PEDF Improves Mitochondrial Function in RPE Cells During Oxidative Stress

Yuan He,1,2 Kar Wah Leung,3 Yuan Ren,2 Jinzhi Pei,2 Jian Ge,4 and Joyce Tombran-Tink1,2,4,5

1Department of Neural and Behavioral Sciences, Pennsylvania State University College of Medicine, Hershey, Pennsylvania, United States
2Department of Ophthalmology, The Second Affiliated Hospital of Xi’an College of Medicine, Xi’an, China
3Division of Life Science, The Hong Kong University of Science and Technology, Hong Kong
4State Key Laboratory of Ophthalmology, Zhongshan Ophthalmic Center, Sun Yat-sen University, Guangzhou, China
5Department of Ophthalmology, Pennsylvania State University College of Medicine, Hershey, Pennsylvania, United States

Correspondence: Joyce Tombran-Tink, Penn State University College of Medicine, 500 University Drive, Hershey, PA 17033, USA; jttink@aol.com; jxt57@psu.edu.
Submitted: April 30, 2014
Accepted: August 30, 2014

PURPOSE. Oxidative stress plays an important role in health and aging. We have shown that oxidative stress impairs mitochondrial function and promotes RPE cell death in an age-dependent manner. This study investigates the role of pigment epithelium-derived factor (PEDF) in limiting oxidative stress–induced damage to RPE cells through mitochondrial pathways.

METHODS. Three groups of early-passaged RPE cells from donors 50 to 55, 60 to 65, and 70 to 75 years old (yo) were either preconditioned with PEDF followed by exposure to sublethal doses of hydrogen peroxide (H2O2) or post-treated with PEDF after H2O2 treatment. Effects of PEDF on mitochondrial function and cell viability were examined.

RESULTS. Oxidative stress induced an age-dependent increase in LDH release, reactive oxygen species (ROS) levels, and cell death and a decrease in adenosine triphosphate (ATP) production and mitochondrial membrane potential (ΔΨm) in human RPE cells. Preconditioning or poststressed treatment with PEDF resulted in increased cell viability, inhibition of cytochrome c release and caspase 3 cleavage, and improved mitochondria function denoted by a decrease in ROS generation and increases in ATP production and ΔΨm. Oxidative stress also disrupted the reticular network, trafficking, and distribution of the mitochondria and blocked activation of phosphatidylinositol 3 kinase (PI3K), Akt, and Erk signaling in the cells. These effects were more pronounced in RPE cells from individuals >60 yo compared to the 50 to 55 yo age group. Pigment epithelium-derived factor mitigated negative effects of oxidative stress on mitochondrial remodeling and cellular distribution and unblocked its control of PI3K/Akt and mitogen-activated protein kinase (MAPK) signaling. Although PEDF potentiated both PI3K/Akt and MAPK signaling in the cells, stabilization of mitochondrial networks and function was dependent on its activation of PI3K/Akt. Specificity of PEDF’s activity was confirmed using the pharmacological inhibitors LY294002, SH6, and U0126. We also show that in the absence of oxidative stress, pharmacological inhibition of the PI3K/Akt pathway alone was sufficient to disrupt mitochondrial structure and function. In addition, PEDF blocked effects of oxidative stress on expression of cyclophilin D and UCP2, genes controlling mitochondrial function, and the apoptotic genes caspase 3, Bax, and Bcl2. Control of ROS levels by PEDF was specifically linked to UCP2 regulation since PEDF-induced expression of this gene in UCP2-deficient cells was associated with a decrease in ROS production.

CONCLUSIONS. We provide evidence that PEDF promotes resilience of aging RPE cells to oxidative stress by stabilizing mitochondrial networks and function and that mitochondrial dynamics in human RPE cells are controlled, in part, through the PI3K/Akt pathway.

Keywords: RPE, aging, mitochondria, ROS, mitochondrial membrane potential, ATP, PEDF, PI3K/Akt, MAPK, UCP2, cyclophilin D, caspase 3, apoptosis, oxidative stress, mitochondrial structure

Oxidative stress is a critical factor in the pathogenesis of chorioretinal disorders, including age-related macular degeneration (AMD) and diabetic retinopathy.1,2 Reactive oxygen species (ROS), generated during phagocytosis of oxidized photoreceptor outer segment,3 contribute to a high-stress environment in the retina and are believed to be a factor in several retinal diseases. Photoreceptor renewal can lead to excessive accumulation of cholesterol ester and oxidized lipids, which are both associated with early pathogenesis of AMD.4 High glucose–driven overproduction of ROS and oxidative stress are also components of diabetic retinopathy.5,6
Several studies demonstrate that nonlethal doses of oxidative stress perturb expression of many important functional groups of proteins including protective and chaperone proteins, antioxidant enzymes, apoptotic factors, and DNA-repair enzymes,5,7–12 many of which are involved in the control of mitochondrial function. This is not surprising since a range of pathological structural events in cells are induced by oxidative stress, including actin reorganization,13 membrane blebbing, 12 apoptosis,14 redistribution of para-cellular junctional proteins,9 and changes in paracellular permeability.9,15

