). GSH contents of perchloric acid extracts of cells were determined by HPLC as described in the Materials and Methods. To enable direct comparisons of responses in the two cell types, results are expressed as percent control (means $\pm$ SE), with control values in kidney cells being 14.2 ± 1.1 nmol/ 10^6 cells (mean $\pm$ SE, $n = 6$) and control values in hepatocytes being 24.8 ± 1.2 nmol/ 10^6 cells (mean $\pm$ SE, $n = 6$). *Significantly different ($P < 0.05$) from the corresponding control cells.

Figure 4. Effect of modulation of P450 status on Tri- and Perc-induced cytotoxicity in isolated kidney cells from male F344 rats

Isolated kidney cells (1×10^6 cells/ml) were preincubated with either medium (= Control) or 0.25 mM Metyr, 0.25 mM SKF-525A, or 1 mM DITC. At the end of the preincubation period, cells were incubated for 1, 2, or 3 hr with either media (= Control), 2 mM Tri (A), 10 mM Tri (B), 2 mM Perc (C), or 10 mM Perc (D). Cells, isolated from PYR-treated rats (E), were incubated with 2 mM or 10 mM Tri or Perc. At indicated times, cell viability was determined by LDH release. Data, expressed as the percent LDH release, are shown for 3-hr incubations and are means $\pm$ SE of measurements from 3-8 separate cell preparations. *Statistically significant ($P < 0.05$) from corresponding control samples. Statistics for panels A-D: †Statistically significant ($P < 0.05$) from corresponding sample with Tri or Perc alone. Statistics for panel E: *Statistically significant ($P < 0.05$) from corresponding control samples. †Statistically significant ($P < 0.05$) from the corresponding sample from non-PYR treated rats.

Figure 5. Effect of modulation of P450 status on Tri- and Perc-induced cytotoxicity in isolated hepatocytes from male F344 rats

Isolated hepatocytes (1×10^6 cells/ml) were preincubated with either medium (= Control) or 0.25 mM Metyr, 0.25 mM SKF-525A, or 1 mM DITC. At the end of the preincubation period, cells were incubated for 1, 2, or 3 hr with either media (= Control), 2 mM Tri (A), 10 mM Tri (B), 2 mM Perc (C), or 10 mM Perc (D). Cells, isolated from PYR-treated rats (E), were incubated with 2 mM or 10 mM Tri or Perc. At indicated times, cell viability was determined by LDH release. Data, expressed as the percent LDH release, are shown for 3-hr incubations and are means $\pm$ SE of measurements from 3-8 separate cell preparations. Statistics for panels A-D: *Statistically significant ($P < 0.05$) from corresponding control samples. †Statistically significant ($P < 0.05$) from corresponding sample with Tri or Perc alone. Statistics for panel E: *Statistically significant ($P < 0.05$) from corresponding control samples. †Statistically significant ($P < 0.05$) from the corresponding sample from non-PYR treated rats.

Figure 6. Effect of modulation of GSH status on Tri- and Perc-induced cytotoxicity in isolated kidney cells from male F344 rats

Isolated kidney cells (1×10^6 cells/ml) were preincubated for 30 min with either medium (= Control) or 2 mM BSO, 1 mM DEM, or 5 mM GSH. At the end of the preincubation period, cells were incubated for 1, 2, or 3 hr with either media (= Control), 2 mM Tri (A), 10 mM Tri (B), 2 mM Perc (C), or 10 mM Perc (D). At indicated times, cell viability was determined by LDH release. Data, expressed as the percent LDH release, are shown for 3-hr incubations and are means $\pm$ SE of measurements from 3-6 separate cell preparations. *Statistically significant ($P < 0.05$) from corresponding control samples. †Statistically significant ($P < 0.05$) from corresponding sample with Tri or Perc alone.

