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Globin Monoadducts and Cross-Links Provide Evidence for the Presence of S-(1,2-Dichlorovinyl)-L-cysteine Sulfoxide, Chlorothioketene, and 2-Chlorothionoacetyl Chloride in the Circulation in Rats Administered S-(1,2-Dichlorovinyl)-L-cysteine

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Abstract

S-(1,2-Dichlorovinyl)-L-cysteine (DCVC), a mutagenic and nephrotoxic metabolite of trichloroethylene is bioactivated to S-(1,2-dichlorovinyl)-L-cysteine sulfoxide (DCVCS) and chlorothioketene and/or 2-chlorothionoacetyl chloride by cysteine conjugate S-oxidase (S-oxidase) and cysteine conjugate β -lyase (β -lyase), respectively. Previously, we identified DCVCS-globin monoadducts and cross-links upon treating rats with DCVCS or incubating erythrocytes with DCVCS. In this study, formation of DCVC-derived reactive intermediates was investigated after rats were given a single (230 or 460 $\mu\text{mol/kg}$, i.p.) or multiple (3 or 30 $\mu\text{mol/kg}$ daily for 5 days) DCVC doses. LC/ESI/MS of trypsin digested globin peptides revealed both S-oxidase and β -lyase-derived globin monoadducts and cross-links consistent with in vivo DCVC bioactivation by both pathways. MS/MS analyses of trypsin-digested fractions of globin from one of the rats treated with multiple 30 $\mu\text{mol/kg}$ DCVC doses led to identification of β -lyase-derived monoadducts on both Cys93 and Cys125 of the β chains. While rats dosed with the 230 $\mu\text{mol/kg}$ DCVC dose exhibited β -lyase-dependent monoadducts and cross-links only (4 out of 4 rats), rats given the 460 $\mu\text{mol/kg}$ DCVC dose (2 out of 4), and rats administered the multiple DCVC doses (2 out of 4) exhibited both β -lyase and S-oxidase-derived monoadducts and cross-links. Since previous incubations of erythrocytes with DCVC did not result in detection of DCVCS-derived monoadducts or cross-links and had only resulted in detection of β -lyase-derived monoadducts and cross-links, the DCVCS-globin monoadducts and cross-links detected in this study are likely the result of DCVC bioactivation outside the circulation and subsequent translocation of DCVCS and N-acetylated DCVCS into the erythrocytes.

Introduction

Hb adducts are used for biomonitoring of toxicant exposure in human epidemiological studies (1–4) and to elucidate the relative contributions of different metabolic pathways in cases where a toxicant can yield multiple reactive metabolites (5–7). Long life span of erythrocytes (corresponds to turnover of Hb; 120 days for humans, 63 days for rats) and stability of adducts allow detection of short-lived reactive metabolites present in the circulation at low levels of toxicant exposure over an extended period of time. Hb interaction with reactive metabolites can also provide insight into the interactions of reactive metabolites with other proteins in target tissues of toxicity.

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To this end, we aimed to gain insight into the *in vivo* bioactivation of S-(1,2-dichlorovinyl)-L-cysteine (DCVC)¹, the cysteine conjugate metabolite of trichloroethylene (TCE), by studying Hb adducts resulting from DCVC bioactivation. TCE, an occupational hazard and environmental contaminant, is listed in the Eleventh Report on Carcinogens as “reasonably anticipated to be a human carcinogen” (8). Renal-cell carcinomas from workers exposed to TCE exhibited mutation in the *von Hippel-Landau* tumor suppressor gene. GSH-dependent metabolism of TCE results in initial formation of S-(1,2-dichlorovinyl)-L-glutathione (DCVG) which can be metabolized to yield DCVC. After human exposure to TCE, DCVG was detected in blood within 30 min and its presence in blood persisted for up to 12 h (9). The evidence for DCVC formation in humans stems from measurements of the urinary mercapturate, N-acetyl-S-(1,2-dichlorovinyl)-L-cysteine (NA-DCVC). Excretion of NA-DCVC has been detected in the urine over 48 h after a 6 h human exposure to TCE (10).

Formation of DCVC is widely believed to be contributing to the nephrotoxicity and carcinogenicity of TCE (9–12). Bioactivation of DCVC via the cysteine conjugate β -lyase (β -lyase) pathway is believed to play a major role in the nephrotoxicity and carcinogenicity of TCE (12–14). β -Lyases are pyridoxal 5'-phosphate-dependent enzymes that catalyze β -elimination reactions (15). β -Elimination reaction with DCVC results in generation of pyruvate, ammonia, and an electrophilic reactive sulfur-containing fragment, 1,2-dichloroethenethiolate, that can tautomerize to yield 2-chlorothionoacetyl chloride and/or lose HCl to form chlorothioketene (Figure 1). The latter two highly reactive intermediates result in covalent modifications of proteins and DNA (16,17).

An additional bioactivation pathway of DCVC is the flavin-containing monooxygenase 3 (FMO3)-dependent formation of S-(1,2-dichlorovinyl)-L-cysteine sulfoxide (DCVCS), which may also play a role in the nephrotoxicity and carcinogenicity of TCE. DCVCS, a highly reactive Michael acceptor (18–20), was a much more potent nephrotoxicant to rats than DCVC both *in vivo* and *in vitro* in primary cultures of human renal proximal tubular cells (20,21). A stable GSH adduct, S-[1-chloro-2-(S-glutathionyl)vinyl]-L-cysteine sulfoxide, has been isolated and characterized from the bile of rats treated with DCVCS, and GSH depletion was observed in both the liver and kidneys (19).

