THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 270, No. 32, Issue of August 11, pp. 18825–18830, 1995
Printed in U.S.A.
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Blue Light-induced Reactivity of Retinal Age Pigment

IN VITRO GENERATION OF OXYGEN-REACTIVE SPECIES*

(Received for publication, April 4, 1995, and in revised form, May 25, 1995)

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Exposure of the eye to intense light, particularly blue light, can cause irreversible, oxygen-dependent damage to the retina. However, no key chromophores that trigger such photooxidative processes have been identified yet. We have found that illumination of human retinal pigment epithelium (RPE) cells with light induces significant uptake of oxygen that is both wavelength- and age-dependent. Analysis of photoreactivity of RPE cells and their age pigment lipofuscin indicates that the observed photoreactivity in RPE cells is primarily due to the presence of lipofuscin, which, under aerobic conditions, generates several oxygen-reactive species including singlet oxygen, superoxide anion, and hydrogen peroxide. We have also found that lipofuscin-photosensitized aerobic reactions lead to enhanced lipid peroxidation as measured by accumulation of lipid hydroperoxides and malondialdehyde in illuminated pigment granules. Hydrogen peroxide is only a minor product of aerobic photoexcitation of lipofuscin. We postulate that lipofuscin is a potential photosensitizer that may increase the risk of retinal photodamage and contribute to the development of age-related maculopathy.

Although the anterior eye tissues, the cornea and lens, filter out the most damaging components of solar radiation (1), the retina can be subjected to intense illumination from focal light that includes the relatively energetic photons from the blue part of the solar spectrum (2). As a result, photic retinopathy may develop (3), and this process will be enhanced by the presence of oxygen (4). Solar radiation has also been implicated in the development of ARM[1] (5), a degenerative disease that is recognized as the predominant cause of blindness in people over 60 in many developed Western countries (6, 7). The primary lesion associated with photoreceptor degeneration and loss of vision in ARM is believed to be located in the RPE (8). However, no key chromophores that trigger primary events in either light-induced phenomena have been identified yet (9). Among several potential photosensitizing molecules that are normally present in human RPE, melanin and lipofuscin deserve special attention. These two pigments, present in the cell exclusively in the particle form, have broad optical absorption bands (10). Their biogenesis is very different; whereas RPE melanin appears very early in the fetal development (11), RPE lipofuscin becomes apparent only during the second decade of life and accumulates with age (12). Even though melanin can photogenerate superoxide anion and hydrogen peroxide (13), it is believed that under typical *in vivo* conditions, melanin acts as an efficient antioxidant (10). In this regard little is known about the photosensitizing abilities of lipofuscin, except that this age pigment stimulates photoreduction of cytochrome *c* under aerobic conditions, which suggests possible photoformation of superoxide anion (14).

To determine whether the human RPE exhibits any substantial photoreactivity that may lead to retinal phototoxicity, we measured action spectra of photoinduced oxygen uptake and photoformation of hydrogen peroxide for RPE cells. In a search for the major retinal chromophores responsible for the observed RPE photoreactivity, we studied aerobic photoreactions in suspensions of purified retinal lipofuscin and melanin granules and identified some products of the interaction of photoexcited lipofuscin with oxygen.

MATERIALS AND METHODS

Chemicals—Chemicals (at least reagent grade, unless otherwise stated) were purchased from Aldrich, Merck, or Sigma and used as supplied. Catalase (thymol-free) and superoxide dismutase (lyophilized powder) from bovine liver were purchased from Sigma. mHCTPO was a gift from Prof. H. J. Halpern (University of Chicago, Chicago, IL) and used as received. To minimize paramagnetic contaminations, DMPO was redistilled *in vacuo* before use. Phosphate buffer was treated with Chelex 100 prior to use.

RPE Cells—RPE cells were removed from the posterior poles of human and bovine eyes, and from flasks of confluent human RPE cultures (4th passage) with a rubber scraper and suspended at 1–3 mg of protein/ml of phosphate-buffered saline (15). All results of experiments with cells were normalized to equal protein content of the RPE cells as determined by the Lowry method (16). Human eyes were obtained from Wisconsin Lion Eye Bank or Manchester Eye Bank (United Kingdom), and bovine eyes from a local slaughterhouse.

