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13. ABSTRACT (Maximum 200 words)

The cellular pigments of the retinal pigment epithelium (RPE) have been shown to catalyze free radical activity, especially when illuminated with visible or ultraviolet light. This activity is sufficient to cause photooxidation of several major cellular components, including proteins, fatty acids, as well as antioxidants, and other small molecules. Similar reactivity of melanin granules has been demonstrated in intact RPE cells by the use of fluorescent oxidation-sensitive vital probes. Experiments have been conducted in cultures of bovine and baboon RPE cells exposed to quantum-equivalent, 488, 514.5 or 647.1 nm emissions from Argon and Krypton ion CW lasers. Based on fluorescence microscopy and biochemical analysis, the blue-green wavelengths, on a quantal basis, most efficiently induced photooxidative stress in the pigmented cells. The fluorescence signal after laser exposure, corresponding to areas of greatest oxidative stress, was restricted to the cells' cytoplasm. These findings indicate that the melanosomes of pigment cells are involved in intracellular photooxidative reactions, and indeed, may be considered as photosensitzers possibly contributing to light-induced, oxidative damage to ocular tissue.

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MECHANISMS OF PHOTO-OXIDATIVE STRESS IN RETINAL PIGMENT EPITHELIUM:
IS MELANIN A PHOTOSENSITIZER?

Final Report for:
AFOSR Grant F49620-95-1-0332

Randolph D Glickman
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ABSTRACT

The cellular pigments of the retinal pigment epithelium (RPE) have been shown to catalyze free radical activity, especially when illuminated with visible or ultraviolet light. This activity is sufficient to cause photooxidation of several major cellular components, including proteins, fatty acids, antioxidants, and enzyme cofactors. The photochemistry of the RPE melanosomes has been studied in considerable detail, although almost entirely in experiments utilizing pigment granules isolated from the RPE cells. The photoactivated reactions of these granules have an action spectrum peaking between 450 and 500 nm. Similar, wavelength-dependent reactions occur within intact RPE cells, as demonstrated with intracellular vital stains, especially the oxidation-sensitive chemical probe, 2',7'-dichlorofluorescein, which is non-fluorescent when chemically reduced and fluorescent when oxidized. Another oxidation-sensitive fluorescent probe, dihydrorhodamine 123, was not oxidized intracellularly in a wavelength-dependent fashion, and in fact, was found to have a higher oxidation potential which may have inhibited its reaction with light-excited reactive species within the cell. The experiments were conducted in cultured bovine and baboon RPE cells that were labeled with these probes, and then exposed to quantum-equivalent, 488, 514.5 or 647.1 nm emissions from Argon and Krypton ion CW lasers. The probes were isolated from the cells by solid phase extraction, and the amount of oxidized probe quantified by HPLC with fluorescence detection. Alternatively, cells were imaged with a fluorescence microscope. Images were acquired at various intervals after the cells were exposed to blue (λ_{max} = 490 nm) and yellow (λ_{max} = 582 nm) light derived from the microscope exciter lamp. The kinetics and amplitude of the fluorescence change in the cells were quantified with image processing software. Both types of experiments yielded the conclusion that blue-green wavelengths, on a quantal basis, most efficiently induced photooxidative stress in the pigmented cells. The microscopy also showed that fluorescence was restricted to the cells' cytoplasm. These findings indicate that the melanosomes of pigment cells are involved in intracellular photooxidative reactions, and indeed, may be considered as photosensitzers possibly contributing to light-induced, oxidative damage to ocular tissue.

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MECHANISMS OF PHOTO-OXIDATIVE STRESS IN RETINAL PIGMENT EPITHELIUM:
IS MELANIN A PHOTOSENSITIZER?

Randolph D. Glickman, Ph.D.

INTRODUCTION

The retinal pigment epithelium (RPE) of the eye contains several pigments, including melanin, lipofuscin, and melanolipofuscin. The amount of pigmentation in the RPE cells changes with age, i.e. melanin tends to decrease and lipofuscin tends to increase [1]. Environmental and systemic factors also affect pigment content. The biological role and significance of these pigments is not entirely understood. For example, melanin is a broadband absorber and is generally thought to protect ocular tissues against excess light. The protection could derive from melanin's ability to absorb and screen light from reaching sensitive target tissues [2], to sequester heavy metals that might otherwise catalyze oxidative reactions [3], or to trap free radicals produced by photochemical reactions or ionizing radiation [4,5]. Lipofuscin is considered an aging pigment resulting from lysosomal accumulation of peroxidative end products of lipid decomposition [6] and, in the eye, polymerization of remnants of retinaldehyde, the chromophore of the visual pigment [7]. Lipofuscin is known to produce reactive oxygen species when illuminated with UV light [8]. Melanolipofuscin also is associated with aging and represents degenerated melanosomes combined with lipofuscin [9]. The accumulation of these substances has been associated with pathological changes [6]. We have previously demonstrated that melanin, as well as the other RPE pigments, all have some photoactivity with respect to the initiation of lipid peroxidation [10,11]. Lipofuscin also is able to promote peroxidative reactions in the dark, probably because the granules contain labile hydroperoxides that themselves are reactive, as well as metal ions (especially iron) that catalyze oxidative (Fenton-type) reactions.

Melanin-mediated photooxidative reactions

In spite of its presumed protective role, melanin (a) induces oxidative changes in physiological substrates such as ascorbic acid, fatty acids, and proteins during irradiation with visible light [12-16], (b) is a photoinducible free radical [17,18], (c) produces reactive oxygen species when irradiated by UV and visible light [19,20], and (d) promotes photochemical oxidations [15,21,22]. While the significance of these findings for photooxidative stress *in vivo* is not entirely clear, the possibility exists that pigments in the RPE cells may have a dual role in both protecting against and promoting photooxidative reactions, depending on the cellular environment and the nature of the oxidizing stressor.

Laser-induced melanosome disruption as a precursor to photochemical damage

Although the role of melanin and melanosomes in mediating thermal damage in the eye following continuous wave (CW) laser exposure is quite well understood, and has been successfully modeled based on the linear absorption of optical radiation by the pigment granules [23], the involvement of pigment granules in mediating damage produced by short

pulse (i.e. relative to the time constant of thermal diffusion or <1 µs approximately) laser exposures is less well known. For extremely short laser pulses (≤ 100 ps), tissue damage (ionization and optical breakdown) results as a function of peak power during the pulse and is less dependent on the specific tissue absorption of the laser wavelength [24]. In the RPE, short pulse laser exposures above the damage threshold do cause disruption of the melanosomes [25]. The explosive disruption of the melanosome is undoubtedly fatal to the cell, and produces immediate damage to surrounding cells by transmission of mechanical forces. Yet, in some cases, the damage may take 24 hours or more to become apparent [25]. Based on the enhanced photochemical activity of isolated melanosomes disrupted by exposure to Q-switched Nd:YAG laser [26,27], Glickman proposed that the elevated photochemical activity of laser-damaged melanosomes may be responsible for the delayed onset of lesions after some short-pulse laser exposures, especially near threshold exposures [28]. Recently, a formal model incorporating the photochemical activity of damaged melanosomes to explain the delayed formation of threshold laser lesions has been proposed [29]. The need to detect or identify increased photooxidative stress in the cytoplasm of RPE cells following laser injury was a major motivation for the present research.

