Inhibition of RPE Lysosomal and Antioxidant Activity by the Age Pigment Lipofuscin

Farrukh A. Shamsi and Mike Boulton

PURPOSE. To determine whether lipofuscin is detrimental to lysosomal and antioxidant function in cultured human retinal pigment epithelial (RPE) cells.

METHODS. Isolated lipofuscin granules were fed to confluent RPE cultures and the cells maintained in basal medium for 7 days. Parallel cultures were established that did not receive lipofuscin. Cultures were either exposed to visible light (390–550 nm) at an irradiance of 2.8 mW/cm^2 or maintained in the dark at 37°C for up to 24 hours. Cells were subsequently assessed for cell viability, lysosomal enzyme activity, and antioxidant capacity.

RESULTS. There was no loss of cell viability during the first 3 hours of light exposure, whereas a 10% loss of viability was observed in lipofuscin-fed cultures after 6 hours' exposure to light. Activities of acid phosphatase, *N*-acetyl-β-glucuronidase, and cathepsin D were decreased by up to 50% in lipofuscin-fed cells exposed to light compared with either unfed cells or cells maintained in the dark. There was also a decrease in the antioxidant potential of RPE cells. Catalase and superoxide dismutase activities decreased by up to 60% and glutathione levels by 28% in light-exposed lipofuscin-fed cells compared with unfed cells or cells maintained in the dark.

CONCLUSIONS. Lipofuscin has the capacity to reduce the efficacy of the lysosomal and antioxidant systems in RPE cells that may play an important role in retinal ageing and the development of age-related macular degeneration. (*Invest Ophthalmol Vis Sci.* 2001;42:3041–3046)

The retinal pigment epithelium (RPE) is essential for the maintenance of retinal function.[1] Two of its important roles are the ingestion and degradation of the spent tips of photoreceptor outer segments[2] and the provision of protection against oxidative stress.[3]

The daily phagocytosis of photoreceptor outer segments and, to a lesser extent, the autophagy of spent organelles requires the RPE to have a comprehensive lysosomal system to ensure the degradation of phagosomal material.[4] Up to 40 hydrolytic enzymes are present within lysosomes of which cathepsin D has been shown to be important in the breakdown of rod outer segments.[5] Despite this extensive lysosomal system in the RPE, there is accumulation of undegradable material within lysosomes that accumulates to form the autofluorescent lipid-protein aggregate lipofuscin.[4] Lipofuscin granules progressively accumulate with age, eventually occupying up to 19% of cytoplasmic volume by the age of 80.[6]

The RPE is located in a region of high oxidative stress. It is constantly exposed to visible light and high oxygen tension and is highly metabolically active, thus providing an ideal environment for the generation of reactive oxygen species (ROS).[3,7] In addition, phagocytosis of rod outer segments causes a burst of ROS, which adds a further oxidative burden.[8] To cope with these toxic oxygen intermediates, the RPE has evolved effective defenses against oxidative damage. It is particularly rich in antioxidants such as vitamin E, superoxide dismutase (SOD), catalase, glutathione, and ascorbate.[7,9] In addition, the RPE is able, within limits, to repair damage incurred by ROS that have evaded the antioxidant defenses.

Lipofuscin provides an additional oxidative burden on the RPE. We have previously demonstrated lipofuscin to be a photoinducible generator of ROS and that this is wavelength dependent.[1,10,11] Furthermore, we have demonstrated in biochemical assays that lipofuscin is capable of photoinducing extragranular lipid peroxidation as well as reduction in the activity of the enzymes catalase and acid phosphatase.[12] More recently, we have confirmed the phototoxicity of lipofuscin in a cellular system in which loss of lysosomal integrity and oxidative damage was a precursor to RPE cell death.[13]

The purpose of this study was to determine whether lipofuscin is detrimental to the normal functioning of the lysosomal and antioxidant systems in the RPE.

METHODS

Chemicals

Ham's F10 and Ham's SF10PF cell culture media were obtained from Gibco BRL (Life Technologies Ltd., Paisley, UK), fetal calf serum (FCS) was from TCS Biologicals Ltd. (Buckingham, UK). Trypsin, antibiotics, fungizone, 3-(4,5-dimethylthtiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), phosphate-buffered saline (PBS) tablets, *p*-nitrophenol, *p*-nitrophenyl phosphate, *p*-nitrophenyl β-D-glucosaminide, tyrosine, hemoglobin, triton X-100, hydrogen peroxide, potassium thiocyanate, catalase, glutathione, 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), nitro blue tetrazolium (NBT), β-nicotinamide adenine dinucleotide (NADH), 5-methyl phenazinium methyl sulfate (phenazine methosulfate; PMS), and SOD were purchased from Sigma Chemical Co. (Poole, UK). The bicinchoninic acid (BCA) protein assay kit was from Pierce & Warriner Ltd. (Chester, UK), and EDTA and sodium bicarbonate were obtained from BDH (Poole, UK). All other chemicals used were of highest purity analytical grade.