* This work was supported by KBN Grant 4 P05A 115 08 from the State Committee for Scientific Research, Poland, by Core Grant P30EY01931 and Grants R01EY06664 and RR01008 from the National Institutes of Health, by Macula Foundation Inc. USA, and by Research into Aging, London, United Kingdom. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *"advertisement"* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: ARM, age-related maculopathy; RPE, retinal pigment epithelium; mHCTPO, 4-protio-3-carbamoyl-2,2,5,5-tetraperdeuteromethyl-3-pyrroline-1-yloxy; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; CTPO, 3-carbamoyl-2,2,5,5-tetramethyl-3-pyridine-1-yloxy; LOOH, lipid hydroperoxide(s); MDA, malondialdehyde; 5α-OOH, 3β-hydroxy-5α-cholest-6-ene-5-hydroperoxide; 6α-OOH, 3β-hydroxycholest-4-ene-6α-hydroperoxide; 6β-OOH, 3β-hydroxycholest-4-ene-6β-hydroperoxide; HPLC, high performance liquid chromatography; ChOOH, cholesterol hydroperoxide(s); DMPC, dimyristoyl phosphatidylcholine; DCP, dicetyl phosphate; EC, electrochemical (detection); 7α-OOH, 3β-hydroxycholest-5-ene-7α-hydroperoxide; 7β-OOH, 3β-hydroxycholest-5-ene-7β-hydroperoxide; G, gauss; W, watt(s).

Lipofuscin, Melanolipofuscin, and Melanosome Granules—Lipofuscin, melanolipofuscin, and melanosome granules were isolated from RPE cells from 60–70-year-old individuals according to a procedure previously described (17) except that mechanical homogenization was used instead of ultrasonication. Isolated granules were suspended in 10 mM phosphate buffer (pH 7.2) and dispersed by forcing them through a narrow gauge needle. Concentration of the granules was determined by counting on a hemeocytometer. Typical concentration of the pigment granules used in experiments was in the range $(0.5 - 1.5) \times 10^9$/ml.

Melanin Quantification—The amount of melanin in the samples studied was determined by the ESR melanin assay (18). ESR spectra were recorded using a Bruker ESP 300E spectrometer operating at 9.5 GHz with 100 kHz field modulation. The ESR signal intensities of acidified (pH 1) samples were compared at liquid nitrogen temperature using a measured amount of synthetic 3,4-dihydroxyphenylalanine melanin as a standard.

Action Spectra—Action spectra of oxygen photoconsumption and hydrogen peroxide photoformation were obtained using monochromatic light from a compact arc, high pressure mercury lamp (Oriel PhotoMax 200 W) equipped with a combination of narrow-band interference and broad band filters (effective fluence rate at the sample surface was 5–29 mW/cm^2 with bandwidth ~10 nm).

Rates of photo-dependent oxygen uptake were obtained by measuring kinetics of oxygen concentration changes in irradiated samples. In these measurements ESR oximetry was employed using 10^{-4} M CTPO or mHCTPO as the nitroxide spin probes (19, 20). Samples in flat quartz cells (0.25 mm) were illuminated *in situ*, in a resonant cavity, at ambient temperature. Instrument settings were: microwave power, 1 mW; modulation amplitude, 0.1 G (for mHCTPO) or 0.05 G (for CTPO).

Rates of H_2O_2 formation were determined at 4 °C in order to minimize decomposition of H_2O_2. Samples were illuminated in a thermostated optical chamber of a miniature oxygen monitor (Instech Laboratories, Horsham, PA). Aliquots of granule suspension were taken during sample illumination, and the concentration of hydrogen peroxide was measured using an oxidase electrode (Yellow Spring Instruments Co. (YSI), Yellow Springs, OH) as described previously (21).

Blue Light Effects—Blue light effects (photo-dependent uptake of oxygen and photoformation of hydrogen peroxide by RPE cells, and photoformation of lipid hydroperoxides by isolated pigment granules) were studied upon illumination derived from a compact arc, high pressure xenon lamp (Varian Eimac VIX 300 UV) with a combination of cut-off and broad band filters; effective spectral range: 408–495 nm, fluence rate ~220 mW/cm^2, unless stated otherwise.

