Modulation of Expression of Rat Mitochondrial 2-Oxoglutarate Carrier in NRK-52E Cells Alters Mitochondrial Transport and Accumulation of Glutathione and Susceptibility to Chemically Induced Apoptosis

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ABSTRACT

We previously showed that two anion carriers of the mitochondrial inner membrane, the dicarboxylate carrier (DIC; Slc25a10) and oxoglutarate carrier (OGC; Slc25a11), transport glutathione (GSH) from cytoplasm into mitochondrial matrix. In the previous study, NRK-52E cells, derived from normal rat kidney proximal tubules, were transfected with the wild-type cDNA for the DIC expressed in rat kidney; DIC transfectants exhibited increased mitochondrial uptake and accumulation of GSH and were markedly protected from chemically induced apoptosis. In the present study, cDNAs for both wild-type (WT) and a double-cysteine mutant of rat OGC (rOGC and rOGC-C221,224S, respectively) were expressed in Escherichia coli, purified, and reconstituted into proteoliposomes to assess their function. Although both WT rOGC and rOGC-C221,224S exhibited transport properties for GSH and 2-oxoglutarate that were similar to those found in mitochondria of rat kidney proximal tubules, rates of transport and mitochondrial accumulation of substrates were reduced by 75% in rOGC-C221,224S compared with the WT carrier. NRK-52E cells were stably transfected with the cDNA for WT-rOGC and exhibited 10- to 20-fold higher GSH transport activity than nontransfected cells and were markedly protected from apoptosis induced by tert-butyl hydroperoxide (tBH) or S-(1,2-dichlorovinyl)-l-cysteine (DCVC). In contrast, cells stably transfected with the cDNA for rOGC-C221,224S were not protected from tBH- or DCVC-induced apoptosis. These results provide further evidence that genetic manipulation of mitochondrial GSH transporter expression alters mitochondrial and cellular GSH status, resulting in markedly altered susceptibility to chemically induced apoptosis.

The mitochondrial pool of GSH in cells such as hepatocytes and renal proximal tubular cells is critical for regulation of thiol and redox status, cellular energetics, and as part of the process involved in induction of apoptosis (Beatrice et al., 1984; Yagi and Hatefi, 1984; Lé Quôc and Lé Quôc, 1985; Reed, 1990; Kroemer et al., 1998). Although free GSH is present at similar millimolar concentrations in both the mitochondrial matrix and cytoplasm, the mitochondrial GSH pool is not passively regulated. Because there is little if any de novo synthesis of GSH within the mitochondria (Griffith and Meister, 1985; Lash et al., 1998), the mitochondrial GSH pool must be derived from transport of GSH from the cytoplasm across the mitochondrial inner membrane (Smith et al., 1996).

Initial investigations of mitochondrial GSH transport in liver or kidney focused on the relationship between transport activity and energetics (Griffith and Meister, 1985; Martensson and Meister, 1989; Schnellmann, 1991). Our first approach toward understanding mitochondrial GSH transport began with the realization that GSH is an anion; hence, it may be a substrate for one or more of the various anion carriers of the mitochondrial inner membrane (McKernan et al., 1991). Several of the anion carriers share a common 30-kDa molecular mass subunit and are believed to belong to

ABBREVIATIONS: GSH, glutathione; DIC, dicarboxylate carrier; 2-OG, 2-oxoglutarate; OGC, oxoglutarate carrier; rDIC, rat dicarboxylate carrier; tBH, tert-butyl hydroperoxide; WT, wild-type; rOGC, rat 2-oxoglutarate carrier; DCVC, S-(1,2-dichlorovinyl)-l-cysteine; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; RT-PCR, reverse transcription-polymerase chain reaction; bp, base pair(s); Mes, 4-morpholineethanesulfonic acid; TMD, transmembrane domain; PLP, pyridoxal 5’-phosphate; Ph Succ, phenylsuccinate.
a carrier “superfamily” (Palmieri et al., 1996). Substrates for these various carriers include inorganic phosphate, citric acid cycle metabolites, and glycolytic intermediates such as lactate and pyruvate. We demonstrated an important role for two of the carriers from rat kidney mitochondria, the dicarboxylate carrier (DIC; Slc25a10) and the oxoglutarate carrier (OGC; Slc25a11), in GSH transport by assessments of substrate specificities, patterns of inhibition, and energetics in suspensions of isolated mitochondria from rat renal cortical homogenates, isolated rat proximal tubular cells, and partially purified and reconstituted carrier proteins from rabbit kidney mitochondria (McKernan et al., 1991; Chen and Lash, 1998; Lash et al., 1998; Chen et al., 2000).