Isolation and Purification of Lipofuscin

Isolation of lipofuscin granules from eyes of human donors aged 50 to 60 years was performed as previously described,[14] except that RPE cells were disrupted by mechanical homogenization rather than ultrasonication. Lipofuscin granules were isolated and purified by differential centrifugation and suspended in PBS, and the lipofuscin concentration was determined by counting granules on a hemocytometer.

Isolation and Culture of Human RPE Cells

RPE cells were isolated and cultured from human eyes, as previously described.[15] The eyes, from donors aged between 40 and 70 years,

were provided by the Bristol Eye Bank, United Kingdom. The corneas had been used for transplantation, and permission had been given to use the poles for research. The RPE cells were grown in Ham's F10 medium supplemented with 20% FCS and antibiotics and, unless otherwise stated, were maintained at 37°C in a 5% CO_2 incubator. All cultures were used between passages 3 and 5, and each cell contained less than five lipofuscin granules before experimentation.

Phototoxicity Experiments

RPE cells were grown to confluence in 24-well culture plates or 25-cm² culture flasks. At confluence the culture medium was replaced with basal medium (Ham's F10 supplemented with 2% FCS) and maintained for a further 24 hours. Cultures received purified lipofuscin granules (~300 granules/cell) suspended in basal medium. After 24 hours the medium was replaced with fresh basal medium, and the cultures were maintained for 7 days, during which the basal medium was changed every 2 days. Before light exposure, the basal medium was replaced with Ham's SF10PF medium, which does not contain the photosensitizers phenol red, tryptophan, riboflavin, and folic acid.[16] For each set of experiments, one set of RPE cultures (with or without lipofuscin) were wrapped in an aluminum-lined black sheet of paper (dark maintained) and the other set was left uncovered (light exposed). Both light- and dark-maintained cultures were exposed to 390- to 550-nm light emitted by a sunlight simulator[16] (Sol 500; Hönle UV Ltd., Birmingham, UK), with appropriate filters for various times, as explained in the figure legends. The irradiance was 2.8 mW/cm², and the light source was positioned at a distance from the cultures, ensuring that the cells were continuously exposed to a temperature of 37°C.

MTT Cell Viability Assay

The assay was performed essentially as described by Mosmann.[17] Briefly, after the RPE cells had been exposed to the experimental conditions the medium was removed and MTT prepared in Ham's SP10PF was added to each well. The cells were incubated for 3 hours at 37°C in a humidified 5% CO_2 incubator. The unreacted MTT was aspirated off and 250 μl of 0.04 N acidified isopropanol was added to solubilize the reduced formazan crystals. Aliquots of solubilized crystals were transferred to a 96-well plate, and absorbance was measured at 570 nm in a microplate reader (Multiskan Ascent; Labsystems, Helsinki, Finland). Absorbances were normalized against control absorbances immediately before light exposure–dark maintenance and are expressed as viability as a percentage of control absorbance.

Lysosomal Enzyme Assays

Immediately subsequent to light exposure or dark maintenance, cells were lysed with 0.2% Triton X-100 in distilled water for 5 minutes. The lysate was then assayed for the activities of the following enzymes.

Acid Phosphatase Assay. The lysate was incubated with 7.5 mM *p*-nitrophenyl phosphate in 0.1 M acetate buffer (pH 5.0) for 45 minutes at 37°C.[18,19] The reaction was stopped by the addition of 100 mM NaOH, (pH 10.5), and the absorption of light by *p*-nitrophenol released in the reaction was measured at 405 nm. By using a calibration curve for *p*-nitrophenol the absorbances were converted to picomoles of reaction product.

***N*-acetyl β-D-glucosaminidase Assay.** The lysate was incubated with 7.5 mM *p*-nitrophenyl β-D-glucosaminide in 0.1 M citrate buffer (pH 5.0) for 45 minutes at 37°C.[19] The reaction was stopped by the addition of 0.4 M glycine-NaOH buffer (pH 10.5) and the absorption of light by *p*-nitrophenol released in the reaction was measured at 405 nm. By using a calibration curve for *p*-nitrophenol, the absorbance values were converted to picomoles of reaction product.

Cathepsin-D Assay. The lysate was assayed for cathepsin D activity using a modification of the assay described by Rakoczy et al.[20] The lysate was incubated with 2% hemoglobin in 0.2 M formate buffer (pH 3.3) at 37°C for 60 minutes, and the reaction was stopped by the addition of ice-chilled 3% trichloroacetic acid. After centrifugation at

100*g* for 5 minutes the proteolytic products from the digested hemoglobin in the supernatant were measured using the BCA assay. The absorption values of BCA complex, colorimetrically measured at 562 nm, were converted to absolute values of tyrosine equivalents using a calibration curve.

