Characterization and Regulation of Carrier Proteins of Mitochondrial Glutathione Uptake in Human Retinal Pigment Epithelium Cells

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PURPOSE. To characterize two mitochondrial membrane transporters 2-oxoglutarate (OGC) and dicarboxylate (DIC) in human RPE (hRPE) and to elucidate their role in the regulation of mitochondrial glutathione (mGSH) uptake and cell death in oxidative stress.

METHODS. The localization of OGC and DIC proteins in confluent hRPE, polarized hRPE monolayers and mouse retina was assessed by immunoblotting and confocal microscopy. Time- and dose-dependent expression of the two carriers were determined after treatment of hRPE with H2O2, phenyl succinate (PS), and butyl malonate (BM), respectively, for 24 hours. The effect of inhibition of OGC and DIC on apoptosis (TUNEL), mGSH, and mtDNA was determined. Silencing of OGC by siRNA knockdown on RPE cell death was studied. Kinetics of caspase 3/7 activation with OGC and DIC inhibitors and effect of cotreatment with glutathione monoethyl ester (GSH-MEE) was determined using the IncuCyte live cell imaging.

RESULTS. OGC and DIC are expressed in hRPE mitochondria and exhibited a time- and dose-dependent decrease with stress. Pharmacologic inhibition caused a decrease in OGC and DIC in mitochondria without changes in mtDNA and resulted in increased apoptosis and mGSH depletion. GSH-MEE prevented apoptosis through restoration of mGSH. OGC siRNA exacerbated apoptotic cell death in stressed RPE which was inhibited by increased mGSH from GSH-MEE cotreatment.

CONCLUSIONS. Characterization and mechanism of action of two carrier proteins of mGSH uptake in RPE are reported. Regulation of OGC and DIC will be of value in devising therapeutic strategies for retinal disorders such as AMD.

Keywords: retinal pigment epithelium (RPE), oxidative stress, mitochondria, apoptosis, 2-oxoglutarate carrier (OGC), dicarboxylate carrier (DIC), glutathione (GSH), mitochondrial GSH (mGSH)

AMD, a progressive degenerative retinal disease, is the leading cause of irreversible blindness in the elderly and is characterized by the decrease in macular function due to the degeneration of RPE cells.1 Multiple cellular mechanisms are involved in the dysfunction and death of RPE cells in AMD, including accumulation of toxic metabolites, endoplasmic reticulum (ER) stress, oxidative stress and inflammation.2,3 Mitochondria is the powerhouse of mammalian cells and the main generators of reactive oxygen species (ROS), the over-generation of which leads to oxidative stress. This phenomenon contributes to mitochondrial damage linked to a number of pathological disorders, including AMD.4 In addition, mitochondria are considered to play a vital role in controlling the fate of cells through regulation of death pathways.5,6

Glutathione (GSH), an endogenous antioxidant abundantly present in retina and in RPE cells, is important for the maintenance of cellular viability.5,7 Intracellular GSH levels are maintained by redox cycling, de novo synthesis, and transmembrane transport. Even though GSH is synthesized in the cytosol, it is distributed in intracellular organelles such as mitochondria, nucleus, ER and Golgi complex.8 However, mitochondrial GSH (mGSH) is mainly found in reduced form and represents 10% to 15% of the total cellular GSH pool. mGSH works as a critical antioxidant reserve that is derived entirely from the larger cytosolic pool by the action of carrier transporters.9 Since mGSH plays a significant role in cellular defense against pro-oxidants, the depletion of mGSH affects cell viability, either by predisposing cells to apoptosis or by modulating mitochondrial membrane potential and subsequent activation of caspases, through regulation of redox pathways.10

Our laboratory has a longstanding interest in the antioxidant defense of the eye and has reported the expression and regulation of several redox proteins and GSH in RPE.11,12 For example, we have presented evidence for the essential role played by GSH in protection of RPE and retina, and how endogenous proteins such as αB crystallin contribute to this
Maintenance of mGSH pool is critical for the health of RPE.7,12 Dependence of mGSH transport on membrane fluidity.19 Changes in membrane dynamics, thus reproducing the release of OGC from rat liver was sensitive to cholesterol-mediated impairment of mitochondria. Our findings uncover a divergent role of mGSH transporters OGC and DIC in RPE/retina and their role in maintaining mGSH status in retinal health and injury. Accordingly, we investigated the role of OGC and DIC, the cells were exposed to H2O2 at varying doses (50, 100, 200, 300 μM) for 24 hours, and varying durations (2, 4, 6, 8, 24 hours) with 200 μM H2O2. To identify dose and time-dependent inhibition of OGC and DIC expression by chemical inhibitors, cells were incubated with phenylsuccinic acid (PS) and butylmalonic acid (BM; Sigma-Aldrich Corp., St. Louis, MO, USA) in varying doses (2, 5, 10 mM) for 24 hours, and varying durations (2, 4, 6, 8, 24 hours) with a single 5 mM dose of either PS or BM, respectively. Cells were also treated with 5 mM PS or BM, in the presence or absence of 2 mM GSH-MEE (Sigma-Aldrich Corp.) for 24 hours. To identify the effect of competitive inhibitors of the two transporters, cells were treated with a 5 mM dose of either dimethyl 2-oxoglutarate or diethyl malate for 24 hours. All inhibition studies were performed in serum-free media for 24 hours before treatments. The protocol for generation of long-term polarized human fetal primary RPE cultures has been described in our previous publication.20 Confluent cell cultures from passages 2 to 4 were used, and they were changed to serum-free media for 24 hours before treatments. The protocol for generation of long-term polarized human fetal primary RPE cultures has been described in our previous publication.20

