

Intrinsic tissue fluorescence in an organotypic perfusion culture of the porcine ocular fundus exposed to blue light and free radicals

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Abstract

Background A wide variety of pathological pathways may result in age-related macular degeneration. Because of its complexity, there is no comprehensive model of the disease yet. One key feature is the accumulation of the autofluorescent pigment lipofuscin in the retinal pigment epithelium (RPE). Thus, we developed an organotypic perfusion culture model of the porcine ocular fundus, generating lipofuscin under exposure to blue light and hydrogen peroxide.

Methods Porcine fundi (choroid, Bruch's membrane, RPE, and retina) were explanted in toto, transferred into a perfusion culture chamber, perfused with cell culture medium and kept at 37°C. Free radical stress was induced by supplementation of H₂O₂, and/or the specimens were exposed to blue light, or kept untreated as controls. After a culture period of 7 days, the specimens were subject to microscopic inspection, histology, fluorescence microscopy, and measurement of fluorescence spectra as well as fluorescence decay times.

Results Histology showed atrophic ganglion cells and rod outer segments. All other tissue structures were morphologically intact. Compared to the controls, RPE and retina exposed to light showed increased fluorescence, which was shifted towards shorter wavelengths. The fluorescence spectra and decays resembled that of lipofuscin granules isolated from human donor eyes. HPLC analysis revealed the abundance of the lipofuscin component *N*-retinylidene-*N*-retinylethanolamine (A2E), its precursor products, as well as two new, green-emitting fluorophores.

Conclusions Porcine ocular fundi were successfully preserved in an organotypic perfusion culture for 7 days, and exhibited remarkable autofluorescence after light and free radical exposure, making the model suitable for investigations of lipofuscinogenesis.

Keywords Ocular fundus · Organ culture · Autofluorescence · Fluorescence spectroscopy · Lipofuscin

Introduction

A key feature of aging, and a hint as to the risk of the development of an age-related macular degeneration (AMD), is the increase in fundus autofluorescence [4, 11, 18, 42, 43]. The principal fluorophore which is observed clinically is lipofuscin [9]. Lipofuscin is a mixture of compounds, and derives from lipids and posttranslational modified proteins which are stored in the lysosomal compartment of the cells. Although a lot of studies have shown that lipofuscin is involved in the aetiology of AMD [37], only a small number of pathogenetic pathways have been clarified recently. Most attention has been paid to the pyridinium bisretinoide A2E, a Schiff-base product of retinaldehyde and ethanolamine which can be synthesised

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easily [29]. A2E is the major fluorophore of the chloroform extractable fraction of lipofuscin [12]. However, the chloroform soluble material constitutes only 20% of the dry weight of lipofuscin [23]. Rozanovska et al. demonstrated that both the chloroform-soluble and the nonsoluble fraction of lipofuscin granules showed photoreactivity [33]. Surprisingly, the photoreactivity of the non-soluble components increased with donor age, whereas that of the chloroform-soluble remained constant. Furthermore, confocal scanning fluorescence microscopy revealed very different fluorescence spectra for single lipofuscin granules [17] as well as for single RPE cells [3]. Taken together, these findings show that different constituents contribute to the observed fundus autofluorescence as well as the photoreactivity and cytotoxicity of lipofuscin.

On the other hand, the generation and accumulation of lipofuscin involves a wide variety of biochemical processes. The high oxygen transport rates and concentrations in the retinal pigment epithelium (RPE) and in Bruch's membrane [6], and the light exposure of these tissues, result in a high generation rate of free oxygen radicals (reactive oxygen intermediates; ROI) [24]. Phagocytosis of the receptor outer segments, an important function of the RPE, results in the uptake of polyunsaturated fatty acids (PUFAs) by the cells. Lipofuscin accumulation by cultured RPE cells fed with rod outer segments has been demonstrated [28, 41, 45]. The PUFAs are oxidised by the ROI to lipid peroxides which decay non-enzymatically to 4-hydroxynonenal (HNE), 4-hydroxyhexenal (HHE) [5], carboxyethyl pyrrole (CEP) [10, 14], and malondialdehyde (MDA) [7]. These compounds form adducts with cysteine, lysine, or histidine residues of the phagocytosed proteins, preventing them from the normal liposomal degradation [34]. Together with A2E and its precursors mentioned above, these modified proteins accumulate in lysosomes of the RPE as lipofuscin. Incomplete hydrolysis by lysosomes results in the deposition of lipofuscin granules in the RPE cells and in the exocytosis of membranous debris, which is discarded onto Bruch's membrane [19]. This debris forms basal-laminar deposits on Bruch's membrane and reduces its permeability, which may contribute to the development of AMD as well [39].

