Acute Glutathione Depletion Restricts Mitochondrial ATP Export in Cerebellar Granule Neurons*

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Decreases in GSH pools detected during ischemia sensitize neurons to excitotoxic damage. Thermodynamic analysis predicts that partial GSH depletion will cause an oxidative shift in the thiol redox potential. To investigate the acute bioenergetic consequences, neurons were exposed to monochlorobimane (mBCl), which depletes GSH by forming a fluorescent conjugate. Neurons transfected with redox-sensitive green fluorescent protein showed a positive shift in thiol redox potential synchronous with the formation of the conjugate. Mitochondria within neurons treated with mBCl for 1 h failed to hyperpolarize upon addition of oligomycin to inhibit their ATP synthesis. A decreased ATP turnover was confirmed by monitoring neuronal oxygen consumption in parallel with mitochondrial membrane potential (Δψm) and GSH-mBCl formation. mBCl progressively decreased cell respiration, with no effect on mitochondrial proton leak or maximal respiratory capacity, suggesting adequate glycolysis and a functional electron transport chain. This approach to “state 4” could be mimicked by the adenine nucleotide translocator inhibitor bongkrekic acid, which did not further decrease respiration when administered after mBCl. The cellular ATP/ADP ratio was decreased by mBCl, and consistent with mitochondrial ATP export failure, respiration could not respond to an increased cytoplasmic ATP demand by plasma membrane Na+ cycling; instead, mitochondria depolarized. More prolonged mBCl exposure induced mitochondrial failure, with Δψm collapse followed by cytoplasmic Ca2+ deregulation. The initial bioenergetic consequence of neuronal GSH depletion in this model is thus an inhibition of ATP export, which precedes other forms of mitochondrial dysfunction.

A balance between the formation of reactive oxygen species, as normal byproducts of mitochondrial respiration (1), and the actions of antioxidants prevents oxidative stress and is crucial to neuronal survival (2). Together with the overactivation of glutamate receptors (excitotoxicity), oxidative stress is a result of the bioenergetic crisis that characterizes ischemia and plays a central role in the pathophysiology of the consequent neuronal damage (3). Neurons are particularly sensitive to oxidative damage and can be strongly sensitized to other injurious stimuli by levels of oxidative stress that are nontoxic per se (4). Similarly, Ca2+ homeostasis is lost more quickly in cerebellar granule neurons (CGNs),2 which show higher superoxide levels prior to the application of toxic concentrations of glutamate (5).

The tripeptide glutathione is a key antioxidant that maintains protein thios in a reduced state and scavenges H2O2 in a reaction catalyzed by glutathione peroxidase (6, 7). In vivo, mitochondrial glutathione is partially lost during ischemia (8), and its supplementation in the form of glutathione ethyl ester can reduce the infarct size (9). Although mitochondria within cells deprived of GSH can eventually release cytochrome c and undergo opening of the permeability transition pore (PTP) (10–12), the initial bioenergetic consequences of GSH depletion remain unclear. Thermodynamic and kinetic factors predict that the steady-state thiol redox potential will be very sensitive to changes in total glutathione pool size (7, 13). This may lead to the oxidative damage of key mitochondrial proteins. Indeed, either an inhibitor of GSH synthesis or the direct removal of reduced GSH with ethacrynic acid causes the inactivation of complexes I (14) and II/III and IV (15, 16) that becomes apparent several hours from the beginning of treatment. An intriguing target for thiol oxidation is represented by the adenine nucleotide translocator (ANT), which functions as a dimer and can be progressively inhibited as the intermolecular oxidation of thiol groups increases (17). This loss of ADP/ATP exchange activity precedes the formation of intramolecular cross-linking, which seems to require stronger oxidative conditions and may be related to mitochondrial PTP opening (18, 19).

In this study, we rapidly depleted neuronal glutathione by conjugation with monochlorobimane (mBCl), a glutathione S-transferase (GST) substrate that is extensively used to assay glutathione pools (20, 21). The intracellular thiol redox potential was detected with a redox-sensitive green fluorescent protein variant (22), whereas population cell respiratory rates were monitored in parallel with cytoplasmic free Ca2+ concentration ([Ca2+]i) and mitochondrial membrane potential (Δψm) (23), allowing glutathione depletion to be correlated with a rapid inhibition of ATP turnover that became evident within the first hour of treatment. The data suggest that a restriction of mitochondrial ATP export to the cytoplasm, consistent with ANT inhibition, is the first event occurring in this model of GSH depletion.

MATERIALS AND METHODS

Reagents—Tetramethylrhodamine methyl ester (TMRM), Fluoro-4FF, Fluoro-5F, and mBCl were from Molecular Probes (Eugene, OR). Redox-sensitive green fluorescent protein-2 (roGFP2) cDNA was a kind gift of S. James Remington (University of Oregon, Eugene). Lipofectamine 2000 was from Invitrogen. All other reagents were from Sigma.