A key cellular target of oxidative stress is the mitochondrion. These organelles control cell death processes and are a major source of ROS, with nearly 4% of the oxygen utilized by the electron transport chain converted to superoxide anions.16,17 While these organelles generate ROS, they are also cellular targets of ROS, and their dysfunction may play a role in aging, tissue health, and disease.18–21 Oxidative insult to the mitochondria leads to perturbation of its metabolic and redox status, altered membrane permeability transition, and cytochrome c leakage, which triggers a cascade of apoptotic events in cells.22,23

We have shown that mitochondrial function declines in RPE cells with increased chronological age and that oxidative stress aggravates this condition, resulting in cell damage, supporting arguments that mitochondrial dysfunction may be linked to RPE injury and the onset of AMD.19,20,24 In this study we tested effects of the neuroprotective factor pigment epithelium-derived factor (PEDF) in limiting vulnerability of aging human RPE cells to oxidative stress by mitochondrial pathways.

**MATERIALS AND METHODS**

Tissue culture reagents were obtained from Gibco BRL (Gaithersburg, MD, USA). 2',7'-dichlorodihydrofluorescin diacetate (H$_2$DCF-DA), 5,5',6,6'-tetrachloro 1,1',3,3'-tetraethylbenzimid azolocarbocyanine iodide (JC-1), Fluo-3/AM, Rhod-2/AM, Mito Tracker Red, and Mitosox Red were obtained from Molecular Probes (Eugene, OR, USA). Hydrogen peroxide (H$_2$O$_2$) and luciferin/luciferase-based adenosine triphosphate (ATP) assay reagents were purchased from Sigma (St. Louis, MO, USA) and lactate dehydrogenase (LDH) reagents from Roche Pharmaceuticals (Nutley, NJ, USA). PEDF monoclonal antibody, LY294002, SH6, and U0126 were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). Goat anti-mouse Alexa Fluor 488-conjugated antibody was purchased from Abcam (Cambridge, MA, USA), and PEDF protein and polyclonal antibody were generated in our lab.
Primary Human RPE Culture

Human eyes from individuals between 50 and 75 years of age were obtained from nondiseased donors at the Zhongshan Ophthalmic Center, Sun Yat-sen University, Guangzhou, China, and RPE was isolated as previously described.34 Five to seven different donor RPE lines were used for each age group. Primary RPE cultures were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% fetal bovine serum (FBS). All cultures were used between passages 3 and 6, and data were generated from the average of triplicate experiments for each donor line.

Characterization of RPE Cells

Expression of RPE-65, an RPE cell marker, was examined to assess RPE homogeneity in the cultures. Cells were grown on polylysine (10 μg/mL)-coated glass coverslips at a density of $1 \times 10^5$ cells/well for 48 hours, fixed with 4% paraformaldehyde for 15 minutes, washed, and incubated with 5% bovine serum albumin (BSA) containing 0.1% Triton X-100 for 30 minutes. The cells were subsequently immunolabeled with mouse monoclonal anti-RPE-65 (1:250) at 4°C overnight, washed with PBS, and incubated for an additional 45 minutes with goat anti-mouse Alexa Fluor 488 (Abcam). Normal mouse and rabbit serum (1:1000) were used instead of the RPE-65 antibody in some experiments to serve as negative controls. Labeled cells were visualized by confocal microscopy (Carl Zeiss Jena GmbH, Jena, Germany).

Treatment With Hydrogen Peroxide and PEDF

The experiments were carried out as follows.