Antioxidant Assays

Immediately subsequent to light exposure or dark maintenance, cells were lysed with 0.2% Triton X-100 in distilled water for 5 minutes. The lysate was then assayed for the following antioxidants.

Catalase Assay. The catalase activity was measured by the modified method of Cohen et al.[21] The cell lysate was incubated for 5 minutes in the presence of 6 mM H_2O_2 at room temperature. The reaction was quenched by the addition of 0.75 N H_2SO_4 and 50 mM $FeSO_4$. The color of the products formed was developed by the addition of potassium thiocyanate (KSCN), and the absorbance of the ferrithiocyanate product was colorimetrically measured at 460 nm. The standard curve of known activity of pure catalase was used to calculate the absolute values of test samples.

SOD Assay. The cell lysate was incubated with 50 mM phosphate buffer (pH 7.4), containing 0.1 mM EDTA, 62 μM NBT, and 98 μM NADH. The reaction was initiated by the addition of 3.3 μM PMS.[22] The absorbance of reduced NBT in the reaction mixture was monitored at 560 nm after a 5-minute incubation. The assay does not distinguish between Cu-Zn-SOD and Mn-SOD. The absolute values of SOD in test samples were calculated from the standard curve of known activity of pure catalase.

Glutathione Assay. Glutathione was determined essentially as described by Cui and Lou.[23] The cell lysate was incubated with 0.1 mM DTNB in the presence of 1 M Tris-HCl buffer (pH 8.2) containing 0.02 M EDTA. The absorbance of the reaction product was measured at 412 nm. The absolute values of glutathione were calculated from the standard curve of known concentrations of pure glutathione.

Statistical Analysis

All assays were performed in triplicate on at least three separate occasions, each with a culture isolated from a different donor. To account for the effect of lipofuscin on absorption as well as interexperimental variation, data were normalized against control absorbances obtained immediately before light exposure-dark maintenance and are expressed as a percentage of control absorbance. The rate of enzyme inactivation was calculated from the standard plot of known values of product or the enzyme itself on computer (Prism, ver. 3.02; GraphPad, San Diego, CA). Analysis of variation (ANOVA) was used to determine interexperimental variation, and a Student's *t*-test was performed to identify variation between time points. Values of significance were taken as $P < 0.01$.

RESULTS

RPE Cell Viability

There was no significant loss of cell viability during the first 3 hours of the study, irrespective of whether cells were fed lipofuscin or exposed to light (Fig. 1). A slight (~10%) but significant decrease ($P < 0.01$) in cell viability was observed at 6 hours in lipofuscin-fed cells, whether exposed to light or not. Lipofuscin-fed cells continued to show an increasing loss of viability that was greatest at 24 hours in lipofuscin-fed cells exposed to light (42% decrease in viability) compared with unfed cells. After these results, we decided to assess enzyme and antioxidant activity at 3 hours (no significant cell loss) and 6 hours (initial phase of cell death). There was no decrease in viability in cells maintained in the absence of lipofuscin.
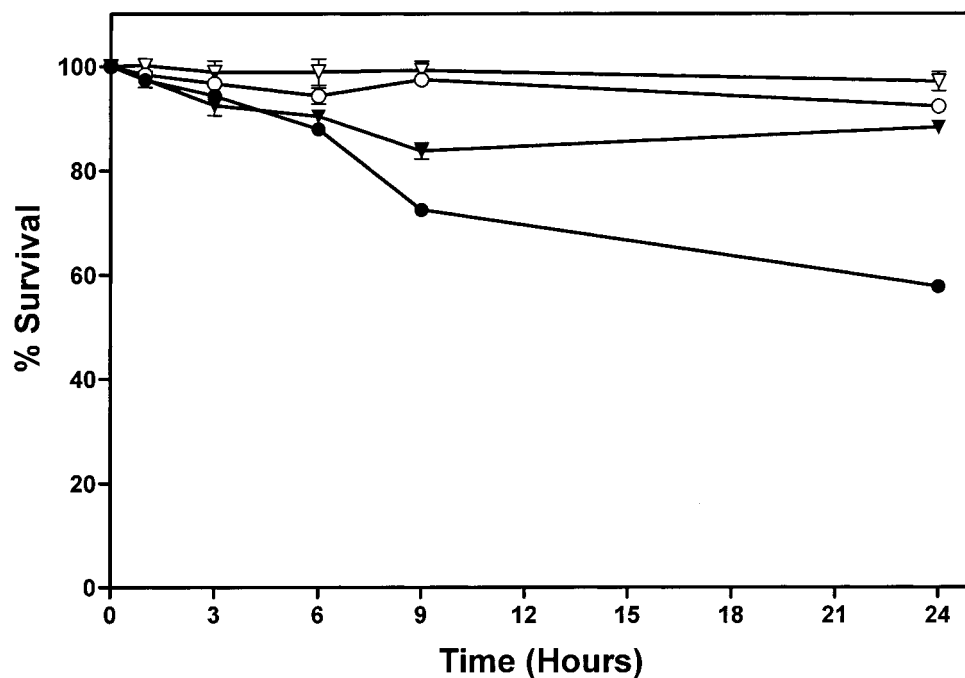
FIGURE 1. The effect of lipofuscin and light on RPE cell viability. RPE cells were cultured under the following conditions over a 24-hour period: with lipofuscin and exposed to light (●), with lipofuscin and maintained in the dark (▼), without lipofuscin and exposed to light (○), and without lipofuscin and maintained in the dark (▽). Viability was assessed by the ability of the cells to reduce MTT to formazan. The absorbance of the product was measured at 570 nm. The results shown are the mean of four experiments; *vertical bars* represent SEM.