Both the DIC and OGC proteins have been purified to homogeneity and reconstituted into proteoliposomes (Bisaccia et al., 1985, 1988; Kaplan and Pedersen, 1985; Landark-Benba et al., 1994). Two difficulties with studying the purified DIC protein from mammalian tissues relate to its low abundance in the inner mitochondrial membrane and its instability in its purified form, because of autoxidation and inactivation (Kaplan and Pedersen, 1985; Bisaccia et al., 1988). To circumvent this limitation, the cDNA for the DIC from Norway rat, Rattus norvegicus, was isolated using related expressed sequence tags homologous to genes in Caenorhabditis elegans and yeast (Kakhkiashvili et al., 1997). The rat DIC was then expressed in Escherichia coli and functionally reconstituted into proteoliposomes (Fiermonte et al., 1998).

In previous work (Lash et al., 2002b), we expressed recombinant rat DIC (rDIC) as the N-terminal polyhistidine (His$_n$)-fusion protein from rat kidney in E. coli, reconstituted the purified DIC into proteoliposomes, and showed that it functions in transport of both dicarboxylates and GSH with properties similar to those of intact mitochondria. We then transiently transfected the cDNA for rDIC in a cell line derived from rat kidney proximal tubules (NRK-52E cells). The recombinant rDIC-His$_n$ in transfected NRK-52E cells localizes in mitochondria whereupon it mediates GSH uptake, thereby demonstrating function of the carrier in an intact renal cell. Furthermore, overexpression of the rDIC in NRK-52E cells markedly protected against apoptosis induced by either DCVC or tert-butyl hydroperoxide (tBH), supporting the toxicological significance of the mitochondrial GSH transport process.

In the present work, we studied the function of the rOGC, as both a bacterially expressed and reconstituted protein and in transfected NRK-52E cells. We then generated a double-cysteine mutant of the rOGC and showed that it exhibits markedly lower transport activity than the wild-type (WT) carrier. cDNAs for both the wild-type and double-cysteine mutant of the rOGC were then stably transfected into NRK-52E cells to assess further the influence of genetic manipulation of mitochondrial GSH transport activity on susceptibility of the cells to chemically induced apoptosis. Our results provide further evidence that manipulation of mitochondrial GSH transport activity in a renal cell line can modulate responses to cytotoxic chemicals.

**Materials and Methods**

**Experimental Design.** The overall objective of this study was to determine whether modulation of mitochondrial concentrations of GSH can alter susceptibility of a renal proximal tubular cell to chemically induced apoptosis. Mitochondrial GSH status was altered by stable transfection of NRK-52E cells, a stable cell line derived from rat proximal tubules, with either the cDNA for rOGC or a double-cysteine mutant rOGC-C221,224S that exhibits markedly reduced transport function. Thus, comparisons were made between wild-type NRK-52E cells, which possess relatively low transport function and levels of mitochondrial GSH; NRK-52E cells stably overexpressing the rOGC, which possess high transport function and levels of mitochondrial GSH; and NRK-52E cells stably overexpressing the rOGC-C221,224S, which possess low-to-moderate transport function and moderate levels of mitochondrial GSH. Two well-characterized mitochondrial toxicants, tBH and DCVC, were used at concentrations that are known to cause apoptosis but little or no necrosis. In addition, incubations were performed for relatively short times because this is also the time frame over which the cells primarily undergo apoptosis.

**Materials.** l-$^{3}$$H$-Glycyl-GSH (44.8 Ci/mmol) and l-$^{3}$$C$2-OG (281 mCi/mmol) were purchased from DuPont NEN (Boston, MA). Restriction enzymes for PCR, other enzymes (e.g., DNA polymerases and T7 RNA polymerase), and plasmids were purchased from New England Biolabs (Beverly, MA), Promega (Madison, WI), and Gibco-BRL/Life Technologies (Gaithersburg, MD). PCR primers were custom synthesized by Integrated DNA Technology, Inc. (Coralville, IA). Cloning vectors (pGEM-T Easy and pRSET, pcDNA3.1/V5-HisTOPO) were purchased from Promega and Invitrogen (Carlsbad, CA). Dowex-1, Triton X-100, Percoll, phospholipids, and digitonin (aqueous type, recrystallized twice from hot ethanol) were purchased from Sigma-Aldrich (St. Louis, MO). Silicone oil (high temperature, $n_D = 1.4950$ at $20^\circ C$, $d = 1.050$) and mineral oil (white, light, paraffin oil, $n_p = 1.4760$ at $20^\circ C$, $d = 0.862$) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Materials for gel electrophoresis (SDS, acrylamide, and agarose, buffers) were purchased from Bio-Rad (Hercules, CA) or Sigma-Aldrich. ProBond nickel-chelating resin was purchased from Invitrogen. Millipore polycarbonate filter inserts (0.2-$\mu m$ pore size, 25-mm diameter) were purchased from Millipore Corporation (Billerica, MA). NRK-52E cells (catalog no. CRL-1571) and cell culture medium (catalog no. 30-2002; Dulbecco’s modified Eagle’s medium with 4 mM l-glutamine adjusted to contain 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, and 1.0 mM sodium pyruvate), and 10% (w/v) bovine calf serum were purchased from the American Type Culture Collection (Manassas, VA). Antibodies to the His$_n$-fusion proteins were purchased from Invitrogen or Qiagen (Valencia, CA). DCVC was synthesized from trichloroethylene and l-cysteine in sodium metal and liquid ammonia (Elfarra et al., 1986). Purity ($>95\%$) was assessed by high-performance liquid chromatography and thin layer chromatography, and identity was confirmed by $^1$H NMR. Double-distilled, deionized water was used for all experiments. All other chemicals and reagents were purchased from commercial vendors and were of the highest purity available.

