The Mitochondrial Complex I Inhibitor Rotenone Induces Endoplasmic Reticulum Stress and Activation of GSK-3β in Cultured Rat Retinal Cells

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PURPOSE. Both mitochondrial dysfunction and endoplasmic reticulum (ER) stress have been implicated in the pathogenesis of neurodegenerative disorders. The purpose of the present study was to investigate the mechanisms of cellular damage resulting from mitochondrial dysfunction induced in cultured retinal cells and, in particular, whether ER stress plays a role in this response.

METHODS. Mixed retinal cell cultures containing neurons and glia were prepared from neonatal rats. The complex I inhibitor rotenone was used to induce mitochondrial dysfunction. Immunocytochemistry, Western blotting, and assays for reactive oxygen species (ROS), adenosine triphosphate (ATP), and TdT-dUTP terminal nick-end labeling (TUNEL) were used as standard to elucidate cellular responses.

RESULTS. Neurons were more rapidly affected by rotenone (1 μM) than glial cells, with significant loss of these cells appearing by 6 hours after application. Glioblastoma death was apparent by 24 hours and was associated with positive nuclear labeling by TUNEL. All cell loss was associated with increased ROS production, but neuronal loss was also concurrent with a significant depletion in cellular ATP. Increased expression of the characteristic ER stress components immunoglobulin heavy-chain binding protein (BiP), ATF-4 (activating transcription factor 4), phospho-PERK (pancreatic endoplasmic reticulum kinase/PKR-like endoplasmic reticulum kinase), and growth arrest and DNA damage-inducible protein/C/EBP homologous protein (CHOP) was evident in the rotenone-treated cultures after 6 hours in glial cells. Furthermore, inhibition of glycogen synthase kinase-3β (GSK-3β) with LiCl was able to protect glia cells, whereas inhibition of the calpain with calpain inhibitor III protected only neurons.

CONCLUSIONS. These data together demonstrate that a mitochondrial dysfunction induced in retinal cells can give rise to pathology via a variety of mechanisms including ATP depletion, ROS elevation, ER stress, or activation of GSK-3β or calpain. Such mechanisms predominantly depend upon the concentration and duration of mitochondrial challenge and the type of cell affected.

Keywords: rotenone, GSK-3β, ER stress, calpain, ATP

Mitochondrial dysfunction has been implicated in the pathogenesis of several different central nervous system diseases, including neurodegenerating conditions in the retina such as Leber’s hereditary optic neuropathy (LHON)1,2 and glaucoma.3,4 Mitochondrial impairment to preparations of retinal cells is known to cause death to both neurons and glia, with the latter cell type dying in a manner characteristic of apoptosis.5 Rotenone is a plant-derived ketonic pesticide that acts as a mitochondrial complex I (NADH-quinone reductase) inhibitor and has therefore been used to experimentally induce cellular metabolic dysfunction. In this way rotenone has been used to experimentally model Parkinson’s disease (PD).6,7 LHON,8 and metabolic compromise in the retina.9 Rotenone-induced toxicity is also associated with oxidative stress10 and adenosine triphosphate (ATP) reduction.5 However, in some instances, cell death occurs before these stressors are evident, suggesting that other cell death triggers are involved.10,11 It has also been shown that rotenone application can cause endoplasmic reticulum (ER) stress in certain cells.12,13 The ER is responsible for many cellular processes, such as synthesis, folding, and transport of proteins, in order to ensure their correct functioning.1,14 Endoplasmic reticulum stress refers to a situation whereby any one of the numerous processes of this organelle malfunctions as a result of a perturbation in cellular homeostasis, for example, in calcium dynamics.14 This can have profound cellular implications; failure in protein folding and targeting, for example, leads to expression of ER-localized chaperones (BiP) and proapoptotic transcription factors.15 This can directly lead a cell toward apoptosis.16 We have recently shown that mitochondrial impairment in mixed rat retinal cultures does disrupt intracellular calcium homeostasis.3 We
therefore sought to determine whether ER stress plays a role in the retinal toxicity seen after rotenone application.

**MATERIALS AND METHODS**

**Materials**

Cell culture media and fetal bovine serum (FBS) were purchased from Invitrogen (Mulgrave, Victoria, Australia). Unless stated, all other chemical reagents were from Sigma-Aldrich (Castle Hill, New South Wales, Australia). Antibodies used for Western blotting and immunocytochemistry are described in the Table. Culture plates and plastic ware were from Sarstedt Pty Ltd (Adelaide, South Australia, Australia). Animals for culture were obtained from the University of Adelaide.

