Protection against Oxidant-Induced Apoptosis by Exogenous Glutathione in Leber Hereditary Optic Neuropathy Cybrids

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Purpose. To use different paradigms of oxidative and metabolic stress in a cellular model of Leber hereditary optic neuropathy (LHON), with the aim of evaluating the efficacy of potentially therapeutic molecules for the treatment of this disease.

Methods. Cybrids bearing one of the three most common LHON pathogenic mutations (11778/ND4, 3460/ND1, 14484/ND6) were incubated with two compounds known to induce oxidative injury, *tert*-butyl hydroperoxide (*t*-BH) and rotenone. To mimic metabolic stress, cells were incubated in a glucose-free medium containing galactose. Cell viability was determined using the MTT assay. To identify the apoptotic type of cell death, nuclear morphology was examined after cell loading with Hoechst. Cellular glutathione (GSH), and oxidized glutathione (GSSG) levels were measured enzymatically.

Results. Incubation with *t*-BH caused apoptotic cell death of control and LHON cybrids, whereas only LHON cybrids were damaged by rotenone concentrations up to 2.5 μM. Both types of stress caused a marked imbalance in the glutathione levels, but an increase in the GSSG/GSH+GSSG ratio was detected only after rotenone treatment. The efficacy of several antioxidant and antiapoptotic compounds was then assessed in cells exposed to these two oxidative paradigms. Only exogenous GSH remarkably protected the *t*-BH- and rotenone-treated cybrids from cell death. In contrast, GSH was unable to increase the viability of cybrids exposed to metabolic stress.

Conclusions. These results suggest that GSH is an effective antioxidant compound to be tested as a potential treatment for LHON. (*Invest Ophthalmol Vis Sci.* 2008;49:671–676) DOI: 10.1167/iovs.07-0880

Leber hereditary optic neuropathy (LHON) is a maternally inherited disease characterized by the selective loss of retinal ganglion cells (RGCs), leading to central vision loss and optic nerve atrophy.[1] Most patients with LHON carry one of three point mutations affecting positions 11778/ND4, 3460/ND1, and 14484/ND6 of mitochondrial DNA (mtDNA).[1] These mutations affect different subunits of complex I, the first site of the respiratory chain, though it is unclear how this dysfunction leads to LHON.[2]

Energetic failure is documented by the measurement of mitochondrial ATP synthesis driven by complex I substrates, which was substantially reduced in digitonin-permeabilized transmitochondrial cytoplasmic hybrid cells (cybrids) with LHON mutations.[3,4] Furthermore, ^{31}P-MRI studies in vivo demonstrated defective ATP production in skeletal muscle.[5] However, evidence also indicates that all LHON mutations may disrupt the interaction of complex I with its substrate ubiquinone, impairing the downstream flow of reducing electrons, which in turn causes a buildup of oxidized ubiquinone and an overproduction of reactive oxygen species (ROS).[6,7] Accordingly, mitochondrial ROS production was significantly enhanced in cybrids bearing LHON mutations,[8] and the antioxidant machinery was consistently altered, with glutathione peroxidase, glutathione reductase, and Mn-superoxide dismutase activities markedly reduced.[9] Overall, despite the large number of studies, the molecular mechanisms linking complex I defect to RGC degeneration are still poorly defined, and no truly effective therapy for LHON has yet been established.

Given the inability to directly investigate RGCs of LHON patients and the lack of animal models for this disease, a widely used approach to study the cellular phenotype of LHON mutations is the cybrid cell model.[10–12] Cybrids are obtained by fusing 143B.TK$^-$ osteosarcoma-derived rho(0) cells devoid of mtDNA, with cytoplasts produced by enucleation of cells derived from LHON patients or controls.[13] In this way, the mtDNA of donor cells can be studied in the context of a "neutral" nuclear background.

