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Transport of *N*-acetyl-*S*-(1,2-dichlorovinyl)-L-cysteine, a metabolite of trichloroethylene, by mouse multidrug resistance associated protein 2 (Mrp2)

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ABSTRACT

N-acetyl-*S*-(1,2-dichlorovinyl)-L-cysteine (Ac-DCVC) and *S*-(1,2-dichlorovinyl)-L-cysteine (DCVC) are the glutathione conjugation pathway metabolites of a common industrial contaminant and potent nephrotoxicant trichloroethylene (TCE). Ac-DCVC and DCVC are accumulated in the renal proximal tubule where they may be secreted into the urine by an unknown apical transporter(s). In this study, we explored the hypothesis that the apical transport of Ac-DCVC and/or DCVC may be mediated by the multidrug resistance associated protein 2 (Mrp2, ABCC2), which is known to mediate proximal tubular apical ATP-dependent transport of glutathione and numerous xenobiotics and endogenous substances conjugated with glutathione. Transport experiments using membrane vesicles prepared from mouse proximal tubule derived cells expressing mouse Mrp2 utilizing ATPase assay and direct measurements of Ac-DCVC/DCVC using liquid chromatography/tandem mass-spectrometry (LC/MS/MS) demonstrated that mouse Mrp2 mediates ATP-dependent transport of Ac-DCVC. Expression of mouse Mrp2 antisense mRNA significantly inhibited the vectorial basolateral to apical transport of Ac-DCVC but not DCVC in mouse proximal tubule derived cells endogenously expressing mouse Mrp2. The results suggest that Mrp2 may be involved in the renal secretion of Ac-DCVC.

Introduction

Trichloroethylene (TCE) is an industrial solvent produced in the United States at a rate of ~130,000 tons per year, and is the most commonly found chemical contaminant in ground water (De Rosa et al., 1996). TCE is especially toxic for kidney, liver, heart, skin, and the central nervous and gastrointestinal systems (Agency for Toxic Substances and Disease Registry, 1993). TCE causes acute renal failure in mammals by selective necrosis of the proximal straight tubule (Silber et al., 1986; Wolfgang et al., 1989; Wallin et al., 1992; Kays et al., 1993; Cooper, 1994; Dekant et al., 1994; Lash et al., 2001).

An important biochemical mechanism of the detoxification and bioactivation of TCE starts with conjugation with glutathione (GSH) (Inoue et al., 1984). A GSH conjugation pathway metabolite, *S*-1,2-dichlorovinyl-L-cysteine (DCVC), is involved in the mediation of TCE toxicity via the formation of toxic compounds in reactions catalyzed by cysteine conjugate β-lyases or/and flavin monooxygenases (Anders et al., 1994; Anders and Dekant, 1998; Lash et al., 2006). The GSH conjugation pathway occurs predominantly in the liver and results in the production of DCVC and further *N*-acetyl-DCVC (Ac-DCVC) that both accumulate in the renal proximal tubule. Ac-DCVC is not directly toxic because it is not a substrate of β-lyases and flavin monooxygenases. Given that Ac-DCVC in the proximal tubule may be deacetylated by aminoacylase 3 (AA3) (Pushkin et al., 2004), thereby providing the substrate for β-lyases and flavin monooxygenases to generate toxic compounds, intracellular levels of both DCVC and Ac-DCVC in the proximal tubule significantly determine the TCE induced toxicity. Therefore, knowledge of the transporter(s) involved in the renal proximal tubule secretion of Ac-

Abbreviations: AA3, aminoacylase 3; DCVC, *S*-(1,2-dichlorovinyl)-L-cysteine; Ac-DCVC, *N*-acetyl-*S*-(1,2-dichlorovinyl)-L-cysteine, Ac-DCVC; DDM, dodecyl β-D-maltopyranoside; E17bG, estradiol 17-β-D-glucuronide; HPLC, high performance liquid chromatography; GSH, reduced glutathione; KLH, keyhole lymphet hemocyanin; LC/MS/MS, liquid chromatography/tandem mass-spectrometry; LTC4, leukotriene C4; mPCT cells, mouse proximal tubule derived cells; Mrp2, multidrug resistance associated protein 2; PBS, phosphate buffered saline; P_i, inorganic phosphate; RT-PCR, reverse transcription polymerase chain reaction; K_m, Michaelis constant; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; TBS, 20 mM Tris–HCl, pH 7.5, containing 140 mM NaCl; TCE, trichloroethylene; V_{max}, maximum reaction rate.

