Mitochondria impairment correlates with increased sensitivity of aging RPE cells to oxidative stress

Yuan He · Jian Ge · Janice M. Burke ·
Roland L. Myers · Zhi Z. Dong · Joyce Tombran-Tink

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Abstract Impairment of mitochondria function and cellular antioxidant systems are linked to aging and neurodegenerative diseases. In the eye, the retinal pigment epithelium (RPE) is exposed to a highly oxidative environment that contributes to age-related visual dysfunction. Here, we examined changes in mitochondrial function in human RPE cells and sensitivity to oxidative stress with increased chronological age. Primary RPE cells from young (9–20)-, mid-age (48–60)-, and >60 (62–76)-year-old donors were grown to confluency and examined by electron microscopy and flow cytometry using several mitochondrial functional assessment tools. Susceptibility of RPE cells to H₂O₂ toxicity was determined by lactate dehydrogenase and cytochrome c release, as well as propidium iodide staining. Reactive oxygen species, cytoplasmic Ca²⁺ [Ca²⁺]_c, and mitochondrial Ca²⁺ [Ca²⁺]_m levels were measured using

2',7'-dichlorodihydrofluorescein diacetate, fluo-3/AM, and Rhod-2/AM, respectively, adenosine triphosphate (ATP) levels were measured by a luciferin/luciferase-based assay and mitochondrial membrane potential ($\Delta\Psi_m$) estimated using 5,5',6,6'-tetrachloro 1,1',3,3'-tetraethylbenzimid azo-carbocyanine iodide. Expression of mitochondrial and antioxidant genes was determined by real-time polymerase chain reaction. RPE cells show greater sensitivity to oxidative stress, reduction in expression of mitochondrial heat shock protein 70, uncoupling protein 2, and superoxide dismutase 3, and greater expression of superoxide dismutase 2 levels with increased chronological age. Changes in mitochondrial number, size, shape, matrix density, cristae architecture, and membrane integrity were more prominent in samples obtained from >60 years old compared to mid-age and younger donors. These mitochondria abnormalities correlated with lower ATP levels, reduced $\Delta\Psi_m$, decreased [Ca²⁺]_c, and increased sequestration of [Ca²⁺]_m in cells with advanced aging. Our study provides evidence for mitochondrial decay, bioenergetic deficiency, weakened antioxidant defenses, and increased sensitivity of RPE cells to oxidative stress with advanced aging. Our findings suggest that with increased severity of mitochondrial decay and oxidative stress, RPE function may be altered in some individuals in a way that makes the retina more susceptible to age-related injury.

Y. He · R. L. Myers · J. Tombran-Tink (✉)
Department of Neural and Behavioral Sciences,
Pennsylvania State University College of Medicine,
500 University Drive,
Hershey, PA 17033, USA
e-mail: jtink@aol.com

Y. He · J. Ge (✉) · Z. Z. Dong
State Key Laboratory of Ophthalmology,
Zhongshan Ophthalmic Center, Sun Yat-sen University,
54 South Xianlie Road,
Guangzhou 510060, China
e-mail: gejian@mail.sysu.edu.cn

Y. He
Department of Ophthalmology, the Second Affiliated Hospital
of Xi'an College of Medicine,
Xi'an, China

J. M. Burke
Department of Ophthalmology, Medical College of Wisconsin,
Milwaukee, WI, USA

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Abbreviations

AMD	Age-related macular degeneration
RPE	Retinal pigment epithelium
H ₂ -DCF-DA	2',7'-Dichlorodihydrofluorescein diacetate

JC-1	5,5',6,6'-Tetrachloro 1,1',3,3'-tetraethylbenzimid azolocarboxyanine iodide
MPTP	Mitochondrial permeability transition pore
ROS	Reactive oxygen species
$\Delta\Psi_m$	Mitochondrial membrane potential
ATP	Adenosine Triphosphate
$[Ca^{2+}]_c$	Cytoplasmic calcium
$[Ca^{2+}]_m$	Mitochondrial calcium
mtHSP70	Mitochondrial heat shock protein 70
SOD	Superoxide dismutase
UCP2	Uncoupling protein 2

Introduction

It is often argued that the metabolic rate of an organism determines its life span [1–3] and that neurodegenerative diseases associated with advanced aging have a common root in mitochondrial dysfunction. This concept is intellectually appealing since mitochondria are key regulators of the cell's metabolic rate. They produce most of the cells adenosine triphosphate (ATP), generate the bulk of reactive oxygen species (ROS) [4–6], and play an important role in the organism's antioxidant defense systems [7–10]. They propagate in response to the cells energy requirements. When energy needs are high, they will grow and divide. When energy requirements are low, they are selected for mitophagy or become inactive. Mitochondria vary in number from cell to cell, are highly prone to oxidative damage, lack efficient mtDNA repair mechanisms, and can accrue mutations and go unnoticed [11–13]. Abnormal accumulation of defective mitochondria in cells can trigger activation of senescence or apoptosis [14–17].

There is compelling evidence that mitochondrial dysfunction is an early event in Alzheimer's and Parkinson's disease supporting claims that age-related mitochondrial dysfunction is an upstream event in many neurodegenerative disorders [18–21]. This is underscored in studies showing that amyloid precursor protein transgenic mice have decreased mitochondrial membrane potential, lowered ATP-levels and higher ROS levels, altered Bcl-xL/Bax ratio, reduced COX IV activity, and an overall decrease in mitochondrial respiratory function at a stage when there is no detectable level of amyloid-beta deposits in the brain [22]. In Parkinson's disease, mitochondrial impairment is a prelude to dopaminergic cell death [23–25].

