Metabolic activation and detoxication of nephrotoxic cysteine and homocysteine S-conjugates

[S-(1,2-dichlorovinyl)-L-cysteine/S-(1,2-dichlorovinyl)-L-homocysteine/cysteine conjugate β-lyase/cystathionine γ-lyase/S-(1,2-dichlorovinyl)-DL-α-methylhomocysteine]

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ABSTRACT S-(1,2-Dichlorovinyl)-L-homocysteine (DCVHcy), an analogue of the nephrotoxin S-(1,2-dichlorovinyl)-L-cysteine (DCVCys), is a much more potent nephrotoxin than DCVCys both in vivo and in isolated renal proximal tubular cells. S-(1,2-Dichlorovinyl)-DL-α-methylhomocysteine, at equimolar doses relative to DCVHcy, is not nephrotoxic. Agents that inhibit pyridoxal phosphate-dependent enzymes (DL-propargylglycine and aminooxyacetic acid) or renal organic anion transport (probenecid) protect against DCVHcy-induced nephrotoxicity. With kidney cytosol, DCVHcy or the analogue S-(2-benzothiazolyl)-L-homocysteine (BTHcy) is not metabolized to 2-ketobutyrate, but 2-mercaptobenzothiazole is a metabolite of BTHcy and the Vₘₐₓ for its formation is enhanced by addition of 2-ketobutyrate. These results are consistent with a bioactivation mechanism for DCVHcy that involves enzymatic deamination followed by a nonenzymatic β-elimination to produce two reactive intermediates—i.e., S-(1,2-dichlorovinyl)thiol and 2-keto-3-butenoic acid. The Kₘ values for the N-acetylation of DCVCys and DCVHcy by kidney microsomal N-acetyltransferase are similar, but the rate of DCVCys N-acetylation is 4-fold greater than the rate measured with DCVHcy as the substrate. Thus, the remarkable nephrotoxic potency of DCVHcy compared with DCVCys may be attributable to intrarenal differences in activation and detoxication.

The bioactivation of nephrotoxic halogenated hydrocarbons involves initial hepatic glutathione S-conjugate formation and subsequent renal metabolism of the glutathione S-conjugates to the corresponding cysteine S-conjugates (1). Nephrotoxic cysteine S-conjugates may form electrophilic episulfonium ions by nonenzymatic intramolecular rearrangements (2) or may be cleaved by renal cysteine conjugate β-lyase (β-lyase) to produce pyruvate, ammonia, and unidentified sulfur-containing reactive intermediates, which initiate renal proximal tubular cell death (3–5). S-(1,2-Dichlorovinyl)-L-cysteine (DCVCys) is a model cysteine S-conjugate and a potent nephrotoxin (5–7), and a role for β-lyase in DCVCys-induced nephrotoxicity has been established (5).

The significant role of various bioactivation processes in chemical toxicity makes it important to identify classes of organic chemicals that may be converted to reactive intermediates. Therefore, in this study, the metabolism and nephrotoxicity of homocysteine analogues (Fig. 1) of nephrotoxic cysteine S-conjugates were evaluated. Homocysteine S-conjugates, if cleaved by cystathionine γ-lyase (8), should produce reactive intermediates similar to those produced by the action of β-lyase on the corresponding cysteine S-conjugates and are, therefore, expected to be nephrotoxic.

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MATERIALS AND METHODS

Materials. L-Homoserine, DL-propargylglycine, pyridoxal phosphate, sodium 2-ketobutyrate, aminooxyacetic acid, probenecid, acetyl-coenzyme A, collagenase (type IV), and sodium pyruvate were purchased from Sigma. Deuterium oxide and deuterium chloride were obtained from Aldrich. Tetrabutylammonium phosphate was supplied by Eastman. Sodium 3-(trimethylsilyl)tertadueteropropionate was obtained from Wilmad Glass (Bueno, NJ). [1-14C]Acetyl-coenzyme A (58.7 mCi/mmol; 1 Ci = 37 GBq) was purchased from New England Nuclear.