In the present study, we designed two models of oxidative stress, *tert*-butyl hydroperoxide (*t*-BH) and rotenone, in LHON cybrids with the three common pathogenic mtDNA mutations (11778/ND4, 3460/ND1, and 14484/ND6). *t*-BH is one of the most common prooxidant agents used to evaluate the effects of oxidative stress.[14] The complex I inhibitor rotenone has been reported to selectively increase O_2^- production within mitochondria in different cell lines.[15–17] In these two oxidative stress paradigms, we tested the efficacy of compounds able to buffer excessive ROS production or to increase respiratory activity and ATP synthesis. We also tested the same compounds in a model of metabolic stress consisting of incubation of LHON cybrids in a glucose-free medium containing galactose.

Materials and Methods

Materials

ATP, L-carnitine, creatine, decylubiquinone, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetra-zoliumbromid (MTT), N-acetylcysteine (NAC), *t*-BH, D-β-hydroxybutyrate, cyclosporin A, methyl succinate, GSH, rotenone, and vitamin E were purchased from Sigma (Milan, Italy). The ATP monitoring kit was from BioOrbit (Turku, Finland). Idebenone was a generous gift from Takeda Farmaceutici Italia (Catania, Italy).

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Cell Lines and Culture Conditions

In the present study, we used the parental human osteosarcoma cells (143B.TK$^-$), control cybrids (HGA) carrying wild-type mtDNA, and LHON cybrids carrying the three pathogenic mutations (11778/ND4, 3460/ND1, 14484/ND6) already characterized in our previous studies.[9,18,19] Cells were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum (FCS; South America source from Gibco, Invitrogen, Turin, Italy), 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.1 mg/mL bromodeoxyuridine in an incubator with a humidified atmosphere of 5% CO_2 at 37°C. For the experiments, 4×10^4 cells were seeded in 24-well dishes and were preincubated for 24 hours in DMEM, in the presence or absence of the various drugs, and then incubated for 16 hours with 250 μM *t*-BH or for 24 hours with 2.5 μM rotenone, unless otherwise indicated. In some experiments, cells were incubated for 24 hours (clones with 3460/ND1 and 14484/ND6 mutations) or 48 hours (clone with 11778/ND4 mutation) in glucose-free DMEM supplemented with 5 mM galactose, 5 mM Na-pyruvate, and 5% FCS (DMEM-galactose).

Cell Viability Measurement

The percentage of viable cells was measured with the colorimetric MTT assay, as previously described.[18] For cells incubated with NAC or GSH, the multiwell plates were centrifuged, and the growth medium was removed and replaced with the same medium without NAC or GSH before the addition of MTT.

Nuclear Morphology

Nuclei were visualized with a digital imaging system after loading cells with 1 μg/mL Hoechst for 30 minutes at 37°C.[19]

Measurement of GSH and GSSG

Cells ($5–6 \times 10^6$) grown in 10-cm diameter dishes were treated with t-BH or rotenone, as described, and cellular GSH and GSSG levels were measured exactly as previously described.[9]

Statistical Analysis

Data were expressed as mean ± SEM and were analyzed using the Student's *t*-test, one-way ANOVA, and Bonferroni post hoc multiple comparison test. Only values of $P < 0.05$ were considered significant.

Results

Viability of Cells Treated with *t*-BH or Rotenone

Figure 1A shows the dose–response effect of t-BH on the viability of parental 143B-TK$^-$ cells, control, and LHON cybrids. At a concentration as low as 50 μM, *t*-BH induced a marked loss of viability in parental 143B.TK$^-$ cells, whereas control and LHON cybrids were only slightly affected (less than 20% of cells were nonviable). In cybrids, the effect of *t*-BH was dose dependent and was maximal at 250 μM (Fig. 1A) and after 16 hours of incubation (not shown). Incubation with up to 1 μM rotenone significantly reduced the viability of LHON cybrids (approximately 50% of cells were nonviable) but not of control cybrids or of the parental 143B-TK$^-$ cell line (Fig. 1B). By increasing rotenone concentrations up to 5 μM, the viability of 143B-TK$^-$ cells and control cybrids decreased, which enabled us to approach values similar to those observed in LHON cybrids at the same concentration. Analysis of nuclear morphology of *t*-BH- and rotenone-treated LHON cybrids revealed a pattern of chromatin condensation and fragmentation suggestive of an apoptotic type of cell death (Figs. 1C, 1D).