Mitochondrial decay is evident during normal aging of tissues as well and causes the cell's anti-stress pathways to operate with less efficiency [26–28]. Specifically, alterations in cytoplasmic Cu/Zn-superoxide dismutase (SOD),

lipid peroxidation, mitochondrial Mn-SOD, and oxidative stress markers have been observed [27, 28]. Knockdown of the mitochondrial heat shock protein 70 promotes progeria-like phenotypes in *Caenorhabditis elegans* implying a direct link between mitochondrial function and aging [29]. These observations reinforce the concept that deficiencies in the cell's bioenergetic machinery are intricately intertwined with the process of aging, a “progressive, generalized impairment of function, resulting in an increased vulnerability to environmental challenge and a growing risk of disease and death” [30].

It is therefore conceivable that mitochondrial dysfunction is a major underlying cause in the progression of age-related retinal diseases such as age-related macular degeneration (AMD), a multifactorial disorder with etiology stemming, in part, from cumulative oxidative damage to the retinal pigment epithelium (RPE) [31–37]. Histological changes in mitochondrial morphology are evident in the RPE at the earliest stages of AMD and precede vision loss, even though the disease has been primarily associated with photoreceptor damage [38–42].

The RPE, which comprises a highly metabolically active monolayer of cuboidal cells, is crucial to the health and function of the retina. Juxtaposed between the photoreceptors apically and the choriocapillaris basally, this epithelium is continuously bombarded by high levels of oxidants [43, 44]. Among its numerous function, it constitutes the blood retinal barrier, facilitates selective transport between the choroidal vasculature and the outer retina, phagocytoses and degrades shed photoreceptor outer segments, regenerates photopigments, secretes neurotrophic, adhesion, and vascular regulatory factors, and contributes to the functional integrity of Bruch's membrane and the choriocapillaris. Disruption in any of these high-energy requiring processes is detrimental to the health of the RPE and retina.

The evidence points to a clear association between RPE health and compromised mitochondrial function. Abnormal regulation of several mitochondrial proteins is noted in AMD retinas including ATP synthase, cytochrome C oxidase, and mitochondrial heat shock protein 70 (mtHsp70) [45]. What is more, experimental findings support a link between mitochondrial impairment and RPE degeneration. Case in point, abnormal mitochondrial features are observed in RPE cells treated with DL-buthionine-(S,R)-sulfoximine, an agent that promotes degeneration of the cells by reducing cellular glutathione levels [46]. When given rod outer segments, preferential damage is seen in mtDNA but not nuclear DNA of RPE [47, 48] and aging promotes mtDNA damage and faulty DNA repair in both epithelium and choroid of rats [49]. In addition, A2E, the major lipofuscin component and byproduct of the visual cycle, can disrupt mitochondrial

function and induce degeneration of cultured RPE cells at concentrations found in the aging human eye [50]. Lipofuscin accumulation is believed to be a major risk factor for AMD.

Given the daily challenges, the RPE faces and its exposure to an onslaught of oxidants in the retina, we asked when in human chronological aging are RPE cells most susceptible to oxidative stress and how does mitochondrial health alter the cells coping mechanisms in a high-stressed environment. We provide ultrastructure, biochemical, and gene expression evidence for mitochondrial decay, attenuation of antioxidant systems, and increased sensitivity of RPE cells to increased oxidative stress as early as age 60 in human aging.

Materials and methods

Materials

Tissue culture reagents were obtained from Gibco BRL (Gaithersburg, MD, USA). Mouse monoclonal anti-RPE65 was purchased from Novus Biologicals (Littleton, CO, USA). 2',7'-Dichlorodihydrofluorescein diacetate (H_2 -DCF-DA), 5,5',6,6'-tetrachloro 1,1',3,3'-tetraethylbenzimidazolylcarbo-cyanine iodide (JC-1), and Mito Tracker Red from Molecular Probes (Eugene, OR, USA), and mouse anti-human cytochrome c from Promega (Madison, WI, USA). The luciferin/luciferase-based ATP, lactate dehydrogenase (LDH), and mitochondria isolation kit assay kits were obtained from Sigma (St. Louis, MO, USA), Roche Pharmaceuticals (Nutley, NJ, USA), and Pierce Chemical Co (Rockford, IL, USA), respectively.

Culture and characterization of primary human RPE cells

Cell culture All human tissues used in this study was procured and managed in accordance with the Tenets of the Declaration of Helsinki and with approval from the Institution Review Board from the Sun Yat-sen University at Guangzhou. We obtained informed written consent from all participants involved in our study. Informed written consent was also received from the parents/guardians of all minor-aged donors used in this research. Human RPE cells were isolated essentially as described by Dr. Janice M Burke [51] from donor samples obtained from either Dr. Burke's lab at the University of Wisconsin or the Zhongshan Ophthalmic Center Eye Bank (Guangzhou) from consenting donors with no known history of ocular diseases. The samples were grouped into three categories: young (age 9–20; $n=7$), mid-age (age 48–60; $n=7$), and >60 (age 62–76; $n=7$) years old. RPE cells were dissected from the central and peripheral regions of the eyes, harvested between 8 and 12 h of death and primary cultures established using identical

conditions for each sample using Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). All experiments described below were carried out in triplicates for each donor sample at three passages—third to fifth—using the same number of RPE cells from 90% confluent monolayer cultures. Histograms generated represent average values of all samples at the three passages within a given group.

