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Detection of Multiple Globin Monoadducts and Cross-Links after in Vitro Exposure of Rat Erythrocytes to S-(1,2-Dichlorovinyl)-L-cysteine Sulfoxide and after in Vivo Treatment of Rats with S-(1,2-Dichlorovinyl)-L-cysteine Sulfoxide

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Abstract

S-(1,2-Dichlorovinyl)-L-cysteine sulfoxide (DCVCS), a Michael acceptor produced by an FMO3-mediated oxidation of the trichloroethylene metabolite S-(1,2-dichlorovinyl)-L-cysteine (DCVC), is a more potent nephrotoxicant than DCVC. Because DCVCS incubations with N-acetyl-L-cysteine at pH 7.4, 37°C resulted in formation of three diastereomeric monoadducts and one diadduct, globin monoadducts and cross-links formed after in vitro incubations of rat erythrocytes with DCVCS (0.9–450 μM) for 2 h and those present at 30 min after in vivo treatment of rats with DCVCS (23 and 230 μmol/kg) were characterized. ESI/MS of intact globin chains revealed adduction of 1 DCVCS moiety on the β2 chain at the three lowest DCVCS concentrations and on the β1 chain after the in vivo treatment with 230 μmol/kg DCVCS. Interestingly, intact globin dimers and trimers were detectable by ESI/MS with all DCVCS concentrations in vitro (also by SDS-PAGE) and in vivo. LC/MS and MALDI/FTICR of trypsin digested peptides from globin samples obtained after in vitro (450 μM DCVCS) or in vivo exposure to DCVCS (230 μmol/kg) suggested formation of DCVCS monoadducts not only with Cys93 and Cys125 of the β chains, but also with Cys13 of the α chains, whereas no monoadducted peptides were detected at lower DCVCS concentrations in vitro or in vivo. However, LC/MS and MALDI-TOF/TOF suggested the presence of several DCVCS-derived peptide cross-links both in vivo and in vitro at all DCVCS exposure levels. Collectively, the results indicate at least 4 out of the 5 cysteine moieties of the rat hemoglobin heterodimer may be alkylated by DCVCS, in reactions that could also lead to formation of multiple cross-links. DCVCS- and N-acetyl-DCVCS (NA-DCVCS)-derived globin cross-links containing GSH and Cys were also detected by mass spectrometry, providing strong evidence for the reactivity and/or cross-linking ability of DCVCS, NA-DCVCS and their GSH or Cys conjugates both in vitro and in vivo. Thus, hemoglobin adducts and cross-links may be useful biomarkers to investigate possible presence of DCVCS in the circulation after DCVC or trichloroethylene exposure.

Introduction

Trichloroethylene (also known as trichloroethene; TCE) [1] is widely used in industry as a metal degreaser and 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 (VHL)* tumor suppressor gene. A metabolic route that is believed to be carcinogenic is the GSH-

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dependent pathway which begins with the formation of S-(1,2-dichlorovinyl)glutathione (DCVG) primarily in the liver (2) (Figure 1). DCVG was detectable in blood of humans within 30 min after exposure to TCE and its presence in blood persisted for up to 12 h (3). After the translocation of DCVG into the circulation or excretion into bile, it can be cleaved by γ-glutamyl transpeptidase and cysteinylglycine dipeptidases present in kidney cells, the luminal membrane of the bile duct epithelium, the bile canalicular membrane of hepatocytes, and the intestinal lumen to yield the cysteine S-conjugate, S-(1,2-dichlorovinyl)-L-cysteine, (DCVC). Formation of DCVC can be followed by urinary excretion of DCVC mercapturic acid, N-acetyl-DCVC (NA-DCVC), or further bioactivation of DCVC by cysteine conjugate β-lyase to yield reactive intermediates (Figure 1). NA-DCVC has been detected in the urine of humans after accidental (4) and occupational exposure to TCE (5,6).

Another pathway of DCVC bioactivation was characterized using recombinant enzymes as well as rat, rabbit, and human kidney and liver microsomes (7,8,9). In this pathway, DCVC serves as a substrate for S-oxidation by FMO3 which is present primarily in the liver. The resulting metabolite is S-(1,2-dichlorovinyl)-L-cysteine sulfoxide (DCVCS) (Figure 1), a reactive electrophile that was a much more potent nephrotoxicant to rats than DCVC in vivo (10). DCVCS produced necrosis and apoptosis, mitochondrial dysfunction, and GSH depletion in primary cultures of human proximal tubular cells (11). In rats treated with DCVCS, a stable GSH adduct, S-[1-chloro-2-(S-glutathionyl)vinyl]-L-cysteine sulfoxide, has been isolated and characterized from bile, and GSH depletion was observed in both liver and kidney (12). The reactivity of DCVCS as a Michael acceptor was recently investigated in vitro by incubating DCVCS with nucleophilic amino acids at physiological conditions (13). Three diastereomeric monoadducts and a novel diadduct containing two moieties of N-acetyl-L-cysteine (NAC) were characterized when DCVCS was incubated with NAC. These adducts were formed by the Michael addition of one molecule of NAC to the terminal vinylic carbon of DCVCS or the DCVCS monoadduct followed by loss of HCl. Because we have further established that DCVCS was not reactive with N-acetyl-L-lysine or L-valinamide, these results provided evidence for the selectivity of DCVCS towards NAC and suggested a potential for formation of DCVCS adducts and cross-links with proteins containing cysteine residues.

Several Michael acceptors, such as hydroxymethylvinyl ketone (14), 4-hydroxy-2-nonenal (15), and crotonaldehyde (16) have been shown to form covalent adducts with nucleophilic amino acids of the blood protein, hemoglobin (Hb). Hb adducts allow detection of short-lived intermediates present in the circulation at low levels over an extended period of time (126 days for humans, 60 days for rats) (17). Therefore, Hb adducts may be useful as biomarkers of exposure because they can provide information about reactive intermediates that may be formed in the liver and transported into the circulation at low exposure conditions.

In this study, we investigated potential formation of DCVCS monoadducts and cross-links with the cysteine residues of Hb after in vitro exposure of rat RBCs to DCVCS and after treatment of rats with DCVCS in vivo. Because DCVCS readily reacts with GSH both in vitro and in vivo (12) and is likely to react with Cys, formation of DCVCS-GSH and DCVCS-Cys conjugates (Figure 2), respectively, could occur in liver or blood due to the presence of these endogenous thiols in hepatocytes and erythrocytes. These conjugates could then undergo further nucleophilic addition-elimination reaction with sulfhydryl groups of Hb to yield the corresponding monoadducts (Figure 2). Therefore, we also investigated formation of Hb

[1]Abbreviations: ACN, acetonitrile; DCVC, S-(1,2-dichlorovinyl)-L-cysteine; DCVG, S-(1,2-dichlorovinyl)glutathione; DCVCS, S-(1,2-dichlorovinyl)-L-cysteine sulfoxide; ddH_2O, double deionized water; ESI-TOF, Electrospray-Time-Of-Flight; ESI-QTOF, Electrospray-Quadrupole Time-Of-Flight; Hb, hemoglobin; ESI-LTQ, Electrospray-Linear Trap Quadrupole; MALDI-FTICR, Matrix Assisted Laser-Desorption Ionization/Fourier Transform Ion Cyclotron Resonance; 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.

adducts and cross-links containing DCVCS-Cys and DCVCS-GSH. Furthermore, DCVCS could undergo N-acetylation (Figure 1) primarily in the liver leading to the formation of the following conjugates: NA-DCVCS, NA-DCVCS-Cys, and NA-DCVCS-GSH (Figure 2). Formation of these N-acetylated conjugates in RBC or their transport into the RBC from the circulation can lead to formation of Hb adducts and cross-links containing NA-DCVCS, NA-DCVCS-Cys, and NA-DCVCS-GSH as shown in Figure 2.