Although both the β -lyase and FMO3 pathways may be contributing to DCVC bioactivation and nephrotoxicity and/or carcinogenicity, the expression levels of FMO3 and β -lyase are low in the human kidney (22–30). This may indicate that either humans are poorly susceptible to DCVC-induced toxicity or that bioactivation of DCVC in humans may primarily occur extrarenally and metabolites translocated into the circulation get distributed to the kidneys. Since the liver is capable of converting a xenobiotic to its mercapturic acid via biliary-hepatic recycling of GSH/cysteine conjugates as was demonstrated with 1-chloro-2,4-dinitrobenzene (31), the process of bioactivation of TCE to DCVCS or to 1,2-dichloroethenethiolate could occur in the liver before translocation of the reactive metabolites via the circulation into the kidney.

Previously, evidence for formation of DCVCS-derived monoadducts and cross-links with cysteine residues of globin was provided both *in vitro* when incubating red blood cells (RBCs) with DCVCS and *in vivo* upon dosing Sprague-Dawley rats with DCVCS (32). Because incubation of RBCs with DCVC had also resulted in detection of β -lyase-derived monoadducts and cross-links (33), globin adducts could be used to investigate the presence of DCVCS and

¹Abbreviations: ACN, acetonitrile; β -lyase, cysteine conjugate β -lyase; DCVC, S-(1,2-dichlorovinyl)-L-cysteine; DCVG, S-(1,2-dichlorovinyl)glutathione; DCVCS, S-(1,2-dichlorovinyl)-L-cysteine sulfoxide; ESI-QTOF, Electrospray-Quadrupole Time-Of-Flight; ESI-LTQ, Electrospray-Linear Trap Quadrupole; FMO3, flavin-containing monooxygenase 3; NAC, N-acetyl-L-cysteine; NA-DCVC, N-acetyl-S-(1,2-dichlorovinyl)-L-cysteine; NA-DCVCS, N-acetyl-S-(1,2-dichlorovinyl)-L-cysteine sulfoxide; RBC, red blood cell; TFA, trifluoroacetic acid; TCE, trichloroethylene.

chlorothioketene and/or 2-chlorothioacetyl chloride in the circulation after DCVC exposure. In the present study, we used mass spectrometry techniques to analyze trypsin digested globin peptides for monoadducts and cross-links due to S-oxidase and β -lyase-derived reactive metabolites after single or multiple dosing of rats with DCVC.

Experimental Procedures

Caution

DCVC is hazardous and should be handled with care. DCVC was shown to be a strong, direct-acting mutagen by the Ames test (34)

Materials

Trifluoroacetic acid (TFA) was purchased from Sigma-Aldrich Research (St. Louis, MO). Acetone was purchased from Fisher Scientific (Pittsburgh, PA). Trypsin (reductively alkylated) was obtained from Promega (Madison, WI). Heparin was supplied by American Pharmaceutical Partners (Schaumburg, IL). DCVC was synthesized and characterized as previously described (18,19).

Animals

Male Sprague-Dawley rats (160–230g), purchased from Harlan (Madison, WI), were maintained on a 12 h light/dark cycle and given water and food ad libitum. For the acute exposure study, 4 rats were each injected i.p. with a single dose of 230 or 460 μ mol/kg of DCVC dissolved in saline to a final concentration of approximately 5 mg/mL and 10 mg/mL, respectively. The high dose was chosen based on nephrotoxicity data showing a 3.1-fold increase in blood urea nitrogen concentrations and a 196-fold increase in urine glucose excretion rate at 24 h after injection (21). The low dose displayed no effects on blood urea nitrogen concentrations or urinary glucose excretion rates, however, histopathological changes (scattered tubular necrosis extending to the deep cortex but not the medulla) in the kidneys were observed (21). For the subacute exposure study, 4 rats were each injected i.p. with 3 or 30 μ mol/kg of DCVC every 24 h for 5 days. The low dose was chosen to reflect the AUC levels of DCVG recovered in the blood of male volunteers over a 4 h exposure to 100 ppm of TCE (9) whereas the high dose was selected to investigate the effect of dose variation on biomarker formation. Two rats were injected i.p. with saline to obtain control globin for MS analyses. Rats were sacrificed 1 h after dosing (1 h after the last dose for the subacute study) by CO₂ asphyxiation. Heparinized whole blood was obtained through cardiac puncture and globin was immediately precipitated (32,35).

Mass Spectra of Trypsin Digest

Trypsin digestion was performed as described previously (32,33). Samples from two rats dosed with 460 μ mol/kg DCVC were loaded onto a Zorbax (Agilent) C₁₈ stable bond column (0.075 mm $\times$ 150 mm, 5 μ , 300 Å) equipped with a Micromass Electrospray Hybrid Quadrupole Orthogonal Time-Of-Flight mass spectrometer (ESI-QTOF/MS) (32,33). Because the use of this ESI-QTOF/MS was later suspended, the rest of the globin samples were analyzed by an Electrospray-Linear Trap Quadrupole (ESI-LTQ) Orbitrap XL mass spectrometer (Thermo Scientific; Waltham, MA) equipped with an HPLC system. Peptides were eluted over 4 h with a flow rate of 200 nL/min on a fused silica MagicC18 (Michrom Bioresources; Auburn, CA) capillary column (75 μ m i.d., 360 μ m o.d. $\times$ 15 cm) packed with beads (3 μ m, 200 Å). Mobile phase A was 0.1 M acetic acid in doubly deionized H₂O and mobile phase B was 0.1 M acetic acid in 95% acetonitrile (ACN). Initial 1% B was maintained for 20 min, then increased to 40% over 195 min, to 60% over 20 min, to 100% over 5 min, then held for 3 min before being

decreased to 1% over 2 min and held for 15 min. Spectral lists of masses were exported and deconvoluted using Excel to be compared to theoretical monoisotopic masses.