Preparation of CGNs—CGNs were prepared from 7-day-old Wistar rats as described previously (24), with modifications. Cells were plated onto Lab-Tek 8-well chambered coverglasses at a density of 380,000 cells/well or onto 22 × 40-mm coverslips at a density of 3 × 10^6 cells/well or onto 22 × 40-mm coverslips at a density of 3 × 10^6 cells/well.
coverslips. Coverslips had previously been coated with 33 μg/ml poly-ethyleneimine. Cultures were maintained in minimal essential medium supplemented with 10% fetal bovine serum, 30 mM glucose, 20 mM KCl, 2 mM glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin. 24 h after plating, 10 μM cytosine arabinoside was added to inhibit growth of non-neuronal cells. Cell cultures were maintained at 37 °C in an incubator with a humidified atmosphere of 5% CO2 and 95% air and used for experiments at 7–9 days in vitro.

Experimental Buffers—Standard "high KCl" buffer consisted of 100 mM NaCl, 25 mM KCl, 20 mM TES, 15 mM glucose, 1.3 mM MgCl2, 1.3 mM CaCl2, 1.2 mM Na2SO4, 0.4 mM KH2PO4, and 0.2 mM NaHCO3 (pH 7.3) at 37 °C. Some experiments were conducted in "low KCl" buffer containing 120 mM NaCl, 3.5 mM KCl, 20 mM TES, 15 mM glucose, 1.3 mM CaCl2, 1.2 mM Na2SO4, 0.4 mM KH2PO4, and 5 mM NaHCO3 (pH 7.3) at 37 °C.

Functional Confocal Microscopy—Imaging of single neurons plated onto Lab-Tek 8-well chambered coverslips was performed in a Pascall confocal system (Carl Zeiss AG, Oberkochen, Germany) using an Axiovert 100 M inverted microscope with a ×20 objective and argon (488 nm) and helium/neon (543 nm) lasers. Control and experimental wells were imaged in parallel using a computer-controlled motorized stage and Physiology software, which allows parallel or sequential acquisition of time courses. For simultaneous detection of [Ca2+]i, and Δψm (see Fig. 7), neurons were loaded with the low affinity Ca2+-indicator Fluo-4-FF (0.5 μM; as acetoxymethyl ester; Ca2+-Kd = 9.7 μM) and TMRM- (5 nM) for 30 min at 37 °C. This concentration of TMRM- was insufficient for aggregation in the matrix (i.e. the experiment was performed in "non-quench mode") (25), meaning that a decrease in either the plasma membrane or mitochondrial membrane potential was reflected in a reduction in whole cell fluorescence. In experiments in which [Ca2+]i, transients following KCl-induced plasma membrane depolarization were detected, neurons were loaded with 0.5 μM Fluo-5F (Ca2+-Kd = 2.3 μM). Fluo-4-FF and Fluo-5F were excited at 488 nm, and emission was collected between 505 and 530 nm. TMRM+ was excited at 543 nm, and the emitted fluorescence was collected between 560 and 615 nm. Under both conditions, TMRM+ was always present in the buffer throughout the experiment (25).

Cell Respiriometer—Respiration of intact neurons in situ was measured as described previously (23). A 22 × 40-mm glass coverslip with attached cells was assembled in a closed RC-30 imaging chamber (Warner Instruments, Hamden, CT) and then mounted on an Olympus IX81 inverted fluorescence microscope equipped with ×20 and ×40 objectives. A miniature polarographic Clark-type oxygen electrode for perfusion systems with 1/16-inch fittings (Microelectrodes Inc., Bedford, NH) was used to monitor the oxygen tension in the eluted buffer. The flow rate was typically kept at 40–60 μl/min to provide sufficient oxygen depletion for detection. In experiments in which brevetoxin was employed, the buffer was also supplemented with 1 μM MK-801 to ensure that the results observed did not originate from the activation of N-methyl-d-aspartate receptors. 3 nM TMRM+ was present in the buffer (non-quench mode conditions). A chart recorder was wired to an OM-4 oxygen meter (Microelectrodes, Inc.) to provide a continuous trace of the oxygen depletion (and hence respiration) of the cells. The microscope was equipped with a CoolSNAP HQ CCD camera (Roper Scientific, Tucson, AZ) and MetaFluor and MetaMorph imaging software (Universal Imaging Corp., Downington, PA). TMRM+ and mBCI were excited via S555/20x and D380/13x filters, respectively (Chroma Technology Corp., Rockingham, VT). The emission from both fluorophores was collected through a 73101 dual band emission filter. roGFP2 was excited via a D480/30x filter, and the emission was collected through the 73101 filter.

Transfection of roGFP2—Cultured neurons were transfected following a published protocol (26), with modifications. On day 0, following attachment to their coverslips, CGNs were transfected to culture medium without penicillin and streptomycin and incubated with 0.8 μg of roGFP2 DNA and the transfection reagent Lipofectamine 2000. After 2 h, neurons were returned to the usual culture medium and used for experiments after day 6.

Monitoring Δψm with Rhodamine 123—Rhodamine 123 in quench mode is the most sensitive means of detecting small changes in Δψm (25). Neurons were incubated in low KCl buffer and exposed to 2.2 μM rhodamine 123 for 15 min at 37 °C (to attain quench mode conditions). Cells were then rinsed with fresh buffer and imaged with the Olympus IX81 microscope. Rhodamine 123 was excited via a D480/20x filter, and the emitted fluorescence was collected through a 51000m filter (Chroma Technology Corp., Rockingham, VT). Given the relatively low membrane permeability, rhodamine 123 leaks out of the cells very slowly and therefore was not present in the media during the experiments.