Lysosomal Enzyme Activity

All three lysosomal enzymes demonstrated a significant decrease in activity in lipofuscin-fed cells in the presence of light at both 3 and 6 hours compared with either unfed cells or cells maintained in the dark (Fig. 2). Unfed cells maintained in the dark showed no significant loss of enzyme activity throughout the time course of the experiment. Lipofuscin-fed cells exposed to light demonstrated a significant ($P < 0.01$) 27% and 53% loss of acid phosphatase activity, a 37% and 53% loss of N-acetyl-β-glucuronidase activity, and a 30% and 48% loss of cathepsin D activity at 3 and 6 hours after light exposure, respectively, compared with unfed cells maintained in the dark. Some enzyme inactivation was observed in lipofuscin-fed cells in the absence of light compared with unfed cells in the absence of light (19%, 21%, and 26% for acid phosphatase, N-acetyl-β-glucuronidase, and cathepsin D, respectively), much less than that observed for lipofuscin-fed cells exposed to light. The enzyme activity in unfed cells exposed to light was not significantly different from that observed in unfed cells maintained in the dark.

Antioxidant Activity

There was a decrease in antioxidant potential of lipofuscin-fed cells compared with unfed cells (Fig. 3). The greatest decrease was observed in light-exposed lipofuscin-fed cells that demonstrated a significant ($P < 0.01$) decrease of 35% and 65% for catalase, 28% and 48% for SOD and 13% and 35% for glutathione at 3 and 6 hours, respectively, compared with unfed cells maintained in the dark, which showed no change in antioxidant potential over the time course of the experiment. By contrast, unfed cells exposed to light showed a significant decrease in antioxidant potential that was greatest at 6 hours after exposure: 33%, 30%, and 17% with catalase, SOD, and glutathione, respectively. The levels of SOD and glutathione in lipofuscin-fed cells maintained in the dark were intermediate between unfed cells exposed to light and lipofuscin-fed cells exposed to light. Although catalase activity in lipofuscin-fed cells maintained in the dark tended to be lower than unfed cells exposed to light, the difference was not significant.

Discussion

In this study, lipofuscin inhibited lysosomal function and reduced antioxidant capacity in RPE cells, confirming our hypothesis that lipofuscin can be detrimental to RPE cell function. That this occurred in a cellular system with its full complement of antioxidants and repair systems demonstrates the potential for such action in vivo. It is not surprising that lipofuscin inhibited biochemical pathways, because light has previously been shown to photogenerate the production of ROS from lipofuscin,[10] and ROS cause oxidant damage to proteins and peptides.[24] Indeed, we have previously shown that the photogeneration of ROS by lipofuscin can cause extracellular damage to acid phosphatase and catalase and that this can be prevented by the addition of antioxidants.[12] An unexpected observation was that lipofuscin-fed cells maintained in the dark also caused some loss of both lysosomal and antioxidant activity, but this was always significantly less than lipofuscin in the presence of light. The dark effect of lipofuscin has been previously reported by us[25] and others[26] who demonstrated the formation of superoxide anions and lipid peroxidation. Whether this dark effect is an inherent property of lipofuscin or induced by the isolation process is unclear.