<p>| Table. Antibodies and Primers Used in the Study |</p>
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Human Primer Sequences

| RT-PCR | OGC1 Forward:5’GGCTGGACGCTGCTTCAAGTTGT 3’ | 5’ | Invitrogen, Carlsbad, CA, USA |
| DIC Reverse:5’TCCCTCAAGAATGCCAAGGACA 3’ | 3’ |
| OGC2 Forward:5’ATGATCACGGGCTTTGTCACCCT 3’ | 3’ |
| Reverse:5’TCCCTCAAGAATGCCAAGGACA 3’ | 3’ |
| DIC2 Reverse:5’ACAAATGAGCCACGGTGTTG 3’ | 3’ |
| Forward:5’TCCAAGGGGGAGATATCCAGG 3’ | 3’ |
| GAPDH- F Forward:5’ACACGTCCGCGATCCTTCTT | 5’ |
| GAPDH- R Reverse:5’CTTGATTTTGGAGGATCTGCG | 3’ |
| OGC (5’ to 3’ | 3’ |

Methods

General

Reagents used in the present study for Western blot analysis, immunofluorescence staining, and primer sequences for RT-PCR are listed in the Table. Cell Culture

All experiments and procedures were conducted in compliance with the tenets of the Declaration of Helsinki and ARVO guidelines. The RPE cells were isolated from human fetal eyes and cultured as previously described.20 Confluent cell cultures from passages 2 to 4 were used, and they were changed to serum-free media for 24 hours before treatments. The protocol for generation of long-term polarized human fetal primary RPE cultures has been described in our previous publication.20 Cell Exposures

To study the effect of oxidative stress on expression of OGC and DIC, the cells were exposed to H2O2 at varying doses (50, 100, 200, 300 μM) for 24 hours, and varying durations (2, 4, 6, 8, 24 hours) with 200 μM H2O2. To identify dose and time-dependent inhibition of OGC and DIC expression by chemical inhibitors, cells were incubated with phenylsuccinic acid (PS) and butylmalonic acid (BM; Sigma-Aldrich Corp., St. Louis, MO, USA) in varying doses (2, 5, 10 mM) for 24 hours, and varying durations (2, 4, 6, 8, 24 hours) with a single 5 mM dose of either PS or BM, respectively. Cells were also treated with 5 mM PS or BM, in the presence or absence of 2 mM GSH-MEE (Sigma-Aldrich Corp.) for 24 hours. To identify the effect of competitive inhibitors of the two transporters, cells were treated with a 5 mM dose of either dimethyl 2-oxoglutarate or diethyl malate for 24 hours. All inhibition studies were performed in serum-free media for 24 hours before treatments.
performed with RPE cells in serum-free medium containing 0.1% dimethyl sulfoxide.

**Reverse Transcribe Polymerase Chain Reaction**

Total RNA was extracted from confluent hRPE cells using an RNA extraction kit (RNeasy Mini Kit; Qiagen, Valencia, CA, USA). We used 1 µg total RNA for cDNA synthesis using a cDNA synthesis kit according to the manufacturer’s instructions (First-Strand cDNA Synthesis Kit; Invitrogen, Carlsbad, CA, USA). PCR was performed using a commercial kit (HiFidelity Polymerase Kit; Qiagen), with two pairs of primers for OGC and DIC listed in the Table, and β-actin served as the internal control. Results are reported as fold change over controls (mean ± SEM).

**Western Blot Analysis**

Protein was extracted from the cells and concentration was determined by a protein assay kit and Western blot was done as previously. Briefly, equal amounts of proteins (30 µg/well) were resolved and transferred to blotting membranes (Millipore, Billerica, MA, USA). Membranes were probed overnight at 4°C with primary antibody (Table). After incubation with the appropriate secondary antibody (Vector Laboratories, Burlingame, CA, USA), protein bands were detected by a chemiluminescence (ECL) detection system (SuperSignal West Pico PLUS; Thermo Fisher Scientific, Rockford, IL, USA). To verify equal loading, membranes were reprobed with β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). We used 721B and MCF7 cell lysates as positive controls for OGC and DIC. Subunit IV of cytochrome c oxidase (COX IV) and α-tubulin were used as mitochondrial and cytosolic markers.

**Localization of OGC and DIC in RPE Cells by Immunofluorescence**

hRPE cells were grown in four-well chamber slides (Falcon, Corning, NY, USA). To visualize the mitochondria, red dye (MitoTracker Red CMXRos 500 nM; Life Technologies, Carlsbad, CA, USA) was added to samples and incubated at 37°C for 10 minutes, prior to fixation with 4% paraformaldehyde. Cells were incubated with primary antibodies (Table) overnight at 4°C and followed by secondary antibodies (Vector Laboratories) was used for 30 min at room temperature. After nuclear staining with DAPI (Vector Laboratories), the slides were examined on a laser-scanning microscope (LSM 710; Carl Zeiss Microscopy, Thornwood, NY, USA).