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DCVC and DCVC is necessary to understand mechanisms involved in the TCE induced nephrotoxicity.

DCVC and Ac-DCVC accumulated in the kidney may be filtered through glomeruli or/and secreted through the renal proximal tubule by the consecutive action of the basolateral amino acid system(s) (Wolfgang et al., 1989) and the basolateral organic acid transporter 1 (Oat1), respectively, and apical transporters. The nature of the apical transporter(s) mediating secretion of DCVC and Ac-DCVC in the proximal tubule is unknown although a Na^+-independent transport of DCVC mediated by isolated rat renal proximal tubule cells has been described (Lash and Anders, 1989) that may be involved in the apical secretion of DCVC in the proximal tubule. The Na^+-dependent transport of DCVC into the rabbit renal brush-border membrane vesicles was described by Wright et al., (1998), which might be responsible for reabsorption of DCVC from the lumen of the proximal straight tubule given the predominant right-side out orientation of the membrane vesicles prepared from renal brush-border membranes (Haase et al., 1978). There are no data on the transport processes involved in reabsorption of Ac-DCVC in renal proximal tubules.

The multidrug resistance associated protein 2 (Mrp2, ABCC2) is a candidate transporter to mediate Ac-DCVC or/and DCVC secretion in the renal proximal tubule because it has been shown to mediate the apical transport of a wide range of endo- and xenobiotics, including bilirubin, hormones, drugs, and carcinogens, primarily as their glucuronide, GSH, or sulfate conjugates (Van Aubel et al., 2000; Nies and Keppler, 2007). Mrp2 is highly expressed at the apical membrane of renal proximal tubules (Schaub et al., 1997), mammalian hepatocytes (Büchler et al., 1996; Paulusma et al., 1996), and small intestinal villi (Van Aubel et al., 2000) where it mediates the ATP-dependent transport efflux of amphiphilic anionic substrates. In the present study, we determined whether Mrp2 mediates the ATP-dependent transport of Ac-DCVC and/or DCVC using (1) membrane vesicles prepared from mouse proximal tubule derived cells expressing mouse Mrp2, and (2) monolayers of polarized mouse proximal tubule derived cells expressing mouse Mrp2.

Membrane vesicles containing Mrp2 provide an opportunity to characterize its transport characteristics. Given the ATP-dependence of this system, the hydrolysis of ATP is often used as an indirect measure of the transport kinetics. The transport characteristics of Mrp2 have been investigated extensively using canalicular membrane vesicles, and hepatocytes isolated from wild type (wt) and Mrp2-deficient rats (Oude Elferink et al., 1995; Suzuki, Sugiyama, 1998). Unfortunately, the predominant right-side out orientation of the membrane vesicles preparations using the renal brush-border proximal tubule membranes (Haase et al., 1978) prevents their use to study the transport kinetics mediated by renal Mrp2. Therefore, membrane vesicles prepared from the animal cells expressing recombinant Mrp2 are an attractive alternative experimental approach that we used in the present study. In addition, we measured the vectorial basolateral to apical and apical to basolateral transport of Ac-DCVC and DCVC through monolayers of the mouse proximal tubule derived mPCT cells endogenously expressing Mrp2. The results indicate that mouse Mrp2 mediates the ATP-dependent transport of Ac-DCVC but not DCVC, and may be involved in renal secretion of Ac-DCVC.

Materials and methods

Cloning of mouse Mrp2. Mouse full-length Mrp2 cDNA was amplified by RT-PCR from a mouse kidney total RNA (Stratagene, La Jolla, CA) using following sense (CGGTACCTATGGACGAATTCTGCAACTCTAC) and antisense (TCTCGAGCTAGAGCTCCGTGTGGTTCACACT) primers. For expression in the mouse proximal tubule derived mPCT-A2 cells, it was inserted into the *Kpn*I and *Xho*I site of the pcDNA3.1/His vector (Invitrogen, Carlsbad, CA).

