

# Photoreactivity of Aged Human RPE Melanosomes: A Comparison with Lipofuscin

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**PURPOSE.** To determine whether aging is accompanied by changes in aerobic photoreactivity of retinal pigment epithelial (RPE) melanosomes isolated from human donors of different ages, and to compare the photoreactivity of aged melanosomes with that of RPE lipofuscin.

**METHODS.** Human RPE pigment granules were isolated from RPE cells pooled into groups according to the age of the donors. Photoreactivity was determined by blue-light-induced oxygen uptake and photogeneration of reactive oxygen species. Short-lived radical intermediates were detected by spin-trapping, hydrogen peroxide by an oxidase electrode, singlet oxygen by cholesterol assay, and lipid hydroperoxides by iodometric assay.

**RESULTS.** Blue-light photoexcitation of melanosomes resulted in age-related increases in both oxygen uptake and the accumulation of superoxide anion spin adducts. The efficiencies of these processes, however, were still significantly lower than that induced by photoexcited lipofuscin. During irradiation of melanosomes, a substantial amount of oxygen was converted into hydrogen peroxide, whereas for lipofuscin, hydrogen peroxide accounted for not more than 3% of oxygen consumed. In contrast to lipofuscin, photoexcited melanosomes did not substantially increase the rate of oxidative reactions in the presence of polyunsaturated lipids or albumin. However, oxygen uptake was significantly elevated in the presence of ascorbate. Thus, the rate of photo-induced oxygen uptake in samples containing both ascorbate and melanosomes approached that observed in lipofuscin samples.

**CONCLUSIONS.** Blue-light-induced photoreactivity of melanosomes increases with age, perhaps providing a source of reactive oxygen species and leading to depletion of vital cellular reductants, which, together with lipofuscin, may contribute to cellular dysfunction. (*Invest Ophthalmol Vis Sci.* 2002;43:2088-2096)

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Supported by Grants 6PO4A 06217 and 4PO5A 03615 from the State Committee for Scientific Research (KNCB), Poland; by Grants R01EY10832, R01EY13722, and P30EY01931 from the National Institutes of Health, Bethesda, MD; and by the Wellcome Trust, United Kingdom.

Submitted for publication October 15, 2001; revised February 25, 2002; accepted March 11, 2001.

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We have demonstrated that human RPE cells exhibit aerobic photoreactivity that significantly increases with increasing age of eye donors and with decreasing excitation wavelength.<sup>1</sup> Adult human RPE contains two types of pigment granules—melanosomes and lipofuscin—both of which are photoreactive and show increasing photoreactivity with decreasing excitation wavelengths.<sup>1</sup>

Melanosomes, the most prominent pigments in young RPE, are membrane-limited organelles containing melanin, mainly eu-melanin, which is a complex polymer—a product of enzymatic oxidation of tyrosine, synthesized in a protein matrix.<sup>2</sup> RPE melanin appears early in fetal development and its biosynthesis occurs in human melanosomes during the first postnatal years. There is evidence of melanogenesis in RPE cells in culture,<sup>3-6</sup> and data suggest the possibility of further melanin production throughout life in adult animals.<sup>2,7</sup> Although melanin is known to be photoreactive, generating superoxide anion and hydrogen peroxide,<sup>8</sup> RPE melanin is usually viewed as a photoprotective agent.<sup>9</sup> It has been shown, however, that the physicochemical properties of melanosomes, such as fluorescence and absorption change with the age of eye donors.<sup>10,11</sup> It has been suggested that age-related changes in these properties are a result of exposure of melanosomes to oxidative conditions, such as light or hydrogen peroxide.<sup>12-14</sup> Changes in the physicochemical properties of melanosomes with age raise the possibility that their photoreactivity also undergoes age-related changes.

Lipofuscin, which accumulates within the RPE during aging, is believed to originate from the incomplete lysosomal digestion of shed photoreceptor outer segments.<sup>15-18</sup> The composition of RPE lipofuscin granules is very different from that of melanosomes. Lipofuscin is a lipid and protein aggregate containing several fluorophores and photosensitizer(s). The chemical identity of the main photosensitizer(s) present in lipofuscin remains unknown, with only one fluorescent component of lipofuscin being isolated and characterized to date.<sup>19-22</sup> With aging, human RPE also accumulates complex granules: melanolipofuscin, containing both melanin and lipofuscin.<sup>17-18</sup> Accumulation of lipofuscin and melanolipofuscin is associated with age-related macular degeneration.<sup>7,23</sup> We have shown that RPE lipofuscin is one of the major factors contributing to the age-related increase in aerobic photoreactivity of RPE.<sup>1</sup> Although the corresponding photochemistry is rather complex, we have identified several reactive oxygen species that are generated on irradiation of retinal lipofuscin with blue light: singlet oxygen, superoxide anion, hydrogen peroxide, and lipid hydroperoxides.<sup>1,24,25</sup>

Although both lipofuscin and melanosomes have been shown to generate reactive oxygen species and to promote photo-oxidation of polyunsaturated fatty acids, when excited with visible light under aerobic conditions,<sup>1,26-28</sup> there is no direct quantitative comparison of photoreactivity of these pigment granules. Therefore, it was of interest to compare and quantify the impact of lipofuscin and melanosomes on the overall photoreactivity of aged RPE and to establish their relative contributions to damage induced by blue light—light of the shortest wavelength transmitted to the adult human ret-

ina,<sup>29-31</sup> which has been implicated in the development of age-related macular degeneration.<sup>32</sup>

The purpose of the present work was to analyze the blue-light-induced photoreactivity of melanosomes isolated from individuals of different age and to compare aged melanosomes with the other major RPE pigment granule, lipofuscin. As a measure of photoreactivity, we used photo-induced oxygen uptake and generation of reactive oxygen species: singlet oxygen, superoxide anion, hydrogen peroxide, and lipid hydroperoxides. In addition, we compared pigment granule photoreactivity in the presence of reduced nicotinamide adenine dinucleotide (NADH) and ascorbate, all biological electron donors. We also compared the efficiency of photo-oxidation of potential physiological substrates, such as polyunsaturated lipids and proteins, under the same experimental conditions.

