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Cysteine Conjugate β -Lyase Activity of Rat Erythrocytes and Formation of β -Lyase-Derived Globin Monoadducts and Cross-Links after in Vitro Exposure of Erythrocytes to 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 can be bioactivated to reactive metabolites, S-(1,2-dichlorovinyl)-L-cysteine sulfoxide (DCVCS) or chlorothioketene and/or 2-chlorothionoacetyl chloride, by cysteine conjugate S-oxidase (S-oxidase) and cysteine conjugate β -lyase (β -lyase), respectively. Previously, we characterized reactivity of DCVCS with Hb upon incubation of erythrocytes with DCVCS and provided evidence for formation of distinct DCVCS-Hb monoadducts and cross-links both in isolated erythrocytes and in rats given DCVCS. In the present study, we investigated DCVC bioactivation and Hb adduct formation in isolated rat erythrocytes incubated with DCVC (9 and 450 μ M) at 37 °C, pH 7.4. The results suggested no DCVCS monoadducts or cross-links were formed; however, LC/ESI/MS and MALDI/MS of trypsin digested globin peptides revealed presence of β -lyase-derived globin monoadducts and cross-links. Adducts and cross-links in which the sulfur atom of the reactive sulfur intermediates were replaced by oxygen have also been detected. Use of SDS-PAGE provided additional evidence for globin cross-link formation in the presence of DCVC. Interestingly, the MS results suggest the observed peptide selectivity of the β -lyase-derived reactive sulfur/oxygen-containing species was different than that previously observed with DCVCS. While these results suggested erythrocytes have β -lyase but not S-oxidase activity, further support for this hypothesis was obtained using S-(2-benzothiazolyl)-L-cysteine, an alternative substrate for β -lyases. Collectively, the results demonstrate the utility of Hb adducts and cross-links to characterize the metabolic pathway responsible for DCVC bioactivation in erythrocytes and to provide distinct biomarkers for each reactive metabolite.

Introduction

Trichloroethylene (TCE) is a common environmental pollutant listed in the Eleventh Report on Carcinogens as “reasonably anticipated to be a human carcinogen” (1). Renal-cell carcinomas from occupationally exposed workers exhibited mutation in the *von Hippel-Landau* tumor suppressor gene. S-(1,2-Dichlorovinyl)-L-cysteine (DCVC), a mutagenic and nephrotoxic metabolite of TCE, has been implicated in the renal toxicity and carcinogenicity of TCE (2–4). Formation of DCVC occurs via a GSH-dependent pathway that primarily occurs in the liver and initially converts TCE to S-(1,2-dichlorovinyl)-L-glutathione (DCVG). Following systemic distribution and/or secretion into the bile duct, DCVG is converted to

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DCVC by γ -glutamyl transpeptidase and cysteinylglycine dipeptidases present in the kidneys, bile duct, and intestines (3–5). After human exposure to TCE, DCVG was detected in blood within 30 min and its presence persisted for up to 12 h (3). DCVC was also detectable in the blood of male rats for up to 48 h after exposure to TCE (5). DCVC can be converted to the mercapturic acid derivative, N-acetyl DCVC, in the liver or the kidneys (4). Excretion of N-acetyl DCVC has been detected in the urine over 48 h after a 6 h human exposure to TCE (6).

Bioactivation of DCVC in the kidneys via the cysteine conjugate β -lyase (β -lyase) pathway was suggested to play a major role in DCVC cytotoxicity in proximal tubule cells of Sprague-Dawley rats (reviewed in 4,7,8). β -Lyases are pyridoxal 5'-phosphate-dependent enzymes that catalyze β -elimination reactions (7). At least eleven β -lyase enzymes that catalyze cysteine S-conjugate β -elimination reactions have been identified (7). β -Elimination proceeds by cleavage of the thioether linkage and results in generation of pyruvate, ammonia, and depending upon the chemical structure of the cysteine-S-conjugate, a stable thiol or an electrophilic reactive sulfur-containing fragment (8,9). In the case of DCVC, β -elimination reaction yields a highly reactive thiol species, 1,2-dichloroethenethiolate that could tautomerize to yield 2-chlorothionoacetyl chloride or lose HCl to form chlorothioketene (Figure 1). Both reactive metabolites could covalently bind macromolecules, such as proteins and DNA (9–11). Renal β -lyase activity was shown to be most abundant in the cytosol of rats and humans, however, overall renal cytosolic β -lyase activity in human samples was approximately 10% that of rats (12,13). Because purified recombinant human kidney glutamine transaminase K (GTK) exhibited higher β -lyase activity with cysteine S-conjugates than rat kidney GTK (14), the low activity detected in human kidney samples warrants further investigation to better characterize the variability of renal cytosolic β -lyase activity in human kidneys.

