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Mitochondrial Glutathione Transport: Physiological, Pathological and Toxicological Implications

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Abstract

Although most cellular glutathione (GSH) is in the cytoplasm, a distinctly regulated pool is present in mitochondria. Inasmuch as GSH synthesis is primarily restricted to the cytoplasm, the mitochondrial pool must derive from transport of cytoplasmic GSH across the mitochondrial inner membrane. Early studies in liver mitochondria primarily focused on the relationship between GSH status and membrane permeability and energetics. Because GSH is an anion at physiological pH, this suggested that some of the organic anion carriers present in the inner membrane could function in GSH transport. Indeed, studies by Lash and colleagues in isolated mitochondria from rat kidney showed that most of the transport (>80%) in that tissue could be accounted for by function of the dicarboxylate carrier (DIC, *Slc25a10*) and the oxoglutarate carrier (OGC, *Slc25a11*), which mediate electroneutral exchange of dicarboxylates for inorganic phosphate and 2-oxoglutarate for other dicarboxylates, respectively. The identity and function of specific carrier proteins in other tissues is less certain, although the OGC is expressed in heart, liver, and brain and the DIC is expressed in liver and kidney. An additional carrier that transports 2-oxoglutarate, the oxodicarboxylate or oxoadipate carrier (ODC; *Slc25a21*), has been described in rat and human liver and its expression has a wide tissue distribution, although its potential function in GSH transport has not been investigated. Overexpression of the cDNA for the DIC and OGC in a renal proximal tubule-derived cell line, NRK-52E cells, showed that enhanced carrier expression and activity protects against oxidative stress and chemically induced apoptosis. This has implications for development of novel therapeutic approaches for treatment of human diseases and pathological states. Several conditions, such as alcoholic liver disease, cirrhosis or other chronic biliary obstructive diseases, and diabetic nephropathy, are associated with depletion or oxidation of the mitochondrial GSH pool in liver or kidney.

Keywords

Glutathione; Mitochondria; Transport; Dicarboxylates; Dicarboxylate carrier; Oxoglutarate carrier; Oxidative stress; Diabetic nephropathy; Alcoholic liver disease

1. Introduction

Glutathione (GSH) is the primary low-molecular-weight thiol in all aerobic cells. Its functional importance lies in the presence of the thiol group on the cysteinyl residue **(**Fig. 1). The thiol enables GSH to function as both a reductant and a nucleophile. Synthesis of GSH occurs by two sequential, ATP-dependent reactions in the cytoplasm and is catalyzed by γ-glutamylcysteine synthetase and GSH synthetase. The available evidence indicates that most if not all of the biosynthetic activity resides in the cytoplasm with little if any activity in other organelles, including the mitochondria [1,2]. Virtually all mammalian cells have some capacity

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to synthesize GSH and activity is widely distributed. Degradation of GSH is mediated by a separate pathway that, in contrast to the synthetic pathway, exhibits a discrete tissue distribution [3]. Thus, the initial step in the breakdown of GSH, which involves cleavage of the γ-glutamyl-peptide bond by γ-glutamyltransferase (GGT), is localized in luminal membranes of epithelial cells, such the renal proximal tubule and enterocytes, and is largely absent from cells such as hepatocytes or cardiac myocytes.

As a reductant, GSH maintains intracellular, sulfhydryl-containing proteins in the reduced and active form by either the reduction of potentially toxic peroxides or by the action of thiol-disulfide exchange reactions. The first process is mediated by the selenium-containing GSH peroxidase and the second process is mediated by thioltransferases. The GSH–glutathione disulfide (GSSG) redox pair has a midpoint electrochemical potential at pH 7.0 ($E_{m,7}$) of −240 mV, which places it well between the most reduced redox couple, H^+/H_2 (−420 mV) and the most oxidized redox couple, O_2/H_2O (+820 mV). Accordingly, the GSH/GSSG redox couple can readily interact with most of the physiologically relevant redox couples, undergoing reversible oxidation or reduction reactions, thereby maintaining the appropriate redox balance in the cell.

As a nucleophile, GSH serves a critical function in the cell by reacting with electrophiles that are generated as a consequence of metabolic processes involving both endogenous compounds and xenobiotics. While GSH can react non-enzymatically with electrophiles, rates of nucleophilic addition reactions are greatly enhanced by the catalytic action of a family of enzymes called the GSH *S*-transferases (GSTs). Most of the GST isoforms are present in the cytoplasm, although isoforms are also present in the endoplasmic reticulum and mitochondrial matrix.

The mitochondrion is an excellent example of a subcellular organelle whose function is closely linked to maintenance of redox balance. As mitochondria are the primary intracellular sites of oxygen consumption, they may also be primary sites of generation of reactive oxygen species (ROS). Although normal electron transport in mitochondria involves four-electron reduction of molecular oxygen to water, partial reduction reactions occur even under physiological conditions, causing release of superoxide anion and hydrogen peroxide. Toxic or pathological conditions, such as oxidative stress, that lead to an impairment of mitochondrial function, can increase release of ROS. A large number of mitochondrial enzymes, including dehydrogenases and transport ATPases, contain critical sulfhydryl groups that must be maintained in the reduced form for proper function [4]. Furthermore, redox-sensitive components in the electron transport chain, such as iron ions on heme prosthetic groups and iron-sulfur centers, may be oxidized during a redox imbalance, thereby producing mitochondrial dysfunction.

The redox status of GSH and other thiols has long been known to be critical for proper mitochondrial function [5–8]. Alterations in GSH concentration and redox status have been associated with oxidative stress induced by peroxides and other oxidants in mitochondria from kidney, liver, brain, and tumor cells [2,9–15], regulation of mitochondrial Ca^{2+} ion distribution and pyridine nucleotide oxidation status [16–21], damage to mitochondrial DNA [22,23], and induction of the membrane permeability transition [21,24–29]. More recently, changes in mitochondrial GSH status have been associated with activation of signaling pathways and expression of genes that regulate apoptosis [30–37] and cell growth and differentiation [38, 39]. Besides ROS, recent attention has focused on the role of reactive nitrogen species, in particular nitric oxide (NO) and peroxynitrite (ONOO−), in the regulation of mitochondrial and cellular function and in mediating certain forms of chemically induced and pathological injury. Although NO may interact directly with proteins and other cellular macromolecules, effects of NO may also be mediated by formation of *S*-nitrosoglutathione (GSNO) [40–43]. Thus, GSH may react with NO to release ROS **(**Fig. 2) or it may serve as an NO donor via

formation of GSNO **(**Fig. 3). The normally high concentration of GSH in the mitochondrial matrix and the presence of a constitutively expressed nitric oxide synthase in the organelle suggest that formation of GSNO plays an important physiological role.