Characterization of RPE cultures Purity of the cultures was determined using an RPE specific marker, RPE 65, a protein involved in the conversion of all-*trans* retinol to 11-*cis* retinal during phototransduction in the retina. Cells were grown on polylysine (10 μ g/ml)-coated glass coverslips at a density of 1×10^5 cells/well in 24-well plates for 48 h. Cell were fixed with 4% paraformaldehyde for 15 min, incubated with 5% bovine serum albumin containing 0.1% Triton X-100 for 30 min, immunolabeled with mouse monoclonal anti-RPE65 (1:250) at 4°C overnight, then goat anti-mouse Alexa Fluor 488-conjugated antibody (1:1,000) for 45 min. Normal mouse serum (1:1,000) was used instead of the RPE65 antibody in some experiments to serve as an antibody control. RPE-65 immunoreactivity was visualized by confocal microscopy.

Age-related sensitivity of RPE to oxidative stress

The age-related sensitivity of RPE cells to oxidative stress was assessed in all samples of the three age groups. Cells were exposed to various concentrations of hydrogen peroxide and LDH levels estimated in the conditioned medium. Suspensions of RPE cells was dispensed at 1×10^5 cells/well in 100 μ l serum-free DMEM (SFM) for 24 h. Cultures were then exposed to 0, 160, or 320 μ M H_2O_2 for 2 h. This treatment was replaced by SF medium for an additional 24 h. Sensitivity of the RPE cells to H_2O_2 toxicity among the groups was then estimated using cell death and gene expression assays as described below. Cell death was estimated using LDH assay, propidium iodide (PI) staining, and cytochrome c release, and expression of oxidative stress and apoptotic genes estimated quantitatively by real-time polymerase chain reaction (PCR).

LDH assay Fifty microliters of culture supernatant from the H_2O_2 -treated samples and controls were incubated with an equal volume of LDH reaction mixture for 30 min. The reaction was terminated and the absorbance measured at 490 nm using a Benchmark Microplate Reader. Cell death, proportional to the LDH activity, was calculated as a percentage of the no-treatment controls. Triplicate measure-

Table 1 The table lists the PCR primers, Genbank accession numbers, sequence, and amplicon sizes for various oxidative stress and apoptotic genes

Gene name	Accession #	Forward	Reverse	Amplicon size (b.p.)
mtHsp70	NM_005345	GCTGACCAAGATGAAGGAGA	CAGCCTGTTGTCAAAGTCCT	343
UCP2	NM_003355	CTACAAGACCATTCACGAGAGG	AGCTGCTCATAGGTGACAAACAT	373
ATPase- α	NM_001001937	GCTGTTGCTTACCGTCAGAT	GGTACCTGCTACCTGCTTCA	345
ATPase- β	NM_001686	CTCTGTGTTTGCTGGTGTG	CCCAATAATGCAGACACCTC	279
ATPase- γ	NM_001001973	ACAGATGAAAAGCGAGGTTG	CGTCAGCATCAATATCGTCA	338
SOD1	NM_000454	CAATTCGAGCAGAAGGAAA	TTCTTCATTCCACCTTTGC	346
SOD2	NM_000636	TGGCTTGCAAAAAGTAAACC	ATCCCTACAAGTCCCCAAAG	329
SOD3	NM_003102	TCCATTTGTACCGAAACACC	GATGCGAAACAGCTGAAGAC	287
COX1	NC_001807	CTTCGTCTGATCCGTCCTAA	ATGGGAGATTATCCGAAGC	220
COX2	NC_001807	AGTCCTGTATGCCCTTTTCC	TGAAGATTAGTCCGCCGTAG	245
Bcl-2	NM_000633	CAGGAAAGGCTCGAAATACA	CTGATCCAAACTTGGAATG	296
Bax	NM_004324	CGGAACTGATCAGAACCATC	GAGGTCAGCAGGGTAGATGA	301

ments were taken for each donor sample at passages 3, 4, and 5; and the histograms generated represent the average values of all samples at the three passages within a given group.

PI staining After H_2O_2 exposure, coverslips were washed with SFM, live cells stained with 4 μ g/ml PI (diluted in

PBS), and nuclear labeling of dead cells estimated by epifluorescence microscopy.

Cytochrome c release Release of cytochrome c, a putative event of the mitochondria apoptotic pathway following loss of $\Delta\psi_m$, was measured as described [52, 53]. After H_2O_2

Fig. 1 Phase-contrast micrographs showing examples of primary cultures of RPE cells from the young, mid-age, and >60 age groups (top panel). Cells appear larger and more elongated in the oldest age

group. Scale bar 30 μ m. Dot plots from flow cytometry analyses indicating increase in cell size (forward scatter) and cytoplasmic complexity (side scatter) with increased chronological age