Detecting photooxidative reactions within the RPE cell

Most of the data on the photochemistry of the RPE pigments has been obtained in studies with isolated pigment granules. There is little published information on photooxidative reactions within the RPE cell itself. Although photochemical damage to the retina and RPE, defined as light-induced tissue damage resulting in the absence of significant tissue heating, is well documented [30,31], there is continuing controversy about the chromophore responsible for these changes [32,33]. In an effort to determine if one or all of the RPE pigments are responsible for photooxidative stress, we utilized oxidation-sensitive vital fluorescent probes to measure the redox state of the RPE cell following exposure to various wavelengths of visible light produced by continuous wave lasers and other light sources.

Scope of the project

The major goals of this project were to determine (1) if photoactivated RPE melanosomes are capable of generating oxidative stress in RPE cells by damaging critical cellular components, (2) if the photochemical characteristics of isolated melanin granules are also present in intact RPE cells, (3) if photooxidative reactions could be detected with the use of oxidation-sensitive, fluorescent, vital probes, (4) if these probes could be taken up by RPE cells in culture, (5) if the probes were tolerated over the 2-3 hr time course of the typical experiment; and (6) if light exposure from laser and other light sources could induce sufficient oxidative stress in the cells to oxidize the probes and thereby produce a fluorescent signal. In addition, the methodology for utilizing these oxidation-sensitive probes for the use of laser-tissue interaction was optimized. The results of this project show that the use of oxidation-sensitive fluorescent probes is a powerful way to study intracellular photochemical reactions, and that photoactivated melanin is a likely contributor to oxidative stress in the RPE.

METHODS

RPE Cell Cultures

Primary cultures of RPE cells were prepared from bovine eyes freshly obtained from a local slaughterhouse, and from baboon eyes generously provided by the Southwest Foundation for Biomedical Research (San Antonio). After opening the globes and removing the retinae, RPE cells were gently brushed off the choroid into a pool of culture medium. Collected cells were centrifuged and plated at a constant density in 24-well plastic culture plates. Cells were grown in Dulbecco's Minimum Essential Medium (DMEM) containing 10% fetal calf serum and maintained at 37 °C in an atmosphere supplemented with 5% CO_2.

Viability testing of the cells by MTT test

The so-called MTT test is a cell viability test that utilizes dehydrogenase enzymes located in the mitochondria or elsewhere in the cell to catalyze the conversion of nitrotetrazolium salts to a colored formazan product. This assay has been validated in a number of cell models [34,35]. A commercial MTT test kit (active reagent: 3-[4,5-Dimethylthiazol-2-yl]-2-5-diphenyltetrazolium bromide, MTT) was obtained from Sigma Chemical Co. (St. Louis, MO). RPE cells of either bovine or baboon origin were used. Each assay used 150,000 - 600,000 cells. Assays were either run in bulk, by carrying out the reactions with the harvested cells in a 1.5-ml Eppendorf plastic microcentrifuge tube and, after spinning out the melanosomes, reading the results in a Beckman DU-640 spectrophotometer, or, by adding the reagents directly to the cells in their 24-well culture plates and monitoring the results in a Packard Instruments Spectracount plate reader. In either case, after adding the MTT reagent (5 mg/ml) and incubating the cells for 3 hr, the reactions were stopped and the formazan product solubilized by addition of 0.1 N HCl in absolute isopropanol. Samples were mixed and the reaction result was read as the difference in optical absorbance between the test wavelength at 570 nm and the reference wavelength at 630 nm. The extent of the MTT reaction in live cells was compared to that of "dead cell" controls, which were aliquots of cells killed by heating at 100 °C for 10 min.

Preparation of Probes

The oxidation-sensitive probes, 2'-7'-dichlorofluorescein (DCFH) and dihydrorhodamine 123 (DHR123) were obtained from Molecular Probes (Portland, OR). These probes have the property of being nonfluorescent when chemically reduced and highly fluorescent when chemically oxidized. DCFH was supplied from the manufacturer as the diacetate ester (DCFH-DA), and a 1 mM stock solution was prepared by dissolving 10 mg in 20 ml of methanol. For studies involving cellular uptake, the ester form was hydrolyzed by the cells themselves (see Results). For studies in cell-free experiments, 0.5 ml of DCFH-DA stock solution was hydrolyzed with 2.0 ml of .01N NaOH for 30 min at room temperature. Hydrolysis was stopped by adding 10 ml of 25 mM NaH_2PO_4 (pH=7.5). DHR123 was supplied as a dry powder of which 10 mg were added to 1 ml of N,N-dimethylformamide and then diluted to a 1 mM stock solution using methanol [36]. Working dilutions (usually 10 µM) of the probes were made by diluting stock solutions with culture

medium. Both probes were stored under nitrogen at -20°C, and working solutions were prepared fresh each day.

Fluorescence Microscopy

RPE cells were plated on Lab-Tek ChamberSlides (Nalge/Nunc) and allowed to attach to the ChamberSlides. Prior to microscopy, the cells were incubated in 10 μM DCFH or 10 μM DHR123 for 1 hr. After incubation, the cells were briefly washed in probe-free medium and cover-slipped. Photooxidative reactions were activated by exposing the probe-labeled RPE cells to the exciter source in an Olympus BX-60 fluorescence microscope. The fluorescein exciter (λ_{max}=490 nm) and the Texas red exciter (λ_{max}=582 nm) were used in different experiments. Fluorescence images were captured on a computer frame grabber at timed intervals during exposure to the excitation source. Image analysis was performed on these images with *Image-Pro* software (Media Cybernetics) by defining a constant Area of Interest (AOI) in the images, and measuring the mean and standard deviation of the AOI pixel intensity in each image. These values were plotted against the time of image acquisition to construct a fluorescence growth curve.

HPLC Analysis

Probes were analyzed by high performance liquid chromatography (HPLC) on a Waters μBondapak-C_{18} or a Phenomenex Bondclone-C_{18} column (which gave equivalent results), with one of the following mobile phases: DCFH and its derivatives were eluted with 8mM ammonium phosphate (pH 8.0) modified with 60% methanol, and DHR123 was eluted with 8mM ammonium phosphate (pH 8.0) modified with 60% acetonitrile. The flow rate was 1 ml/min. Fluorescence was measured by a Waters 474 detector with λ_{ex} at 488 nm and λ_{em} at 530 nm. For determination of the oxidation potential of the probes, chromatographic separation was carried out with these same conditions, except that electrochemical detection was performed with a Waters 460 electrochemical detector. Hydrodynamic voltammograms were constructed by systematically increasing the detection potential for successive sample injections, and measuring the resulting oxidation current. The half-wave potential, $E_{1/2}$, and the threshold response, E_0, were determined from the data by fitting a sigmoidal function of the form

$$y = a_0 + \left(a_1 \Big/ \left(1 + e^{(-(x-a_2)/a_3)}\right)\right) \qquad \text{(equation 1)}$$

to the data set. The Y-axis intercept of this function was taken as the threshold for oxidation (E_0) and the coefficient, a_2 (which corresponds to the value of X at which the function amplitude is at half-maximum), as the value for $E_{1/2}$.