Analyses. Melting points (uncorrected) were determined in open glass capillary tubes. Proton NMR spectra were recorded on a Bruker 270-MHz spectrometer. The elemental analyses (C, H, N, Cl, S) for S-(1,2-dichlorovinyl)-DL-α-methylhomocysteine (DCVMeHcy, Fig. 1) were done by Midwest Microlab (Indianapolis, IN).

Syntheses. DCVCys and S-(1,2-dichlorovinyl)-L-homocysteine (DCVHcy, Fig. 1) were synthesized by published procedures (5, 6). S-(2-Benzothiazolyl)-L-homocysteine (BTHcy) was synthesized by the reaction of the disodium salt of L-homocysteine with 2-chlorobenzothiazole, as described for S-(2-benzothiazolyl)-L-cysteine (9); the details of the synthesis will be described elsewhere. S-Benzyl-DL-α-methylhomocysteine was prepared from 4-(benzylthio)-2-butanone by the Bucherer-Bergs synthesis, and the resulting hydantoin was hydrolyzed with barium hydroxide (10). The crude product S-benzyl-DL-α-methylhomocysteine [mp 217–220°C with decomposition, literature 252–254°C (10); NMR (D₂O, 2HCl) δ (ppm): 7.40 (m, 5H), 3.80 (s, 2H), 2.47]

Abbreviations: DCVCys, S-(1,2-dichlorovinyl)-L-cysteine; DCVHcy, S-(1,2-dichlorovinyl)-L-homocysteine; DCVMeHcy, S-(1,2-dichlorovinyl)-DL-α-methylhomocysteine; BTHcy, S-(2-benzothiazolyl)-L-homocysteine; β-lyase, cysteine conjugate β-lyase.

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Preparation of DCVMeHcy. Sodium metal was added to a solution of S-benzyl-DL-α-methylhomocysteine (0.67 g, 2.8 mmol) in liquid ammonia (25-30 ml) until a blue color persisted for 7.5 min. Trichloroethylene (0.39 g, 2.9 mmol) was added to the liquid ammonia, and the mixture was stirred for 45 min. The cooling bath was then removed, and the liquid ammonia was evaporated with a stream of nitrogen. The residue was dissolved in hot water (5 ml), and the pH of the solution was adjusted to 5.4 with 5% HCl. The resulting copious precipitate was diluted with 1 vol of ethanol, cooled, and filtered. The crude precipitate was dissolved in hot water, treated with activated charcoal, and filtered.

The colorless filtrate was refrigerated overnight, and the solid crystals (89 mg) that formed were isolated by filtration and dried under reduced pressure. An additional 100 mg of product was obtained upon addition of alcohol to the mother liquor (total yield 28%). The compound changed color upon heating to 160°C and melted at 186-189°C with decomposition.

Analysis: Calculated for C_{3}H_{11}NSO_{2}Cl: C, 34.44; H, 4.54; N, 5.74; S, 13.13; Cl, 29.04. Found: C, 34.66; H, 4.50; N, 5.78; S, 13.30; Cl, 28.76. Proton NMR (D_{2}O, 2HCl) δ (ppm): 6.76 (s, 1H, trans vinylic proton); 3.02 (m, 2H); 2.22 (m, 2H); 1.61 (s, 3H).

Enzymatic Assays. Male Fischer 344 rats (175-225 g; Charles River Breeding Laboratories) were killed by decapitation, and renal subcellular fractions were prepared as previously described (5). Protein concentrations were determined according to published methods (11, 12). Renal cystathionine γ-lyase activity with l-homoserine (16 mM) as the substrate was determined by measuring the formation of 2-ketobutyric acid (13). The production of 2-mercaptopentobenzothiazole from BTHC (0.3-0.8 mM) was measured as described for the metabolism of S-(2-benzothiazolyl)-L-cysteine (5, 9). Incubation mixtures contained 1 ml of potassium borate buffer (0.1 M, pH 8.6), 1.5-2 mg of rat kidney cytosolic protein in 0.4 ml of potassium chloride solution (1.15% wt/vol), and 0.1 ml of BTHC solution in a final volume of 1.5 ml; in some experiments, the incubation mixture also contained 5 mM 2-ketobutyrate. Pyruvate formation from DCVCys (0.5-2 mM) was determined with kidney cytosol as previously described (9). Rates of N-acetylation of DCVCys (0.25-2 mM) and DCVHcy (0.5-2 mM) were measured with [1-Methyl]acetyle-cysteine A as described by Duff and Jakoby (14).

