Chapter 20
Mitochondrial Decay and Impairment of Antioxidant Defenses in Aging RPE Cells

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Abstract In the eye, the retinal pigment epithelium (RPE) is exposed to a highly oxidative environment, partly due to elevated oxygen partial pressure from the choriocapillaris and to digestion of polyunsaturated fatty acid laden photoreceptor outer segments. Here we examined the vulnerability of RPE cells to stress and changes in their mitochondria with increased chronological aging and showed that there is greater sensitivity of the cells to oxidative stress, alterations in their mitochondrial number, size, shape, matrix density, cristae architecture, and membrane integrity as a function of age. These features correlate with reduced cellular levels of ATP, ROS, and $[Ca^{2+}]_c$, lower $\Delta\psi m$, increased $[Ca^{2+}]_m$ sequestration and decreased expression of mtHsp70, UCP2, and SOD3. Mitochondrial decay, bioenergetic deficiencies, and weakened antioxidant defenses in RPE cells occur as early as age 62. With increased severity, these conditions may significantly reduce RPE function in the retina and contribute to age related retinal anomalies.

20.1 Summary

In the eye, the retinal pigment epithelium (RPE) is exposed to a highly oxidative environment, partly due to elevated oxygen partial pressure from the choriocapillaris and to digestion of polyunsaturated fatty acid laden photoreceptor outer segments. Here we examined the vulnerability of RPE cells to stress and changes in their mitochondria with increased chronological aging and showed that there is greater sensitivity of the cells to oxidative stress, alterations in their mitochondrial number, size, shape, matrix density, cristae architecture, and membrane integrity as a function of age. These features correlate with reduced cellular levels of ATP, ROS, and $[Ca^{2+}]_c$, lower $\Delta\psi m$, increased $[Ca^{2+}]_m$ sequestration and decreased expression

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of mtHsp70, UCP2, and SOD3. Mitochondrial decay, bioenergetic deficiencies, and weakened antioxidant defenses in RPE cells occur as early as age 62. With increased severity, these conditions may significantly reduce RPE function in the retina and contribute to age related retinal anomalies.

20.2 Introduction

It is often argued that the metabolic rate of an organism determines its life span (Beckman and Ames 1998; Sohal et al. 2002; Pamplona et al. 2002) and that neurodegenerative diseases that occur with advanced aging have a common root in mitochondrial dysfunction. The mitochondria divide continuously throughout the life of a cell and their numbers in the cell varies according to organism, tissue type, and energy demands. They control a range of processes including cell signaling, differentiation, death, proliferation, and cell cycle. They produce most of the cells ATP, generate the bulk of ROS (Viña et al. 2006; Duchen 1999; Lane 2006), and are important to the organism's antioxidant defense systems (Mancuso 2007; Jezek and Hlavatá 2005; Czarna and Jarmuszkiewicz 2006; Inoue et al. 2003). These organelles are highly prone to oxidative damage, can accumulate mutations because they lack efficient mtDNA repair mechanisms, and can pass these mutations on to daughter cells (Passos et al. 2007; Chen et al. 2007; Stuart and Brown 2006). A shift in the balance of the number of normal and defective mitochondria in cells can influence senescence and apoptotic programs (Koopman et al. 2007; Chen et al. 2006; Hauptmann et al. 2008; Kwong et al. 2007).

There is compelling evidence that mitochondrial dysfunction is an early event in many neurodegenerative diseases including Alzheimer's disease (Lin and Beal 2006; Takuma et al. 2005; Beal 1998; Krieger and Duchen 2002; Eckert et al. 2008; Song et al. 2004; Schapira 1999; Valente et al. 2004) and that mitochondrial decay causes the cell's anti-stress pathways to operate with less efficiency (Wenzel et al. 2008; Hayakawa et al. 2008; Sasaki et al. 2008; Kimura et al. 2007). It is therefore conceivable that unchecked propagation and accumulation of dysfunctional mitochondria in aging RPE cells is also an underlying cause in the progression of age-related retinal diseases such as age AMD, a multifactorial disorder with etiology stemming, in part, from cumulative oxidative damage to the RPE (D'Cruz et al. 2000; Gal et al. 2000; Dorey et al. 1989; Green and Enger 1993; Beatty et al. 2000; Dunaief et al. 2002; Winkler et al. 1999). Histological changes are evident in the RPE and mitochondria of these cells at the earliest stages of AMD and precede vision loss, even though the disease has been primarily associated with photoreceptor damage (Green et al. 1985; Young 1987; Hageman et al. 2001; Penfold et al. 2001; Feher et al. 2006).

The RPE, a metabolically active epithelium crucial to maintaining the health of the retina, is continuously bombarded by high levels of oxidants (Weiter 1987; Zareba et al. 2006). Among its numerous responsibilities, this epithelium constitutes the blood retinal barrier, facilitates selective transport between the choroidal

vasculature and outer retina, phagocytose and degrade shed photoreceptor outer segments, regenerate photopigments, secrete neurotrophic, adhesion, and vascular regulatory factors, and contributes to the integrity of Bruch's membrane and the choriocapillaris. Disruption in any of these high-energy requiring processes is detrimental to the health of the retina.