Extraction of Probes for HPLC Analysis

For cellular uptake experiments, aliquots of 10^6 RPE cells were incubated in 10 μM probe for varying times. After the desired incubation time, the cells were centrifuged and the resulting pellet washed once in probe-free culture medium. The probe contained within the cells was isolated using solid phase extraction (SPE). This technique of sample preparation employs the retention of sample and impurities on a sorbent material similar to that used in reverse-phase HPLC analytical columns, followed by selective elution of the sample molecule(s) with a specific solvent. The impurities

are retained on the extraction cartridge. Cells were lysed with 500 µl of 0.5% Triton X dissolved in Tris buffered saline and then incubated for 10 min. The samples were centrifuged, and the supernatant containing the probe and soluble components of the cytosol were processed with Waters Oasis HLB SPE cartridges using the following protocol: the cartridge was initially washed and the sorbent wetted by the passage of 1 ml each of methanol and distilled H_2O. The cartridge was not allowed to dry out before the sample was introduced into it. Then, 0.5 ml of the supernatant was passed through the cartridge. Finally, 1 ml of methanol was passed through the cartridge to elute the probes. Recovery efficiency for the probes was about 85%. Aliquots of this eluant were analyzed by HPLC using the conditions described above.

Chemical oxidation of probes for estimation of total uptake

In order to measure total probe content in the uptake experiments, probes extracted from RPE cells were subjected to chemical oxidation to convert all the probe molecules to the oxidized, highly fluorescent moiety. This was accomplished by combining the eluants obtained from SPE extraction with 5 µl each of horseradish peroxidase (HRP, 5 mg/ml) and H_2O_2, (20 mM) for 30 min at 37°C [36]. These processed samples were then analyzed by HPLC with fluorescence detection.

Statistical Analysis

Routine statistical analysis, including the Bonferroni test for multiple comparison between groups, was performed with the *ProStat* statistical computer program (Poly Software International, Sandy, UT).

RESULTS

Viability of RPE cells following labeling with fluorescent probes

A classic test of cellular viability is the uptake of the dye, Trypan Blue. Live cells tend to exclude this dye, while dead or dying cells allow the penetration of the dye into the cytoplasm. The pigmentation, however, of the RPE cells interferes with the interpretation of this test, rendering it useless for evaluating cellular viability in these cells. We then adopted the so-called MTT test which measures metabolic activity in the cells. This test was also rendered more difficult to read by the presence of pigment granules, however, sufficient replications indicated that while cell viability was somewhat reduced by exposure to DCFH (Table I), over the two hour duration of the experiment, this reduction did not reach statistical significance (Table II: comparison of No Probe condition to 1-hour and 2-hour probe incubation conditions).

Table I. Effect of 1- and 2-hour incubations in DCFH
on MTT test of RPE cell viability.

Treatment	# Expts.	MTT, mean ± s.d.
Killed Cells	5	.167 ± .017
No Probe	4	.240 ± .024
+Probe, 1 h incubation	4	.232 ± .023
+Probe, 2 h incubation	4	.216 ± .018

Table II. Probability matrix. Statistical significance of effect of DCFH on
RPE cell viability (Bonferroni multiple comparison test).

	Killed Cells	No Probe	1 Hour incubation
No Probe	0.000	****	****
1 Hour incubation	0.000	0.588	****
2 Hour incubation	0.003	0.119	0.286

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Fluorescence microscopy studies of photooxidative stress in RPE cells

The use of a vital, fluorescent stain initially offered the possibility of obtaining direct visualization of photooxidative stress within the RPE cells, especially the spatial localization of oxidative reactions. Although the early data were encouraging, two factors prevented this imaging approach from yielding the quantitative data desired. One was the difficulty in calibrating the fluorescence signal so that meaningful comparisons could be made between experiments. The second problem resulted from the cells' pigmentation. Many of the primary RPE cell cultures used in these studies were heavily pigmented, retaining most if not all of their melanosome content. We found that the presence of the heavy pigment tended to quench or block the fluorescence signal from the cells. In many experiments, only a bright, fluorescent halo could be observed around the cell. However, one salient observation was made when intracellular fluorescence was clearly observed: that fluorescence was limited to the cytoplasm, and did not extend into the nucleus (Figure 1). This was of interest because it implied that photooxidative reactions occurred in the vicinity of the melanosomes. In these experiments, the photooxidative stress was not produced with laser exposure, but with the exciter of the fluorescence microscope which was simply allowed to illuminate the cells for various lengths of time. As described in the Methods, two exciter wavelengths were used, one with a spectrum centered at 490 nm and the other at 582 nm. Several cells, such as the group of cells pictured in Figure 2, were imaged clearly enough to measure the relative increase in fluorescence with increasing exposure time. The images of five RPE cells were selected for this type of image analysis. The changes in pixel intensity of a defined AOI in each image, during exposure to the microscope exciter lamp, are plotted in Figure 3. These observations indicated that the 490 nm light caused an increase in the intracellular fluorescence, while there was little or no increase when cells were exposed to the 582 nm light.
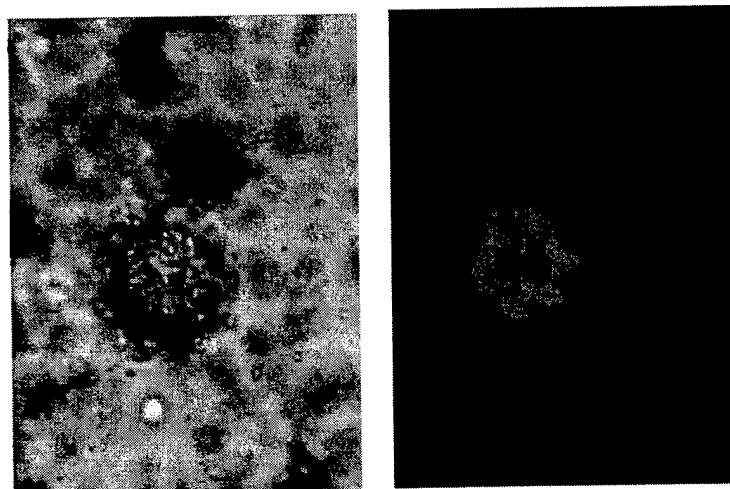