Determination of ATP/ADP Ratios—Total cellular ATP/ADP ratios were determined using a luciferase chemiluminescence assay (Calbiochem). Cells were incubated in low KCl buffer under the relevant conditions and lysed, and extracts were assayed for ATP according to the manufacturer’s instructions. Pyruvate kinase (2 units/assay) was added to the buffer supplied with the kit. The increase in chemiluminescence was recorded in a TD-20/20 luminometer (Turner BioSystems, Sunnyvale, CA). Once the ATP signal was detected, 0.5 mM phosphoenolpyruvate was added, and the further increase in chemiluminescence due to the conversion of ADP to ATP was determined.

Immunocytochemistry—Neurons plated onto Lab-Tek 4-well chambered slides were washed with 50 mM Tris and 150 mM NaCl (pH 7.5), fixed with 4% paraformaldehyde, and permeabilized in 0.1% Triton X-100. A purified mouse anti-cytochrome c monoclonal antibody (Pharminet) that recognizes the native form of cytochrome c (1:100 dilution) was added to the coverslips (overnight, 4 °C). The cells were then incubated with a fluorescein isothiocyanate-conjugated anti-mouse secondary antibody. Nuclei were stained with TOTO-3 before mounting the slides on ProLong Gold medium (both reagents from Molecular Probes). The samples were imaged with a Nikon PCM 2000 confocal system equipped with argon (488 nm) and helium/neon (633 nm) lasers.

Statistical Analysis—All data analyzed were collected from at least three independent experiments and are expressed as means ± S.E. Data from two populations were compared with the unpaired two-tailed Student’s t test. One-way analysis of variance followed by Tukey’s test was used in the analysis of three or more experimental groups. p values <0.05 were considered significant.

RESULTS

Conjugation of Endogenous GSH with mBCI Oxidizes the Thiol Redox Potential—mBCI becomes fluorescent when conjugated to GSH by intracellular GST. The reaction is highly selective for glutathione over other intracellular thiols (27), but is not a good indicator of subcellular distribution because GSTs are present in both the cytoplasm and the mitochondrion (28), and the probe eventually accumulates in the nucleus (Fig. 1) (29). Another substrate for GST, ethacrynic acid, has been frequently used to deplete cells of GSH (16, 30). However, ethacrynic acid also directly inhibits mitochondrial complex II (31) and, in our hands, caused a partial drop in Δψm when added to CGNs. An
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A major advance in the ability to monitor thiol redox potentials has come as a result of the development of roGFP analogs by substituting surface-exposed residues of the chromophore with cysteines (22). Notably, an analog of roGFP2 (redox-sensitive yellow fluorescent protein) expressed in the cytoplasm of yeast cells appears to directly equilibrate with the cytoplasmic glutathione couple (32). Although the redox state of roGFPs can be monitored ratiometrically by excitation at 400/480 nm, oxidation of roGFP2 will result in a lower fluorescence emission, whereas its reduction will cause fluorescence enhancement.

The validity and sensitivity of the construct in CGNs were established by applying 500 μM H2O2 to elicit exogenous oxidative stress. The treatment resulted in a rapid decrease in roGFP2 emission (Fig. 1A) that was reversed by dithiothreitol (DTT). A reduction in roGFP2 emission corresponds to oxidation of the protein. The time courses are the means of the emission from eight cells from seven independent fields. div, division.

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An additional advantage of mBCl is that the reaction course can be followed by the development of fluorescence, as mBCl depletes GSH by forming the adduct GSH-mBCl (Fig. 1A), with the reaction usually reaching completion within 60 min.

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This case, oligomycin will depolarize the inner membrane. This “oligomycin null point” assay (33, 34) can therefore be used to investigate the bioenergetic status of the mitochondrial population within a single cell body. Fig. 2A (panels i–iii) shows responses of representative neurons whose ΔΨm was hyperpolarized, was unchanged, or depolarized in response to the inhibitor, respectively. Because rhodamine 123 is in “quench mode,” hyperpolarization will cause further sequestration and quenching of the dye inside the matrix and decrease the whole cell fluorescence. The final addition of carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) to totally release the dye into the cytoplasm confirms that the experiment is performed under quench mode conditions.

As expected, oligomycin decreased the fluorescence in a majority of control neurons (53%) (Fig. 2A, panel i). By contrast, the large majority of mBCl-treated cells (83%) did not undergo a detectable change in fluorescence following oligomycin addition, whereas 15% of the cells were exposed to 100 μM mBCl, followed by 2 mM dithiothreitol (DTT). A reduction in roGFP2 emission corresponds to oxidation of the protein. The time courses are the means of the emission from eight cells from seven independent fields. div, division.