DCVC is also a substrate for S-oxidation (S-oxidase) by flavin-containing monooxygenase 3 (FMO3) (15,16). The resulting metabolite, S-(1,2-dichlorovinyl)-L-cysteine sulfoxide (DCVCS), is a reactive electrophile that was a much more potent nephrotoxicant to rats in vivo than DCVC (2). DCVCS induced necrosis, apoptosis, GSH depletion, and mitochondrial dysfunction in primary cultures of human proximal tubule cells (17). DCVCS treatment depleted hepatic and renal GSH and a DCVCS-GSH adduct was detected in the bile of rats given DCVCS (18). Although the FMO3 bioactivation pathway may be contributing to DCVC nephrotoxicity and/or mutagenicity, the expression levels of FMO3 in the human kidney are low in comparison to human liver (16,19,20). Incubations of DCVC with human liver microsomes in the presence of NADPH resulted in formation of DCVCS, whereas DCVCS was not detectable with human kidney microsomes (16). FMO3 mRNA levels in human kidney were also detected at much lower levels than in liver (19). Low levels of S-oxidase and possibly cytosolic β -lyase (see above) in the human kidney suggest that humans are poorly susceptible to DCVC-induced toxicity. Alternatively, bioactivation of DCVC may occur primarily extrarenally, and reactive metabolites may get translocated from tissues such as the liver to the kidney via the circulation.

Because currently there is no evidence for translocation of DCVC-derived reactive metabolites among tissues, we recently developed methods that would allow us to investigate the presence of DCVCS in the circulation after exposure of rats to DCVC or TCE. These methods were based upon the expected reactivity of DCVCS as a Michael acceptor with Hb. Several globin cysteine-containing peptides monoadducted and cross-linked by DCVCS were identified both in vitro in red blood cells (RBCs) and in vivo in rats after exposure to DCVCS (21). Because DCVCS readily formed three monoadducts and one cross-linked diadduct upon incubation with N-acetyl-L-cysteine (NAC) at physiological conditions but not with N-acetyl-L-lysine or L-valinamide (22), cysteine residues of Hb have been implicated as primary reaction sites. While the results suggested Hb adducts can serve as biomarkers for DCVCS in the circulation, there was no prior information on whether RBCs have the ability to bioactivate DCVC.

Therefore, in the present study we characterized Hb adducts formed after incubation of rat erythrocytes with DCVC. We also investigated β -lyase activity in RBCs using the model cysteine S-conjugate β -lyase substrate, S-(2-benzothiazolyl)-L-cysteine (BTC).

Experimental Procedures

Caution

DCVC and DCVCS are hazardous and should be handled with care. DCVC was shown to be a strong, direct-acting mutagen by the Ames test (23).

Materials

Trifluoroacetic acid (TFA), alpha-ketobutyric acid sodium salt monohydrate (KBA), aminooxyacetic acid (AOAA), 2-mercaptobenzothiazole (2-MBT), horseradish peroxidase (Type II, 224 purpurogallin units/mg solid), cytochrome P450 reductase, and human Hb were purchased from Sigma-Aldrich Research (St. Louis, MO). Sodium borate decahydrate was purchased from ICN Biomedicals (Aurora, OH). Acetone and 30 % H_2O_2 were purchased from Fisher Scientific (Pittsburgh, PA). Trypsin (reductively alkylated) was obtained from Promega (Madison, WI). SDS, 15 % Tris-HCl Criterion precast gels, DTT, glycine, Kaleidoscope Prestained Standards were purchased from Bio-Rad Laboratories (Hercules, CA). SilverSnap Stain Kit II was obtained from Pierce (Rockford, IL). Heparin was supplied by American Pharmaceutical Partners (Schaumburg, IL). DCVC, DCVCS, and BTC were synthesized and characterized as previously described (15,18,24,25). Purity of all synthetic compounds was determined to be >95% by HPLC analysis.

Animals

Male Sprague-Dawley rats (180–220 g), purchased from Harlan (Madison, WI), were maintained on a 12 h light/dark cycle and given water and feed ad libitum. Heparinized whole blood was obtained through cardiac puncture and processed immediately.

Incubations of Erythrocytes with DCVC

After removal of the plasma fraction from the whole blood, the red blood cells were washed three times with an equal volume of saline and centrifuged at $1,855 \times g$ for 5 min in between washes. Erythrocytes (approximately $3.52 \times 10^6/220 \mu\text{L}$) were resuspended in an equal volume of PBS (10 mM, pH 7.4) before incubation with and without DCVC (9, 90, 450 μM) in a shaking water bath for 2 h at 37°C. At the end of the incubation, RBCs were lysed with equal volume of cold doubly deionized H_2O and globin was isolated using acidified acetone as described previously (26).

Hemolysis

To ascertain that the DCVC concentrations used to investigate globin adduct formation were not associated with RBC toxicity, the extent of hemolysis was measured for erythrocytes incubated alone and with DCVC (90 or 450 μM) at 0–2 h using a spectrophotometric assay as previously described (21).