In the AMD retina there is abnormal regulation of several mitochondrial proteins including ATP synthase, cytochome C oxidase complex, and mtHsp70 (Nordgaard et al. 2008) and a link between mitochondrial dysfunction and RPE degeneration (Jin et al. 2005; Liang and Godley 2003; Jin et al. 2001; Wang et al. 2008; Suter et al. 2000). This is not surprising given the daily challenges the RPE faces. Here we provide evidence for structural and functional modifications in the mitochondria of RPE cells, attenuation of the cells antioxidant system, and increased sensitivity of the RPE to oxidative stress with increased chronological age. We propose that increased accumulation of defective mitochondria in RPE cells with aging contributes to reduced function of these cells and increased pathological consequences in the retina.

20.3 Materials and Methods

20.3.1 Primary Human RPE Cell Culture

Human RPE cells were isolated from non-diseased donors as previously described (McKay and Burke 1994) and cultures maintained in DMEM supplemented with 5% FBS. Cultures in third to sixth passages from normal human donors, ages 9, 52, 62, and 76 years, were used for the experiments below.

20.3.2 Hydrogen Peroxide Toxicity – PI Assays

Cells were seeded at a density of 1×10^5 for 24 h in serum free medium (SFM), then exposed to 320 μM H_2O_2 for 2 h. Cultures were washed and cell death estimated by propidium iodide (PI) (4 μg/ml) staining.

20.3.3 Mitochondrial Morphometrics

RPE cells were seeded onto fibronectin coated Thermanox cover slips, fixed and processed for electron microscopy (Pavlovic et al. 2008; Zagon et al. 2007). Twenty individual RPE cell EM micrographs were randomly taken along different planes of cells from each donor and for each group, a carbon grating replica was photographically recorded to calibrate the magnification of the cells (Laguens 1971). Micrographs were enlarged to 16,200X to count the mitochondria in three 49 μm^2 perinuclear areas of the cell (Fig. 20.3). The counting template consisted of

Area 1, placed closest to the nucleus in the mitochondrial dense region and Area 2 and 3 located at a 45° angle to area 1 and at the same distance of 10.5 μm from the nucleus. The mitochondria morphology was examined at 35,000x and morphometric analysis performed using the NIH Image J program. Data is presented at the mean for each individual area/20 cells and the mean of all three areas/20 cells.

RPE cell morphology was also examined using phase-contrast and confocal microscopy and cell size estimated by flow cytometry. Mitochondrial population was estimated by labeling detached cultures with 50 nM MitoTracker Red and analysis of 10,000 cells by flow cytometry (BD FACS AriaTM, Becton Dickinson, USA) at an excitation wavelength of 488 nm and emission of 590 nm.

20.3.4 Protein and Weight Estimation of RPE Cells and Mitochondria

RPE cells (1×10^6) for each donor age were harvested by centrifugation. Pellets were weighed, lysed, and protein concentrations estimated. Mitochondria were isolated using reagents from the Pierce mitochondria isolation kit for cultured cells (Pierce Chemical Co Rockford, Illinois, USA) (Hauptmann et al. 2008). Mitochondrial pellet weights were estimated, then the organelles were lysed, centrifuged, and concentration of protein in the supernatant estimated.

20.3.5 Measurement of ROS, ATP and Mitochondrial Membrane Potential ($\Delta\Psi$m)

Cellular oxidative stress was determined by the amount of ROS in the cytoplasm (Degli Esposti 2002; Amer et al. 2003) of the RPE cells essentially as we have described previously (He et al. 2008a). Cells were harvested by centrifugation, washed, and 2×10^6 cells/ml incubated at 37°C for 30 min with 0.4 μM ROS indicator H_2-DCF-DA. Excess H_2-DCF-DA was removed from the samples and cells analyzed by flow cytometry using 488 nm excitation and 530 nm emission wavelengths. ATP levels were determined using a luciferin/luciferase-based assay essentially as described (He et al. 2008a) and luminescence measured using a luminometer (Orion II Luminometer, Berthold Detection Systems, TN).

$\Delta\psi$m measurements were carried out as we have previously described (He et al. 2008a) 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 fluorescence green in cells with low $\Delta\Psi$m. JC-1 (1 μg/ml) was added to 2×10^6 cells/ml in suspension and samples incubated for 20 min at 37°C, washed, and analyzed by flow cytometry. Data were collected at an emission wavelength of 530 nm for green fluorescence and 590 nm for red fluorescence.

20.3.6 Measurement of ($[Ca^{2+}]_c$) and ($[Ca^{2+}]_m$)

Cytoplasmic calcium $[Ca^{2+}]_c$ levels were measured with the fluorescent probe fluo-3/AM and mitochondrial calcium $[Ca^{2+}]_m$ with Rhod-2/AM (K_d ~570 nM) as described (He et al. 2008b; Deng et al. 2006; Mironov et al. 2005) using 1×10^5 cells/well in SFM. The cells were loaded with either 1 μM fluo-3/AM for 30 min, or 1 μM rhod-2/AM for 1 h, trypsinized, washed twice, resuspended in 200 μl PBS, then analyzed by flow cytometry at excitation and emission wavelengths of 488 and 525 nm respectively, for fluo-3/AM, and 549 nm and 581 nm respectively, for Rhod-2/AM.

20.3.7 Expression of Mitochondrial Associated Genes

Total mRNA from RPE cultures was isolated, RT-PCR and real time-PCR performed at an annealing temperature of 58°C for 35 cycles for the mitochondria associated genes, mtHsp70, UCP2, ATPase-α, β, γ, SOD1, SOD2, SOD3, Bax, Bcl-2, COX1 and COX2 using their respective primers (Invitrogen, Carlsbad, CA). GAPDH was used as an internal RNA loading control and no reverse transcriptase (NRTs) reactions as negative controls to confirm that amplification was RNA dependent. For real-time PCR, the 2-step amplifying protocol was used with iQ SYBR green supermix solution (BioRad). Both the melting curve and gel electrophoretic analyses were used to determine amplification homogeneity and quality of the reaction.