**Amplification of Rat Kidney Mitochondrial rOGC cDNA by RT-PCR and Bacterial Expression.** Total RNA was previously isolated from renal cortical homogenates of male Fischer-344 rats (200–300 g body weight; Charles River Laboratories, Inc., Wilmington, MA) by acid phenol extraction using the TRIzol extraction kit (Gibco-BRL/Life Technologies) and was stored at −80°C until needed. Total rat kidney RNA was reverse transcribed with SuperScript II reverse transcriptase and amplified with forward and reverse primers based on the complete cDNA sequence (1149 bp) for the heart mitochondrial OGC protein from the Norway rat (sequence reported by Dolce et al. (1994); also see GenBank accession no. NM_022998, which reports cDNA sequence corresponding to positions 42 to 987 of the above-mentioned sequence). PCR primers were designed with the aid of Oligo 7.0 (Molecular Biology Insights, Inc., Cascade, CO), based on the knowledge that the coding region is from positions 42 to 986, and were as follows: upper primer, 5’-GCC GAG GCC CAT CAA GGG ACG ATT-3’; positions 21 to 43; and lower primer, 5’-CTT TGA TGA GCT GCT GCT GTG GGT-3’. Conditions were: 94°C for 2 min, followed by 40 cycles of 94°C for 30 sec, 54°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 5 min.
primer, 5'-ACT TGG AAA CCC TGGCAC ACG AGT CTC A-3', positions 1000 to 1027. RT-PCR was conducted by running 30 cycles of 94°C x 3 min for denaturation and 70°C x 2 min for annealing/extension. Sample was then loaded onto a 1% (w/v) agarose gel, and the gel was stained with ethidium bromide, and the bands were visualized under UV light. The 1149-bp product was ligated into a T-A cloning vector (pGEM-T Easy) for transformation. Confirmation of the PCR product was by automated DNA sequencing.

The full-length cDNA for rat kidney OGC was subcloned into the pRSSET T7 expression vector for high-level expression in E. coli as a His<sub>6</sub> fusion product. Bacterial cells containing the overexpressed protein were harvested by centrifugation (5000g x 5 min at 4°C), supernatants were discarded, and pellets were resuspended in 20 ml of TE-1 buffer (50 mM Tris-HCl and 2 mM EDTA, pH 8.0). Cells were then lysed by incubation for 15 min at 30°C and occasional mixing of TE-2 buffer (10 mM 2-OG) in a final volume of 1 ml. After mixing on a vortex mixer, approximately 60 min and was stored at room temperature for approximately 5 min before being used in transport assays. This freeze-thaw cycle and sonication pretreatment Measurements. Uptake rates were calculated by linear curve-fitting as described previously (Chen and Lash, 1998; Chen et al., 2000), plotting ln[P<sub>total</sub>/(P<sub>total</sub> - P<sub>i</sub>)] versus time according to Halestrap (1975). P<sub>total</sub> represents the total uptake of substrate at equilibrium (estimated by exponential decay curve fitting of time-course data), and P<sub>i</sub> represents substrate uptake at time t (0–5 min). The initial rates of uptake were then determined from the first-order rate equation v = k (P<sub>total</sub>).

As a control, liposomes were reconstituted with either buffer and no protein or protein that was inactivated by boiling for 5 min. These control liposomes exhibited less than 5% of the measured uptake of either GSH or 2-OG (data not shown), similar to previous reconstitution studies (Chen et al., 2000), indicating that nearly all of the measured substrate transport was because of the function of the reconstituted carrier protein.

Site-Directed Mutagenesis for Generation of a Double-Cysteine Mutant of rOGC. A double-cysteine mutant of the rOGC (rOGC-C221,224S), in which C221 and C224 were converted to S, was generated by nested PCR using two sets of upstream and downstream primers: upstream primer 1, 5'-GCC GAG GGG CAT CAA GGG AGC AT-3'; and downstream primer 2, 5'-GGC GAG AAG TGG GAC AGA TTG TTG TCA G-3'. Protein was purified using a Ni<sup>2+</sup>-affinity chromatography, and reconstituted into proteoliposomes as described above for the wild-type rOGC.