Cell Death Measurements. Retinal pigment epithelial cells were seeded at $1 \times 10^5$ cells/well for 24 hours. Effects of PEDF were examined by adding 100 ng/mL PEDF to the cells 48 hours prior to or 24 hours post H$_2$O$_2$ treatment. This dose of PEDF was selected because 50 to 100 ng/mL PEDF is considered to be an effective dose range for neuroprotective activity in cell cultures.26-29,31-33 In mitochondrial function studies, we also tested effects of 250 ng/mL PEDF. H$_2$O$_2$ was added at concentrations of 0, 80, 160, or 320 μM for 2 hours. Sensitivity of the RPE cells to H$_2$O$_2$ toxicity was estimated using an LDH assay, staining with propidium iodide (PI), and phase-contrast microscopy (Carl Zeiss Jena GmbH).
PEDF Limits Oxidative Stress Damage in RPE Cells

LDH Assay. Culture supernatant (50 μL) was incubated with an equal volume of LDH reaction mixture for 30 minutes. The reaction was terminated and absorbance measured at 490 nm using a Benchmark Microplate Reader (BioRad, Hercules, CA, USA). Cell death, proportional to the LDH activity, was calculated as a percentage of nontreatment controls in each age group. Triplicate measurements were taken for the samples.

Propidium Iodide Staining. Cells seeded on coverslips were incubated in 4 μg/mL PI solution to label the nuclei of dying cells. Percentages of labeled cells were estimated under epifluorescence microscopy (Carl Zeiss Jena GmbH).

Measurement of Cytochrome c Release in the RPE Cells

Cytochrome c release was visualized using confocal microscopy. Cells were seeded onto polylysine (10 μg/mL)-coated glass chamber slides at a density of 2000 cells/chamber. After washing with PBS, cells were fixed in ice-cold 4% paraformaldehyde for 15 minutes and permeabilized in 4 minutes in 100 mM phosphate buffer, 1 mg/mL bovine serum albumin (BSA), and 0.2% Triton X-100. Endogenous peroxidase activity was quenched with 3% H2O2, then cells were incubated with 0.5% blocking reagent for 30 minutes (TSA-Direct kit; Dupont-NEN, Boston, MA, USA) and immunolabeled with mouse monoclonal anti-cytochrome c (1:1000 dilution) at room temperature for 1 hour. Normal mouse IgG was used instead of anti-cytochrome c in some experiments to serve as negative control. After incubation with the primary antibody, cells were washed and incubated for 45 minutes with horseradish peroxidase-conjugated goat anti-mouse IgG (1:50), followed by fluorescein isothiocyanate (FITC)-tyramide solution (1:50) for 10 minutes. Cells were subsequently washed and mounted using fluorescent mounting medium, and labeling was visualized by a Zeiss 100 M confocal microscope (Carl Zeiss Jena GmbH).

For morphological analysis of mitochondria, treated and nontreated cells were labeled with 50 nM Mito Tracker Red (Molecular Probes) for 30 minutes at 37°C, and mitochondrial distribution and organization in the cells were visualized by confocal microscopy at an excitation wavelength of 488 nm and emission of 590 nm.

Mitochondrial Function

Reactive oxygen species, mitochondrial membrane potential (ΔΨm), and ATP levels were measured in H2O2-treated cells after pre- and postconditioning with PEDF for 24 or 48 hours. Measurements were carried out essentially as we have described previously.19,20 For all flow cytometry measurements, 10,000 cells were analyzed.

ROS Measurements. Retinal pigment epithelium cells were detached by trypsinization, and 2 × 106 cells/mL were incubated with 0.4 μM H2-DCF-DA at 37°C for 30 minutes. Cells (10,000/treatment) were analyzed by flow cytometry (Becton, Dickinson and Co., Franklin Lakes, NJ, USA) using 488-nm excitation and 530-nm emission wavelengths, and data were processed using the FCS Express software (Becton, Dickinson and Co.). Reactive oxygen species generation was also estimated by confocal microscopy of cells labeled with 5 μM Mitosox Red (Molecular Probes) for 10 minutes at 37°C.

Mitochondrial Membrane Potential. ΔΨm measurements were calculated after incubating cells with 1 μg/mL JC-1 for 20 minutes at 37°C. Labeled cells were analyzed by flow cytometry at an excitation wavelength of 488 nm. Data were collected at an emission wavelength of 530 nm for green fluorescence and 590 nm for red fluorescence. Results are expressed in arbitrary units as mean fluorescence intensity of 10,000 cells per sample.

ATP Levels. Adenosine triphosphate levels were determined using a luciferin/luciferase-based assay. 1 × 105 cells/well in 96-well plates were permeabilized after treatments using 50 μL somatic cell ATP-releasing reagent, and samples were incubated with 50 μL ATP reagent containing luciferin and luciferase. Luminescence was measured using a luminescence spectrometer (Orion II Luminometer; Berthold Detection Systems, Oak Ridge, TN, USA), and cellular ATP levels were expressed as the percentage of luminescence intensity compared to nontreated controls. Data are expressed as arbitrary units as the mean fluorescence intensity.