20.4 Results

20.4.1 Age Related Sensitivity of RPE Cells to Oxidative Stress

Phase-contrast micrographs of primary cultures of RPE cells show that RPE cells from the 9 yo donor grow as a monolayer of tightly packed cobblestone-like cells in culture whereas those from individuals >62 yo are larger and more fibroblastic in appearance (Fig. 20.1). The identity of the cells in the cultures was established by visual observation of the pigmented cells by phase contrast microscopy and using RPE65 as an expression marker. Visual observation and immunocytochmistry indicate that greater than 99% of the cells in the primary cultures were pigmented and expressed RPE65.

In Fig. 20.2, we show that there are age related differences in the susceptibility of RPE cells to oxidative stress. When treated with 160–320 uM H2O2, approximately 90% of cells were PI positive in cultures >62 yo compared to the 9 and 52 yo donors. Flow cytometry for PI fluorescence intensity also confirms greater cell death in the older cultures.

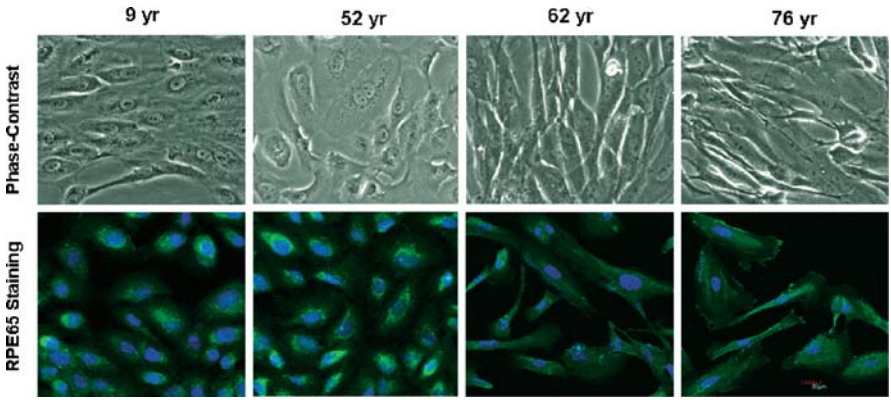

Fig. 20.1 Phase-contrast micrographs of primary cultures of RPE cells obtained from various donors ages (*upper*). Cells from donors >60 yo are larger and more fibroblastic in appearance compared to those obtained from 9 and 52 yo individuals. >99% of the cells in all cultures are pigmented and express RPE 65. Scale bar = 30 μm

20.4.2 Variation in Mitochondrial Number, Structure, and Size

We used a template that consisted of three identical counting areas to sample the number of mitochondria per unit area in the mitochondria polarized region of the RPE cells. RPE cells of all ages contain a mitochondrial-polarized region found in a crown-like shape in the perinuclear area although there is some distribution of these organelle throughout the cytoplasm of the cells (Fig. 20.3). In Table 20.1, we show that there are fewer mitochondria per unit area of the mitochondrial polarized cytoplasm of the RPE cells with increased donor age with >2 fold differences between cells from the youngest and oldest donors (p <0.05). The average number of mitochondria in all three areas/cell (mitochondria/147uM^2/cell) is 46.22 ± 12.86, 37.75 ± 13.78, 25.68 ± 8.69 and 20.15 ± 5.30 for the 9, 52, 62 and 76 yo RPE, respectively.

Electron microscopic comparison of the various RPE cultures shows very marked differences in the mitochondrial populations of the cells (Fig. 20.4). Those from the 9 and 52 yo individuals are numerous, more regular in size, and are either round or oval in shape. The cristae are distinctly visible and outer membranes intact. Cells from the 62 and 76 yo donors have mitochondria that are sparsely distributed in the cytoplasm, irregular in size, tubular in shape, have highly electron-dense matrices, less distinct cristae, and disrupted outer membranes. There is a higher density of mitochondria in the 9 and 52 yo cells compared to those from older individuals in the mitochondrial polarized perinuclear area of the cells.

These findings were also confirmed by confocal microscopy (Fig. 20.5) where we show that Mito Tracker Red (Hauptmann et al. 2008) labeling intensity decreases as a function of RPE age. Labeling was perinuclear and discrete in the 9 yo samples compared to the diffused, branching pattern of the 62 and 76 yo cells.