Stoichiometry of O_2 Consumption and Hydrogen Peroxide Formation—Stoichiometry of O_2 consumption and hydrogen peroxide formation by isolated pigment granules was determined by monitoring initial depletion of oxygen and formation of H_2O_2 following onset of the sample illumination. Consumption of oxygen was measured at 4 °C using a miniature oxygen electrode (Instech Laboratories, Horsham, PA) and a thermostated closed optical chamber. Rates of H_2O_2 formation were determined in the same way as for the action spectrum of hydrogen peroxide formation. To minimize enzymatic decomposition of formed H_2O_2, cells were pretreated for 3 h at 4 °C with 1.0 mM sodium azide.

Superoxide Anion Detection—The DMPO spin probe (200 mM) was used as a spin trap for the detection of superoxide anion (22). Time course of DMPO adducts formation, and their decay, was followed to distinguish between primary and secondary products. Superoxide dismutase was used as an additional test for superoxide anion formation (23), and the involvement of hydrogen peroxide was assessed by carrying out measurements in the presence of catalase.

Determination of Lipid Peroxidation—Total concentration of lipofuscin-derived LOOH was measured by iodometric assay (24), after chloroform/methanol (2:1, v/v), extraction of lipids from irradiated and control samples.

MDA was analyzed using the thiobarbituric acid assay followed by HPLC separation and fluorometric determination (λ_{exc} = 523 nm, λ_{em} = 550 nm) of MDA-thiobarbituric acid complex, as described elsewhere (25).

Detection of Singlet Oxygen—Cholesterol was used as an acceptor of singlet oxygen that gives characteristic oxidation products with $O_2(^1\Delta_g)$: 5α-OOH, 6α-OOH, and 6β-OOH (26). Lipofuscin (6×10^8 granules/ml) was preincubated with unilamellar liposomes (5 mM total lipids) for 48 h at 4 °C. Liposomes, composed of DMPC/cholesterol/DCP (1:1:0.05) were prepared as described previously (27). Complete samples containing desferrioxamine (0.1 mM) to minimize metal-ion catalyzed free radical processes were irradiated at 4 °C with a halogen lamp. Incident light intensity was 2000 W/m^2 as measured at the surface of the sample. Copper sulfate (100 g/ml) and 408 nm cut-off filters were used to eliminate light below 408 nm and infrared. At selected irradiation times, samples were collected, and lipids were extracted with chloroform/methanol (2:1, v/v) in the presence of 1 mM EDTA and analyzed by reversed-phase HPLC-EC as described (28).

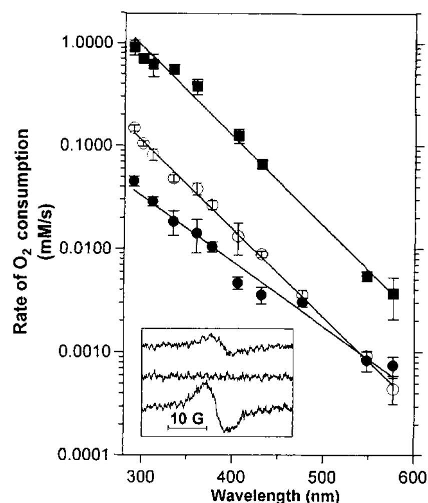

FIG. 1. **Action spectra for photo-dependent oxygen uptake in suspensions of isolated RPE cells from human donors (60–70-year-olds, ■), and bovine (the non-pigmented tapetal fundus (○) and the pigmented non-tapetal fundus (●).** Samples were equilibrated with a mixture of 50% N_2 and 50% air. Rates of photo-dependent oxygen uptake were obtained by ESR oximetry using CTPO as a spin probe. Data were normalized to equal protein content (mg/ml) and fluxes of incident photons (einstein/s/m^2). Values are the means ± S.D. of triplicate experiments. Dark consumption of oxygen, under the conditions employed, was negligible. *Inset*, ESR spectra of melanin radicals in human (*top*) and bovine non-pigmented (*middle*) and pigmented (*bottom*) RPE cells. Instrument settings: microwave power, 20 μW; modulation amplitude, 3 G.

Measurements of Light Intensity—Fluence rates were routinely measured using a model 65A YSI radiometer and/or a calibrated silicon photodiode (Hamamatsu, Photonics, K.K.).