Additional information about mitochondrial ATP synthesis can be obtained by utilizing the proton current, it follows that addition of the ATP synthase inhibitor oligomycin will acutely hyperpolarize the mitochondrial membrane (Fig. 2B). A, 2 μg/ml oligomycin (O) and 1 μM FCCP (F) were added sequentially. Three mitochondrial responses to oligomycin were observed from the single cell traces: hyperpolarization (panel i), no change (panel ii), and depolarization (panel iii). The number of control or treated cells that showed each type of response is shown in the histograms. Note that the majority of cells treated with mBCl (83%, n = 178) failed to hyperpolarize upon addition of oligomycin, whereas in 27 cells (15%), ΔΨm decreased, suggesting ATP synthase reversal. The addition of FCCP to collapse ΔΨm confirmed that rhodamine 123 remained in quench mode. B, cells were preincubated in standard high KCl buffer at 37 °C with 0.1% Me2SO (Control, 100 μM mBCl (for 75 min), 1 μg/ml oligomycin (for 30 min), 750 nm rotenone (for 30 min), or 1 μM FCCP (for 30 min) prior to extraction and adenine nucleotide determination. Data collected from three independent experiments were analyzed. *, p < 0.01 versus the control and FCCP. Rhod-123 fluoro, rhodamine 123 fluorescence.
showed mitochondrial depolarization upon addition of oligomycin, indicating that, in some mitochondria, the membrane potential was sustained by the ATP synthase running in reverse.

The unchanged rhodamine 123 fluorescence observed in most mBCI-treated cells upon oligomycin addition suggests that the mitochondria are close to respiratory state 4 (35), i.e. with little ATP turnover. This could be due either to a decreased cellular ATP demand or to an inhibition of the generation or export of ATP from the mitochondrion to the cytoplasm. The former would result in an increased total cellular ATP/ADP ratio, whereas the latter would decrease the ratio. A 75-min exposure to mBCI caused a significant decrease in the ATP/ADP ratio compared with control cells (2.76 ± 0.29 versus 4.83 ± 0.36) (Fig. 2B). This decrease was similar to that induced by oligomycin (2.54 ± 0.18). ATP synthase reversal induced by the inhibition of complex I with rotenone and by the protonophore FCCP caused a greater reduction in the ATP/ADP ratio (to 1.9 ± 0.16 and 1.2 ± 0.23, respectively). The decreased ratio is thus consistent with inhibited generation or export of ATP from the matrix.

Mitochondria in CGNs Exposed to mBCI Approach State 4—To confirm and quantify the above observations, the O2 consumption of intact neurons attached to their coverslips was determined (Fig. 3) with a cell respirometer assembly as described previously (23). Cells were either perfused with standard high KCl buffer + 0.1% Me2SO (control) until mitochondrial inhibitors were added or switched to buffer additionally containing 100 μM mBCI after basal respiration was assessed. Rates of respiration were calculated from the perfusion rate times the difference between the downstream oxygen tension during chamber perfusion and that recorded when the chamber was bypassed with a shunt.

The basal respiration of control neurons was stable until oligomycin-containing medium was perfused. The inhibitor decreased respiration to 44 ± 6% of the basal level, the residual value reflecting the inherent proton leak across the inner mitochondrial membrane (Fig. 3A). Thus, almost half of the basal respiration was used to compensate for the endogenous mitochondrial proton leak, whereas the remainder reflected basal ATP turnover. The effect of 100 μM mBCI was to initiate within 15 min a slowing of the basal respiration that progressed as GSH was conjugated to mBCI over a 60-min period (Fig. 3B). At that point, ATP turnover in the cells (calculated as the oligomycin-sensitive component of respiration) had decreased to <50% of that in control cells. Notably, mBCI caused no significant change in state 4 respiration (following oligomycin addition); thus, there was no uncoupling or permeability transition induction following 60 min of mBCI exposure, and the decrease in total respiration could be ascribed to an inhibition of mitochondrial ATP synthesis.

The respirometer also allows maximal respiratory capacity to be quantified. Following the addition of the protonophore FCCP, mBCI-exposed neurons increased their respiration to the same extent as control cells (203 ± 42% over the state 4 rate in control cells versus 180 ± 30% in mBCI-treated cells) (Fig. 3D), demonstrating that this acute GSH depletion did not detectably restrict substrate delivery or electron transfer activity. This contrasts with long-term partial glutathione depletion that manifests as an inhibition of complex I activity in PC12 cells (36).

The respirometer was mounted on an epifluorescence microscope, which allows imaging of multiple fluorescence indicators at a single cell level (see “Materials and Methods”). Thus, when 100 μM mBCI was added, we could confirm that the fluorescence of the intracellular adduct GSH-mBCI reached a plateau within 1 h (see Fig. 1A). Also the fluorescence from cells equilibrated with 3 nM TMRRM+ indicated that there was no detectable depolarization of either plasma or mitochondrial membrane potentials during this period (Fig. 4).

Because respiratory capacity is not rate-limiting, the approach to state 4 following mBCI addition could be due to a decreased capacity for mitochondrial ATP synthesis (ATP synthase inhibition) or to inhibited export to the cytoplasm (ANT inhibition), whereas a decreased cellular ATP demand (e.g. inhibited ion pump activity) would be inconsistent with the decreased ATP/ADP ratio observed (Fig. 2B). Because of reports showing ANT sensitivity to thiol oxidation (18, 19, 37), a partial inhibition of this protein appears a plausible mechanism for the observed effects. In support of this view, incubation with the cell-permeant ANT inhibitor bongkrekic acid (10 μM) induced a decline in
basal respiration; but, as with 60 min mBCl exposure, the approach to state 4 was incomplete, and an additional effect of oligomycin was apparent (Fig. 3C). If bongkrekic acid and mBCl were affecting cell respiration via alternative mechanisms, their combined effects should be additive and produce a greater decrease in respiration. To investigate this, we perfused cells with mBCl for 30 min, switched to buffer containing mBCl + bongkrekic acid for an additional 30 min, and then assessed the oligomycin-insensitive respiration. Interestingly, oxygen consumption was reduced to a similar extent by bongkrekic acid alone or in combination with mBCl. (Respiration was 55.4 ± 7.9% above the oligomycin rate in cells exposed to bongkrekic acid versus 53.1 ± 13.8% in cells treated with both mBCl and bongkrekic acid.)