treatment, the cells were washed in PBS, resuspended in 1 ml of mitochondrial medium containing 250 mM sucrose, 10 mM KCl, 20 mM HEPES-KOH, pH 7.5, 1 mM EGTA, 1 mM EDTA, 1.5 mM $MgCl_2$, and 1% protease inhibitors mixture. The cells were permeabilized for 30 s by mild vortexing with 0.001% digitonin, followed by centrifugation for 3 min at $1,000\times g$. Pellets were resuspended in 4% paraformaldehyde for 20 min, washed, then incubated for 15 min in labeling medium containing 2% FBS, 0.2% sodium azide, and 0.5% Triton X-100 in PBS. The suspension was centrifuged at $3,000\times g$ for 5 min, incubated for 1 h with 1 $\mu g/ml$ mouse monoclonal anti-cytochrome c diluted in 200 μl of labeling medium followed by incubation for an additional hour in 5 $\mu g/ml$ goat anti-mouse AlexaFluor 488-conjugated cytochrome c antibody diluted in 200 μl of labeling medium. Labeled cells were washed, resuspended in 200 μl PBS, and 10,000 cells/sample analyzed by flow cytometry. Analyses for individual RPE samples were carried out as described above and data expressed as the mean of the anti-cytochrome c fluorescence in RPE samples within a given age group. Fluorescent images of the labeled cells were also collected using confocal microscopy.

Expression of oxidative stress and apoptotic genes Total mRNAs from confluent RPE cultures were isolated and real time-PCR performed at an annealing temperature of $58^\circ C$

and 35 cycles for the following genes: mtHsp70, UCP2, ATPase- α , β , γ , superoxide dismutase 1 (SOD1), superoxide dismutase 2 (SOD2), superoxide dismutase 3 (SOD3), Bax, Bcl-2, COX1, and COX2. Primer sequences are listed in Table 1 and were synthesized by Invitrogen (Carlsbad, CA, USA). GAPDH was used as an internal RNA loading control and no reverse transcriptase reactions as negative controls to confirm that amplification was RNA dependent. For real-time PCR, the two-step amplifying protocol was used with iQ SYBR green supermix solution (BioRad). Both the melting curve and gel electrophoretic analyses were used to determine amplicon homogeneity and quality of the reaction.

Morphological changes in RPE mitochondria with increased aging

Electron microscopy RPE cells from all donor samples within the three age groups were processed identically for electron microscopy (EM) analysis. Cells were seeded onto fibronectin-coated Thermanox cover slips, cultured for 48 h in DMEM containing 2% FBS, and fixed in a solution containing 0.5% glutaraldehyde, 4% paraformaldehyde, and 85 mM cacodylate buffer. Specimens were post-fixed overnight in buffered osmium tetroxide (1%) and potassium ferrocyanide (1.5%), dehydrated in a graded series of ethanol, then infiltrated and embedded following standard

Fig. 2 Greater than 99% of cells in all primary RPE cultures express the RPE specific marker, RPE-65 (green fluorescence). Scale bar 30 μm

Fig. 3 a Phase-contrast light micrographs and PI staining (red labeling) show that cultures from the oldest age groups are more sensitive to H₂O₂ toxicity. PI staining was carried out on unfixed cells after H₂O₂ treatment. Scale bar 30 μ m. **b** Age-related increase in

LDH release at all concentrations of H₂O₂. Data are expressed as percentage of cell death as the mean $\pm$ SE of all samples within an age group ($n=7$; $p<0.05$)

EM procedures. Ultra thin sections ($\sim 800\text{\AA}$) were mounted onto square 200 mesh copper grids, stained with uranyl

acetate and lead citrate, and examined using a Philips 400 transmission electron microscope.

Mitochondrial morphology and morphometrics Twenty individual RPE cell EM micrographs were randomly taken along different planes of cells from each donor sample. The images were captured on 8.3×10.2 cm films at an original magnification of 1,650. After each group of 20 micrographs, a carbon-grating replica was photographically recorded to accurately calibrate the magnification of the cells. Micrographs were enlarged to $16,200\times$ to count the mitochondria in three perinuclear areas of $49 \mu\text{m}^2$ each in the cells. The counting template consisted of area 1, closest to the nucleus in the mitochondrial dense region. Areas 2 and 3 were located at a 45° angle to area 1 and at $10.5 \mu\text{m}$ from the nucleus. The mitochondria morphology was examined at $35,000\times$. Morphometric analysis was performed using the NIH Image J program by two observers. When discrepancies resulted, a third observer was used. Data is presented at the mean for each individual area/20 cells and the mean of all three areas/20 cells.

RPE cell morphology among the various age groups was also examined using phase-contrast and confocal microscopy and cell size estimated by flow cytometry. Cells from confluent RPE cultures were harvested and number of mitochondria estimated after labeling with 50 nM of the mitochondrial markers, MitoTracker Red and Mitotracker Green for 30 min at 37°C . Flow cytometric using BD FACS (AriaTM, Becton Dickinson, Franklin Lakes, NJ, USA) analysis were carried out using 10,000 cells/sample and an excitation wavelength of 488 nm and emission of 590 nm. Results are expressed in arbitrary units as the mean fluorescence intensity of all samples within a group. Cells labeled similarly in culture were also analyzed by confocal microscopy to validate flow cytometry findings.

Age-related changes in mitochondrial function

ROS production Cellular oxidative stress was determined by the amount of ROS in the RPE cytoplasm [54, 55] essentially as we have described [57]. Cells from confluent cultures were harvested by centrifugation and 2×10^6 cells/ml from each age incubated at 37°C for 30 min with $0.4 \mu\text{M}$ of the ROS indicator, $\text{H}_2\text{-DCF-DA}$, diluted in SFM. $\text{H}_2\text{-DCF-DA}$ penetrates cells and emits green fluorescence upon oxidation through reactions with H_2O_2 . Excess $\text{H}_2\text{-DCF-DA}$ was removed from the samples and cells analyzed by flow cytometry using 488 nm excitation and 530 nm emission wavelengths. For each sample 10,000 cells were analyzed and data processed using the FCS Express software.