Globin Chain Cross-link Analysis by SDS-PAGE

Globin samples from in vitro incubations (9–450 μM DCVC and control) were investigated for globin chain cross-link formation using SDS-PAGE as described previously (21). Briefly, 2.5 μg of globin dissolved in 2.5 μL doubly deionized H_2O was added to treatment buffer (0.5 M Tris-HCl, 10% glycerol, 10% SDS, and 0.01 g/mL bromophenol blue, 10% doubly deionized H_2O). Samples were incubated at room temperature for 30 min after addition of 200 mM DTT to reduce disulfide bonds before being loaded onto a 15 % Tris-HCl Criterion Precast Gel.

Kaleidoscope Prestained Standards (1 μ L diluted 50 fold with the treatment buffer) were used for molecular weight determination. Silver stained gels were analyzed for dimer formation using density measurements with Quantity One software (Bio-Rad Laboratories; Hercules, CA).

Mass Spectra of Trypsin Digest

Trypsin digest of globin was obtained as described previously (21). Samples (control and 450 μ M incubation) were desalted using C-18 solid-phase zip tips before being 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) (21). Trypsin digests from 450 μ M DCVC and control incubations were also subjected to a MALDI-TOF² 4800 mass spectrometer (Applied Biosystems; Foster City, CA) (21). The digest samples with and without 9 μ M DCVC were subjected to an HPLC system carrying a Zorbax (Agilent) C₁₈ stable bond column (0.075 mm $\times$ 100 mm, 3 μ , 300 Å) and analyzed by a Linear Trap Quadrupole (LTQ) Orbitrap XL mass spectrometer (Thermo Scientific; Waltham, MA) (21). The latter instrument was used due to suspension of ESI-QTOF/MS availability.

Mass Spectral Analyses of Tryptic Peptides

Peak lists of experimental monoisotopic peptide masses were imported into Microsoft Access for comparison with the theoretical masses of modified peptides generated from the virtual digest of rat globin chains (α 1, α 2, β 1, and β 2) using Protein Prospector (www.prospector.ucsf.edu). Same methodology and search criteria were applied as described previously (21). Calculations were based on the ³⁵Cl monoisotopic masses for adducts containing one Cl atom (monoadducts). The isotopic pattern was then evaluated for the presence of ³⁷Cl. We limited our analyses to cysteine-containing peptides (including one and two missed cleavages) since DCVCS preferentially reacts with sulfhydryl groups (21,22). Theoretical lists of monoisotopic masses for modified peptides were calculated for the following S-oxidase-derived monoadducts as described previously (20): 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), and NA-DCVCS (+236.9862 Da, addition of NA-DCVCS and loss of HCl). Data were also analyzed for masses that matched formation of dimers between cysteine-containing 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).

Trypsin digests were also analyzed for peptides and peptide dimers modified by β -lyase-derived metabolites as shown in Figure 1. Theoretical monoisotopic masses were calculated for peptides modified by β -lyase-derived sulfur-containing monoadducts (type 1; +91.9488 Da) and by monoadducts formed between GSH and the reactive thiol species (type 2; +363.0559 Da, addition of GSH conjugate and loss of HCl). 2-Chlorothionoacetyl chloride and chlorothioketene could also give rise to 2-chlorothiolic acid (ClCH₂C=OSH), an oxygen-containing reactive intermediate, in the presence of water (27). Hydrolysis could occur before and/or after reaction with Hb resulting in substitution of sulfur for oxygen. Therefore, formation of peptides modified by the oxygen-containing fragments (type 3; +75.9716 Da) and by monoadducts formed between GSH and the oxygen-containing fragment (type 4; +347.0787 Da, addition of GSH conjugate and loss of HCl) were also investigated. Due to the expected reactivity of β -lyase reactive intermediates with nucleophilic residues besides cysteines, we extended our search to include up to 4 monoadducts (reactive intermediates and their GSH conjugates) on the same cysteine-containing peptides.

We also analyzed for the presence of cross-links formed by the sulfur-containing reactive intermediate (cross-link type 1; +55.9721 Da, addition of the sulfur-containing fragment and loss of 2 HCl), and cross-links formed due to the oxygen-containing reactive intermediate (cross-link type 2; +39.9949 Da, addition of the oxygen-containing fragment and loss of 2 HCl).