The lysosomal system of the RPE is integral to the degradation and turnover of intracellular organelles and ingested photoreceptor outer segments. A decline in the efficiency of this system leads to the build up of nondegradable material and cellular congestion, culminating in cell dysfunction. Our observation that lipofuscin inhibited lysosomal enzyme activity is in keeping with previous results[12] and is likely to be predominantly due to the photogeneration of ROS.[10,11] However, it is possible that N-retinylidene-N-rentinylethanolamine (A2E), a major fluorophore of lipofuscin, may also contribute to this inhibition, because some enzyme inactivation was observed in the absence of light. Holz et al.[27] have previously demonstrated that A2E reduces lysosomal activity in the absence of light. Whether this explains the reactivity of lipofuscin under dark conditions has yet to be determined. However, the concentration of A2E contained within the lipofuscin fed to our cells[28]
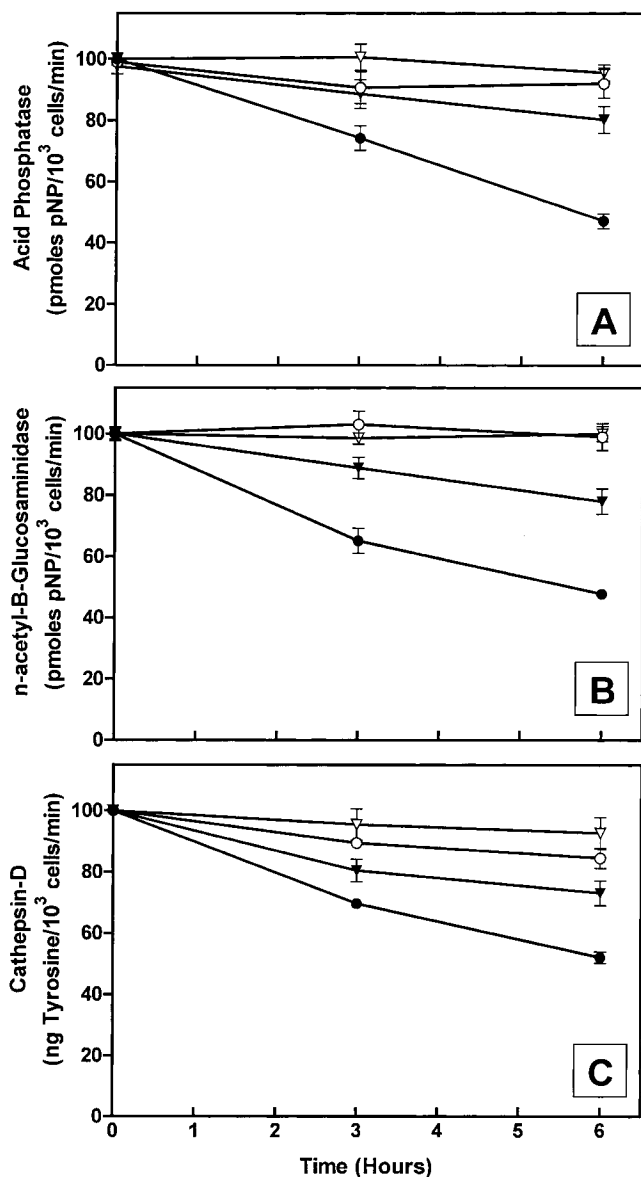
FIGURE 2. The effect of lipofuscin and light on lysosomal enzyme activity. RPE cells were cultured under the conditions described in Figure 1 over a 6-hour period and the lysates assayed for acid phosphatase (**A**), *N*-acetyl-β-glucuronidase (**B**), or cathepsin D (**C**). Acid phosphatase and *N*-acetyl-β-glucuronidase activities are presented as picomoles of *p*-nitrophenol produced from the phenol phosphate substrate per 10^3 cells/min. Cathepsin D activity is presented as nanograms tyrosine released from the hemoglobin substrate per 10^3 cells/min. The data were normalized against untreated RPE cells at time 0, to account for experimental variations due to different cell conditions. The results shown are the mean of three experiments; *vertical bars* represent SEM.

greater the number of lipofuscin granules the less the number of free lysosomes[35,36]; thus, the capacity for lysosomes to efficiently degrade ingested outer segments is reduced. Furthermore, the chronic inactivation of lysosomal enzymes through lipofuscin may result in newly synthesized enzymes being directed away from primary lysosomes. Cingle et al.[37] have reported a decrease in the specific activity of a number of lysosomal enzymes with age, and Rakoczy et al.[5] have demonstrated downregulation of cathepsin D in association with retinal ageing changes. The recent observation that heme oxygenase (a marker for oxidative stress) is increased within lysosomes in the macular RPE is further support for oxidative stress occurring within the lysosomal vacuome.[38] Brunk et al.[39] and Hellquist et al.[40] have previously shown that exposure

was more than 100 times less than that used to inhibit lysosomal enzyme activity in the study by Shütt et al.[16]