**Rat Retinal Cell Cultures**

This research was approved by both the University of Adelaide (M-2011-070) and the Animal Ethics Committees of SA Pathology and conformed to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, 7th edition (2004), as well as the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Rat retinal cell cultures comprising both neurons and glia were prepared using a trypsin–mechanical digest procedure previously described.17,18 Briefly, retinas were enucleated from 1- to 2-day-old rat pups and incubated in physiological buffer (solution medium; 120 mM NaCl, 5.4 mM KCl, 24 mM NaHCO3, 0.1 mM NaH2PO4, 3 g/L BSA, 20 mM glucose, and 0.15 mM MgSO4, 28 μM phenol red) containing 0.1 mg/mL trypsin (Sigma-Aldrich) at 37°C for 8 minutes. After the reaction was stopped, cells were resuspended in minimal essential medium (MEM) containing 10% FBS, 10 mg/mL gentamicin sulfate, 200 μM glutamine, and 25 mM glucose and applied to 13-mm-diameter borosilicate glass coverslips (immunocytochemistry), 6-well plates (Western blot), or 12-well plates (ATP assay and reactive oxygen species determination), all of which had previously been coated with 10 μg/mL poly-D-lysine, for 2 hours. Cells were maintained in saturating humidity with 5% CO2 at 37°C and were used 7 days after culture. Some cultures were maintained for up to 28 days with medium being changed every 2 days. These cultures were identified as comprising predominantly Müller glial cells as shown previously.19

**Treatment of Cultured Rat Retinal Cells**

For mitochondrial inhibition, cultures were incubated for either 2, 6, or 24 hours in media containing rotenone (100 nM, 1 μM) and thapsigargin (100 nM, 1 μM). In some instances, when experiments were performed to determine potential neuroprotective effect, test compounds were applied to cultures 1 hour before rotenone to allow sufficient time for target interaction before induction of mitochondrial impairment.

**Immunocytochemistry**

Cells were fixed with neutral buffered formalin for 15 minutes, washed in phosphate-buffered saline (PBS; 137 mM NaCl, 5.4 mM KCl, 1.28 mM Na2HPO4, 7 mM NaH2PO4; pH 7.4) and then permeabilized with PBS containing 0.1% Triton X-100 (PBS-T). Preparations then underwent blocking in normal horse serum (NHS; 3.3% vol/vol in PBS; PBS-HS) and were subsequently labeled with a range of neuron- and glia-specific antibodies, diluted in PBS-HS, at 4°C overnight. Labeling was visualized by consecutive incubations with appropriate biotinylated second antibodies (Vector Laboratories, ALS, Brisbane, Queensland,

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**Table. Antibodies Used in This Research**

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* Clone number, as indicated in column heading. ICC, immunocytochemistry; WB, Western blotting; CST, Cell Signaling Technology.
Rotenone Induces ER Stress and Activates GSK-3β

in the cultures (e.g., Tau, GABA, and PGP9.5 for neurons; glial fibrillary acidic protein [GFAP] for astrocytes; vimentin for

with 50 μL ATP assay mix reagent (FLAA) containing luciferin and luciferase. After incubation at room temperature for 10 minutes, luminescence was assessed by luminometry (Fluostar Optima; BMG Labtech, Mornington, Victoria, Australia). The ATP levels were compared to a standard curve in each measurement for actual quantification. Data were collected from four independent experiments and related to total protein content as assessed according to the method of Bradford. 21

TdT-dUTP Terminal Nick-End Labeling (TUNEL)

For the TUNEL procedure, treated cells on coverslips were fixed as described for immunocytochemistry, washed in PBS-T for 10 minutes, and immersed in PBS. The labeling procedure was carried out as described previously. 5 Briefly, samples were rinsed in Tris buffer (10 mM Tris-HCl, pH 8.0) for 5 minutes and then equilibrated in TdT buffer (30 mM Tris-HCl, pH 7.2 containing 140 mM sodium cacodylate, 1 mM cobalt chloride) for 10 minutes. After that, the cells were incubated in the same buffer containing TdT (0.15 U/mL; Promega, Madison, WI, USA) and biotin-16-dUTP (10 mM; Roche, Castle Hill, New South Wales, Australia) for 60 minutes at 37°C. The reaction was finished by two washes in saline sodium citrate solution (30 mM sodium citrate containing 300 mM NaCl) for 15 minutes. The sections were rinsed in PBS for 5 minutes, and nonspecific binding sites were blocked by washing in PBS containing 2% BSA for 10 minutes. Finally, cell labeling was visualized using streptavidin Alexa Fluor 594 (1:1000; Molecular Probes, Eugene, OR, USA) containing 2% BSA for 30 minutes at 37°C. In order to quantify the numbers of TUNEL-labeled nuclei, counts were obtained and averaged from five different randomly selected fields of at least four coverslips from six separate cultures.