Mass Spectral Analyses of Tryptic Peptides

The analyses of the digest for the specific monoadducts and cross-links shown in Figure 1 were performed as described previously (32,33). The search for modified cysteine-containing peptides (including those with one and two missed cleavages) included the following DCVCS-derived monoadducts: DCVCS (+194.9757 Da, addition of DCVCS moiety and loss of HCl), DCVCS-GSH (+466.0828 Da, addition of DCVCS-GSH conjugate and loss of 2 HCl), NA-DCVCS (+236.9862 Da, addition of NA-DCVCS and loss of HCl), and NA-DCVCS-GSH (+508.0933 Da, addition of NA-DCVCS-GSH conjugate and loss of 2 HCl). Data were also analyzed for cross-linked peptides with DCVCS as a cross-linker (+158.9990 Da; addition of DCVCS and loss of 2 HCl) or NA-DCVCS as a cross-linker (+201.0096 Da; addition of NA-DCVCS and loss of 2 HCl).

Because DCVC can also be bioactivated by β -lyases, our analyses extended to β -lyase-derived monoadducts with the sulfur-containing fragments: type 1 (+91.9488 Da, addition of chlorothioketene or chlorothionoacetyl chloride and loss of HCl) and type 2 (+363.0559 Da, addition of GSH conjugate and loss of 2 HCl) (Figure 1) (33). Thiol reactive intermediates (2-chlorothionoacetyl chloride and chlorothioketene) (Figure 1) could undergo hydrolysis before and/or after reaction with Hb resulting in substitution of sulfur for oxygen (36). Therefore, we also analyzed for the formation of peptides modified by the oxygen-containing fragments: monoadduct type 3 (+75.9716 Da, addition of chloroketene or 2-chloroacetyl chloride and loss of HCl) and monoadduct type 4 (+347.0787 Da, addition of GSH conjugate and loss of 2 HCl) (Figure 1). Due to the expected reactivity of β -lyase-derived reactive intermediates with nucleophilic residues besides cysteines, we extended our search to include up to 4 monoadducts types 1–4 on the same cysteine-containing peptides. We also analyzed for the presence of cross-links formed due to the sulfur-containing intermediate (cross-link type 1; +55.9721 Da, addition of sulfur-containing fragment and loss of 2 HCl) and due to the oxygen-containing intermediate (cross-link type 2; +39.9949 Da, addition of oxygen fragment and loss of 2 HCl).

All Cys-containing peptides have identical sequences and masses between $\alpha 1$ and $\alpha 2$ chains [except for peptide 1–16 where Asp5 ($\alpha 1$ chain) is substituted for Ala5 ($\alpha 2$ chain)] and between $\beta 1$ and $\beta 2$ globin chains. This lack of distinction between peptides prevents exact assignment of the specific modified chain. Therefore, non-specific (generic) α and β chain assignments were used for most peptides. The allowable mass error for identifying adducts based on monoisotopic masses was set to ± 50 ppm for ESI-QTOF/MS and to ± 3 ppm for ESI-LTQ Orbitrap/MS based on instrument accuracy. For peptide cross-links with masses above 5000 Da analyzed on ESI-LTQ Orbitrap/MS, the third peak of the isotope envelope (monoisotopic mass +2 Da) was also considered with allowable mass error set to ± 5 ppm.

HPLC Separation of Tryptic Digests

To enrich the concentration of modified peptides in order to localize specific sites of modification through tandem MS analyses, globin (22 mg) from one of the rats treated with 30 $\mu\text{mol/kg}$ DCVC for 5 days was trypsin digested (32). Separation of peptides was then accomplished using Vydac C_{18} protein and peptide HPLC column (5 μ , 4.6 mm $\times$ 25 cm) at a flow rate of 1 mL/min and monitored at A_{220} as previously described (32). Briefly, mobile phases were: pump A, 0.1% TFA in doubly deionized H_2O and pump B, 0.1% TFA in ACN. Initial 9% B was maintained for 5 min, then increased to 65% over 63 min and held for 5 min. The percent B was then decreased to 9 over 2 min and held for 5 min. Three peptide fractions were collected in 2–3 min increments between the 18–25 min time interval and lyophilized to

dryness. This particular time interval has been previously shown to contain the most modified cysteine-containing peptides (6,32).

MS/MS of Trypsin Digested Fractions

Fractions of trypsin-digested peptides (redissolved in 500 μ L of 0.1 M acetic acid) were subjected to MS/MS analyses using LTQ Orbitrap as described above. MS/MS spectra were acquired after a 3 μ L injection using data-dependent scanning which begins with the MS scan (300 and 2000 m/z) followed by 5 MS/MS scans. Dynamic exclusion of previously analyzed precursors was 60 s. MS/MS data were then converted to mgf format by Transproteomic Pipeline software package from Institute for Systems Biology (Seattle, WA) to enable Mascot (Matrix Science; London, UK) search against a customized amino acid sequence database retrieved from the National Center for Biotechnology Information for known Sprague-Dawley rat Hb sequences. The following Hb modifications were specified in the search: DCVCS, NA-DCVCS, DCVCS-GSH, NA-DCVCS-GSH, and monoadduct types 1–4 on peptides containing cysteines, lysines, arginines, N-terminal valines and histidines. Peptide and fragment mass tolerances were set to ± 2.5 Da and ± 0.5 Da, respectively. We focused on sequencing monoadducted peptides as opposed to cross-linked peptides due to the challenges associated with assigning sequences to cross-links (37).

Results

S-Oxidase-derived Monoadducts on Globin Peptides

Acute DCVC Exposure—In order to determine the presence of DCVCS in RBCs as a result of sulfoxidation of DCVC, we analyzed trypsin-digested globin peptides from rats treated with 230 and 460 μ mol/kg DCVC and sacrificed at 1 h. LC/MS data were analyzed for the presence of peptides modified by DCVCS, DCVCS-GSH, NA-DCVCS, or NA-DCVCS-GSH monoadducts. However, monoadducted peptides were not detected with either of the acute DCVC doses, possibly because of preferential formation of cross-links (see below) and/or formation of peptides modified by both S-oxidase and β -lyase-derived reactive intermediates.