Cell Signaling

Effects of PEDF and acute oxidative stress on various signaling pathways were also examined. Cultures were treated with 320 μM H2O2 for 1 hour followed by treatment with PEDF for 1 hour. Some cultures were treated for 1 hour prior to addition of H2O2 with the following pharmacological inhibitors: PI3K inhibitor LY294002 (25 μM), the Akt inhibitor SH6 (25 μM), or the MAPK/Erk inhibitor U0126 (10 μM). Controls were cultured in medium supplemented with 0.2% dimethyl sulfoxide, which was used as the solvent for the inhibitors. Samples were processed for mitochondrial labeling, microscopy, and Western blot analysis.

Western Blot Analysis

Retinal pigment epithelial cells were lysed using Cytobuster lysis buffer (Novagen, Madison, WI, USA) and protein concentrations estimated using DC Protein Assay Kit (BioRad). Protein (30 μg) was separated by SDS-PAGE and transferred onto nitrocellulose membranes (BioRad). After blocking with
5% (wt/vol) nonfat dried milk, membranes were incubated with primary antibodies (1:1000) for 3 hours at room temperature (RT), followed by washing and incubation with horseradish peroxidase–conjugated secondary antibodies for 1 hour at RT. Bound antibody was determined using the Bio-Rad ECL detection system, and ImageJ software (National Institutes of Health, Bethesda, MD, USA) was used to quantitate protein levels.

**RT- and Real-Time PCR**

Total mRNA was isolated using RNeasy kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s protocol. First-strand cDNA was synthesized using iScript cDNA synthesis kit (Bio-Rad), and RT-PCR was carried out using 300 ng cDNA with iTaq polymerase (Bio-Rad) at an annealing temperature of 58°C for 35 cycles for cyclophilin D, UCP2, caspase 3, Bcl2, and Bax. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the internal RNA loading control, and samples for which no reverse transcriptase (NRTs) was added to the PCR experiments were used as negative controls. The PCR products were resolved by 1% agarose gel electrophoresis. For quantitative real-time PCR, the two-step amplifying protocol was used with IQ SYBR Green Supermix solution (Bio-Rad). Both the melting curve and gel electrophoretic analyses were used to determine amplicon homogeneity and quality of the data.
in fluorescence levels compared to controls, arbitrarily set as 1, and results are expressed as the mean H2O2-induced reduction in ATP levels. ATP levels, quantified by a luciferin/luciferase-based assay, were 35% to 45% lower in the two oldest RPE age groups compared to the youngest. Oxidative stress further decreased ATP levels from the baseline by 15% (P < 0.05) (8). PEDF preconditioning promoted ATP generation above baseline and blocked H2O2 effects on ATP levels. Data are shown as fold changes in fluorescence levels compared to untreated controls, and results are expressed as the mean ± SE (n = 3; P < 0.05). (B) PEDF prevents H2O2-induced reduction in ATP levels. ATP levels, quantified by a luciferin/luciferase-based assay, were 35% to 45% lower in the two oldest RPE age groups compared to the youngest. Oxidative stress further decreased ATP levels from the baseline by 15% (P < 0.05) (8). PEDF preconditioning promoted ATP generation above baseline and blocked H2O2 effects on ATP levels. Data are shown as fold changes in fluorescence levels compared to untreated controls, and results are expressed as the mean ± SE (n = 3; P < 0.05).

UCP2 Knockdown

The UCP2-siRNA was purchased from GeneCopoeia (Rockville, MD, USA). Transfection was carried out according to the manufacturer’s recommendations. The human ARPE19 cell line (purchased from ATCC, Manassas, VA, USA) was used in these experiments since these cells have the same sensitivity to oxidative stress as 50 to 55 yo RPE cells with respect to mitochondrial pathology, can be rescued by PEDF, and can withstand the transfection process much better than primary cultures of human RPE cells (He Y, Tombran-Tink J, unpublished data, 2014). Cells were grown to ~80% to 85% confluence and incubated with 1.5 μg scrambled siRNA or UCP2 siRNA in the presence of EndoFectin Lenti transfection reagent (3 μL/mL) (GeneCopoeia). Transfection was carried out in the absence of serum in growth medium. Three hours after transfection, one-half volume of DMEM containing 30% FBS was added to the cells, and cultures were grown for 48 hours. The UCP2 siRNA efficacy was estimated by confocal microscopy and real-time PCR. ARPE19 cells transfected with control (Mock) siRNA were used as a negative control. Pigment epithelium-derived factor was added to some UCP2 siRNA-treated cultures 6 hours after transfection for 48 hours. PEDF limits oxidative stress damage in RPE cells.