Culture of NRK-52E Cells. NRK-52E cells were cultured on collagen-coated, polystyrene T-25 or T-175 culture flasks with Dulbecco's modified Eagle’s medium containing 4% (v/v) glutamine, 1.5 g/l sodium bicarbonate, 4.5 g glucose/l, 1 mM sodium pyruvate, and 10% (v/v) bovine calf serum in an atmosphere of 5% CO<sub>2</sub>, 95% air at 37°C. On reaching confluence (5–9 days), subcultures were prepared by a 15-min treatment with 0.02% (w/v) EDTA, 0.05% (w/v) trypsin solution, and replating the cells at a density of 4 x 10<sup>4</sup> cells/cm<sup>2</sup>.

Transfection of NRK-52E Cells. Plasmid DNA was purified, following amplification in E. coli cells, using the Promega Wizard Prep purification kit. cDNAs for either wild-type rOGC or rOGC-C221,224S were subcloned into the pcDNA3.1/V5-His-TOPO vector and stably transfected into the NRK-52E cells with FuGENE 6 from Roche Applied Science (Indianapolis, IN). Transfections were carried out as described by the manufacturer using a 3:1 ratio of FuGENE 6 reagent to plasmid DNA. After 24 h, cells were harvested by incubating with Cellstripper (Mediacech, Herndon, VA) for 5 min and gentle scraping. Stable transfectants were selected with Geneticin (G418).

Isolation of Mitochondria from NRK-52E Cells and Transport Measurements. Mitochondria were prepared from seven confluent T-175 flasks, which contained approximately 70 to 85 x 10<sup>6</sup>
concentrations were adjusted to 1 to 3. For gel electrophoresis, 10 or 20 mg protein was loaded per well onto a 10% (w/v) or a 12% (w/v) polyacrylamide gel (Bio-Rad), and separation was achieved according to the method of Laemmli (1970). Total protein was visualized by staining with Comassie Brilliant Blue G. For Western blot analysis, protein was transferred by electroblotting to a nitrocellulose membrane (MSI, Westborough, MA), blocked with 3% (w/v) bovine serum albumin (Promega), washed with Tris-buffered saline containing Tween 20, probed with a monoclonal anti-RRGGHHHH tag antibody (QIAGEN), washed with Tris-buffered saline containing Tween 20, and then probed with an anti-mouse IgG antibody conjugated to alkaline phosphatase (Jackson ImmunoResearch Laboratories Inc., West Grove, PA). Immunoreactive bands were visualized following incubation with a solution containing 5-bromo-4-chloro-3-indolyl-phosphatase (Jackson ImmunoResearch Laboratories Inc., West Grove, PA). The PCR reaction was performed on 10 separate occasions, and 6 bp nucleotide differences were observed for tissues from which the mitochondrial OGC cDNA sequence is available in either the literature or in GenBank (Table 1).

Results

Tissue-Specific Variations in rOGC cDNA and Amino Acid Sequences. In the course of amplifying the cDNA of mitochondrial OGC using total rat kidney RNA as a template, we discovered that the sequence of our PCR products exhibited significant differences from the rat heart and brain mitochondrial OGC cDNAs, which are the only two rat tissues from which the mitochondrial OGC cDNA sequence is available in either the literature or in GenBank (Table 1). The PCR reaction was performed on 10 separate occasions, each time with the identical sequence being obtained for the product. Nucleotide differences were observed for 6 bp between kidney and heart and for 10 bp between kidney and brain. Amino acid differences for deduced sequences include two residues between kidney and heart and six residues between kidney and brain. Although neither of the two amino acid differences between kidney and heart OGC is in predicted transmembrane domains (TMDs) and both are substitutions of amino acids that are similar in terms of charge and polarity (T for A and T for I), two of the amino acid differences between kidney and brain OGC are in predicted TMDs (TMD2 and TMD3), and one difference is very close to TMD1. Two of these differences are substitutions of amino acids that differ by charge or polarity (K for N and T for P) and therefore could have a significant effect on protein tertiary structure.

There is also uncertainty about the identity of transporters for 2-OG and GSH in rat liver. Although rat liver OGC may have a high degree of homology with rat brain OGC (Coll et al., 2003), its sequence has not been reported. Although many of the
reported properties of GSH and 2-OG transport in rat liver mitochondria are similar to what we have found in rat kidney mitochondria (Coll et al., 2003). GSH transport in rat liver mitochondria was inhibited by L-glutamate, which differs from our previous findings. A thorough search of the GenBank database and the published literature, however, failed to find any cDNA or amino acid sequences for the OGC from rat liver mitochondria. Fiermonte et al. (2001), however, reported cloning and expression of a rat liver oxodicarboxylate carrier that transports 2-OG and other C2-C5 dicarboxylates. They isolated a 1456-bp cDNA with a 99-bp 5'-untranslated region, an open reading frame of 897 bp, and a 460-bp 3'-untranslated region. The cDNA encodes a polypeptide of 298 amino acids with a molecular mass of 33,276 kDa, which contrasts with the OGC from rat kidney, heart, lung, spleen, testes, skeletal muscle, and brain. The protein was detected by immunodetection in mitochondria of rat heart, kidney, liver, lung, spleen, and brain. The relationship of the ODC with the OGC, however, is unclear.