Subacute DCVC Exposure—LC/MS data from rats treated with either 3 or 30 μ mol/kg DCVC daily for 5 days and sacrificed one hour after the last treatment were analyzed for the same monoadducts as in the acute study. Although no adducted peptides were detected at the lowest dose, one peptide on each α and β chain was detected with NA-DCVCS or NA-DCVCS-GSH modification, respectively, at 30 μ mol/kg subacute DCVC dose (Table 1). Overall, two out of four rats exhibited S-oxidase derived adducts at the high subacute dose (Table 1).

S-Oxidase-derived Globin Peptide Cross-links

Acute DCVC Exposure—Trypsin digests from rats treated with 230 and 460 μ mol/kg DCVC were also analyzed for peptide cross-link formation with DCVCS (+158.9990 Da) or NA-DCVCS (+201.0096 Da) as cross-linkers. Although no cross-links were detected with the low dose, 7 overall cross-links consisting of peptides from α chains, β chains, and between α and β chains were detected at 460 μ mol/kg DCVC (Table 2). Interestingly, 4 out of the 7 peptide dimers were cross-linked by NA-DCVCS suggesting that N-acetylation plays an important role in DCVC metabolism. In our analyses we have accounted for the presence of two reactive sites (Cys104 and Cys111) on long α chains peptides, (93–127) and (100–127). A dimer between peptides containing (Cys13 + Cys104/111) was detected with NA-DCVCS as cross-linker also had one DCVCS as a monoadduct (Table 2) implicating both Cys104 and Cys111 in reactions with DCVCS and NA-DCVCS. Our results suggest that all 5 cysteine positions (Cys 13, 104, 111- α chains; Cys93, 125- β chains) of the rat Hb $\alpha\beta$ -heterodimer are involved in DCVCS/NA-DCVCS-derived cross-link formation. Although our data does not allow differentiation among intra- or inter-chain cross-linking between peptides of the same chains

(i.e. between $\alpha 1$ or $\alpha 2$ and between $\beta 1$ or $\beta 2$), the inter-chain cross-linking is evident from the three peptide dimers between α and β chains. Overall, two out of four rats exhibited S-oxidase-derived cross-links at the high acute DCVC dose (Table 2).

Subacute DCVC Exposure—Rats treated with 3 $\mu\text{mol/kg}$ DCVC revealed four globin peptide cross-links, involving both α and β chains with mostly NA-DCVCS as the cross-linker (Table 3), whereas globin cross-links were not detected at 30 $\mu\text{mol/kg}$ DCVC, possibly because of preferential formation of peptides modified by a combination of reactive intermediates (S-oxidase and β -lyase-derived) and/or formation of cross-links between globin peptides and GSH (Table 1). As indicated above (Table 2), cross-links with modified positions $\alpha(\text{Cys}13 + \text{Cys}104/111)$ and $\beta(\text{Cys}93 + \text{Cys}125)$ were also present at the high acute dose (460 $\mu\text{mol/kg}$ DCVC). Overall, three out of four rats exhibited S-oxidase-derived cross-links at the 3 $\mu\text{mol/kg}$ subacute DCVC dose (Table 3).

β -Lyase-derived Monoadducts on Globin Peptides

Acute DCVC Exposure—In order to investigate β -lyase dependent bioactivation of DCVC (Figure 1), formation of globin peptides modified by sulfur- (monoadduct types 1 and 2) and oxygen (monoadduct types 3 and 4)-containing reactive fragments after dosing of rats with DCVC (230 and 460 $\mu\text{mol/kg}$) was analyzed. The $\alpha(\text{Cys}13)$ -containing peptide was modified by either sulfur- or oxygen-containing reactive fragment at both doses (Table 4). Multiple number of β -lyase-derived monoadducts on cysteine-containing peptides suggested that sites other than cysteines were modified. Overall, one out of 4 rats exhibited β -lyase-derived monoadducts at both the high and low acute DCVC doses (Table 4).

Subacute DCVC Exposure—The β -lyase pathway was also investigated after multiple treatments of rats with 3 or 30 $\mu\text{mol/kg}$ DCVC daily for 5 days and then studying globin adduct formation with β -lyase-derived sulfur or oxygen-containing intermediates. One out of 4 rats dosed with 30 $\mu\text{mol/kg}$ DCVC revealed peptide $\beta(121-144)$ modified by two reactive moieties (monoadducts type 1; $\text{Mass}_{\text{theo}}$ of 2628.1735 Da, Mass_{obs} of 2628.1713 Da), whereas no β -lyase-derived monoadducted peptides were detected at the 3 $\mu\text{mol/kg}$ DCVC dose.

β -Lyase-derived Globin Peptide Cross-links

Acute DCVC Exposure—LC/MS analyses of peptide dimers cross-linked by the sulfur-containing fragment (cross-link type 1) or an oxygen-containing fragment (cross-link type 2) (Figure 1) revealed 4 and 6 unique cross-links between different Cys-containing peptides at 230 $\mu\text{mol/kg}$ and 460 $\mu\text{mol/kg}$ DCVC doses, respectively (Table 5). Cross-links detected at the low DCVC dose were exclusively between β chains, whereas at the high DCVC dose both α and β chains were involved suggesting inter-chain cross-linking. The majority of rats at 230 $\mu\text{mol/kg}$ DCVC displayed cross-links between $\beta(\text{Cys}93)$ and $\beta(\text{Cys}125)$ -containing peptides and the most prevalent cross-links at 460 $\mu\text{mol/kg}$ DCVC were between $\alpha(\text{Cys}13)$ and $\beta(\text{Cys}125)$ -containing peptides implicating His116, His117, Lys120, Cys125, and/or Lys132 as preferred nucleophilic sites for β -lyase-derived crosslink formation. Overall, three out of four rats exhibited β -lyase-derived crosslinks at both high and low acute doses (Table 5).