To what extent lipofuscin contributes to changes in RPE lysosomal enzyme activity with age is unclear. There are conflicting reports of both an age-related increase and decrease of lysosomal enzyme activity in the body with data showing differences between species, the type of tissue analyzed, and the methodology.[29–32] In general, studies indicate an increase in lysosomal enzyme activity with age in the RPE.[32–34] This apparent contradiction of our data can be easily explained by the steady accumulation of lipofuscin granules with age. The
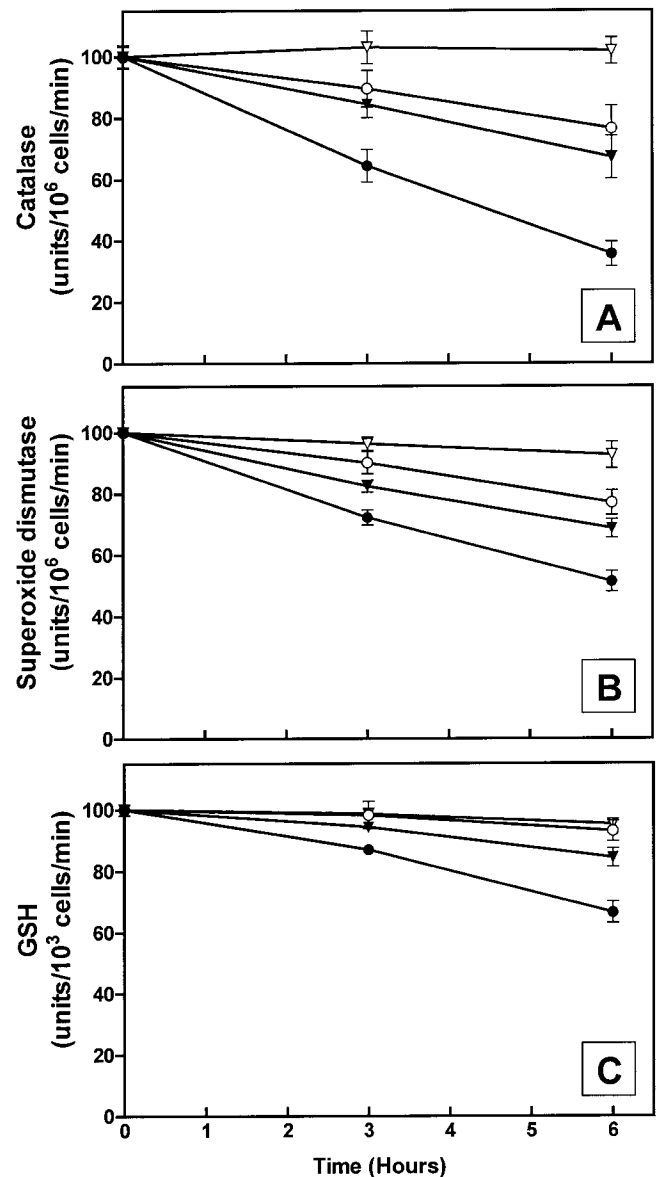

FIGURE 3. The effect of lipofuscin and light on the antioxidant potential of RPE cells. RPE cells were cultured under the conditions described in Figure 1 over a 6-hour period and the lysates assayed for catalase (**A**), SOD (**B**), or glutathione (**C**). Catalase and SOD activities are presented as units/10^6 cells/min and glutathione as units/10^3 cells/min. The data were normalized against untreated RPE cells at time 0, to account for experimental variations due to different cell conditions. The results shown are the mean of three experiments; *vertical bars* represent SEM.

of cells to nonlethal concentrations of oxidative stressors induces degeneration–repair mechanisms involving lysosomal stabilization and that this may in part involve interaction with reactive ferrous iron through Fenton-type mechanisms.[41]

The observation that irradiation of lipofuscin-fed cells resulted in a decrease in antioxidant status was unexpected. It had been hypothesized that antioxidants' enzyme activity would increase, to neutralize the increased presence of ROS. However, it is likely that lipofuscin-derived ROS directly inhibit the action of SOD and catalase as well as regulating the availability of glutathione.[12] Reports on age-related changes to the antioxidant system are generally conflicting and often tissue specific.[42–44] In general, one or more of the antioxidant enzymes decrease with age, but this varies with both species and tissue. This decrease in antioxidant protection is believed to contribute to the ageing process, because life span can be significantly increased by the use of SOD-catalase mimetics[45] and transgenics.[46] Analysis of RPE samples has demonstrated an age-related decrease in catalase activity but no change in SOD activity[47] in the macular RPE, whereas De La Paz et al.[48] demonstrated a decrease in SOD in the peripheral RPE with no change in the macula; no changes were observed in either the periphery or macula for other antioxidants tested. Our results indicate that lysosome-centered generation of ROS results in both intra and extralysosomal damage.

In our studies lipofuscin had the capacity to inhibit the action of both the lysosomal and antioxidant systems in the RPE. To what extent this chronic low-level damage contributes to the ageing process and the development of AMD is unclear. Decreased catalase activity in the cytoplasm and lysosomes and decreased plasma glutathione reductase, glutathione peroxidase, and SOD have all been reported in patients with AMD.[38,49,50] Further studies are essential to confirm the contribution oxidative damage makes to RPE ageing and dysfunction.

References

1. Boulton ME. Ageing of the retinal pigment epithelium. In: Osborne N, Chader G, eds. *Progress in Retinal Research*. Vol. 11. Oxford, UK: Pergamon; 1991:125–151.

2. Young RW. Shedding of discs from rod outer segments in the rhesus monkey. *J Ultrastructure Res.* 1971;34:190–203.

3. Cai J, Nelson KC, Wu M, Sternberg P Jr, Jones DP. Oxidative damage and protection of the RPE. *Prog Retinal Eye Res.* 2000;19:205–221.