The published nucleotide and amino acid sequences from the heart, brain, and liver of the rat noted are compared with those of the rat kidney that we obtained in our current study. Sequences were aligned using BLAST.

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<th>Amino Acid Sequences</th>
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<td>vs. Brain OGC GenBank accession nos: DNA: NM_022388, U84727 (source); Protein: AAB41797, NP_071785</td>
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<td>vs. Liver OGC GenBank accession nos: DNA: AJ289714; Protein: CAC277162</td>
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The carrier cDNA with accession number AJ289714 is listed in GenBank as the rat mitochondrial oxodicarboxylate carrier. It is described in the original reference (Fiermonte et al., 2001) as an exchanger that transports 2-OG as well as several other C2-C5 dicarboxylates. mRNA for the carrier was detected by RT-PCR in mitochondria of rat liver, kidney, heart, lung, spleen, testes, skeletal muscle, and brain. The protein was detected by immunodetection in mitochondria of rat heart, kidney, liver, lung, spleen, and brain. The relationship of the ODC with the OGC, however, is unclear.

**TABLE 1**

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A) rOGC-His6; SDS Gel.  
B) rOGC-C221,224S-His6; SDS Gel.  
C) rOGC-His6; Western.  
D) rOGC-C221,224S-His6; Western.  

**Fig. 1.** Coomassie-stained SDS gels (A and B) and immunobLOTS (C and D) of wild-type (A and C) and double-cysteine mutant (B and D) rOGC expressed in E. coli. Positive clones from BL21 Lys S cells were transformed with the pRSET B-OGC-WT (A and C) or pRSET C-OGC-C221,224S (B and D) cDNA inserts and were grown for 2 h (A and B) or for 0, 1, or 2 h (C and D) in the presence of isopropyl β-D-thiogalactoside. Cells were then solubilized and put through several freeze-thaw cycles. Lysed cells were centrifuged at 10,000g × 5 min, and 20-μg protein was electrophoresed on 12% (w/v) polyacrylamide gels in the presence of SDS. A, lane 1, flow-through from Ni2+-affinity column; lane 2, standards; lanes 3 and 4, column wash; and lanes 5–7, column elution fractions. B, lane 1, E. coli extract; lane 2, standards; lane 3, flow-through from Ni2+-affinity column; lanes 4 and 5, column wash; and lanes 6–8, column elution fractions. Arrow, rOGC band. C and D, the presence of either the OGC-WT-His6 or OGC-C221,224S-His6 fusion proteins in inclusion bodies were confirmed by Western blots using a monoclonal anti-RGSHHHH tag antibody with chemiluminescence detection. Expected products of 34 to 37 kDa were obtained.
protein expression, as shown by Western blot analysis using the monoclonal anti-RGSHHHH tag antibody, with the expected molecular mass of 34 to 37 kDa, were observed for both the WT (Fig. 1C) and double-cysteine mutant (Fig. 1D) carriers. The overall yield of both the WT and double-cysteine mutant proteins from a 1-liter culture of bacteria was typically 20 to 25 mg of protein.

Reconstitution and Transport Function of rOGC and rOGC-C221,224S. The bacterially expressed and purified rOGC was reconstituted in proteoliposomes for assessment of its ability to transport 2-OG and GSH. Similar to results with the partially purified and reconstituted inner membrane transporter preparation (Chen et al., 2000), the expressed and reconstituted rOGC exhibited time- and concentration-dependent uptake of both 2-OG (Fig. 2A) and GSH (Fig. 2B) with maximal accumulation of substrate after 2 to 3 min of incubation, followed by a decrease at later time points. This spike is consistent with heteroexchange of trans- for cis-substrate. As two controls, liposomes were either reconstituted without added purified protein and uptake of substrates was measured or protein was boiled before incorporation into proteoliposomes and measurement of uptake. As found in our previous studies (Chen et al., 2000; Lash et al., 2002b), both controls exhibited <5% of the apparent uptake of radiolabeled 2-OG or GSH, indicating that transport in the proteoliposomes is because of a catalytically active protein (data not shown).

Initial rates of uptake were derived as described under Materials and Methods, and Eadie-Hofstee plots were constructed to derive kinetic parameters (Table 2). The results show that 2-OG is a much better substrate for the rOGC than is GSH, with the carrier having a 6.9-fold lower $K_m$ value for 2-OG than for GSH, although the $K_m$ value for GSH is well within the range of normal concentrations of GSH in proximal tubular cytoplasm. $V_{max}$ values for 2-OG or GSH as substrates were similar.