Glutathione Content

Glutathione plays many roles in the cell. It has important functions as an antioxidant and is a transport and storage form of cysteine. It also plays an essential role in the maintenance of intracellular protein thiols in a reduced state and in scavenging H_2O_2 in the reaction catalyzed by glutathione peroxidase.[20] Under resting conditions, the GSH levels of all the cells were not significantly different, though a slight reduction was apparent in parental 143B-TK$^-$ cells and cybrids with the 3460/ND1 mutation (Fig. 2A). The product of GSH oxidation, glutathione

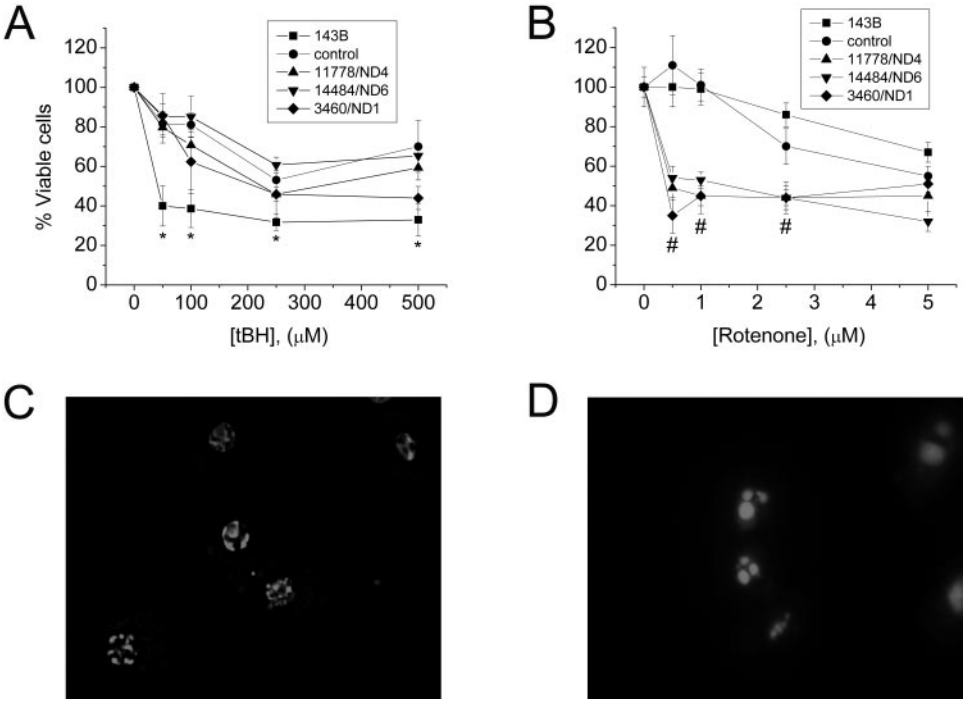

Figure 1. Effect of *t*-BH and rotenone on parental 143B.TK$^-$ cells, control, LHON cybrid viability, and nuclear morphology. Cells were incubated for 16 hours in DMEM-glucose containing different concentrations of *t*-BH (**A**, **C**) or for 24 hours with different concentrations of rotenone (**B**, **D**). Cell viability was determined with the MTT assay. Data represent mean ± SEM of at least three determinations. *Significant difference in the comparison of 143B.TK$^-$ cells with all other cybrids ($P < 0.05$). #Significant difference in the comparison of all LHON cybrids with 143B.TK$^-$ cells and control cybrids. Statistical evaluation was carried out using the one-way ANOVA and Bonferroni post hoc multiple comparison test. Nuclear morphology was determined by Hoechst staining (**C**, **D**). Representative images are shown.