ATP levels ATP levels were determined using a luciferin/luciferase-based assay as described [56]. Cells from confluent RPE cultures were harvested and 1×10^5 cells

from each sample in an age group seeded into wells of a 96-well plate in SFM for 24 h. Culture medium was then removed, cell membranes permeabilized using $50 \mu\text{l}$ of somatic cell ATP-releasing reagent, and samples incubated with $50 \mu\text{l}$ ATP Assay Mix Reagent containing luciferin and luciferase. Luminescence was measured immediately using a luminometer (Orion II Luminometer, Berthold Detection Systems, Oak Ridge, TN, USA). Cellular ATP levels are expressed in arbitrary units as the mean luminescence intensity of all samples within a group.

Mitochondrial membrane potential Mitochondrial membrane potential ($\Delta\psi\text{m}$) measurements were carried out as we have described [56] using the indicator JC-1, a lipophilic, and cationic dye which fluoresces red when it aggregates in the matrix of healthy, high-potential mitochondria, and green in cells with low $\Delta\psi\text{m}$. Cells from confluent RPE cultures were harvested and JC-1 ($1 \mu\text{g/ml}$) added to 2×10^6 cells/ml in suspension. Samples were incubated for 20 min at 37°C , washed, and 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. Ten thousand cells were analyzed and results expressed in arbitrary units as the mean fluorescence intensity for all samples within a group.

$[\text{Ca}^{2+}]_c$ and $[\text{Ca}^{2+}]_m$ levels Cytoplasmic calcium $[\text{Ca}^{2+}]_c$ was measured using the fluorescent probe fluo-3/AM and mitochondrial calcium $[\text{Ca}^{2+}]_m$ with Rhod-2/AM ($K_d \sim 570$ nM) as described [57–59]. Cells from confluent RPE cultures were seeded into 6-well plates at a density of 1×10^5 cells/well in SFM for 24 h, then loaded with either $1 \mu\text{M}$ fluo-3/AM for 30 min or $1 \mu\text{M}$ rhod-2/AM for 1 h. Cells were harvested, washed, resuspended in $200 \mu\text{l}$ PBS, then analyzed by flow cytometry at excitation and emission wavelengths of 488 and 525 nm, respectively, for fluo-3/AM, and 549 and 581 nm, respectively, for Rhod-2/AM. Ten thousand cells were analyzed and results expressed in arbitrary units as mean fluorescence intensity for all samples within a group.

Statistical analysis

RPE cells of all age groups were cultured under identical conditions and values for each experiment determined using confluent monolayers. Experiments for each donor sample were carried out in triplicates at passages 3, 4, and 5 and the data for each sample calculated as an average of the nine values. Histograms generated represents the average values of the total number of donor samples within a given group ($n=7$ per group) and the data expressed as the mean $\pm$ standard

error (SE). One-way analysis of variance test was performed and statistical significance was set at $p < 0.05$.

Results

In this study, we measured age-related changes in mitochondrial function of human primary RPE cells from three age groups, young, mid-age, and >60 years old using seven donors per group and estimated how these cells respond to oxidative stress.

All RPE cultures were obtained from relatively healthy donors with no known ocular pathology. All eyes were harvested by 8–12 h post-mortem, RPE cells isolated, and cultures of all three groups maintained in identical growth conditions. Triplicate assays were carried out for the experiments described at passages 3, 4, and 5 for each of the seven samples within the group and the data presented as an average of all the samples within that age group.

Morphological changes in RPE cells

Phase-contrast microscopic observation indicated a size shift in RPE cells with increasing age (Fig. 1), which was confirmed by flow cytometry (Fig. 1) and electron microscopy (Fig. 7). All seven donors in the >60 group were larger and more elongated in appearance compared to all 14 cultures obtained from the <60-year-old samples as shown in the representative micrographs for each group (Fig. 1).

Cell size resolution by flow cytometry clearly shows size differences with increasing age (Fig. 1). Although we confirmed that cultures were enriched for RPE cells with >99% of cells expressing RPE65, an RPE specific marker (Fig. 2), there was considerable heterogeneity and complexity in cultures obtained from individuals >60 years old. This is indicated by the shift in side and forward scatter in the dot plot for these samples compared to those for the young and mid-age ranges. Dot plots for all ages below 60 years old show overlapping forward and side scatter profiles suggesting that these cells were similar in size complexity.

Age-related sensitivity of RPE cells to oxidative stress

Here, we were interested in understanding how RPE cells coped with oxidative stress and how the aging process affected their coping mechanisms.

Cell death Susceptibility of confluent cultures from the various groups to oxidative stress was examined using measures that detected cell death and gene expression changes. In Fig. 3, we show that H_2O_2 had a significant effect on the viability of RPE cells in the oldest age group.

Morphologically, these cells maintained an unhealthy, degenerative appearance after treatment. Fewer cells were visible on coverslips in part because of cell death and in part because of cell detachment although cells from all age groups were plated at the same seeding density. Both PI staining of the cells and LDH release into the culture medium indicated an increase in cell death with advanced aging (Fig. 3a, b).