Figure 1. Micrographs of DCFH-labeled RPE cells. Left: bright field image. Right: fluorescence micrograph of same cell using fluorescein exciter, showing fluorescence in cell's cytoplasm.
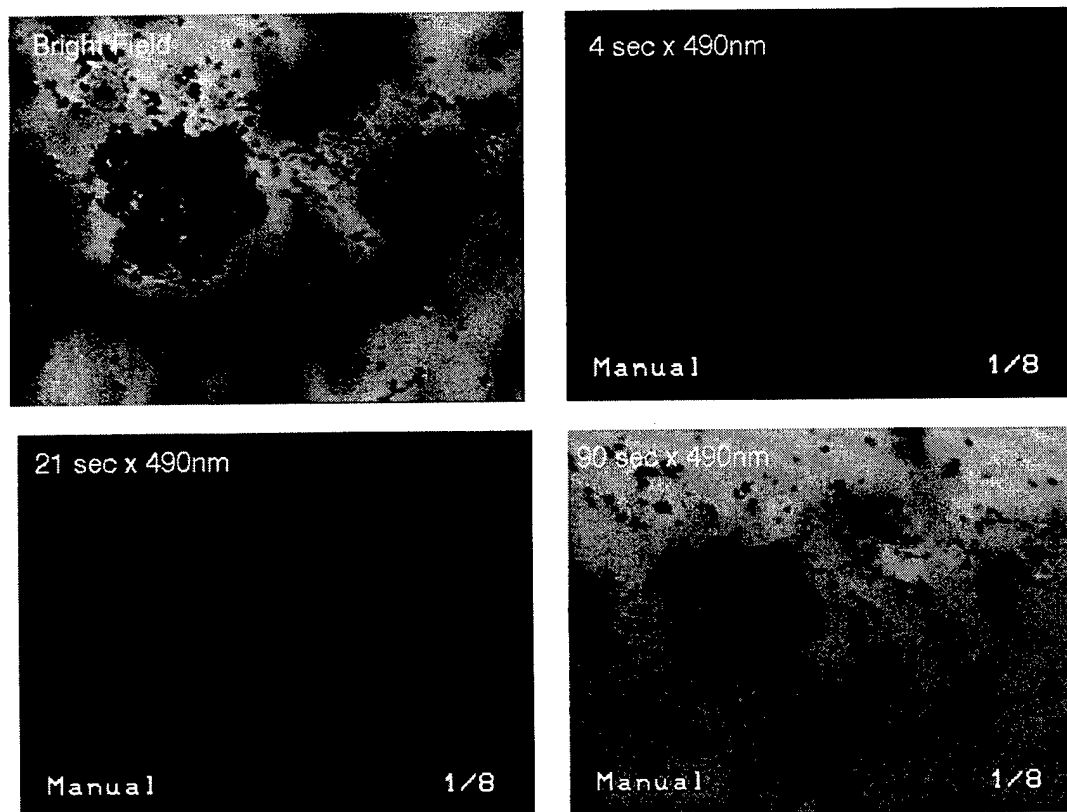
Figure 2. Fluorescence micrographs of a group of RPE cells loaded with DCFH and exposed to the fluorescein exciter lamp of the microscope (λ_{max} = 490 nm). Top row: (left) bright field view of the cells; (right) 4 sec exposure to exciter lamp. Bottom row: (left) 21 sec exposure; (right) 90 sec exposure to the exciter lamp.

Fluorescence Microscopy of RPE Cells
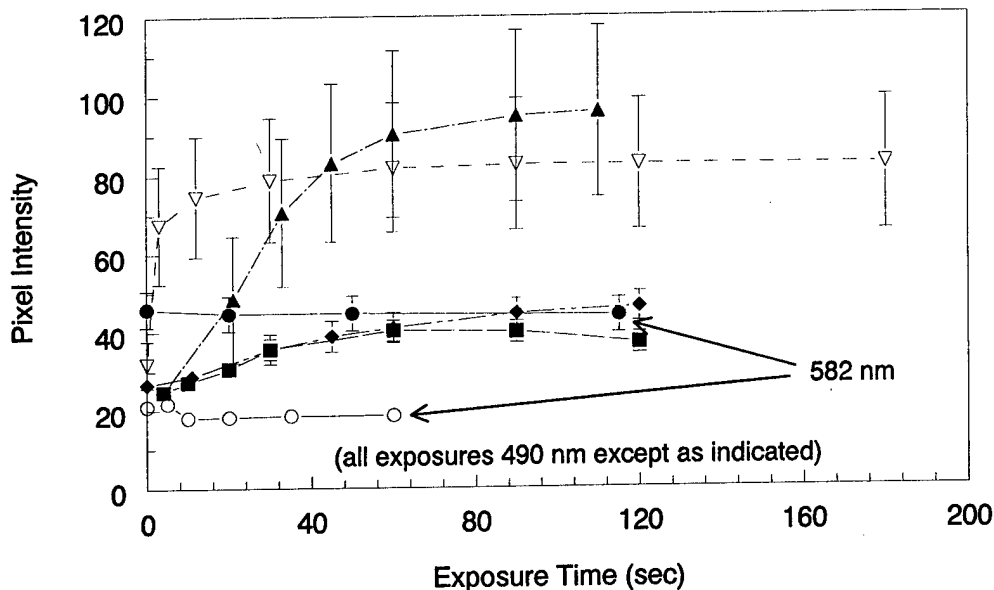## Photooxidation of probe-labelled cells

Figure 3. Increase in fluorescence of probe-labeled RPE cells continuously exposed to microscope exciter lamp (wavelengths indicated in figure). Data points are the mean ± s.d. of the pixel intensity of a defined AOI positioned within the image of a cell and followed over time in successive images.

Nevertheless, the limited, qualitative nature of the imaging data led us to develop a biochemical methodology for the direct analysis by HPLC of the amount of the DCFH and DHR123 probes oxidized by various wavelengths of light. The results of these studies are described in the following sections.

HPLC method for analysis of DCFH and DHR123

Development of a suitable HPLC method for the separation of each probe allowed us to isolate the probe from cell lysates, and quantify the probe fluorescence with precision. Figures 4 and 5 show, respectively, chromatograms of DCF (oxidized DCFH) and DHR123 (also oxidized) standards. Under our analytical conditions, DCF had a retention time of about 4.1 min, while that of DHR123 was 5.5 min. Each figure also shows a sample of "aged" probe, indicating that each probe was susceptible to autoxidation leading to the formation of oxidative breakdown products. For this reason, working dilutions of the probes were made up fresh for each day's experiments.
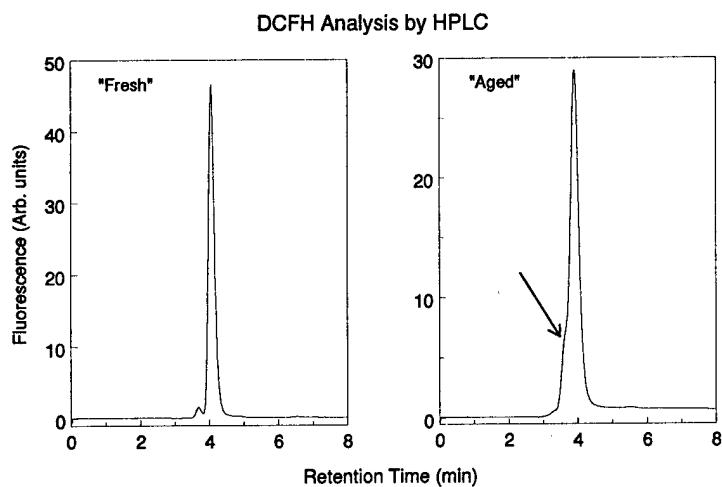
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DCFH Analysis by HPLC

Figure 4. HPLC chromatograms of fresh DCF (left) and autoxidized ("aged") DCF (right). Arrow points to appearance of oxidative breakdown products
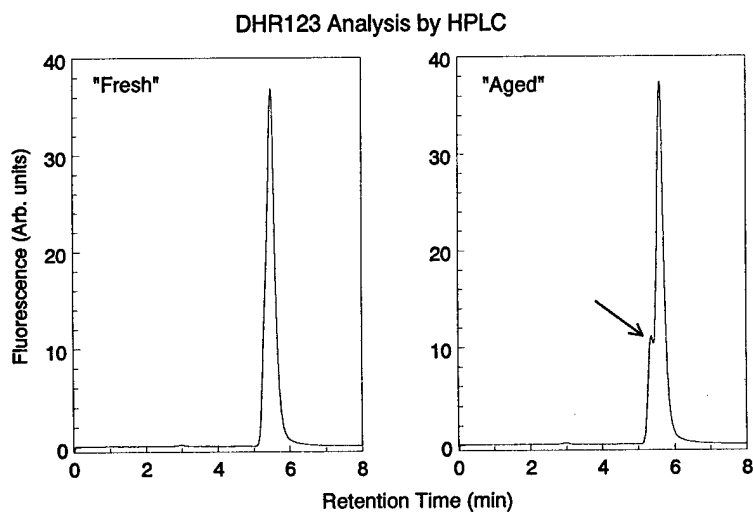
DHR123 Analysis by HPLC

Figure 5. HPLC chromatograms of DHR123. Freshly oxidized probe is shown on the left, and autoxidized ("aged") probe on the right. Arrow points to appearance of oxidative breakdown products.