Cell Viability Assay

To determine the total dead (necrotic, apoptotic) cells, a Live/Dead assay kit was employed (Invitrogen). In this assay, ethidium homodimer (EthD-1) was used to produce fluorescence in dead cells. After treatment, adherent sample cells on glass coverslips were washed with PBS and then incubated as described in the kit protocol for 30 minutes at room temperature. The resulting fluorescence was determined using a fluorescence microplate reader (Fluostar Optima; BMG Labtech; excitation 485–530 nm, emission 530–645 nm). The percentage of dead cells was calculated from the fluorescence readings. Counts were obtained and quantified from four separate experiments in triplicate.

Statistical Analysis

Experiments were carried out with \( n = 4 \) for the Western blotting densitometry and \( n = 6 \) to 8 for immunohistochemistry. Data were analyzed for significance using a one-way ANOVA followed by a post hoc Tukey multiple-comparison test with commercially available statistical software (Stata/IC 12.1; Stata, College Station, TX, USA); \( P < 0.05 \) was considered significant. Data are expressed as mean ± standard error of the mean (SEM).

RESULTS

Cell Culture

After 7 days in vitro, different types of neurons and/or glia could be disseminated by immunofluorescence labeling (Figs. 1A–I) in the cultures (e.g., Tau, GABA, and PGP9.5 for neurons; glial fibrillary acidic protein [GFAP] for astrocytes; vimentin for

Australia; 1:250 in PBS-HS, 1 hour); nuclei were counterstained using 4',6-diamidino-2-phenylindole (DAPI; 500 ng/mL in PBS, 5 minutes). Finally, cells on coverslips were mounted using antifade mounting medium (Dako, Botany, New South Wales, Australia) and examined under a confocal fluorescence microscope (Olympus, Mount Waverly, Victoria, Australia).

Quantification of γ-aminobutyric acid (GABA)-immunoreactive (-IR) neurons was performed by manually counting positively labeled cells on five different randomly chosen fields per coverslip from four to six independent experiments. Vimentin-IR glial cells and Tau-IR neurons were quantified by using ImageJ Software (http://rsb.info.nih.gov/ij/index.html; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA).

Western Blotting

Cells were harvested from plates by scraping into PBS; samples were then sonicated in homogenization buffer (20 mM Tris-HCl, pH 7.4, containing 2 mM EDTA, 0.5 mM EGTA, 1 mM dithiothreitol, 50 μg/mL leupeptin, 50 μg/mL pepstatin A, 10 μg/mL aprotinin, and 0.1 mM phenylmethylsulfonyl fluoride). An equal volume of sample buffer (62.5 mM Tris-HCl, pH 7.4, containing 4% wt/vol SDS, 10% vol/vol glycerol, 10% vol/vol β-mercaptoethanol, and 0.002% wt/vol bromophenol blue) was added, and samples were heated to 70°C for 3 minutes. Electrophoresis was performed as reported previously 8 using 10% or 12% polyacrylamide gels (as appropriate) containing 0.1% (wt/vol) SDS. Proteins were transferred to polyvinylidene difluoride membrane, and blots were subsequently labeled with antibodies diluted as described in the Table. Membrane labeling was carried out with a two-step procedure using appropriate biotinylated secondary antibodies (Vector Laboratories, AL: 1:500, 30 minutes) followed by a streptavidin–Alexa Fluor 594 (1:1000; Molecular Probes, Eugene, OR, USA) reaction as described previously. 20 β-actin was assessed in cell extracts as a positive control, and quantification was performed using Adobe Photoshop, version CS2 (Adobe, Australia, Sydney, New South Wales, Australia).
FIGURE 1. The effect of 6- and 24-hour incubations with rotenone (1 μM) on 7-day-old rat retinal cultures, as demonstrated by immunocytochemistry and TUNEL. Five antibodies were used to assess the influence on three different neuronal and two different glial markers: (A–C) GABA (neurons; red) and PGP9.5 (amacrine; green); (D–F) Tau (neurons; green) and GFAP (astrocytes; red); (G–I) vimentin (Müller glial cells; green) and TUNEL (apoptotic cells; red). Control labeling is seen for the requisite antibodies in (A, D, G), and the effects of increasing incubation time of rotenone (1 μM for 6 hours in [B, E, H]; 1 μM for 24 hours in [C, F, I]) are also shown. Quantification of representative neuronal and glial cell...
Müller glial cells). In contrast, after 28 days in culture with changing of medium every 2 days, predominant vimentin-positive Müller cells could be identified; no neurons were observed (data not shown; see Wood et al. 17).