β -Lyase Activity in RBCs

A time course enzymatic assay was performed to determine if a prototype cysteine S-conjugate β -lyase substrate, BTC, can be metabolized by the β -lyase activity in RBCs (24). Formation of its stable metabolite, 2-MBT, was monitored over 60 min by HPLC. Freshly isolated washed RBCs from rats ($n=3$) were lysed with equal volume of cold doubly deionized H₂O and hemolysate was centrifuged twice ($10,621 \times g$, 20 min at 4 °C) (28). The supernatant was removed and dialyzed with 10 mM PBS (8.4 mM Na₂HPO₄, 1.6 mM KH₂PO₄, 154 mM NaCl, pH 7.4) against Spectrapor 7 dialysis membrane with the molecular mass cut-off of 15,000 Da (Spectrum Laboratories; Rancho Dominguez, CA). RBC hemolysate (RBCh) was used in incubations after being stored overnight at 4 °C. Protein concentrations were determined according to the method of Lowry et al (29). RBCh (7–9 mg protein/ml) was incubated with 1 mM BTC in the presence and absence of 1 mM KBA, an α -ketoacid used to enhance β -lyase activity (30). Incubations were also performed in the presence and absence of AOAA (0.2 mM), a potent inhibitor of renal β -lyases (31). All chemicals were initially dissolved in 10 mM PBS pH 7.4 except for BTC which was dissolved as described by Dohn and Anders (25). Briefly, initial dissolution of BTC (10 mM) in doubly deionized H₂O (40% of final volume) with addition of 10 μ L of 1 M NaOH was followed by addition of sodium borate decahydrate buffer (0.2 M pH 8.6) (50% of final volume) and final volume was adjusted with doubly deionized H₂O. Incubations were initiated by addition of BTC (30 μ L) after a 5 min preincubation time in the shaking bath at 37°C. The final pH of the reaction mixture was 8.3. At each time point protein from reaction aliquots (60 μ L) was precipitated with ice cold ethanol (90 μ L) and the samples were subjected to centrifugation ($20,817 \times g$, 10 min at 4 °C). The resulting supernatant was filtered through a 0.2 μ M Acrodisc LC13 membrane filters (Pall; East Hills, NY) before HPLC analyses as described below.

S-Oxidase Activity in RBCs

In order to determine if S-oxidase activity could arise due to monooxygenase-like activity of Hb in the presence of H₂O₂ which could form in RBCs during oxidative stress (32), we set up several reactions to model these catalytic processes that may result in DCVC oxidation. Experimental designs similar to those used to demonstrate dibenzothiophene and aniline oxidations by Hb were followed (33,34). Briefly, DCVC (1 mM) was incubated with H₂O₂ (1–5 mM) in 10 mM PBS at pH 7.4, 37°C in the presence and absence of human Hb (0.1–2 mM) and formation of DCVCS over 1 h was monitored by HPLC as described below. To further investigate DCVC oxidation over 1 h by Hb catalysis we incubated human MetHb (1 μ M), P450 reductase (0.02 units, 1 unit equals 1 μ mole of cytochrome c reduced per minute), DCVC (10 mM) with and without NADPH (0.2 mM) in KH₂PO₄ buffer (20 mM, pH 6.8) (34). The concentration of MetHb was determined by the method of Winterbourn et al (35). Reactions (200 μ L) were stopped by the addition of ice cold acetone (600 μ L), centrifuged as described above, and dried using a stream of N₂ before being reconstituted in PBS (10 mM, pH 7.4) and analyzed by HPLC.

Alternatively, horseradish peroxidase (0.002 mM) was used as a model for catalytic activity of Hb in the presence of H₂O₂ (0.5 mM) and DCVC (1 mM) and DCVCS formation was monitored at 37 °C over 1 h. The buffer used with horseradish peroxidase experiments contained 0.1 M KH₂PO₄, 0.15 M KCl, and 2 mM EDTA adjusted to pH 7.4 (33).

HPLC Analyses

HPLC analyses were performed using a Gilson gradient controlled HPLC system (Model 306 pumps) equipped with a Beckman Ultrasphere 5- μ m analytical (4.6 mm $\times$ 25 cm) column. HPLC methods listed below were developed from the original methods described by Elfarra and Hwang for 2-MBT analyses and by Ripp et al. for DCVCS analyses (15,24). UV detection was set at 321 nm (for 2-MBT analyses) and 220 nm (for DCVCS analyses).

Mobile phases for 2-MBT analyses were: pump A, doubly deionized H₂O, pH adjusted to 2.5 with TFA and pump B, 50% ACN, pH adjusted to 2.5 with TFA. Initial 70% B was maintained for 5 min, then increased to 100% over 2 min and held for 4 min. The percent B was then decreased to 70 over 1 min and held for 3 min. Quantitation of 2-MBT was based on peak areas of the standard curve ($r > 0.99$) generated using synthetic 2-MBT (limit of detection was 0.39 μ M).

Mobile phases for DCVCS analyses were: pump A, 1% acetonitrile (ACN), 0.1% TFA and pump B, 20% ACN, 0.1% TFA. Initial 12.5% B was maintained for 5 min, then increased to 90% over 3 min and held for 4 min. The percent B was then decreased to 12.5 over 3 min and held for 3 min. Quantitation of DCVCS diastereomers I and II was based on peak areas of the standard curve ($r > 0.99$) generated using synthesized DCVCS (limit of detection was 2.16 μ M for each diastereomer).

Statistics

Statistical analyses were executed using One Way ANOVA test followed by Student-Newman-Keuls method using SigmaStat (San Jose, CA). Significance was assigned if the values were < 0.05 .

Results

Hemolysis

In vitro incubations of rat RBCs with DCVC (90 and 450 μ M) at physiological conditions (pH 7.4, 37 $^{\circ}$ C) did not cause hemolysis as determined by the hemolysis assay (data not shown). Erythrocytes remained intact after 2 h similar to control incubations without DCVC.

