Dicarboxylate carrier-mediated glutathione transport is essential for reactive oxygen species homeostasis and normal respiration in rat brain mitochondria

Christelle K. Kamga,1,2 Shelley X. Zhang,2 and Yang Wang1,2

1Department of Pharmacology and Toxicology, University of Louisville, Louisville, Kentucky; and 2Department of Pediatrics, University of Chicago, Chicago, Illinois

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Kamga CK, Zhang SX, Wang Y. Dicarboxylate carrier-mediated glutathione transport is essential for reactive oxygen species homeostasis and normal respiration in rat brain mitochondria. Am J Physiol Cell Physiol 299: C497–C505, 2010. First published June 10, 2010; doi:10.1152/ajpcell.00058.2010.—Glutathione transport into mitochondria is mediated by oxoglutarate (OGC) and dicarboxylate carrier (DIC) in the kidney and liver. However, transport mechanisms in brain mitochondria are unknown. We found that both carriers were expressed in the brain. Using cortical mitochondria incubated with physiological levels of glutathione, we found that butylmalonate, a DIC inhibitor, reduced mitochondrial glutathione to levels similar to those seen in mitochondria incubated without extramitochondrial glutathione (59% of control). In contrast, phenylsuccinate, an OGC inhibitor, had no effect (97% of control). Additional experiments with DIC and OGC short hairpin RNA in neuronal-like PC12 cells resulted in similar findings. Significantly, DIC inhibition resulted in increased reactive oxygen species (ROS) content in and H2O2 release from mitochondria. It also led to decreased membrane potential, increased basal respiration rates, and decreased phosphorus-to-oxygen (P/O) ratios, especially when electron transport was initiated from complex I. Accordingly, we found that DIC inhibition impaired complex I activity, but not those for complexes II and III. This impairment was not associated with dislodgment of complex subunits. These results suggest that DIC is the main glutathione transporter in cortical mitochondria and that DIC-mediated glutathione transport is essential for these mitochondria to maintain ROS homeostasis and normal respiratory functions.

oxidative stress; electron transport chain; complex I; oxoglutarate carrier

GLUTATHIONE (GSH), the most abundant non-protein thiol in mammalian cells, is essential for reactive oxygen species (ROS) homeostasis and mitochondrial function. ROS are produced in mitochondria as obligatory by-products of cellular respiration. While electrons from energy substrates are passed along the respiratory chain to establish the proton gradient, which in turn drives the synthesis of ATP from ADP, leakage of electrons often occurs at complexes I and III, leading to the formation of superoxide (O2−) through one-electron reduction of oxygen (18). O2− is usually readily converted to hydrogen peroxide (H2O2) by manganese superoxide dismutase (MnSOD) in the matrix or copper/zinc SOD (Cu/ZnSOD) in the intermembrane space (18, 47). Although H2O2 is further reduced to water by peroxiredoxins, glutathione peroxidase, or, in some organs, catalase (2), it may also react with reduced transition metal ions, such as Fe2+, to form the hydroxyl radical (·OH), especially when there is an imbalance between its production and the capacity of antioxidants that reduce it as specified above. ·OH is a highly reactive radical that causes peroxidation of all cellular constituents, including lipids, proteins, and nucleic acids, and is believed to be mainly responsible for oxidative stress-induced cellular damage (38). Importantly, both peroxiredoxins and glutathione peroxidase, the main antioxidant mechanisms that reduce H2O2, depend on GSH for reducing equivalent and in turn their H2O2-reducing capabilities. In addition, GSH is also essential in glutathione S-transferase-mediated antioxidant effects (34). GSH is therefore a key element in the overall antioxidant defense (15).