Metabolite Isolation and Characterization. For 2-mercaptopentobenzothiazole, the incubation mixture (final volume 1.5 ml) contained BTHC (4 mM) and kidney cytosol (2 mg/ml) in potassium borate buffer (0.1 M, pH 8.6). The enzymatic reaction was terminated after 4 hr by addition of 0.3 ml of 10% (wt/vol) trichloroacetic acid. The acid-soluble supernatant was extracted with ethyl acetate (5 ml), the ethyl acetate extract was evaporated to dryness under a stream of nitrogen, and the residue was dissolved in methanol (2 ml) and analyzed by HPLC with an authentic sample of 2-mercaptopentobenzothiazole as a reference. For the characterization of 2-keto acids, the incubation mixture (final volume 7.5 ml) contained substrate (L-homoserine, DCVHcy, or BTHC); 8 mM and kidney cytosol (2 mg/ml) in potassium borate buffer (0.1 M, pH 8.6). Incubation flask were shaken at 37°C, and samples (1.5 ml) were taken every hour. The enzymatic reaction was terminated by the addition of 0.3 ml of 10% trichloroacetic acid. A portion (0.5 ml) of the acid-soluble supernatant was mixed with 0.5 ml of 0.2% (wt/vol) 2,4-dinitrophenylhydrazine in 2 M HCl, diluted with methanol, and analyzed by HPLC as described by Hemming and Gubler (15) with authentic samples of 2-ketobutyrate and pyruvate as references.

HPLC Analyses. A 4.6 × 250 mM Altex Spherisorb ODS 10-μm column with a 2-cm-long precolumn (Anspec, Ann Arbor, MI) packed with 37- to 53-μm Pellicular ODS (Whatman) was used with an Altex 110A liquid chromatograph (Altex). For 2-mercaptopentobenzothiazole, the eluant was methanol/water (7:3, vol/vol), the flow rate was 1 ml/min, and the UV detection was at 321 nm. For 2-keto acids, the eluent was methanol/0.05 M tetrabutylammonium phosphate (6:4, vol/vol), the flow rate was 2 ml/min, and the UV detection was at 366 nm.

In Vitro Toxicity. Isolated kidney cells were prepared by the collagenase perfusion method of Jones et al. (16). Cell viability and concentration were estimated in the presence of 0.2% trypan blue in a hemacytometer. Cell viability was also assessed by measurement of leakage of lactate dehydrogenase into the medium (17). Cell viability by either method was typically 85-95%, and the yield was 30-50 × 10^6 cells per rat.

In Vivo Experiments. Male Fischer 344 rats were housed and fed as described previously (5). Rats were given isotonic saline or DCVCys, DCVHcy, or DCVMeHcy in isotonic saline i.p. In some experiments, rats were given DL-propargylglycine (0.4 mmol/kg i.p.) 12 hr before treatment or aminoxyacetic acid (0.5 mmol/kg i.p.) or probenecid (0.18 mmol/kg i.p.) 1 hr before treatment and were killed 24 hr after DCVHcy had been given. Morphological examination of kidney and liver as well as the analyses of blood urea nitrogen, of blood and urine glucose concentrations, and of serum glutamate-pyruvate transaminase activity were done as previously described (5).

Statistics. Results were examined by analysis of variance with the MDCSTAT computer program (Micro Data Collection, Novato, CA). A level of P ≤ 0.05 was chosen for acceptance or rejection of the null hypothesis.