Bioenergetic Failure of mBCl-treated Neurons following Increased Cytoplasmic ATP Demand—The plasma membrane Na\(^+\)/K\(^+\)- ATPase is potentially the major ATP-utilizing reaction in the neuron (38). However, its activity is usually restrained by the limited Na\(^+\) cycling due to the low basal Na\(^+\) conductance of the plasma membrane. To activate Na\(^+\) cycling and to increase the cytoplasmic ATP demand, the voltage-dependent Na\(^+\) channel activator brevetoxin was used. Because of its ability to promote extensive release of vesicular glutamate and thus activate N-methyl-D-aspartate receptors (39), brevetoxin was perfused in the presence of 1 \(\mu\)M MK-801. In control neurons, brevetoxin robustly enhanced respiration, utilizing ~50% of the "reserve capacity" of the mitochondria and increasing respiration by 32 ± 5% above basal levels (Fig. 4A). After washout of the neurotoxin, basal respiration was restored, and the neurons responded to FCCP, confirming that the mitochondria possessed sufficient ATP-generating capacity to meet this enhanced demand. In contrast, when brevetoxin was added to neurons that had been incubated with mBCl for 60 min, little respiratory enhancement was seen (11 ± 2% above that immediately prior to toxin addition) (Fig. 4B). The loss of TMRM\(^{+}\) signal immediately upon addition of the toxin indicates a loss of plasma or mitochondrial membrane potential, and the subsequent minimal response to FCCP, together with examination of cell morphology (data not shown), indicates extensive cell damage consistent with a cellular ATP deficit.

To further investigate the ability of mBCl-treated neurons to handle higher cellular ATP demand caused by increased Na\(^+\) cycling, the neurons were exposed to the excitoxin kainate (Fig. 5), which induces massive Na\(^+\) entry into the cell through non-inactivating \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (34). As in the above experiments, cells were loaded with 3 nM TMRM\(^{+}\) to monitor \(\Delta\psi_m\), in parallel with cell respiration. In control neurons, 50 \(\mu\)M kainate induced a substantial increase in cell respiration (+76 ± 7.4%) that returned to basal values once the excitoxin was washed out (Fig. 5A). Partly because of plasma membrane depolarization, TMRM\(^{+}\) fluorescence decreased significantly upon perfusion with kainate, but recovered almost completely after the washout. In contrast to control cells, neurons perfused with 100 \(\mu\)M mBCl for 60 min increased respiration only slightly when exposed to kainate (+30.1 ± 10.5%; \(p < 0.05\) versus kainate effect in control cells) (Fig. 5B). Moreover, mitochondria in mBCl-treated cells profoundly depolarized in the presence of kainate, failed to repolarize after the washout, and showed virtually no response to subsequent FCCP addition. Thus, as with brevetoxin (Fig. 4B), the experiments with kainate suggest that mitochondria within mBCl-treated cells cannot increase ATP production sufficiently to cope with the increased cytoplasmic demand of Na\(^+\) cycling at the plasma membrane, leading to membrane depolarization and cell death.

Mitochondrial Ca\(^{2+}\) Uptake Is Preserved in mBCl-treated Neurons—When plasma membrane Ca\(^{2+}\) channels are activated by plasma membrane depolarization in elevated KCl, Ca\(^{2+}\) entry elevates \([\text{Ca}^{2+}]\), sufficiently for the in situ mitochondria to accumulate the cation (40). This accumulation can be detected following the addition of protonophore as a transient “spike” in \([\text{Ca}^{2+}]\), as Ca\(^{2+}\) is released into the cytoplasm and pumped out across the plasma membrane. Mitochondrial Ca\(^{2+}\) accumulation is not dependent upon ATP and thus should be unimpaired if the only mBCl-induced modification to the mitochondrion is an inhibited ATP export. Fig. 6 shows that mitochondria accumulated Ca\(^{2+}\) under these conditions and released it into the cytoplasm when the collapse of \(\Delta\psi_m\) was induced by 250 nM FCCP. A similar pattern was observed after treatments with mBCl for 75 min, indicating that these
neurons were still able to accumulate Ca\(^{2+}\) within the mitochondrial matrix. FCCP induced a 1.76 ± 0.29-fold increase in Fluo-5F fluorescence in control cells compared with a 1.65 ± 0.25-fold increase in mBCI-treated cultures. Ca\(^{2+}\) accumulation is largely dependent upon the parallel uptake of phosphate (41). Although the mitochondrial phosphate transporter is sensitive to thiol-oxidizing agents, the more subtle effects elicited here by GSH depletion did not appear to affect the transporter sufficiently to influence Ca\(^{2+}\) transport. However, in mBCI-treated cells, the [Ca\(^{2+}\)]\(_{\text{plateau}}\) that followed the application of KCl was slightly elevated. This might reflect a reduced extrusion capacity of the plasma membrane Ca\(^{2+}\)-ATPase due either to direct oxidative damage or to the fact that less ATP is available in mBCI-treated neurons.