It is, therefore, clear that maintenance of adequate concentrations of GSH within the mitochondrial matrix is essential for regulation and proper function of numerous critical processes. This review will focus on the current state of knowledge about how the mitochondrial pool of GSH is determined and regulated and some approaches that the author's laboratory has taken to modulate this pool to alter susceptibility to chemically induced injury or disease.

2. Early studies on mitochondrial GSH homeostasis and energetics

The earliest studies that focused specifically on the mitochondrial GSH transport process studied the relationships between mitochondrial respiratory state and transport activity in mitochondria from rat liver. In 1990, Kurosawa et al. [44] found that GSH was transported into rat liver mitochondria at highest rates under state 4 conditions and that transport was diminished by a protonophore, by antimycin A, or under state 3 conditions. Fasted rats were used in their studies, indicating that mitochondria were likely in an energy-depleted state. In that same year, Martensson et al. [45] defined two kinetic components for uptake of GSH by isolated mitochondria from rat liver: A high-affinity component (K_m = 60 μM, V_{max} = 0.5 nmol/min per mg protein) and a low-affinity component (K_m = 5.4 mM, V_{max} = 5.9 nmol/min per mg protein). Both components were inhibited by a protonophore, glutamate, and by the GSH-analogue ophthalmic acid. Fasted rats were also used in their studies. In 1995, Kaplowitz and colleagues [46] expressed GSH transport activity in mitochondria of *Xenopus laevis* oocytes that were microinjected with total liver mRNA. The transport activity exhibited similar properties to those observed in mitochondria from rat liver and was distinct from those present in the canalicular or sinusoidal plasma membranes. Fractionation of poly(A)$^+$ RNA identified a single mRNA species of 3 to 3.5 kb. There is some concern, however, about the identity of the activity measured because the oocytes exhibit some endogenous GSH transport activity and no attempt was made to identify the potential function of specific carriers in GSH uptake.

Using rat kidney mitochondria, Schnellmann [47] concluded that GSH was taken up by both a carrier-mediated process and by diffusion. The carrier-mediated process was modestly (30%) inhibited by glycine, serine, and ophthalmic acid, but not by glutamate, cysteine, γ-glutamylglutamate or proline.

Other studies that are described below provided insight into the relationships between mitochondrial GSH status and several pathological or disease states. Because we know that transport of cytoplasmic GSH into the mitochondrial matrix is the primary, if not sole, determinant of GSH status in the organelle, the implication from these studies is that GSH transport is inhibited or somehow defective. Direct evidence for this suggestion, however, has only recently become available. Although these studies and those described above provided mostly indirect information about GSH transport activity under various physiological or pathological conditions, they did not address the crucial questions of what specific carrier protein(s) mediate(s) the transport process and the specific energy sources that are used to maintain the tightly regulated mitochondrial GSH pool. In the section that follows, we present summaries of the studies that identified and confirmed the function of specific carrier proteins in the mitochondrial inner membrane of rat kidney in transport of cytoplasmic GSH into the matrix.

3. Role of anion carriers in GSH uptake

3.1. GSH as an organic anion: Potential carriers in mitochondrial inner membrane

The charged nature of the GSH molecule suggests that it cannot passively diffuse across the mitochondrial inner membrane. Rather, because mitochondria possess a membrane potential with the matrix space negative relative to the cytoplasm and because GSH is a negatively charged molecule at physiological pH, GSH must be transported actively or in exchange for another anion. Possible net charges for GSH are determined by four functional groups (cf. Fig. 1): There is one free amino group (pK_a = 8.6; predominantly protonated = +1), two free carboxyl groups (pK_a = 3.53 and 2.12; predominantly deprotonated = −2), and the thiol group (pK_a = 9.2; predominantly protonated = 0). Whereas the pH of the cytoplasm is typically near 7.0, that of the mitochondrial matrix is slightly alkaline (approximately 7.8). Hence, more of the −SH groups will be deprotonated in the matrix. Additionally, a microenvironment may exist near the active sites of the carrier proteins that effectively lowers the pK_a of the thiol group so that a much higher proportion of GSH molecules would be in the thiolate form. Overall then, the GSH pool available for mitochondrial carriers is likely to have a net charge of between −1 and −2.

Considering the anionic nature of GSH, our first approach to determining the function of specific, inner membrane carrier proteins in mitochondrial GSH transport was to examine the potential activity of known carriers [2]. Eight known anion carriers are present in the mitochondrial inner membrane that could conceivably play a role in the uptake of GSH from the cytoplasm **(**Table 1). These carriers are involved in the translocation of citric acid cycle intermediates, amino acids, and gluconeogenesis precursors across the mitochondrial inner membrane and thus play critical roles in mitochondrial and cellular energetics [48,49]. High activity of these carriers is expected in cells such as those of the renal proximal tubule because of high rates of mitochondrial respiration, active transport, and gluconeogenesis. These carriers are presumably expressed in mitochondria from all tissues, although tissue-specific differences in expression levels and activities also presumably exist (see section 3.2.4 below).

Based on substrate specificities, potential candidates among these carriers for a role in GSH uptake are the monocarboxylate (MCC), dicarboxylate (DIC; *Slc25a10*), 2-oxoglutarate (OGC; *Slc25a11*), tricarboxylate or citrate (CIC; *Slc25a1*), glutamate–hydroxide (GC1/2;*Slc25a22/18)*, and glutamate-aspartate (AGC1/2;*Slc25a12/13*) carriers. Because the adenine nucleotide translocase and phosphate–hydroxide carriers have fairly restricted substrate specificities, they are not likely to catalyze GSH transport. Although the MCC, DIC, OGC, and CIC all differ with respect to substrate specificity and inhibitor sensitivity, they share a common ~30 kDa molecular weight subunit and are believed to belong to a carrier "superfamily" [50]. Each of these carriers are electroneutral, meaning that they catalyze exchange of anions or a combination of anions and a proton, so that there is no net transfer of charge across the inner membrane. The glutamate–hydroxide carrier is similarly electroneutral, exchanging glutamate for an hydroxide ion. In contrast, the glutamate–aspartate carrier is electrogenic, catalyzing net transfer of one positive charge into the mitochondrial matrix. Although the two glutamate carriers are not likely candidates to mediate transport of GSH, the presence of a glutamyl residue on the GSH molecule suggests the possibility that GSH may interact with these carriers.