Kinetics of DCFH and DHR123 uptake by RPE cells

To characterize the uptake of the probes, RPE cells were incubated for various time periods in 10 µM DCFH-DA or DHR123, and then the probes were extracted from the cells using SPE. The probe content in the cell extracts was

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oxidized (in order to make it fluorescent) by reaction with HRP and H_2O_2, and then aliquots were analyzed on HPLC with fluorescence detection. The time course of probe uptake versus time is shown in Figure 6. DCFH-DA was generally taken up quickly and reached a maximum in about 20 min, after which the intracellular content of the probe gradually declined (Figure 6, left). DHR123 was accumulated more slowly, reaching a plateau at about 60 min which was maintained for up to another hour (Figure 6, right)
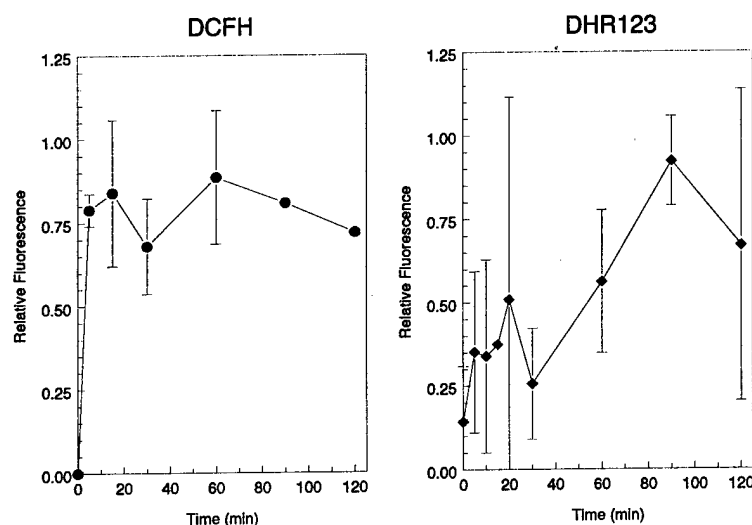

Figure 6. Kinetics of fluorescent probe uptake into cultured RPE cells, determined by HPLC analysis of cell lysates. Left panel: DCFH; right panel: DHR123. Each time point represents the mean ± s.d. of at least 4 measurements.

DCFH-DA Uptake and Hydrolysis

DCFH-DA and its two principal metabolites, the hydrolyzed-reduced form DCFH, and the hydrolyzed-oxidized form DCF, exhibited distinct retention times in HPLC analysis (Figure 7). In this figure, the left-hand panel shows a chromatogram of the stock probe, DCFH-DA. This form elutes as a single peak at about 6.2 min under our HPLC conditions. After the probe is chemically hydrolyzed and oxidized, HPLC analysis reveals a single, highly fluorescent peak (note difference in vertical scale between the right hand panel and the other two) eluting at slightly over 4 min. HPLC analysis of a sample of probe that was chemically hydrolyzed, but **not** oxidized, is shown in the central panel. Note that all forms of DCFH have some degree of fluorescence, which enables the detection of each moiety. The hydrolyzed-reduced form, DCFH, elutes at about 5 min, between DCF and DCFH-DA.

Because the three principal forms of DCFH can be separated and identified on HPLC, the uptake and processing of DCFH-DA by RPE cells could be followed, which proved that, following cellular uptake, the probe is hydrolyzed to
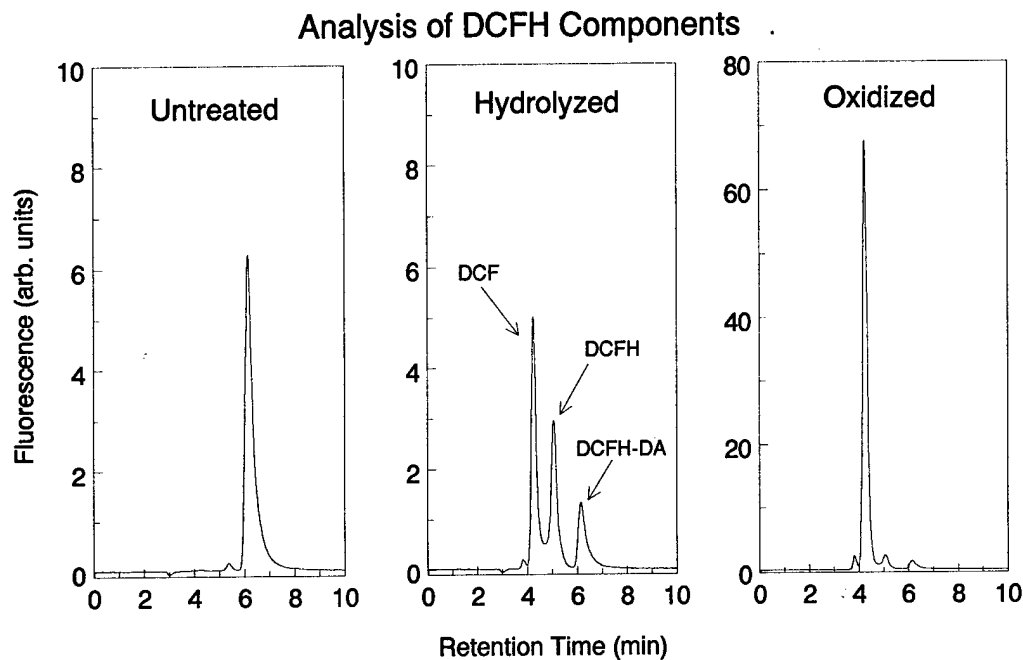
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Figure 7. HPLC analysis of DCFH components. Left panel: DCFH-DA (i.e. working dilution of the probe as suppled by manufacturer). Center panel: DCFH-DA + DCFH + DCF (analysis of probe following hydrolysis with NaOH). Right panel: DCF (analysis of hydrolyzed probe following chemical oxidation with HRP and H_2O_2; note difference in vertical scale).