When 250 nM FCCP was administered in the absence of 50 mM KCl, neither control nor mBCI-treated cells showed [Ca\(^{2+}\)]\(_{\text{plateau}}\), changes (data not shown), consistent with a lack of mitochondrial Ca\(^{2+}\) accumulation in polarized cells (40).

**DISCUSSION**

These experiments allowed us to detect and quantify the earliest bioenergetic consequences of acute neuronal glutathione depletion by combining several novel techniques such as the cell respirometer (Figs. 3–5); membrane potential determinations, including the oligomycin null point test for mitochondrial ATP synthesis (Fig. 2); and fluorescent monitoring of the time course of GSH depletion and verification of oxidative shifts in thiol redox potential (Fig. 1). The initial events of mBCI-mediated glutathione depletion appeared to be focused at the mitochondrion, even though the GSH-mBCl conjugate concentrated in the nucleus (Fig. 1). In culture, removal of GSH by conjugation to a variety of GST substrates affects the mitochondrial pool first (30). Although the reasons for this differential depletion are unknown, one explanation might be that the matrix is slower to replenish its GSH pools, as has been reported for COS cells exposed to the GST substrate 4-hydroxynonenal (28). Mitochondria do not synthesize GSH, but replenish their pool from the cytoplasm (42). The two pools are interconnected; but, in the brain of adult rats, the GSH content of mitochondria is ~4-fold lower than that detected in the total tissue (8). Even greater differences have been observed between mitochondrial and cytoplasmic fractions in primary cultures (30, 43). Mitochondrial GSH pools are preferentially reduced when GST substrates are used, as seen with ethacrynic acid in CGNs (43) and cortical astrocytes (30) and with 4-hydroxynonenal in COS cells (28). Mitochondrial levels of GSH are reduced following focal ischemia (8) and supplementation with glutathione ethyl ester, which increases GSH levels in mitochondria, reduces the infarct zone caused by a similar treatment (9).
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The enzyme that maintains glutathione reduction (glutathione reductase) operates away from thermodynamic equilibrium because redox potentials of the matrix and cytoplasmic NADPH pools at approximately −350 mV (44) are much lower than the glutathione redox potential in the cell at about −240 mV (7, 45, 46). This disequilibrium implies that the glutathione pool rapidly cycles in the cell and that glutathione reductase exerts considerable control over the thiol redox potential. There is evidence that glutathione reductase is limited by the low GSSG concentration in the cell; thus, oxidation of the GSH pool by t-butyl hydroperoxide leads to a rapid and selective oxidation of NADPH both in cultured cells (47) and in vivo (48). Although it is intuitive that reductions in the GSH pool will exacerbate the rate limitation due to glutathione reductase, the distinctive thermodynamics of the glutathione couple, where two GSH molecules condense upon oxidation to form GSSG, mean that the midpoint potential becomes progressively more oxidized as the pool decreases. To maintain the same thiol redox potential when the pool size is halved would require a 4-fold decrease in GSSG concentration (35), which is clearly at odds with the kinetic constraints discussed above.

Although mBCl has been extensively used to assay glutathione in cells (29, 49, 50), the simultaneous monitoring of the bioenergetic consequences during the resulting glutathione depletion is novel. A major advantage over "blind" techniques such as incubation with ethacrynic acid or l-buthionine (SR)-sulfoximine (51) is that the extent and time course of depletion can be monitored continuously by the formation of the fluorescent adduct. In addition, the fluorescent signal from transfected roGFP2 (22), a member of a class of thiol-modified fluorescent proteins that appear to equilibrate directly with the cytoplasmic thiol proteomes that was mostly lost after 3.5 h with mBCl. Apoptotic cells in staurosporine-treated coverslips (condensed nuclei; white arrows) showed no punctate cytochrome c staining.

**FIGURE 7.** Prolonged treatment with mBCl induces Δψm collapse and cytochrome c release. A, cells were incubated in standard high KCl buffer, exposed to 100 μM mBCl when appropriate, and loaded with Fluo-4FF + 5 μM TMRM+. Cells were treated with mBCl for a total of 90 min before confocal imaging was started. (For clarity, single cell traces are shown as beginning at the 90-min point.) Cells began losing TMRM+ signal at ~150 min after mBCl was applied. Note that the increases in [Ca2+]i occurred after mitochondrial depolarization. B, this is most clearly seen when [Ca2+]i and TMRM+ signals are shown for single cells. C, shown are the results from immunocytochemistry. Cells were treated with 100 μM mBCl (for 1 or 3.5 h) or 1 μM staurosporine (5 h), fixed, and stained for native cytochrome c (green). TOTO-3 was also used to stain the nuclei (blue). Control and 1-h mBCl-treated cells showed a punctate mitochondrial pattern for cytochrome c that was mostly lost after 3.5 h with mBCl. Apoptotic cells in staurosporine-treated coverslips (condensed nuclei; white arrows) showed no punctate cytochrome c staining.