Several mass spectrometry techniques were used to detect Hb adducts and cross-links following in vitro exposure of RBCs to DCVCS and after dosing of Sprague-Dawley (SD) rats with high (nephrotoxic) and low doses of DCVCS. Because DCVCS and its metabolites could react with multiple sites of both α and β chains of Hb making the concentration of each specific adduct small, multiple ionization techniques were used to corroborate the evidence and to maximize the potential for detecting DCVCS monoadducts and cross-links both before and after the globin chains were hydrolyzed with trypsin. SDS-PAGE was also used to detect presence of Hb dimers and trimers after in vitro and in vivo exposure to DCVCS.

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 Ames test (18).

Materials

Trifluoroacetic acid 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). SDS, Tris-HCl Criterion precast gels (12.5% and 15%), DTT, glycine, low range molecular weight standard for silver staining 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 and DCVCS were synthesized and characterized as previously described (8,12). Purity of DCVCS was determined to be >99% by HPLC analysis.

Animals

Male Sprague-Dawley rats (190–330g), purchased from Harlan (Madison, WI), were maintained on a 12 h light/dark cycle and given water and food ad libitum. This strain was chosen because the amino acid sequences for its globin chains are known (www.ncbi.nih.gov, 19). For in vivo experiments, three rats were each injected i.p. with a single dose of 230 μmol/kg of DCVCS dissolved in saline to a final concentration of approximately 5 mg/mL. Previously, this DCVCS dose caused an 8-fold increase in BUN concentrations at 24 h after injection and caused anuria and severe proximal tubular necrosis (10). In addition, 4 rats were each injected i.p. with a single dose of 23 μmol/kg of DCVCS. Two rats were injected i.p. with saline to obtain control globin samples for the different analyses. Rats were sacrificed 30 min after dosing by CO_2 asphyxiation because the half-life of DCVCS in the presence of GSH was estimated to be 1 min (8). In reacting DCVCS with NAC in vitro, the half-lives of DCVCS diastereomers I and II were very short (13.8 and 9.4 min, respectively) as well (13). For the in vitro experiments, rats did not undergo any injections before being sacrificed. Heparinized whole blood was obtained through cardiac puncture and processed immediately to obtain RBCs for in vitro experiments.

In vitro incubations of DCVCS with erythrocytes

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 3,000 rpm for 5 min in between washes. Erythrocytes were resuspended in an equal volume of PBS (8.4 mM Na_2HPO_4, 1.6 mM KH_2PO_4, 154 mM NaCl; pH 7.4) before incubation with DCVCS (0.9, 4.5, 9, 90, 450 μM - final concentrations) in a Dubnoff metabolic shaking water bath for 2 h at 37°C. These concentrations were chosen because in primary cultures of human renal proximal tubular cells more than 50% GSH depletion was observed with 500 μM DCVCS (11). An incubation containing buffer rather than DCVCS served as the control. At the end of the incubation, RBCs were lysed with equal volume of cold doubly deionized H_2O (dd H_2O) and globin was isolated using acidified acetone as described previously (20).

Hemolysis

To be certain that the DCVCS 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 DCVCS (90 or 450 μM) at 0–2 h using a spectrophotometric assay (21,22). Briefly, a 10% erythrocyte stock solution was prepared with PBS at each time point and with each concentration. An aliquot (0.125 mL) was removed and 1.75 mL of PBS added each time. To the 0.125 mL of 10% erythrocyte solution containing no DCVCS, 1.75 mL of PBS or ddH_2O was added and used as a negative control and positive control, respectively. All samples were centrifuged at 2,000 rpm for 5 min and the supernatant analyzed at 543 nm. Percent hemolysis at each time point was determined using the following formula: $((Abs^{DCVCS}\text{-}Abs^{(-)\ control})/Abs^{(+)control}) \times 100$ (23).

Globin chain cross-link analysis by SDS-PAGE

Globin samples from in vitro incubations (9–450 μM DCVCS and control) and from rats treated with DCVCS or saline only were investigated for globin chain cross-link formation using SDS-PAGE. Globin (2.5 μg) dissolved in 2.5 μL ddH2O was added to treatment buffer (0.5 M Tris-HCl, 10% glycerol, 10% SDS, and 0.01 g/mL bromophenol blue, 10% 2-mercaptoethanol). After addition of DTT (200 mM- final concentration) (24) to reduce dimer or trimer formation between globin chains that may be due to disulfide bonds, the samples were kept for 30 min at room temperature before being loaded onto a 12.5 % Tris-HCl Criterion Precast Gel (15% for in vivo samples) and run for 75 min at 200 V. Samples without DTT treatment were used as control. Gels were silver stained for visualization. Dimer density measurements were analyzed using Quantity One software (Bio-Rad Laboratories; Hercules, CA).

Electrospray (ESI)/MS of intact globin chains

Acetone precipitated globin was dissolved in 0.1% TFA (10 mg/mL) containing 200 mM DTT. Samples were desalted with C-18 solid-phase zip tips (Millipore; Billerica, MA), dried, and redissolved in ACN:H_2O (50:50) before analysis on ESI-Time-Of-Flight (TOF) mass spectrometer (Agilent; Santa Clara, CA). The spectra were scanned with a mass range 200–1700 m/z and charge series of ions were deconvoluted using Analyst software (Applied Biosystems version 1.3.1).

Analysis of intact globin chains for monoadducts and cross-links

A peak list of observed masses was imported into Microsoft Access for comparison to the lists of theoretical masses of modified globin chains (α1, α2, β1, and β2). All theoretical mass lists accounted for the number of reactive sites (i.e. cysteines) present in each chain (3 for the αchains, 2 for the β chains). Parameters to identify adducted chains included masses that were within ± 2 Da for monomers (15,000–18,000 Da), ± 4 Da for dimers (30,000–33,000 Da), and ± 5 Da for trimers (45,000–49,000 Da) to reflect the expected instrument error. A modified

chain or cross-link was identified when the matched peaks were above the noise level that was assigned based on spectral windows for each mass range. Intact single globin chains from in vitro samples were analyzed for the following monoadducts: DCVCS (+195 Da; addition of DCVCS moiety and loss of HCl), DCVCS-GSH (+466 Da; addition of DCVCS-GSH conjugate and loss of HCl), DCVCS-Cys (+280 Da; addition of DCVCS-Cys conjugate and loss of HCl), and NA-DCVCS (+237 Da; addition of NA-DCVCS and loss of HCl) (Figure 2). The intact single globin chains from in vivo samples were also analyzed for the same monoadducts as well as for NA-DCVCS-GSH (+508 Da; addition of NA-DCVCS-GSH conjugate and loss of HCl) and NA-DCVCS-Cys (+322 Da; addition of NA-DCVCS-Cys conjugate and loss of HCl) (Figure 2). In vitro data were also analyzed for masses that matched formation of dimers and trimers between all 4 globin chains with DCVCS as a cross-linker (+159 Da; addition of DCVCS and loss of 2 HCl molecules) of globin chains with and without monoadducts of DCVCS, DCVCS-GSH, DCVCS-Cys, and NA-DCVCS (Figure 2). In vivo data were also analyzed for dimers and trimers with both DCVCS (+159 Da) or NA-DCVCS as a cross-linker (+201 Da; addition of NA-DCVCS and loss of 2 HCl molecules) of globin chains with and without the monoadducts listed above for in vivo analysis of intact single chains.

Trypsin digestion of globin

Globin from in vivo samples and from RBCs incubated with 450 μM DCVCS along with their respective controls was trypsin digested for modified peptide analysis. Proteolytic digestion of precipitated globin samples was carried out in 6 M urea/100 mM ammonium bicarbonate (pH 8.2) at 37 °C for 16 h. The enzyme/substrate ratio was 1/30 (w/w). The reaction was stopped by adding TFA to bring the solution to pH <3 before analysis by LC/ESI/MS and MALDI-TOF/TOF/MS.