**Confocal Immunofluorescence of ZO-1 Staining in Polarized RPE**

The morphologic features of polarization were visualized by immunolocalization of ZO-1.20,21 Highly differentiated polarized hRPE (TER = 380 ± 60 Ω·cm²) were treated with either 15 mM PS or 15 mM BM with or without cotreatment with 2 mM GSH-MEE. As described earlier,21 RPE monolayers were fixed and blocked before incubation with ZO-1 rabbit polyclonal antibody (Table) at 4°C overnight, and followed by incubation with FITC conjugated anti-rabbit secondary antibody (Vector Laboratories) for 30 minutes. After the immunostaining procedure, membranes were cut, mounted on micro slides (VWR) and viewed on a laser-scanning microscope (Carl Zeiss Microscopy).

**Mitochondrial DNA Isolation**

Mitochondrial DNA (mtDNA) was isolated from cells treated with 5 mM of PS or BM for 24 hours using a commercial kit (Mitochondria DNA Isolation Kit, BioVision, Mountain View, CA, USA) following the instructions of the manufacturer. The concentration of mtDNA was measured by a multi-mode microplate reader (Spectramax iD5; Molecular Devices, San Jose, CA, USA).

**TUNEL Staining**

Apoptosis was detected by the TUNEL method according to the manufacturer’s protocol (In Situ Cell Death Detection Kit, Roche Applied Science, Indianapolis, IN, USA). Briefly, RPE cells were treated with inhibitors, in the presence or absence of GSH-MEE for 24 hours. H2O2 (500 µM) was used as a positive control.

**Silencing of OGC by siRNA Knockdown**

hRPE cells were cultured in 6-well plates or 4-well chamber slides to a confluence of 50% to 70%. Cells were switched to DMEM containing 0.5% FBS shortly before transfection. The siRNA-targeting human OGC sequences (HSIS112214) was purchased from Invitrogen.19 Control siRNA (Santa Cruz Biotechnology, Inc., CA, USA) or OGC siRNA were mixed with RNA transfection reagent (Lipofectamine RNAiMAX; Life Technologies). The mixture was added to the RPE cells and incubated for 6 hours. The composite transfection mixture was removed and replaced with DMEM containing 10% FBS. Cells were processed after 24 hours transfection for mRNA and 48 hours for the protein in mitochondrial fraction. To study the effect of OGC knockdown on cell death under oxidative stress, the OGC silenced cells were exposed to 300 µM H2O2 for 24 hours and assayed for cell death.

**RNA Isolation and Real-Time Quantitative RT-PCR**

Total RNA was isolated from cells using an RNA extraction kit (Qiagen), and RT-PCR was performed as described previously.7 Gene expression levels were normalized relative to GAPDH mRNA and reported as fold-change over control. The sequen-ces of primers used are presented in the Table. Relative multiples of change in mRNA expression was determined by calculating 2^[-ΔΔCt]. Results are reported as mean difference in relative multiples of change in mRNA expression ± SEM.

**Caspase 3/7 Activation Using IncuCyte Cell Apoptosis Assay**

To evaluate the inhibitors of OGC and DIC effects on cell apoptosis, RPE cells (5500 cells/well) were plated in 96-well plates (Corning, NY, USA). The treatment reagents were added to each well already containing SYTOX Green dye caspase 3/7 reagent diluted in cell culture media (1:1000 dilution) to make a total volume of 100 µl/well. Each condition was run in five replicate wells. The following four groups were used in total analysis: control, 5 mM PS, 5 mM BM, 5 mM PS + 2 mM GSH-MEE, 5 mM BM + 2 mM GSH-MEE. H2O2-treated cells served as positive control. Cell apoptosis was monitored for 24 hours using a live cell analysis system (IncuCyte ZOOM; Essen Bioscience, Ann Arbor, MI, USA), which allows an automated incubator method of monitoring live cells. Four image sets from distinct regions per well were taken every 30 minutes. Automated real-time assessment by live cell analysis (Essen Bioscience), measured as green object count for all cells stained green with SYTOX Green, which was allowed to
generate graphics of the data as soon as image analysis was complete. Graphics were generated with basic software graph/export functions (Essen Bioscience).

**Preparation of Mitochondrial and Cytosolic Fractions and Assay for GSH Content**

hRPE cells were harvested after the specified experimental period, mitochondrial and cytosolic proteins were isolated from the freshly collected cells according to the method described previously. The supernatant from centrifugation was collected as the cytosol portion while the mitochondrial fraction was resuspended in 150 μL of mitochondrial buffer. Samples were analyzed immediately for GSH content. Equal amount of proteins were loaded to 96-well plates, and the total cellular, mitochondrial, and cytosolic GSH were measured using a glutathione assay kit (BioVision) following the manufacturer's protocol. Total GSH levels were calculated using a standard curve and GSH concentrations were expressed as μg/10⁶ cells and were normalized to percent of control. GSH measurements were performed in at least three independent experiments.

**Localization of OGC and DIC in Mouse Retinal Layers by Immunofluorescence**

We purchased 129-S6/SvEvTac male mice (6–8 weeks old) from Taconic Farms (Germantown, NY, USA) and eye cups were prepared as described. Cryosections at 8-μm thick were cut and collected for immunochemistry using antibodies presented in the Table overnight at 4°C. Negative control sections were incubated with blocking buffer without primary antibody and then processed as described above. The sections were photographed with a confocal laser microscope (Carl Zeiss Microscopy).

**Statistical Analysis**

All data are expressed as mean ± SEM. Statistical analysis was performed using ANOVA followed by Tukey post-test using graphing software (GraphPad Prism, version 5; GraphPad Software, Inc., La Jolla, CA, USA). *P* < 0.05 was considered significant.