**FIGURE 8.** Glutathione ethyl ester protects CGNs against delayed cell death induced by 1-h mBCl exposure. CGNs were switched to standard high KCl buffer and either left untreated (Control) or incubated with mBCl in the presence or absence of 200 μM glutathione ethyl ester (GSH-EE) for 1 h. Transmitted light images were collected at that point, and cells were returned to the original culture medium. 200 μM glutathione ethyl ester was also present in the appropriate culture medium. 24 h later, cells were exposed to 1 μM propidium iodide, and the visual fields that had been selected the day before were imaged a second time.

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Instead, the initial event appeared to be a progressive decrease in mitochondrial ATP turnover.

The candidate targets for the decreased ATP export from the mitochondrion include the ATP synthase and ANT. Any inhibition was incomplete at 60 min because oligomycin still inhibited respiration (Fig. 3). However, it is well established that ANT is vulnerable to thiol oxidation, and ATP/ADP translocation is progressively inhibited by reaction of specific cysteines with thiol reagents (17, 37, 52). This inhibition is distinct from the change in ANT conformation that opens a channel at much lower ATP levels (54, 55) in our experiments. Thus, any inhibition observed in the delayed Ca\(^{2+}\) release may be due to thiol oxidation at a subcellular level.

In conclusion, our work provides the first analysis of mitochondrial function in intact neurons acutely depleted of GSH. We have shown that the restriction of ATP turnover is the first sign of mitochondrial dysfunction, preceding depolarization and cytochrome c release. In this experimental model, GSH depletion induces an early energy shortage that may transform the use of ATP by rapidly working plasma membrane ion pumps into a toxic challenge.

REFERENCES

1. Droge, W. (2002) Physiol. Rev. 82, 47–95
2. Andersen, J. K. (2004) Nat. Med. 10, (suppl.) 18–25
3. Fiskum, G., Rosenthal, R. E., Verecki, Y., Martin, E., Hoffman, G. E., Chinopoulous, C., and Kowaltowski, A. (2004) J. Bioenerg. Biomembr. 36, 347–352
4. Arundine, M., Aarts, M., Lau, A., and Tymianski, M. (2004) J. Neurosci. 24, 8106–8123
5. Vese, S., Kirk, L., and Nicholls, D. G. (2004) J. Neurochem. 90, 683–693
6. Dringen, R., Kussmaul, L., Gutterer, J. M., Hirlinger, J., and Hamprecht, B. (1999) J. Neurochem. 72, 2523–2530
7. Schafer, F. Q., and Buettner, G. R. (2001) Free Radic. Biol. Med. 30, 1191–1212
8. Anderson, M. F., and Sims, N. R. (2002) J. Neurochem. 81, 541–549
9. Anderson, M. F., Nilsson, M., Eriksson, P. S., and Sims, N. R. (2004) Neurosci. Lett. 354, 163–165
10. Ghibelli, L., Coppola, S., Fanelli, C., Rotilio, G., Civitareale, P., Scovassi, A. I., and Cirillo, M. R. (1999) FEBS Lett. 13, 2031–2036
11. Kowaltowski, A. J., Castillo, R. F., and Vercesi, A. E. (2001) FEBS Lett. 495, 12–15
12. Armstrong, J. S., Yang, H. Y., Duan, W., and Whitehead, M. (2004) J. Biol. Chem. 279, 50420–50428
13. Nicholls, D. G. (2004) Curr. Mol. Med. 4, 149–177
14. Hsu, M., Srinivas, B., Kumar, J., Subramanian, R., and Andersen, J. (2005) J. Neurosci. 92, 1091–1103
15. Heales, S. J., Davies, S. E., Bates, T. E., and Clark, J. B. (1995) Neurochem. Res. 20, 31–38
16. Woodruff, J., Soldern, F., Schulz, J. B., Klockgether, T., Kovar, K. A., and Wüller, U. (1999) Neurosci. Lett. 264, 1–4
17. Majima, E., Ikawa, K., Takeda, M., Hashimoto, M., Shinozaki, Y., and Terada, H. (1995) J. Biol. Chem. 270, 29545–29554
18. Costantini, P., Belzacq, A. S., Vieira, H. L., Larochette, N., de Pablo, M. A., Zamzami, N., Susin, S. A., Brenner, C., and Kroemer, G. (2000) Oncogene 19, 307–314
19. McStay, G. P., Clarke, S. J., and Halestrap, A. P. (2002) Biochem. J. 367, 541–548
20. Barhoumi, R., Bailey, R. H., and Burghardt, R. C. (1995) Cytotherapy 19, 226–234
21. Kamencic, H., Lyon, A., Paterson, P. G., and Jurlin, B. H. (2000) Anal. Biochem. 286, 35–378. H. I.
22. Dooley, C. M., Dore, T. M., Hanson, G. T., Jackson, W. C., Remington, S. J., and Tsiens, R. Y. (2004) J. Biol. Chem. 279, 22284–22293
23. Jekabsons, M. B., and Nicholls, D. G. (2004) J. Biol. Chem. 279, 32989–33000
24. Courtney, M. J., Lambert, J. J., and Nicholls, D. G. (1990) J. Neurosci. 10, 3873–3879
25. Ward, M. W., Rege, A. C., Frenquelli, B. G., and Nicholls, D. G. (2000) J. Neurosci. 20, 7208–7219
26. Dalby, B., Cates, S., Harris, A., Oliki, E. C., Tilkins, M. L., Price, P. J., and Ciccarelli, V. C. (2004) Methods 33, 95–103
27. Fernandez-Checa, J. C., and Kaplowitz, N. (1990) Annu. Rev. Biochem. 59, 212–219
28. Raza, H., Robin, M. A., Fang, J. K., and Avadhani, N. G. (2002) Biochem. J. 366, 45–55
29. Kedan, I., Allen, N. J., Antcliff, D., Pal, S., and Duchen, M. R. (2001) J. Neurosci. Res. 66, 873–884
30. Muday, H., Nilsen, H., and Sims, N. R. (2004) J. Neurosci. 24, 8019–8028
31. McMillan, A. M., and Weiner, M. W. (1976) J. Neurochem. 22, 199–221
32. Ostergaard, H., Tachibana, C., and Winther, J. R. (2004) J. Neurochem. 86, 337–345
33. Rigo, A. C., Vese, S., and Nicholls, D. G. (2001) Cell Death Differ. 8, 995–1003
34. Rigo, A. C., Ward, M. W., and Nicholls, D. G. (2001) J. Neurosci. 21, 1893–1901
35. Nicholls, D. G., and Ferguson, S. J. (2002) Bioenergetics 3, Academic Press Ltd., London
36. Iba, N., Jurma, O., Lalli, G., Liu, Y., Petrus, H. E., Greenamyere, J. T., Liu, R. M., Forman, H. J., and Andersen, J. K. (2000) J. Biol. Chem. 275, 26096–26101
37. Majima, E., Koihe, H., Hong, Y. M., Shinozaki, Y., and Terada, H. (1993) J. Biol. Chem. 268, 22181–22187
38. Scott, I. D., and Nicholls, D. G. (1980) Biochem. J. 186, 21–33
39. Berman, F. W., and Murray, T. F. (1999) J. Pharmacol. Exp. Ther. 299, 439–444
40. Budd, S. L., and Nicholls, D. G. (1996) J. Neurochem. 66, 403–411
41. Nicholls, D. G., and Chalmers, S. (2004) J. Bioenerg. Biomembr. 36, 277–281
42. Martensson, J., Lai, J. C., and Meister, A. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 7185–7189
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43. Wülner, U., Seyfried, J., Groscurth, P., Beinroth, S., Winter, S., Gleichmann, M., Heneka, M., Löschmann, P. A., Schulz, J. B., Weller, M., and Klockgether, T. (1999) *Brain Res.* **826**, 53–62