## MATERIALS AND METHODS

### Chemicals

Chemicals, at least reagent grade, were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany), Sigma Chemical Co. (St. Louis, MO), or Merck (Darmstadt, Germany) and used as supplied. 4-Protio-3-carbamoyl-2,2,5,5-tetraprodeuteromethyl-3-pyrroline-1-yloxy (mHCTPO) was a gift from Howard J. Halpern (University of Chicago, Chicago, IL) and was used as received. Phosphate-buffered saline without calcium and magnesium (PBSA) was treated with chelating resin (Chelex 100; Sigma Chemical Co.) before use to minimize the content of metal ions.

### Isolation of Lipofuscin, Melanolipofuscin, and Melanosome Granules

Human eyes were obtained from the Wisconsin Lions Eye Bank and the Manchester (UK) Eye Bank. RPE cells were isolated as described previously<sup>1</sup> and kept frozen at -70°C until sufficient material was collected for isolation of pigment granules. For study of age-related changes in melanosome photoreactivity, RPE cells were pooled according to donor age into four age groups (AGs). To allow sufficient pigment for analysis, the 33- or 40-year cutoff was chosen, because of limited numbers of eyes of young human donors. For determination of photo-induced oxygen uptake, hydrogen peroxide photogeneration, stoichiometry of oxygen consumption, and formation of hydrogen peroxide, RPE cells were divided into the following AGs: 1 to 33 years old (AG<33; 16 pairs of eyes), 35 to 57 years old (AG35-57; 22 pairs), 63 to 73 years old (AG63-73; 17 pairs), 78 to 100 years old (AG>78; 22 pairs). For another series of experiments on photo-induced oxygen uptake and electron spin resonance (ESR) spectroscopy and spin-trapping, RPE cells were divided into the following AGs: 3 to 39 years old (AG<40; 20 pairs), 42 to 60 years old (AG42-60; 33 pairs), 61 to 80 years old (AG61-80; 29 pairs), and 81 to 98 years old (AG>80; 34 pairs).

For comparative experiments between melanosomes, melanolipofuscin, and lipofuscin, pigment granules were isolated in three independent preparations from pooled RPE cells from donors more than 60 years old. Pigment granules were isolated and purified from RPE cells collected from pooled samples, as previously described.<sup>1</sup> Isolated granules were suspended in PBSA (pH 7.2) and dispersed by forcing them through a narrow-gauge needle. Concentration of the pigment granules was determined by counting on a hemocytometer. Typically, a concentration range of 0.5 to 4.0 × 10<sup>9</sup> pigment granules/mL was used in experiments.

### Preparation of Liposomes

Multilamellar liposomes for photo-induced lipid hydroperoxides and oxygen uptake measurements consisted of 2 mM dimyristoyl phosphatidylcholine (DMPC), 2 mM cholesterol, 2 mM linoleic acid methyl ester, and 1 mM docosatetraenoic methyl ester (PUFA liposomes).

Unilamellar liposomes for singlet oxygen detection consisted of 2.5 mM DMPC, 2.5 mM cholesterol, and 0.125 mM dicyl phosphate (DCP) (DMPC/cholesterol/DCP liposomes). Liposomes were prepared under anaerobic conditions. A chloroform solution of lipids in appropriate concentrations was dried under argon and then in vacuum conditions. To obtain unilamellar vesicles, after hydration of the lipid film in PBSA, followed by five cycles of freezing and thawing, the resulting multilamellar vesicles were passed through two 0.1-μm polycarbonate filters (Nucleopore Corp., Pleasanton, CA) in an extruder apparatus (Lipex Biomembranes, Vancouver, British Columbia, Canada).<sup>33,34</sup>

### Blue-Light Irradiation

Suspensions of pigment granules were irradiated either with broadband blue light derived from a compact-arc, high-pressure Xenon lamp (Eimac VIX 300 UV; Varian, Sunnyvale, CA) with a combination of cutoff and broadband filters; effective spectral range, 408 to 495 nm; fluence rate, approximately 22 mW/cm<sup>2</sup>, or with a monochromatic light derived from a compact-arc, high-pressure mercury lamp (PhotoMax 200 W; Oriol, Stratford, CT) equipped with a combination of narrowband interference and broadband filters (effective fluence rate at the sample surface was 5 to 12 mW/cm<sup>2</sup>, with half-peak bandwidth of 10 nm and maximum of transmission at 406 nm). Fluence rates were routinely measured by radiometer (model 65A; Yellow Spring Instruments Co., Yellow Springs, OH) and/or a calibrated silicon photodiode (Photonics, KK; Hamamatsu, Hamanatsu City, Japan).

### Photo-dependent Oxygen Uptake

Kinetics of oxygen concentration changes in irradiated samples were measured, either using an oxygen electrode or by ESR oximetry.<sup>1,35,36</sup> In ESR oximetry, a sample, with an addition of 0.1 mM mHCTPO used as the nitroxide spin probe, was placed in a flat quartz cell (optical path length, 0.25 mm) in a resonant cavity, and ESR spectra of mHCTPO were collected during their illumination *in situ*, at ambient temperature.<sup>1</sup> Rates of oxygen uptake were obtained by determining the slope of initial oxygen uptake, where the oxygen concentration decreased linearly with time.

### Hydrogen Peroxide

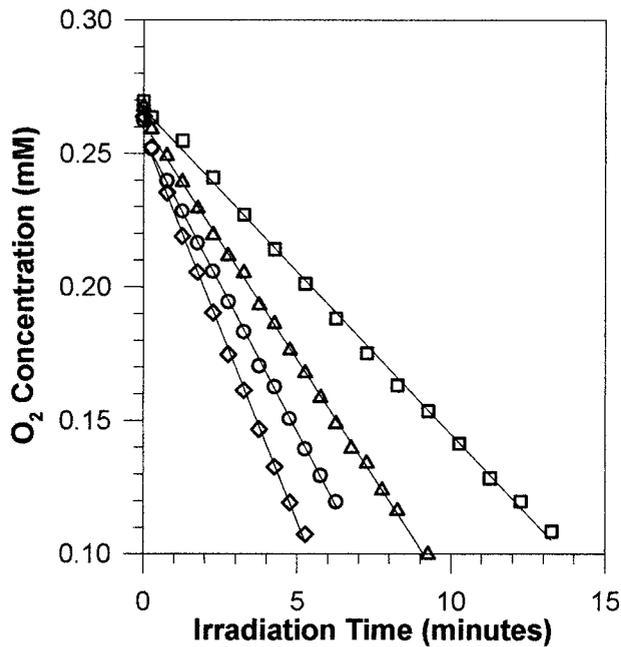
Concentrations of accumulated hydrogen peroxide during irradiation of suspensions of pigment granules were determined at 4°C to minimize decomposition of hydrogen peroxide. Samples were illuminated in a thermostat-controlled chamber with stirring, and aliquots of the granule suspension were taken during sample illumination. The concentration of hydrogen peroxide was measured using an oxidase electrode (YSI, Yellow Springs, OH) as described previously.<sup>1,37</sup>