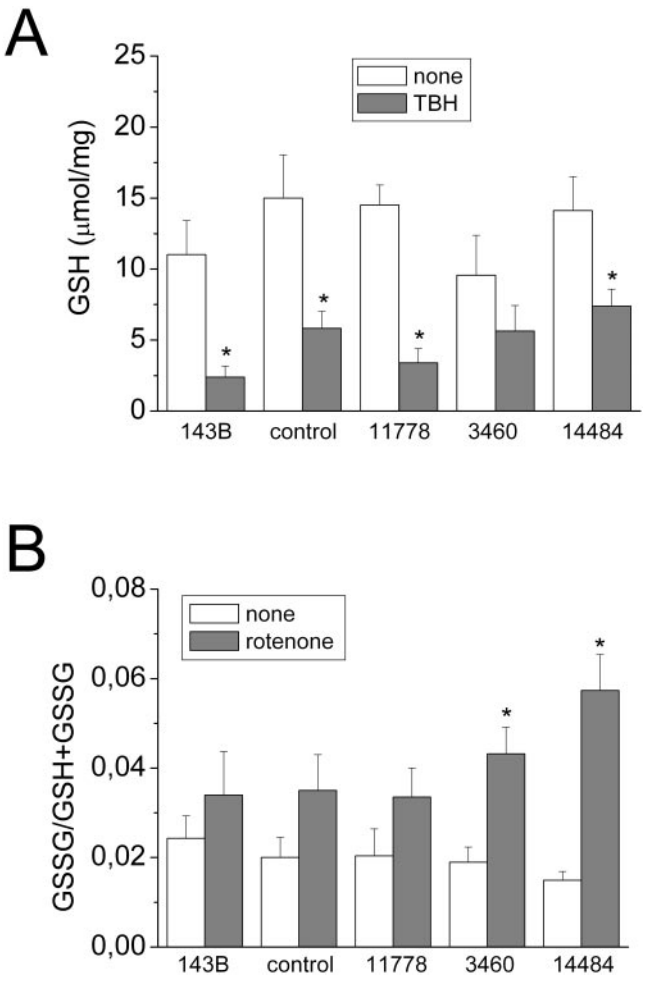

FIGURE 2. GSH depletion in parental 143B.TK$^-$ cells, control, and LHON cybrids treated with *t*-BH and rotenone. Cells were incubated for 16 hours with 250 μM *t*-BH (**A**) or for 24 hours with 2.5 μM rotenone (**B**) in DMEM-glucose, and GSH and GSSG were determined as previously described.[9] GSH is expressed as nmol/mg protein and represents the mean ± SEM of at least three determinations. Values of the GSSG/GSH+GSSG ratio are also mean ± SEM of at least three determinations. *Values significantly different from untreated cells ($P < 0.05$).

disulfide (GSSG), was also detected in similar amount in all cell lines, in the range of 0.15 to 0.2 nmol/mg protein. This finding is in agreement with previous reports.[9] Incubation with 250 μM *t*-BH caused a significant reduction in GSH content in all cell lines except cybrids with the 3460/ND1 mutation (Fig. 2A). However, the decrease in GSH content was not associated with a simultaneous increase in cellular GSSG, whose level was below that of the method detection limit. The possibility that GSSG might be extruded from the cells was not tested because of its high dilution in the extracellular medium. On the contrary, the decrease in GSH levels induced by incubation with rotenone was associated with an increase in GSSG, resulting in a significant elevation of the GSSG/GSH+GSSG ratio in cybrids with 3460 and 14484 LHON mutations (Fig. 2B).

Effect of Antioxidant Compounds

We then evaluated whether various antioxidant compounds were able to counteract the toxic effects of the two oxidative stress paradigms. CoQ_{10} and its short chain derivative, idebenone, together with decylubiquinone, are used in clinical therapy for LHON patients. The aims are to restore electron flow through the respiratory chain downstream of complex I and to increase the overall antioxidant defenses.[21] None of these quinone derivatives was able to ameliorate the viability of control and LHON cybrids treated with *t*-BH (Fig. 3A) or rotenone (Fig. 3B). These antioxidant molecules may be distributed throughout the cell membranes; only a small portion accumulated within the mitochondria, where ROS overproduction should be preferentially localized, certainly after rotenone exposure. Mitochondrial-targeted antioxidants have been recently developed by covalently coupling the antioxidant moieties to the lipophilic cation triphenyl phosphonium. Mitoquinone (MitoQ; Antipodean Pharmaceuticals), one such mitochondrial-targeted antioxidant, has been shown to efficiently reach mitochondria,[22] affording effective antioxidant protection in fibroblasts derived from patients with Friedreich ataxia.[23] However, as illustrated in Figure 3A-B, mitoquinone (MitoQ; Antipodean Pharmaceuticals) was also unable to protect LHON cybrids from cell death induced by *t*-BH or rotenone. We then evaluated the effect of vitamin E (α-tocopherol), of exogenous GSH, and of the precursor of GSH synthesis, NAC. Figure 3C shows that NAC and vitamin E had no effect, whereas GSH remarkably protected all cell lines from death induced by *t*-BH, except those bearing the 3460/ND1 mutation. GSH was also the only compound able to increase the viability of rotenone-treated cybrids with the 3460/ND1 and 14484/ND6 LHON mutations but not with the 11778/ND4 mutation (Fig. 3D).