Effect of Rotenone on the Viability of Retinal Neurons and Glia

Rat retinal cell cultures were exposed to rotenone (100 nM, 1 μM), and cell viability was assessed after 6 and 24 hours by immunocytochemistry as described above (Figs. 1J–M). Rotenone produced no obvious toxicity at the concentration of 100 nM when applied for 6 hours. In contrast, there was a significant loss of GABA-IR, PGP9.5-IR, and Tau-IR neurons but not vimentin-IR glial cells after treatment with 1 μM rotenone for 6 hours (Figs. 1J–M). After 24 hours, however, 1 μM rotenone had significantly reduced numbers of both neurons and glia (Fig. 1M).

Modes of Cell Death Associated With Rotenone Treatment

After 6-hour treatment of 7-day retinal cultures with 1 μM rotenone, cell death by high-affinity nucleic acid stain as determined by enhanced EthD-1 labeling of cells, and by apoptosis as shown by the increased presence of TUNEL-positive nuclei (Figs. 1, 2), was evident. The apoptotic cells were almost exclusively revealed to be glia (Figs. 1G–I). The reductions in numbers of neurons but not glia at this time point are in agreement with glial death via the relatively slow

**FIGURE 2.** Temporal characterization of cell viability, free radical levels, and metabolic alteration following treatment of 7-day-old retinal cultures with rotenone (1 μM). (A) The viability of cells was characterized by TUNEL staining (for apoptosis) and by (B) EthD-1 staining (dead cells). Here, 7-day-old rat retinal cultures were incubated with rotenone (100 nM and 1 μM) and thapsigargin (100 nM and 1 μM) for 0, 2, 6, and 24 hours. Cell death was measured by TUNEL and EthD-1 assays as described in Methods. (C) Quantitative analysis of mitochondrial ROS production was assessed with DHE (expressed as fluorophore dihydroethidium units/μg cell protein). Cell culture was incubated with rotenone (100 nM and 1 μM) as indicated. (D) Intracellular concentrations of ATP. Data are expressed as percentages of untreated (vehicle-treated) control cells. Values represent mean ± SEM in separate experiments (n = 3–5). *P < 0.05, **P < 0.01 compared to control cultures. Scale bar: 20 μm.
process of apoptosis and neuron death via more rapid nonapoptotic processes. Apoptosis became more prevalent either as the incubation time was increased to 24 hours or when glial cells dominated the cultures (28-day mixed retinal cell cultures).

Rotenone-Stimulated Reactive Oxygen Species Production

Rotenone treatment (1 μM) led to a time-dependent decrease in the level of cellular ATP in 7-day retinal cultures (Fig. 2D). The ATP levels started to decrease after incubation with rotenone for 2 hours and then dropped to 69% at 6 hours. In contrast, in the Müller cell-enriched 28-day-old cultures, glia cells were relatively more resistant to rotenone and maintained their ATP level at 87% of the untreated value at 24 hours.

Rotenone Induces CHOP Expression in Glial Cells

Since previous research has shown that mitochondrial disease and rotenone treatment can increase ER stress marker transcripts,22 we aimed to determine whether rotenone could induce ER stress-related protein expression in rat retinal cell cultures. Rat retinal cultures treated with 1 μM rotenone for 6 hours showed significant growth arrest and DNA damage-inducible protein/C/EBP homologous protein (CHOP) expression compared to untreated cells (data not shown). In the 28-day glial-only cultures, treatment with 1 μM rotenone for 6 hours also induced a significant expression in CHOP, and this was predominantly noted in vimentin-IR glia (Supplementary Fig. S5). Quantification of these data is shown in Figures 3Q and 3R. As with rotenone, the known ER stress inducer thapsigargin (100 nM) also stimulated both apoptotic species levels, as stimulated by 1 μM rotenone, peaked at 24 hours, at which time they were quantified as 5.1-fold higher than in the control, untreated group. In particular, there was a marked increase in ROS labeling in neurons after incubation with rotenone for 6 hours, whereas the increase was also observed in glial cells after 24 hours.