### Stoichiometry of Oxygen Consumption and Hydrogen Peroxide Formation

Stoichiometry of oxygen consumption and hydrogen peroxide formation by isolated pigment granules was determined by monitoring the initial depletion of oxygen and the formation of hydrogen peroxide after onset of sample illumination.<sup>1,37</sup> Consumption of oxygen was measured at 4°C with a miniature oxygen electrode (Instech Laboratories, Horsham, PA) in a thermostat-controlled, closed optical chamber with stirring (optical path length, 0.5 cm). After consuming 5%, 10%, 15%, 20%, 30%, or 50% of oxygen, sample aliquots were withdrawn, and concentration of hydrogen peroxide was determined by an oxidase electrode. The determination of hydrogen peroxide concentration was repeated after selected times of further irradiation in an open optical chamber.

### ESR Spectroscopy and Spin-Trapping

For detection of long-lived or persistent radicals, such as the melanin radical, direct ESR spectroscopy was used.<sup>38</sup> ESR spectra were recorded using a spectrometer (ESP 300E; Bruker, Billerica, MA) operat-



**FIGURE 1.** Broadband, blue-light-induced oxygen uptake in suspensions of melanosomes isolated from donors in the following AGs: AG less than 40 (□), AG42 to 60 (△), AG61 to 80 (○), AG more than 80 (◇). Oxygen uptake in the dark was negligible in all samples studied. The concentration of pigment granules was adjusted to  $4 \times 10^9$  granules/mL. Oxygen uptake was measured with ESR oximetry.

ing at 9.5 GHz with 100-kHz field modulation. For the detection of the superoxide anion and other short-lived radicals, 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO; 200 mM) was used as a spin trap for radicals formed by photoexcited melanosomes or lipofuscin granules in dimethyl sulfoxide (DMSO) or a mixture of DMSO and PBSA.<sup>39</sup> The time course of formation of DMPO adducts and their decay was followed in the dark and during irradiation with blue light, to distinguish between primary and secondary products.

### Lipid Peroxidation Assay

Total concentration of accumulated lipid hydroperoxides (LOOH) was measured by the iodometric assay,<sup>40</sup> after chloroform-methanol (2:1, vol/vol), extraction of lipids, according to the procedure described by Folch et al.<sup>41</sup>

### Detection of Singlet Oxygen

Cholesterol was used as an acceptor of singlet oxygen that gives characteristic oxidation products.<sup>42</sup> Pigment granules were preincubated with DMPC-cholesterol-DCP unilamellar liposomes, for 1 to 48 hours at 4°C. Samples were irradiated in a thermostat-controlled chamber with blue light, and, after selected time intervals, aliquots were withdrawn and lipids were extracted according to the Folch procedure. HPLC separation and electrochemical detection of cholesterol hydroperoxides were performed as described previously.<sup>1,43</sup>

### Statistical Analysis

Statistical analysis was performed by Student's *t*-test, and linear regression was performed by the method of least squares.

## RESULTS

Irradiation of melanosomes with blue light demonstrated age-dependent oxygen uptake (Fig. 1), and the rate of this process consistently increased with age of melanosome donors. Comparing the youngest and the oldest melanosomes the rate of

blue-light-induced oxygen uptake increased by a factor of 2.4 between melanosomes from donors less than 40 and more than 80 years old.

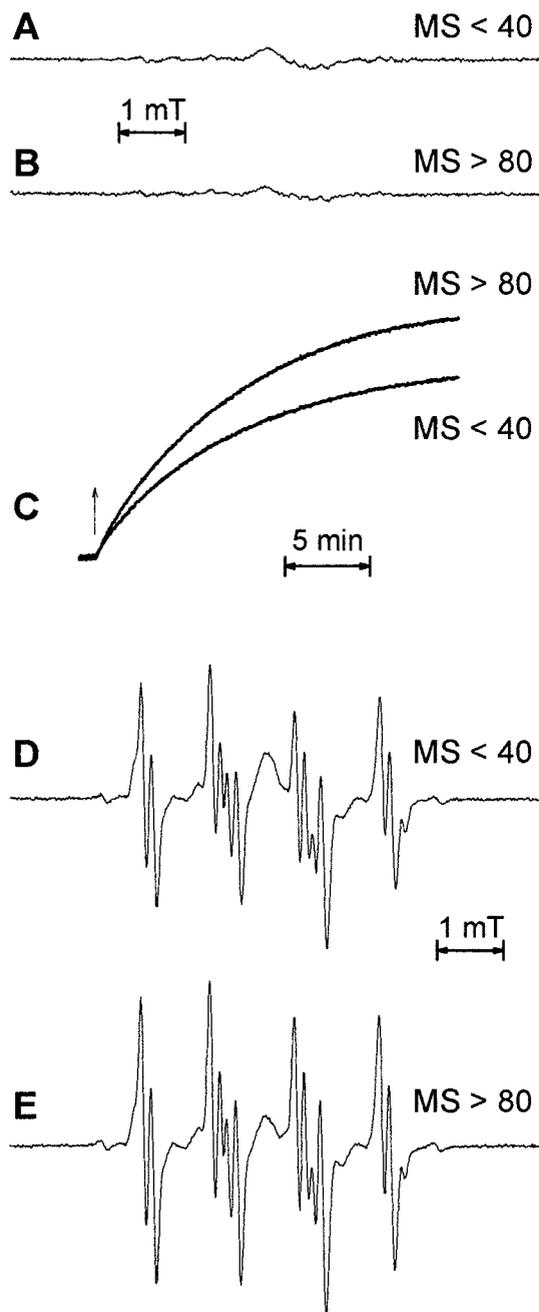
To determine whether photoexcitation of pigment granules under aerobic conditions leads to the formation of free radicals, ESR spin-trapping was used. During blue-light irradiation of melanosomes in DMSO in the presence of DMPO, spin adducts were formed, for which the spectra could be assigned to DMPO-OOH adducts (Fig. 2).<sup>39</sup> The rate of accumulation of DMPO-OOH adducts photogenerated by melanosomes was concentration dependent and monotonically increased with donor age in the four AGs studied, being greater by a factor of 1.4 between the groups less than 40 and more than 80 years of age.