Thus, the pathogenesis of AMD is much too complex to be modelled in a cell culture model. On the other hand, there is no sufficient animal model yet to study the generation of lipofuscin and its pathologic effects at the ocular fundus. Although lipofuscin accumulation is reported for RPE65 - [21] as well as ABCR-deficient [31, 32] mice, these transgenic animals lack only specific proteins responsible for binding and transport of all-trans retinyl ester in the RPE [26] or all-trans retinal in the ROS respectively. Therefore, this lipofuscin may be very different from that accumulated in humans during the aging

process. Another AMD model, the Ccl-2- or Ccr-2 deficient mouse, is published by Ambati et al. [1]. Mutation in the Ccl-2- or Ccr-2-gene results in defects in macrophage mobilization. Though macrophage dysfunction may play a role in AMD, macrophage activation is always a secondary reaction on a pathologic stimulus such as the deposition of cell debris or macromolecules onto Bruch's membrane or in drusen. The primary events in the genesis of AMD, however, are still not known, but probably are associated with lipofuscin. The goal of the investigations presented here was to study the lipofuscin accumulation in an in vitro organ culture model of the ocular fundus exposed to blue light and oxidative stress.

Material and methods

Dulbecco's modified Eagle medium (DMEM) with L-Glutamin, 4.5 g/l D-Glucose, and 25 mM HEPES was obtained from Invitrogen (Karlsruhe, Germany). Procine serum and penicillin/streptomycin (10.000 units/10 mg/ml) were from PAA Laboratories (Pasching, Austria) and hydrogen peroxide (30 wt.% in water) was from Sigma-Aldrich (Steinheim, Germany).

Tissue preparation and organ culture

Porcine eyes were used in this study because their fundus is most similar to that of primates: pigs do not have a tapetum, and the retina resembles that of humans and is well-endowed with cones. Although they lack a fovea, as primates have, pigs have a macula-like area free of retinal vessels at the posterior pole [30]. The pigs were butchered at the age of about 6 months. Analysis in non-cultured specimens showed trace amounts of A2E, A2PE, and A2PE-H₂.

Porcine ocular fundus in toto (excluding sclera) was kept in a perfusion organ culture system (Minucells and Minutissue GmbH, Bad Abbach, Germany) for 7 days. Procedures of tissue preparation and cultivation are described in detail elsewhere [22]. Briefly, porcine eyes were obtained from a local slaughter house, transported and stored at 4°C and processed within 3 hours post mortem. The anterior segment of the eye was cut off 3 mm posterior to the limbus, and was removed from the eyecup together with the adherent vitreous after careful dissection of vitreous attachments to the ora serrata and the optic disc under the microscope. A segment of the eye cup, including the optic disc, was cut out and flat mounted using 23-gauge needles. Neural retina, RPE, and choroids were dissected from the sclera carefully and clamped in a ring-holder system. Thus, a piece of full-thickness ocular fundus (except sclera) of 13 mm in diameter was mounted in that ring holder and transferred into a perfusion culture

container. All microsurgical procedures were performed under sterile conditions.

In the container, the tissue was perfused with DMEM supplemented with 15% adult porcine serum and 1% penicillin/streptomycin by a peristaltic pump (Ismatec Laboratories, Zurich, Switzerland, perfusion rate: 2 ml/h) on the retinal as well as the choroidal side for 7 days. In experiments investigating the fluorescence of the RPE by laser-scanning microscopy, the cultures were terminated after 3 days because of a decline in cell adhesion to Bruch's membrane after that time. The temperature of the tissue culture was maintained at 37°C using a warming table (Medax GmbH, Kiel, Germany).