Figure 7. Effect of modulation of GSH status on Tri- and Perc-induced cytotoxicity in isolated hepatocytes from male F344 rats

Isolated hepatocytes (1×10^6 cells/ml) were preincubated for 30 min with either medium (= Control) or 2 mM BSO, 1 mM DEM, or 5 mM Met. At the end of the preincubation period, cells were incubated for 1, 2, or 3 hr with either media (= Control), 2 mM Tri (A), 10 mM Tri (B), 2 mM Perc (C), or 10 mM Perc (D). At indicated times, cell viability was determined by LDH release. Data, expressed as the percent LDH release, are shown for 3-hr incubations and are means $\pm$ SE of measurements from 3-6 separate cell preparations. *Statistically significant ($P < 0.05$) from corresponding control samples. †Statistically significant ($P < 0.05$) from corresponding sample with Tri or Perc alone.

Figure 8. Summary scheme showing effects of modulation of P450 and GSH status on metabolism and acute cytotoxicity of Tri

The figure summarizes effects of P450 inhibitors (both the non-specific inhibitors Metyr and SKF-525A and the CYP2E1-selective inhibitors CHX and DITC), P450 inducers (PYR, CLO), GSH depletors (BSO, DEM), and intracellular GSH enhancers (GSH for kidney cells and Met for hepatocytes) on metabolism and acute cytotoxicity of Tri. + or -, increase or decrease, respectively, in activity, pathway flux, or response.

Figure 9. Summary scheme showing effects of modulation of P450 and GSH status on metabolism and acute cytotoxicity of Perc

The figure summarizes effects of P450 inhibitors and inducers and modulators of GSH status on metabolism and acute cytotoxicity of Perc. Modulators and effects are as in Fig. 8.

Table 1

Modulation of P450-dependent metabolism of Tri and Perc in rat kidney and liver microsomes.

Microsomes from rat kidney and liver (1 mg protein/ml), isolated from either untreated rats or rats treated for 3 days with either CLO (0.2 g ip/kg body weight) or PYR (0.1 g ip/kg body weight), were preincubated for 15 min at 37°C with either buffer, 0.25 mM SKF-525A, or 1 mM CLX, preincubated for an additional 3 min at 37°C with 2 mM Tri or 2 mM Perc, and then incubated for 15 min at 37°C with 1 mM NADPH to initiate the reaction. Samples were then processed for analysis of P450-derived metabolites by GC. Results are means $\pm$ SE of measurements from 3-5 separate microsomal preparations.

Addition	P450-derived metabolites (nmol/15 min per mg protein)			
	Tri		Perc	
	Kidney	Liver	Kidney	Liver
Substrate	0.068 $\pm$ 0.027	1.78 $\pm$ 0.18	0.036 $\pm$ 0.004	1.15 $\pm$ 0.08
+ SKF-525A	0.066 $\pm$ 0.009	0.75 $\pm$ 0.05*	0.042 $\pm$ 0.002	1.04 $\pm$ 0.12
+ CLX	0.092 $\pm$ 0.033	0.81 $\pm$ 0.06*	0.044 $\pm$ 0.003	0.97 $\pm$ 0.04*
+ CLO	0.142 $\pm$ 0.021*	2.84 $\pm$ 0.16*	0.088 $\pm$ 0.006*	2.14 $\pm$ 0.13*
+ CLO + SKF-525A	0.060 $\pm$ 0.005 [†]	0.82 $\pm$ 0.07,* [†]	0.075 $\pm$ 0.005,* [†]	1.95 $\pm$ 0.09*
+ CLO + CLX	0.187 $\pm$ 0.022,* [†]	0.68 $\pm$ 0.05,* [†]	0.087 $\pm$ 0.012*	1.54 $\pm$ 0.10,* [†]
+ PYR	0.278 $\pm$ 0.019*	1.83 $\pm$ 0.10	0.094 $\pm$ 0.007*	1.22 $\pm$ 0.11

* Significantly different ($P < 0.05$) from incubations with the corresponding substrate alone.

[†] Significantly different ($P < 0.05$) from incubations with substrate from CLO-pretreated rats.