Cell Viability in Galactose Medium

We have previously reported that LHON cybrids undergo apoptotic cell death when incubated in glucose-free medium containing galactose and hence are forced to use oxidative phosphorylation. Under these conditions, the parental 143B.TK$^-$ cells and control cybrids remained viable, whereas LHON cybrids carrying the 3460/ND1 and 14484/ND6 mutations exhibited an approximate loss of 40% in viability after 24 hours and cybrids with the 11778/ND4 mutation after 48 hours.[18] In the very same LHON cybrid clones, Floreani et al.[9] reported a marked decrease in MnSOD and glutathione reductase activities after incubation in galactose medium. We decided, therefore, to test whether the viability of LHON cybrids in galactose medium could be improved by the same set of antioxidant molecules—CoQ_{10}, idebenone and decylubiquinone, vitamin E, NAC, and GSH. Figures 4A and 4B show that neither of these compounds was able to enhance the survival of LHON cybrids.

Supplementation of cells with substrates theoretically able to fuel electrons downstream of complex I, such as D-β-hydroxybutyrate[24] and the membrane-permeable succinate derivative dimethyl-succinate,[25] has been equally unsuccessful. Compounds capable of improving the mitochondrial energetic metabolism, such as creatine[26] and L-carnitine,[27] were also ineffective (Fig. 4C). Similarly, the antiapoptotic drugs cyclosporin A[28] and brimonidine[29,30] were unable to enhance cell survival (Fig. 4D).

DISCUSSION

This study is the first systematic evaluation of potentially therapeutic molecules for the treatment of LHON using different paradigms of stress on cybrids bearing the most common pathogenic mtDNA mutations as a model. When exposed to exogenous or mitochondrially generated oxidative injury, LHON cybrids underwent a remarkable loss of cell viability and showed prevalently apoptotic cell death. Based on the current knowledge of cellular dysfunction induced by the most com-