3.2. Identification of the DIC and OGC as major GSH transporters

3.2.1. Keys to accurate measurement of mitochondrial GSH transport—One of the most straightforward and simple approaches to determining the potential function of individual membrane carriers in the transport of a given substrate across the inner membrane is to use the experimental model of suspensions of freshly isolated mitochondria [51]. The

mitochondrial suspensions are prepared in a standard buffer, mixed with the appropriate substrate solutions, incubated for various time periods, typically at temperatures no higher than 25°C, and then the intramitochondrial and extramitochondrial compartments are separated by either rapid filtration under vacuum or rapid centrifugation procedures. Although measurement of transport activity in this experimental model is technically simple, the procedures used are subject to a large number of potential artifacts that may compromise the accuracy of measurements [48,52]. Some of these potential artifacts are specific to studies involving GSH, some of specific to studies involving suspensions of isolated mitochondria, and others are relevant to any transport study regardless of the experimental model being used. To convey the complexity of the mitochondrial transport process despite the simplicity of the assay methods, a brief discussion of these artifacts and suggested approaches to minimize or otherwise account for them are presented. We have previously discussed these considerations with regard to accurate measurement of mitochondrial GSH transport [52,53].

Potential artifacts in measurement of mitochondrial GSH transport that are important for any transport study, regardless of substrate, tissue of origin, or specific experimental model being used, include the following: 1) Loss of transported substrate during sample processing; or 2) contamination of compartment of interest with substrate from outside the compartment. The first concern, that of potential loss of transported substrate during sample processing, can be minimized by processing in such a way that the compartments are rapidly separated and metabolic and transport processes inactivated. This is typically accomplished by methods such as filtration under vacuum or rapid centrifugation through a medium that separates compartments and delivers the compartment of interest into a medium that inhibits metabolic and transport processes. Examples of such a stop medium include a buffer that contains a high concentration of an inhibitor of metabolism or transport and/or an acid to effect precipitation of proteins. The second concern, that of contamination of material in the outside of the compartment, can be handled methodologically by the filtration or centrifugation methods just described. In spite of these methods, however, a small fraction of fluid from outside the compartment can still contaminate the sample. This is particularly significant for uptake measurements because of the high concentration of substrate in the medium and the small fraction of total solution volume occupied by the compartment of interest. Such contamination can be monitored by the use of radiolabeled markers of the extra- and intracompartmental space. Typically, an impermeant molecule, such as [^{14}C]-sucrose or [^{3}H]-inulin, is used as a marker for the extracompartmental space and $^{3}H_2O$ is used as a measure of total volume. In this manner, corrections can be made for carry-over of extracompartmental substrate that occurs during sample processing.

Potential artifacts that are unique to studies with isolated mitochondria include: 1) Changes in mitochondrial matrix volume that occur during the transport incubation; and 2) induction of the membrane permeability transition (MPT) during transport incubation. The MPT is defined as a voltage-dependent, cyclosporine A (CsA)-sensitive, high-conductance inner membrane channel. Pore opening is favored by increases in matrix calcium ion concentrations and is strongly promoted by a diverse array of agents, including many that oxidize pyridine nucleotides and thiols. Regarding the first potential artifact, radiolabeled markers of extra- and intramitochondrial space can be used to quantitatively determine matrix volume, similar to the approaches used to account for contamination with extracompartmental medium. Additionally, mitochondrial incubations can be performed in the presence of antimycin A (typically 1–5 μM). Caution must be exercised in the use of antimycin A because it can produce oxidative stress and mitochondrial injury under certain conditions [54,55]. Regarding the second potential artifact, a role for the MPT can be assessed by use of the permeability transition pore inhibitor CsA (e.g., at 0.5 nmol/mg protein); inhibition of apparent uptake by CsA would indicate involvement of the MPT. Similarly, one can distinguish between the mechanism of apparent inhibition of transport by a specific compound as occurring by competitive inhibition

or by induction of the MPT by use of CsA. For example, in our study of substrate specificity of GSH uptake in suspensions of mitochondria isolated from rat renal cortex ([53]; see below), we found that the apparent inhibition of GSH uptake by phosphoenolpyruvate, which is a substrate for the CIC, was not due to competitive inhibition but was due to its well known ability to induce the MPT as its effect was eliminated by CsA.

Finally, a potential artifact that is specific to measurement of GSH transport is degradation of GSH during the transport process or during sample processing by contaminating GGT. As explained above, GGT is the sole enzyme that cleaves the γ-glutamyl peptide bond of GSH, thereby initiating its turnover. GGT is present on the luminal membrane, with its active site facing the extracellular space, of many epithelial cells, including the renal proximal tubule, choroid plexus, retinal pigment epithelium, and small-intestinal jejunal epithelium. Although there is a considerable degree of variation in activity levels among species, GGT activity is always highest in the renal proximal tubule [3]. In fact, GGT activity on the luminal or brush-border plasma membrane of renal proximal tubular cells is so high that even a very modest (e.g., < 1%) contamination of an isolated mitochondria preparation with brush-border membranes can result in a significant capacity to degrade GSH that is comparable to rates of transport. Consequently, it is critical to inhibit GGT activity as completely as possible. Although several compounds have been used to inhibit GGT, the most widespread and effective inhibitor is acivicin (L-(αS,5S)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid) [56].

3.2.2. Substrate specificity, sensitivity to inhibitors, and energetics of GSH transport—In our initial study of mitochondrial GSH transport [2], we found that uptake of GSH by rat kidney mitochondria was saturable (K_m = 1.3 mM, V_{max} = 5.59 nmol/min per mg protein), was markedly inhibited by γ-glutamylglutamate, certain *S*-alkyl derivatives of GSH, glutamate, and by dicarboxylates, but was not inhibited by monocarboxylates. Furthermore, the rate of GSH uptake was not altered by uncouplers or a protonophore. These findings suggested that GSH uptake in rat kidney mitochondria is mediated by an electroneutral exchange with dicarboxylates. More recent studies of ours using isolated mitochondria from rat renal cortex [53], assessed substrate specificity, inhibitor sensitivity, and energetics in more depth: We provided additional evidence that of the substrates for the various citric acid cycle carriers, only dicarboxylates specifically interacted with GSH for transport across the mitochondrial inner membrane. Although L-glutamate was inhibitory, the interaction is not due to the two compounds being transported by the same carrier. Rather, the glutamyl residue of the GSH molecule appears to play a role in binding to the carrier(s). Whereas L-glutamate inhibited GSH uptake, GSH did not affect rates of L-glutamate uptake, which were approximately 5-fold higher than those for GSH. Mitochondrial uptake of GSH was also found to be significantly diminished in a nominally phosphate-free or low (≤1 mM) phosphate-containing buffer, consistent with the function of the DIC in GSH uptake.