Consequently, GSH depletion or oxidation is a major determinant of pathological cellular processes, such as excessive oxidative stress (19), mitochondrial transition pore opening (4), respiratory complex inhibition (21), and apoptosis (5, 15). Interestingly, the brain has been believed to be particularly vulnerable to oxidative stress-induced damage presumably because of the dependency of neurons on oxidative phosphorylation for energy supply, which is accompanied by production of large amounts of ROS (18). Perhaps more importantly, however, is the fact that the brain has considerably less catalase activity in comparison with other major organs, including the liver, the kidney, the lung, and the heart (20). Moreover, parts of the brain contain high levels of Fe2+, which favors the formation of ·OH through the Fenton reaction (18). Thus, it is possible that GSH plays an even more significant role in the maintenance of ROS homeostasis in the brain. Indeed, a deficit in cellular GSH has been implicated in the pathogenesis of several neurological disorders such as Parkinson’s disease (10, 42), schizophrenia (13), stroke (18), and trauma (3).

Cellular GSH exists as two separate pools in the mitochondrial and the cytosolic compartments (24). Recent evidence suggests that the mitochondrial pool of GSH (mGSH) is more critical than cytosolic GSH for the survival of neuronal cells under various pathological conditions. For instance, selective depletion of mGSH has been observed during ischemia and reperfusion (1, 44), while ROS overload and neuronal degeneration occur only when mGSH levels fall below 60% of control levels in neurons subjected to GSH synthesis inhibition (51). Furthermore, mGSH, rather than cytosolic GSH, has been found to determine amyloid-β peptide susceptibility in human neuronal and glial cell lines (14) and a decrease in mGSH has been shown to result in selective inhibition of complex I that precedes dopaminergic cell death in Parkinson’s disease (23).

Because GSH synthesis activities are not found in mitochondria (16), transport of GSH from the cytosol into the mitochondrial matrix is believed to be a key mechanism that sustains the mGSH. This process was thought to be mediated by organic anion transporters located in the inner mitochondrial membrane (IMM) since GSH was negatively charged (8, 35).
Recent studies have confirmed that out of the eight protein carriers in this category that are known to reside in the IMM, the dicarboxylate carrier (slc25a10, DIC) and the oxoglutarate carrier (slc25a11, OGC) possess GSH transport capabilities and act as main mitochondrial GSH transporters in renal and hepatic mitochondria (8, 9, 11). In particular, DIC and OGC together account for >80% and at least 50% of the total GSH transport across the mitochondrial membrane in rat and hepatic mitochondria, respectively (8, 54). Despite the expanding body of evidence that suggests an essential role of the mGSH in the brain, however, mechanisms underlying mitochondrial GSH transport in this important organ remain unclear. A recent study suggests that Bcl-2 may carry GSH to the mitochondrial membrane and the intermembrane space through direct binding of GSH to its BH3 groove (55). Whether Bcl-2 transports GSH into mitochondria is nevertheless uncertain, given the outer membrane localization of this protein (22, 37). Accordingly, the purpose of the current study was to investigate the mechanisms of GSH transport into mitochondria and their relevance to mitochondrial function in the rat brain. We hypothesized that DIC and/or OGC would be important to the integrity of the mGSH in the rat brain and that inhibition of their carrier activities would lead to functional impairment of brain mitochondria.

**MATERIALS AND METHODS**

**Reagents.** Unless otherwise specified, all reagents were purchased from Sigma Chemical (St. Louis, MO).

**Animals.** Male Sprague-Dawley rats (Charles River, Portage, MI) and C57BL/6 mice (Taconic Farms, Germantown, NY) were used in this study. All procedures were approved by the Animal Care and Use Committee of the University of Louisville.

**Primary cell cultures.** Primary cultures of cortical neurons were prepared from the cortex of 12- to 24-h-old neonatal C57BL/6 mice as previously described (45).

**Isolation of rat cortical mitochondria.** Rat cortical mitochondria were isolated using gradient and differential centrifugations, according to the method of Sims (43). The isolation buffer contained 10 mM HEPES (pH 7.5), 200 mM mannitol, 70 mM sucrose, and 1 mM EGTA. BSA (2 mg/ml) was added to this buffer prior to homogenization (isolation buffer A). Mitochondria isolated using this method had high purity, since >95% of particles were positively stained with MitoTracker Green (Molecular Probes, Eugene, OR).