Mass spectra of whole digest

Tryptic digest samples were desalted using C-18 solid-phase zip tips before being loaded onto a Zorbax (Agilent) C_{18} stable bond column (0.075 mm × 150 mm, 5μ, 300 Å) equipped with a Micromass Electrospray Hybrid Quadrupole Orthogonal Time-Of-Flight mass spectrometer (ESI-QTOF/MS). The LC method was as follows: mobile phase A was 0.1% formic acid in H_2O and mobile phase B was 0.1% formic acid in ACN. Peptides were eluted with a linear gradient of mobile phase B over 120 min with a flow rate of 200 nL/min. Data were collected in survey mode via data-dependent switching using charge state recognition with ions up to 4 + charge state. Data were deconvoluted by the MaxEnt function of MassLynx 4.0. Some samples were analyzed using a Linear Trap Quadrupole (LTQ) Orbitrap XL mass spectrometer (Thermo Scientific; Waltham, MA) coupled to an HPLC system carrying a Zorbax (Agilent) C_{18} stable bond column (0.075 mm × 100 mm, 3μ, 300 Å). Mobile phase A was 0.1% formic acid in H_2O, and mobile phase B was 95% ACN, 0.1% formic acid. Initially, 1% B was maintained for 20 min. The percent B was then increased to 12% over 10 min, to 50% over 105 min, to 60% over 5min, to 100% over 5 min, then held for 5 min before being decreased to 1% over 2 min and held for 40 min.

Whole digests from 450 μM DCVCS and control incubations were also subjected to a MALDI-TOF/TOF 4800 mass spectrometer (Applied Biosystems; Foster City, CA) and analyzed in a linear mode with parameters determined to be optimum for proteins. Solutions of tryptic digest (0.5 uL each) were applied to a target plate followed by the addition of 0.5 uL of a saturated matrix solution of α-cyano-4-hydroxy-cinnamic acid (6 mg/ml in ACN:H_2O (70:30) with 0.2% TFA). Spectrum was collected using 1,500 laser shots and cytochrome c (12,361 Da) was used as a calibration standard.

HPLC separation of tryptic digests

To enrich the concentration of modified peptides in order to localize specific sites of peptide modification through tandem MS analysis, some globin from incubation of RBCs with 450 μM DCVCS and the corresponding control were trypsin digested for 7 h. The digest was not acidified before fraction collection by HPLC. Separation of peptides was accomplished using Vydac C_{18} protein and peptide HPLC column (5μ, 4.6 mm × 25 cm) at a flow rate of 1 mL/min and monitored at $A_{220.}$ Mobile phase A was 0.1% TFA in H_2O, and mobile phase B was 0.1% TFA in ACN. Initially, 9% B was maintained for 5 min. The percent B was then increased from 9 to 65 from 5 min to 68 min and held for 5 min. The percent B was then decreased from 65 to 9 from 73 to 75 min and held for 5 min. Peptide fractions were collected in 2–3 min windows between the 8–36 min time interval and lyophilized to dryness. Peptide fractions were redissolved in 40 μL methanol:H_2O (50:50) before being subjected to MS.

Mass spectra of trypsin digested fractions

Mass spectrum of each fraction was obtained on a Varian (Palo Alto, CA) IonSpec ProMALDI FTICR mass spectrometer equipped with a 7 tesla superconducting magnet. IonSpec Omega software version 8.0 was used for data acquisition and processing. The sample solution (0.4 μL) was mixed with (0.4 μL) of dihydroxybutyric acid matrix solution, spotted on a MALDI target plate and allowed to dry. External calibration was achieved using a standard peptide mixture (Angiotensin I, and II, arginine vasopressin, substance P, Phe-Met-Arg-Phe-NH_2, and somatostatin). Fractions that contained modified peptides were also subjected to MALDI-TOF/TOF.

Mass spectral analysis of tryptic peptides

Theoretical lists of monoisotopic masses for modified peptides (including one and two missed cleavages) were calculated for the following monoadducts: DCVCS (+194.9757 Da), DCVCS-GSH (+466.0828 Da), DCVCS-Cys (+280.0188 Da), and NA-DCVCS (+236.9862 Da). Cross-linked peptides with DCVCS as cross-linker (+158.9990 Da) were also investigated. These modifications were analyzed for in both the in vitro and the in vivo samples. Additional peptides that contained NA-DCVCS-Cys (+322.0293 Da) and NA-DCVCS-GSH (+508.0933 Da) as well as cross-linked peptides with NA-DCVCS (+201.0096 Da) as a cross-linker were searched for in the in vivo samples. The list of Cys-containing peptides was built from the virtual digest of Hb chains by Protein Prospector (www.prospector.ucsf.edu). The generated peak list containing high resolution monoisotopic masses of peptides in the mixture as well as theoretical mass lists were imported into Microsoft Access where queries were set up to search for matches. The criteria for finding modified peptides were as follows: the peak representing monoisotopic mass that was a match was absent in control samples and allowable mass error was set to ±0.25 Da for LC/ESI-QTOF/MS, ±3 ppm (for average mass ±1.2 Da) for LC/ESI-LTQ Orbitrap/MS, and ±25 ppm for MALDI-TOF/TOF and MALDI-FTICR based on the expected instrument error. Masses and their intensities from LC/MS results were plotted on SigmaPlot (San Jose, CA) to establish relative level of modification based on signal to noise ratio within various mass ranges.

Results

Mass Spectral Analyses -In vitro Samples

DCVCS monoadducts on intact globin chains—Freshly isolated washed RBCs were incubated with 0.9, 4.5, 9, 90, and 450 μM DCVCS at pH 7.4, 37°C for 2 h. After globin was acetone precipitated all samples were subjected to ESI/MS for intact protein analysis. The presence of adducts was determined based on a mass shift of the unmodified chain. Theoretical mass shifts were (+195 Da) for 1 monoadduct of DCVCS, (+390 Da) for 2 DCVCS

monoadducts, and (+585 Da) for 3 DCVCS monoadducts. Since we previously established that DCVCS forms adducts preferentially with NAC (9), the expected alkylation sites were cysteines: three on each a chain (Cys13, Cys104, and Cys111) and two on each β chain (Cys93 and Cys125). Our analysis of all incubations revealed the presence of 1 DCVCS monoadduct on β2 chain at the three lowest DCVCS concentrations (0.9, 4.5, and 9.0 μM; data not shown). These results suggest selective DCVCS alkylation of β2 chains. With our limited criteria (up to 3 DCVCS monoadducts), we did not detect DCVCS monoadducts at 90 and 450 μM concentrations of DCVCS, but this could also be due to preferential formation of DCVCS-GSH monoadducts, DCVCS-Cys monoadducts, NA-DCVCS monoadducts, and/or formation of cross-links at these concentrations (see below).

DCVCS monoadducts on peptides—In order to identify which cysteine-containing peptides were modified by DCVCS (+194.9757 Da), globin from control, 9 μM and 450 μM DCVCS incubations was trypsin digested and subjected to LC/ESI/MS. Observed masses were matched with the theoretical masses of DCVCS modified cysteine-containing peptides, however, no DCVCS monoadducts were detectable by this method. The globin from 450 μM DCVCS incubation was then subjected to MALDI-TOF/TOF MS (Table 1A) which revealed a modified Cys125-containing peptide on β1/β2 chain consistent with the monoadduct detected on intact β2 chain at low DCVCS concentrations (0.9, 4.5, and 9 μM). Digest of the 9 μM DCVCS incubation, however, did not reveal the presence of DCVCS monoadducts.

In order to enrich the concentration of modified peptides for identifying specific reactive cysteines, the digested globin from control and 450 μM DCVCS incubations were fraction collected in specified time intervals using HPLC. Concentrated fractions were subjected to MALDI-FTICR MS and analyzed for matches to the theoretical masses of cysteine-containing peptides modified by DCVCS that were not in control globin. Fractions at retention times 20.5–22.5 min and 26.6–28.5 min revealed masses consistent with a DCVCS monoadduct on Cys13-containing peptides on α1 and α2 chains, respectively (Table 1B). As expected, unmodified peptides eluted earlier than their modified counterparts (data not shown).