Bacterially expressed, purified, and reconstituted rOGC-C221,224S likewise exhibited time- and concentration-dependent uptake of both 2-OG (Fig. 3A) and GSH (Fig. 3B). Similar to transport function for the rOGC-WT, intravesicular accumulation of either substrate exhibited maximal values at approximately 2 min, with modest decreases thereafter. Analysis of kinetic parameters for each substrate by Eadie-Hofstee plots using calculated initial rates (Table 2) showed that the double-cysteine mutant carrier exhibited a 70 and 81% decrease in $V_{max}$ for 2-OG and GSH, respectively, and a 13.6-fold and 14.4% increase in $K_m$ for 2-OG and GSH, respectively. In spite of the modest effect on the $K_m$ value for GSH, it is clear that the transport function of the double-cysteine mutant is markedly diminished compared with that of the wild-type carrier. Transport activity of both the rOGC-WT and rOGC-C221,224S with either 2-OG or GSH as substrate was markedly (>80%) inhibited by either PLP or phenylsuccinate (PhSucc) (Figs. 4 and 5), which is consistent with the expected sensitivity of the carrier.

Effect of Stable Transfection of NRK-52E Cells with cDNAs for rOGC-WT and rOGC-C221,224S on Susceptibility to Chemically Induced Apoptosis. NRK-52E cells were stably transfected with either the cDNA for rOGC-WT or rOGC-C221,224S by selection with G418. Expression of the rOGC-WT protein (Fig. 6A) and the rOGC-C221,224S protein (Fig. 6B) in mitochondrial extracts from stably transfected NRK-52E cells was confirmed by Western blot analyses using the monoclonal anti-RGSHHHH tag antibody.

Before determining the influence of expression of either wild-type or mutant rOGC in the NRK-52E cells on susceptibility to chemically induced apoptosis, we compared the ability of mitochondria from the three cell populations (i.e., nontransfected cells, NRK-52E-WT; cells transfected to overexpress rOGC, NRK-52E-rOGC; and cells transfected to overexpress the double-cysteine mutant carrier, NRK-52E-rOGC-C221,224S) to accumulate either GSH (Fig. 7) or 2-OG (Fig. 8). Each population of NRK-52E cells was incubated with the indicated concentrations of either substrate for various times up to 60 min, and cell samples were washed and fractionated by digitonin treatment to separate cytoplasmic...
and mitochondrial compartments. Results shown for 30-min incubations indicate that cells overexpressing rOGC exhibited markedly higher contents of GSH in mitochondria than either NRK-52E-WT or NRK-52E-rOGC-C221,224S cells, with the latter cell population exhibiting the lowest content of GSH in mitochondria (Fig. 7A). The increase in contents of mitochondrial GSH were prevented by including the rOGC inhibitor PhSucc (Fig. 7B), demonstrating that the alterations in mitochondrial contents of GSH were because of rOGC. Concentrations of GSH in the cytoplasm of the three populations of NRK-52E cells incubated with exogenous GSH were also measured (data not shown). Although differences between the three cell populations were observed, these differences were smaller than those for mitochondrial GSH contents and exhibited an inverse pattern among the three cell populations compared with the pattern for mitochondria. Thus, the highest GSH concentrations in cytoplasm were observed in NRK-52E-WT cells, whereas the lowest GSH concentrations in cytoplasm were observed in NRK-52E-rOGC cells. The NRK-52E cells overexpressing the double-cysteine mutant exhibited intermediate levels of GSH in the cytoplasm.

Similar incubations using 2-OG as transporter substrate also demonstrated markedly increased mitochondrial contents of 2-OG in NRK-52E-rOGC cells compared with either nontransfectants or cells expressing the double-cysteine mutant (Fig. 8A). Coincubation with 10 mM PhSucc also markedly diminished most of the increased contents of
mitochondrial 2-OG (Fig. 8B), indicating that the altered mitochondrial contents of 2-OG were largely because of the different levels of activity of the OGC.

The three populations of NRK-52E cells were then incubated for 1, 2, or 4 h with either media (control), 10 or 50 μM tBH, or 50 or 200 μM DCVC for assessment of the fraction of cells undergoing apoptosis (Fig. 9). Although all three populations of control cells exhibited <1.5% apoptosis throughout the 4-h time course, NRK-52E-WT cells exhibited dose-dependent increases in the fraction of cells undergoing apoptosis after exposure to either tBH or DCVC at all time points, with increases after 1- to 2-h incubations and slightly less apoptosis after 4-h incubations (Fig. 9, A–C). This decrease by the 4-h time point is probably because of cells losing competence to undergo apoptosis and switching to necrosis as a mode of cell death (also see Lash et al., 2002a,b). NRK-52E-rOGC cells, in contrast, exhibited very
small increases in apoptosis after toxicant exposures, indicating significant protection (Fig. 9, D–F). NRK-52E-rOGC-C221,224S cells, however, exhibited comparable amounts of apoptosis as NRK-52E-WT cells. Maximal amounts of apoptotic cells (12–13%) were observed with NRK-52E-rOGC-C221,224S cells incubated for 2 h with either 50 μM tBH or 200 μM DCVC (Fig. 9, G–I).