RESULTS

Photo-dependent Oxygen Uptake and Photoformation of Hydrogen Peroxide

Retinal Pigment Epithelium—The rate of photo-dependent O_2 uptake in suspensions of RPE cells in phosphate-buffered saline was wavelength-dependent, the oxygen uptake being greatest at the shortest wavelength studied (290 nm) and decreasing by more than 50-fold at 578 nm (Fig. 1). Isolated human RPE cells demonstrated approximately 40–60-fold greater rate of uptake of oxygen in the range 406–432 nm than cultured depigmented human RPE cells (data not shown). When normalized to equal protein content and illuminated at 406 nm, RPE cells isolated from human eyes photoconsumed oxygen 8 times faster than bovine RPE cells isolated from the hypomelanotic tapetal region and about 15 times faster than those from the pigmented peripheral fundus (Fig. 1). Different slopes of the lines, drawn through data points obtained for photo-dependent oxygen uptake for pigmented and nonpigmented bovine RPE cells, may indicate that different chromophores are responsible for the observed phenomena in both cell types. Alternatively, the observed difference may be due to melanin-related optical screening in the pigmented cells. The ESR assay demonstrated that pigmented bovine RPE cells from the peripheral fundus contained at least 100 times more melanin than tapetal non-pigmented cells and 3–5 times more than human RPE cells (Fig. 1, *inset*). Therefore, the rate of oxygen consumption in RPE cells does not correlate with the amount of melanin present in the cells. Neither catalase (0.15 mg/ml) nor superoxide dismutase (0.10 mg/ml) had any meas-

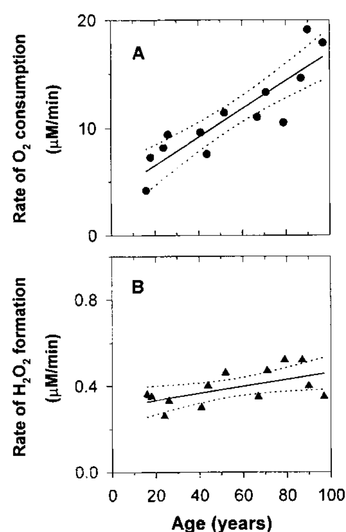

FIG. 2. **The effect of donor age on the consumption of oxygen (*A*) and the formation of hydrogen peroxide (*B*) in irradiated samples (408–495 nm, 220 mW/cm²) of isolated human RPE cells.** Data were normalized to 1 mg of protein/ml of phosphate-buffered saline. *Solid line* represents the best linear approximation of the experimental data; *dotted lines*, 95% confidence interval.

urable effect on the rate of photo-dependent oxygen uptake, while lipid-soluble antioxidants such as butylated hydroxytoluene and α-tocopherol (0.2 mM each) only partially inhibited photo-dependent uptake of oxygen.

Analysis of blue light photoreactivity observed in human RPE cells, isolated from individuals 16–97 years of age, demonstrated a marked increase in the rate of photo-dependent oxygen uptake as the donor age increased (Fig. 2*A*), and only a modest change in the corresponding rate of hydrogen peroxide photoproduction (Fig. 2*B*). The data also indicate that hydrogen peroxide is only a minor product of aerobic photoactivation of human RPE cells, accounting for no more than 6% of the oxygen consumed.

Isolated Pigment Granules—Since lipofuscin and melanin granules are major chromophores that efficiently absorb visible and near-ultraviolet radiation in the adult human RPE, we compared the rates of light-induced oxygen consumption and H_2O_2 formation in purified populations of these pigment granules (Fig. 3), isolated from donors 60–70 years of age. The efficiency of both photoprocesses in either pigment granule type exhibited strong wavelength dependence. When normalized to an equal concentration of pigment granules and number of incident photons at 406 nm, lipofuscin induced almost 6 times faster uptake of O_2 than melanin, with melanolipofuscin being intermediate. Rates of photoinduced oxygen consumption (mM/s) were as follows: 0.547 ± 0.110 (lipofuscin), 0.095 ± 0.022 (melanosomes), and 0.297 ± 0.071 (melanolipofuscin). It should be noted that the wavelength dependence of the rate of oxygen consumption for purified lipofuscin granules is similar to that determined for RPE cells (Fig. 1).