DCVCS-GSH monoadducts

Since the presence of GSH in RBCs could lead to its conjugation with DCVCS and the formed GSH conjugate could still act as a Michael acceptor, we investigated formation of DCVCS-GSH monoadducts with Hb chains (Figure 2). Similar to DCVCS monoadduct analysis, theoretical masses of adducted single globin chains were calculated according to the following mass shifts: (+466 Da) for 1 monoadduct of DCVCS-GSH, (+932 Da) for 2 DCVCS-GSH monoadducts, and (+1,398 Da) for 3 DCVCS-GSH monoadducts. There were no DCVCS-GSH adducts detected on any single chain of Hb suggesting preferential reactivity of DCVCS with globin chains. When globin from 450 μM DCVCS incubation was trypsin digested, neither LC/MS nor MALDI-TOF/TOF MS revealed any adducted peptides with the mass shift of 466.0828 Da in the whole digest. However, fraction 20.5–22.5 min analyzed by MALDI-TOF/TOF MS revealed a mass of 3083.3322 Da (Table 1B) corresponding to a Cys13-containing peptide on the α1/α2 chain modified by DCVCS-GSH.

DCVCS-Cys monoadducts

Cysteine is another biological nucleophile that is present in the circulation and could potentially react with DCVCS; therefore, we investigated the addition of DCVCS-Cys conjugate to Hb chains (Figure 2). Theoretical mass shifts of unmodified chains were (+280 Da) for 1 monoadduct of DCVCS-Cys, (+560 Da) for 2 DCVCS-Cys monoadducts, and (+840 Da) for 3 DCVCS-Cys monoadducts. No monoadducts were detected on any intact single chain, and neither LC/MS nor MALDI-TOF/TOF analysis of the whole digest revealed any adducts with a mass shift of 280.0187 Da. However, a modified peptide (83–95) on β1/β2 chain was detected

by both MALDI-TOF/TOF and MALDI-FTICR in the two sequential fractions of the digest (Table 1B).

NA-DCVCS monoadducts

We also investigated the addition of NA-DCVCS to Hb chains (Figure 2). Theoretical mass shifts of unmodified chains were: (+237 Da) for 1 monoadduct of NA-DCVCS, (+474 Da) for 2 NA-DCVCS monoadducts, and (+711 Da) for 3 NA-DCVCS monoadducts. Globin was also analyzed for cross-links containing multiple NA-DCVCS monoadducts with multiple DCVCS moieties as cross-linkers (+159 Da). Although no monoadducted single chains were detectable, LC/ESI/MS of the whole digest did reveal a Cys93-containing peptide (77–104) on β1/β2 chain with a mass shift of 237.9863 Da consistent with an NA-DCVCS monoadduct (Table 1A). When fractions of digest were analyzed by MALDI-TOF/TOF and MALDI- FTICR, no additional adducts were revealed.

Intramolecular cross-linking

We have previously shown that DCVCS can form a diadduct with two molecules of NAC which suggested cross-linking potential with two Cys residues of Hb chains (Figure 2). Therefore, we investigated intramolecular adduct formation due to DCVCS within each globin chain. A mass shift of +159 Da indicated cross-linking by DCVCS within β1 chains at 0.9 and 9 μM DCVCS concentrations (data not shown) suggesting that both Cys93 and Cys125 may be reactive toward DCVCS.

Intermolecular cross-links

Globin from all RBC incubations with DCVCS was analyzed using ESI/MS for insight into the composition of cross-links. A chart of theoretical masses was created that included all possible combinations of dimers and trimers between α1, α2, β1, and β2 chains formed due to DCVCS. The list of theoretical masses accounted for multiple additions of DCVCS as a cross-linker (+159 Da) between various globin chains based on the number of cysteine residues in each chain. The dimer between α1 and β1 was detected at all but the 90 μM DCVCS concentration (Table 2) but the negative results with the 90 μM DCVCS concentration could be due to preferential formation of trimer cross-links at this concentration (Table S1). Trimers consisting of α1α1α2, α2α2β1, α2α2β2, and α1α2β2 were present at more than one DCVCS concentration (Table S1).

Globin chain cross-links with additional DCVCS monoadducts

ESI/MS data was also analyzed for cross-links containing multiple DCVCS moieties as cross-linkers (+159 Da) with multiple DCVCS monoadducts (+195 Da) according to the number of cysteines in each chain. Dimers consisting of α1α2 and α2β2 (Table 2) and trimers consisting of α1β2β2, α2β1β1, α2α2β1, α2α2β2, and α1α2β2 (Table S1) were detected at more than one DCVCS concentration.

Globin chain cross-links with additional DCVCS-GSH monoadducts

We have further investigated formation of globin cross-links containing DCVCS-GSH monoadducts. The theoretical list of masses accounted for multiple additions of DCVCS as a cross-linking agent (+159 Da) and multiple additions of DCVCS-GSH monoadducts (+466 Da) based on the number of cysteine positions in a chain. Dimer cross-links with additional DCVCS-GSH monoadducts were detected only at 9 and 90 μM DCVCS (Table 2). All DCVCS concentrations exhibited trimer α1α1α2 cross-links with additional DCVCS-GSH monoadducts (Table S1). Trimers α2α2β1 and α2β2α2 cross-links with additional DCVCS-GSH monoadducts were also detectable with at least two DCVCS concentrations (Table S1).

Globin chain cross-links with additional DCVCS-Cys monoadducts

Globin was also analyzed for cross-links containing multiple DCVCS-Cys monoadducts (+280 Da) with multiple DCVCS moieties as cross-linkers (+159 Da) based on the number of cysteines in each chain. Dimers consisting of α1β2 with additional DCVCS-Cys monoadducts were present at the two highest DCVCS concentrations (Table 2) and all detected trimers involved both α1 and β2 chains at every DCVCS concentration (Table S1).

Peptide cross-link analysis

Whole trypsin digests from 9 and 450 μM DCVCS samples were analyzed for peptide dimer formation with DCVCS as the cross-linker (+158.9990 Da). LC/ESI/MS of 9 μM DCVCS sample revealed three dimers that included four out of five possible cysteine positions modified (Cys13, Cys93, Cys104/111, and Cys125) (Table 3A). A 450 μM DCVCS sample revealed 4 cross-links with three out of five cysteine sites modified (Cys13, Cys93, and Cys125) (Table 3B). A dimer containing Cys13 and Cys125 was present at both the high (by both LC-ESI-MS and MALDI-TOF/TOF-MS) and low DCVCS concentrations (Table 3).

Globin chain cross-link analysis by SDS-PAGE

Cross-link formation between various chains of Hb was also investigated using SDS-PAGE. The presence of dimers and trimers was evaluated in globin from incubations of RBCs with and without DCVCS (9–450 μM) using SDS-PAGE. In order to reduce disulfide bond formation between the chains, 200 mM DTT was added to each sample. The dimer and trimer bands corresponding to treated Hb remained darker than control at all DCVCS concentrations after the DTT treatment (Figure 3). Comparison of dimer density measurements indicated a dose-dependent loss of disulfide cross-links (see Figure 3) suggesting that with increasing DCVCS concentrations dimer formation is primarily due to DCVCS acting as a cross-linker rather than due to the disulfide bond formation. Thin bands of trimers appeared darker than in control after DTT treatment, however, density measurements could not be determined.

Hemolysis

DCVCS did not cause hemolysis after its in vitro incubations with rat RBCs. Erythrocytes remained intact after 2 h as determined by hemolysis assay of incubations with 90 and 450 μM DCVCS compared to the incubation without DCVCS at physiological conditions (pH 7.4, 37 °C) (data not shown).

Mass Spectral Analyses- In vivo Samples

DCVCS monoadducts, DCVCS-GSH monoadducts, DCVCS-Cys monoadducts, and other monoadducts on globin chains and peptides—Globin from rats that were treated with 23 μmol/kg DCVCS, 230 μmol/kg DCVCS or saline was subjected to ESI/MS for intact globin chain analysis of modifications by DCVCS, DCVCS-GSH, and DCVCS-Cys. Theoretical masses used for in vitro analysis described above were also used to analyze in vivo samples. β1 intact chain was detected with 1 DCVCS monoadduct (+195 Da) in one of the three rats (data not shown). LC/MS also revealed DCVCS monoadducts present on peptides containing Cys93 and Cys125 of the β chain (Table 4). Although no DCVCS-GSH monoadducts (+466 Da) or DCVCS-Cys monoadducts (+280 Da) were detected on any single globin chain, LC/ESI/MS of the tryptic digest of globin revealed modified Cys13-containing peptides consistent with a DCVCS-GSH monoadduct or a DCVCS-Cys monoadduct on a chain in two out of three rats (Table 4). One of the three dosed rats also revealed a modified Cy93-containing peptide with DCVCS-GSH monoadduct from the β chain.