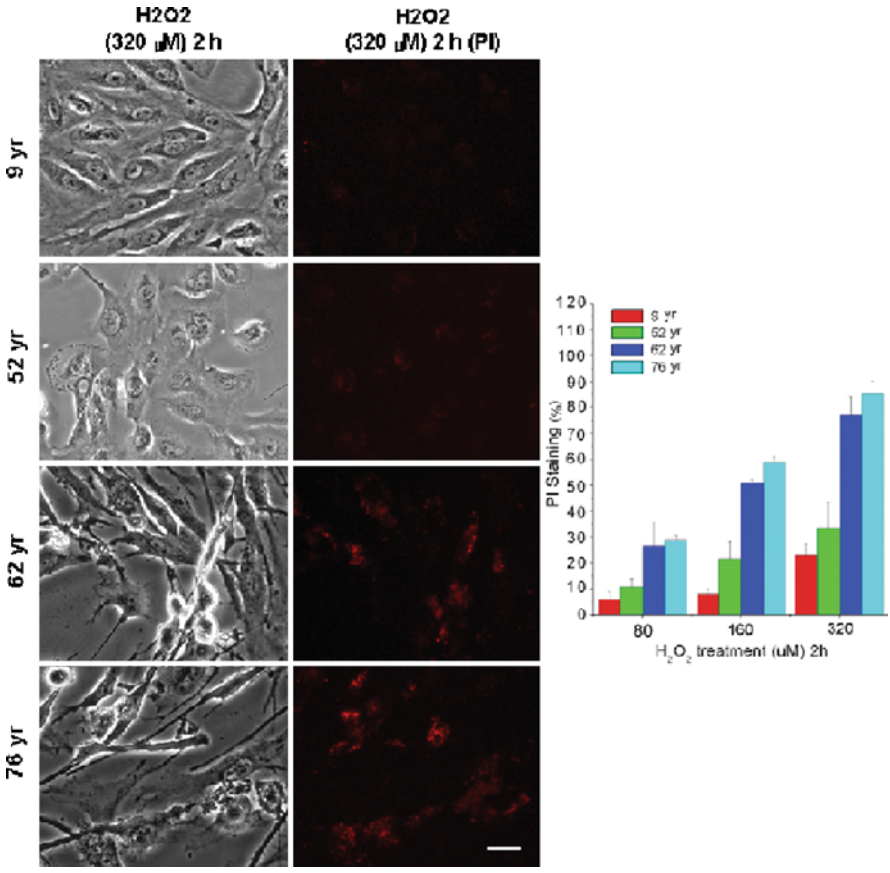

Fig. 20.2 Phase-contrast light micrographs of cells treated with 320 μM H2O2 for 2 h showing sensitivity to oxidative stress. Scale bar = 30 μm. PI staining indicates that there is ~90% cell death in the 76 yo cultures compared to 26% in 9 yo after exposure the H2O2

Table 20.1 Results summarized from EM analyses indicate that mitochondria number decreases as a function of age in RPE cells

	Cytoplasm area			
Number of mitochondria/age	1(close to nucleus)	2	3	1 + 2 + 3
9 year	22.11 ± 3.62	13.78 ± 7.43	10.33 ± 5.55	46.22 ± 12.86
52 year	13.25 ± 5.22*	13.25 ± 6.51	11.25 ± 7.99	37.75 ± 13.78
62 year	10.00 ± 4.27*	7.63 ± 4.70*	8.05 ± 4.17	25.68 ± 8.69*
76 year	7.38 ± 2.93*	5.92 ± 4.82*	6.85 ± 3.48	20.15 ± 5.30*

$n = 20$ cells × 3 areas; area = 49 μm^2; $^*p < 0.05$

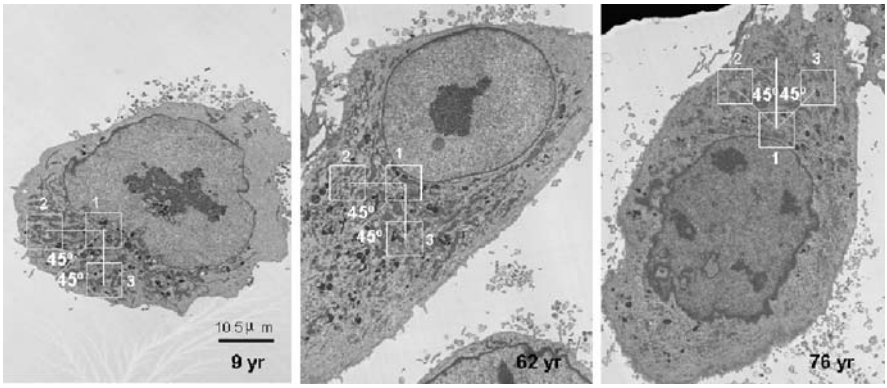

Fig. 20.3 EM micrographs showing mitochondria in perinuclear regions of cells from various donor ages and position of templates used for mitochondrial counting. Scale bar = 10.5 μm

In Table 20.2, we provide our results for cellular and mitochondrial weight and protein estimations for equal number of cells in each sample. There is a ~1.6 fold increase in both wet weight and protein per 1×10^6 RPE cells from the 62 and 72 yo individuals compared to the 52 and 9 yo culures. Although there are fewer mitochondria in the aging cells, there is a ~2 fold increase in wet weight and amount of protein in mitochondrial samples isolated from the two older donor samples supporting the EM observations that mitochondria increase in size in the RPE with increased donor age.

20.4.3 ROS and ATP Production, and $\Delta\Psi m$ *Decrease in RPE Cells with Aging*

Data collected from flow cytometric acquisition/analyses indicate that the amount of ROS generated by the 62, and 76 yo RPE cultures decreases by 3.23-fold (± 0.18) and 4.76-fold (± 0.21) respectively, when compared to the 9 yo cultures (Fig. 20.6) (p <0.05) (Fig. 20.6). There is also an early and consistent decrease in ATP levels in 52, 62 and 76 yrs RPE cells by 31, 35 and 45%, respectively, compared to 9 yr old samples (p <0.05) (Fig. 20.7). The 31% deficiency in energy production at donor age 52 may account for the lower levels of ROS generation by the cells at later stages of aging. The bioenergetic profiles of the various aged RPE cells also correlate well with the $\Delta\psi m$ in the cells. There is a 1.2-fold (±0.1), 1.52-fold (±0.2) and 2.1-fold (±0.3) decrease in $\Delta\psi m$ in the 52, 62 and 76 yo cells, respectively compared to the 9 yo cultures (Fig. 20.8). Together, these analyses suggest increased impairment in mitochondrial function with chronological aging of RPE cells.