**RESULTS**

**OGC and DIC Expression in hRPE Cells**

To determine whether hRPE cells express OGC and DIC, we used PCR, Western blot analysis, and immunofluorescent staining. Both carrier proteins were expressed prominently in hRPE cells (Figs. 1A, 1B). PCR with two specific primer pairs for both OGC and DIC in human RPE from two donors showed that hRPE cells expressed OGC and DIC transcripts (Fig. 1A).

To study subcellular localization of OGC and DIC, Western blot analysis was performed in RPE cell lysates along with positive control cell lysates, 721B (OGC) and MCF7 (DIC). Subunit IV of cytochrome c oxidase (COX IV) was used as the mitochondrial marker, and α-tubulin was used as the cytosolic marker.
revealed the purity of the mitochondrial fraction. OGC and DIC were expressed selectively in mitochondria as a ~32 kDa protein (Fig. 1B, left lanes) and a ~28 kDa protein (Fig. 1B, right lanes), respectively. The complete gel images for OGC and DIC along with the positive controls are presented in Supplementary Figure S2. Furthermore, we costained hRPE cells with red-fluorescent dye (MitoTracker Red; Thermo Fisher Scientific Inc., Waltham, MA, USA) and OGC and DIC antibodies. The double immunofluorescence staining confirmed that both transporters were colocalized in mitochondria in RPE cells (Figs. 2A, 2B, upper lanes). ‘‘No primary antibody’’ control is included in Figures 2A, 2B (bottom lanes). Taken together, these findings firmly establish the mitochondrial localization of OGC and DIC.

Effect of Polarity on OGC and DIC Expression and the Effect of Inhibition on ZO-1

Our laboratory has documented the role of polarity of RPE on the cellular expression and secretion of several growth factors. We investigated the effect of polarization of hRPE cells on the expression of OGC and DIC, and compared polarized RPE to that of nonpolarized RPE cells. The morphology of nonpolarized confluent RPE and the integrity of polarized RPE using ZO-1 marker are shown in Supplementary Figure S1. The polarization of RPE was confirmed by transepithelial electrical resistance (TER = 380 ± 60 Ω-cm²). As shown in Figures 3A through 3D, OGC and DIC protein expression was significantly increased in polarized RPE cells than that in nonpolarized RPE cells (2.5-fold versus nonpolarized; P < 0.05). Cells with a higher TER (TER = 380 ± 60 Ω-cm²) exhibited a >2.5-fold increase in the expression of both carrier proteins as compared to nonpolarized RPE. On the other hand, cells with lower TER (160 ± 57.1 Ω-cm²) upregulated OGC and DIC expression only by 1.4-fold. Next, we analyzed the expression of the tight junction protein ZO-1 in polarized RPE cells with and without PS/BM treatment in the presence or absence of GSH-MEE (Figs. 3E, 3F). While disruption of ZO-1 as seen in the breaks in the hexagonal shape (within the yellow line) is observed in the PS inhibitor group, cotreatment of the inhibitors with GSH-MEE restored the tight junctions close to that of controls. Similar morphologic changes of cell–cell junctions of ZO-1 was also observed in BM treatment group.

OGC and DIC Expression Decreases in hRPE Cells Under Oxidative Stress

Oxidative stress from H₂O₂ (50, 100, 200, 300 μM for 24 hours) caused a dose-dependent decrease of OGC and DIC protein expression of RPE cells (Figs. 4A, 4E). A significant reduction of OGC was observed at and above 100 μM dose in the H₂O₂-treated group (P < 0.01, P < 0.001; Fig. 4B). As compared to untreated control, OGC also exhibited a time-dependent decrease in expression for up to 24 hours with a fixed dose of 200 μM H₂O₂, with a 50% decrease occurring as early as 4 hours (Fig. 4C). Decreased DIC expression was also observed with
FIGURE 3. Effect of polarity on OGC and DIC expression in hRPE cells and confocal microscopy images of ZO-1 staining showing the breaks in tight junction induced by OGC and DIC inhibitors. The restoration of tight junction with GSH-MEE cotreatment was also shown. Western blot analysis were from confluent nonpolarized and polarized RPE cells (A–D), and ZO-1 confocal images were from polarized RPE cells (E, F). The area within the yellow line shows the breaks in tight junction which decreased in the GSH-MEE cotreatment groups as compared to PS/BM treated alone. Data are shown as mean ± SEM (n = 3), the mean TER of polarized cells was 380 ± 60 Ω·cm². **P < 0.001. Scale bar: 50 μm. GSH-MEE, GSH monoethyl ester.
increasing doses of H$_2$O$_2$ as compared to the control group, especially at 200 and 300 μM (P < 0.01; Figs. 4E, 4F). As with OGC, a time-dependent decrease in DIC expression was seen which remained unchanged for up to 24 hours (Figs. 4G, 4H).

**Dose and Time-Dependent Inhibition of OGC and DIC by Chemical Inhibitors**

To validate the relevance of OGC and DIC in RPE, the effect of specific inhibitors, PS, and BM were studied. Exposure to three different concentrations (2, 5, 10 mM) of PS and BM respectively for 24 hours, caused a significant depletions and dose-dependent downregulation of OGC and DIC expression during the treatment period (P < 0.01, P < 0.001; Figs. 5A, 5E).