Because the typical product of dismutation of the superoxide anion is hydrogen peroxide, and hydrogen peroxide is a known product of aerobic photoexcitation of melanin and bovine RPE melanosomes,<sup>44</sup> we compared the rates of blue-light-induced accumulation of hydrogen peroxide in suspension of melanosomes from human donors of different age (Fig. 3A). There were no statistically significant age-related changes in the rates of accumulation of hydrogen peroxide induced by blue-light irradiation of melanosomes from different AGs. However, the percentage of consumed oxygen that is converted to hydrogen peroxide significantly decreased with aging (Fig. 3B).

Because photoreactivity of melanosomes increased with aging, the next step was to compare the photoreactivity of aged melanosomes with lipofuscin and melanolipofuscin isolated from donors more than 60 years old. The rate of blue-light-induced oxygen uptake by aged melanosomes was still approximately six times lower than for lipofuscin (Fig. 4A). Photo-induced formation of hydrogen peroxide mediated by melanosomes, melanolipofuscin, and lipofuscin (Fig. 5) shows that accumulation of hydrogen peroxide was observed during irradiation of all pigment granules, with the initial rate highest for melanosomes, approximately six times higher than for lipofuscin, and intermediate for melanolipofuscin. For melanosomes, approximately 27% of consumed oxygen was accumulated as hydrogen peroxide, less for melanolipofuscin (approximately 11%), and only approximately 1% for lipofuscin (Fig. 4B). The rate of accumulation of DMPO-OOH spin adducts in the presence of photoexcited melanosomes was lower by a factor of 0.5 to 0.7 than for lipofuscin (Fig. 6).

Because of the unknown rate of decomposition of lipid hydroperoxides, it is not possible to determine the stoichiometry between oxygen uptake and formation of lipid hydroperoxides. Thus, only comparative measurements of their concentration were undertaken (Fig. 4C). The yield of lipid hydroperoxides generated after irradiation with blue light was the highest for lipofuscin, followed by melanolipofuscin. The production of lipid hydroperoxides by melanosomes was only slightly above the limit of detection. The yields of photoformation of LOOH correlated positively with the rates of photo-induced oxygen uptake observed during irradiation of different pigment granules (Fig. 4A, C).

To determine whether the absence of detectable amounts of LOOH in irradiated melanosomes was due to the availability of the substrate for peroxidation, the photogeneration of lipid hydroperoxides was compared in the presence and absence of added exogenous polyunsaturated lipids in the form of liposomes (Fig. 7). For melanosomes, the presence of extragranular substrate for lipid peroxidation did not significantly increase the level of lipid hydroperoxides. In contrast, the concentration of lipid hydroperoxides was markedly higher in samples containing lipofuscin in the presence of liposomes than without them. Some increase in the concentration of lipid hydroperoxides was also observed for melanolipofuscin irradiated in the presence of liposomes.

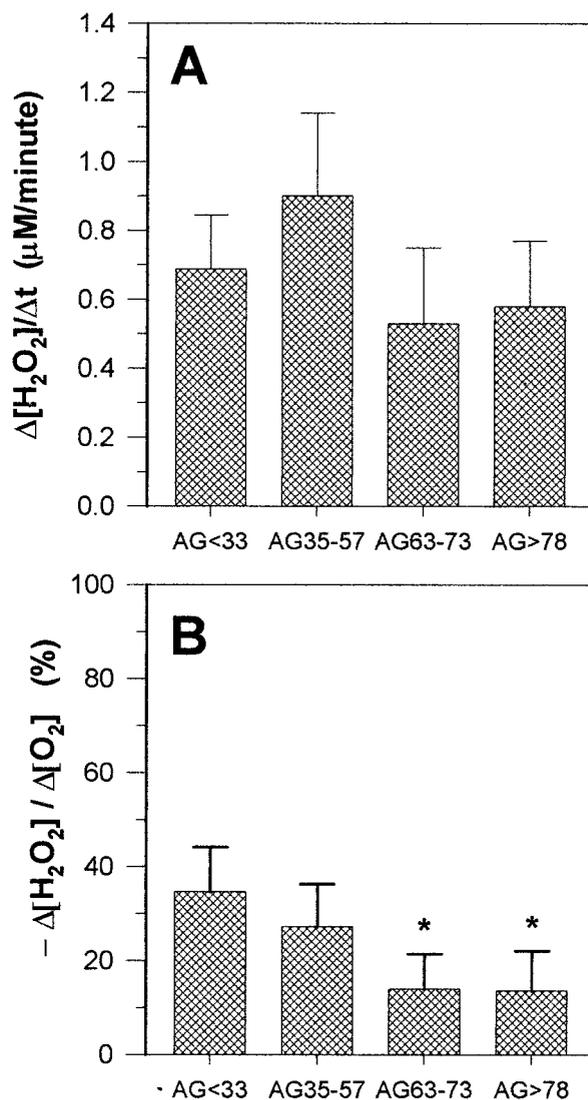


**FIGURE 2.** ESR spectra recorded for melanosomes isolated from donors less than 40 (A, D; AG<40) or more than 80 (B, E; AG>80) years old in the presence of 0.2 M DMPO in DMSO before (A, B), and after (D, E) 22 minutes of irradiation with broadband blue light. (C) Kinetics of changes in amplitude of spin adduct formed during irradiation. Arrow: beginning of irradiation. Incubation of melanosomes with DMPO in the dark did not result in the appearance of an ESR signal of spin adducts. The concentration of granules was adjusted to  $0.9 \times 10^9$  granules/mL. Instrument settings: time constant 0.328 seconds; sweep time 160 seconds; microwave power 10 mW; modulation amplitude 1.0 G.