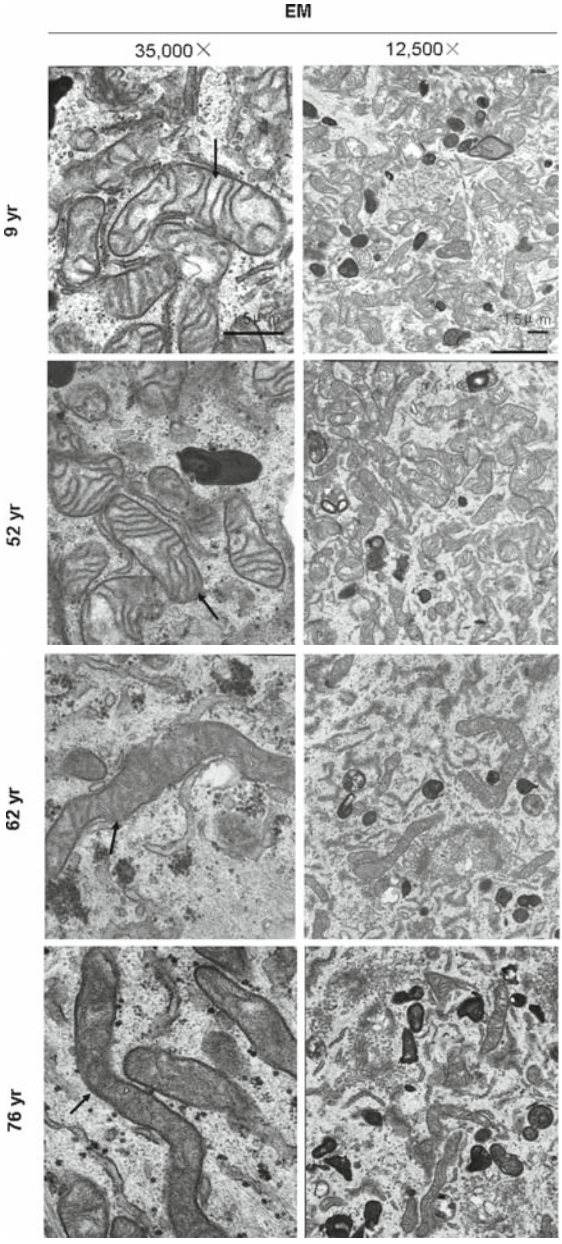

Fig. 20.4 Electron micrographs (magnification 35,000× and 12,500×) of primary RPE cultures. Mitochondria in 9 yo RPE cells are *oval* and *regular* in shape and contain intact membranes with visibly distinct *inner* and *outer membranes* and cristae. Those in 62 and 76 yo are fewer, larger, irregular in size, *tubular* in shape, have highly electron dense matrices, and disruption in membranes and cristae. Scale bar = 1.5 μm

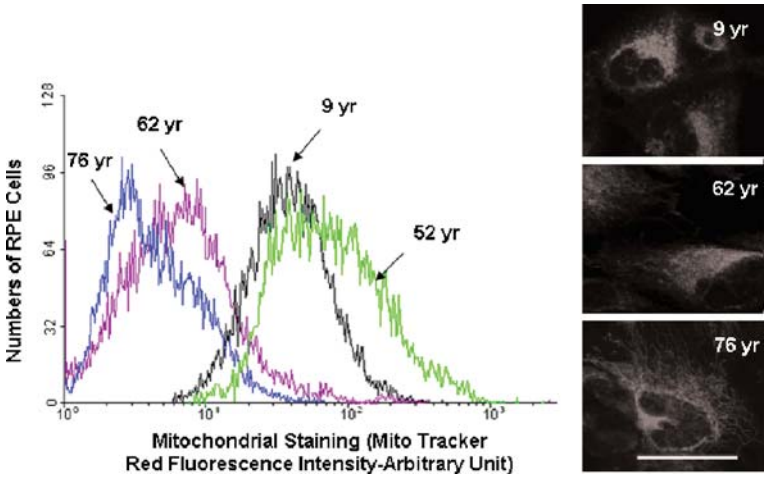

Fig. 20.5 Fluorescence intensity of Mito Tracker Red is decreased with aging of the RPE cells seen here by flow cytometry and confocal microscopy. Scale bar = 30 μm

Table 20.2 Mitochondria weight and protein concentration/1 × 10^6 RPE cells from each donor age

	9	52	62	76
Cell weight/10^6 cells(mg)	14.34 ± 0.62	13.81 ± 0.33	24.11 ± 1.27*	22.33 ± 1.81*
Cytoplasmic protein/10^6 cells(mg)	0.41 ± 0.08	0.39 ± 0.15	0.64 ± 0.09*	0.61 ± 0.05*
Mitochondria weight/10^6 cells(mg)	1.73 ± 0.14	1.51 ± 0.11	2.87 ± 0.18*	2.95 ± 0.35*
Mitochondria protein/10^6 cells(mg)	0.08 ± 0.01	0.08 ± 0.01	0.14 ± 0.02*	0.13 ± 0.01*