RESULTS

RPE Cell Cultures

Retinal pigment epithelium cell homogeneity in primary cultures was confirmed by the expression of RPE-65 and by morphological appearance of the cells. Greater than 99% of cultured cells from all age groups expressed RPE-65 in their cytoplasmic compartments (Fig. 1). With increased donor age, RPE cells appeared larger in size and showed greater cytoplasmic spreading. PEDF reduces H2O2-induced ROS generation in RPE cells. Comparative differences in ROS production as measured by H2-DCF-DA fluorescence using flow cytometry are shown in nonpreconditioned and PEDF-preconditioned cells exposed to oxidative stress. ROS levels increased by 112% (P < 0.05). 120% (P < 8.4), and 201% (P < 7.3) in 50 to 55, 60 to 65, and 70 to 75 yo cells, respectively, compared to untreated controls. PEDF suppressed H2O2-induced ROS elevation with 100 ng/mL PEDF by 32% (P < 8.2), 45% (P < 6.4), and 37% (P < 2.5) in the 50 to 55, 60 to 65, and 70 to 75 yo RPE cultures, respectively (P < 0.05). Data are shown as fold changes in fluorescence levels compared to controls, arbitrarily set as 1, and results are expressed as the mean ± SE (n = 3; P < 0.05). (B) PEDF prevents H2O2-induced reduction in ATP levels. ATP levels, quantified by a luciferin/luciferase-based assay, were 35% to 45% lower in the two oldest RPE age groups compared to the youngest. Oxidative stress further decreased ATP levels from the baseline by 15% (P < 0.05) (8). PEDF preconditioning promoted ATP generation above baseline and blocked H2O2 effects on ATP levels. Data are shown as fold changes in fluorescence levels compared to untreated controls, and results are expressed as the mean ± SE (n = 3; P < 0.05).

Statistical Analysis

All assays were performed using five to seven independent primary cultures of RPE cells between passages 3 and 6, and each experiment was done in triplicate. Data are expressed as means ± standard error (SE). A one-way ANOVA test was performed, and statistical significance was set at P < 0.05.
release of cytochrome c from the mitochondria into the cytosol of RPE cells. This was evident by overlapping green (cytochrome c staining) and red (mitochondria staining) fluorescence labeling in the cells after PEDF treatment and distinct green and red labeling in the H2O2 samples (Fig. 3). We also show that the 17-kDa cleaved caspase 3 product associated with apoptosis was induced by H2O2 treatment (Fig. 4). Cleavage of the 37-kDa caspase 3 protein was not detected in controls or PEDF-treated samples, providing evidence that H2O2-induced RPE cell death was caspase 3 dependent and that PEDF was effective in reversing this effect.

Oxidative Stress Disrupts Mitochondrial Reticular Networks: Reversal by PEDF

A correlation between oxidative stress and disruption of the mitochondrial reticular networks in the RPE cells was observed. Oxidative stress effects on mitochondrial structure and organization were more pronounced with increased chronological age of the RPE cells (Fig. 5). In nonstressed cultures the mitochondria formed fine branching, interconnected networks throughout the cell body and at the leading cytoplasmic edges of the cells. Within an hour of treatment, oxidative stress disrupted the branching mitochondrial networks and reorganized them into large, punctate perinuclear aggregates. This was especially prominent in the >60 yo RPE cells. In all age groups, pretreatment with PEDF promoted resilience to mitochondrial fragmentation and redistribution under stress conditions. This was apparent in the extent of reduced punctate, perinuclear clusters of mitochondria after PEDF treatment and redistribution of these organelles throughout the cytoplasmic compartment of the cells.