Discussion

The present study sought to further define the role of mitochondrial GSH transporters in determining the susceptibility of renal proximal tubular cells to chemically induced apoptosis, focusing on the OGC and using NRK-52E cells as a model system. NRK-52E cells are an excellent model in
which to examine these processes because they exhibit many properties of in vivo proximal tubules, including retention of epithelial morphology and expression of high activities of the (Na\(^{+} + K^{+}\))\(-\)ATPase and several enzymes of the GSH redox cycle (Lash et al., 2002a). Unlike the in vivo proximal tubule and similar to other epithelial-derived, immortalized cell lines, NRK-52E cells exhibit relatively low activities of brush-border membrane enzymes and relatively low activities of mitochondrial respiration, transport, and dehydrogenase enzymes (Lash et al., 2002a). Whereas mitochondrial GSH content represents approximately 30% of the total cellular GSH in the in vivo rat proximal tubule (Schnellmann et al., 1988; Lash et al., 1998), it comprises only 8 to 10% of the total cellular GSH pool in NRK-52E cells. In spite of this, the relatively high activity of the (Na\(^{+} + K^{+}\))\(-\)ATPase argues that cellular supplies of ATP, and hence mitochondrial function, are adequate for cellular work under normal, physiological conditions. These differences between NRK-52E cells and the in vivo proximal tubule actually facilitate our transport measurements because they provide a model that has low activity of GSH degradation and a low baseline level of mitochondrial transport activity. Although it can be argued that the NRK-52E cells are inherently more sensitive than the in vivo rat proximal tubule to toxicants because of their lower mitochondrial GSH content, the ability to modulate mitochondrial GSH contents over a wide range of values, which include those that greatly exceed those in the in vivo proximal tubule, allows us to directly test the hypotheses we have posed.

After amplification of the cDNA for the rOGC using total rat kidney cortex RNA as a template, comparison of the kidney rOGC DNA sequence with those of the rOGC from rat heart and brain showed differences between these published sequences and that for our PCR product from rat kidney. Moreover, comparison of the deduced amino acid sequences from the rat brain rat kidney PCR product with that from rat heart and brain showed two- and six-amino acid residue differences, respectively, two of which between our rat kidney product and that from rat brain differed by polarity or charge. Because these two differences are in TMDs and probably lead to changes in protein three-dimensional structure, catalytic function of the rOGC from rat kidney and brain may differ. This suggests that tissue-specific differences may exist in the rOGC.

Although the cDNA sequence for rat liver OGC is not published in GenBank, Coll et al. (2003) used the cDNA sequence from rat brain OGC to design primers to detect expression of the OGC in rat liver. From this, one would conclude that the cDNA sequence for rat liver OGC is very similar to or the same as that from rat brain. To complicate matters, however, Fieryntone et al. (2001) identified a carrier, called the oxodicarboxylate carrier, that transports 2-OG but exhibits no significant cDNA sequence homology and only 25 to 27% amino acid homology with the deduced amino acid sequences of our cDNA clone or the two published sequences of rOGC from heart or brain. Repetition of the PCR reaction 10 times, with confirmation by sequencing the PCR cDNA product and expression, purification, and functional reconstitution of the OGC protein, provides confidence that the sequence differences among tissues are real and not because of PCR artifacts. Although additional work is required to clarify the identity and function of carriers for 2-OG and GSH in rat liver, the possibility that the cDNA and deduced amino acid sequences of our OGC cDNA from rat kidney differ significantly from those of other tissues warrants further study.