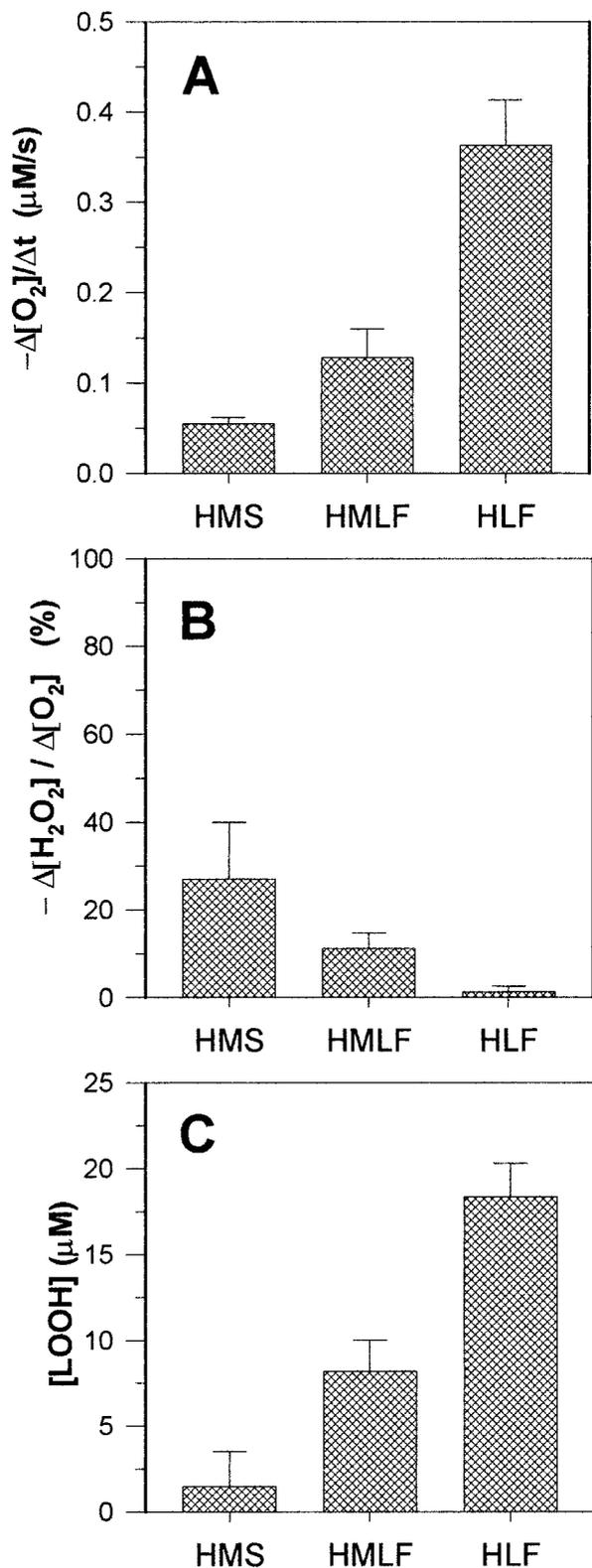
During irradiation, the rate of accumulation of LOOH was decreased, and after longer irradiation times, even the concentration of LOOH decreased. This indicates that the observed kinetics of accumulation of LOOH is a superposition of two processes: photo-induced generation and decomposition of hydroperoxides. Therefore, to determine whether extragranular lipids undergo photo-induced oxidation mediated by lipo-

fuscin or melanosomes, we monitored photo-induced oxidation by measuring photo-induced oxygen uptake in a closed system containing pigment granules, with and without added PUFA liposomes (Fig. 8). In the presence of polyunsaturated lipids, the rate of blue-light-induced oxygen uptake increased approximately 1.6 times in samples containing melanosomes, but was still much lower than for lipofuscin. In contrast, the addition of polyunsaturated lipids to the suspension of lipofuscin induced an increase in the rate of oxygen uptake by a factor of 2.4.

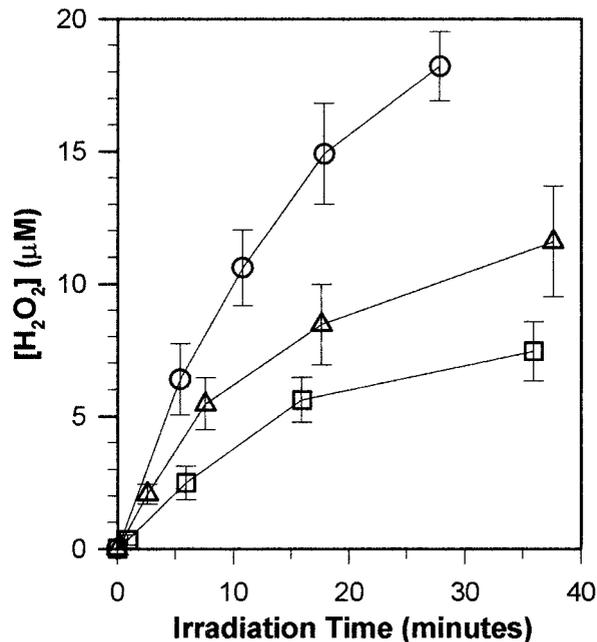
Lipid peroxidation may be induced by singlet oxygen, and therefore we compared the ability of photoexcited melanosomes and lipofuscin to generate singlet oxygen. Irradiation of lipofuscin granules with blue light, in the presence of cholesterol, leads to generation of 5 $\alpha$ -cholesterol hydroperoxides, which are characteristic products of the interaction of cholesterol with singlet oxygen.<sup>1</sup> However, when we used the same approach with melanosomes, we could not detect any traces of



**FIGURE 3.** Initial rates of narrow-band blue-light-induced accumulation of hydrogen peroxide (A) and the ratios of hydrogen peroxide accumulated to the oxygen consumed (B) in suspension of melanosomes from indicated AGs. No oxygen uptake and hydrogen peroxide generation was detected in the dark. The concentrations of melanosomes were adjusted to  $1.2 \times 10^9$  granules/mL. Results are the mean  $\pm$  SD of at least three replicates, and significant differences ( $P < 0.05$ ) are relative to the youngest age group (AG<33).