Preparation of Mrp2-containing membrane vesicles. The mPCT-A2 cell line that does not endogenously express Mrp2 (Fig. 1A) was used in these experiments. Cells were grown on plastic dishes with mouse renal tubular epithelium medium, which contained 12 ml of a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium, and 2 mM glutamine, 10 ng ml^{-1} epidermal growth factor, 5 μg ml^{-1} insulin, 5 μg ml^{-1} transferrin, 4 μg ml^{-1} dexamethasone, 10 units ml^{-1} interferon-γ, and 5 % fetal bovine serum. This medium is identical to the medium used for growth of the mouse proximal tubule derived mPCT cell line (Gross et al., 2001). Cells were transfected with 3 μg μl^{-1} of purified pcDNA3.1/His vector (Qiagen, Santa Clarita, CA) containing the coding sequence of mouse full-length Mrp2 using the Lipofectamine method (Invitrogen) as recommended by the manufacturer. The cells collected from 10 plates 48 h after transfecton were washed 2 times with phosphate buffered saline (PBS) and resuspended in 0.5 mM sodium phosphate, pH 7.4, containing 0.1 mM EDTA and tablet/20 ml of complete protease inhibitors cocktail and 1 μg ml^{-1} pepstatin (both from Roche, Indianapolis, IN). After incubation at 4 °C for 90 min, the suspension was centrifuged at 14,000×*g* for 15 min, and then the supernatant was centrifuged at 100,000×*g* for 40 min, and the pellet was homogenized in ice-cold 50 mM Tris–HCl, pH 7.5, containing 250 mM sucrose, in a tight-fitting Dounce homogenizer. After centrifugation at 500$^-$*g* for 10 min at 4 °C, the supernatant was centrifuged at 15,000×*g* for 10 min, and then the supernatant was centrifuged at 4 °C for 40 min at 100,000×*g*. The pellet was resuspended in 50 mM Tris–HCl, pH 7.5, containing 250 mM sucrose, and passed 25 times through a 27-gauge needle. The vesicles were dispensed in aliquots, frozen in liquid nitrogen, and stored at −80 °C until use but not for more than 3 months. The content of Mrp2 in the vesicles was analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting using our RP-4 antibody specific to mouse Mrp2.

Vesicles were also prepared using an identical procedure from mock-transfected and untransfected mPCT-A2 cells.

Mouse Mrp2-specfic antibody RP-4. The mouse Mrp2-specific antibody RP-4 was generated in rabbit using a specific peptide (KKSQQSPEGTSHGL, amino acids 261–274 in mouse Mrp2) coupled to keyhole lymphet hemocyanin (KLH). The antibody was affinity purified using the immunizing peptide coupled to Sepharose 6B beads (GE HealthCare, Piscataway, NJ). The antibody worked well on immunoblotting, immunohistochemistry of frozen mouse kidney and liver tissues, and on immunoelectron microscopy of mouse kidney.

Immunoblotting. Protein samples were resolved by SDS-PAGE using 7.5% polyacrylamide ready gels from Bio-Rad (Hercules, CA) and then electrotransferred onto PVDF membranes (GE HealthCare). Non-specific binding was blocked by incubation for 1 h in TBS (20 mM Tris–HCl, pH 7.5, 140 mM NaCl) containing 5% dry milk and 0.05% Tween 20 (Bio-Rad). The primary RP-4 antibody and secondary horseradish peroxidase-conjugated mouse anti-rabbit antibody (Jackson ImmunoResearch, West Grove, PA) were used at a dilution 1:1000 and 1:20,000, respectively. Bands were visualized using an ECL kit and ECL Hyperfilm (both GE HealthCare).

Vesicular ATPase activity. Vesicle ATPase activity was measured in an assay of a total volume 200 μl containing 5 mM ATP, 10 mM $MgCl_2$, 50 mM KCl, 2 mM GSH, 2 mM dithiothreitol, 50 mM HEPES, pH 7.5, 3 μl of membrane vesicles and up to 1 mM Ac-DCVC or other compound. After incubation at 37 °C for 30 min, inorganic phosphate (P_i) was quantified with ammonium molybdate (Bencini et al., 1983) by measuring the absorbance at 350 nm on a VMax Kinetic Microplate Reader (Molecular Devices, Sunnyvale, CA). The amount of P_i was calculated using a standard curve generated with potassium