In addition, there was an increase in the release of cytochrome c in the >60-year-old cultures exposed to oxidative stress compared to younger counterparts. Cytochrome c is a component of the electron transport chain of the mitochondria. In response to apoptotic stimuli, cytochrome c is released into the cell cytoplasm by the mitochondria [60, 61]. In RPE cultures from all ages, we show colocalization of cytochrome c (green fluorescence) with mitotracker red (the mitochondrial marker) in control, nonstressed growth conditions (Fig. 4). However, when the cultures were challenged with oxidative stress, there was an almost complete lack of colocalization of the two fluorescent markers in the >60-year-old group while colocalization will still seen in the young and mid-age cultures.

Gene expression changes Given the structural, biochemical, and functional changes in RPE cells with increased aging, we examined expression of several genes important to mitochondrial health and function to assess mechanistic link with increased vulnerability of the cells to oxidative stress. The genes examined included the mtHsp70, mitochondria uncoupling protein 2 (UCP2), SOD2, and SOD3, part of the cells antioxidant defense repertoire, and the apoptosis regulators Bcl-2 (antiapoptotic) and Bax (proapoptotic). The data presented in Fig. 5 provide evidence for a consistent decrease in transcriptional levels of mtHsp70, UCP2, SOD3, Bcl-2, and Bax with an approximate 69-, 27-, 65-, 30-, and 11-fold changes, respectively, and an increase in SOD2 expression by ~21-fold between the youngest and oldest samples, supporting the hypothesis that aging RPE cells have a lower antioxidant defense threshold. Taken together, our data suggest that RPE cells have reduced oxidative stress coping mechanisms with increased aging and that severe stress can induce mitochondrial collapse and death in these cells.

Decreased levels of the anti- and pro-apoptotic genes with increased aging in cultures not exposed to oxidative stress are not surprising since the expression of these gene are usually triggered by an apoptotic challenge. This result is supported by the lack of cytochrome c release we observed in nonstressed cultures indicating that the cells were not undergoing apoptosis (Fig. 4). There were no significant changes in the expression of other genes involved in mitochondrial function including ATPase- α , b, γ , SOD1, COX1, and COX2 among cells of the various donor ages (data not shown).

Fig. 4 In the control groups grown in standard growth medium, cytochrome c (green) colocalizes with mitochondrial staining (Mito-tracker red) in RPE cells of all age groups (merged images) indicating that the cells in all control groups were healthy. With H₂O₂ treatment, however, colocalization of cytochrome c was only evident in the young and mid-age cells (merged images). The discrete green and red labeling in cells of the >60-year-old group suggest that cytochrome c was released from the mitochondrial into the cytoplasm of these cells. Scale bar 30 μ m

Structural changes in the mitochondria

EM The EM comparisons indicated marked differences in the structural features of the mitochondria with aging. Those of the two younger groups were numerous, regular in size, and maintained a round or oval shape. Cristae were distinctly visible and the outer membranes appeared intact. Cells from the >60 age group contained mitochondria that were sparsely distributed in the cytoplasm, irregular in size, tubular in shape, larger, and had electron-dense matrices, less distinct cristae, and disrupted outer membranes (Fig. 6a). Length of the mitochondria in this group (width ratio) was almost sevenfold greater compared to the other ages (Fig. 6b).

Given the above findings of weakened antioxidant defenses with increased aging, we examined whether there were age-related structural and functional differences in the mitochondrial populations of RPE cells that may contribute to the cells increased susceptibility to oxidative stress. Using electron microscopy and rigorous cell counting with a standardized template, we identified a perinuclear “mitochondrial polarized” region in cells of all ages (Fig. 7a). There were fewer mitochondria per unit area in this region with increased donor age with greater than twofold difference between cells from the youngest and oldest groups

Fig. 5 Expression of antioxidant and apoptotic genes in RPE cells with increasing chronological age. Real-time PCR shows that there is a significant increase in transcripts for SOD 2 and decrease for mtHsp70, UCP2, SOD3, Bcl-2, and Bax genes with increasing age. The histogram represents mRNA fold changes to the mean of the “young” group. Results are expressed as the mean $\pm$ SE. *Significantly different from young group ($p<0.05$)

($p<0.05$; Fig. 7b). The average number of mitochondria in all three template areas/cell (mitochondria/147 μ M²/cell) was 48.63 ± 10.16 , 38.36 ± 9.51 , and 19.9 ± 4.13 for the young, mid-age, >60-year-old RPE, respectively.

Mitotracker labeling We extended the EM studies to confirm our findings of fewer mitochondria in the older cell population by labeling these organelles with two mitochondrial specific fluorescent dyes [16] and examining the distribution and intensity of the label by microscopic and flow cytometric analyses (Fig. 8a–c). The confocal images in Fig. 8a show that the Mito Tracker Red labeling intensity was decreased in the oldest population especially in the perinuclear region. Highly branching networks of labeled structures were also seen in this group compared to the discrete punctate perinuclear labeling in the youngest and mid-age groups. Fluorescent intensity of the two different mitochondrial labels measured by flow cytometry, indicated a 0.37 (± 0.15) (Mito tracker green)- and 0.28 (± 0.17) (Mito tracker red)-fold difference in the >60 years old compared to younger aged groups (Fig. 8b, c; $p<0.05$). These results support the EM evidence for numerical, size, and distribution differences of the mitochondria among the three groups. Our results suggest that the response of the older cells to stress correlates with morphological and numerical changes in the mitochondrial population of these cells.