Several other monoadducts (NA-DCVCS, NA-DCVCS-GSH, and NA-DCVCS-Cys) shown in Figure 2 were analyzed for in the in vivo samples to determine if N-acetylated DCVCS and

its conjugates with GSH and Cys were formed after DCVCS treatment and resulted in adducts with Hb. No intact globin chains were detected with these modifications, however, LC/MS revealed peptides containing Cys104/111 of the a chain consistent with a NA-DCVCS monoadduct or a NA-DCVCS-GSH monoadduct in two out of three rats (Table 4).

Collectively, at least four out of five cysteine sites of Hb heterodimer seem to be involved in DCVCS-derived monoadduct formation at the high dose of DCVCS whereas no monoadducts were detected at the low dose (23 μmol/kg).

Peptide cross-link analysis—Trypsin digested globin prepared from each rat treated with 23 or 230 μmol/kg DCVCS was subjected to LC/ESI/MS for cross-link analysis with DCVCS (+158.9990 Da) and NA-DCVCS (+201.0096 Da) as cross-linkers. Eighteen different cross-links were detected with DCVCS or NA-DCVCS as cross-linkers at 230 μmol/kg DCVCS (Table 5A) and eleven cross-links were detected at 23 μmol/kg DCVCS (Table 5B). Peptide dimers containing Cys13 and Cys93 were detected in two out of three rats at high dose (Table 5A) and in three out of four rats given the lower dose of DCVCS (Table 5B). Peptide dimers containing Cys104/111 and Cys125 were present in all three rats at the high dose, but were only present in one rat at the lower DCVCS dose. Three cross-links between Cys13 containing peptides of the a chains and two cross-links with peptides containing Cys93 of the β chains were detected with two out of three rats at the high DCVCS dose (Table 5A), but these cross-links were only detected in one rat each with the 23 μmol/kg DCVCS dose (Table 5B). Cross-links with peptides containing Cys125 of the β chains and cross-links between Cys93 and Cys125 containing peptides of the β chains were detected at both the high and low dose of DCVCS. Collectively, all cross-links at low DCVCS dose contained the same modified sites as the cross-links detected at high dose (Table 5). At least four cysteine moieties of the Hb heterodimer may be involved in formation of peptide dimers with DCVCS or NA-DCVCS as cross-linkers after treatment with the high or low dose of DCVCS.

Globin chain cross-link analysis by SDS-PAGE

The presence of dimers and trimers was evaluated in globin samples from rats treated with 230 μmoles/kg DCVCS, however, we were unable to obtain reproducible results, possibly due to the lower sensitivity of the method in comparison to MS methods.

Discussion

The use of mass spectrometry with different ionization methods provided complimentary evidence for adduct identification in our study. Our data for cross-linked intact globin chains demonstrates the presence of multiple dimers and trimers after DCVCS exposure (Tables 2, S1, S2, and S3). However, due to suboptimal mass accuracy with ESI/TOF which may potentiate ambiguities in some mass assignments, the main purpose of this data is to corroborate the mass spectral evidence for peptide adducts and cross-links obtained after trypsin digestion. MALDI-FTICR provides high mass accuracy, high dynamic range, and enhanced sensitivity (25,26). However, one limitation with FTICR is the drop-off in resolution with increase in m/z (25). Therefore, high mass peptide dimers were less detectable. MALDI-TOF/TOF is also a high accuracy instrument that allowed analysis of high mass peptides. However, with MALDI method of ionization, the matrix may cause significant ion suppression that is less likely to occur with ESI that is coupled to the chromatographic separation. Although less accurate than TOF/TOF or FTICR, ESI permits high-mass ion distribution as multi-charged ions allowing detection of high-mass peptides and cross-links. Attempts at retrieving an MS/MS spectrum of a modified peptide were not successful possibly due to complexity of the mixture. Presence of multiple signals representing various adducts may have caused coelution and signal suppression and, therefore, had caused inadequate MS/MS fragmentation. SDS-PAGE of

globin provided another form of evidence for the presence of dimers and trimers due to DCVCS along with insight into the structure of each cross-link provided by mass spectrometry. Collectively, methods used in this study and the generated data allow qualitative assessment of formation of multiple adducts and cross-links after DCVCS treatment. Quantitative methodology could, however, be developed in the future based upon some of the results.

In this study, we incubated RBCs with DCVCS (0.9–450 μM) for 2h at physiological conditions and analyzed intact globin chains and trypsin digested peptides for formation of multiple DCVCS-containing adducts and cross-links at cysteine residues. Our hemolysis assay results indicate that the adducts described in this study were formed at DCVCS concentrations not associated with RBC hemolysis.

The presence of GSH and Cys in RBCs provides another source of nucleophilic sulfhydryl groups in addition to Hb that may react with DCVCS and form either monoadducted Hb or globin cross-links containing DCVCS-GSH or DCVCS-Cys monoadducts. GSH is abundant in RBC (2,338 nmol/mL) whereas Cys levels are higher in plasma than in erythrocytes (246 nmol/mL vs 59 nmol/mL) of SD rats (27). In humans 97% of blood cysteine is present in plasma, of which 60% is bound (28). More cross-links were detected with DCVCS-GSH monoadducts than with DCVCS-Cys monoadducts (Table 2 and S1). These results are consistent with the overall amount of GSH and Cys, respectively, present in RBCs. Lack of DCVCS-GSH or DCVCS-Cys adducts on single intact globin chains as compared to DCVCS monoadducts suggests initial addition of DCVCS monoadducts to globin chains followed by formation of cross-links and a subsequent addition of DCVCS-GSH or DCVCS-Cys resulting in cross-links containing DCVCS-GSH or DCVCS-Cys monoadducts. Evidence for the high nucleophilicity of Hb cysteine moieties in comparison with that of GSH was provided by the finding that reaction of 5,5-dithio-bis(2,2-nitrobenzoic acid) with rat Hb, specifically Cys125, occured 100 times faster than with GSH (29). Detection of these cross-links and monoadducts in freshly isolated RBCs provides evidence for formation of these conjugates inside erythrocytes. Cross-links with additional DCVCS-GSH and DCVCS-Cys moieties that were detected with in vivo samples may also be due to DCVCS reacting with GSH and Cys, respectively, inside the liver and plasma before translocation into the RBC to react with Hb. Previously, depletions of both hepatic and renal reduced non-protein thiols were detected in rats dosed with DCVCS (12).

Intact globin modification of β chains both in vivo and in vitro was further supported by analysis of trypsin digested globin which revealed a modified Cys125-containing peptide on the β1/β2 chain. In addition, Cys13-containing peptides consistent with a DCVCS-GSH and DCVCS-Cys monoadducts on the α chain were detected with two out of three rats (Table 4) and also at 450 μM DCVCS in vitro (Table 1). Interestingly, no DCVCS-derived peptide monoadducts were detected at 9 μM DCVCS concentration in vitro or at the low dose (23 μmol/kg DCVCS) in vivo possibly because of preferential formation of cross-links at low DCVCS concentrations.

Consistent with the dimer formation between α1 and β1 chains (Table 2), the tryptic digest of globin from the 9 and 450 μM DCVCS incubations revealed several cross-linked peptides on α1/α2 and β1/β2 chains with DCVCS as a cross-linking agent (Table 3). The prevalent involvement of Cys13 and Cys125-containing peptides suggests them as preferential sites for DCVCS reactivity as a cross-linker between Hb chains in vitro.