In particular, higher dose of PS and BM caused significantly reduced OGC and DIC protein expression, especially at 10 mM resulting in a ~70% and 60% decrease over control, respectively (Figs. 5B, 5F). As compared to untreated control, OGC also exhibited a time-dependent decrease in expression for up to 24 hours with 5 mM PS, a ~60% decrease occurring as early as 2 hours (Fig. 5C). Similar trend in time-dependent decrease was found with DIC (Figs. 5G, 5H).

**Inhibition by PS and BM of OGC and DIC Is Selective to Mitochondrial Fraction**

Next, we studied the effect of the chemical inhibitors and H$_2$O$_2$ on OGC and DIC in mitochondrial fraction of hRPE cells.
FIGURE 5. Dose- and time-dependent inhibition of OGC and DIC expression by chemical inhibitors. hRPE cells were incubated with PS and BM, the chemical inhibitors of OGC and DIC respectively, at varying doses (2, 5, 10 mM) for 24 hours. The right panels show representative gels while the left panels show fold changes calculated by normalization of band density with β-actin from three independent experiments. Western blot analysis shows that the chemical inhibitors caused a depletion of OGC and DIC expression, especially at higher doses. Both OGC and DIC expression decreased significantly at 2 hours after treatment with the chemical inhibitors, respectively ($P < 0.001$ versus control). Data are mean ± SEM; $n = 3$. ** $P < 0.01$. *** $P < 0.001$. 

**Mitochondrial GSH Uptake in RPE**
A significant reduction of OGC protein expression was observed in mitochondrial fraction with 5 mM PS treatment and 200 μM H2O2, compared to control, respectively (P < 0.001 versus control; Figs. 6A, 6B). These analyses were performed with COX IV as the mitochondrial specific marker. The inhibition effect is consistent with the whole cell lysate expression data in Figures 4 and 5. Similarly, the reduction of DIC protein expression also occurred with BM and H2O2 treatment groups (P < 0.001 versus control; Figs. 6C, 6D). The mitochondrial protein COX IV was unchanged under these conditions (Figs. 6A, 6C).

To ensure that the PS and BM inhibition effect is specific to OGC and DIC and does not cause any loss of mitochondria, we quantified mtDNA under these conditions. The concentration of mitochondrial DNA was not significantly different between untreated control versus 24 hours in 5 mM PS–BM-treated cells (P > 0.05; Figs. 6E, 6F). This is also evident from the COX IV expression which remained identical in all these groups (Figs. 6A, 6C).

GSH-MEE Protects Against RPE Apoptosis Induced by OGC and DIC Inhibitors

Next, the apoptotic effect of inhibitors of OGC and DIC in hRPE cells was analyzed using TUNEL assay as shown in Figures 7A
and 7C. Quantification is presented as fold changes which is the ratio of TUNEL positive cells to the total cells (Figs. 7B, 7D). Both PS and BM significantly increased TUNEL-positive cells as compared to the control group. PS and BM treatment caused a significant ($P < 0.001$) 4.3-fold increase over control (Fig. 7B), and a significant ($P < 0.001$) 3.5-fold over control (Fig. 7D), respectively. GSH-MEE is well-known to protect cells from oxidant-induced cell death.\footnote{25,26 We investigated the effects of cotreatment with GSH-MEE on inhibitor-induced apoptosis in RPE (Figs. 7A, 7C). The apoptotic cells were significantly decreased in GSH-MEE cotreatment groups compared to the inhibitor-only treatment groups ($P < 0.001$; Figs. 7B, 7D). These results demonstrated the protective effect of GSH-MEE in RPE cells in vitro by preventing apoptotic cell death.}

**Exacerbation of Cell Death in OGC Knockdown RPE and Reversal With GSH-MEE**

In addition to the effect of the chemical inhibitor PS on OGC, we also studied the effect of OGC-siRNA knockdown on hRPE. Treatment with siRNA OGC significantly (~75%) downregulated OGC mRNA and protein levels compared to control siRNA treatment, ($P < 0.001$; Figs. 8A, 8B). A significant increase in cell death following OGC silencing compared to control siRNA was observed ($P < 0.001$), suggesting OGC deficiency can augment cell death in RPE (Fig. 8C). Moreover, cotreatment with GSH-MEE significantly inhibited cell death following OGC silencing, ($P < 0.001$; Fig. 8C), consistent with PS inhibition (Fig. 7A). We also examined whether $\text{H}_2\text{O}_2$ further potentiated cell death. The OGC silenced cells treated with 300 $\mu\text{M} \text{H}_2\text{O}_2$ significantly increased cell death compared to $\text{H}_2\text{O}_2$ treated alone ($P < 0.001$; Fig. 8D), while no significant difference observed between $\text{H}_2\text{O}_2$ treated alone and cotreated with control siRNA ($P > 0.05$; Fig. 8D).

**Time-Dependent Kinetics of Caspase 3/7 Activation With OGC and DIC Inhibitors and its Suppression by GSH-MEE**

The kinetics of cell death induced by OGC and DIC inhibitors and suppression by GSH-MEE were evaluated by live cell analysis (Essen Bioscience). The live cell analysis system (Essen Bioscience) allows for real-time monitoring of cell apoptosis by determining the number of caspase 3/7 positive cells (green fluorescent labeling) at various treatment intervals as shown in Figure 9A for up to 8 hours. Cells undergoing apoptosis with treatments at 6 hours were stained with SYTOX Green dye, which is shown in Figure 9B. Cells were treated with 5 mM PS (Fig. 9A, green), 5 mM BM (Fig. 9A, yellow), PS + GSH-MEE (Fig. 9A, blue), BM + GSH-MEE (Fig. 9A, gray), respectively. Activation of caspase-3/7 increased in a time-dependent manner in both PS and BM treatment groups (Fig. 9A, green and yellow), with peak cell death up to 50-fold over the control condition achieved in 6 hours (Fig. 9C). In addition, cotreatment with GSH-MEE resulted in a significant reduction of apoptosis triggered by the inhibitors (Fig. 9A, gray and blue), that was consistent with the findings of the TUNEL assay. Compared with the inhibitor-treatment groups, inhibitor cotreatment with GSH-MEE groups showed a significant reduction in apoptosis respectively ($P < 0.0001$; Fig. 9C), indicating that GSH-MEE could rescue cells from the injury caused by OGC and DIC inhibitors.