44. Hoek, J. B., and Rydstrom, J. (1988) *Biochem. J.* **254**, 1–10

45. Ashlund, F., Berndt, K. D., and Holmgren, A. (1997) *J. Biol. Chem.* **272**, 30780–30786

46. Kirkin, W. G., Cai, J., Thompson, S. A., Diaz, D., Kavanagh, T. J., and Jones, D. P. (1999) *Free Radic. Biol. Med.* **27**, 1208–1218

47. Nieminen, A. L., Byrne, A. M., Herman, B., and Lemasters, J. J. (1997) *Am. J. Physiol.* **272**, C1286–C1294

48. Klaidman, L. K., Mukherjee, S. K., and Adams, J. D., Jr. (2001) *Biochim. Biophys. Acta* **1525**, 136–148

49. Stevenson, D., Wokosin, D., Girkin, J., and Grant, M. H. (2002) *Toxicol. In Vitro* **16**, 609–619

50. Sebastia, J., Cristofol, R., Martin, M., Rodriguez-Farre, E., and Sanfeliu, C. (2003) *Cytometry* **51**, 16–25

51. Canals, S., Casarejos, M. J., De Bernardo, S., Rodriguez-Martín, E., and Mena, M. A. (2001) *J. Neurochem.* **79**, 1183–1195

52. Majima, E., Shinohara, Y., Yamaguchi, N., Hong, Y. M., and Terada, H. (1994) *Biochemistry* **33**, 9530–9536

53. Halestrap, A. P., and Brennerb, C. (2003) *Curr. Med. Chem.* **10**, 1507–1525

54. Koshkin, V., Bikopoulos, G., Chan, C. B., and Wheeler, M. B. (2004) *J. Biol. Chem.* **279**, 41368–41376

55. Toninello, A., Salvi, M., Schweizer, M., and Richter, C. (2004) *Free Radic. Biol. Med.* **37**, 1073–1080

56. Nicholls, D. G., and Budd, S. L. (2000) *Physiol. Rev.* **80**, 315–360

57. Kim, J. S., He, L. H., and Lemasters, J. J. (2003) *Biochem. Biophys. Res. Commun.* **304**, 463–470

58. Kokoszka, J. E., Waymire, K. G., Levy, S. E., Sligh, J. E., Cai, J. Y., Jones, D. P., MacGregor, G. R., and Wallace, D. C. (2004) *Nature* **427**, 461–465

59. Novelli, A., Reilly, J. A., Lysko, P. G., and Henneberry, R. C. (1988) *Brain Res.* **451**, 205–212

60. Kirkland, R. A., and Franklin, J. L. (2001) *J. Neurosci.* **21**, 1949–1963