Photogeneration of Oxygen-reactive Species

Singlet Oxygen $O_2(^1\Delta_g)$—The involvement of $O_2(^1\Delta_g)$ in photo-oxidation of lipofuscin has unambiguously been shown by product analysis using cholesterol as an acceptor of $O_2(^1\Delta_g)$. HPLC-EC of ChOOHs, obtained after illumination of DMPC/cholesterol/DCP liposomes, in the presence of lipofuscin, showed five major ChOOH products formed in a dose dependent manner (Fig. 4). Three of them: 5α-OOH, 6α-OOH, and 6β-OOH, eluted as single peaks, are characteristic products of the interaction of cholesterol with singlet oxygen. Two others,

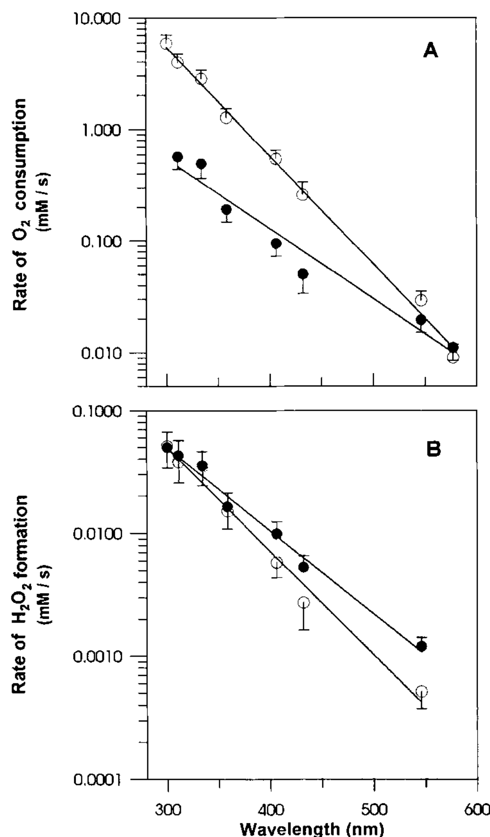

FIG. 3. **Action spectra for photo-dependent oxygen uptake (*A*) and photoformation of hydrogen peroxide (*B*) in air-equilibrated suspensions of lipofuscin (○) and melanosomes (●) isolated from human RPE cells.** Rates of photo-dependent oxygen uptake were obtained by ESR oximetry using mHCTPO as a spin probe. Data were normalized to equal concentration of granules (10^9 granules/ml) and fluxes of incident photons (einstein/s/m²). Values are mean ± S.D. of at least four experiments. Dark consumption of oxygen, under the conditions employed, was negligible.

eluted as partially resolved peaks, can be identified as 7α/7β-OOH epimers. The latter can be formed via rearrangement of 5α-ChOOH or as a result of free radical chain-reactions (27). None of these products has been detected in liposomes exposed to light in the absence of lipofuscin (Fig. 4*D*) or in unirradiated sample (Fig. 4*A*).

Superoxide Anion—Using the ESR spin-trapping technique and DMPO as the spin trap, characteristic spin adducts were detected when a suspension of lipofuscin was illuminated with blue light (Fig. 5*A*). The ESR spectrum, observed after 6 min of illumination time, has been simulated assuming superposition of two nitroxide species, one with the corresponding hyperfine coupling constants being A_N = 14.3 G, A^β_H = 11.7 G, A^γ_H = 1.25 G, and the other with $A_N = A_H$ = 14.9 G (Fig. 5*B*). Aside from noise, which is apparent only in the experimental spectrum, the simulated and experimental spectra match quite well. Clearly, the formed spin adducts can be identified as DMPO-˙OH and DMPO-˙OOH, respectively (29). The latter can be ascribed as being due to the interaction of superoxide anion ($O_2^{\cdot-}$) with DMPO. This relatively unstable spin adduct decays within minutes, via complex reactions, to a long-lived DMPO-˙OH adduct (22). Consistently with the proposed scheme, the DMPO-˙OOH concentration depended on the presence of oxygen (no signal detected in absence of oxygen) (Fig. 6*C*) and could not be detected in samples with added superoxide dismutase (Fig. 6*A*). Catalase alone, on the other hand, had no effect on the intensity of the DMPO-˙OOH signal formed during aerobic illumination of the lipofuscin samples (data not shown).