Mass Spectral Analyses. Monoadducted Peptides Modified by β -Lyase-derived Reactive Intermediates

Formation of β -lyase-derived monoadducts with globin was investigated using trypsin digested peptides from 9 and 450 μ M DCVC incubations that were subjected to LC/ESI/MS. In the event that reactive thiol species undergo hydrolysis and the sulfur is replaced by the oxygen before or after reaction with Hb (27), we have also analyzed globin peptides for the corresponding monoadduct type 3 (Figure 1). In addition, we analyzed for peptides modified by GSH conjugates of reactive sulfur and oxygen-containing intermediates (monoadducts types 2 and 4) because GSH is abundant (0.8–2 mM) in RBCs (32,36). At 450 μ M DCVC we detected three modified peptides on both α and β chains (Table 1). The majority of those peptides also displayed the characteristic ^{35}Cl , ^{37}Cl isotopic pattern. Peptide β (121-132) was modified by both monoadducts types 2 and 3. The overlapping sequence between two modified peptides (8-31 and 12-40 on α chains) suggests that four nucleophilic sites (Cys13, Lys16, His20, Arg31) may be involved in reactivity with monoadducts 1 or 2. When RBCs were incubated with 9 μ M DCVC, no monoadducts were detected.

Cross-linked Peptides Modified by β -Lyase-derived Reactive Intermediates

We also analyzed data for cross-links between two globin peptides with sulfur or oxygen-containing intermediates as cross-linkers (cross-links types 1 or 2, respectively) (Figure 1). Two unique peptide cross-links were formed between α chains, β chains, and α and β chains at 450 μ M DCVC (Table 2). Peptide (1-16) (or the short peptide 8-16) is predominantly involved in cross-link type 1 or 2 formation between α chains suggesting that Lys11, Cys13, and Lys16 may be potential targets for these electrophiles. Short β chain peptides [(83-95) and (121-132)] and peptides containing missed cleavages, such as (77-104) and (121-144)/(121-146) were also detected in cross-link types 1 and 2. When globin from 9 μ M DCVC incubation was analyzed, one cross-link was detected each between the two β chains and between α and β chains. The cross-link containing peptides (12-31) and (121-146) has an overlapping amino acid sequence with the cross-link detected at 450 μ M DCVC suggesting that reactivity of the β -lyase-derived fragment is selective toward these peptides. Target nucleophilic amino acids involved in this cross-link formation may be Cys13 and Lys16 of the α chains and Cys125, Lys132, His143, Lys144, or His146 of the β chains.

Peptides Modified by S-Oxidase-derived Monoadducts and Cross-links

Formation of DCVCS in RBCs incubated with DCVC was assessed by examining trypsin digested cysteine-containing peptides of globin from 450 μ M DCVC incubation with RBCs. Digest subjected to LC/ESI/MS and MALDI/MS was analyzed for peptides modified by DCVCS, NA-DCVCS, and DCVCS-GSH monoadducts as described before (21). Since a diadduct of DCVCS with two moieties of NAC was characterized previously and globin cross-links were detected with DCVCS in vitro and in vivo (21,22), peptide cross-link formation with DCVCS or NA-DCVCS as cross-linkers was also analyzed with DCVC incubation. However, no monoadducted or cross-linked peptides containing DCVCS-derived intermediates were detected with either instrument suggesting that S-oxidation of DCVC does not occur in RBCs.

Globin Chain Cross-link Analysis by SDS-PAGE

To determine if there is any cross-link formation between Hb chains, we evaluated cross-link formation in globin from incubations of RBCs with DCVC (9–450 μ M) using SDS-PAGE. Upon addition of 200 mM DTT to reduce disulfide bond formation, dimer bands persisted at all DCVC concentrations and not in the control (Figure 2) which suggested that treatment of RBCs with DCVC gave rise to cross-linking of globin chains that is not due to disulfide bonds.

β -Lyase Activity in RBCs

In order to assess β -lyase activity with cysteine S-conjugates in RBCs, we used a prototype β -lyase substrate, BTC, that generates a stable thiol, 2-MBT, (24) eluting at 7.6 min on HPLC. After cell lysis and dialysis to remove extraneous protein, RBCh was incubated with and without 1 mM BTC and in the presence or absence of 1 mM KBA and 0.2 mM AOAA and formation of 2-MBT was monitored over 60 min. Control reaction with RBCh incubated alone did not give rise to any coeluting peaks. A time-dependent product formation was observed when RBCh was incubated with BTC with and without KBA (Figure 3) corresponding to the elution time of 2-MBT standard suggesting β -lyase presence in RBCs. Formation of 2-MBT in the presence of AOAA was significantly inhibited in reactions containing KBA at 30 and 60 min ($p < 0.05$) (75 and 83%, respectively) as compared to the corresponding 2-MBT levels in reactions without AOAA providing additional evidence for the activity being mediated by a β -lyase present in RBCs.