**Cell culture and gene silencing.** PC12 cells were grown in a humidified incubator (5% CO2, 37°C), in growth medium supplemented with 1-glutamine, 25 mM HEPES, and 10% fetal bovine serum. They were transfected with SureSilencing shRNA plasmids (SABioscience, Frederick, MD) using the Amaxa Nucleofector (Amaxa, Cologne, Germany). These shRNAs were designed to target rat DIC (5'-CACCTCTCTCCAGATTCAT-3') and OGC (5'-GGAAAGTTCCCATCACCACAT-3'), respectively, under the control of the U1 promoter. The shRNA plasmid with a scrambled sequence (5'-GGAAATCCATCGATCGTAT-3') was used as a negative control.

**Preparation of mitochondria from PC12 cells.** Cell pellet was collected 72 h after transfection, washed twice with cold PBS, and then spun down at 800 g (3 min, 4°C). The packed cell volume was determined and five volumes of the hypotonic buffer (10 mM HEPES, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT, and 0.2 mM phenylmethylsulfonyl fluoride (PMSF)) were added. Cells were pelleted, incubated, and washed with the hypotonic buffer for several rounds and were then homogenized in 800 μl of isolation buffer A with 30 strokes of pestle B (Contes Glass, Vineland, NJ). The homogenate was centrifuged at 1,000 g (4°C, 5 min). The mitochondrial pellet was obtained by spinning the supernatant at 10,000 g (4°C, 10 min), and then was washed twice with the isolation buffer.

**Primary antibodies for immunoblotting.** The following antibodies were used: polyclonal rabbit anti-mouse DIC (Affinity Bioreagents, Golden, CO), polyclonal rabbit anti-mouse OGC (Abcam, Cambridge, MA), monoclonal mouse anti-bovine complexes I, II, and III (BD Biosciences, Franklin Parks, NJ), monoclonal mouse anti-pig-eon cytochrome c (BD Biosciences), monoclonal mouse anti-mouse neuronal nuclear antigen (NeuN; Chemicon International, Temecula, CA) and polyclonal rabbit anti-bovine glial fibrillary acid protein (GFAP; Dako, Carpinteria, CA).

**Assessment of GSH transport.** Transport assays were carried out as previously reported (8, 54), with slight modifications. In brief, mitochondria from the cortex or from PC12 cells (0.5 mg/ml) were incubated in the base mitochondrial buffer (20 mM triethanolamine-HCl [pH 7.4], 225 mM sucrose, 3 mM potassium phosphate [pH 7.4], 5 mM MgCl2, 20 mM KCl, and 0.1 mM PMSF) with various additions (detailed in figure legends) in 1.5 ml microfuge tubes at 20°C. α-Glycero-phosphate (α-GP, 7 mM) was added to all tubes as the respiratory substrate. After incubation for 30 min or 1 h, microfuge tubes were centrifuged at 18,000 g for 30 s. The supernatant was removed and the pellet was washed twice with an equal volume of ice-cold phosphate-free buffer. Potential artifacts, including loss of GSH during sample processing and contamination of matrix GSH by inner membrane-bound GSH, were minimized by using the centrifugation-resuspension method.

**GSH measurement.** This procedure takes advantage of the reaction of naphthalene-2,3-dicarboxaldehyde (NDA) with GSH (but not GSSG) that forms cyclized products that are highly fluorescent (39, 50). Mitochondrial samples from the GSH transport assay were resuspended in 5% sulfosalicylic acid, subjected to three freeze/thaw cycles, and then centrifuged at 10,000 g for 10 min. The supernatant was transferred to empty tubes, and 20-μl aliquots were transferred in triplicates to a 96-well plate. NDA derivatization solution (180 μl) [50 mM Tris (pH 10), 0.5 N NaOH, and 10 mM NDA in DMSO, vol/vol/vol = 1.40/0.2/0.2] was added to all wells. The plate was covered from lights and incubated at room temperature for 25 min. NDA-GSH fluorescence intensity was measured (472-nm excitation and 528-nm emission) using a Wallac Victor3 microplate reader (Perkin Elmer LAS, Waltham, MA). GSH levels were calculated using standard curves for GSH.