PEDF Improves Mitochondrial Function in Oxidative Stress Conditions

Not only did oxidative stress alter mitochondria structure and distribution in RPE cells; it reduced mitochondrial function as well, with severity more prominent in RPE cells obtained from donors >60 yo (Fig. 6). Treatment of H2O2-exposed cultures with 100 ng/mL PEDF reduced stress-induced effects on ROS levels by 32% (±3.1), 47% (±2.3), and 27% (±2.3), and with 250 ng/mL PEDF by 50% (±3.3), 45% (±2.5), and 37% (±2.5) in the 50 to 55, 60 to 65, and 70 to 75 yo RPE cultures, respectively (Fig. 6A) (P < 0.05). Relative ATP levels were increased in H2O2 conditions after treatment with 100 ng/mL PEDF by 97% (±2.2), 23% (±2.1), and 17% (±2.0) and after 250 ng/mL PEDF by 106% (±1.6), 35% (±1.5), and 28% (±1.7).
FIGURE 8. (A, B) PEDF reduces H$_2$O$_2$ effects on mitochondrial membrane potential ($\Delta$Ψ$_m$). The $\Delta$Ψ$_m$ measured by JC-1 flow cytometry were 32% (± 5.1) and 90% (± 6.5) lower in 60 to 65 and in 70 to 75 yo RPE cells, respectively, compared to 50 to 55 yo cells. Relative amounts of red-to-green fluorescence ratio are shown. H$_2$O$_2$ treatment led to an additional decline in $\Delta$Ψ$_m$ by 25% (± 3.9), 70% (± 4.1), and 70% (± 4.3) in 50 to 55, 60 to 65, and 70 to 75 yo cells, respectively, compared to controls. 100 ng/mL PEDF preconditioning reduced H$_2$O$_2$ effects on the $\Delta$Ψ$_m$ in the oldest to youngest samples by 100% (± 4.5), 19% (± 3.9), and 19% (± 4.0) and treatment with 250 ng/mL PEDF by 116% (± 4.9), 32% (± 7.7), and 27% (± 7.7) compared to H$_2$O$_2$ alone. Results are expressed as mean fold decrease in fluorescence levels compared to untreated samples ± SE ($n = 3$; $P < 0.05$).

FIGURE 9. (A) Western blot and (B–D) densitometry analysis of Western blots for pPI3K, p-Akt, and p-Erk. The data show that PEDF induced phosphorylation of PI3K, Akt, and Erk and that its effects on activation of these signaling molecules were blocked by pharmacological inhibition of the associated pathways. Data are shown as fold changes in band intensity compared to the controls, arbitrarily set as 1 ($n = 6$; $P < 0.05$). *Significantly different from control; #, significantly different from H$_2$O$_2$ treatment ($n = 6$; $P < 0.05$); ###, significantly different from PEDF treatment ($n = 6$; $P < 0.05$); ####, significantly different from H$_2$O$_2$+PEDF treatment ($n = 6$; $P < 0.05$).
in the 50 to 55, 60 to 65, and 70 to 75 yo RPE cultures, respectively, compared to cultures treated with H₂O₂ alone (Fig. 6B) (P < 0.05). Stress-induced regulation of mitochondrial ROS levels was confirmed using the mitochondrial-specific ROS detector, Mitosox Red, which showed greater labeling in oxidative stress–induced fragmented mitochondria especially in the >60 yo cell population (Fig. 7). Pretreatment with PEDF showed a reduction in mitochondrial ROS levels compared to cells challenged with H₂O₂ alone. Similarly, stress-induced changes in the ΔΨm were more severe in RPE cells with increased donor age (Fig. 8). H₂O₂ treatment led to a decline in ΔΨm by 25% (±3.9), 70% (±4.1), and 70% (±4.3) in 50 to 55, 60 to 65, and 70 to 75 yo RPE cells, respectively (P < 0.05). The addition of PEDF to the cultures resulted in a marked reduction in H₂O₂ effects on ΔΨm in all cultures. Treatment of H₂O₂-exposed cells with 100 ng/mL PEDF increased ΔΨm by 100% (±4.5), 19% (±3.9), and 19% (±4.0), and treatment with 250 ng/mL PEDF by 116% (±4.9), 32% (±7.7), and 27% (±7.7) in the 50 to 55, 60 to 65, and 70 to 75 yo RPE cells, respectively, above the comparative H₂O₂-treated cultures (P < 0.05).

Differences between the two doses of PEDF were more pronounced in the 50 to 55 yo cultures. Pigment epithelium-derived factor treatment did not fully return mitochondrial function to nonstressed baseline levels in the >60 yo RPE cultures. Both doses of PEDF returned ATP levels and ΔΨm to baseline in the 50 to 55 yo samples. Increasing PEDF dose had an overall slightly better effect on mitochondrial function.

These studies suggest that a possible mechanism for PEDF’s neuroprotective actions is through restoring mitochondrial dynamics perturbed by both aging and oxidative stress conditions.

**PEDF Effects on Mitochondrial Structure and Function Are Mediated Through the PI3K/Akt**

Oxidative stress modulated two important signaling mechanisms in RPE cells, the PI3K/Akt and the MAPK pathways (Fig. 9). In addition, an inverse relationship between aging and activation of these pathways was noted. Treatment with PEDF prevented negative effects of oxidative stress on activation of both pathways, and the specificity of its actions was confirmed using pharmacological inhibitors (Fig. 9). Inhibition of the PI3K/Akt pathway blocked the actions of PEDF on stress-induced changes in mitochondrial branching and redistribution in the RPE cells (Fig. 10), as well as function of these organelles (Figs. 11, 12). Effects of PEDF on ROS production, ATP levels, and the ΔΨm were all blocked in the presence of the PI3K inhibitor, LY294002, and the Akt inhibitor, SH6. Pharmacologically blocking the MAPK pathway with U0126, however, was not effective in reducing effects of PEDF on mitochondria reorganization or function. These findings suggest that increased aging and stress reduce activation of the PI3K/Akt and MAPK pathways in RPE cells and that the actions of PEDF on mitochondrial dynamics are mediated, in part, through the PI3K/Akt pathway.