Rotenone Inhibition of ATP Production

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Rotenone treatment (1 μM) led to a time-dependent decrease in the level of cellular ATP in 7-day retinal cultures (Fig. 2D). The ATP levels started to decrease after incubation with rotenone for 2 hours and then dropped to 69% at 6 hours. In contrast, in the Müller cell-enriched 28-day-old cultures, glia cells were relatively more resistant to rotenone and maintained their ATP level at 87% of the untreated value at 24 hours.

Rotenone Induces the ER Stress Marker, CHOP, Which Predominates in Vimentin-Positive Glia

Since previous research has shown that mitochondrial disease and rotenone treatment can increase ER stress marker transcripts,22 we aimed to determine whether rotenone could induce ER stress-related protein expression in rat retinal cell cultures. Rat retinal cultures treated with 1 μM rotenone for 6 hours showed significant growth arrest and DNA damage-inducible protein/C/EBP homologous protein (CHOP) expression compared to untreated cells (P < 0.05; Supplementary Figs. 3S, ST). In addition, it was clear that CHOP labeling was not colocalized with Tau-IR neurons, but with vimentin-IR glia (Figs. 3A–P, arrow). This was also the case during the early phase (2 hours, 4 hours) of rotenone treatment (data not shown). In the 28-day glial-only cultures, treatment with 1 μM rotenone for 6 hours also induced a significant expression in CHOP, and this was predominantly noted in vimentin-IR glia (Supplementary Fig. S5). Quantification of these data is shown in Figures 3Q and 3R. As with rotenone, the known ER stress inducer thapsigargin (100 nM) also stimulated both apoptotic
Rotenone Induces ER Stress and Activates GSK-3β

Further Evidence for the Induction of ER Stress by Rotenone

We further sought to determine whether rotenone treatment of retinal cultures caused changes in other known markers of cellular ER stress, namely, increases in the expression levels of BiP or activating transcription factor 4 (ATF-4) or phosphorylation of pancreatic endoplasmic reticulum kinase/PERK-like endoplasmic reticulum kinase (PERK). In 7-day-old cultures, treatment with the known ER stress inducer thapsigargin (100 nM) for 6 hours significantly elevated BiP expression (2.1-fold), phosphorylation of PERK at threonine 980 (1.7-fold), and ATF-4 induction (2.4-fold; Figs. 4A, 4C). Similarly to thapsigargin, rotenone (1 μM) also significantly enhanced BiP (2.6-fold) and ATF-4 (4.6-fold) expression, but this compound had only a modest effect on phosphorylation of PERK (1.7-fold; Figs. 4B, 4D). When the incubation time with rotenone was prolonged to 24 hours, phosphorylation of PERK, as determined by the levels of phospho-PERK (p-PERK), was reduced to its original level (1.1-fold); in contrast, expression of BiP and ATF-4 was still at elevated levels, 3.0-fold and 4.8-fold of the untreated control group values, respectively. In 28-day-old cultures (glial cell cultures), thapsigargin did not cause an increase in BiP, ATF-4, or CHOP until 24 hours (Supplementary Figs. S1A, S1C). In comparison, CHOP was increased to 2.9 times its basal level at 6 hours in the rotenone-treated group in these cultures, and all tested ER stress markers were elevated by 24 hours (Supplementary Figs. S1B, S1D).

In order to prove that the ER stress was associated with the rotenone-induced cell death, we applied the chaperone PBA (sodium 4-phenylbutyrate), which is known to protect cells against this cellular mechanism.23 Supplementary Figure S8 shows the protective effect of PBA on retinal glia in both 7- and 28-day-old cultures. In addition, the cellular distribution of p-PERK was determined in cultures by investigating coexpression with different glial markers (data not shown). The results also indicated that these transmembrane receptor proteins colocalized with vimentin-IR and GFAP-IR glia.

Phosphorylation of GSK-3β

In 7-day-old cultures treated with rotenone for 2 hours, the inactive form of glycogen synthase kinase-3β (GSK-3β), p-GSK-3β (Ser9), was detected at 65% of its basal level, whereas the active form, p-GSK-3β (Thr390), was present at 180% of its original level (Figs. 5Q, 5R). Furthermore, immunocytochemistry confirmed that p-GSK-3β (Thr390) was elevated after treatment for 6 hours with 1 μM rotenone in vimentin-positive glial cells rather than PGP9.5-positive neurons (Fig. 5). In 28-day-old cultures (glial cell cultures), p-GSK-3β (Thr390) was elevated to 185% of its basal level after 24-hour treatment with 1 μM rotenone. Consistent with the data in 7-day-old cultures, the p-GSK-3β (Ser9) level was reduced to 70% and 48% of the basal amount by 6- and 24-hour rotenone treatment, respectively (Supplementary Figs. S2A, S2B).