**Mitochondrial ROS measurements.** Following the transport assays, mitochondrial suspensions were incubated at 30°C with 5 μM dihydrorhodamine-123 (DHR-123) (Molecular Probes) and either 20 mM succinate or 10/5 mM glutamate/malate. After 20 min, DHR-123 fluorescence intensity was evaluated with a FACSAtara flow cytometer (BD Biosciences). Kinetic release of H2O2 in the assay medium was detected using the Amplex Red fluorescent dye (Molecular Probes). The assay medium contained 125 mM KCl, 20 mM HEPES, 2 mM potassium succinate. State 3 was initiated by adding either 20 mM succinate or 10/5 mM glutamate/malate. After transport, mitochondrial samples were added to this solution in a 96-well plate, and H2O2 formation was initiated by adding either 20 mM succinate or 10/5 mM glutamate/malate. Fluorescence was detected at 30°C in the Wallac Victor3 microplate reader.

**Detection of mitochondrial membrane potential.** After transport assays, mitochondrial samples were incubated with 100 nM tetramethylrhodamine methyl ester (TMRM, Molecular Probes) for 20 min at 30°C, and red fluorescence was measured by flow cytometry.

**Measurement of mitochondrial respiratory rates.** Oxygen consumption was measured with a Clark-type electrode (Strathkelvin Instruments, Glasgow, UK) at 30°C. State 4 respiration was determined in the presence of either 5/2.5 glutamate/malate or 10 mM succinate. State 3 was initiated by adding 18 nmol ADP.
butylmalonate; M/GSH/PS, buffer containing 2.5 mM GSH and 7 mM phenylsuccinate. M0 is freshly isolated mitochondria. mGSH content decreased in M in comparison with M0, indicating the importance of GSH transport across the inner mitochondrial membrane (IMM) even under totally unchallenged conditions.

Furthermore, mGSH content decreased in M/GSH/BM but not in M/GSH/PS in comparison with M/GSH, indicating that DIC was responsible for GSH transport across the IMM. We found that a brief incubation of these mitochondria without cytosolic GSH pool at physiological levels when compared with those freshly isolated (M0) (mGSH, 3.00 ± 0.42 and 4.25 ± 0.35 nmol/mg protein in M and M0, respectively) (Fig. 2B). In contrast, phenylsuccinate (M/GSH/PS), an OGC inhibitor, at a molar ratio similar to what was shown to effectively inhibit OGC-mediated GSH transport in renal mitochondria (8), had no effect in M/GSH/PS and M/GSH, respectively (Fig. 2B). These results indicate that rat cortical mitochondria rely on GSH transport across the IMM to maintain their mGSH.

RESULTS

Expression of DIC and OGC proteins in cortical mitochondria. Using Western blot analysis, we found that both DIC and OGC were expressed in rat cortical mitochondria (Fig. 1A). These two transporters were also expressed at comparable levels in primary cultures of cortical neurons and cortical glial cells (Fig. 1B). The band at ~37 kDa on the OGC blot in Fig. 1B was probably a nonspecific band derived from nonmitochondrial compartments, because it disappeared in blots using mitochondrial protein preparations, as shown in Fig. 1A.

DIC inhibition depletes mGSH. GSH transport assays were performed using high-quality mitochondria that had minimal contamination from synaptosomes and myelin and routinely exhibited respiratory control ratio (RCR) > 4. mGSH content was measured with the NDA method, which included an independent standard curve for each experiment and was shown to be linear and highly reproducible within a large range in previous publications (50, 52) and in this study (Fig. 2A).