4. Eldred G. Lipofuscin and other storage deposits in the RPE. In: Marmor M, Wolensberger T, eds. *The Retinal Pigment Epithelium.* Oxford, UK: Oxford University Press; 1998:651–668.

5. Rakoczy PE, Sarks SH, Daw N, Constable IJ. Distribution of cathepsin D in human eyes with or without age-related maculopathy. *Exp Eye Res.* 1999;69:367–374.

6. Feeney-Burns L, Hilderbrand ES, Eldridge S. Aging human RPE: morphometric analysis of macular, equatorial, and peripheral cells. *Invest Ophthalmol Vis Sci.* 1984;25:195–200.

7. Beatty S, Koh HH, Henson D, Boulton ME. The role of oxidative stress in the pathogenesis of age-related macular degeneration. *Surv Ophthalmol.* 2000;45:115–134.

8. Miceli M, Liles M, Newsome D. Evaluation of oxidative processes in human pigment epithelial cells associated with retinal outer segment phagocytosis. *Exp Cell Res.* 1994;214:242–249.

9. Newsome D, Miceli M, Liles M, Tate D, Oliver P. Antioxidants in the retinal pigment epithelium. *Prog Retinal Res.* 1994;13:101–123.

10. Rozanowska M, Jarvis-Evans J, Korytowski W, Boulton ME, Burke JM, Sarna T. Blue light-induced reactivity of retinal age pigment: in vitro generation of oxygen-reactive species. *J Biol Chem.* 1995;270:18825–18830.

11. Rozanowska M, Wessel J, Boulton M, et al. Blue light induced singlet oxygen generation by retinal lipofuscin in non polar media. *Free Radic Biol Med.* 1998;24:1107–1112.

12. Wassell J, Sallyanne D, Bardsley W, Boulton M. The photoreactivity of retinal age pigment lipofuscin. *J Biol Chem.* 1999;274:23828–23832.

13. Winkler B, Boulton M, Gottsch J, Sternberg P. Oxidative damage and age-related macular degeneration. *Mol Vis.* 1999;5:32, available at http://www.molvis.org/molvis/v5/p32/

14. Boulton M, Docchio F, Dayhaw-Barker P, Ramponi R, Cubeddu R. Age related changes in the morphology, absorption and fluorescence of melanosomes and lipofuscin granules of the retinal pigment epithelium. *Vision Res.* 1990;30:1291–1303.

15. Boulton ME, Marshall J, Mellario J. Retinitis pigmentosa: a preliminary report on tissue culture studies of retinal pigment epithelial cells from eight affected human eyes. *Exp Eye Res.* 1983;37:307–313.

16. Schütt F, Davies S, Kopitz J, Holz F, Boulton ME. Photodamage to human RPE cells by A2-E, a retinoid component of lipofuscin. *Invest Ophthalmol Vis Sci.* 2000;41:2303–2308.

17. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxic assays. *J Immunol Methods.* 1983;65:55–63.

18. Si F, Shin SH, Biedermann A, Ross GM. Estimation of PC12 cell numbers with acid phosphatase assay and mitochondrial dehydrogenase assay: dopamine interferes with assay based on tetrazolium. *Exp Brain Res.* 1999;124:145–150.

19. Cabral L, Unger W, Boulton M, et al. Regional distribution of lysosomal enzymes in the canine retinal pigment epithelium. *Invest Ophthalmol Vis Sci.* 1990;31:670–676.

20. Rakoczy PE, Lai CM, Baines M, Grandi SD, Fitton JH, Constable J. Modulation of cathepsin D activity in retinal pigment epithelial cells. *Biochem J.* 1997;324:935–940.

21. Cohen G, Kim M, Ogwa V. A modified catalase assay suitable for a plate reader and for analysis of brain cell cultures. *J Neurosci Methods.* 1996;67:53–56.

22. Ewing JF, Janero DR. Microplate superoxide dismutase assay employing a nonenzymatic superoxide generator. *Anal Biochem.* 1995;232:243–248.

23. Cui XL, Lou MF. The effect and recovery of long tern H_2O_2 exposure on lens morphology and biochemistry. *Exp Eye Res.* 1993;57:157–167.

24. Halliwell B, Gutteridge J, Cross C. Free radicals, antioxidants and human disease: where are we now? *J Lab Clin Med.* 1995;119:598–620.

25. Boulton M, Dontsov A, Jarvis-Evans J, Ostrovsky M, Svistunenko D. Lipofuscin is a photoinducible free radical generator. *J Photochem Photobiol B Biol.* 1993;19:201–204.

26. Dontsov AE, Glickman RD, Ostrovsky AA. Retinal pigment epithelium pigment granules stimulate the photo-oxidation of unsaturated fatty acids. *Free Radic Biol Med.* 1999;26:1436–1446.

27. Holz FG, Schütt F, Kopitz J, et al. Inhibition of lysosomal degradative functions in RPE cells by a retinoid component of lipofuscin. *Invest Ophthalmol Vis Sci.* 1999;40:737–743.