Subacute DCVC Exposure—LC/MS analyses of cross-links due to β -lyase-derived intermediates revealed four and three peptide cross-links involving both α and β chains in rats dosed with multiple 3 and 30 $\mu\text{mol/kg}$ DCVC doses, respectively (Table 6). The majority of rats at 3 $\mu\text{mol/kg}$ DCVC dose exhibited inter-chain cross-links between $\alpha(\text{Cys}104/111)$ and $\beta(\text{Cys}125)$ -containing peptides. Cross-link consisting of $\alpha(8-31)$ and $\alpha(12-31)$ and cross-link consisting of $\beta(77-104)$ and $\beta(105-132)$ peptide sequences detected at 30 $\mu\text{mol/kg}$ DCVC (Table 6) were also observed at acute DCVC exposure (Table 5). Overall, three and two out

of four rats exhibited β -lyase-derived cross-links at 3 and 30 $\mu\text{mol/kg}$ subacute DCVC doses, respectively (Table 6).

MS/MS Analyses of Peptide Digest Fractions—Globin from the rat that exhibited β -lyase-derived monoadducts on the β (Cys125)-containing peptide at the subacute 30 $\mu\text{mol/kg}$ DCVC dose was trypsin digested and fraction collected using HPLC before ms/ms analysis. Fraction eluting at 21–23 min displayed an ms/ms fragmentation pattern consistent with a β (Cys125)-containing peptide (121–132) with type 3 modification on Cys125 (Figure 2, Table 7A). The spectrum of a doubly charged precursor ion (m/z 708.8185) displayed 7 total b-type ions (4 b-ions and 3 b^0 -ions, corresponding to additional water loss) and 9 y-ions. Fragmentation between Thr123 and Pro124 gave rise to a singly charged y_9 -ion (m/z 1039) and a doubly charged y_9 -ion (m/z 520). The high intensity of these ions along with the presence of the corresponding b_3 -ion (m/z 378) confirmed the presence of type 3 modification on the cysteine-containing fragment of this peptide. Additional y-ions produced due to cleavage of the modified portion of the peptide were: y_{10} (m/z 1140), its doubly charged ion (m/z 570), and y_8 (m/z 942). The b-ions that corresponded to the cleaved fragments of the modified portion of the peptide were: b_{10} (m/z 1142), b_{11} (m/z 1270), and ions (b_7^0 , b_8^0 , b_9^0) resulting from additional water loss. Fragmentation of the unmodified portion of the peptide gave rise to several expected y- and b-ions confirming the identity of the peptide.

Another cysteine-containing peptide that displayed good ms/ms fragmentation pattern was β (83–95) with type 3 modification on Cys93, detected in fraction eluting at 23–25 min (Figure 3, Table 7B). The spectrum of a triply charged precursor ion (m/z 511.8978) displayed 4 b-ions and 9 y-ions. Fragmentation between Thr84 and Phe85 gave rise to the most intense doubly charged y_{11} -ion (m/z 688.6) and its corresponding b_2 -ion (m/z 159.2), which confirm the presence of type 3 modification on the cysteine-containing fragment of this peptide. Further evidence of modification at Cys93 was provided by the presence of y_{3-10} -ions and b_{12} -ion produced due to cleavage of the modified portion of the peptide. Several expected b-ions (b_2 , b_8 , b_9) that correspond to the unmodified fragments of the peptide confirm identity of the peptide.

Discussion

In the present study, we investigated S-oxidase and β -lyase dependent bioactivation of DCVC by characterizing globin monoadducts and cross-links with the respective reactive intermediates after dosing rats with single and multiple doses of DCVC. Single dose treatments were performed to determine if DCVCS and/or β -lyase-derived metabolites form in vivo at nephrotoxic doses. Subacute dosing regimen was implemented to mimic low levels of DCVC that may be transient in the blood upon workplace or environmental TCE exposure over an extended period of time. The chosen doses (3 and 30 $\mu\text{mol/kg}$ DCVC for 5 days) were based on the amount of DCVG recovered in the blood after a 4 h inhalation exposure of human volunteers to 100 ppm of TCE (9). The American Conference of Governmental Industrial Hygienists set threshold limit value of exposure in the workplace at 50 ppm TCE as an 8-hour time-weighted average with a ceiling value of 100 ppm (8). Our results indicate that modifications of macromolecules may occur with consistent low level DCVC exposure over time as well as with high acute exposure levels.

Formation of DCVCS-derived monoadducts and cross-links suggests the presence of DCVCS in the circulation and provides evidence for the FMO3-dependent metabolism of DCVC. Although our previous DCVCS study led to detecting globin adducts with 4 out of 5 cysteine residues per $\alpha\beta$ -heterodimer (32), all 5 cysteine sites appeared to be involved in DCVCS-derived adduct formation after DCVC administration. Overall, two and three out of four rats exhibited S-oxidase-derived globin modifications at both high acute and subacute DCVC doses

(460 and 30 $\mu\text{mol/kg}$) and at low subacute DCVC dose (3 $\mu\text{mol/kg}$), respectively (Table 8). DCVCS-derived globin cross-links were more prevalent than DCVCS-derived monoadducts (none detected) in rats treated with high acute and low subacute doses of DCVC. These results are consistent with our previous results where DCVCS-derived cross-links were more prevalent than monoadducts after exposure of rats to 23 and 230 $\mu\text{mol/kg}$ DCVCS (32).

NA-DCVCS was frequently observed in monoadducts and cross-links in single and multiple dosing regimens suggesting that after bioactivation of DCVC to DCVCS by FMO3, N-acetylation of DCVCS occurs in vivo before translocation into RBCs. These results are consistent with our previous findings when we detected globin cross-links due to N-acetylation of DCVCS after rats were dosed with DCVCS (32). Although the presence of DCVCS monoadducts and cross-links provided evidence for bioactivation of DCVC by FMO3 in vivo, formation of NA-DCVCS may also be dependent on another S-oxidase (CYP450 3A1/2) (38), i.e. N-acetylation of DCVC could be followed by oxidation via CYP450 3A1/2. The latter metabolic pathway was demonstrated in vitro in rat liver microsomes (38).