Electron transport chain enzyme activities. Complexes I (rotenone-sensitive NADH-ubiquinone oxidoreductase) and II (succinate ubiquinone oxidoreductase) activities were determined according to Birch-Machin and Turnbull (6). Complex III (antimycin A-sensitive ubiquinol-cytochrome c reductase) specific activity was measured by following the reduction of cytochrome c (III) at 550 nm (ε = 18.5 mM/cm) (6, 32). Decylubiquinol was freshly prepared by reduction of decylubiquinone with crystals of sodium borohydride. Mitochondria (20 μg) were preincubated (3 min at 30°C) in a reaction medium containing 0.25 M Tris·HCl (pH 7.4), 2 mM KCN, 0.01% BSA, 1 mM EDTA, 0.6 mM n-dodecyl β-D-maltoside, and 2 μg/ml rotenone; 15 μM cytochrome c was added and the baseline was recorded for 50 s. The reaction was initiated by adding decylubiquinol to a final concentration of 50 μM, and reduction of cytochrome c (III) was measured for 300 s with and without antimycin A.

Statistical analysis. Comparisons between groups were performed using Student’s t-tests with the SigmaStat software (Systat Software, San Jose, CA). P < 0.05 was considered statistically significant as indicated in individual figures.

Expression of DIC and OGC proteins in cortical mitochondria.

Fig. 1. Dicarboxylate carrier (DIC) and oxoglutarate carrier (OGC) protein expression patterns. Protein samples from rat cortical mitochondria (A) and mouse neurons and glial cells (B) were assayed for DIC and OGC expression. The neuronal nuclear antigen (NeuN) was used as a neuronal marker, and the glial fibrillary acidic protein (GFAP) was used as a glial marker. Shown are representative Western blots.

Fig. 2. Glutathione (GSH) transport through DIC is essential for the maintenance of the mitochondrial (m)GSH pool. A: standard curve for naphthalene-2,3-dicarboxaldehyde (NDA)-GSH fluorescence assay, showing linearity and consistency. Data are means ± SE, n = 11. B: rat cortical mitochondria were incubated at 20°C for 30 min in 4 different conditions: M, buffer alone; M/GSH, buffer containing 2.5 mM GSH; M/GSH/PS, buffer containing 2.5 mM GSH and 7 mM phenylsuccinate. M0 is freshly isolated mitochondria. mGSH content decreased in M in comparison with M0, indicating the importance of GSH transport across the IMM. Furthermore, mGSH content decreased in M/GSH/BM but not in M/GSH/PS in comparison with M/GSH, indicating that DIC was responsible for GSH transport across the IMM. Data are means ± SE.
levels and that DIC appears to be the main GSH transporter in these mitochondria under normal conditions.

**Inhibition of GSH transport increases oxidative stress in rat cortical mitochondria.** To assess the role of DIC-mediated GSH transport in maintaining mitochondrial ROS homeostasis, we examined intramitochondrial ROS levels under various conditions, using flow cytometry. We found that in comparison with those incubated with GSH (M/GSH), mitochondria incubated without GSH (M) or with both GSH and the DIC inhibitor (M/GSH/BM) had substantially higher levels of intramitochondrial ROS when complex I substrates (glutamate/malate) were used as the electron donor (168% and 229% of M/GSH, respectively) (Fig. 3, A and C). In contrast, no increase in ROS levels was detected when complex II substrate (succinate) was used (Fig. 3, B and C). We also examined kinetic release of H$_2$O$_2$ from these mitochondria. We found that, in line with intramitochondrial ROS levels, rates of H$_2$O$_2$ release from mitochondria incubated without GSH (M) and from those incubated with GSH and the DIC inhibitor (M/GSH/BM) were significantly higher than that from mitochondria incubated with GSH (M/GSH) when complex I substrates (glutamate + malate) were used (213.4 ± 68.3% and 192.2 ± 34.2% of M/GSH, respectively) (Fig. 4, A and C). Again, no increase in the rate of H$_2$O$_2$ release was detected when complex II substrate (succinate) was used (Fig. 4, B and C). These results indicate that DIC-mediated GSH transport and an intact mGSH pool are crucial for ROS homeostasis in rat cortical mitochondria. Inhibition of DIC-mediated GSH transport increases mitochondrial oxidative stress, especially when electron transport is initiated from complex I.