Rotenone-Induced Activation of Calpain-μ

Analysis of α-spectrin (150 kD) proteolysis by Western blotting after a particular stimulus or treatment to a system can specifically detect whether caspase or calpain activation has been induced. This is because proteolytic cleavage of this cytoskeletal component can occur in both a calpain-specific (yields 145 kD product) and a caspase-specific (yields 120 kD product) manner.24 In the present study (Fig. 6), it was clear that rotenone predominantly induced α-spectrin cleavage by activation of calpain. In addition, treatment with 1 μM rotenone for 6 hours showed the greatest induction of calpain-μ: a 2.4-fold-level increase compared to untreated cells, which then decreased to 75% of the original level by 24 hours. Furthermore, immunocytochemical analysis of cultures after rotenone treatment demonstrated that calpain-μ was colocalized with PGP9.5-positive neurons after rotenone treatment (Figs. 6A–H). In 28-day-old cultures (glial cell cultures), no calpain-specific (150–145 kDa) spectrin breakdown products (SBDP) could be detected in rotenone-treated cells (Supplementary Fig. S3).

Inhibition of GSK-3β and Calpain

Western blotting and TUNEL staining were employed to determine whether the GSK-3β inhibitor, LiCl, or the calpain inhibitor, calpain inhibitor III, was able to protect glia or neurons from rotenone-induced toxicity and apoptosis (Figs. 7, 8). In 7-day cultures, treatment with rotenone (1 μM) for 24 hours led to significant losses of neuronal- and glial-specific proteins (Figs. 1, 7). As stated previously, there was also a significant increase in detection of TUNEL-positive nuclei after rotenone treatment (Fig. 2A). The presence of the GSK-3β inhibitor LiCl (20 mM) for 1 hour before rotenone administration significantly preserved GFAP- and vimentin-positive glial cells, as well as reducing TUNEL detection. In contrast, the calpain inhibitor, calpain inhibitor III, protected neurons (Fig. 7) but not glia (Figs. 8, 9). This result was supported by TUNEL-positive cell detection in 28-day-old cultures: Calpain inhibitor III did not prevent rotenone-induced apoptosis in the glial-dominant cultures (Fig. 9).

Linking ER Stress to Activation of GSK-3β

Since both ER stress and activation of GSK-3β contributed to rotenone cytotoxicity, we sought to determine the possible interaction between them. Western blot data (Supplementary Fig. S7) showed that p-GSK-3β (Thr390) declined to almost its control level (1.1-fold) with the administration of LiCl. Furthermore, LiCl strongly decreased the expression of CHOP (Fig. 8).

DISCUSSION

In the present study the complex I inhibitor rotenone was employed to impair mitochondrial function in mixed rat retinal cell cultures. The rationale behind using this approach was to subject retinal cells to moderate insults with relevance to pathological situations involving metabolic deficiency,25 anoxia,3 or the degeneration of retinal ganglion cells.26 Consistent with previous research,2 the present data demonstrated that treatment with 1 μM rotenone for 6 hours caused an increase in the production of ROS and a reduction in the total ATP levels in retinal cell cultures. Data in the present study also proved that rotenone stimulated ER stress at the same concentration and incubation time as alterations in both ROS and ATP levels. The date shown herein, therefore, delineate that toxicity of rotenone to retinal cells was mediated through a variety of mechanisms.