The DCVCS-derived cross-links that were present at both acute and subacute dosing regimens consisted of α (Cys13) and α (Cys104/111)-containing peptides as well as β (Cys93) and β (Cys125)-containing peptides. Because such cross-links were also detected after treatment of rats with DCVCS (23 and 230 $\mu\text{mol/kg}$) (32), these peptide cross-links could serve as reliable biomarkers of TCE exposure. Interestingly, both α (Cys104) and β (Cys93) residues of rat Hb occupy the same positions in human Hb with β (Cys93) considered as the most reactive sulfhydryl site (39).

Detection of monoadducts and cross-links with β -lyase-derived sulfur/oxygen-containing fragments indicated involvement of the β -lyase pathway in bioactivation of DCVC in vivo. Overall, rats exhibited β -lyase-derived adducts at both high acute and subacute (two out of four), at low subacute (three out of four), and at low acute (four out of four) dosing regimens (Table 8). Although we were unable to obtain MS/MS fragmentation of peptides modified by the S-oxidase-derived intermediates, we obtained conclusive evidence for the presence of the β -lyase-derived monoadduct type 3 (oxygen-containing intermediate) on Cys93 and Cys125 of β chains in one of the rats treated subacutely with 30 $\mu\text{mol/kg}$ DCVC. Similar to DCVCS, cross-linking between globin chains was more prevalent than formation of monoadducts at all dosing regimens consistent with our in vitro results when RBCs were incubated with 9 μM DCVC (33). Involvement of nucleophilic residues other than cysteines, (i. e. lysines, histidines, methionines, or arginines) is evident because of the presence of multiple monoadducts on the same peptides suggesting less selectivity than DCVCS which is only reactive with cysteines (19,40). A β -lyase-derived metabolite of S-(1,2,3-trichlorovinyl)-L-cysteine (TCVC), dichlorothioketene, gave rise to a N^ε-(dichloroacetyl)-L-lysine adduct after rat treatments with tetrachloroethene and TCVC (41,42) suggesting that β -lyase-derived metabolites of DCVC may also react with ϵ -amino groups of proteins. The extent of cysteine binding can vary depending on the reactive intermediate and the extent at which other nucleophilic residues are involved (43,44).

Because we detected S-oxidase- and β -lyase-derived globin adducts upon administration of DCVC to rats (two out of four rats at the subacute 3 and 30 $\mu\text{mol/kg}$ doses and the 460 $\mu\text{mol/kg}$ acute dose exhibited both; Table 8), both the S-oxidase and β -lyase pathways play an important role in DCVC bioactivation in vivo. Previously, we established the presence of β -lyase activity in RBCs (33). Because RBCs represent an additional compartment for DCVC metabolism via the β -lyase pathway, the detected adducts with chlorothioketene/chloroketene and 2-chlorothionoacetyl chloride/chloroacetyl chloride could be due to bioactivation of DCVC within RBCs or outside of the circulation. Since we also previously established the lack of S-oxidase activity in RBCs (33), the presence of DCVCS-derived globin adducts is likely

due to formation of DCVCS outside of the circulation. Hepatic secretion of S-benzylcysteine mercapturate across the sinusoidal membrane into the plasma was demonstrated to occur via a probenecid-sensitive transport system in the perfused rat liver (45), suggesting that intermediates resulting from the S-oxidase-dependent metabolism of DCVC can also be actively transported into the circulation. The amount that gets translocated into RBCs and binds directly to Hb is presently unknown. Because DCVCS is highly reactive toward sulfhydryl groups (19,32,40), most of it is likely to conjugate with GSH spontaneously or via GSH S-transferase in the liver. DCVCS could, however, be freed from its GSH conjugate for reactivity within RBCs if the DCVCS-GSH conjugate undergoes a spontaneous or a GSH S-transferase-catalyzed retro-Michael reaction as was demonstrated previously with the GSH conjugates of trans-4-phenyl-3-buten-2-one and 4-hydroxy-2-nonenal (46,47).

FMO3 bioactivation of DCVC can be extended to humans since S-oxidase activity in the liver is similar between rats and humans (23). However, several factors make it difficult to delineate the exact contribution of FMO3, CYP450 3A, and β -lyase in DCVC bioactivation. Although FMO3 expression is predominant in the liver, a 10 fold and 5–6 fold interindividual differences were observed in human liver and kidney, respectively (24,25). Significant variations in enzyme activities of CYP450 3A and β -lyase between species, tissues, and individuals also exist (22,48). In addition, interindividual differences in enzyme activities may influence the ratio between detoxification via N-acetylation and bioactivation via β -lyase or FMO3-dependent pathways.

All three cysteine positions per human Hb $\alpha\beta$ -heterodimer (α 104, β 93, β 112) could potentially be reactive toward DCVCS and NA-DCVCS even though α (Cys104) and β (Cys112) are located internally between the α 1 β 1/ α 2 β 2-interface (49,50). Both Cys93 and Cys104 were implicated in reaction with methyl bromide and Cys112 was implicated in reaction with epichlorohydrin upon exposure of human erythrocytes to methyl bromide and epichlorohydrin, respectively (51,52). Furthermore, human β chain was modified by lewisite due to formation of cross-link between Cys93 and Cys112 when erythrocytes were exposed to lewisite (49, 53). Since rat Hb contains more sulfhydryl groups (5 per α,β -heterodimer) of which Cys13, Cys93, and Cys125 are more reactive than cysteine residues in human Hb (3 per α,β -heterodimer) (39), further studies using human erythrocytes are warranted to develop DCVC biomarkers with human globin.

Globin adducts formed due to S-oxidase and/or β -lyase-derived intermediates may also provide insight into formation of DCVC after TCE exposure. The plasma half-lives of DCVC in mice and rats are short (23 min and 2.8 h, respectively), therefore, Hb adducts could serve as persistent biomarkers for monitoring the presence of DCVC in the circulation over an extended period (54,55).