RESULTS

In Vivo Nephrotoxicity of DCVCys and DCVHcy. Treatment of rats with DCVHcy produced dose-dependent nephrotoxicity (Fig. 2). Doses of DCVHcy as low as 2.5 and 5 μmol/kg increased urine glucose excretion rates and blood urea nitrogen concentrations, respectively. By comparison, doses of 230 and 460 μmol/kg of DCVCys were required to alter these renal function parameters. Morphological examination of kidneys from DCVHcy-treated rats (>60 μmol/kg) showed acute proximal tubular cell swelling and acidophilic juxtaglomerular nephrons. The proximal convoluted tubular epithelium was virtually destroyed, with only remnants of eosinophilic debris and occasional pyknotic nuclei present.

Doses of DCVHcy ≤230 μmol/kg failed to alter blood urea nitrogen concentrations or urine glucose excretion rates. Treatment of rats with DL-propargylglycine, aminoxyacetic acid, or probenecid protected against DCVHcy-induced increases in urine glucose excretion rates (Table 1).

No morphologically detectable hepatic lesions were apparent after giving DCVHcy (460 μmol/kg), and serum glutamate-pyruvate transaminase activities were only slightly elevated [19 ± 16 and 82 ± 9 international units/liter (mean ± SD, n = 4) for control and DCVHcy-treated rats, respectively].

In Vivo Nephrotoxicity of DCVCys and DCVHcy. The dose-dependent effects of DCVCys and DCVHcy on the viability of isolated rat kidney cells were measured (Fig. 3). As was found in the in vivo studies, DCVHcy was a more potent toxin than was DCVCys; approximately equivalent losses of viability were produced with 0.1 mM DCVHcy and 1 mM DCVCys. Both aminoxyacetic acid and DL-propargylglycine protected against DCVHcy toxicity, and the α-methyl analogue DCVMeHcy was not toxic to the isolated cells (Table 2).
Fig. 2. Blood urea nitrogen concentrations (Upper) and urine glucose excretion rates (Lower) after administration of DCVHcy (●●●) or DCVCys (○○○). Male Fischer 344 rats (175-225 g; ≥4 rats per group) were given DCVHcy or DCVCys in isotonic saline i.p. or saline alone. Urine was collected and analyzed for glucose concentrations, rats were killed 24 hr after treatment, and blood samples were analyzed for blood urea nitrogen concentrations. Rats given DCVHcy at doses higher than 115 μmol/kg were anuric 6 hr after treatment. Treatment with DCVHcy or DCVCys did not alter plasma glucose concentrations. Error bars represent SEM.

HPLC Analyses. Incubation of BTHcy with kidney cytosol led to the isolation of a metabolite with a retention time (2.8 min) identical to that of authentic 2-mercaptobenzothiazole; samples prepared from control incubations did not contain 2-mercaptobenzothiazole. Incubation of L-homoserine with kidney cytosol for periods of 1 hr or longer led to the formation of a metabolite with retention times identical to those obtained with 2-ketobutyrate (syn- and anti-2,4-dinitrophenylhydrazones gave retention times of 3.8 and 6.9 min). No 2-ketobutyrate was detected in control incubations or in incubations (up to 8 hr) including DCVHcy or BTHcy.

γ-Lyase Activity. Most of the enzymatic activity responsible for the metabolism of L-homoserine (16 mM) to 2-ketobutyrate and of BTHcy (0.4 mM) to 2-mercaptobenzothiazole is located in the renal cytosolic fraction (data not shown). The $K_m$ and $V_{max}$ for the metabolism of L-homoserine to 2-ketobutyrate were 16.7 mM and 55.6 mmol/min per mg of protein, respectively; the $K_m$ and $V_{max}$ for the metabolism of BTHcy to 2-mercaptobenzothiazole were 0.37

Table 1. Effect of DL-propargylglycine, aminoxyacetic acid, or probenecid on DCVHcy-induced increases in urine glucose excretion rates

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Urine glucose, mg/24 hr</th>
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<tbody>
<tr>
<td>DCVHcy (60 μmol/kg)</td>
<td>293 ± 77</td>
</tr>
<tr>
<td>DCVHcy (60 μmol/kg) + DL-</td>
<td>64 ± 7*</td>
</tr>
<tr>
<td>propargylglycine (0.4 mmol/kg)</td>
<td></td>
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<tr>
<td>DCVHcy (5 μmol/kg)</td>
<td>268 ± 35</td>
</tr>
<tr>
<td>DCVHcy (5 μmol/kg) + aminoxyacetic acid (0.5 mmol/kg)</td>
<td>112 ± 15*</td>
</tr>
<tr>
<td>DCVHcy (5 μmol/kg) + probenecid (0.18 mmol/kg)</td>
<td>32 ± 13*</td>
</tr>
</tbody>
</table>

Male Fischer 344 rats (175-225 g) were given DCVHcy in saline i.p. alone or after treatment with the compounds shown. Values are mean ± SEM for 4–6 rats per group.