Endoplasmic reticulum stress has been implicated in a broad range of chronic human diseases, including neurodegenerative disease such as glaucoma.27 In a rat model of chronic glaucoma, both phospho-PERK and CHOP were detected in ganglion cells, accompanied by TUNEL-positive cells and decreased number of ganglion cells.28 Similar results could also be found in a rat retinal ischemia–reperfusion...
FIGURE 5. Effect of rotenone (1 μM) on GSK-3β phosphorylation in 7-day-old rat retinal cell culture. Double labeling immunofluorescence of p-GSK-3β (Thr390) with the amacrine marker PGP9.5 and the Müller cell marker vimentin (arrow in [N, P]) after incubation with rotenone (1 μM) for 6 hours in 7-day-old rat retinal cell cultures. Active p-GSK-3β (Thr390) was not found to increase or colocalize with PGP9.5 (E-H) in the presence of rotenone (1 μM), compared with control cells (A-D). (M–P) Active p-GSK-3β (Thr390) was increased and found to colocalize with vimentin (arrow in [N, P]) compared with the control group (I-L). Scale bar: 20 μm. (Q) Temporal characterization of upregulation of active p-GSK-3β (Thr390) and downregulation of inactive p-GSK-3β (Ser9) following treatment with rotenone (1 μM) for 0, 1, 2, 3, 6, and 24 hours as determined by Western blotting. (R) Phosphorylated GSK-3β and total GSK-3β was measured by densitometry, normalized to β-actin, and expressed relative to control cells. *P < 0.05, **P < 0.01 compared to control cells. The results shown represent the mean ± SEM of three independent experiments.
model\textsuperscript{29} and an excitotoxic paradigm of ganglion cell death.\textsuperscript{27} All these animal models have a relatively chronic or moderate ATP loss. However, in our acute mitochondrial injury model, it is interesting to note that even though mixed retinal cell cultures containing both glia and neurons\textsuperscript{30} were severely depleted of their ATP levels by rotenone, the vimentin-rich retinal glial cell cultures (28 days in vitro) were relatively unaffected. These data are therefore in agreement with previous studies and suggest that retinal neurons produce the majority of their energy via mitochondrial reactions, whereas glial cells produce much of their ATP through the glycolytic pathway.\textsuperscript{31–33} Since the ER depends on ATP to correct misfolding of protein errors, ATP reduction can lead to ER stress. Reduction in cellular ATP de-energizes the ER-based calcium ATPase, thereby disrupting the function of the ER,\textsuperscript{34–36} initiating the so-called unfolded protein response (UPR). During the early phase of ER stress, the UPR plays a crucial role both by increasing the availability of BiP\textsuperscript{37} and by promoting autophosphorylation of PERK; these events contribute to the cellular defense against incidences of protein misfolding.\textsuperscript{38} If cellular stress is prolonged, however, the UPR triggers apoptosis by causing an upregulation of ATF-4 (a member of the CREB family of DNA-binding proteins).\textsuperscript{39}

Conversely, dramatic and sudden ATP depletion results in the rapid death of neurons via nonapoptotic processes.\textsuperscript{40} Therefore, the actual intracellular ATP level in any given situation in different retinal cell types likely acts as the major determinant of the mode of cell death. So, which type of cell

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**FIGURE 6.** Effect of rotenone (1 \(\mu\)M) on 7-day-old rat retinal cells, as assessed by immunocytochemistry and Western blotting of the appearance of \(\alpha\)-spectrin breakdown products and calpain-\(\mu\) expression. Representative immunocytochemistry photomicrographs of calpain-\(\mu\) activation are shown for control and rotenone (1 \(\mu\)M) for 6 hours in 7-day-old cultures, respectively. Calpain-\(\mu\) was increased and found to colocalize with PGP9.5 (arrow in [E, F, H]), compared with the control group (A–D). Scale bar: 20 \(\mu\)m. (I) Western blotting analyses for \(\alpha\)-spectrin and calpain-\(\mu\) in rat retinal cultures treated with rotenone (1 \(\mu\)M) for 0, 6, and 24 hours. Analysis showed that the calpain-specific (150–145 kDa) spectrin breakdown products (SBDP) were increased in rotenone-treated cells at 6 and 24 hours. Results shown are representative of three independent experiments. (J) Densitometric quantification of \(\alpha\)-spectrin breakdown at 145 kDa and calpain-\(\mu\) levels normalized to \(\beta\)-actin protein levels. **P < 0.01 compared to untreated.

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**FIGURE 7.** Protective effect of GSK-3\(\beta\) inhibitor LiCl and the calpain inhibitor, calpain inhibitor III, against rotenone (1 \(\mu\)M) toxicity on retinal neuronal cell markers. Rat retinal cells (7 days in vitro) were pretreated with LiCl (20 mM) or calpain inhibitor III (25 \(\mu\)g/mL) for 1 hour and then exposed to rotenone (1 \(\mu\)M) for 6 hours. (A) Cell viability was determined by Western blotting, and data are expressed as a percentage of the control. Effect was normalized for \(\beta\)-actin levels in each sample and was quantified (B). It was obvious that treatment with calpain inhibitor III rather than LiCl led to protection of neuronal cells compared with the rotenone-treated group. The results shown represent the mean ± SEM of three independent experiments. *P < 0.05, **P < 0.01 compared to control cells. #P < 0.05 compared to rotenone-treated group. Ct, control; R, rotenone; R/L, LiCl plus rotenone; R/C, calpain inhibitor III plus rotenone.
death was involved in the present study? Retinal neurons are known to rely predominantly on mitochondria for their ATP production. Thus, rotenone treatment may be expected to cause a rapid nonapoptotic death of neurons almost solely due to drastic cellular energy failure. This was, indeed, observed. In the case of retinal glia, however, which rely more upon aerobic glycolysis for their ATP production, mitochondrial inhibition will not have such drastic effects upon cellular ATP levels, causing a reduction but not a complete depletion. This would likely favor the more ATP-dependent apoptosis, driven by events such as ER stress, as noted in the present study.