DCFH by intracellular esterases. RPE cells were incubated with a 10 μM solution of DCFH-DA, and aliquots were removed for assay at 20 minute intervals, beginning at 0 min of incubation (the probe was added to the cell cultures, and the sample was then immediately processed). The sample was extracted using SPE, chemically oxidized to enhance detection of the compounds, and analyzed by HPLC with fluorescence detection. The peaks in the chromatograms obtained were identified by comparison to the known retention times of the identified probe components shown in Figure 7. The chromatograms obtained at t = 0', 15', 20', and 60' are shown in Figure 8. At time = 0', all of the probe is present as DCFH-DA. By 15', probe has accumulated in the cells and is starting to be hydrolyzed. (Note that because these samples have been manually oxidized prior to HPLC analysis, little or no DCFH is found, because all DCFH produced by the action of cellular esterases is oxidized to DCF by our procedure). By 20', most accumulated probe has been hydrolyzed, and by 60' virtually all detectable probe in the cells has been hydrolyzed. Apparently, the total probe content in the cells declines by 60' either because of efflux from the cell, or metabolic breakdown to a form that is either non-fluorescent or not captured by our isolation procedure.
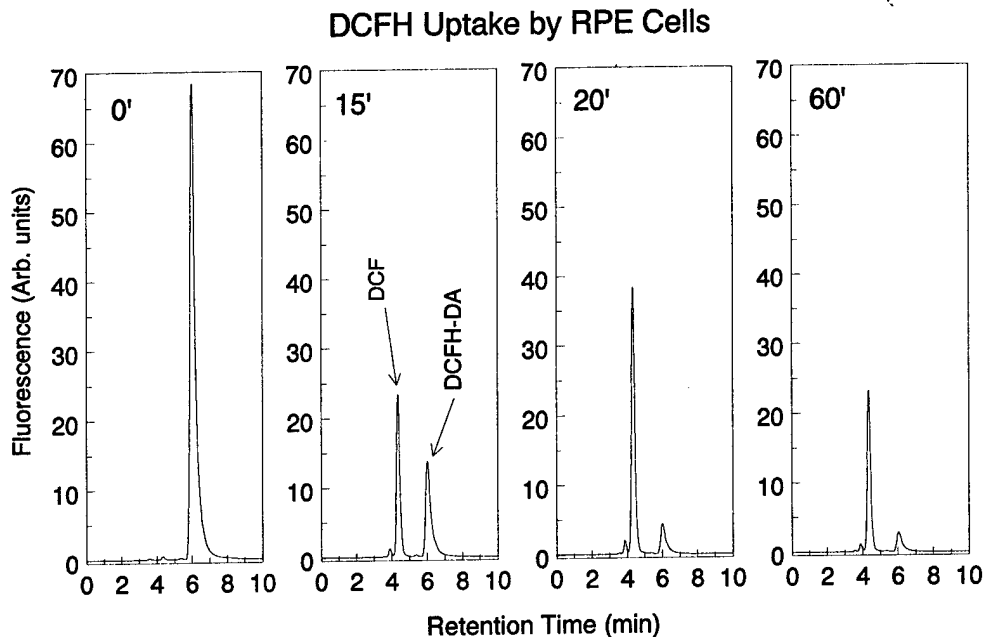
DCFH Uptake by RPE Cells

Figure 8. Uptake and processing of DCFH-DA by RPE cells. HPLC analysis of cell lysates following incubation for the indicated times in 10 μM DCFH. Note that at 0', all the recovered probe is in the diacetate form, but by 20' of incubation, most of the probe has been hydrolyzed by the action of cellular esterases. See text for further details.

Photooxidation of Intracellular Probe

Aliquots of RPE cells were incubated with 10 μM DCFH or DHR123 for 1 hr, and then were either exposed to light or maintained in the dark as a control. Ten-minute light exposures were made with the 488 or 514.5 nm emissions of the Argon ion laser, or the 647.1 nm emission of the Krypton ion laser. All exposures were quantum-equivalent, delivering $\sim 4.99 \times 10^{18}$ photons/cm²-sec in the 10 min period. Following the laser exposure, the cells were lysed and the probe isolated from the lysates by SPE. Twenty-μl aliquots were injected into the HPLC column and the probe content quantified by fluorescence detection. DCFH exhibited a clear pattern of photooxidation by laser exposure, as shown in Figure 9. In the dark, or in cell free media, there was a low level of DCF fluorescence detected, indicating that most of the probe remained in the reduced form. All three laser wavelengths resulted in DCFH oxidation, but the degree to which the probe was oxidized was clearly wavelength dependent. On a quantal basis, the Argon 488 nm output was the most efficient in producing photooxidation. In contrast, DHR123 was not consistently photooxidized (Figure 10). The amount of fluorescence recovered from exposed RPE cells labeled with DHR123 did not bear any clear relationship to the wavelength or intensity of the laser irradiation.
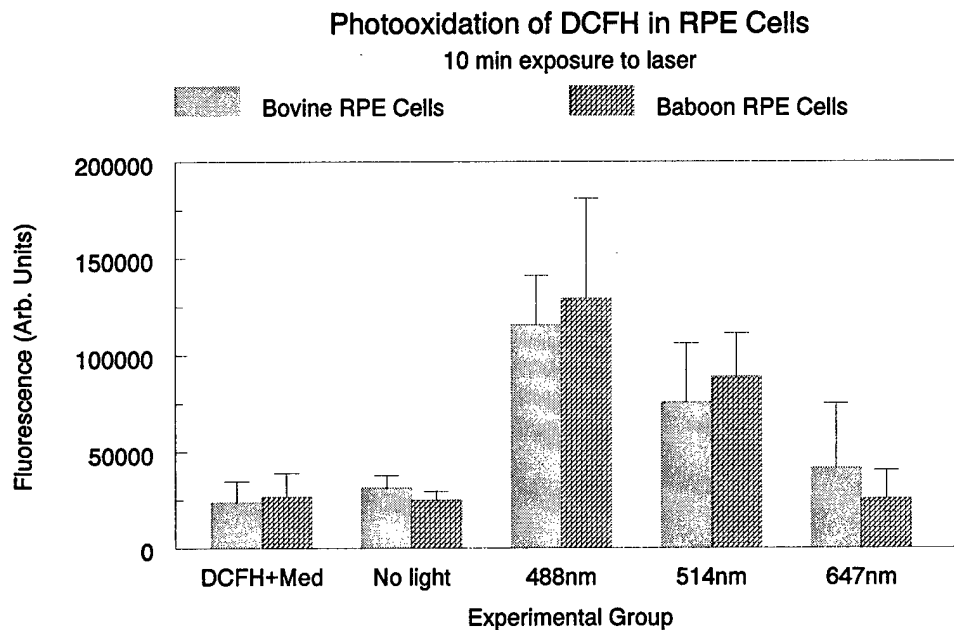
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Photooxidation of DCFH in RPE Cells
10 min exposure to laser

Bovine RPE Cells   Baboon RPE Cells

Figure 9. Photooxidation of DCFH incorporated into cultured bovine and baboon RPE cells and exposed to the indicated argon ion (488 and 514 nm) and krypton ion (647 nm) laser lines. All exposures were quantum equivalent, delivering ~4.99 × 10^{18} photons/cm^2-sec. Further experimental details are given in the text.