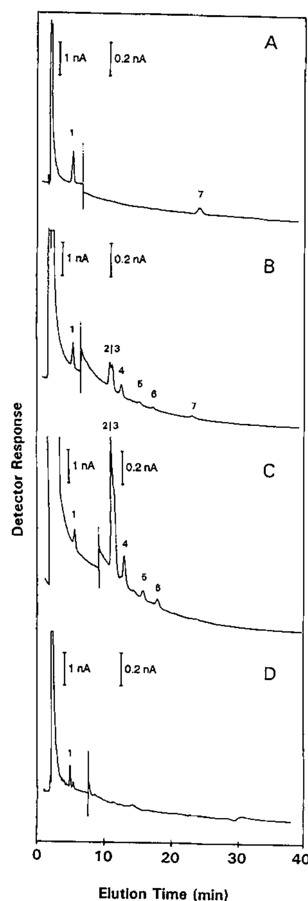

FIG. 4. **Chromatographic separation of cholesterol hydroperoxides from photooxidized liposomes fused with lipofuscin granules: no illumination (*a*) and after illumination with blue light for 18 min (*b*), 65 min (*c*) or 65 min in the absence of lipofuscin (*d*).** Peak identities are as follows: *1*, hydroperoxide of cis-11-eicosenoic acid methyl ester as internal standard; *2*, 7β-OOH; *3*, 7α-OOH; *4*, 5α-OOH; *5*, 6α-OOH; *6*, 6β-OOH; *7*, not identified lipofuscin derived solute. Samples were illuminated with blue light, as described under "Materials and Methods."

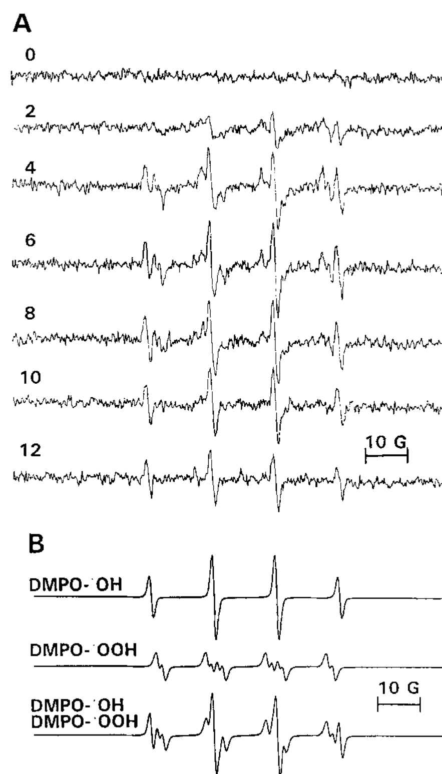

FIG. 5. **ESR signals of DMPO spin adducts in illuminated aqueous suspension of human RPE lipofuscin (*A*), and simulated spectra of DMPO-˙OH, DMPO-˙OOH, and their superposition (1: 0.7 ratio of the corresponding species) (*B*).** In *A*, the first (*top*) signal was recorded in dark (0 min illumination time), while the consecutive signals were recorded after indicated illumination time (in minutes). Lipofuscin was isolated from human donors 60–70 years of age and suspended in 50 mM phosphate buffer with 200 mM DMPO. ESR samples were irradiated with blue light (408–480 nm, irradiance, 38 mW/cm^2). Instrumental settings: time constant, 0.128 s; sweep time, 120 s; microwave power, 20 mW; modulation amplitude, 1.25 G.

However, our data suggest that at least 50% of the observed ESR signal, assigned to DMPO-˙OH, is not related to the formation of DMPO-˙OOH. This is evident from data shown in Fig. 6*A*, in which the formation of DMPO-˙OH is apparent even in the presence of superoxide dismutase, where no superoxide anion could be detected.

The mechanism of DMPO-˙OH generation during aerobic illumination of lipofuscin is unknown at present. Interestingly, the spin adduct appears to be H_2O_2-independent, since it is also formed in the presence of catalase (Fig. 6*B*).