Abnormalities in mitochondrial function

ROS levels The free radical theory that the higher the metabolic rate, the greater the production of ROS, and the shorter the life span of the organism [1–6] may not hold true for all cells and species. Changes in the structure and distribution of the mitochondria in aging RPE cell populations led us to examine the function of the organelles in these samples. To our surprise, endogenous ROS production, measured by H₂-DCF-DA oxidation and flow cytometric acquisition/analyses, was lower in the older samples. Levels decreased by 0.30-fold (± 0.13) when compared to cells from the youngest donors (Fig. 9a; $p<0.05$). One possibility that may account for this is that the older population of cells reduced metabolic activity and hence ROS production in a strategic maneuver to maintain function and prevent premature degeneration.

ATP production There was also an early and consistent decline in ATP levels by 30% and 44% in the mid-age and >60-year-old RPE cells, respectively, compared to younger aged cells ($p<0.05$; Fig. 9b). The utilization of energy in the various aged RPE may, in part, explain the differences in vulnerability of the cells to oxidative stress and in their levels of ROS production.

Fig. 6 a Electron micrographs of primary RPE cultures. The mitochondria in the two youngest age groups are regular in shape and size and contain intact membranes with visibly distinct inner and outer membranes and cristae. Those from the >60-year-old samples are fewer, larger, irregular in size, tubular in shape, have highly

electron dense matrices and show architectural disruption in the membranes and cristae. *Scale bar* 1.5 μm. **b** Length/width ratio of these organelles is almost sevenfold different compared to young and mid-age samples (using NIH image J software)

$\Delta\psi_m$ The bioenergetic profiles of the three age groups correlated well with the $\Delta\psi_m$ as would be expected. A 0.85-fold (± 0.15) and 0.48-fold (± 0.17) decrease in $\Delta\psi_m$ was seen in the mid-age and >60-year-old RPE cells,

respectively, compared to their younger counterparts (Fig. 9c). The decrease in the $\Delta\psi_m$ is a key indicator of cell viability and would account for the increase in cytochrome c released into the cytoplasm of the cells

Fig. 7 **a** The mitochondria in young and mid-age samples are in discrete perinuclear regions of the cells and appear more tightly packed compared to those in the >60-year-old samples. Scale bar 10.5 μ m. **b** The histogram confirms visual observation of decreased numbers of individual mitochondria with increased aging. The data represent the mean of the average counts in all three areas of 20 cells per sample within the group

followed by the apoptotic events noted in the aging cultures.

[Ca²⁺]_c and [Ca²⁺]_m levels There is abundant evidence for altered calcium dynamics in the mitochondrial and cytoplasmic compartments of cells with aging in ways that render cells more vulnerable to degenerative events. When the resting levels of calcium in the cytoplasm [Ca²⁺]_c and in the mitochondria [Ca²⁺]_m of RPE cells were measured using the fluorescence Ca²⁺ indicators fluo-3/AM and Rhod-2/AM, respectively, we noted a significant decrease in [Ca²⁺]_c and increase in mitochondrial sequestration of Ca²⁺ in RPE cells from older individuals compared to those in the younger age groups (Fig. 10). The relative amounts of total fluo-3 AM and Rhod-2 fluorescence intensity was decreased by 0.60-fold (± 0.23) in [Ca²⁺]_c and increased by

2.51-fold (± 0.70) in [Ca²⁺]_m levels in the >60-year-old cultures, respectively, compared to the young cultures (Fig. 10a, b; $p < 0.05$). The disruption in calcium homeostatic mechanisms and the lower energy levels in the aging RPE cells are certain to impose limits in their response to environmental stress.

We present several lines of evidence for structural, functional, and gene expression changes in the mitochondria of RPE cells with increased chronological age that may impose constraints on the cells that render them vulnerable to oxidative stress. Our findings imply that this process is progressive and detectable as early as age 60 at a time when a number of visual abnormalities become evident in the eye. We propose that decrease in synthesis of antioxidant enzymes and bioenergetic deficits in progressively aging RPE cells are likely to increase their sensitivity to oxidative

Fig. 8 **a** Fluorescence intensity of the mitochondria indicator, Mito Tracker Red, is decreased with increasing age. The confocal images confirm discrete mitochondrial labeling in the perinuclear region in young and mid-age samples and branching labeled structures throughout cells of the >60-year-old samples. *Scale bar* 30 μm . **b, c** The histograms generated from flow cytometry (10,000 cells) show decrease fluorescence intensity with both Mitotracker red and green. Data are expressed as a fold change in fluorescence levels to the young group and as the mean $\pm$ SE of all samples in the group ($n=7$). *Significantly different from young group ($p<0.05$)

stress. Such events may contribute to the development and/or progression of age related visual dysfunction.

Discussion

The retinal pigment epithelium is exposed to an oxygen-rich environment primarily due to elevated oxygen partial pressure from the choriocapillaries. RPE cells phagocytose and digest photoreceptor outer segments, which provide an additional oxidative burden to the cells since outer segments are extremely rich in polyunsaturated fatty acids (PUFA) [62]. Thus, ROS can be generated in RPE cells by oxidation of

PUFAs [63, 64], the process of phagocytosis [48, 65], and RPE photosensitizers exposed to intense visible light [66, 67]. The production of excess ROS by normal metabolic processes and photochemical injury often lead to cumulative oxidative damage to the RPE [35–37], an event believed to be an underlying cause in some visual disorders. Here, we examined how oxidative stress and increased chronological aging alter the function of these organelles.

We show that with increased chronological age, RPE cells become larger and have more complex cytoplasmic compartments and an unusually intricate branching network of mitochondria compared to their younger counterparts, which likely reflects increased cell surface spreading.