**Inhibition of GSH transport damages IMM function.** We further examined mitochondrial respiration under various conditions to determine the biological relevance of DIC-mediated GSH transport to fundamental mitochondrial functions. We found that mitochondria incubated with GSH (M/GSH) maintained their mitochondrial membrane potential (MMP) as compared with those freshly isolated; however, mitochondria incubated with GSH and the DIC inhibitor (M/GSH/BM) had drastically decreased MMP (Fig. 5A). Furthermore, we found that inhibition of DIC-mediated GSH transport with butylmalonate significantly increased the basal rate of mitochondrial oxygen consumption, as shown by the increase in state 4 respiration rate in the M/GSH/BM group (Fig. 5B). Similar to the increase in mitochondrial oxidative stress, state 4 respiration rate increased more prominently when complex I substrates were used as the electron donor (Fig. 5B). State 3...
respiration rate also increased but to a lesser percentage, resulting in a slightly lower RCR in these mitochondria (data not shown). In line with these signs of IMM injury, inhibition of DIC-mediated GSH transport also decreased oxidative phosphorylation efficiency in rat cortical mitochondria, because the phosphorus-to-oxygen (P/O) ratio was significantly lower in the M/GSH/BM group in comparison with that of freshly isolated mitochondria (M0) regardless of the electron donors used (~ 81% and 74% of M0 with complex I and II substrates, respectively) (Fig. 5C). These findings appear to indicate that mitochondrial respiration is critically dependent on the presence of a normal mGSH pool that is maintained through DIC-mediated GSH transport across the mitochondrial membrane.

Inhibition of GSH transport impairs complex I activity. Because disturbance to mitochondrial ROS homeostasis and damage to IMM functions following inhibition of DIC-mediated GSH transport seemed to occur consistently when electron transfer was initiated from complex I, we wondered whether this important IMM component was particularly vulnerable to mGSH depletion. We found that the most basic complex I function, the NADH-ubiquinone oxidoreductase enzymatic activity, was intact in mitochondria incubated with GSH (M/GSH). However, it was significantly impaired when the DIC inhibitor butylmalonate was added to block GSH transport across the mitochondrial membrane, as shown by a ~17% decrease in the rotenone-sensitive NADH-ubiquinone oxidoreductase activity in the M/GSH/BM group, in comparison with freshly isolated mitochondria (M0) (Fig. 6, A and D). On the contrary, neither complex II functionality, as reflected by succinate-ubiquinone oxidoreductase activity (Fig. 6, B and E), nor complex III functionality, as reflected by ubiquinol-cytochrome c reductase activity (Fig. 6, C and F), was significantly altered by inhibition of DIC-mediated GSH transport. Interestingly, DIC inhibition-induced impairment in complex I functionality did not appear to be associated with dislodgment of complex subunits from the IMM, as suggested by unaltered levels of the 39-kDa subunit protein in mitochondria incubated with various conditions (Fig. 7). Complexes II, III, and cytochrome c also appeared intact (Fig. 7). Thus, inhibition of GSH transport damages mitochondrial respiration through preferential impairment of complex I functionality, which involves a mechanism other than complex protein loss from the IMM.