**FIGURE 4.** The rates of oxygen uptake (A), ratios of accumulated hydrogen peroxide to the consumed oxygen (B), and the concentrations of accumulated lipid hydroperoxides (C), measured after 15% of oxygen was consumed during irradiation with broadband blue light of suspensions of RPE melanosomes (HMS), melanolipofuscin (HMLF), or lipofuscin (HLF), isolated from human donors more than 60 year old. No oxygen uptake or lipid and hydrogen peroxide generation was detected after corresponding incubation times in the dark under the conditions used. The concentrations of pigment granules were ad-



**FIGURE 5.** Accumulation of hydrogen peroxide during broadband blue-light irradiation of RPE pigment granules: melanosomes ( $\circ$ ), melanolipofuscin ( $\Delta$ ) and lipofuscin ( $\square$ ), isolated from human donors more than 60 year old. The concentrations of hydrogen peroxide in samples kept in the dark were undetectable. The concentrations of pigment granules were adjusted to  $1.2 \times 10^9$  granules/mL.

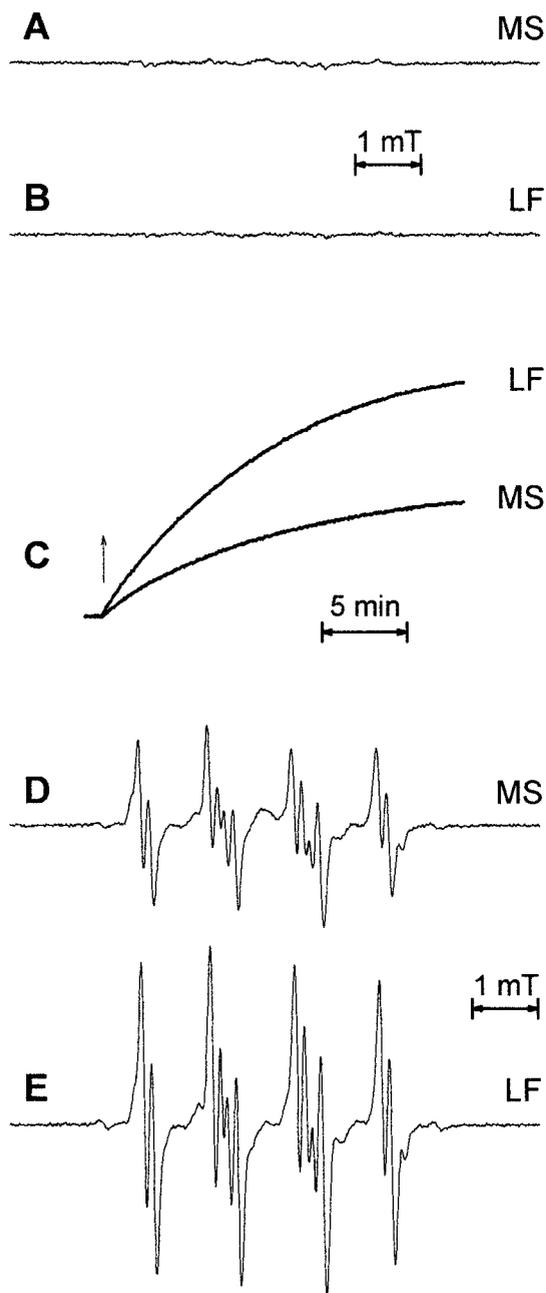
5 $\alpha$ -cholesterol hydroperoxides and therefore, from the threshold of 5 $\alpha$ -cholesterol hydroperoxide detection in the system used, the generation singlet oxygen by photoexcited melanosomes, if any, was estimated to be at least an order of magnitude lower than for lipofuscin (data not shown).

To test whether other physiological compounds may become substrates of oxidation mediated by photoexcited melanosomes or lipofuscin, we measured photo-induced oxygen uptake in samples containing pigment granules and bovine serum albumin (BSA), NADH, or ascorbate (Fig. 8). The presence of BSA resulted in an approximately 50% increase in the rate of oxygen uptake in samples containing lipofuscin, but not melanosomes. In contrast, addition of either ascorbate or NADH did not increase the rate of photo-induced oxygen uptake in samples containing lipofuscin. However, they substantially increased oxygen uptake in samples containing melanosomes, in a concentration dependent manner, being greater by a factor of approximately 1.4 and 3.1 in the presence of 2 mM NADH and 0.2 mM ascorbate, respectively (Fig. 8).

## DISCUSSION

The results obtained in this study show clearly that photoreactivity of human melanosomes increases with donor age; the photo-induced oxygen uptake mediated by melanosomes increased by a factor of 2.4 between young and old melanosomes, whereas the rate of accumulation of DMPO-OOH spin adduct increased with age by a factor of 1.4. Despite this, accumulation of DMPO-OOH spin adduct was still 1.5 to 2.0 times lower than for lipofuscin, even though lipofuscin is less

justed to  $1.2 \times 10^9$  granules/mL. Results are expressed as the mean  $\pm$  SD of at least three replicates, and differences between all three types of granules are significant ( $P < 0.01$ ).