In order to prove the assumption that ER stress was involved in glial cell toxicity only, we also investigated whether the chemical chaperone PBA could protect cells in our model. Interestingly, administration of 4-PBA rescued glial cells but not neurons, in agreement with the notion that ER stress affects only these cells and not neurons.

Previous research has suggested that the activation of calpain plays an important role in rat retinal cell death in response to anoxia or ischemia-reperfusion. In our experiments, we provided evidence that in the first 6 hours after application of rotenone, when toxicity to neurons was noted, calpain- activation was also observed in cultures. Mitochondrial inhibition will not have such drastic effects upon cellular ATP levels, causing a reduction but not a complete depletion. This would favor the more ATP-dependent apoptosis, driven by events such as ER stress, as noted in the present study.

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Rotenone Induces ER Stress and Activates GSK-3β

Figure 10. Hypothetical mechanisms by which rotenone induced rat retinal cell death in culture.

calpain-μ contributed to nonapoptotic neuronal death but not to apoptotic glial cell death. Indeed, the prevention of cell death by calpain inhibition has been demonstrated in human neuroblastoma and hypoxic-ischemic brain injury, where nonapoptotic modes of cell death are involved. Accordingly, one limitation of the present investigations is that we did not determine the proportion of cell death due to necrosis.

Glycogen synthase kinase-3β is a multifactorial negative regulator of the cell cycle that also plays a role in cell death/survival signaling, as well as control of the opening of the mitochondrial permeability transition pore (mPTP) in situations of cellular energy depletion. Even though GSK-3β can regulate cell death in response to cellular stress, the biological function of this enzyme has not been explored in detail in the retina. The present data indicate that rotenone treatment caused an activation of GSK-3β after 24-hour incubation, as shown by upregulation of active phospho-GSK-3β (Thr196) as well as the downregulation of inactive phospho-GSK-3β (Ser21). The immunocytochemistry results and the ability of the GSK-3β inhibitor, LiCl, to protect glia but not neurons supported the notion that this enzyme played a role in the death of only the former cells in the present study. The reduced ATP depletion in glia with respect to neurons is important here, since not only is GSK-3β known to require ATP for activation but also ATP-derived phosphate is instrumental for its catalytic action on substrate proteins.

Research has suggested that GSK-3β inhibitors can protect different cell preparations from ER stress-associated apoptosis. The underlying mechanism by which GSK-3β promotes cell apoptosis, however, via ER stress remains unclear. Possible mechanisms that have been proposed for the protection of GSK-3β inhibitors from ER stress include inhibition of Bax activation or a decrease in intracellular calcium levels. Here we show in our retinal cell preparation that ER stress-induced upregulation of the proapoptotic transcription factor CHOP is inhibited by GSK-3β inhibition. Therefore, it is therefore likely that inhibition of this enzyme raises the threshold for apoptosis as induced by ER.

In the present study, toxicity of rotenone to neurons and glia in retinal cultures has been shown to be mediated through multiple mechanisms, according to the cell type involved. Given that apoptosis is energy dependent and that rotenone affects mitochondria, the death pathways are likely to be related to the prefered ATP generation pathways in different cell types (i.e., via glycolysis in glial cells and via mitochondrial oxidative phosphorylation in neurons). This provides a clear link between the degree of reduction in ATP caused by different forms of metabolic dysfunction and the cell death pathway taken by the affected cell. The pathways to cell death in the current model, summarized in Figure 10, show that neuronal death is induced by rapid loss of ATP, concurrent generation of ROS, and the activation of calpain, whereas the delayed, apoptotic death of glia is mediated through a combination of ER stress induction and activation of the enzyme GSK-3β. This process is characterized by moderate ATP loss and increasing ROS generation. These data may provide useful therapeutic avenues for treatment of different cells in retinas exposed to situations of mitochondrial dysfunction.

In conclusion, rotenone treatment initiates a variety of distinct cell signaling pathways in rat retinal cell cultures. These processes include rapid GSK-3β activation, initiation of ER stress, and upregulation of calpain-μ. Further investigations are needed to clearly delineate the role that each of these events plays in the response of different retinal cells to metabolic compromise.

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