**GSH-MEE Supplementation Attenuates Inhibitor-Induced Depletion of mGSH in hRPE Cells**

Next, we sought to investigate whether cell death from OGC and DIC inhibition is a result of decreased antioxidant defense particularly that of GSH. The results show that there was no significant difference in GSH levels either in whole cell lysates (Figs. 10A, 10B) or the cytosolic fractions (Figs. 10C, 10D), in the treatment groups. On the other hand, mGSH exhibited significant differences among the treatment groups (Figs. 10E, 10F). As shown in Figure 10E, mGSH level was 60% lower in PS treatment group than that in the control group ($P < 0.05$), and BM treatment also induced a 70% depletion of mGSH level compared to the control condition ($P < 0.05$; Fig. 10F). These results are consistent with the known increase in ROS and a pro-oxidative environment in OGC and DIC-deficient cells.\footnote{13,17,25 Earlier studies have provided evidence that GSH-MEE freely crosses membrane bilayers and diffuses into mitochondria, contributes to mGSH replenishment, and}
protects against oxidative stress in several cell types.\textsuperscript{27–29} Therefore, we determined whether GSH-MEE treatment attenuated inhibitor-induced depletion of mGSH concentration in hRPE cells. GSH level was approximately 3-fold elevated in the (PS + GSH-MEE) group over PS group ($P < 0.001$) and a 70% decrease of OGC protein compared to siRNA control, $P < 0.001$ (B), respectively. (C) After 24 hours transfection with either control siRNA or OGC siRNA, cells were treated with or without GSH-MEE (2 mM) for 24 hours. (D) Cells were treated with 300 mM H$_2$O$_2$ for 24 hours after transfection with either control siRNA or OGC siRNA. Cell death was determined by TUNEL staining (red). Nuclei were stained with DAPI (blue). Quantification of the TUNEL-positive cells in the experimental groups was shown in right lanes of (C, D). (C) Compared with control siRNA-treated cells, OGC knockdown significantly increased cell death while GSH-MEE cotreatment protected cells from apoptosis. (D) No significant difference of cell death between H$_2$O$_2$ treated alone and cotreated with control siRNA, while cotreatment with OGC silencing siRNA significantly increased cell death. Data are mean ± SEM from three independent experiments performed in duplicate. **$P < 0.01$. ***$P < 0.001$.

**Selective Depletion of Mitochondrial GSH by Competitive Inhibition by Dimethyl 2-Oxoglutarate and Diethyl Malate**

Furthermore, to obtain additional evidence that PS and BM are specific inhibitors of OGC and DIC, we carried out competitive inhibition experiments using substrates of OGC, dimethyl ester of oxoglutarate, a cell-permeable analog of oxoglutarate and diethyl malate. As shown in Figure 11A, mGSH level was ~0.7-fold lower in 2-oxoglutarate treatment group than that in the control group ($P < 0.01$), and malate treatment group also showed a ~0.5-fold depletion of mGSH level compared to the control ($P < 0.05$; Fig. 11B). This significant depletion of mGSH observed in either 2-oxoglutarate or malate treatment group was consistent with the inhibition with PS and BM on OGC and DIC (Figs. 10E, 10F). Determination of mGSH with excess (5 mM) of the above two substrates significantly reduced mGSH levels in 24-hour incubations suggesting that GSH uptake mitochondria undergo competitive inhibition similar to what has been shown in other cells.\textsuperscript{30}

**Localization of OGC and DIC in Mouse Retina**

To determine the distribution of OGC and DIC in the mouse retina, especially in the RPE and choroid layer, immunostaining was used. OGC and DIC expression was prominent as evidenced by strong green fluorescence in the photoreceptor inner segments, which harbor abundant mitochondria. Furthermore, immunofluorescent labeling in mouse retinas with OGC and DIC antibodies showed clear evidence for localization of the two transporters in the inner segments (IS) and photoreceptors (Fig. 12). Next, in order to measure the expression of OGC and DIC in retinal tissue, mitochondrial and cytosolic fractions were isolated from RPE and choroid. 