Lipid Hydroperoxides—Illumination of lipofuscin granules with blue light yielded measurable concentrations of hydroperoxides (Fig. 7*A*). An apparent decrease in the hydroperoxide content, observed at longer illumination times, indicates that the resulting kinetics is a superposition of two processes: photo-induced formation and decomposition of hydroperoxides. This conclusion is consistent with the observed monotonic accumulation of MDA, a nonspecific indicator of lipid peroxidation (30), that accompanied the formation of hydroperoxides (Fig. 7*B*).

DISCUSSION

The observed photoreactivity of human RPE cells is, to a significant extent, determined by their lipofuscin content. This is evident from the comparison of action spectra of photo-dependent oxygen uptake in RPE cells and in purified lipofuscin granules (Figs. 1 and 3). Both action spectra exhibit significant similarities in that the efficiency of illuminating light, to induce oxygen uptake and generation of H_2O_2, falls steeply with the wavelength between 300 and 600 nm. Thus, blue light seems to be the most efficient radiation under physiologically relevant conditions. This is because ultraviolet radiation is completely filtered out by the cornea and lens of the adult human eye and, as a result, virtually no light below 400 nm is transmitted to the retina (1).

Retinal melanin does not seem to be the major contributor to photo-dependent oxygen uptake in either human RPE cells or bovine pigment epithelium, which may prove that lipofuscin is a major retinal chromophore that photoexcitation leads to consumption of oxygen. Such a conclusion about the dominant role of lipofuscin in aerobic photoreactions of human RPE is further supported by the distinct changes of the RPE photoreactivity with age of the donors (Fig. 2). The increase in the rate of photo-dependent oxygen uptake between the second and ninth decade of life, observed in this work, is consistent with the reported accumulation of retinal lipofuscin with age (12). The presence of lipofuscin, or its precursors, may also account for photo-dependent oxygen uptake, albeit reduced, in bovine RPE cells. It must be stressed that the molecular nature of chromophores of human RPE lipofuscin responsible for its photoreactivity remains unknown.

Melanolipofuscin granules exhibit aerobic photoreactivity which is intermediate between that of lipofuscin and melanin. However, the contribution of melanolipofuscin to photo-induced consumption of oxygen, observed in RPE cells, is difficult

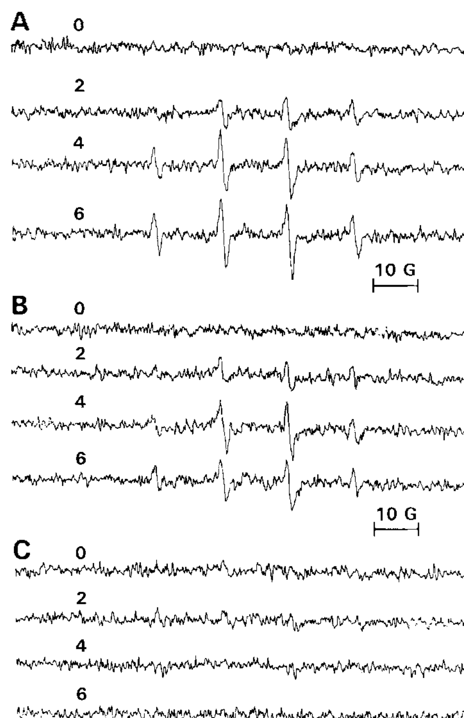

FIG. 6. **The effect of inclusion of superoxide dismutase (0.10 mg/ml) (*A*), both superoxide dismutase and catalase (0.15 mg/ml, about 1900 units/ml) (*B*) and oxygen removal (*C*) on formation of DMPO spin adduct during illumination of ESR sample containing lipofuscin suspension in the presence of DMPO.** Experimental conditions and instrumental settings the same as in Fig. 5.

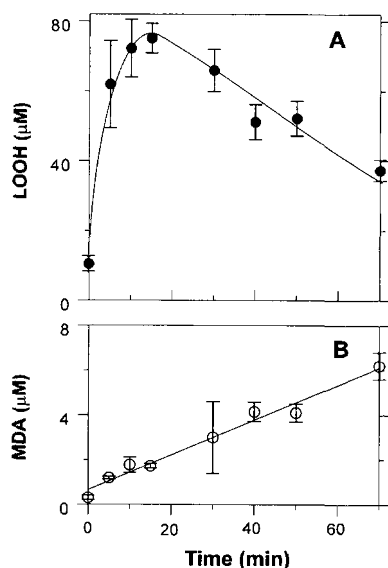

FIG. 7. **Formation of hydroperoxides (*A*) and malondialdehyde (*B*) during aerobic illumination with blue light (408–495 nm, 220 mW/cm^2) of human lipofuscin isolated from 60–70-year-old donors.** Values are means ± S.D. of triplicate experiments.

to evaluate. One has to consider that in human RPE a significant number of these complex granules become apparent in individuals 60–100 years of age, when lipofuscin granules are more numerous than any other pigment granules (31).