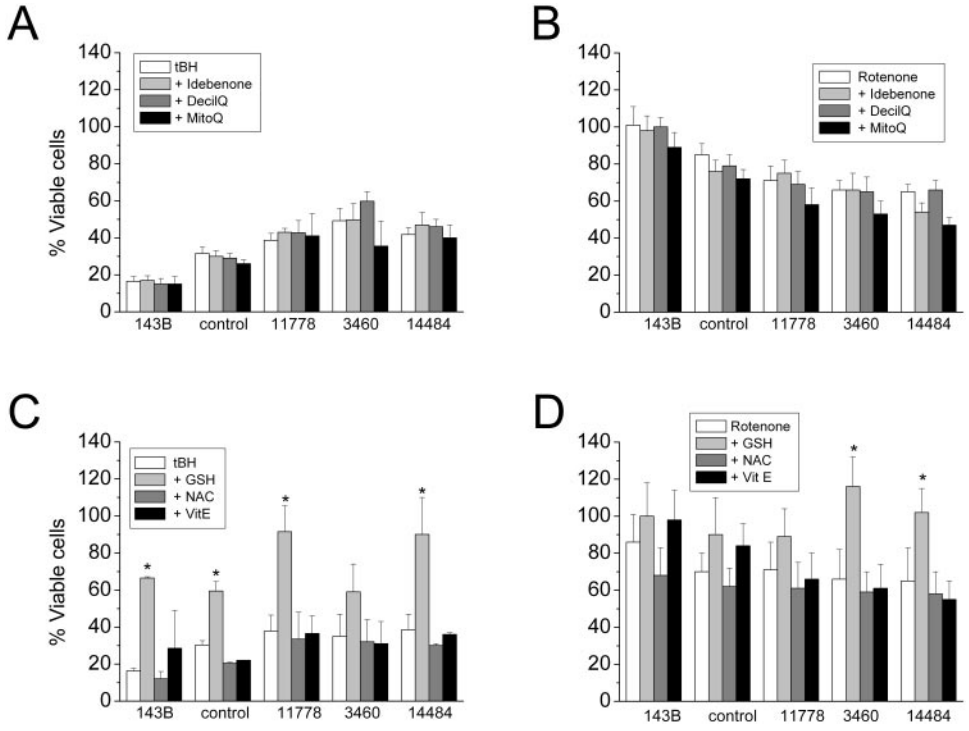

FIGURE 3. Effect of antioxidants on viability of the parental 143B-TK$^-$ cells, control, and LHON cybrids treated with *t*-BH or rotenone. Cells were preincubated for 24 hours with 1 μM idebenone, 1 μM decylubiquinone (decylQ), or 0.1 μM mitoquinone (**A**, **B**) or 10 mM GSH, 10 mM NAC, or 20 μM vitamin E (**C**, **D**). After that, 250 μM *t*-BH (16 hours; **A**, **C**) or 2.5 μM rotenone (24 hours; **B**, **D**) was added. Data represent mean ± SEM of at least three determinations. *Significantly different from *t*-BH or rotenone alone ($P < 0.05$).

mon LHON mutations, the analysis of a wide range of compounds theoretically capable of counteracting the loss of viability of LHON cybrids proved disappointing. GSH was the only compound able to promote a significant increase of cell viability after incubation with both *t*-BH and rotenone. GSH was, however, unable to improve the survival of LHON cells exposed to the metabolic stress represented by incubation in galactose medium.

The oxidative imbalance induced by the complex I inhibitor rotenone led to a significant loss of viability in LHON cybrids but not in control cybrids or in the parental 143B.TK$^-$ cell line. These findings are consistent with previous findings showing an increased sensitivity of cybrids with the 11778/ND4 mutation to oxidative stress provoked by H_2O_2 treatment.[28] In fact, it has been proposed that pharmacologic or genetic complex I inhibition increases the soluble pool of cytochrome *c* in the intermembrane space, possibly through enhanced cardiolipin peroxidation,[31] thus making cells more prone to undergo apoptosis.[18,32] In the presence of rotenone, a nonsignificant tendency toward an increased GSSG/GSSG+GSH ratio was observed in all cell lines that reached statistical significance for the LHON cybrids carrying the 3460/ND1 and 14484/ND6 mutations. Accordingly, exogenous GSH markedly enhanced the viability of these two cybrid cell lines when treated with