28. Davies S, Elliott M, Floor E, et al. Photocytotoxicity of lipofuscin in cultured human RPE. *Free Radic Biol Med.* 2001;21:256–265.

29. Asano S, Komoriya h, Hayyshi E, Sawada H. Changes in intracellular activities of lysosomal enzymes in tissues of rats during aging. *Mech Ageing Dev.* 1979;10:81–92.

30. Ivy GO, Schottler F, Wenzel J, Baudry M, Lynch G. Inhibition of lysosomal enzymes: accumulation of lipofuscin-like dense bodies in the brain. *Science.* 1984;226:985–987.

31. De Priester W, Van Manen R, Knook DL. Lysosomal activity in the aging rat liver. II: morphometry of acid phosphatase positive dense bodies. *Mech Ageing Dev.* 1984;26:205–216.

32. Verdugo ME, Ray J. Age-related increase in activity of specific lysosomal enzymes in human retinal pigment epithelium. *Exp Eye Res.* 1997;65:231–240.

33. Penn JS, Baker BN, Howard AG, Williams TP. Retinal light-damage in albino rats: lysosomal enzymes, rhodopsin, and age. *Exp Eye Res.* 1985;41:275–284.

34. Boulton M, Moriarty P, Jarvis-Evans J, Marcyniuk B. Regional variation and age-related changes of human lysosomal enzymes in the

human retinal pigment epithelium. *Br J Ophthalmol.* 1994;78: 125–129.

35. Juang H, Lee EY, Lee SI. Age-related changes in the ultrastructural features of cathepsinB- and D-containing neurons in rat cerebral cortex. *Brain Res.* 1999;844:43–54.

36. Nakanishi H, Sastradipura DF, Yoshimine Y, et al. Increased expression of cathepsin E and D in neurons of the aged rat brain and their colocalization with lipofuscin and carboxy-terminal fragments of Alzheimer amyloid precursor protein. *J Neurochem.* 1997;68:739–749.

37. Cingle KA, Kalski RS, Bruner WE, O'Brian CM, Erhard P, Wyszynski RE. Age-related changes of glycosidases in human retinal pigment epithelium. *Curr Eye Res.* 1996;15:433–438.

38. Frank RN, Amin RH, Puklin JE. Antioxidant enzymes in the macular retinal pigment epithelium of eyes with neovascular age-related macular degeneration. *Am J Ophthalmol.* 1999;127:694–709.

39. Brunk U, Zhang H, Dalen H, Ollinger K. Exposure of cells to nonlethal concentrations of hydrogen peroxide induces degeneration-repair mechanisms involving lysosomal destabilisation. *Free Radic Biol Med.* 1995;19:813–822.

40. Hellquist H, Svensson I, Brunk U. Oxidant-induced apoptosis: a consequence of lethal lysosomal leak? *Redox Rep.* 1997;3:65–70.

41. Brunk U, Jones C, Sohal R. A novel hypothesis of lipofuscinogenesis and cellular aging based on interactions between oxidative stress and autophagocytosis. *Mutat Res.* 1992;275:395–403.

42. Rikans LE, Hornbrook KR. Lipid peroxidation, antioxidant protection and aging. *Biochem Biophys Acta.* 1997;1362:116–127.

43. Pansarasa O, Bertorelli L, Vecchiet J, Felzani G, Marzatico F. Age-dependent changes of antioxidant activities and markers of free radical damage in human skeletal muscle. *Free Radic Biol Med.* 1999;27:617–622.

44. Akcetin Z, Erdemli G, Bromme HJ. Experimental study showing a diminished cytosolic antioxidant capacity in kidneys of aged rats. *Urol Int.* 2000;64:70–73.

45. Melov S, Ravenscroft J, Malik S, et al. Extension of life-span with superoxide dismutase/catalase mimetics. *Science.* 2000;289:1567–1569.

46. Parkes TL, Elia AJ, Dickinson D, Hilliker AJ, Phillips JP, Boulianne GL. Extension of drosophila lifespan by overexpression of human SOD1 in motoneurons. *Nat Genet.* 1998;19:171–174.

47. Liles MR, Newsome DA, Oliver PD. Antioxidant enzymes in the aging human retinal pigment epithelium. *Arch Ophthalmol.* 1991; 109:1285–1288.

48. De La Paz MA, Zhang J, Fridovich I. Antioxidant enzymes of the human retina: effect of age on enzyme activity of macula and periphery. *Curr Eye Res.* 1996;15:273–278.

49. Cohen S, Olin K, Feuer W, Hjelmeland I, Keen C, Morse L. Low glutathione reductase and peroxidase activity in age-related macular degeneration. *Br J Ophthalmol.* 1994;78:791–794.

50. Prashar S, Pandav S, Gupta A, Nath R. Antioxidant enzymes in RBCs as a biological index of age-related macular degeneration. *Acta Ophthalmol.* 1993;71:214–218.