S-Oxidation in RBCs

We assessed the extent of DCVC oxidation over 1 h when human Hb (0.1–2 mM) or horseradish peroxidase (0.002 mM) was incubated with DCVC (1 mM) in the presence of H₂O₂ (1–5 mM) and (0.5 mM), respectively. We also incubated metHb (1 μ M) and DCVC (10 mM) in the presence of P450 reductase (0.02 units) and NADPH (0.2 mM) to monitor formation of DCVCS over 1 h. Increase in area of DCVCS peaks eluting at 4.0 min (diastereomer I) and 4.3 min (diastereomer II) was not detected in any of these systems suggesting that DCVC is not a substrate for Hb or horseradish peroxidase catalysis. Collectively, these results suggest that oxidation of DCVC to DCVCS in RBCs is unlikely to occur under physiological conditions.

Discussion

In the present study we have used Hb adducts to characterize DCVC bioactivation in intact erythrocytes. Our detection of Hb adducts with β -lyase-derived reactive intermediates after incubation of RBCs with DCVC suggested that DCVC is bioactivated in RBC via β -lyase. β -Lyase activity has been detected in a variety of mammalian tissues (testes, pancreas, spleen, heart, muscle, and brain) besides liver and kidney (37). The presence of aspartate aminotransferase has been previously characterized in erythrocytes (38). Bioactivation of DCVC by aspartate aminotransferases has been demonstrated in the liver, kidneys, and brain (7,39,40). In this study we report data showing that cysteine conjugates (BTC and DCVC) are substrates for β -lyase activity in RBCs. The finding that BTC β -lyase activity was inhibited by AOAA provided further support for BTC being a substrate for β -lyase in male Sprague-Dawley rat erythrocytes. Because β -lyases have different selectivities for cysteine conjugates (41) and BTC was shown to display a much lower activity than DCVC with mitochondrial aspartate aminotransferase (39), BTC may not fully portray total β -lyase activity in RBCs. Bioactivation of DCVC to DCVCS via S-oxidase pathway in RBCs was also studied by investigating the presence of Hb adducts modified by DCVCS and its related products (NA-DCVCS, DCVCS-GSH). Due to high reactivity of DCVCS with N-acetyl-L-cysteine, GSH, and Hb (18,21,22) we expected formation of monoadducts or cross-links between DCVCS and Hb if DCVCS was formed after RBCs were incubated with DCVC. Lack of detectable DCVCS-derived Hb adducts or cross-links by the various MS techniques used suggested lack of formation of DCVCS and no S-oxidase activity in erythrocytes. These results suggest that RBCs are capable of contributing to further DCVC metabolism via the β -lyase pathway, but not via the S-oxidase pathway. Therefore, DCVC bioactivation to DCVCS may only occur outside of the circulation.

Formation of monoadducts (types 1–4) occurs due to nucleophilic addition reaction with nucleophilic residues of Hb resulting in a corresponding mass shift for the unmodified peptide. Although we limited our analyses to cysteine-containing peptides, presence of multiple monoadducts on the same peptides suggests that other nucleophilic residues, such as lysines, histidines, or arginines are also reactive toward sulfur/oxygen-containing reactive species generated by the β -lyase pathway. A β -lyase-dependent reactive metabolite of S-(1,2,2-trichlorovinyl)-L-cysteine (TCVC), dichlorothioketene, was shown to covalently modify renal and hepatic proteins in vivo and an N^ε-(dichloroacetyl)-L-lysine adduct was detected after rat treatment with tetrachloroethene or TCVC (42,43). In addition, upon treatment of rats with fluorinated ethylenes, thioanoyl halide derivative of the resulting cysteine-S-conjugate was shown to react with lysine groups of mitochondrial proteins (44). These results suggest that both chlorothioketene and 2-chlorothioanoyl chloride of DCVC may also react with ϵ -amino groups of proteins. Our study revealed that Hb adducts containing GSH were formed much less than globin monoadducts and cross-links at 450 μ M DCVC. This could be due to the higher concentration of Hb (8–10 mM) in RBC in comparison with that of GSH (0.8–2 mM) (32, 36) making Hb more accessible to reactive electrophiles than GSH. Preference of β -lyase-derived intermediates for amino groups over sulfhydryl groups may have also contributed to

their selectivity. Thus, β -lyase-derived metabolites of DCVC appear to be less selective in their reactivity with proteins than DCVCS which reacts only with cysteines (21,22) suggesting that these species have a higher potential than DCVCS for reactivity with a wider variety of macromolecules.

A second nucleophilic addition reaction between another nucleophilic residue of Hb and a monoadducted β -lyase-derived intermediate followed by elimination of HCl results in formation of a cross-link as evidenced by SDS-PAGE and determined by a corresponding mass shift on LC/ESI/MS and MALDI/MS. Cross-linking between globin chains (due to β -lyase-derived intermediates) was prevalent in contrast to the lack of monoadducted peptides detected at low DCVC concentration (9 μ M) consistent with our previous study where cross-links (due to DCVCS) but not monoadducts were detected at low DCVCS concentration (9 μ M) (21). It is possible that some cross-linking may occur after the DTT treatment of globin during the trypsin digestion step. However, that would only concern sulfhydryl residues of Hb that remained unreacted in intact RBCs and formed disulfide bonds upon globin isolation procedure. Although reactivity of chlorothioketene with DNA base (cytosine) and formation of a monoadduct, N⁴-(chlorothioacetyl)cytosine, and an intramolecular cross-link, (3-N⁴-thioacetyl)cytosine, was previously characterized in vitro in organic solvents (45), to our knowledge cross-linking due to β -lyase-derived intermediates has not been previously demonstrated under physiological conditions.