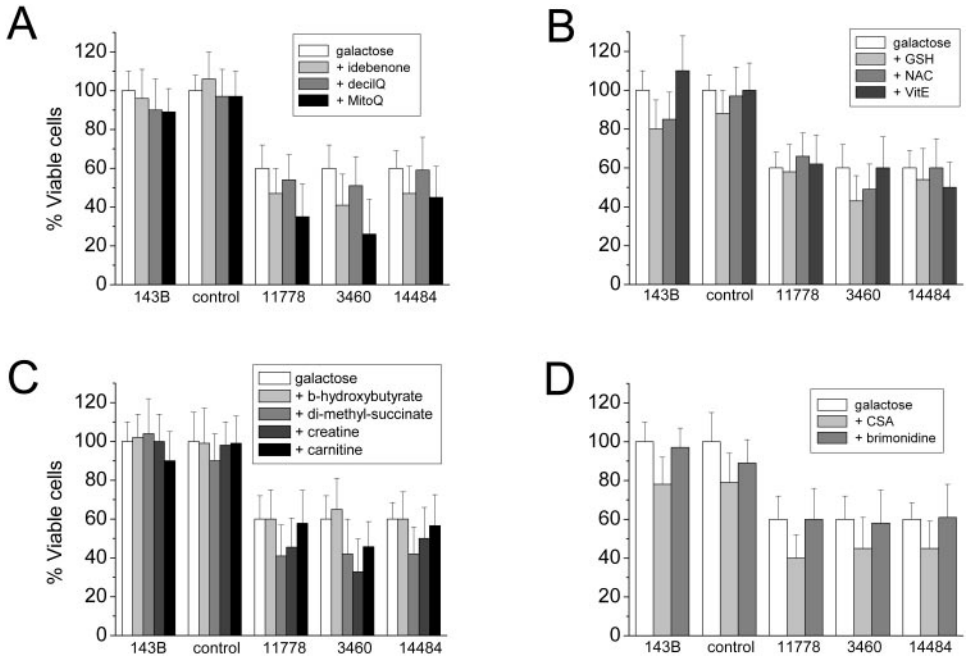

FIGURE 4. Effect of antioxidants on viability of parental 143B.TK$^-$ cells, control, and LHON cybrids incubated in galactose medium. Cells were preincubated for 24 hours in DMEM-glucose with 1 μM idebenone, 1 μM decylubiquinone (decylQ), or 0.1 μM mitoquinone (**A**); with 10 mM GSH, 10 mM NAC, or 20 μM vitamin E (**B**); 4 mM D-β-hydroxybutyrate, 1.5 mM creatine, 1.5 mM L-carnitine, or 10 mM dimethylsuccinate (**C**); or 1 μM cyclosporin A (CSA) or 10 μM brimonidine (**D**). Cells were then incubated in DMEM-galactose medium with the same compounds for 24 hours (clones with 3460/ND1 and 14484/ND6 mutations) or 48 hours (clone with 11778/ND4 mutation). Data represent mean ± SEM of at least three determinations.

the complex I inhibitor. The GSSG/GSH+GSSG ratio of cybrids bearing the 11778/ND4 LHON mutation was increased by rotenone similar to the increase in control cybrids and parental 143B-TK$^-$ cells, and exogenous GSH was unable to increase the survival of all these cell lines. This finding cannot be easily explained given that the 11778/ND4 LHON mutation is frequently associated with a severe phenotype, characterized by a reduced rate of spontaneous recovery of visual function. A possible explanation for these discrepancies may have to do with the variable biochemical signatures of the different LHON mutations.[2]

Conversely, we found that the oxidative imbalance induced by *t*-BH affected control and LHON cybrids similarly, with the parental cell line 143B.TK$^-$ even more sensitive than the cybrids to low concentrations of *t*-BH. This latter result could be related to the slightly reduced content of GSH (seen also in the present study) and the poor glutathione peroxidase activity previously reported in the 143B.TK$^-$ cell line.[33] The fact that the observed decrease in GSH content by *t*-BH was not associated with an increase in GSSG levels might be a consequence of the extrusion of excess GSSG into the extracellular medium, where it could act as a powerful prooxidant molecule.[34]

The different effects of *t*-BH and rotenone on cellular GSH content is not easily explained. However, this difference may be related to the chemical reaction of the two compounds. In fact, based on current knowledge, it is likely that *t*-BH and rotenone act at different intracellular compartments. *t*-BH has been reported to generate peroxy radicals in the cytosol through its reaction with ferrous iron, similar to the Fenton reaction,[35] whereas rotenone is known to act at the mitochondrial respiratory chain, particularly at the complex I site, generating superoxide anions.[15–17]

Another noticeable observation is the different biochemical behavior of the LHON cell line bearing the 3460/ND1 mutation. The GSH content of these latter cells was less severely decreased by *t*-BH treatment than other LHON cybrids, and adding exogenous GSH could not counteract *t*-BH–induced cell death. Although the GSH content of control and LHON cybrids was similar, a nonsignificant tendency toward a reduction was observed in cells with the 3460/ND1 mutation. This is in agreement with previous findings showing that cybrids with this mutation exhibited the highest basal ROS production[8] and an inefficient antioxidant machinery.[9] The 3460/ND1 LHON mutation consistently reduces complex I activity[36] and is coupled to a severe biochemical phenotype.[2] Our results confirm that the 3460/ND1 LHON mutation is associated with chronic impairment of the antioxidant machinery, at least in the cybrid cell model.