Transport properties of the amplified, bacterially expressed, and reconstituted rOGC from kidney were consistent with the function of this carrier that we observed previously in suspensions of rat kidney mitochondria (Chen and Lash, 1998) and in proteoliposomes reconstituted with a partially purified preparation of mitochondrial inner membrane carriers from rabbit kidney mitoplasts (Chen et al., 2000). These similarities included a lower \(K_m\) but a higher \(V_{\text{max}}\) value for 2-OG than for GSH as transport substrate and inhibition of transport by PLP and PhSucc. In a previous study (Lash et al., 2002b), we demonstrated that transient overexpression of the cDNA for the rDIC in NRK-52E cells produced a 3- to 11-fold increase in mitochondrial GSH content and protected the cells from tBH- and DCVC-induced apoptosis. Because our previous work (Chen and Lash, 1998) concluded that the rDIC and rOGC contributed approximately 60 and 40%, respectively, to the uptake of GSH from cytoplasm into mitochondrial matrix, our expectation was that overexpression of the rOGC in NRK-52E cells would also provide significant protection from tBH- and DCVC-induced apoptosis. Indeed, this expectation was realized because stably transfected NRK-52E cells that overexpressed the rOGC were almost completely protected and significantly protected, respectively, from tBH- and DCVC-induced apoptosis. The difference in the ability to protect from tBH- and DCVC-induced apoptosis probably lies in the distinct mechanisms of action of the two toxicants. Whereas tBH can be described as a “pure” oxidant that causes lipid peroxidation and GSH oxidation in mitochondria (McKernan et al., 1991), DCVC exerts mitochondrial toxicity by at least two mechanisms, including both an oxidative stress with increased lipid peroxidation and GSH oxidation (Lash and Anders, 1987), and inhibition of mitochondrial dehydrogenases by alkylation (Lash and Stevens, 1990; van de Water et al., 1995; Cooper et al., 2002). The NRK-52E-rOGC cells also exhibited markedly higher contents of mitochondrial GSH than NRK-52E-WT cells when each population was incubated with exogenous GSH (cf. Fig. 7), indicating that the protection was associated with an enhanced ability of the transfectants to accumulate GSH in their mitochondria.

Although most previous studies of tBH- and DCVC-induced cellular injury have focused on the association of their biochemical effects with cellular necrosis, this occurs at much higher doses of toxicants than were used in the present study. Thus, although both tBH and DCVC can cause oxidation of mitochondrial GSH and cellular necrosis (Lash and Anders, 1987; McKernan et al., 1991; Lash et al., 1998), these same toxicants can result in apoptosis under appropriate exposure conditions (Lash et al., 2001). The pronounced oxidation of the mitochondrial GSH pool and lipid peroxidation described previously occurred at much higher concentrations than those used in the present study.

To explore how much mitochondrial GSH is necessary and sufficient to protect NRK-52E cells, we constructed a double-cysteine mutant of the renal rOGC by site-directed mutagenesis. Although amplified, bacterially expressed, and reconstituted rOGC-C221,224S exhibited similar sensitivity to PLP and PhSucc as the wild-type carrier, it exhibited markedly
lower catalytic efficiency (i.e., $V_{\text{max}}/K_v$ values) for transport of 2-OG and GSH. NRK-52E-ROG-C221,224S cells were no less sensitive than NRK-52E-WT cells to TBH- and DCVC-induced apoptosis. This lack of protection suggests that there is a threshold in the mitochondrial content of GSH that is needed to prevent cell death from oxidants or other mitochondrial toxins. Consistent with this conclusion, mitochondrial accumulation of GSH was modestly lower in NRK-52E-ROG-C221,224S cells than in NRK-52E-WT cells (cf. Fig. 7). This conclusion is also consistent with observations that certain forms of chemically induced cytotoxicity, notably oxidative stress, are dependent on depletion of mitochondrial GSH below a certain level (Martensson and Meister, 1989; Reed, 1990; Shan et al., 1993; Fernandez-Checa et al., 1997). Comparison of the extent of protection by overexpression of the rOGC-C221,224S cells than in NRK-52E-WT cells (cf. Fig. 7).

In summary, the present study has provided additional evidence that manipulation of mitochondrial GSH transport function can modulate renal cellular susceptibility to toxicants and has provided insight into the limitations of GSH levels required to elicit protection, consistent with the existence of a threshold level of mitochondrial GSH that is required for protection. Determination of this threshold concentration will require a more systematic analysis of correlations between transport rates, mitochondrial GSH content, and cellular susceptibility. The reported association of depletion or oxidation of hepatic mitochondrial GSH with several disease states, including chronic alcohol consumption and alcoholic liver disease (Fernandez-Checa et al., 1993) and cirrhosis and other forms of biliary obstruction (Krahnenhuß et al., 1992, 1995), emphasizes the importance of understanding how mitochondrial GSH is regulated. In the kidneys, studies have not directly addressed the question of mitochondrial GSH and disease, although diabetic nephropathy is associated with oxidative stress and GSH oxidation (Obrosova et al., 2003). The potential clinical significance of modulating mitochondrial GSH content is further highlighted by a study using mouse B16 melanoma cells, in which the authors proposed that maintenance of mitochondrial GSH content delays the progress of cellular injury.

References


van de Water B, Zoeteweij JP, de Bent HJGM, and Nagelkerke JF (1995) Inhi-
bition of succinate:ubiquinone reductase and decrease of ubiquinol in nephro-
toxic cysteine S-conjugate-induced oxidative cell injury. Mol Pharmacol 48:
928–937.


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