RNA interference-mediated silencing of DIC decreases mGSH. To confirm the specificity of the pharmacological approach, we studied cross-mitochondrial GSH transport in PC12 cells, a neuronal-like cell line, following DIC or OGC gene silencing using an shRNA approach. Expression of DIC and OGC proteins in these cells was significantly decreased 72 h after transfection with a plasmid encoding a DIC shRNA (40–60% of control DIC levels) or an OGC shRNA (20–50% of control OGC levels) (Fig. 8A). Mitochondria derived from transfected cells were then assayed for GSH transport the same way as described for cortical mitochondria. We found that silencing of DIC diminished the GSH transport process (mGSH, 2.21 ± 0.28 and 2.77 ± 0.36 nmol/mg protein in DIC and control shRNA, respectively; P < 0.01) (Fig. 8B). In contrast, silencing of OGC had no effect (mGSH, 3.23 ± 0.66 and 2.77 ± 0.36 nmol/mg protein in OGC and control shRNA, respectively) (Fig. 8B). These results seem to support the finding that DIC, but not OGC, is involved in GSH transport in mitochondria of neuronal cells under normal conditions.

DISCUSSION

The present study demonstrates that GSH transport across the IMM is important in maintaining the mGSH pool in rat cortical mitochondria and that the mitochondrial dicarboxylate carrier (DIC) accounts for the majority of this transport activity under normal conditions, because inhibition of DIC reduces mGSH to a level similar to that seen in mitochondria incubated without extra-mitochondrial GSH. In contrast, the oxoglutarate carrier (OGC), which has been shown to be important for GSH transport in renal and hepatic mitochondria, does not play a significant role under the same conditions. This notion is further supported by the fact that silencing of DIC, but not OGC, results in a reduction of the mGSH pool in cultured PC12 cells. Involvement of other anion transporters, such as the tricarboxylate carrier (CIC), is unclear but seems doubtful. CIC was reported to be capable of transporting GSH across the IMM in rat brain mitochondria when facilitated by high levels of intramitochondrial malate (49), an exchange substrate for CIC (29). However, the actual amount of GSH
transported into brain mitochondria through this carrier must be less significant under normal conditions, where intramitochondrial malate is at a much lower level (41). Additionally, the fact that CIC-mediated GSH transport activity was inhibited by citrate or isocitrate at a substrate-to-inhibitor concentration ratio of 5:1 (49), which was similar to the ratio of these substances in normal cells (38, 41), would suggest that CIC plays, at most, a minor role in transporting GSH in brain mitochondria. Of note, CIC was shown previously to play no role in GSH transport in renal cortical mitochondria (9).

Our finding that the dominant GSH transporter in cortical mitochondria is different from those in renal and hepatic mitochondria (8, 54) supports the assessment by Lash (30) that diverse mechanisms might be involved in GSH transport into mitochondria in various organs. While it is unknown at present whether other carriers are involved in this important function in other organs/cells and what determines the preferential usage of a particular carrier or carrier set in a particular organ/cell type, the concept of organ/cell-specific GSH transporter itself is significant in several aspects. For example, although the importance of mGSH in antioxidant defense is probably common in all organs/cells, mechanisms maintaining mGSH in different organs/cell types may need to be individually defined. Consequently, therapies targeting mGSH and GSH transport in a particular organ/cell type may need to be individually devised and tested.

Given the well-established role of mGSH in multiple aspects of mitochondrial antioxidant defense (27) and the lack of GSH-synthetic machinery inside mitochondria (16), it was surprising that the significance of GSH-transporting mechanisms and the consequence of their deficiencies in the brain had...
never been studied before. The current study therefore represents the first attempt in addressing these fundamental issues. It seems that the mGSH pool in cortical mitochondria is a small reserve in comparison with its turnover, since a significant portion (~30–35%) of it was consumed within 30 min under an unchallenged condition as seen in mitochondria incubated without extramitochondrial GSH at a mild temperature (20°C). Accordingly, maintenance of mGSH in these mitochondria is critically dependent on active GSH transport across the IMM, because inhibition of DIC, the major GSH transporter in cortical mitochondria, for a short period of time resulted in depletion of the mGSH pool, disturbance of ROS homeostasis, and impairment of respiratory functions in these otherwise unchallenged mitochondria. The high sensitivity of cortical mitochondria to DIC inhibition suggests that DIC-mediated GSH transport is an essential part of and perhaps a major rate-limiting component in the entire mitochondrial antioxidant defense in the brain. Additional results from DIC silencing experiments using shRNA in PC12 cells are in line with this notion (Fig. 8).