*Figure 10.* PEDF regulates mitochondrial branching through PI3K/Akt signaling. Effects of PEDF to reduce H₂O₂-induced mitochondrial fragmentation and perinuclear aggregation were blocked by pharmacological inhibition of the PI3K/Akt pathway. Inhibition of MAPK/Erk pathway did not block effects of PEDF to promote mitochondrial reassembly throughout cells exposed to oxidative stress (60-65 yo RPE cells; scale bar: 15 μm).
FIGURE 11. Effects of PEDF on mitochondria function are mediated through the PI3K/Akt pathway. (A) Flow cytometry measurements of ROS levels by H2-DCF-DA fluorescence; (B) ATP levels by luciferin/luciferase-based assay. (C) Mitosox Red uptake of cells treated with H2O2, PEDF, or both in the presence of PI3K (LY294002), Akt (SH6), and MAPK/Erk (U0126) inhibitors shows specificity of PEDF’s action on mitochondrial function through the PI3K/Akt pathway (60-65 yo RPE cells; n = 3; P < 0.05).
PEDF Regulates Genes Involved in Mitochondrial Function and Apoptosis

Since our studies show that both oxidative stress and PEDF target mitochondrial health, we examined how these agents control expression of genes associated with mitochondrial function (Fig. 13). Transcription of cyclophilin D, a regulator of mitochondrial permeability transition, was not significantly altered among the three RPE aged groups but was induced by oxidative stress in all RPE cultures in an age-dependent manner ($P = 0.035, 50-55\ yo; P = 0.026, 60-65\ yo; P = 0.023, 70-75\ yo$). Cyclophilin D expression was reduced to below baseline levels in all cultures when PEDF was added ($P = 0.048, 50-55\ yo; P = 0.029, 60-65\ yo; P = 0.037, 70-75\ yo$). Transcription of the mitochondrial uncoupling protein UCP2, on the other hand, was elevated by both $H_2O_2$ ($P = 0.012, 50-55\ yo; P = 0.009, 60-65\ yo; P = 0.013, 70-75\ yo$) and PEDF ($P = 0.009, 50-55\ yo; P = 0.008, 60-65\ yo; P = 0.008, 70-75\ yo$). Pigment epithelium-derived factor, however, induced much higher levels of UCP2 expression in the presence of stress compared to $H_2O_2$ ($P = 0.009, 50-55\ yo; P = 0.007, 60-65\ yo; P = 0.008, 70-75\ yo$).

Oxidative stress also induced expression of the proapoptotic genes caspase 3 ($P = 0.036, 50-55\ yo; P = 0.028, 60-65\ yo; P = 0.025, 70-75\ yo$) and Bax ($P = 0.048, 50-55\ yo; P = 0.009, 60-65\ yo; P = 0.016, 70-75\ yo$) and reduced levels of the antiapoptotic Bcl2 ($P = 0.033, 50-55\ yo; P = 0.037, 60-65\ yo; P = 0.008, 70-75\ yo$), while PEDF had the opposite effects on these genes and reversed their regulation by $H_2O_2$ ($P = 0.038, 50-55\ yo; P = 0.032, 60-65\ yo; P = 0.015, 70-75\ yo$) (Figs. 8A, 8B). The severity of the effects of oxidative stress on expression of apoptotic genes was age dependent.

PEDF Modulates Mitochondrial Function by Regulating UCP2 Expression

Functional knockdown of UCP2 by siRNA (50-55 yo RPE cells) resulted in an 83.66% ($P = 0.023$) knockdown efficacy of the gene (Fig. 14). The UCP2-deficient cells showed more robust generation of ROS under stressed conditions compared to control ($P < 0.05$) (Fig. 15). Treatment of UCP2-depleted cells with PEDF increased levels of the UCP2 mRNA over those in the untreated depleted cells ($P < 0.05$) (Fig. 14), which was associated with reduced production of mitochondrial ROS below levels seen in wild-type ($P < 0.05$) and in deficient cells that were exposed to stress ($P < 0.05$) (Fig. 15).
Taken together, our results suggest that PEDF counteracted effects of stress on RPE cells by modulating activation of PI3K/Akt and expression of UCP2.