Function of the DIC and OGC in GSH uptake were further demonstrated in studies where the inner membrane carriers from mitochondria of rabbit kidney cortex were enriched and reconstituted into proteoliposomes [57], using reconstitution methods previously developed for study of the DIC and OGC [58–61]. This approach has the advantage of eliminating the potentially confounding effects of matrix metabolism or volume change or induction of the MPT. Kinetics, substrate specificity, and inhibitor sensitivity were similar to those properties observed in intact mitochondria from rat renal cortex. Based on effects of butylmalonate (selective DIC inhibitor) and phenylsuccinate (selective OGC inhibitor), both singly and in combination, we estimated that of the total amount of GSH transport in renal cortical mitochondria, ~60% is mediated by the DIC and ~40% is mediated by the OGC. At least 80% of the total observable GSH transport across the mitochondrial inner membrane of kidney mitochondria could be attributed to function of the DIC and OGC [53,57]. A basic scheme illustrating the function of the two carriers in renal mitochondrial GSH transport and their

relationship to both the citric acid and GSH redox cycles, is shown **(**Fig. 4). It should be emphasized that although the DIC and OGC account for most of the observable transport of GSH in mitochondria from renal cortex, other thus far unidentified carriers may also contribute and that the contribution of these two carriers in mitochondria from other tissues may be less than that in kidney.

Kaplowitz and colleagues [62] identified decreased mRNA expression and transport activity of the OGC in rat liver mitochondria as being linked to the decreased mitochondrial GSH content characteristic of alcoholic liver disease, confirming the role of this carrier in mitochondrial GSH transport. More detailed mechanistic studies of the kinetics and properties of GSH transport in liver mitochondria, however, are not available. As discussed below, we have unpublished data suggesting that tissue-specific differences exist in the function of the various carriers for mitochondrial GSH transport. Fernandez-Checa and Kaplowitz [63] recently reviewed the role of hepatic mitochondrial GSH transport in disease and chemically induced toxicity. As briefly discussed below (see section 4), studies from these investigators highlight the human health and therapeutic significance of mitochondrial GSH transport.

3.2.3. Structural and functional studies of the DIC and OGC—As noted above, both the DIC and OGC appear to belong to a “superfamily” of mitochondrial inner membrane transporters with similar three-dimensional structure, as predicted by hydropathy analysis [64]. The TMpred program predicts three-dimensional structure and transmembrane-spanning domains (TMDs) using amino acid polarity and charge. Based on this program, both the DIC and OGC, as well as other carriers in the superfamily (e.g., the MCC, CIC, PiC), are predicted to have three TMDs. Each TMD is comprised of two hydrophobic stretches that span the membrane presumably as α-helices, each separated by hydrophilic loops. Both the N- and C-terminal ends of each monomer are on the cytoplasmic side of the inner membrane. The hydropathy plots can then be used to predict which amino acid residues may be essential for proper three-dimensional structure and insertion into the membrane. Both carriers are similar in size, with the DIC containing 286 or 287 amino acids, depending on species, and a molecular mass of approximately 31 kDa, and the OGC containing 314 to 322 amino acids, depending on species, and a molecular mass of 34–37 kDa. All the members of the Slc25 transporter family are believed to exist as homodimers.

Various approaches have been used to identify essential residues for proper carrier function, including inhibition by amino acid-specific reagents, site-directed and cysteine-scanning mutagenesis, and spin labeling. For example, Palmieri’s group used Arg-specific reagents [65] and different mutagenesis and spin-labeling techniques [66,67] to demonstrate the function of an Arg residue near the substrate-binding site and two other Arg residues in TMD1 and TMD2 of the OGC. Cys residues are often critical to protein function. Although the DIC and OGC exhibit some homology, their content of Cys residues and the apparent role of these residues in carrier function appear to differ. Thus, the DIC from rat or bovine liver contains five Cys residues at positions 17, 21, 22, 211, and 216. Conversion of any one of these residues in the bacterially expressed DIC from rat kidney mitochondria to Ser or Ala by site-directed mutagenesis, however, failed to significantly alter transport activity (J. Wang, F. Xu, D.A. Putt, L.H. Matherly, and L.H. Lash, unpublished observations). In contrast, the OGC contains only three Cys residues at positions 184, 221, and 224, with the latter two forming an intramolecular disulfide bond [68–71]. Conversion of C221 and C224 to Ser results in a marked reduction in transport activity, indicating that formation of the intramolecular disulfide bridge is critical for function [71].

For the OGC, therefore, the Cys residues appear to be critical for proper three-dimensional structure and function. For the DIC, however, the functional significance of the cysteine residues is unclear. In studies to purify and reconstitute the DIC [58,60], it was noted that in

marked contrast with the OGC, the DIC is relatively unstable when isolated, undergoing relatively facile oxidative inactivation. Thus, although replacement of any of the five Cys residues of the DIC with a Ser or Ala does not markedly affect activity, oxidation of the thiol groups in the native protein inhibits activity.

3.2.4. Species and tissue specificity of transport—The DIC appears to be invariant across tissues and exhibits a high degree of amino acid sequence homology across species. Protein sequences from the liver mitochondrial carriers are known for three species: mouse, rat, and human. Whereas mouse and human DIC have 287 amino acid residues, rat DIC has 286. The extent of sequence homology between mouse and rat is 96% whereas that between mouse and human is 89%. The OGC, in contrast, exhibits much broader cDNA and amino acid sequence differences across both species and tissues. Using the cDNA sequence from brain as the basis for comparison, rat and mouse exhibit the highest degree of homology (95%; 919/964), with those from rat and bovine and rat and human exhibiting somewhat lower degrees of homology (rat vs. bovine: 90% or 866/964; rat vs. human: 89% or 862/964). Comparison of deduced amino acid sequences, however, shows greater species differences, with that from the mouse differing the greatest amounts from those from rat, human, or bovine. Thus, whereas the OGC protein from bovine, rat, and human contain 314 amino acid residues, that from mouse contains 322 amino acid residues. Moreover, whereas amino acid sequence homologies between the OGC from rat, bovine, and human are 95% to 96%, those between mouse and the other species is only 34%. The functional implications for these differences are unknown.

cDNA sequences for the OGC from rat that are published in GenBank^{TM} are only available from brain and heart mitochondria. Although Coll et al. [62] quantified OGC expression in rat liver, the published cDNA sequence from rat brain was used as the template for their PCR primer design. In our study of OGC from rat kidney [71], we used total rat kidney RNA as a template. The sequence of our PCR product, which was repeated numerous times with identical results, exhibited significant differences from that of the mitochondrial OGC from rat heart and brain. Nucleotide differences in the cDNA sequences were observed for 6 bp or 10 bp between kidney and heart or kidney and brain, respectively. Amino acid differences for the deduced sequences did not appear to be significant between kidney and heart (2 amino acid residues) but were significant between kidney and brain (6 amino acid residues), with 2 differences involving changes in charge or polarity and 3 differences involving residues in or near TMDs. The nature of these differences suggests that the OGC proteins in rat liver and kidney mitochondria exhibit differences in structure that may translate into functional differences.