Since superoxide anion has been detected in irradiated lipofuscin samples (Fig. 5), it is conceivable that $O_2^{\bar{}}$ may be a major intermediate in lipofuscin-photosensitized generation of H_2O_2. However, our data clearly show that in isolated pigment granules photo-induced consumption of oxygen is not accompanied by stoichiometric production of H_2O_2 (Fig. 3). Since similar results have been observed for RPE cells, we may conclude that an identical or closely related mechanism of photo-dependent oxygen uptake occurs in isolated pigment granules and in RPE cells.

Molecular oxygen consumed during illumination of RPE cells may in part be utilized in lipid peroxidation processes. This can be inferred from the detection of hydroperoxides that were induced by blue light in purified lipofuscin granules (Fig. 4), even though the yield of photoformation of lipofuscin-derived hydroperoxides, as well as that of the accompanying accumulation of MDA, has not been determined in this work.

An important question is the identity of reactive species that initiate lipofuscin-sensitized photoperoxidation, and lead to the observable photoconsumption of O_2. Two major mechanisms of lipid peroxidation should be considered: the free radical and singlet oxygen pathways (27). Our ESR spin-trapping data and the observed formation of both 7α-OOH and 7β-OOH epimers, seem to indicate that the free radical mechanism operates. Although superoxide anion is rather a nonreactive species, and in aqueous media acts predominantly as a moderate reductant (32), its protonated form, the hydroperoxyl radical, can attack double-allylic hydrogen atoms in a number of unsaturated fatty acids (33). However, powerful antioxidants, such as butylated hydroxytoluene and α-tocopherol, only partially inhibited the photo-dependent uptake of O_2. It may suggest that peroxidation, induced in lipofuscin granules by light, is mostly a nonradical process or a mixed-type process, in which singlet oxygen is involved. In such processes oxygen is consumed during the primary formation of lipid hydroperoxides, while the secondary chain-propagating reactions are less efficient (34). However, at this point it cannot be ruled out that the relatively small inhibitory effect of tested antioxidants on photo-dependent uptake of oxygen, is also due to their inefficient penetration of the lipofuscin granule.

Due to the particle character of lipofuscin and the molecular heterogeneity of its main constituents, quantitative determination of reactive species, which may be generated by lipofuscin granules, is rather difficult. One has to realize that any short-lived species, generated within the lipofuscin granule, are likely to be scavenged or quenched by several reactive lipofuscin components, before having chance to diffuse out of the granule. Therefore, the yield of photoformation of reactive species, such as singlet oxygen, determined by its interaction with a selective acceptor, homogeneously distributed in the entire sample volume, would be severely underestimated. The situation can be improved if a suitable acceptor of $O_2(^1\Delta_g)$ is preferentially located in the granule or in its close proximity. Indeed, singlet oxygen photoformation has unambiguously been detected by the chemical method using cholesterol as an exogenous singlet oxygen acceptor (Fig. 4). However, at this stage we cannot determine quantum yield of the formation of $O_2(^1\Delta_g)$ by lipofuscin.

In conclusion, human RPE lipofuscin exhibits substantial photoreactivity and, under aerobic conditions, is able to form several potentially cytotoxic species. It is important to stress that these are typical conditions for a normally functioning eye; the RPE *in vivo* is constantly exposed to high oxygen tension and must endure substantial fluxes of light that reach the posterior eye segments (35). The extent to which the long term effects of lipofuscin photoreactivity affect the structural integrity and function of the RPE-retinal complex *in vivo* has yet to be elucidated, as has its role in ARM.

Acknowledgements—We thank Christine Skunatz for technical assistance. We are grateful to Professor H. J. Halpern for providing us with the mHCTPO nitroxide probe.

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