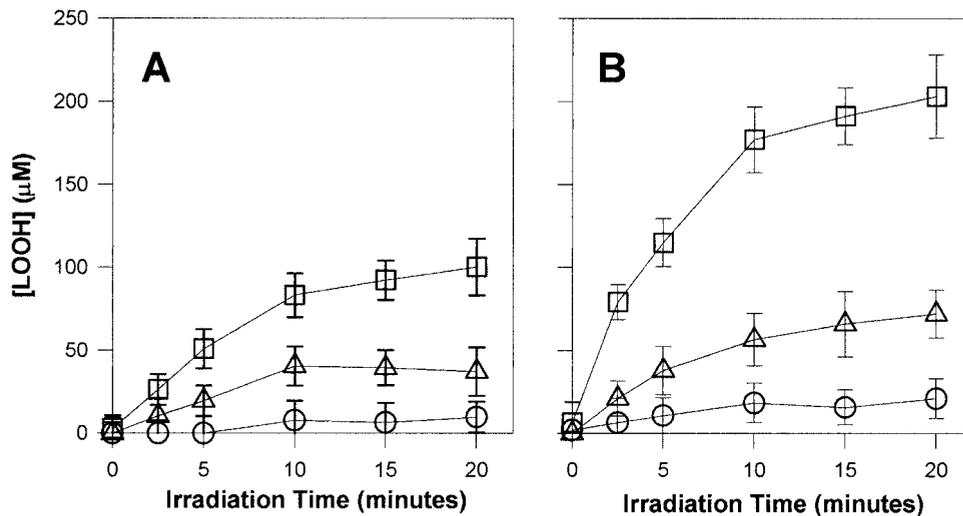
**FIGURE 6.** ESR spectra for melanosomes (MS; **A**, **D**) and lipofuscin (LF; **B**, **E**) from donors more than 60 years old in the presence of 0.2 M DMPO in DMSO before (**A**, **B**) and after (**D**, **E**) 22 minutes of irradiation with broadband blue light. (**C**) Kinetics of changes in amplitude of spin adduct during irradiation. *Arrow*: beginning of irradiation. Incubation of pigment granules with DMPO in the dark did not result in the appearance of an ESR signal of spin adducts. Concentration of granules was adjusted to  $1.6 \times 10^9$  granule/mL. Other experimental conditions are as in Figure 2.

efficient than melanosomes in the photogeneration of hydrogen peroxide. These observations may be explained in terms of the ability of melanin to act as a pseudodismutase.<sup>44</sup> Melanin is able to oxidize and reduce the superoxide anion, to oxygen and hydrogen peroxide, respectively. It has been estimated that synthetic dihydroxyphenylalanine (DOPA)-melanin mainly oxidizes the superoxide anion, which accounts for approximately 80% of the reactions between superoxide anion and DOPA-melanin, and only approximately 20% of superoxide is

reduced to hydrogen peroxide.<sup>44</sup> The scavenging of superoxide by human RPE melanosomes has been implicated also in the decreasing rates of the reduction of cytochrome *c* in the xanthine-xanthine oxidase system in the presence of increasing concentrations of RPE melanosomes.<sup>45</sup> Therefore, it is tempting to speculate that most of the superoxide anion photogenerated by melanosomes, is effectively scavenged by melanin and transformed into oxygen or hydrogen peroxide, whereas only a small fraction is able to escape and interact with DMPO. Nonetheless, with aging, melanosomes either become more efficient in generating and/or less efficient in scavenging superoxide. The efficiency of photogeneration of hydrogen peroxide by melanosomes does not significantly change with aging, which indicates that both processes may occur. Superoxide anion may be involved in damaging key cellular constituents.<sup>46</sup> In particular, if it undergoes protonization in lysosomal compartments of the cell, it may initiate a chain of lipid peroxidation and/or dismutate more effectively to hydrogen peroxide. Generation of hydrogen peroxide by photoexcited pigment granules may have more deleterious effects in aged RPE, because catalase activity in RPE decreases with aging.<sup>47,48</sup> Catalase activity also decreases during irradiation with blue light, especially in the presence of lipofuscin.<sup>26,49</sup> Therefore, photogeneration of hydrogen peroxide by photoexcited pigment granules may impose a risk of accumulation of hydrogen peroxide, particularly in the aged RPE. Hydrogen peroxide may in turn take part in Fenton type reactions to generate a very oxidizing hydroxyl radical.<sup>46</sup>

Irradiation of aged melanosomes with blue light results in oxygen uptake, and approximately 25% of oxygen consumed is used for the generation of hydrogen peroxide. Irradiation of lipofuscin induces about six times faster oxygen uptake, but accumulation of hydrogen peroxide does not exceed 3% of the oxygen consumed. The other part of the oxygen consumed must therefore lead to oxidation of intragranular components of lipofuscin and melanosomes. Although the oxidation products of melanosomes remain unidentified, one of the oxidation products formed within lipofuscin granules is lipid hydroperoxide. It might be speculated that intragranular proteins become another substrate of oxidation both in melanosomes and lipofuscin.

The results also indicate that photoexcitation of pigment granules leads not only to photo-oxidation of lipids confined within lipofuscin granules, but also to oxidation of extragranular lipids and proteins. For melanosomes, however, photoexcitation leads only to a minor increase in photo-induced oxygen uptake in the presence of extragranular unsaturated lipids compared with lipofuscin. It can be assumed that it is singlet oxygen, that is responsible, at least in part, for oxidation of exogenous lipids and proteins in the presence of photoexcited lipofuscin. This excited form of molecular oxygen, with a lifetime in aqueous solution of approximately 3  $\mu$ s, would be able to diffuse outside the granule and oxidize polyunsaturated lipids or selected amino acid residues of proteins. However, only photoexcited lipofuscin granules, and not melanosomes, are capable of efficient singlet oxygen generation, which may explain why oxidation of extragranular lipids and proteins is observed mainly with lipofuscin. In contrast, photoexcited melanosomes apparently do not generate measurable amounts of singlet oxygen, and are unlikely to produce other products with the ability to diffuse out of the granule and induce substantial oxidation of extragranular lipids and proteins. These results differ from those of Dontsov et al.,<sup>27</sup> and Glickman and Lam,<sup>28</sup> who demonstrated that RPE melanosomes efficiently induce peroxidation of lipids. These differences may be explained by the different experimental conditions used. In our study, all the samples were virtually free from metal ions, such as iron or copper. In contrast, iron chelated by EDTA (the