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**Figure 8.** Exacerbation of RPE cell death by silencing of OGC and attenuation of cell death by cotreatment with GSH-MEE. (A, B) Expression of OGC mRNA and protein levels in hRPE cells following knockdown of OGC. The mRNA was extracted 24 hours after transfection and the protein in mitochondrial fraction was quantitated by Western blotting using COX IV as a mitochondrial biomarker (B). Gene silencing significantly decreased OGC expression, a 75% decrease of OGC mRNA compared to that of siRNA control, $P < 0.001$ (A) and a 70% decrease of OGC protein compared to siRNA control, $P < 0.001$ (B), respectively. (C) After 24 hours transfection with either control siRNA or OGC siRNA, cells were treated with or without GSH-MEE (2 mM) for 24 hours. (D) Cells were treated with 300 mM H$_2$O$_2$ for 24 hours after transfection with either control siRNA or OGC siRNA. Cell death was determined by TUNEL staining (red). Nuclei were stained with DAPI (blue). Quantification of the TUNEL-positive cells in the experimental groups was shown in right lanes of (C, D). (C) Compared with control siRNA-treated cells, OGC knockdown significantly increased cell death while GSH-MEE cotreatment protected cells from apoptosis. (D) No significant difference of cell death between H$_2$O$_2$ treated alone and cotreated with control siRNA, while cotreatment with OGC silencing siRNA significantly increased cell death. Data are mean ± SEM from three independent experiments performed in duplicate. **$P < 0.01$. ***$P < 0.001$. 

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**Mitochondrial GSH Uptake in RPE**

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FIGURE 9. Time-dependent increase in caspase-3/7 generation with OGC and DIC inhibitors and suppression by GSH-MEE. (A) Kinetic measure of the number of caspase 3/7 positive cells shown over time in response to specified treatments. hRPE cells were plated and either left unstimulated or stimulated with 5 mM PS (green), 5 mM BM (yellow), or cotreated with 2 mM GSH-MEE (PS + GSH-MEE: blue and BM + GSH-MEE: gray). Images were acquired every 15 minutes. Automated real-time assessment by IncuCyte ZOOM, measured as green object count for all cells stained green with SYTOX Green, which was allowed to generate graphics of the data as soon as image analysis was complete. PS and BM induced significant cell
apoptosis compared with control, which was markedly suppressed by GSH-MEE. (A) Caspase-3/7 activation increased for the first 2 hours with PS and BM which was inhibited by cotreatment with GSH-MEE. (B) IncuCyte ZOOM image (phase contrast and green fluorescence overlaid) of hRPE cells with stimulus at 6 hours. Cells undergoing apoptosis have membrane compromise, and their DNA are stained with SYTOX Green dye that is already present in the media along with the stimuli (original magnification $\times 20$). (C) Quantification of the activation of caspase-3/7 (green fluorescent staining of nuclear DNA) at 6 hours in the treatment groups showing the suppression of inhibitor-induced caspase-3/7 activation by GSH-MEE. H$_2$O$_2$ treatment of RPE cells was used as a positive control. Data are shown as mean ± SEM ($n=4$). ****$P<0.0001$.

**FIGURE 10.** Selective depletion of mitochondrial GSH with OGC and DIC inhibitors and repletion with GSH-MEE cotreatment. GSH levels were measured in whole cell lysates (A, B), cytosolic fraction (C, D), and mitochondrial fraction (E, F). GSH levels were significantly decreased with inhibitors but is restored to above control levels with GSH-MEE (E, F). Whole cell and cytosolic fractions do not show such a change (A-D). The GSH levels were normalized to control level (=1). All experiments were carried out in triplicate. Data are shown as mean ± SEM of four independent experiments, *$P<0.05$, **$P<0.01$. 

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The role of GSH in retinal neuroprotection is well known. Unlike forms a critical pool in the mitochondrial compartment and the ameliorate the progression to vision loss.

Protecting mitochondrial DNA integrity via therapeutics mentioned previously, we have shown that mGSH plays a key specific process exhibiting dual kinetic components. As transport of cytosolic GSH into mitochondria by a carrier-

Mitochondria harbor transporters or channels that facilitate entry of GSH into bilayer, both outer and inner mitochondrial membranes must negatively charged and is unable to pass freely across the lipid membrane.

Chronic damage to mitochondria with oxidative, environmental, and genetic factors can lead to damage to mitochondrial DNA. Evidence implicates mitochondrial damage in the AMD disease process. Ferrington’s laboratory found a potential link between mitochondrial dysfunction due to increased mitochondrial DNA lesions and AMD. Thus, the findings from Booty et al. may arise from biochemical and biophysical properties of L. Lactis or OGC or OGC may require other partners under their experimental conditions.

Mitochondria possess several detoxifying enzymes and antioxidants such as MnSOD, glutaredoxin, and GSH. GSH forms a critical pool in the mitochondrial compartment and the role of GSH in retinal neuroprotection is well known. Unlike cytosol, mitochondria do not synthesize GSH de novo from its constituent amino acids and mGSH originates from the transport of cytosolic GSH into mitochondria by a carrier-specific process exhibiting dual kinetic components. As mentioned previously, we have shown that mGSH plays a key role in the removal of ROS in injured RPE. Since GSH is negatively charged and is unable to pass freely across the lipid bilayer, both outer and inner mitochondrial membranes must harbor transporters or channels that facilitate entry of GSH into mitochondria.

In the current study, we have identified OGC and DIC as the two solute carrier proteins that mediate the uptake of GSH by RPE mitochondria. These two proteins are members of the SLC25 transporter family and vary greatly in the nature and size of the transported substrates, modes of transport and driving forces although the mechanism of substrate translocation may not differ. We could establish that both proteins are expressed selectively in mitochondria of human RPE cells as well as in RPE/choroid of young mouse retina. A wide body of data suggests that these transporters reside in the inner mitochondrial membrane in several cell lines and tissues and mediate GSH import to mitochondria. OGC-mediated transport was not found in bacteria, contrasting the functional expression of mitochondrial-targeted OGC from HepG2 transport in Xenopus oocytes and the reconstitution of partially purified OGC in proteoliposomes from kidney mitoplasts. Thus, the findings from Booty et al. may arise from biochemical and biophysical properties of L. Lactis or OGC or OGC may require other partners under their experimental conditions.