Collectively, detection of globin adducts with DCVCS and NA-DCVCS provides the first evidence for the presence of DCVCS and NA-DCVCS in the circulation after prerenal bioactivation of DCVC in vivo. Since DCVCS is a potent nephrotoxicant and we have shown that DCVCS can react with Hb to form adducts and cross-links, DCVCS could potentially react with kidney proteins in the same manner playing a role in DCVC toxicity. Detection of globin adducts with sulfur/oxygen-containing fragments generated by β -lyase suggests the presence of the reactive thiol species in the circulation formed by prerenal bioactivation of DCVC in vivo. Our data presents the first in vivo evidence for the formation of protein cross-links by β -lyase-derived intermediates which could also play a role in DCVC toxicity. Quantitative assessment of these adducts should further our understanding of the flux through these bioactivation pathways and the potential roles of circulating metabolites in nephrotoxicity and/or nephrocarcinogenicity. S-Oxidase (FMO3 and P450 3A)- and β -lyase-derived cross-links

detected at such low DCVC exposure levels in the subacute study suggest that they could serve as biomarkers of chronic low level TCE exposure.

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Figure 1.

Proposed mechanism for formation of Hb monoadducts and cross-links derived from the S-oxidase and β-lyase metabolic pathways of DCVC. For S-oxidase pathway, globin monoadduct formation includes DCVCS, DCVCS-GSH, NA-DCVCS, and NA-DCVCS-GSH and globin cross-link formation involves DCVCS and NA-DCVCS as cross-linkers. For β-lyase pathway, globin monoadduct and cross-link formation involves sulfur- and oxygen (formed upon hydrolysis)-containing reactive intermediates. ^aMonoadducts (types 2 and 4) can also arise by initial conjugation with GSH followed by Hb. Hb is used here to describe general types of adducted globins without specification of which globin chain is involved.

Figure 2. LC/MS/MS of peptide β (121–132) with type 3 modification at Cys125. Superscript 0 indicates a fragmented ion with additional water loss. Superscript +2 indicates a doubly charged fragmented ion.

Figure 3. LC/MS/MS of peptide β (83–95) with type 3 modification at Cys93. Superscript +2 indicates a doubly charged fragmented ion.

Table 1

LC/ESI/MS Results of Peptides Modified by DCVCS-derived Monoadducts after Rats were Dosed Subacutely (every 24 h for 5 days) with DCVC (30 μ mol/kg).

Peptide	Mass _{theo} (Da)	Mass _{obs} (Da)	Monoadducts ^a	Modified positions	n ^b
α chains					
α 1 (1–16)	2028.8926	2028.8929	NA-DCVCS	Cys13	1/4 ^d
β chains					
β (105–132)	3543.7261	3543.7258 ^c	NA-DCVCS-GSH	Cys125	2/4 ^d

^a NA-DCVCS (+236.9862 Da), NA-DCVCS-GSH (+508.0933 Da)

^b n= number of rats that exhibited the monoadduct over total number of rats

^c Mass_{obs} for one other rat was 3543.7239 Da

^d One of the four rats exhibited both monoadducts

Table 2

LC/ESI/MS Results of Peptide Cross-links Formed by DCVCS or NA-DCVCS after Rats were Dosed Acutely with DCVC (460 μ mol/kg).

Cross-linked peptides	Mass _{theo} (Da)	Mass _{obs} (Da)	Cross-linker ^a	Modified positions	n ^b
α chains					
$\alpha 1$ (1–16) + α (100–127)	5206.3913	5206.1807	DCVCS	Cys13+Cys104/111	1/4
$\alpha 1$ (1–16) + α (100–127)	5248.3913	5248.4468	NA-DCVCS	Cys13+Cys104/111	1/4
+ 1 DCVCS monoadduct					
β chains					
β (83–104) + β (121–144)	5167.5069	5167.3667	DCVCS	Cys93+Cys125	1/4
β (83–95) + β (121–144)	4101.9607	4101.9922	NA-DCVCS	Cys93+Cys125	1/4
α and β chains					
$\alpha 2$ (1–16) + β (121–144)	4392.1925	4392.3628	NA-DCVCS	Cys13+Cys125	1/4
$\alpha 1$ (1–16) + β (77–104)	5235.5290	5235.6816	DCVCS	Cys13+Cys93	1/4
α (100–127) + β (121–132)	4602.1159	4602.2178	NA-DCVCS	Cys104/111+Cys125	1/4

^a DCVCS (+158.9990 Da), NA-DCVCS (+201.0096 Da)

^b n= number of rats that exhibited the cross-link over total number of rats. Overall, two different rats exhibited S-oxidase-derived cross-links.

Table 3
LC/ESI/MS Results of Peptide Cross-links Formed by DCVCS or NA-DCVCS after Rats were Dosed Subcutely (every 24 h for 5 days) with DCVC (3 μ mol/kg).

Cross-linked peptides	Mass _{theo} (Da)	Mass _{obs} (Da)	Cross-linker ^a	Modified positions	n ^b
α chains					
$\alpha 2$ (1–16) + α (93–127)	5808.8340	5808.8376 ^c	NA-DCVCS	Cys13+Cys104/111	2/4
$\alpha 2$ (1–16) + α (8–31)	4523.1736	4523.1835	DCVCS	Cys13+Cys13	1/4
β chains					
β (77–104) + β (121–144)	5929.9083	5929.9128	NA-DCVCS	Cys93+Cys125	1/4
β (105–132) + β (121–132)	4576.2732	4576.2738	NA-DCVCS	Cys125+Cys125	1/4

^aDCVCS (+158.9990 Da) or NA-DCVCS (+201.0096 Da)

^bn= number of rats that exhibited the cross-link over total number of rats. Overall, three different rats exhibited S-oxidase-derived cross-links

^cMassObs for one other rat was 5808.8372 Da

Table 4
LC/ESI/MS Results of β -lyase-derived Globin Peptide Monoadducts after Rats were Dosed Acutely with DCVC