While extensive studies of transport kinetics and substrate specificity for the OGC have been conducted in rat kidney mitochondria [53], no such studies have as yet been published for rat liver mitochondria. We have, however, conducted preliminary studies in isolated rat liver mitochondria (Q. Zhong, L.H. Lash, unpublished observations), and one clear difference between GSH transport in mitochondria from the two tissues is that whereas function of the DIC and OGC can account for at least 80% of the total transport activity in kidney mitochondria, these two carriers account for at most 50% of total transport activity in liver mitochondria. This suggests that at least one other carrier besides the DIC and OGC plays a quantitatively significant role in GSH transport into rat liver mitochondria. Additional studies are needed to clarify the function of various carriers for GSH in rat liver mitochondria.

Besides the potential function of tissue-specific carriers, differences also exist in the level of expression and activity of carriers in various tissues. It is known, for example, that deficiencies exist in certain mitochondrial enzymes or transmembrane carriers, resulting in so-called mitochondriocytopathies [72]. Further, these disorders most severely affect those tissues that are most dependent on mitochondrial energy production, such as skeletal muscle and heart.

Huizing et al. [72] determined the human tissue distribution of several key carriers involved in either oxidative phosphorylation (i.e., the AAC, PiC, and voltage-dependent anion channel) or metabolite transport (i.e., OGC, carnitine-acylcarnitine carrier, CIC). Levels of mRNA expression in various tissues generally correlated with tissue dependence on mitochondrial energy, with skeletal muscle and heart exhibiting much higher levels of mRNA for several carriers than other tissues, including brain, pancreas, lung, liver, placenta, and kidney. Of interest for GSH transport, OGC mRNA expression was by far the highest in skeletal muscle and heart, but was also relatively high in brain, liver, and kidney. Pancreas and placenta exhibited very low levels of OGC mRNA expression and that in lung was barely detectable.

3.2.5. Potential role of other carriers—As indicated above, less specific, mechanistic information on mitochondrial GSH transport is available in liver than in kidney. Although rat liver OGC may have a high degree of homology with rat brain OGC [62], its sequence has not been reported. A thorough search of the GenBank™ database and the published literature failed to find any cDNA or amino acid sequences for the OGC from rat liver mitochondria [71]. Fiermonte et al. [73], however, reported cloning and expression of a rat liver oxodicarboxylate (oxoadipate) carrier (ODC; *Slc25a21*) that transports 2-OG and other C5–C7 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, which contrasts with the OGC from rat kidney, heart, and brain mitochondria, which are all 314 amino acids in length. The relationship between the rat liver ODC and OGC is unclear as is the potential for the ODC to transport GSH. Clearly, further investigation is needed to establish the roles of the OGC versus ODC in rat liver, to determine the significance of the OGC sequence variants in relation to kidney and liver GSH transport, and to assess the functional implications of these sequence differences for mitochondrial GSH transport.

3.2.6. Regulation of mitochondrial GSH transport—The identification of the DIC and OGC as the primary membrane carriers responsible for transport of GSH into renal mitochondria suggests that mitochondrial GSH status is closely regulated by mitochondrial energetics. Certainly, the fact that GSH is transported by the same carriers as and, therefore, competes with, dicarboxylates, suggests that nutritional status can directly influence GSH transport ability. Indeed, as described above, some of the earliest studies in liver and kidney mitochondria found marked differences in transport activity dependent on respiratory state [44,45,47]. Indeed, the principal function of the DIC is to transport dicarboxylates from the cytoplasm into the mitochondria, thereby supplying substrates for the citric acid cycle [48, 49]. Interestingly, the DIC also transports thiosulfate into mitochondria, delivering it to rhodanese and thiosulfate reductase. Although the OGC can also deliver substrates to the enzymes of the citric acid cycle, it is primarily viewed as functioning in the malate-aspartate and 2-OG-isocitrate shuttles, nitrogen metabolism, and gluconeogenesis from lactate.

Correlation between mitochondrial GSH status and energetics makes sense because the mitochondria are the primary sites within the cell for oxygen consumption and, hence, for endogenous generation of ROS. One would expect, therefore, that higher concentrations of GSH in the matrix would be advantageous in rapidly respiring mitochondria to minimize the potential release of toxic ROS generated during electron transport. Although some investigation of the relationship between rates of GSH transport and respiratory state has been done, the hypothesis or expectation stated above has not been tested directly.

There is, therefore, some expectation that ROS or RNS may directly affect activity of GSH carriers by interaction with key Cys residues. For the DIC, while the five Cys residues do not seem to be essential for activity (as replacement with Ser did not affect activity), they may serve as regulatory sites or sensors of oxidative and/or nitrosative stress. Our hypothesis is that

relatively low levels of ROS or RNS can up-regulate expression of one or more of the mitochondrial GSH carriers. Higher levels of these species, however, may lead to in activation of the carriers. The relatively facile autoxidation and inactivation of the DIC upon purification and isolation [58,60] supports this notion. For the OGC, its three Cys residues appear to function much differently than those of the DIC. Whereas C221 and C224 are important for intramolecular disulfide bond formation, C184 may function similarly to the Cys residues of the DIC as a redox sensor, although the relative stability of the purified OGC to autoxidation and inactivation could be interpreted as arguing against this hypothesis. Additional studies are needed to directly assess the influence of ROS and RNS on GSH carrier expression and function.

4. Mitochondrial GSH status and disease or pathological states

An increasing number of toxic or pathological states are being recognized for being associated with marked depletion and/or oxidation of the mitochondrial GSH pool. These observations highlight the importance of this pool. Additionally, our increasing knowledge about the carriers that are responsible for GSH transport into mitochondria provides us with therapeutic targets. For example, hydrogen peroxide generation in liver mitochondria, which leads to oxidative injury, is stimulated only when mitochondrial GSH is depleted below a critical level of approximately 40% of normal [74,75]. Chronic ethanol ingestion and alcoholic liver disease are associated with marked decreases in liver mitochondrial GSH content [76–81]. One consequence of this ethanol-induced decrease is an increase in susceptibility to certain toxicants, such as acetaminophen [82,83], and to toxic cytokines such as TNFα [84–86]. Cirrhosis and other forms of biliary obstruction are also characterized by mitochondrial dysfunction and depletion of the matrix GSH pool [87,88]. Type II diabetes has been reported to be associated with mitochondrial oxidative stress and a depleted and/or oxidized state of mitochondrial GSH in rat heart, brain, and kidney [89–91].