ESI/MS of globin from rats treated with 230 μmol/kg DCVCS revealed formation of dimers and trimers due to DCVCS and NA-DCVCS as cross-linkers (Tables 2 and S3). Rats contained cross-links, with and without additional DCVCS monoadducts that primarily involved α1 chains consistent with the in vitro data. Cross-links between peptides with DCVCS and NA-DCVCS as cross-linkers were detectable at both 23 and 230 μmol/kg DCVCS doses (Tables

5A and 5B). Although less peptide dimers were detected overall at 23 μmol/kg DCVCS, cross-links containing Cys13 and Cys93 modified sites with DCVCS or NA-DCVCS as a cross-linker and cross-links containing Cys104/111 and Cys125 with NA-DCVCS as a cross-linker were detectable with both high and low DCVCS doses with majority of the animals tested. These data indicate that these specific peptide dimers may provide a useful biomarker for DCVCS exposure.

Our data indicate that at least 4 out of 5 cysteines sites of Hb heterodimer may be involved in formation of DCVCS cross-linked peptides. Cys125 is the obvious target since it is located externally at the α1β1 contact of Hb (30). Cys13 is located internally on the a chains, and Cy93 on the β chains is less exposed to the solvent than Cys125; it should be noted, however, that both Cys13 and Cys93 were shown to be reactive with 5,5-dithio-bis(2-nitrobenzoic acid) (29) suggesting limited steric hinderance for the reaction of DCVCS or 5,5-dithio-bis(2-nitrobenzoic acid) with the various cysteine residues of Hb. Although Cys104/111 is located internally at the α1β1 contact of Hb (29,30), it was suggested that glutathionylation of Hb a chains may cause dissociation of tetramers into dimers or monomers giving access to Cys104/111 (31). Similarly, formation of DCVCS-derived monoadducts with Hb may lead to tetramer dissociation resulting in formation of dimers and trimers.

A search for adducts with N-acetylated DCVCS and its derivatives was performed because DCVCS may undergo metabolic N-acetylation reactions in the liver similar to DCVC (4,5,6) before possible translocation into the RBC. The finding of several cross-links containing NA-DCVCS as a cross-linker and adducts with NA-DCVCS-GSH with in vivo samples suggest that some NA-DCVCS could cross the RBC membrane. Small amount of DCVCS could have been N-acetylated in RBCs before or after reaction of DCVCS with Hb, because N-acetylation of p-aminobenzoic acid, sulfanilamide, and procainamide has been shown to occur in RBCs (32). The enzyme responsible for these activities in erythrocytes has not been characterized. LC/MS of globin samples obtained after in vitro incubations revealed a Cys93-containing peptide modified by NA-DCVCS. Interestingly, a peptide dimer formed due to DCVCS with Cys13 and Cys93 as the modified sites in vitro, was also detected in vivo with both doses with NA-DCVCS as the cross-linker, suggesting same reactive sites may be involved with DCVCS and NA-DCVCS.

Collectively, our previous in vitro study of the reactivity of DCVCS with NAC (13), results of SDS-PAGE, and adduct analysis by various MS techniques provide strong evidence for DCVCS monoadduct and cross-link formation both in vitro and in vivo. Specific peptides that were modified indicate that DCVCS forms monoadducts and cross-links with Cys13 and Cys104/111 of α chains and Cys93 and Cys125 of β chains both in vivo and in vitro. Cross-links rather than monoadducts preferentially form with lower doses of DCVCS both in vivo and in vitro, suggesting that cross-linked peptides would provide more reliable biomarkers. DCVCS and NA-DCVCS-derived monoadducted peptides and globin cross-links containing GSH and Cys indicate that DCVCS/NA-DCVCS is reactive with Hb, GSH, Cys, and potentially other biological nucleophiles. Consistency of cross-links at low and high DCVCS doses may allow development of biomarkers to investigate possible presence of DCVCS in the circulation after DCVC or TCE exposure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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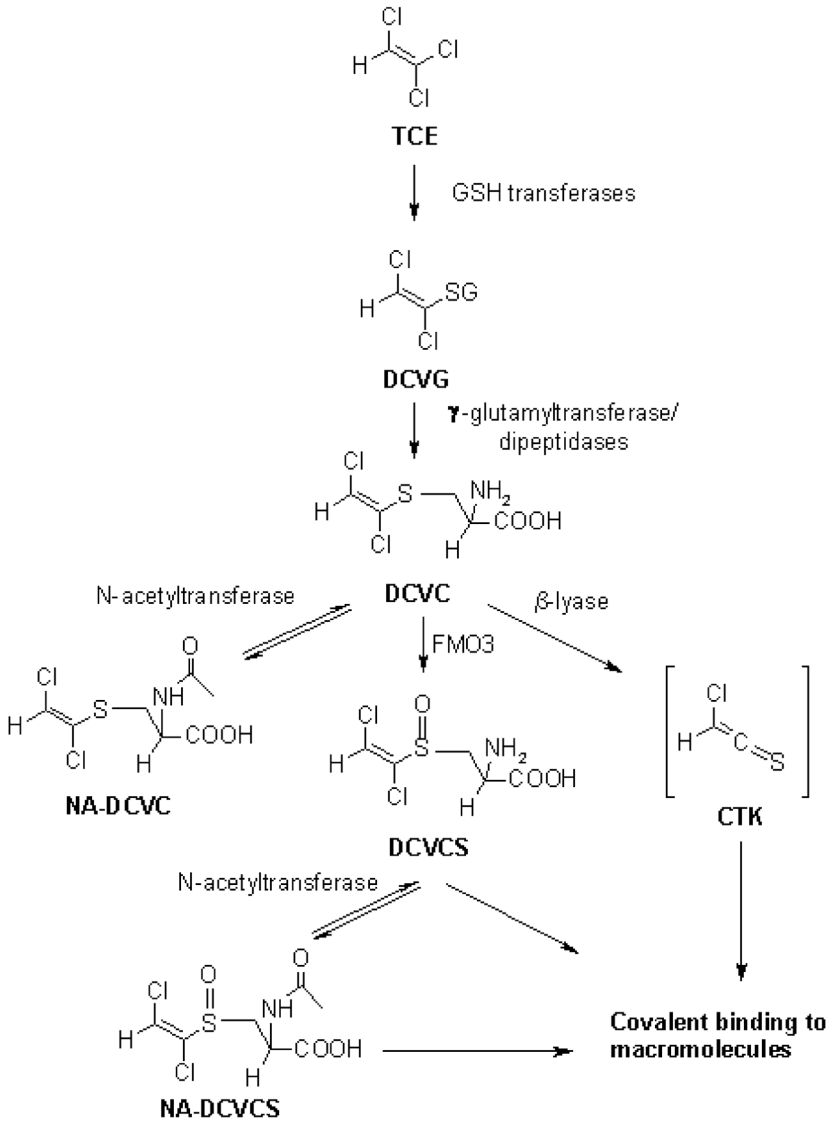

Figure 1.

Glutathione-dependent metabolism of trichloroethylene (TCE) including proposed mechanism for N-acetylation of S-(1,2-dichlorovinyl)-L-cysteine sulfoxide (DCVCS). S-(1,2-dichlorovinyl)glutathione (DCVG), S-(1,2-dichlorovinyl)-L-cysteine (DCVC), N-acetyl-S-(1,2-dichlorovinyl)-L-cysteine (NA-DCVC), N-acetyl-S-(1,2-dichlorovinyl)-L-cysteine sulfoxide (NA-DCVCS), and chlorothioketene (CTK).