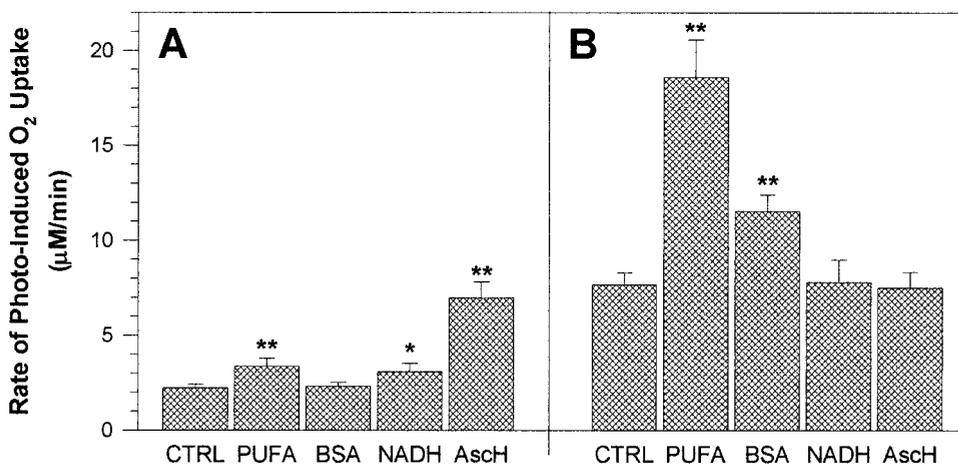
**FIGURE 7.** Broadband, blue-light-induced generation of lipid hydroperoxides in suspensions of RPE melanosomes (○), melanolipofuscin (△), and lipofuscin (□), without exogenous substrate oxidation (A), or in the presence of PUFA liposomes (B). Data are the mean  $\pm$  SD of at least three experiments. Dark generation of LOOH under the conditions used was negligible.

protocol used by others) may be reduced, and therefore activated, directly by melanin or by superoxide anion and hence take part in Fenton-type reactions leading to decomposition of hydrogen peroxide or lipid hydroperoxides.<sup>44,46</sup>

Photoexcited melanosomes induce oxidation of important cellular reductants, such as ascorbate and NADH,<sup>38,50-52</sup> whereas photoexcited lipofuscin has only a minor effect on the rate of their oxidation<sup>38</sup> and their presence does not alter the rate of oxygen uptake. The photoreactions of melanosomes with ascorbate are accompanied by an increased rate of oxygen uptake, which, in the presence of a physiological concentration of ascorbate for RPE (0.2 mM),<sup>53,54</sup> becomes similar to the rate of oxygen uptake in the presence of photoexcited lipofuscin. It should be stressed, however, that the main products of the reactions mediated by photoexcited melanosomes, in the presence of ascorbate, and lipofuscin are different. For melanosomes, the main products include superoxide anion, hydrogen peroxide, and dehydroascorbate, whereas for lipofuscin, they include products of lipid peroxidation. In our previous study we determined the mechanism responsible for photo-induced ascorbate oxidation mediated by melanin, including RPE melanosomes, under aerobic conditions.<sup>38</sup> Melanin acts as an electron transfer agent, whereas the ultimate electron acceptor is molecular oxygen, which reoxidizes the photo-reduced melanin. During this process substantial amounts of superoxide anion and hydrogen peroxide are generated, and ascorbate is oxidized to ascorbate radical and dehydroascorbate. These processes are likely to take place in

RPE, which contains high concentrations of ascorbate.<sup>53,54</sup> The depletion of ascorbate and NADH, induced by photoexcited melanin, may result in a change in the redox state of the RPE cell. It has been reported that exposure to light increases the concentrations of dehydroascorbate in primate retina.<sup>54</sup> Oxidative stress as well as abnormal redox state are believed to contribute to cellular death by apoptosis, or, at higher doses of reactive oxygen species, to necrosis.<sup>55</sup> It also indicates a possibility that ascorbate may act as a pro-oxidant during irradiation of pigmented RPE.

The irradiances received at the retinal surface from the sun or from cool-white fluorescent lights under typical lighting conditions (0.1–0.2 mW/cm<sup>2</sup>)<sup>29</sup> are one to two orders of magnitude lower than that used in our experiments. Therefore, the rates of oxidation and the fluxes of reactive oxygen species generated by photoexcited pigment granules under typical conditions would be expected to be one to two orders of magnitude lower than we observed. However, under extreme conditions during accidental or even intended exposure of the retina to higher irradiances, such as during gazing at the sun at noonday, the pigment granules may generate significant fluxes of reactive oxygen species. If the capacity of antioxidant and repair mechanisms of RPE cells is exceeded, cytotoxicity may result. The efficiency of antioxidant and repair mechanisms may be tested *in vitro* in RPE cells loaded with pigment granules and irradiated with light under controlled conditions. Recent data using ARPE-19 cells in culture indicate that, indeed, irradiation with blue light of RPE cells fed aged human



**FIGURE 8.** The effect of the addition of PUFA liposomes (PUFA), 4 mg/mL of bovine serum albumin (BSA), 2 mM NADH, or 0.2 mM ascorbate (Asch) on the rate of broadband, blue-light-induced oxygen uptake in suspensions of lipofuscin (A) or melanosomes (B). Control samples without additional substrate of oxidation (CTRL). The rates of oxygen uptake in the dark were negligible for all samples studied. Data are the mean  $\pm$  SD of at least three replicates, and significant differences are relative to the control experiments. \* $P < 0.05$ , \*\* $P < 0.01$ .

melanosomes or lipofuscin resulted in toxicity, whereas viability of RPE cells fed young bovine melanosomes was not diminished.<sup>56</sup> Due to experimental limitations, it may be only speculated that chronic exposure to reactive species generated by photoexcited pigment granules throughout life in postmitotic RPE cells would lead to build up of damage, which would become manifested only at older age.

In conclusion, in RPE of older human donors, exposed to light, both melanosomes and lipofuscin, may become a substantial source of reactive oxygen species and lead to depletion of vital cellular reductants. The consequences of increased photoreactivity of aged melanosomes for RPE cells are unknown, and also unknown is whether their antioxidant properties<sup>57</sup> undergo age-related changes. It may be speculated that the decrease of the RPE melanin content<sup>13</sup> decreases RPE antioxidant potential and together with the increased photoreactivity of melanosomes and accumulated lipofuscin granules contributes to the aging process, as well as to the pathogenesis of age-related macular degeneration.

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