There are a few interesting issues from this study that warrant additional discussion. The first is the location(s) in these cortical mitochondria where increased ROS production occurs after DIC inhibition. ROS are known to be produced at complex I and complex III through the forward electron flow when electron transport is initiated from complex I (17). It can also be produced at a separate location in complex I at the reverse electron flow site and at complex III through the forward electron flow that is for the most part sensitive to membrane potential (48). Even a small depolarization has been shown to significantly decrease ROS production in succinate-supported mitochondria (48). It is thus possible that significant damage exists at the reverse electron flow site in complex I, but production of ROS from this location is suppressed by the severe collapse of membrane potential in these mitochondria (Fig. 5A). Additional studies need to be performed to further clarify this interesting issue and to eventually map the sites of structural damage.

The second interesting issue that warrants additional discussion is the relationship between increased oxidative stress and impairment of respiratory functions after DIC inhibition. It seems only logical to postulate that increased mitochondrial oxidative stress associated with mGSH depletion leads to impairment of respiratory functions. However, the fact that the impairment of respiratory functions observed in our study was more severe than the increase in ROS production made one wonder whether this dogma was correct. Indeed, significant damage to respiratory functions occurred in cortical mitochondria following DIC inhibition. For instance, complex I enzymatic activities decreased by 17%. While such a number may appear small, it is important to note that complex I activities are reduced by 25–40% among the most severe Parkinson’s disease cases in several postmortem studies (25, 53). Another example was MMP, which decreased by >65%. Intriguingly, both of these changes, which were not proportional to the increase in ROS production, could be ascribed to direct effects of intramitochondrial GSH redox status. In particular, recent studies have shown that thiol redox status of complex I is critical for its proper functioning and that depletion of mGSH results in selective inactivation of this large complex, likely through modifications of thiol residues of its subunit proteins (23, 33). Other studies have revealed that impaired GSH redox status is correlated with increased membrane permeability, leading to membrane potential collapse (36, 40). Thus, based on these considerations, we have formed a new paradigm that defines complex I and other protein components of the IMM as primary targets of altered GSH redox status following DIC inhibition. Modifications of thiol residues may reduce protein-protein and protein-substrate interactions and may cause disassembly and altered conformation of large complex proteins, but not severe enough to cause protein dislodgement from the IMM. These pathological changes of the IMM decrease its normal functionality and increase electron leakage that, combined with a compromised mitochondrial antioxidant defense secondary to mGSH depletion, lead to increased oxidative stress as seen in cortical mitochondria after DIC inhibition. It is expected that increased oxidative stress will in turn attack the IMM, accelerating its damage. Obviously, details of this paradigm need to be worked out in future studies.

The third issue that merits mentioning is the use of α-GP as the electron donor for mitochondria during GSH transport assays. To assess GSH transport in coupled mitochondria, it

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was necessary that a respiratory substrate be included in the assay buffer. However, commonly used substrates such as succinate and malate are dicarboxylates and their addition to the assay buffer would have interfered with the assessment of GSH transport. For this reason, we decided to use α-GP. This respiratory substrate does not require an IMM transporter, because it is oxidized to dihydroxyacetone phosphate by glycerol-3-phosphate dehydrogenase located on the outer surface of the IMM. FADH₂ produced by this reaction donates its electrons to the respiratory chain through coenzyme Q (26). RCR values of brain and liver mitochondria with α-GP as the electron donor were relatively low (2.7–3.0) (28, 46). However, α-GP appeared to be sufficient as a respiratory substrate, because it maintained normal membrane potential and supported ROS production in these mitochondria (28, 46). Results from rat cortical mitochondria in the current study were similar to those reported in the literature.

In conclusion, DIC is the main GSH transporter in rat cortical mitochondria. Furthermore, DIC-mediated GSH transport is essential for these mitochondria to maintain ROS homeostasis and normal respiratory functions.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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