*Significantly different (P < 0.05) from rats given DCVHcy alone.

Fig. 3. Cytotoxicity of DCVHcy and DCVCys for isolated proximal tubular cells from rat kidney. Cell viability was measured by trypan blue exclusion (filled symbols) or by lactate dehydrogenase leakage (open symbols). Cells (1 × 10^6 per ml) were incubated at 37°C for 3 hr with the indicated concentrations of DCVHcy (●, ○) or DCVCys (○, ●). Results are the means of three cell preparations; SEMs were less than 5% of the means.
Table 2. Effect of DCVHcy, DCVMeHcy, and metabolic inhibitors on viability in isolated proximal tubular cells from rat kidney

<table>
<thead>
<tr>
<th>Addition</th>
<th>Trypan blue exclusion</th>
<th>LDH leakage</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>80 ± 1*</td>
<td>80 ± 1*</td>
</tr>
<tr>
<td>DCVHcy (1 mM)</td>
<td>28 ± 1</td>
<td>25 ± 4</td>
</tr>
<tr>
<td>DCVHcy (1 mM) + aminooxycetic acid (0.1 mM)</td>
<td>59 ± 3*</td>
<td>55 ± 2*</td>
</tr>
<tr>
<td>DCVHcy (1 mM) + DL-propargylglycine (1 mM)</td>
<td>64 ± 1*</td>
<td>64 ± 2*</td>
</tr>
<tr>
<td>DCVMMeHcy (1 mM)</td>
<td>77 ± 1*</td>
<td>80 ± 1*</td>
</tr>
</tbody>
</table>

Cell viability was measured by trypan blue exclusion or by leakage of lactate dehydrogenase (LDH) into the extracellular medium. Cells (1 × 10⁶ per ml) were incubated at 37°C for 3 hr with the indicated additions. Results are means ± SEs of three cell preparations. *Significantly different (P < 0.05) from incubation mixtures containing DCVHcy alone.

mM and 5.2 nmol/min per mg of protein, respectively, and the V_max was increased 145% by addition of 5 mM 2-ketobutyrate.

Figure 4. Proposed mechanism for the renal metabolism and bioactivation of homocysteine S-conjugates.

DISCUSSION

In the present study, the homocysteine S-conjugate DCVHcy was a potent and selective nephrotoxin both in vivo and in isolated renal proximal tubular cells; indeed, DCVHcy is much more nephrotoxic than the corresponding cysteine S-conjugate DCVCys. The greater potency of DCVHcy compared to DCVCys in isolated renal proximal tubular cells demonstrates that the large difference in the in vivo nephrotoxicity of DCVHcy and DCVCys is not due to extrarenal factors but is due to differences in the intrarenal metabolism of the two compounds, differences in the target sites within the cell, or both. Experiments were carried out to examine the role of cystathionine γ-lyase in the bioactivation and nephrotoxicity of DCVHcy. The catalytic mechanism of cystathionine γ-lyase, a pyridoxal phosphate-dependent enzyme, involves Schiff base formation with the α-amino group and subsequent abstraction of the proton at the α-carbon atom (18). Hence DCVMeHcy, which lacks a proton on the α-carbon atom, cannot be cleaved by cystathionine γ-lyase, and DCVMeHcy was not nephrotoxic. DL-Propargylglycine (19, 20), and aminooxycetic acid (21), a “suicide substrate” for and a known inhibitor of cystathionine γ-lyase, both protected against DCVHcy-induced nephrotoxicity. Although these findings are compatible with a role for cystathionine γ-lyase in DCVHcy-induced nephrotoxicity, the observation that no 2-ketobutyrate is formed from DCVHcy by renal cytosol does not support such a role; L-homoserine, a known substrate for cystathionine γ-lyase (13), was metabolized to 2-ketobutyrate (Fig. 4, pathway b).