Photooxidation of DHR123 in RPE Cells
10 min exposure to Argon laser
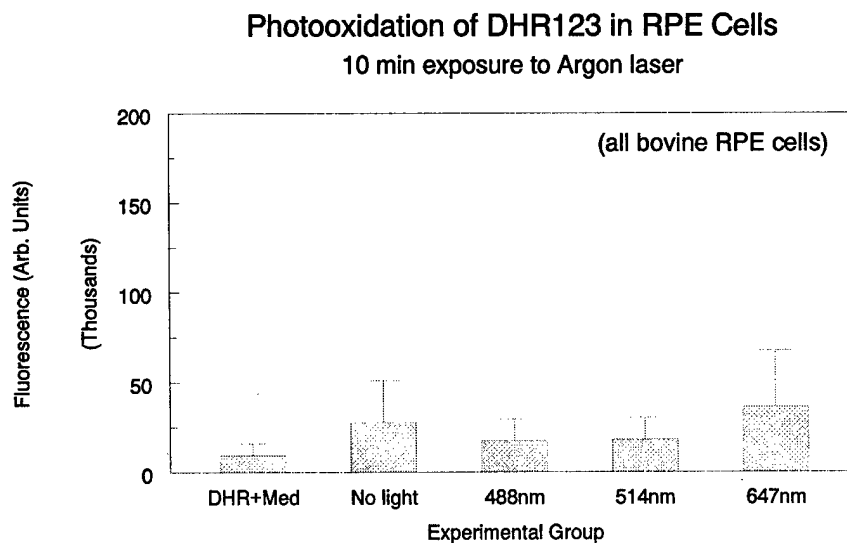
(all bovine RPE cells)

Figure 10. Photooxidation of DHR123 in RPE cells exposed to quantum-equivalent exposures at several laser lines. DHR123 is not oxidized in a wavelength-dependent manner. Experiment conducted as in Figure 9.

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The failure *in situ* of DHR123 to be oxidized consistently by light exposure was puzzling, especially because it had been oxidized by laser-excited isolated melanosomes in the original pilot study. However, in the intact cell (= *in situ*), the mode of interaction between the photoactivated melanosome and the probe may be different from the *in vitro* case, perhaps requiring the intervention of a reactive intermediate that may or may not be present in adequate quantities to mediate the reaction. Another possibility is that the oxidation potential of the probes may differ enough to lead to different reaction pathways. Because there was no published information on the oxidation potential of the probes, nor was any data available from the manufacturer, we determined the half-wave oxidation potential of both probes by use of HPLC analysis with electrochemical detection. The hydrodynamic voltammograms produced by this technique are shown in Figure 11. A sigmoidal mathematical function was fit to these data, and the $E_{1/2}$ and E_0 parameters were calculated as described in the Methods. The values of these parameters are shown in Table III.

Table III

Oxidation potentials of DCFH and DHR123

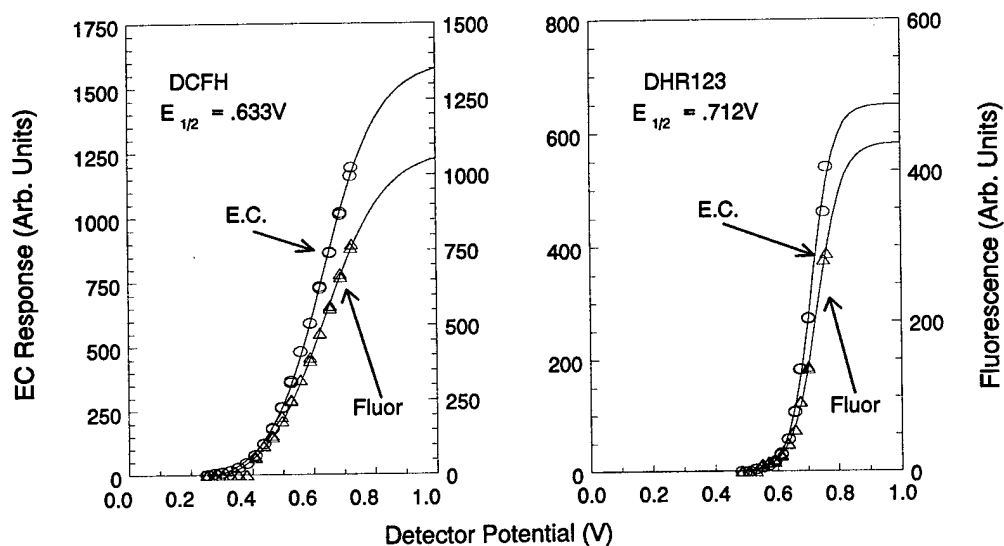| (potentials in V)	DCFH	DHR123
E_0	.279	.513
$E_{1/2}$	.633	.712

Figure 11. Hydrodynamic voltammograms of DCFH (left) and DHR123 (right). The oxidation potentials were determined by HPLC with electrochemical (E.C.) detection. A downstream fluorescence detector measured the fluorescence of the probes oxidized by the E.C. detector. As the probes were increasingly oxidized by higher working potentials in the E.C. flow cell, probe fluorescence increased. The values of the half-wave and threshold oxidation potentials were calculated from the sigmoidal functions fit to the data as described in the text.

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DISCUSSION

Utility of oxidation-sensitive probes for the study of laser-induced cellular photooxidative stress

At least in the case of DCFH, the results of the present investigation show that an oxidation-sensitive probe can be introduced into a living cell, and that the fluorescence signal derived from the cell is related to the cell's recent light exposure history. Moreover, the intensity of the fluorescence is related to the wavelength of the incident light, which points to the involvement of a specific chromophore with a characteristic action spectrum. A biochemical approach, using HPLC analysis of extracted probe, proved to be the most productive method for studying photooxidative stress in RPE cells because of interference of the pigmentation itself with a strictly optical approach. Although fluorescence microscopy was not as useful as originally hoped in studying the photochemical reactions of these probes in the pigmented cells, it did reveal that the spatial extent of the fluorescence increase in the cell was restricted to the cytoplasm. While the actual cellular distribution of the probe was not determined in this study - it is possible that the probe simply did not penetrate the nucleus - there is no published data that suggests that the nucleus excludes these molecules. Additional work is necessary to resolve this issue. However, the increase in fluorescence in the cytoplasm indicates that the photoactive chromophore is located there. Further discussion below will argue that this chromophore is melanin.
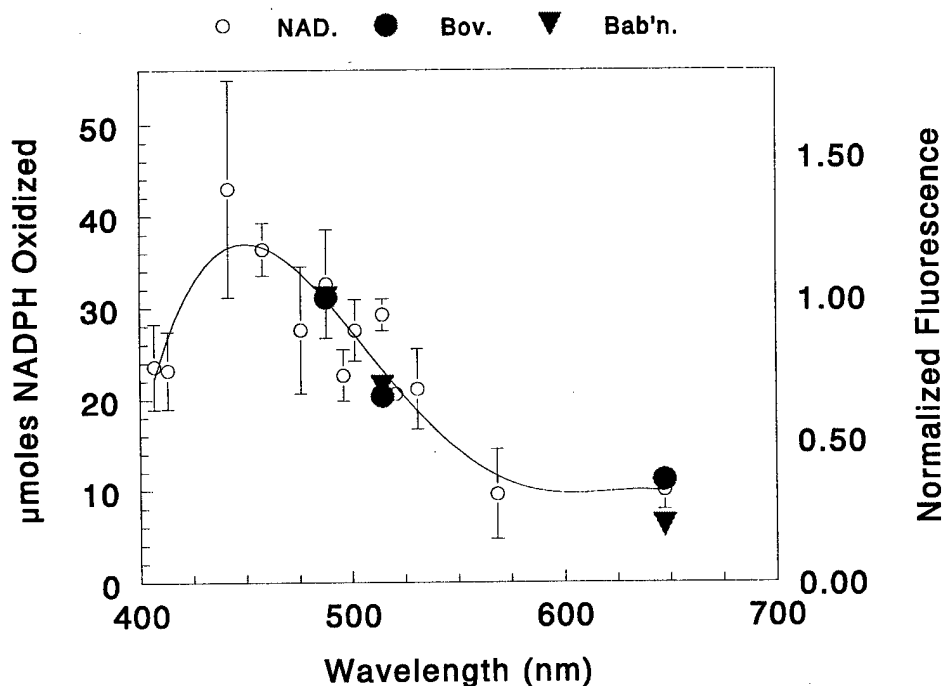