In evaluating similarities between predominant α chain monoadducted and cross-linked peptides modified by β -lyase-derived reactive intermediates at 9 and 450 μ M DCVC, potential target nucleophilic sites could be narrowed down to Cys13 and Lys16 because these residues overlap in detected peptides (1-16, 8-16, 8-31, 12-31, and 12-40) (Tables 1 and 2). Similarly, Cys125 and Lys132 of β chains may be involved because majority of modified peptides (121-132 and 121-146) at high DCVC concentration contain these residues. Peptides from the β chains involved in cross-link formation at 9 and 450 μ M DCVC contained amino acid sequence 83–95 suggesting that His87, His92, Cys93, or Lys95 could be potential targets for electrophilic attack by β -lyase-derived sulfur/oxygen-containing intermediates at high and low DCVC concentrations. Overall, the relative amount of peptides modified by the sulfur-containing fragment (monoadduct types 1 and 3) was similar to peptides modified by the oxygen-containing fragment (monoadduct types 2 and 4) as indicated by β -lyase-derived monoadduct and cross-link data (Tables 1 and 2). Attempts to obtain adequate MS/MS fragmentation of modified peptides were not successful possibly due to complexity of the mixture, low abundance of these ions, and the presence of multiple signals representing different types of adducts/cross-links. However, the use of mass spectrometry with different ionization methods provided corroboration for the presence of multiple monoadducted and cross-linked peptides.

To confirm lack of detectable DCVCS-Hb adducts with lack of S-oxidase activity in RBCs, we investigated the presence of S-oxidase activity in RBCs by investigating monooxygenative-like properties of Hb. Environment inside erythrocytes is rich in oxygen and heme-containing Hb (8–10 mM) predisposing these cells to constant oxidative stress (36). Autooxidation of Hb (2–3% a day) to methHb followed by dismutation of superoxide is a major source of H₂O₂ generation in RBCs (32). In the presence of H₂O₂ certain substrates, such as dibenzothiophene and chlorpromazine, are oxidized by monooxygenative and peroxidative catalysis of Hb and peroxidases, respectively, in vitro (33,46). In addition, because P450 can mimic monooxygenase activity of Hb in RBCs, a reconstituted system containing rat liver cytochrome P450 reductase, NADPH, and human Hb with aniline as a substrate gave rise to an oxidation product of aniline (47,48). Failure to detect DCVCS formation with any of these experimental designs suggested that RBCs do not contribute to bioactivation of DCVC via S-oxidation.

In the present study, we detected multiple globin monoadducts and cross-links with reactive intermediates generated by the β -lyase pathway and characterized β -lyase activity in RBCs which suggests that RBCs represent an additional compartment for DCVC metabolism via the β -lyase pathway. Because we did not identify any DCVCS-Hb adducts and due to the lack of S-oxidase activity in RBCs, bioactivation of DCVC to DCVCS is not likely to occur in the circulation. Thus, any detection of DCVCS in the circulation would likely be due to its formation and translocation from other tissues, i.e. liver. Unlike DCVCS which reacts only with cysteine residues (21,22), identification of β -lyase-derived monoadducts and cross-links with different nucleophilic residues of Hb in this study suggests that DCVC bioactivated via β -lyases could yield reactive sulfur/oxygen-containing species that react with a wide spectrum of nucleophilic residues on macromolecules forming monoadducts and cross-links.

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Abbreviations

ACN	acetonitrile
AOAA	aminoxyacetic acid
BTC	S-(2-benzothiazolyl)-L-cysteine
β-lyase	cysteine conjugate β -lyase
DCVG	S-(1,2-dichlorovinyl)-L-glutathione
DCVC	S-(1,2-dichlorovinyl)-L-cysteine
DCVCS	S-(1,2-dichlorovinyl)-L-cysteine sulfoxide
ESI-QTOF	Electrospray-Quadrupole Time-Of-Flight
FMO3	flavin-containing monooxygenase 3
GTK	glutamine transaminase K
KBA	alpha-ketobutyric acid sodium salt monohydrate
MALDI-TOF²	Matrix Assisted Laser-Desorption Ionization/Time-Of-Flight ²
2-MBT	2-mercaptobenzothiazole

NAC	N-acetyl-L-cysteine
NA-DCVCS	N-acetyl-S-(1,2-dichlorovinyl)-L-cysteine sulfoxide
RBC	red blood cell
RBCh	red blood cell hemolysate
TFA	trifluoroacetic acid
TCE	trichloroethylene.

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Figure 1.