*$p < 0.05$

20.4.4 Age-Related Variations in ($[Ca^{2+}]_c$) and ($[Ca^{2+}]_m$) in RPE Cells

There is abundant evidence of altered calcium dynamics in cells with increased aging, a condition that renders the cells more vulnerable to degenerative events (Toescu et al. 2004). We noted a correlation between lower $[Ca^{2+}]_c$ levels and increased mitochondrial sequestration of Ca^{2+} in RPE cells with aging (Fig. 20.9). The relative amounts of fluo-3AM and Rhod-2 fluorescence intensity in the cultures reflect a 1.52-fold (± 0.33) and 1.85-fold (± 0.28) decrease in $[Ca^{2+}]_c$ and a 2.32-fold (± 1.49) and 2.75-fold (± 1.88) increase in $[Ca^{2+}]_m$ levels in the 62 and 76

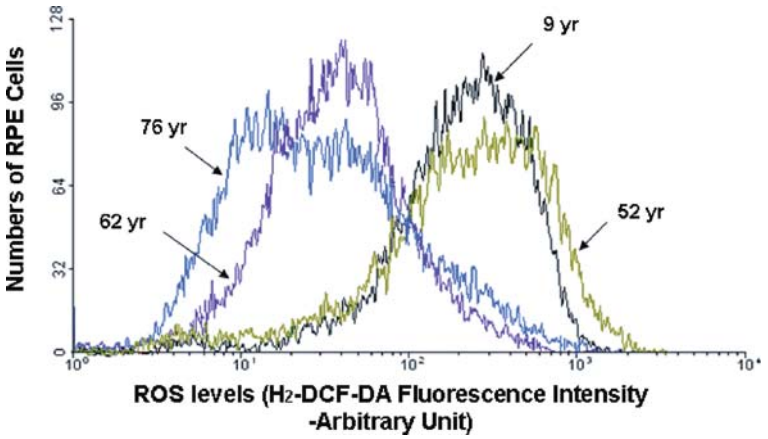

Fig. 20.6 Distribution of the fluorescent intensity for the ROS indicator, H_2-DCF-DA, in RPE cultures using flow cytometry. A decrease in ROS levels is seen with increased aging

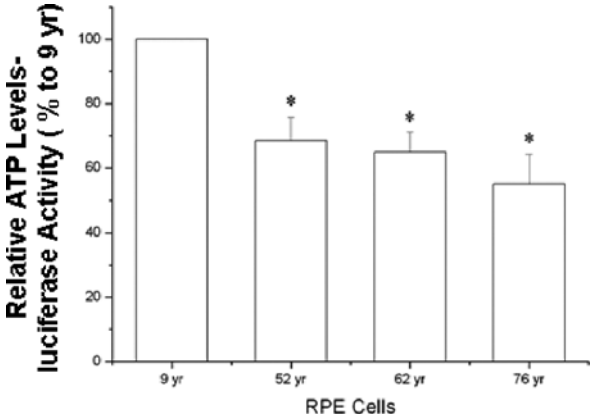

Fig. 20.7 ATP levels are decreased in RPE cells with increased donor age

yo cells, respectively compared to the youngest counterpart (Fig. 20.9) (p <0.05). Disruption in calcium homeostatic mechanisms together with lower energy levels in the aging RPE cells may certainly impose limits on these cells in their response to environmental stress.

20.4.5 Expression of Genes Associated with Mitochondrial Function

Given the structural, biochemical, and functional changes in the mitochondria with increased donor age, we examined the expression of several genes important to mitochondrial health and function to ascertain if any may have a mechanistic link with

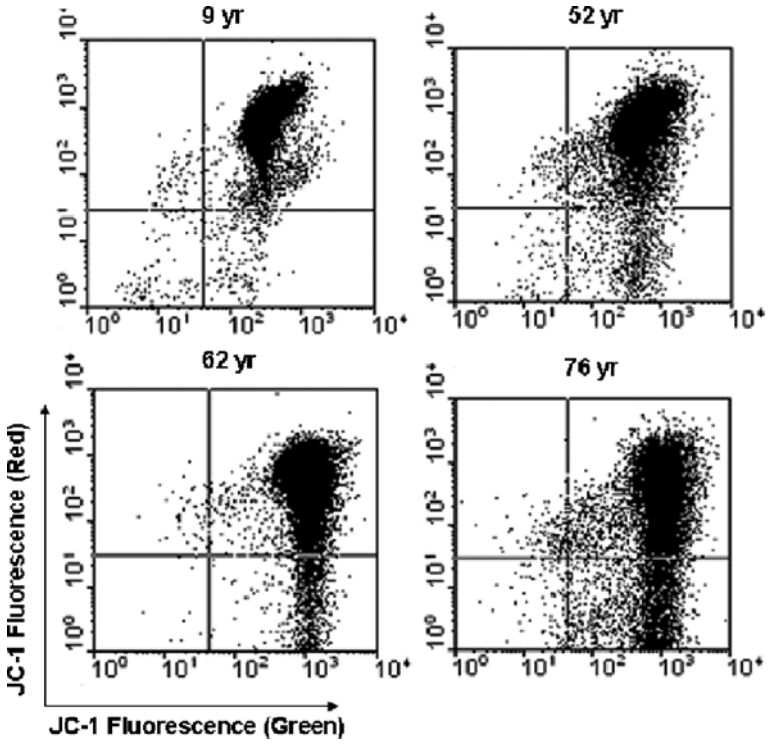

Fig. 20.8 $\Delta\Psi$m are decreased in RPE cells with increased donor age

the changes observed above. In our studies we found a consistent decrease in the mRNA levels of mtHsp70, UCP2, SOD3, Bcl-2 and Bax and increase in SOD2 expression with increased aging of the RPE cells (Fig. 20.10) but no significant differences in the expression of ATPase-α, b, γ, SOD1, COX1 and COX2 between cells of the various donor ages (data not show). This data suggest that there are variations in expression of genes important to mitochondrial function that may alter the threshold level of the cells to environmental hazards.