Figure 12. Action spectrum of NADPH oxidation by laser-excited melanin (all exposures $\approx 3.18 \times 10^{21}$ photons/cm^2/300 sec). Open circles: photooxidation of NADPH. Superimposed data: normalized fluorescence of DCFH in bovine (filled circles) and baboon (filled triangles) RPE cells following CW laser exposure.

Significance of the wavelength dependence of DCFH photooxidation

Although the intracellular photooxidative conversion of DCFH to DCF has been characterized for only three major laser wavelengths, the results obtained are very consistent with the action spectrum reported for the photochemical oxidation of NADPH by laser-excited RPE melanin [15]. The oxidation of NADPH in that study was used as an assay for the excitation of reactive free radical sites in the melanin heteropolymer, and the resulting action spectrum exhibited a peak in the visible spectrum between 450 and 500 nm (see Figure 12). For comparison, the data shown in Figure 9 has been normalized and superimposed on the melanin action spectrum in Figure 12. It is clear that photooxidation of DCFH in laser-exposed RPE cells follows a very similar wavelength dependence as that of NADPH. It is unclear why DHR123 was not similarly photooxidized in the RPE cell, but recently we have found that this probe has a higher oxidation potential than that of DCFH (.712 V as compared to .633 V) [37]. The higher redox potential may prevent it from reacting with the principal oxidizing species in the cell. Another possibility is that because DHR123 is more lipophilic than DCFH, the probe may be selectively taken up in membranous structures such as mitochondria where it is inaccessible to the pigment granules.The localization of DCF fluorescence only in the cytoplasm of the cell argues strongly that the chromophore responsible for this photochemical reaction is one of the RPE pigment granules. Melanin is the most likely candidate; one, because of the overlap of the DCFH photooxidation data with the melanin action spectrum, and two, because lipofuscin and melanolipofuscin, the other two RPE pigment granules, are not found in appreciable quantities in the young bovine eyes used in some of the present experiments [11]. The baboon eyes were from older animals that may have contained larger amounts of lipofuscin, therefore the contribution of lipofuscin cannot be totally excluded. There was insufficient tissue available from the baboon eyes to analyze for lipofuscin. Nevertheless, because lipofuscin is capable of inducing oxidative reactions even in the dark [11], the low level of dark oxidation in our preparations suggests that the contribution of lipofuscin granules was not significant.

Consequences of photooxidation in the RPE cell

The oxidation of DCFH in laser-exposed RPE cells serves only as a beacon of oxidative stress. In the absence of any other information, it would have limited significance. However, there are several reports in the literature concerning oxidative damage to physiological molecules due to light-activated RPE pigment granules [8,11,13,14,16,38]. Because most of these observations have been made with isolated pigments, the significance of these findings for oxidative stress *in vivo* are not yet known. The importance of the present finding is that an intracellular reporter of oxidative stress (i.e. the fluorescent probe DCFH) yields a positive signal in light-exposed pigmented cells. It is known that these probes can be oxidized by hydroxyl radical ($E^{\circ'} = +1.060V$ for $HOO\bullet \rightarrow H_2O_2$), and probably by singlet oxygen - at least for DCFH ($E^{\circ'} = +.650V$ for $O_2^1 \rightarrow O_2\bullet^-$). These reactive oxygen species can be produced by photochemical reactions, and indeed have been reported in studies on light-activated melanin [19,20]. Normally the RPE cells have antioxidants that quench reactive species produced directly or indirectly by light-activated pigment granules, preventing cellular damage. The cumulative effects of oxidative damage may be the basis of some of the age-related retinal degenerations. The melanosome breaks down in age, leading to the accumulation of melanolipofuscin granules along with the lipofuscin

granules [1]. The melanosome is also damaged by short-pulse laser exposures [25]. The breakdown of the melanosome structure has been shown to increase its photochemical reactivity [26]. Taken together, all of these observations lead to the general conclusion that the RPE pigments may play dual roles in the eye: protecting against light damage as long as sufficient antioxidants such as ascorbic acid are present to quench the light-excited pigment radicals, but promoting light damage in the event of melanosome structural damage, antioxidant depletion, or the inability to recycle antioxidants, which is an energy-dependent process. Examination of these related aspects of RPE cell metabolism may yield new insights into the prevention of oxidative stress in the retina and its supportive tissues. The use of oxidation-sensitive fluorescent probes to provide an intracellular measure of oxidative stress ("redox status") will greatly aid research on oxidative stress in the eye resulting from laser injury, as well as from metabolic and environmental factors.

CONCLUSIONS

The oxidation-sensitive fluorescent probe, 2',7'-dichlorofluorescein diacetate, can be used to detect laser- and light-induced oxidative stress in retinal pigment epithelial (RPE) cells. This probe, which becomes fluorescent when chemically oxidized, interacts with melanosomes in the cytoplasm of the RPE cells in a wavelength-dependent fashion. Wavelengths in the region between 440 and 500 nm are the most effective in producing a fluorescence increase; longer wavelengths in particular do not induce these reactions. Another fluorescent probe, dihydrorhodamine 123, is not oxidized in a wavelength-dependent manner. DCFH, at least for short term experiments, is relatively nontoxic and appears to be a novel and sensitive reporter for intracellular photochemical damage produced as a consequence of laser and light exposures. The DCFH data, along with the evidence that photoactivated melanosomes produce oxidative damage to physiologically important intracellular components, support the conclusion that melanin is indeed a photosensitizer, and that under conditions of antioxidant depletion, it likely contributes to photochemical damage to the RPE and retina.

ACKNOWLEDGMENTS

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Appendix 1. PERSONNEL SUPPORTED/COLLABORATIVE ARRANGEMENTS

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Appendix 2. PUBLICATIONS

[Books Published]

Glickman RD. Biological responses to laser radiation. In: Hardy K, Meltz M, Glickman R (Eds.): *Non-ionizing radiation: An overview of the physics and biology*. Medical Physics Publishing: Madison, WI, pp 63-80, 1997.

[Articles Published]
(Related to RPE Photooxidative stress)

Glickman RD and Lam K-W. Melanin may promote photooxidation of linoleic acid. In *Laser-Tissue Interaction VI*, SL Jacques, Ed., Proc. SPIE 2391:254-261, 1995.

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