As noted above, Kaplowitz and colleagues [62] provided direct evidence that the altered state of mitochondrial GSH in alcoholic liver disease is due, at least in part, to decreased expression and activity of the OGC. This was the first evidence of this type for any disease or pathological state that is characterized by mitochondrial damage or oxidative stress. The specific involvement of defects in or decreased expression of GSH carriers in other diseases has not been established. As discussed above, Fernandez-Checa and Kaplowitz [63] recently reviewed the role of alterations in hepatic mitochondrial GSH in various pathological and toxic states.

5. Manipulation of mitochondrial GSH

Numerous approaches can and have been used to alter concentrations of GSH in cells. For therapeutic objectives, an increase in either GSH content or GSH/GSSG redox state would typically be desired. Due to feedback inhibition of γ-glutamylcysteine synthetase (GCS) by GSH, however, achievable levels of GSH inside cells have an upper limit. The most common approach to increasing cellular GSH concentrations to bypass this limit is to incubate cells with either GSH, GSH ethyl ester, or amino acid precursors, depending on whether or not intact GSH is transported across the plasma membrane [56]. Another approach would involve induction or overexpression of GCS. If one wants to specifically increase GSH content in a particular subcellular organelle (e.g., the mitochondria), then simply increasing GCS activity or expression or providing the extracellular medium with GSH or GSH precursors will not be very effective. Rather, because the mitochondrial GSH pool appears to be largely, if not entirely, determined by transport from the cytoplasm, the most logical method is to alter expression of the DIC or OGC to effect the desired changes in mitochondrial GSH status [71,92].

In our initial study [92], we transiently transfected NRK-52E cells, an immortalized cell line derived from rat proximal tubules that exhibits many properties that are favorable for study of mitochondrial GSH transport [93], with the cDNA for the rat DIC. Besides exhibiting many transport and metabolic functions that are normally found in the proximal tubular cell, NRK-52E cells exhibit relatively high activities of GSH synthesis and other GSH-dependent metabolic reactions with the exception of GGT. As is typical with immortalized epithelial cell lines, the brush-border membrane is largely lost so that activities of enzymes normally found there will be low. For our purposes, this is an advantage as it largely removes the possibly confounding effect of degradation during measurement of transport.

Three clones that transiently overexpressed the DIC exhibited 3- to 11-fold increases (mean = 5.5-fold) in rates of GSH uptake into mitochondria **(**Fig. 5A). Similarly, NRK-52E cells that stably expressed the OGC exhibited 6.1-fold increases in mitochondrial GSH uptake as compared to the wild-type cells. Mutation of the two Cys residues of the OGC involved in an intramolecular disulfide bond to Ser (OGC-C221,224S) resulted in a modified carrier protein that exhibited a marked reduction in GSH transport so that mitochondrial accumulation of GSH in these cells was actually slightly less than that in non-transfected, wild-type cells.

To investigate the toxicological consequences of altering mitochondrial GSH transport capability, we incubated the different populations of NRK-52E cells with either of two well-characterized mitochondrial toxicants, tert-butyl hydroperoxide (tBH) or *S*-(1,2-dichlorovinyl)-L-cysteine (DCVC), and determined the fraction of cells undergoing apoptosis **(**Fig. 5B). Dramatic differences were observed in the sensitivity of the different cell populations to the two agents, with the cells overexpressing either the DIC or OGC exhibiting resistance to cell injury. In contrast, cells overexpressing the double-cysteine mutant of the OGC exhibited similar sensitivity to tBH or DCVC as the wild-type cells. These results support the use of approaches to enhance mitochondrial GSH to protect cells from oxidants and other cytotoxic chemicals. The absence of protection with cells overexpressing the double-cysteine mutant of the OGC suggests that a threshold level of mitochondrial GSH exists for optimal mitochondrial function and resistance to chemically induced toxicity.

6. Summary and conclusions

Studies using a variety of experimental models, including isolated mitochondria, purified and reconstituted carrier proteins, bacterial-expressed and reconstituted carrier proteins, and cell lines overexpressing specific carrier cDNAs, have established that transport of GSH from the cytoplasm into mitochondria in rat kidney proximal tubule is primarily mediated by the DIC and OGC. Some tissue-specific differences have been identified, so that additional carriers may be more important in some tissues. Genetic manipulation of carrier activity and expression has been demonstrated to be an effective means of producing severalfold increases in mitochondrial contents of GSH, thereby protecting cells from oxidants and other mitochondrial toxicants. The existence of several diseases that are characterized by oxidation or depletion of the mitochondrial GSH pool in specific tissues illustrates the potential human health significance of approaches that enhance mitochondrial GSH transport.

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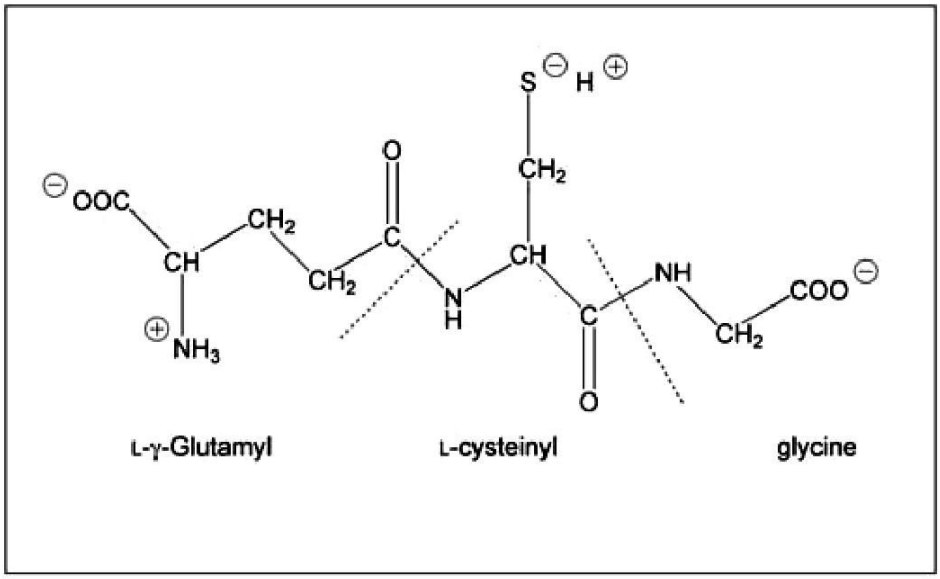

Fig. 1.
Structure of GSH.