Although, we recognize that a limitation of this study is the size of the RPE donor samples used, we showed a longitudinal decrease in structural and functional integrity of the mitochondria in RPE cells with aging. Since this presentation was made at the XIIIth International Symposium on Retinal Degeneration in China (September 2008), we have analyzed RPE samples from 4 other individuals >60 year old and have confirmed these findings. There is still the difficulty in obtaining samples from younger donors, but the human ARPE19 cell line derived from a 19 year old male showed features of cell growth, morphology, and mitochondrial structure and function that were similar to primary cultures from the 9 yo donor (data not shown).

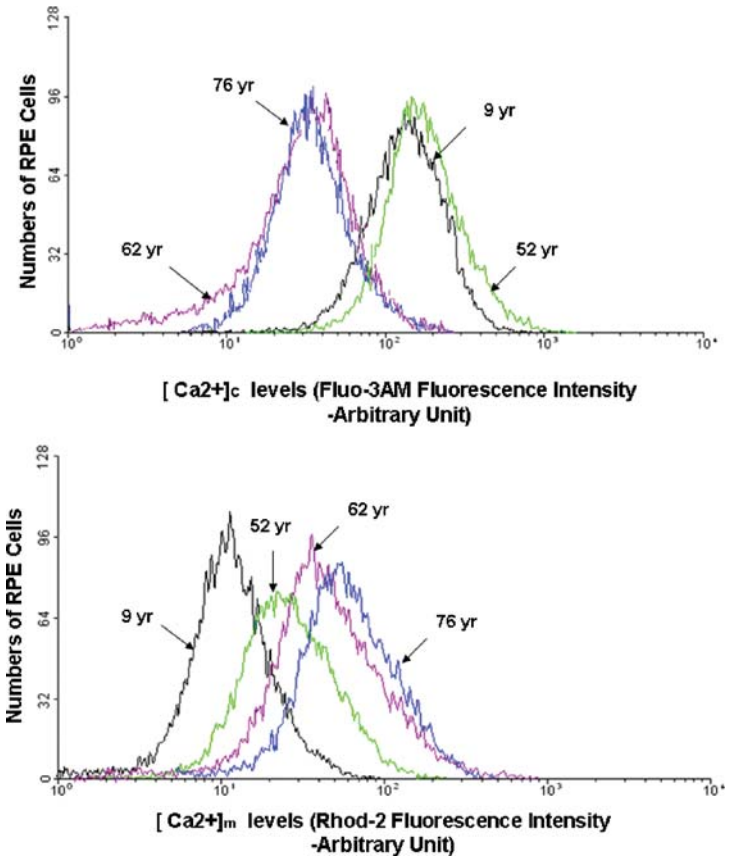

Fig. 20.9 Flow cytometry showing that RPE cells sequester more $[Ca^{2+}]_m$ and have lower levels of $[Ca^{2+}]_c$ with increased aging

20.5 Discussion

The RPE is in a location in the retina where it is constantly bombarded by reactive oxygen species. Cumulative oxidative damage can cause this tissue to degenerate (Beatty et al. 2000; Dunaief et al. 2002; Winkler et al. 1999). We examined the function of these organelles in RPE cells and the susceptibility of these cells to oxidative stress with increased chronological aging. Our study showed that with increased aging there are numerous structural abnormalities in the mitochondria of RPE cells which correlate with decreased bioenergetic levels of the cells, attenuation of the cell's antioxidant system, and increased sensitivity to oxidative stress. Our work is supported by the findings of Feher et al (Feher et al. 2006) who showed, in a larger sampling of individuals, that there are structural abnormalities in the mitochondria of the RPE with advancing age and that these abnormalities increase in severity in individuals with AMD.

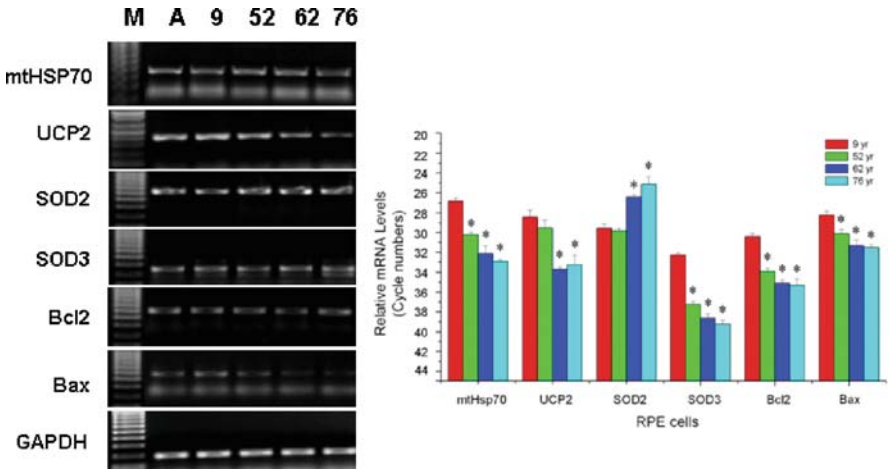

Fig. 20.10 Expression of antioxidant and apoptotic genes in RPE cells of various donor ages