Our laboratory has shown that the expression of growth factors can be regulated by the polarity of the RPE. For example, we have shown that amount of secretion of PEDF varies several folds based on the polarity of human RPE in the order: nonpolarized < polarized < stem cell RPE. Consistent with this, we found that both OGC and DIC expression increased in polarized RPE as compared to nonpolarized RPE making polarized RPE more resistant to oxidative injury. We also observed that expression of the carrier proteins increased with increased TER as anticipated.

Our experiments revealed that inhibition of OGC and DIC caused apoptosis in RPE cells (Figs. 7, 8). However, this sensitization to apoptosis may be partially and not solely due to the inhibition of GSH transporters. It is likely other substrates carried by the two transporters may contribute to this effect and further investigation will be needed in this regard. Live-cell imaging of caspase 3/7 activation established that the induction of apoptosis is rapid and occurs as early as 2 hours (Fig. 9). Under these conditions, mGSH is decreased significantly while whole cell and cytosolic GSH remained unaltered. Our studies also revealed that the decrease in GSH and induction of apoptosis could be overcome by coinubcation with GSH-MEE indicating the significance of OGC and DIC in...
maintaining proper RPE function. GSH-MEE being a membrane permeable GSH analog, will result in the distribution of GSH in all cellular compartments and can trigger activation of other protective factors in the cytosol. In addition, GSH feedback inhibition will be operative in the cytosol when the GSH status is altered. Thus, the increase in mGSH and role of mGSH in cellular protection becomes critical under these conditions. Depletion of mGSH has been reported to impair s-glutathionylation of proteins,44,45 enzymes of the TCA cycle and also complex I, III, V of the electron transport chain are functionally altered by s-glutathionylation. Thus, augmentation of GSH through GSH carrier proteins might support mitochondrial respiration.46

We also obtained evidence to show that apoptotic cell death in RPE with OGC and DIC inhibitors arises from the specific, functional effect of these transporters and not from the loss or degeneration of mitochondria. No significant changes in mitochondrial DNA content was seen with PS and BM inhibition of OGC and DIC (Fig. 6). It will be interesting to establish the correlation of OGC/DIC depletion with mitochondrial function such as ATP generation and respiratory complex proteins and restoration of GSH levels by known GSH replenishing agents in RPE/retina. In addition, it would be worthwhile to identify novel interacting partners for the two carriers. For example, Wilkins et al.47 have identified Bcl-2 interacts with OGC and found that this interaction is enhanced by GSH. The substrate specificity of the two transporters has been studied extensively by Lash’s laboratory.14,39 Using substrates for monocarboxylate transporter (lactate, pyruvate) and tricarboxylate transporter (citrate, isocitrate), they estab-

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**FIGURE 12.** Expression of OGC and DIC (green) in mouse retinal layers in mouse RPE/choroid. (A) Immunofluorescence staining of OGC and DIC (green) of the posterior eye cup of mice. DAPI (blue) was used to counterstain nuclei. In upper panels diffuse green fluorescence can be seen in RPE. Strong green fluorescence was noted at the photoreceptor inner segment which harbor abundant mitochondria. Bottom panels show negative control devoid of primary antibody for OGC and DIC. Scale bar: 20 μm. (B) Protein expression of OGC and DIC in RPE/choroid of mice. Mitochondrial and cytosolic fractions of RPE/choroid from mice were isolated and immunoblotted for DIC and OGC. Both carriers are expressed in mitochondrial fraction of RPE/choroid and are absent in the cytosol. IS: photoreceptor inner segments; ONL, outer nuclear layer; OS, photoreceptor outer segments.
lished that OGC and DIC transport of GSH is unaltered by the above transporters in kidney mitochondria. In the present work, we show that, in the presence of excess amount of oxoglutarate and malate, mGSH levels are significantly decreased in RPE as compared to controls (Fig. 11) indicating the above two competitively inhibit GSH transporters. This finding is consistent with previous work,\textsuperscript{18,37} which showed similar inhibition in other cell types.

Mechanism of cellular protection by the two mitochondrial GSH transporters has been studied to a large extent in cultured cells. In recent studies, Kamga et al.\textsuperscript{38} showed that DIC and not OGC is essential for reactive oxygen species homeostasis and normal respiratory functions using rat brain mitochondria. Furthermore, upregulation of DIC was shown to improve cellular redox homeostasis and energy metabolism in a cancer cell line.\textsuperscript{46} Working with germline mutations in the mitochondrial OGC (SLA25A11) gene, Buffet et al.\textsuperscript{49} reported that OGC acts as a tumor suppressor gene. Our present study shows both OGC and DIC are prominently expressed in cultured hRPE cells and mouse retina layers. Assessing the relative contribution of each of these transporters in the retina would require studies with knockout tissues. To our knowledge, knockout animal models are not available at the present time to address this question.

The expression and localization of OGC and DIC in RPE/choroid and in the inner nuclear layer of the mouse retina suggests that OGC and DIC may also contribute to the health of photoreceptors and the neural retina, though additional supportive evidence using astroglial cells and Müller cells will be necessary to confirm this hypothesis. It is hoped that the information presented here will be of use in studies on the expression of mGSH transporters in pathophysiological mouse models and in devising strategies for restoring mitochondrial and cellular integrity through increasing GSH status.

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