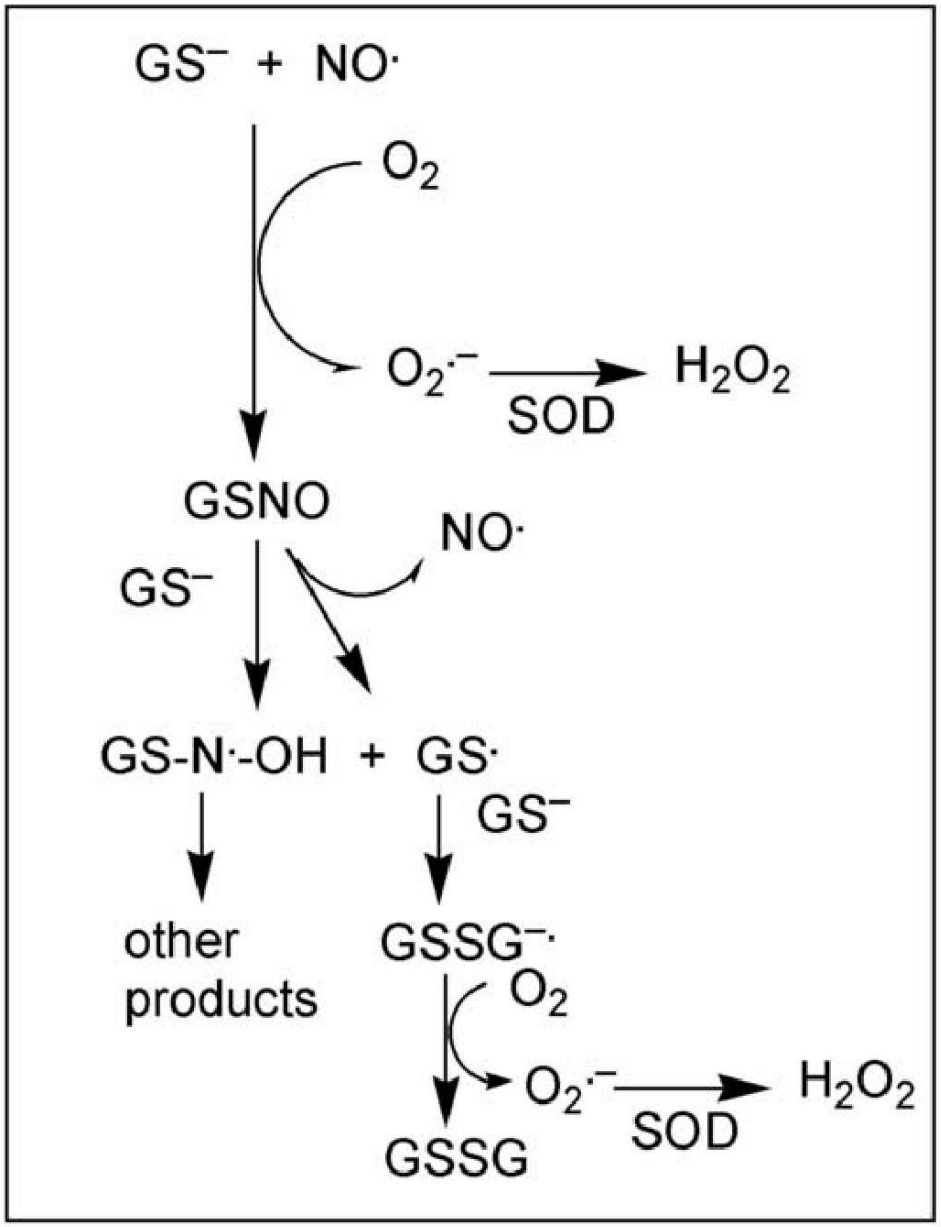

Fig. 2.

Reactions by which GSH reacts with NO to form GSNO. GSH, as the thiolate, reacts with NO in the presence of O_2 and forms GSNO. GSNO can release NO (function of GSNO as an NO donor) or it may react in the presence of the thiolate to form a species that can glutathionylate protein sulfhydryl groups. SOD, superoxide dismutase. Adapted from [43].

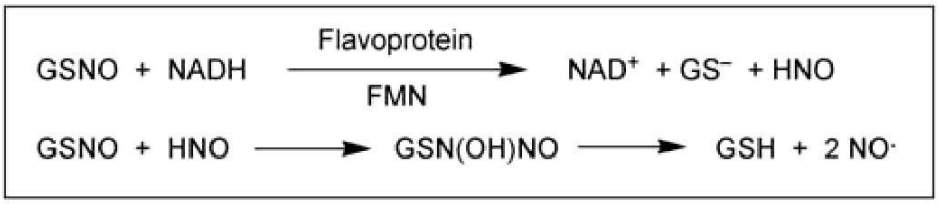

Fig. 3.
NO formation from GSNO. GSNO acts as an NO donor in two reactions, the first of which is mediated by a flavoprotein containing FMN. The HNO generated from the first reaction can react with another molecule of GSNO to form an intermediate that decomposes to GSH and NO.