**DISCUSSION**

Accumulated oxidative injury is an underlying mechanism in aging and diseases, including Alzheimer’s disease, amyotrophic lateral sclerosis, muscular dystrophy, Parkinson’s disease, and cataract. These are all late- or slow-onset diseases in which oxidative damage accumulates over time and is associated with mitochondrial pathologies.

Cumulative oxidative damage to the RPE is a causal factor in retinal injury. We previously reported that susceptibility of human RPE cells to oxidative stress increases with chronological human aging and that mitochondrial dysfunction was central to the vulnerability of these cells to stress.

Oxidative stress decreased $\Delta \Psi m$ and ATP output and increased ROS levels in an age-dependent manner, suggesting that aging compromises mitochondrial function, making RPE cells less resilient to stress.

We have expanded our studies to determine whether the function of the mitochondria in aging RPE cells can be improved by neuroprotective intervention and to examine the impact of such treatment on the responsiveness of aging cells to oxidative stress.

The data obtained from this work indicate that oxidative stress promotes mitochondria remodeling, trafficking, and loss of function and that PEDF promotes reassembly of mitochondrial reticular networks and optimizes function of these organelles in stressed RPE cells. With increased aging, RPE cells showed reduced activation of the PI3K/Akt and MAPK pathways and were less tolerant to mitochondrial and cellular damage by oxidative stress, suggesting that optimizing signaling through these pathways is beneficial to survival of aging RPE cells. These results support studies showing that the life span of Akt1$^{-/-}$ mice compared to wild-type littermates was much shorter when they were exposed to genotoxic stress. Pigment epithelium-derived factor restored mitochondrial dynamics that were perturbed in aging and oxidative stress conditions; and although it activated both PI3K/Akt and MAPK signaling in RPE cells, its protective effects were specifically coupled to its activation of PI3K/Akt. Our findings are novel in their implications, first, that the neuroprotective actions of PEDF are linked to its effects on mitochondrial remodeling and function, and secondly, that mitochondrial dynamics are regulated in part through the PI3K/Akt pathway.

Given that oxidative stress induced structural remodeling and functional pathologies in mitochondria, it was not entirely surprising to see upregulation of cyclophilin D expression, a protein regulating mitochondrial transition permeability pore (MPTP). However, it is unclear whether reduction of cyclophilin D expression by PEDF is linked to mitochondrial remodeling, but preventing MPTP opening is certain to have beneficial consequences on mitochondrial function.
The mitochondrial uncoupling protein UCP2 controls production of mitochondria-derived ROS. Absence of this protein is implicated in accumulation of ROS in Ucp2-/- mice. In our study, UCP2 transcription was reduced with increased aging of RPE cells, and this was further compromised in oxidative stress conditions. Our results, showing that UCP-deficient RPE cells had increased ROS levels and that increasing UCP2 expression with PEDF dampened ROS production in aging and deficient cells exposed to oxidative stress, suggest that modulation of UCP2 function is another factor influencing vulnerability of aging RPE to stress.

The effects of aging on mitochondrial dynamics in human retinas are supported by studies showing that structural abnormalities in the mitochondrial populations of the RPE increase with aging and worsen in individuals with AMD. Other lines of evidence implicate specific mitochondrial DNA haplogroups in perturbation of mitochondrial function and as risk factors for AMD development.

Our observations that perturbations in mitochondrial dynamics increase vulnerability of aging RPE cells to oxidative stress and are a risk factor for developing pathologies make a strong case that the mitochondria are a viable target in retinal diseases.

The effects of PEDF in mitigating stress-induced cellular damage, largely as a consequence of attenuating mitochondrial structural and functional deficits, have implications for this—and other neuroprotective molecules that control mitochondrial dynamics—as rational targets for valuable research in retinal diseases.

Acknowledgments

Supported by grants from the Ben Franklin Foundation, Pennsylvania, United States; The Juvenile Diabetes Research Foundation, United States; the National Natural Science Foundation of China (No. 81100665); the Departments of Health (No. 2010D56), Education (No. 2013J0798), and Science and Technology (No. 2011K14-02-07) and No. 2013KJXX-31 of Shaanxi Province, China; grants from Xi’an Medical University (10FC01) and The Second Affiliated Hospital of Xi’an Medical University (10EYFPC01).

Disclosure: Y. He, None; K.W. Leung, None; Y. Ren, None; J. Pei, None; J. Ge, None; J. Tombran-Tink, None

References

PEDF Limits Oxidative Stress Damage in RPE Cells