Considering the other compound tested in this study, NAC, the metabolic precursor of GSH, was completely ineffective at preventing *t*-BH- and rotenone-induced cell death. It is likely that the pathway for de novo synthesis of GSH was too slow for compensation in our experimental conditions, possibly because of energetic impairment or because of the inhibition of the two ATP-consuming enzymes involved in the process, γ-glutamylcysteine synthetase and glutathione synthetase.

Remarkably, all the other antioxidant molecules tested in the present study were ineffective, even mitoquinone (MitoQ; Antipodean Pharmaceuticals), the quinone analogue specifically targeted to mitochondria. The lack of effect of mitoquinone (MitoQ; Antipodean Pharmaceuticals) on rotenone-treated cells is surprising because it has been predicted that this antioxidant molecule is inserted within the hydrophobic core of the inner mitochondrial membrane, close to the site of ROS production.[22] Nevertheless, our results are in agreement with previous reports on fibroblasts that showed mitoquinone (MitoQ; Antipodean Pharmaceuticals) was unable to reduce rotenone-induced mitochondrial O_2^- overproduction. Although its exact site of action remains to be defined, it has been suggested that mitoquinone (MitoQ; Antipodean Pharmaceuticals) exhibits its effect downstream of O_2^- formation, reacting mainly with lipid peroxidation products.[17]

Supplementation with exogenous GSH was unable to protect LHON cybrids from death induced by metabolic stress. This finding supports the notion that ROS production is not a major determinant for cell death in galactose medium, in agreement with previous data showing that the increase in GSSG measured in LHON cybrids under these conditions was associated with an enhanced amount of GSH.[9] According to this study, LHON cybrids increase GSH synthesis to counteract the overproduction of ROS in galactose medium, though it remains unclear how cells with a significant ATP depletion[19] may sustain de novo GSH synthesis. In addition, compounds theoretically able to improve the mitochondrial energetic metabolism, such as cell-permeant succinate, β-hydroxybutyrate, creatine, and carnitine, were unable to promote LHON cybrid survival in galactose medium, indicating that they cannot circumvent the complex I dysfunction. The finding that cyclosporin A was also ineffective in protecting against galactose-induced cell death of LHON cybrids suggests that the opening of the permeability transition pore is not involved in this type of cell death. It must be taken into consideration that the inefficacy of all these compounds might depend on the drastic metabolic conditions established in cells by the galactose model, which may not necessarily match what occurs in vivo during the acute phase of the disease.

The question arises as to whether a glutathione imbalance could be involved in LHON in vivo, considering that even moderate GSH depletion impairs neuronal ATP supply when energy demand is increased,[37] as can occur, for example, in the unmyelinated section of RGC axons, which strongly depend on oxidative ATP synthesis to drive their Na^+/K^+-ATPase.[2] On the other hand, the ATP-consuming de novo GSH synthesis might not take place under conditions of dramatic energy failure, as is suggested to occur in NT2-differentiated cybrids,[38] indicating a close interplay between energetic derangement and oxidative stress.

We conclude that, based on the data presented in this study, GSH and possibly other novel, related antioxidant compounds may represent new pharmacologic tools to mitigate complex I–related neurodegeneration originating from an oxidative injury. Although the oral bioavailability of GSH has been reported to be negligible in humans,[39] recently dietary supplementation with a whey-based product has been reported to increase glutathione levels in blood cells of patients with cystic fibrosis[40] and HIV.[41] A larger long-term trial is warranted to evaluate whether this positive influence on GSH metabolism translates into a more favorable course of these latter diseases. In addition, it is reasonable to propose such a clinical development for LHON, in which topical application of GSH to the eye may be a possible therapy. These considerations may be true not only for LHON but also for other complex I–related disorders, such as Parkinson disease and hereditary spastic paraplegia caused by paraplegin mutations.

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