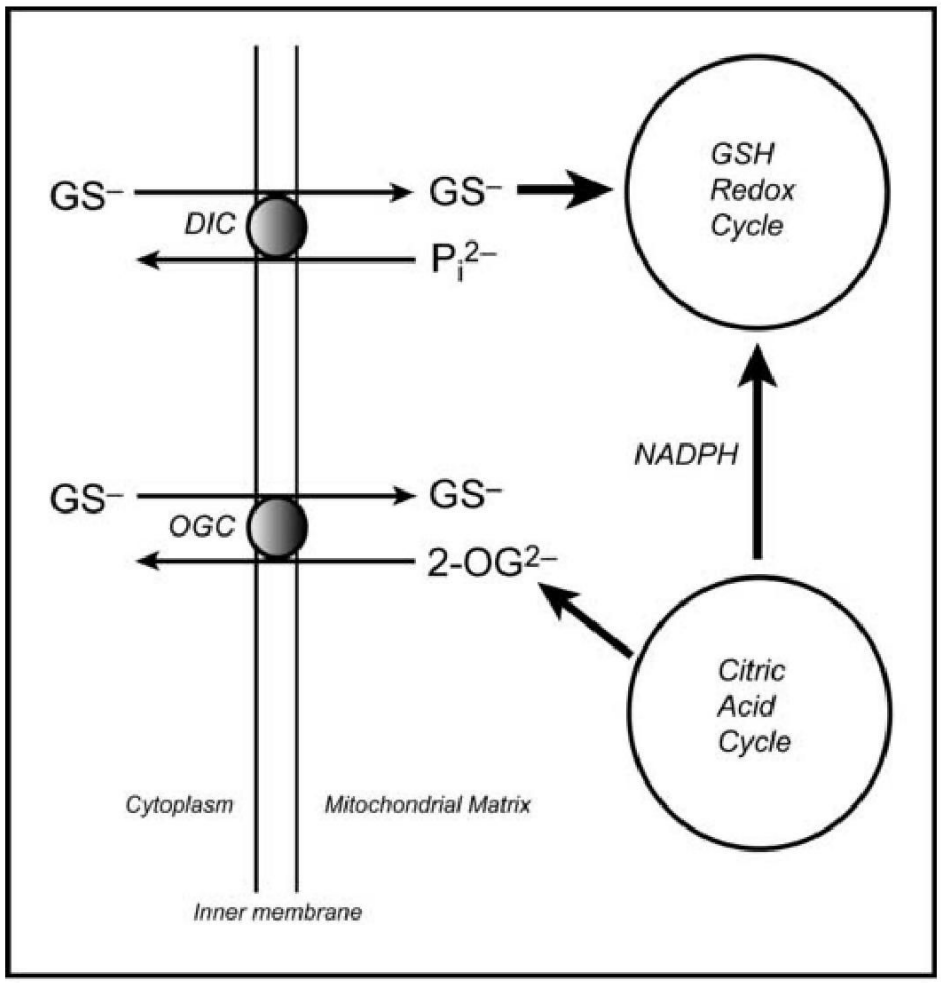

Fig. 4.

Mitochondrial transport of GSH. Generalized summary scheme, simplified from [56], illustrating the basic function of the DIC and OGC in GSH transport and their relationships with the citric acid and GSH redox cycles.

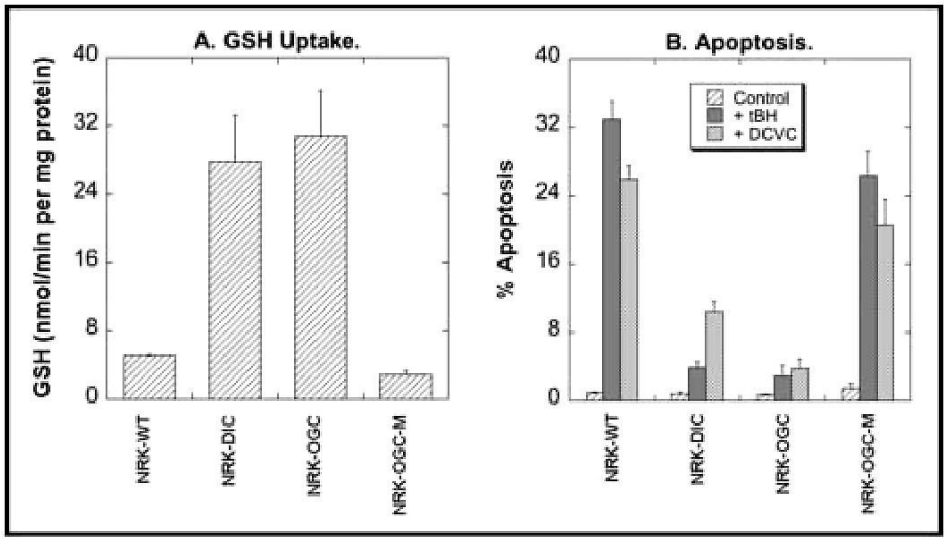

Fig. 5.

Genetic modulation of mitochondrial GSH transport in NRK-52E cells and susceptibility to chemically induced apoptosis. The cDNA for the DIC was transiently overexpressed in NRK-52E cells (NRK-DIC) and the cDNA for either the OGC or a double-cysteine mutant of the OGC (NRK-OGC or NRK-OGC-M, respectively) was stably overexpressed in NRK-52E cells. A. Uptake rates for GSH into mitochondria from different genetically modified NRK-52E cell populations. Mitochondria from each cell population were incubated with [^{3}H]-GSH (final concentration = 5 mM). Data are expressed as uptake rates and are means ± SEM of measurements from 3–5 separate experiments. B. Fraction of apoptotic cells. Each cell population was incubated for 4 hr with either medium (= Control), 10 μM tert-butyl hydroperoxide (tBH), or 50 μM *S*-(1,2-dichlorovinyl)-L-cysteine (DCVC). The fraction of cells undergoing apoptosis was estimated by propidium iodide staining, flow cytometry and FACS analysis. Results are means ± SEM of 4–5 separate experiments. These data were derived from studies originally presented in refs. 71 and 92, and were combined to illustrate toxicologic effects of genetic manipulation of mitochondrial GSH carriers.

Table 1

Mitochondrial anion transporters

Carrier	Function	Charge Transfer	Inhibitors
Adenine nucleotide translocase (AAC1–3; *Slc25a4–6*)	ADP^{3-} in, ATP^{4-} out	Electrogenic	Atractyloside, Carboxyatractyloside Bongkrekic acid
Phosphate (PiC; *Slc25a3*)	$H_2PO_4^-$ in, OH^- out	Electroneutral	SH-reagents
Dicarboxylate (DIC; *Slc25a10*)	$Malate^{2-}$ in, HPO_4^{2-} out	Electroneutral	Butylmalonate
2-Oxoglutarate (OGC; *Slc25a11*)	$2\text{-Oxoglutarate}^{2-}$ in, $malate^{2-}$ out	Electroneutral	Phenylsuccinate
Glutamate–Aspartate (AGC1/2; *Slc25a12/13*)	$Glutamate^- + H^+$ in, $Aspartate^-$ out	Electrogenic	—
Glutamate–Hydroxide (GC1/2; *Slc25a22/18*)	$Glutamate^-$ in, OH^- out	Electroneutral	—
Tricarboxylate / Citrate (CIC; *Slc25a1*)	$Citrate^{3-} + H^+$ in, $malate^{2-}$ out	Electroneutral	1,2,3-Benzenetricarboxylate, triethyl citrate
Monocarboxylate(MCC)	$Pyruvate^-$ in, OH^- out	Electroneutral	Cyanohydroxycinnamate