Fig. 9 **a** Relative amount of total H₂-DCF-DA fluorescence. There is a 0.30-fold (± 0.13) decrease in ROS levels in the >60-year-old RPE group compared to young group. Data are expressed as a fold change in fluorescence levels to the young group and as the mean $\pm$ SE all samples within the group ($n=7$). *Significantly different from young group ($p<0.05$). **b** Similar comparisons are shown for ATP levels when the mean for the young samples were arbitrarily set at 100%. Results are expressed as a mean percentage of the ATP levels $\pm$ SE. *Significantly different from young samples ($n=7$; $p<0.05$). **c** Comparative $\Delta\Psi_m$ measurements using the fluorescence indicator JC-1 and flow cytometry. The $\Delta\Psi_m$ is 0.85-fold (± 0.15) and 0.48-fold (± 0.17) lower in mid-age, and >60 RPE cells, respectively, compared to the young samples. Results are expressed as the mean fold decrease in fluorescence levels to the young group, arbitrarily set at 100% $\pm$ SE. *Significantly different from young group ($n=7$; $p<0.05$)

Mitochondria often undergo ultrastructural remodeling to tailor energy output to meet the demands on the cell and their number can vary between cells of the same tissue [68–71]. The “megamitochondria” we observed have disruptions in their cristae architecture, a condition which was previously noted with cross-linking ATP synthase com-

plexes [72, 73]. A general trend in cells of older organisms is a decrease in number and increase in size of the mitochondrial population [70, 74]. Marked increase in the percentage of oversized mitochondria with structural abnormalities have been reported in several instances including in RPE cells of advance aged individuals with

Fig. 10 **a** Relative amounts of total fluo-3 AM fluorescence ($[Ca^{2+}]_c$). **b** Relative amount of total Rhod-2 fluorescence ($[Ca^{2+}]_m$) using flow cytometry. Data are expressed as a fold changes in fluorescence levels to the young samples and as the mean $\pm$ SE of all samples within the group. *Significantly different from young group ($n=7$; $p<0.05$)

increasing severity [42] of AMD at the synaptic terminals of old rats [75] and in other adverse conditions where cells are exposed to large amounts of free radicals over an extended period [76, 77]. The speculation is that numeric loss of mitochondria is due, in part, to impaired duplicative capacity of these organelles and that a shift in size compensates for numbers to keep volume density and area involved in cellular respiration constant during the lifespan of the individual [70, 78–80]. mtDNA is highly susceptible to oxidative stress and unlike nuclear DNA, mutations in mtDNA are not repaired. These mitochondria can go undetected, duplicate, and increase in cells [81, 82]. It is, therefore, not surprising that aging RPE cells accumulate a subpopulation of impaired mitochondria which may weaken their response to stress and promote untimely cell degeneration.

To cope with toxic oxygen intermediates, cells evolve effective defenses against oxidative damage. RPE cells are particularly rich in anti-oxidants such as vitamin E, superoxide dismutase, catalase, glutathione-S-transferases, glutathione, and ascorbate [83, 84]. Catalase activity and hemeoxygenase-1 levels are known to decrease in aging RPE cells [85, 86]. We show that several antioxidant enzymes, including mtHsp70, UCP2, and SOD3 have reduced expression with increased aging of the cells, a possible explanation for why these cells are less viable when exposed to increased oxidative stress compared to younger cells. The structural and gene changes we observed correlated well with alterations in the mitochondria membrane potential, ATP production, ROS generation, and mitochondrial calcium levels clearly indicating that with increased aging RPE cells accumulate a subpopulation of dysfunctional mitochondria, which could increase their susceptibility to oxidative stress.

The decrease in ROS production in the aging cells contradicts popular findings that ROS increases in aging tissues. One explanation is that upregulation of SOD2 expression we noted in these cells may be a compensatory mechanism that attenuates ROS production with life span extension as a primary goal. Our finding of lower ATP levels in the cells underscores the “low metabolic rate–high life expectancy” principle.

Apoptosis is generally accompanied by loss of $\Delta\Psi_m$, induction of MPT opening, and cytosolic translocation of cytochrome c [60, 61] by the mitochondria in response to proapoptotic stimuli. Although the aging cells in normal growth conditions showed a decreased in $\Delta\Psi_m$ compared to the younger cells, we did not detect release of cytochrome c or increased expression of Bax in these cells indicating that apoptotic mechanisms have not been triggered in the normal aging RPE population. However, mitochondrial collapse and cytochrome c release readily occurred when these cells were exposed to oxidative stress. Our results suggest that though aging RPE cells are viable

they were not able to cope with oxidative stress compared to cells from younger donors.

Our observation of higher $[Ca^{2+}]_m$ in the aging RPE follows physiological principles that $[Ca^{2+}]_m$ overload can trigger MPTP opening resulting in lowered $\Delta\Psi_m$ and cytochrome c release [58, 87–89] which was evident in these cultures. The logical progression then is that released cytochrome c interacts with the IP3 receptors inducing further calcium release from ER stores followed by massive cytochrome c release from the mitochondria and activation of apoptotic pathways. This study presents evidence that aging RPE cells are less likely to cope with high levels of oxidative stress because of abnormalities in a subpopulation of their mitochondria.

We hypothesize that RPE cell function may be severely compromised if abnormal mitochondria populations are not efficiently destroyed by the cells and accumulate above the normal aging threshold.

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