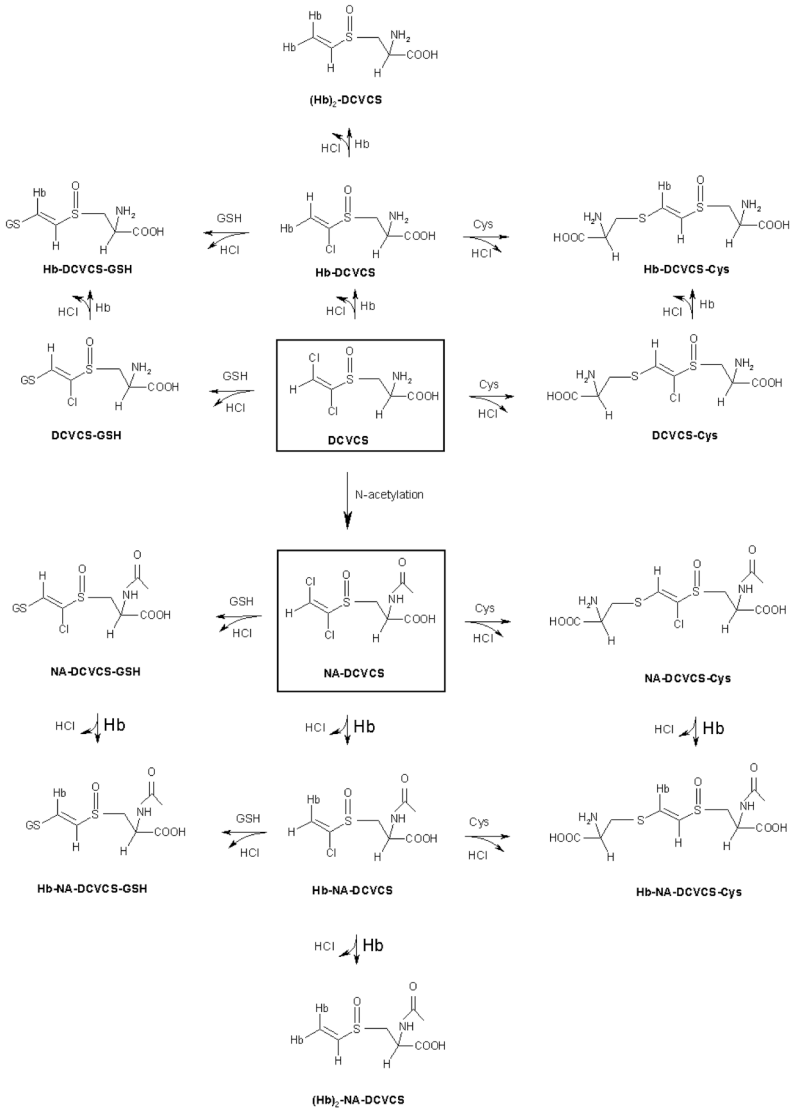

Figure 2.

Proposed mechanism for formation of Hb monoadducts with DCVCS, DCVCS-GSH, DCVCS-Cys, NA-DCVCS, NA-DCVCS-GSH, and NA-DCVCS-Cys as well as cross-links involving DCVCS and NA-DCVCS as cross-linkers.

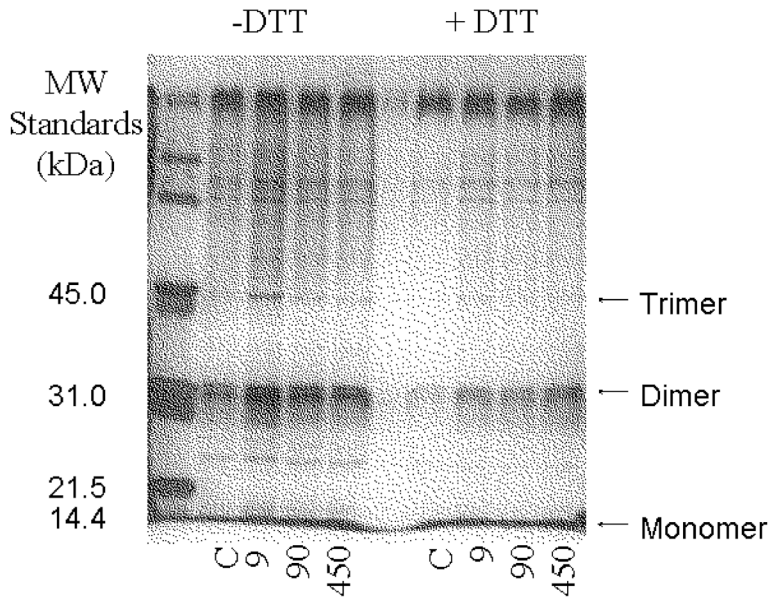

Figure 3.

SDS-PAGE of globin after incubation of erythrocytes with and without DCVCS (9–450 μM) for 2h at pH 7.4, 37°C. Dimer band intensities using optical density measurements in the absence and presence of 200 mM DTT are shown below.

DCVCS [μM]	Dimer band intensity (ODu/mm^2) −DTT	+DTT	Loss of intensity (%)
0	3.89	1.09	72
9	5.13	2.21	57
90	4.74	2.11	55
450	4.25	3.33	22

Table 1

Peptides Modified by the Following monoadducts: DCVCS (+194.9757 Da), DCVCS-GSH (+466.0828 Da), DCVCS-Cys (+280.0188 Da), and NA-DCVCS (+236.9862 Da) after Incubation of RBCs with DCVCS (450 μM)

(A) Whole Digest Hb chain	peptide	monoadduct	modified position	$mass_{theo}$ (Da)	$mass_{obs}$ (Da)	MS	
β1	(121–146)[a]	DCVCS	Cys125	2939.3739	2939.3923	MALDI-TOF/TOF	
β1	(77–104)[a]	NA-DCVCS	Cys93	4097.8942	4097.9722	LC-ESI	
(B) Digest Fractions Hb chain	peptide	monoadduct	modified position	$mass_{theo}$ (Da)	$mass_{obs}$ (Da)	MS	fraction (min)
α1	(1–16)	DCVCS	Cys13	1986.8819	1986.9026	MALDI-FTICR	20.5–22.5
α2	(1–16)	DCVCS	Cys13	1942.8921	1942.9323	MALDI-FTICR	26.6–28.5
α1	(8–31)[b]	DCVCS-GSH	Cys13	3083.3409	3083.3322	MALDI-TOF/TOF	20.5–22.5
β1	(83–95)[a]	DCVCS-Cys	Cys93	1737.7034	1737.6735	MALDI-TOF/TOF[c]	26.6–28.6

[a] Peptides (77–104), (83–95), and (121–146) can also be from β2.

[b] Peptide (8–31) can also be from α2.

[c] Also detected with MALDI-FTICR in the 24.0–26.6 min fraction.

Table 2

Summary of Dimers Between Intact Globin Chains for in Vitro Samples (RBCs Incubated with DCVCS 0.9–450 μM)

	DCVCS as cross-linker without additional monoadducts	DCVCS as cross-linker plus following monoadducts		
		DCVCS	DCVCS-GSH	DCVCS-Cys
DCVCS 0.9 μM				
$Mass_{theo}$ (Da)	31204	32064	-	-
$Mass_{obs}$ (Da)	31207	32065		
Cross-links	α1β1+1cl	β1β2+1cl+1		
DCVCS 4.5 μM				
$Mass_{theo}$ (Da)	31204	31138; 31759	-	-
$Mass_{obs}$ (Da)	31205	31140; 31758		
Cross-links	α1β1+1cl	α1α2+2cl+2; α2β2+1cl+3		
DCVCS 9 μM				
$Mass_{theo}$ (Da)	31204	-	31843; 32616	30992
$Mass_{obs}$ (Da)	31206		31840; 32620	30995
Cross-links	α1β1+1cl		α1β2+2cl+1; α1β2+1cl+3	α1α1+2cl+1
DCVCS 90 μM				
$Mass_{theo}$ (Da)	-	31759; 31138; 31745	31785	31657
$Mass_{obs}$ (Da)		31762; 31138; 31744	31785	31662
Cross-links		α2β2+1cl+3; α1α2+2cl+2; 2β1+1cl+3	α2β1+2cl+1	α1β2+2cl+1
DCVCS 450 μM				
$Mass_{theo}$ (Da)	31204	31759; 31138	-	31657
$Mass_{obs}$ (Da)	31206	31762; 31138		31660
Cross-links	α1β1+1cl	α2β2+1cl+3; α1α2+2cl+2		α1β2+2cl+1

[a] 1cl (+159 Da) = 1 DCVCS cross-linker (between chains), 2cl (+159*2 Da) = 2 DCVCS cross-linkers, 3cl (+159*3 Da) = 3 DCVCS cross-linkers. Cross-links with and without monoadducts are shown. Monoadducts include: DCVCS (+195 Da; +195*2 Da; +195*3 Da), DCVCS-GSH (+466 Da, with up to 3 adducts as described above for DCVCS), DCVCS-Cys (+280 Da, with up to 3 adducts as described above for DCVCS). Example: αβ+1cl+2 defines a cross-link between the stated chains with 1 cross-linker (DCVCS) and 2 monoadducts.