An alternative mechanism for the bioactivation of DCVHcy, which is consistent with the results obtained but does not require the participation of cystathionine γ-lyase, is shown in Fig. 4. Transamination of DCVHcy, which is a known metabolic pathway for methionine, cysteine, and homocysteine (22–24), should convert DCVHcy to the corresponding 2-keto acid. This intermediate is expected to be unstable due to the acidity of the protons on the β-carbon atom and should readily undergo a nonenzymatic β-elimination to yield a sulfur-containing reactive intermediate, S-(1,2-dichlorovinyl)thiol, and a Michael acceptor, 2-oxo-3-butenolic acid. This bioactivation scheme accounts for the observed lack of toxicity of DCVMeHcy, which cannot be deaminated by transamination. In addition, the formation of the Michael acceptor may contribute to the enhanced toxicity of DCVHcy; other Michael acceptors are toxic to renal epithelial cells (25). Furthermore, DL-propargylglycine and aminooxycetic acid inhibit a variety of pyridoxal phosphate-dependent enzymes (5, 19–21, 26) and block the toxicity of DCVHcy, which supports the involvement of pyridoxal phosphate-dependent enzymes in the bioactivation of DCVHcy.

Additional supportive evidence for this proposed bioactivation mechanism of DCVHcy is available. In the present study, the DCVHcy analogue BTHcy was metabolized by kidney cytosol to 2-mercaptobenzothiazole, and this reaction was enhanced by addition of 2-ketobutyrate, an amino group acceptor. No 2-ketobutyrate was detected as a metabolite of DCVHcy or BTHcy; these results indicate that the trans-
amination of BTHcy and subsequent nonenzymatic β-elimination lead to the formation of 2-mercaptopbenzothiazole.

The protective effect of probenecid against DCVHcy-induced nephrotoxicity indicates a role for renal organic anion transport in DCVHcy-induced nephrotoxicity and may partially explain the target organ selectivity of DCVHcy. Probenecid inhibits the active accumulation of N-acetyl-S-benzyl-L-cysteine by isolated renal cortical tubules and retards the plasma clearance of N-acetyl-S-benzyl-L-cysteine (27, 28) and also blocks the nephrotoxicity of DCVCys and other nephrotoxic cysteine S-conjugates (2, 5).

The bioactivation of DCVCys by β-lyase and of DCVHcy by transamination requires a primary amino group; therefore, differences in rates of mercapturic acid formation from DCVCys and DCVHcy may explain, in part, the enhanced nephrotoxicity of DCVHcy. Although the & values for the N-acetylation of DCVCys and DCVHcy by kidney microsomal N-acetyltransferase were similar, the rate of DCVCys N-acetylation was 4-fold greater than that of DCVHcy. Hence mercapturic acid formation is a much more important detoxication mechanism for cysteine S-conjugates than for homocysteine S-conjugates. Thus, intrarenal dispositional factors, such as N-acetylation, may contribute to the differences in nephrotoxicity between DCVHcy and DCVCys.

In summary, the present study describes a bioactivation mechanism for the homocysteine S-conjugate DCVHcy that involves an enzymatic deamination followed by a nonenzymatic β-elimination to produce two reactive intermediates [S-(1,2-dichlorovinyl)thiol and 2-oxo-3-butenolic acid] that are apparently responsible for the cytotoxicity of DCVHcy (Fig. 4). Other toxic and carcinogenic homocysteine conjugates have been reported (29–32) and may undergo bioactivation by a mechanism similar to that shown in Fig. 4. The remarkable nephrotoxic potency of DCVHcy as compared to DCVCys may be attributable to intrarenal differences in bioactivation and detoxication. The role of alternative bioactivation and detoxication mechanisms for toxic and carcinogenic homocysteine S-conjugates warrants further investigation.

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