Peptide/Cys position	Sequence ^a	Mass _{theo} (Da)	Mass _{obs} (Da)	Monoadduct type (#) ^b	n ^c
(A) 230 μmol/kg DCVC					
α chains					
α (12–40)/Cys13	NCWGGKIGGGHGGEGYGEELQRMFAAFPTTK	3307.4263	3307.4330	3 (2)	1/4
(B) 460 μmol/kg DCVC					
α chains					
α (8–31)/Cys13	TNINKCWGKIGGGHGGEGYGEELQR	3706.4258	3706.3284	2 (3)	1/4
β chains					
β (77–104)/Cys93	HLDNLKGTFAHLSEL H CDK L HVDPENFR	3469.5202	3469.3931	1 (2)	1/4

^a Potential nucleophilic reactive sites are marked in bold
^b (#) refers to the number of monoadducts on a peptide; see Figure 1 for structures of monoadduct types 1, 2, and 3
^c n= number of rats that exhibited the monoadduct over total number of rats. The same rat exhibited all monoadducts.

Table 5
LC/ESI/MS Results of β -lyase-derived Globin Peptide Cross-links after Rats were Dosed Acutely with DCVC.

Cross-linked peptides/Cys position	Mass _{theo} (Da)	Mass _{obs} (Da)	Cross-linker type ^a	n ^b
(A) 230 μmol/kg DCVC				
β chains				
β (77–104)/Cys93 + β (105–132)/Cys125	6378.2275	6378.2425 ^c	1	3/4
β (77–104)/Cys93 + β (105–132)/Cys125	6362.2503	6362.2521 ^d	2	
β (83–104)/Cys93 + β (83–104)/Cys93	5169.4567	5169.4613	2	2/4
β (77–104)/Cys93 + β (83–104)/Cys93	5907.8257	5907.8505	1	
(B) 460 μmol/kg DCVC				
α chains				
α 2 (1–16)/Cys13 + α 2 (1–16)/Cys13	3534.8279	3534.6614	2	2/4
α (8–31)/Cys13 + α (12–31)/Cys13	4817.2415	4817.2372	2	
β chains				
β (83–104)/Cys93 + β (83–104)/Cys93	5169.4567	5169.2656	2	1/4
α and β chains				
α (8–31)/Cys13 + β (121–132)/Cys125	4012.8611	4012.9927	1	3/4
α (12–31)/Cys13 + β (105–132)/Cys125	5235.6161	5235.6816	2	
α 1 (1–16)/Cys13 + β (105–132)/Cys125	4866.5339	4866.5478	2	

^a see Figure 1 for structures of cross-linker types 1 and 2

^b n= number of rats that exhibited the cross-link over total number of rats. Overall, three different rats exhibited β -lyase-derived cross-links at each dose

^c Mass_{obs} for two other rats were 6378.2561 and 6378.2441 Da

^d Mass_{obs} for two other rats were 6362.2586 and 6362.2505 Da

Table 6

LC/ESI/MS Results of β -lyase-derived Globin Peptide Cross-links after Rats were Dosed Subacutely (every 24 h for 5 days) with DCVC.

Cross-linked peptides/Cys position	Mass _{theor} (Da)	Mass _{obs} (Da)	Cross-linker type ^a	n ^b
(A) 3 μmol/kg DCVC				
α chains				
α (8–16)/Cys13 + α (93–127)/Cys104, 111	4963.4387	4963.4410	2	1/4
β chains				
β (121–144)/Cys125 + β (121–146)/Cys125	5243.6464	5243.6375	1	1/4
α and β chains				
α (100–127)/Cys104, 111 + β (121–146)/Cys125	5860.8554	5860.8547 ^c	1	3/4
α (100–127)/Cys104, 111 + β (121–132)/Cys125	4444.1485	4444.1489	2	
(B) 30 μmol/kg DCVC				
α chains				
α (8–31)/Cys13 + α (12–31)/Cys13	4817.2415	4817.2373	2	1/4
β chains				
β (77–104)/Cys93 + β (105–132)/Cys125	6376.2275	6376.2276	1	1/4
β (83–95)/Cys93 + β (105–132)/Cys125	4532.3123	4532.3085	2	1/4

^a see Figure 1 for structures of cross-linker types 1 and 2

^b n= number of rats that exhibited the cross-link over total number of rats. Overall, two different rats exhibited β -lyase-derived cross-links at each dose.

^c Mass_{obs} for one other rat was 5860.8520 Da

Table 7

The m/z of y-ions for unmodified and modified peptide fragments. Modification is monoadduct type 3 (see structure in Figure 1; +76 Da).

	Mass _{theo}	Mass _{obs}	Mass _{obs} ^a
(A) peptide β (121–132), see Figure 2			
y1	147.2		
y2	275.3	275.2	
y3	422.5	422.3	
y4	493.6	493.3	
y5	564.7	564.3	
y6	692.8	691.0	
y7	763.9	763.4	
y8	867.0	942.3^b	
y9	964.1	1039.4	520.4
y10	1065.2	1140.4	570.8
(B) peptide β (83–95), see Figure 3			
y1	147.2		
y2	262.3		
y3	365.4	441.3	
y4	502.6		289.6
y5	615.7		346.2
y6	744.8	820.4	
y7	831.9	907.3	
y8	945.1	1020.5	
y9	1082.2		579.4
y10	1153.3		614.9
y11	1300.5		688.6

^a Observed mass of the doubly charged fragmented ion

^b Highlighted masses represent ions from the modified portions of the peptides

Table 8

Summary of the number of rats that exhibited S-oxidase- and β -lyase-derived adducts (monoadducts and cross-links).

	S-Oxidase	β -Lyase	Both Pathways
Acute Doses			
230 μ mol/kg DCVC	-	4/4 ^a	-
460 μ mol/kg DCVC	2/4	2/4	2/4
Subacute Doses			
3 μ mol/kg DCVC	3/4	3/4	2/4
30 μ mol/kg DCVC	2/4	2/4	2/4

^a number of rats that exhibited the adducts over total number of rats