It is well known that the numbers of mitochondria vary between cells in a tissue (Knott et al. 2008) and that this organelle can undergo ultrastructural remodelling to tailor energy output to meet environmental demands on the cell (Bereiter-Hahn and Vöth 1994; Bertoni-Freddari et al. 1993, 2001). mtDNA are highly susceptible to oxidative stress and unlike nuclear DNA, mutations in mtDNA are not repaired and can be inherited or acquired by individual cells (Sastre et al. 2000; Barja 2004; Melov 2004). A cell can have several populations of mtDNA (Kmiec et al. 2006). Over 80% of mtDNA codes for functional proteins, thus most mtDNA mutations lead to functional problems (Knott et al. 2008; Reeve et al. 2008). For example, Leber's hereditary optic neuropathy is associated with mutation in NADH-COQ reductase, ragged muscle fibers with mutation in mt lysine tRNA, and Kaerns-Sayre syndrome with several large deletions in mtDNA (Pätsi et al. 2008; Finsterer 2007). Diabetes, stroke, Alzheimer's and Parkinson's diseases, lactic acidosis, myopathy, osteoporosis, cancer, cardiovascular diseases, and aging, all show strong associations with mitochondrial dysfunction (Knott et al. 2008; Beal 2007; Lin and Beal 2006). The oxidative stress theory of aging, an expansion of the mitochondrial theory of aging, is based on the idea that somatic mutations in mtDNA provoke respiratory chain dysfunction, which leads to enhanced ROS production which, in turn, promotes further mtDNA mutations and cell function collapse (Passos et al. 2007; Ishikawa et al. 2008). This vicious cycle is amplified in mitochondrial biogenesis, which occurs in a cell cycle-independent manner. It is, therefore, not surprising that aging cells accumulate a subpopulation of dysfunctional mitochondria which, in excess, may weaken the cell's response to environmental hazards and promote cellular aging and untimely degeneration of the cell.

A general trend in mitochondria structure is seen with aging: in older organisms, there are studies showing a decrease in mitochondrial numbers but an increase in the

organelle's size due to fusion with other mitochondria or lack of fission (Bertoni-Freddari et al. 1993, 2008). This is seen at the synaptic terminals of old rats where there is a marked increase in the percentage of oversized organelles, often referred to as megamitochondria (Melov 2004). These are found in some adverse conditions as well, such as in cells exposed to large amounts of free radicals over an extended period (Karbowski et al. 1999; Wakabayashi 2002). The speculation is that numeric loss of mitochondria is due to impaired duplicative capacity of these organelles and that a shift in size compensates for numbers (Bertoni-Freddari et al. 1993; Solmi et al. 1994; Bertoni-Freddari et al. 2003, 2005). Our ultrastructural studies indicate that there are 'megamitochondria' in the aging RPE cells and that these are abnormal in appearance and have disruptions in the cristae architecture, a condition previously reported with cross-linking ATP synthase complexes (Ko et al. 2003; Gavin et al. 2004).

To cope with toxic oxygen intermediates, the RPE evolved effective defenses against oxidative damage and is particularly rich in anti-oxidants. However, several antioxidant enzymes, including mtHsp70, UCP2, and SOD3 have reduced expression in the RPE with aging. This may be one explanation why the primary cultures of RPE cells from older donors are more susceptible to oxidative stress. Strangely, however, there is a decrease in ROS production in the RPE from donor samples >60 yo which contradicts popular findings that ROS increases in aging tissues. One explanation is that the increase in SOD2 expression that we noted in these cells with aging may have a compensatory effect on attenuating ROS production with life span extension as a primary goal. Our finding of significantly lower ATP levels in the aging RPE cells underscores the 'low metabolic rate – high life expectancy' principle. However, one can argue that these in vitro studies do not predict organismal ageing or disease progression.

Some mitochondrial-specific actions leading to apoptosis include loss of $\Delta\Psi m$, induction of MPT opening, increased mitochondrial calcium levels, and cytosolic translocation of apoptogenic factors, such as cytochrome c (Armstrong 2006; Green and Kroemer 2004). Although the aging RPE cells have decreased $\Delta\Psi m$, as would be expected from the lower ATP levels they generate, there was no cytochrome c released by the cells or increased expression of the proapoptotic Bax gene. It is known that mitochondria Ca^{2+} overload triggers mitochondrial permeability transition pore (MPTP) opening, which lowers $\Delta\Psi m$ (Jackson and Thayer 2006; Dahlem et al. 2006). The high $[Ca^{2+}]_m$ in the aging RPE cells may, therefore, account for the lower $\Delta\Psi m$ in the cells.

Reduction in mitochondrial function and increased susceptibility of RPE cells to oxidative stress is likely to be part of the normal aging process in the retina. While accumulation of a relatively small number of defective mitochondria by these cells may compromise epithelial function but not trigger apoptosis, acute degeneration signals may be propagated when there is an excess in number of these dysfunctional organelles in any RPE cell of the epithelium.

In conclusion, we present strong evidence for structural and biochemical abnormalities in the mitochondria of RPE cells as a function of normal aging. We

propose that impairment of mitochondrial function makes RPE cells more vulnerable to oxidative damage and that excess accumulation of dysfunctional mitochondria in some RPE cells may trigger a degeneration cascade in a focal region of the epithelium that could be an underlying event in the onset of some retinal pathologies.

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