Table 3

Peptide Cross-links formed by DCVCS (+158.9990 Da) after Incubation of RBCs with DCVCS

cross-linked peptides	$mass_{theo}$ (Da)	$mass_{obs}$ (Da)	modified positions	MS
(A) 9 μM DCVCS				
α2 (1–16) + β1 (121–146)[a]	4650.3148	4650.2544	Cys13+Cys125	LC/ESI
α1 (93–127)[b] + β1 (83–95)[a]	5476.5926	5476.4819	Cys104/111+Cys93	LC/ESI
α1 (100–127)[b] + α1 (12–40)[a]	6374.9681	6375.1782	Cys104/111+Cys13	LC/ESI
(B) 450 μM DCVCS				
α1 (12–16)[b] + β1 (121–146)[a]	3509.6645	3509.7559	Cys13+Cys125	LC/ESI[c]
α1 (8–31)[b] + β1 (121–144)[a]	5219.5341	5219.7422	Cys13+Cys125	LC/ESI
β1 (83–104)[a] + β1 (121–144)[a]	5167.5069	5167.3677	Cys93+Cys125	LC/ESI
α1 (1–16) + β1 (83–95)[a]	3407.5910	3407.6748	Cys13+Cys93	MALDI-TOF/TOF

[a] Peptides β1 (83–95), (83–104), (121–144) and (121–146) can also be from β2.

[b] Peptides α1 (8–31), (12–16), (12–40), (93–127), and (100–127) can also be from α2.

[c] Also detected with MALDI-TOF/TOF MS.

Table 4

LC/ESI/MS Results of Peptides Modified by the Following Monoadducts after Dosing of Rats with DCVCS (230 μmol/kg): DCVCS (+194.9757 Da), DCVCS-GSH (+466.0828 Da), DCVCS-Cys (+280.0188 Da), NA-DCVCS (+236.9862 Da), and NA-DCVCS-GSH (+508.0933 Da)

Hb chain	Peptide	$mass_{theo}$ (Da)	$mass_{obs}$ (Da)	monoadduct	modified position	n[a]
α1	(8–31)[b]	3083.3409	3083.3987	DCVCS-GSH	Cys13	2/3
α1	(12–40)[b]	3435.5018	3435.6680	DCVCS-Cys	Cys13	
β1	(77–104)[c]	3482.6835	3482.5944	DCVCS	Cys93	2/3
		3751.7055	3751.9128	DCVCS-GSH	Cys93	
α1	(93–127)[b]	4295.4977	4295.8805	NA-DCVCS	Cys104/111	2/3
α1	(100–127)[b]	3569.5784	3569.8267	NA-DCVCS-GSH	Cys104/111	1/3
β1	(105–132)[c]	3232.6618	3232.5779	DCVCS	Cys125	

[a] n= number of rats detected with cross-link over total number of rats.

[b] Peptides α1 (8–31), (12–40), (93–127), and (100–127) can also be from α2.

[c] Peptides β1 (77–104) and (105–132) can also be from β2.

Table 5

LC/ESI/MS Results of Peptide Cross-links Formed by DCVCS (+158.9990 Da) or NA-DCVCS (+201.0096 Da) after Dosing Rats

cross-linked peptides	$mass_{theo}$ (Da)	$mass_{obs}$ (Da)	cross-linker	modified position	n[a]
(A) 230 μmol/kg DCVCS					
α1 (12–40)[b] + β1 (83–95)[c]	4813.1678	4813.2031	NA-DCVCS	Cys13+Cys93	2/3
α1 (8–31)[b] + β1 (67–95)[c]	6070.6264	6070.8096	NA-DCVCS	Cys13+Cys93	
α1 (100–127)[b] + β1 (121–132)[c]	4605.1559	4606.2055	NA-DCVCS	Cys104/111+Cys125	3/3
α1 (93–127)[b] + β1 (121–132)[c]	5401.5388	5401.7339	NA-DCVCS	Cys104/111+Cys125	
α1 (12–16)[b] + α1 (12–31)[b]	2926.2547	2926.1982	DCVCS	Cys13+Cys13	2/3
α1 (12–16)[b] + α1 (12–40)[b]	3962.7493	3962.5723	NA-DCVCS	Cys13+Cys13	
α1 (12–31)[b] + α2 (1–16)	4108.9050	4108.7754	NA-DCVCS	Cys13+Cys13	
β1 (83–95)[c] + β1 (83–104)[c]	4180.9156	4180.8003	DCVCS	Cys93+Cys93	2/3
β1 (77–95)[c] + β1 (67–95)[c]	5585.7246	5585.7383	DCVCS	Cys93+Cys93	
β1 (105–132)[c] + β1 (105–132)[c]	6229.2571	6229.2702	DCVCS	Cys125+Cys125	2/3
β1 (121–132)[c] + β1 (105–132)[c]	4537.2160	4536.2302	DCVCS	Cys125+Cys125	
β1 (121–144)[c] + β1 (121–144)[c]	5088.5520	5088.7539	NA-DCVCS	Cys125+Cys125	
β1 (77–104)[c] + β1 (105–132)[c]	6479.2471	6479.2661	DCVCS	Cys93+Cys125	2/3
β1 (67–95)[c] + β1 (121–146)[c]	6194.0462	6193.8677	NA-DCVCS	Cys93+Cys125	
β1 (77–95)[c] + α1 (100–127)[b]	5397.5616	5397.4292	DCVCS	Cys93+Cys104/111	1/3
α1 (8–16)[b] + α1 (100–127)[b]	4478.0209	4477.7983	DCVCS	Cys13+Cys104/111	2/3
α2 (1–16) + α1 (93–127)[b]	5812.6036	5812.8316	NA-DCVCS	Cys13+Cys104/111	1/3
α1 (12–31)[b] + β1 (105–132)[c]	5396.6212	5396.7607	NA-DCVCS	Cys13+Cys125	
(B) 23 μmol/kg DCVCS					
α1 (12–16)[b] + β1 (67–95)[c]	4014.9141	4015.1177	DCVCS	Cys13+Cys93	3/4
α1 (1–16) + β1 (77–104)[c]	5235.5290	5235.6445	DCVCS	Cys13+Cys93	
α1 (8–16)[b] + β1 (83–104)[c]	3828.7668	3828.9341	NA-DCVCS	Cys13+Cys93	
α2 (1–16) + β1 (83–95)[c]	3407.7036	3406.6116	NA-DCVCS	Cys13+Cys93	
α1 (100–127)[b] + β1 (121–144)[c]	5705.7610	5705.7770	NA-DCVCS	Cys104/111+Cys125	1/4
α1 (1–16) + α2 (1–16)	3697.8228	3697.9409	DCVCS	Cys13+Cys13	1/4
β1 (83–95)[c] + β1 (83–104)[c]	4180.9156	4180.1304	DCVCS	Cys93+Cys93	1/4
β1 (77–104)[c] + β1 (83–104)[c]	6008.8536	6009.0044	DCVCS	Cys93+Cys93	
β1 (121–144)[c] + β1 (121–144)[2]	5088.5520	5088.7450	NA-DCVCS	Cys125+Cys125	1/4
β1 (83–104)[c]+ β1 (105–132)[c]	5800.8636	5800.8906	NA-DCVCS	Cys93+Cys125	1/4
β1 (67–95)[c] + α1 (100–127)[b]	4478.0209	4477.7983	DCVCS	Cys93+Cys104/111	1/4

[a] n=number of rats detected with cross-link over total number of rats.

[b] Peptides α1 (8–16), (8–31), (12–16), (12–31), (12–40), (93–127), and (100–127) can also be from α2.

[c] Peptides β1 (67–95), (77–95), (77–104), (83–95), (83–104), (105–132), (121–132), (121–144), and (121–146) can also be from β2.