Cysteine conjugate β -lyase dependent metabolism of S-(1,2-dichlorovinyl)-L-cysteine (DCVC) and proposed mechanism for formation of Hb monoadducts and cross-links with sulfur (oxygen)-containing reactive intermediates. Hydrolysis that could occur before and/or after reaction with Hb results in substitution of sulfur for oxygen (27) and formation of the corresponding Hb monoadducts (types 3 and 4) and cross-link (type 2) could then result where the sulfur atom is replaced by the oxygen. Hb is used here to describe general types of adducted globins without specification of which globin chain is involved. ^aMonoadducts (types 2 and 4) can also arise by initial conjugation with GSH followed by reaction of GSH conjugate with Hb.

Figure 2. SDS-PAGE of globin (2.5 µg loaded) after incubation of RBCs with and without DCVC (9–450 µM) for 2 h at pH 7.4 and 37 °C. The table below contains typical values for dimer band intensities using optical density measurements. Fold values refer to densities with DCVC over density without DCVC. Monomers display two bands (α chains- 15153 (α_1), 15197 (α_2) Da and β chains- 15848 (β_1), 15861 (β_2) Da). Standards and globin were run on the same gel.

Figure 3.

Formation of 2-MBT over time when RBC hemolysates (RBCh) were incubated with 1 mM BTC in the presence and absence of 1 mM KBA and 0.2 mM AOAA. Values are means $\pm$ SD from 3 experiments. ^aSignificantly higher ($p < 0.05$) than the corresponding values in the presence of AOAA at 30 (with KBA) and at 60 min (with and without KBA).

LC/ESI/MS and MALDI/MS Results of β -lyase-derived Globin Peptide Monoadducts (types 1, 2, 3, and 4; see Figure 1 for structures) after Incubation of RBCs with 450 μ M DCVC for 2 h at 37 °C.

Table 1

α chain peptides	Sequence	Monoadduct type (#) ^a	Mass _{theo} (Da)	Mass _{obs} (Da)	MS
(8-31) ^b	TNKNCWG K IGGHGGEYGEEALQR ^d	2 (3)	3706.4258	3706.2383	LC/ESI
(12-40) ^b	NCWG K IGGHGGEYGEEALQRMFAAFPTTK	1 (4)	3523.2781	3523.1331 ^e	LC/ESI
(93-127) ^b	VDPVNF K FLSHCLLVTLA CH HPGDFTFAMHASLDK	3 (1)	3936.8795	3936.7815	LC/ESI
β chain peptides					
(83-95) ^c	GTFAHLSELHCDK	3 (3)	1717.5538	1717.6759 ^e	LC/ESI
(121-132) ^c	EFTPCAQAQAFQK	3 (2)	1508.5512	1508.6398 ^e	LC/ESI
(121-132) ^c	EFTPCAQAQAFQK	2 (1)	1703.6868	1703.5432 ^e	LC/ESI

^a # refers to the number of monoadducts on a peptide

^b Peptides α 1 (8-31), (12-40), and (93-127) can also be from α 2.

^c Peptides β 1 (83-95) and (121-132) can also be from β 2.

^d Residues in bold represent possible nucleophilic sites where reaction may occur

^e Monoisotopic masses displayed characteristic ³⁵Cl, ³⁷Cl isotopic pattern

Table 2

LC/ESI/MS and MALDI/MS Results of β -lyase-derived Globin Peptide Cross-links (types 1 and 2; see Figure 1 for structures) after Incubation of RBCs with DCVC for 2 h at 37 °C.

peptides cross-linked between	mass _{theo} (Da)	mass _{obs} (Da)	cross-linker type	MS
450 μM DCVC				
α chains				
$\alpha 1$ (1-16) + $\alpha 1$ (1-16)	3622.8075	3622.7271	2	LC/ESI
$\alpha 1$ (1-16) + $\alpha 1$ (100-127) ^a	5000.3121	5000.5464	1	LC/ESI
β chains				
$\beta 1$ (121-132) ^b + $\beta 1$ (121-144) ^b	3823.9018	3823.9792	2	LC/ESI
$\beta 1$ (83-95) ^b + $\beta 1$ (121-132) ^b	2853.2877	2853.2858	1	MALDI
$\beta 1$ (83-95) ^b + $\beta 1$ (121-132) ^b	2837.3105	2837.3266	2	MALDI
α and β chains				
$\alpha 1$ (1-16) + $\beta 1$ (121-146) ^b	4591.2767	4591.3613	1	LC/ESI
$\alpha 1$ (8-16) ^a + $\beta 1$ (77-104) ^b	4388.1535	4388.1172	2	LC/ESI
9 μM DCVC				
β chains				
$\beta 1$ (83-104) ^b + $\beta 1$ (77-104) ^b	5893.5787	5893.8281	2	LC/ESI
α and β chains				
$\alpha 1$ (12-31) ^a + $\beta 1$ (121-146) ^b	4963.5187	4963.4291	1	LC/ESI

^aPeptides $\alpha 1$ (8-16), (12-31), and (100-127) can also be from $\alpha 2$

^bPeptides $\beta 1$ (77-104), (83-95), (83-104), (121-132), (121-144), and (121-146) can also be from $\beta 2$