Irradiation and oxidative treatment

The effect of light exposure was investigated by irradiation of the cultures from the retinal side with blue light (415 nm–480 nm, peak wavelength 467 nm). Two irradiance levels, 0.053 mW/mm² and 0.41 mW/mm², were applied. Since photoreceptor outer segments are known to be phagocytosed mainly at night, in some experiments the irradiation was used in a diurnal rhythm (irradiation from 6 P.M. to 6 A.M., cultures kept in dark from 6 A.M. to 6 P.M.). A 150 W tungsten halogen lamp (Stocker & Yale Inc., Salem, NH, USA) was used as light source. The light was guided by a glass fibre bundle to a special perfusion culture chamber equipped with windows. It was filtered to the spectral range denoted above and imaged onto the retina. In order to enhance the oxidative stress to the tissue, hydrogen peroxide was added to the culture medium in 150 µM concentration and, thus, continuously supplied to the culture.

In each perfusion culture experiment, three samples were processed in parallel: one culture exposed to blue light and to H₂O₂ in some of the experiments (see results), one exposed to H₂O₂ but kept in dark, and a control exposed neither to light nor to H₂O₂.

Microscopy

The auto-fluorescence of the RPE was inspected after 3 days of culture. Immediately after the end of the culture, the retina was removed and the RPE, backed by Bruch's membrane and choroid, was placed on a coverslip and observed under an inverse laser scanning microscope (LSM 510, Carl Zeiss, Germany). The fluorescence was excited by an Argon laser (wavelength 458 nm) and recorded at two spectral channels: 505–550 nm and 585–700 nm. An inverted microscope Eclipse TS-100-F (Nikon, Tokyo, Japan) was used in conjunction with a CCD-camera F-view from Soft Imaging Systems (SIS, Münster, Germany) for the inspection of the histological sections. The auto-

fluorescence of unstained histological sections was measured after 7 days in culture.

Measurement of fluorescence spectra and lifetimes

The auto-fluorescence of the tissue was excited using a filter with a transmission band of 450–490 nm. The fluorescence emission spectra were recorded for wavelengths >530 nm by an imaging spectrograph CP 200 (Jobin Yvon, Longjumeau, France) equipped with an intensified CCD-Camera (Princeton Instruments, Trenton, NJ, USA) attached to the microscope. The fluorescence decay was observed using excitation at 446 nm by a short-pulse (100 ps FWHM) laser diode LDH 440 (Picoquant, Berlin, Germany) and a time-correlated single photon counting (TCSPC) detection system SPC 530 (Becker & Hickl, Berlin, Germany) as described in detail elsewhere [35].

Fluorescence measurements were performed on the Nikon microscope equipped with the light sources and detectors mentioned above, and fluorescence spectra as well as decay times were recorded immediately after the end of the 7-day culture period. Measurements were taken from the fundus in toto, with illumination from the retinal side. Furthermore, the retina was detached from the RPE, and separate measurements of both structures were taken.

Absorption and emission spectra of extracted fluorophores, purified by HPLC, were measured using a Shimadzu UV-2401 (Shimadzu/Cole Scientific, Moorpark, CA, USA) or a Fluorolog FL3–22 spectrophotometer (Jobin Yvon, Edison, NJ, USA).

Histology

Fundus specimens were embedded in paraffin, cut to 2-µm or 5-µm slices, and stained with haematoxylin and eosin. The slices were microscopically inspected and photographed for the evaluation of the morphologic integrity of tissues. Unstained slices (thickness: 8 µm) were inspected by fluorescence microscopy, in order to localize auto-fluorescent anatomical structures.

Extraction

Chloroform soluble compounds were extracted from either the cultured tissues in toto or from retina and RPE–choroid complex separately, as described previously [27]. The extracts were washed with 4 ml of distilled water/methanol (1:1, v/v) and the organic phase was then taken to dryness under a stream of N₂. Sample residues were dissolved in 500 µl of 2-propanol for analysis by HPLC. All procedures were carried out on ice under dim red light according to a standardised protocol [27], in order to minimise sample loss.

HPLC

The tissue extracts were analyzed by normal-phase HPLC on a silica column (Zorbax Rx-Sil 5 μm , 250 X 4.6 mm, Agilent; Palo Alto, CA, USA) using an Agilent model 1100 liquid chromatograph equipped with photodiode array and fluorescence detectors (Agilent Technologies, Wilmington, DE, USA). The mobile phase (hexane/2-propanol/ethanol/25 mM potassium phosphate/acetic acid, 485:376:100:37:0.275, v/v) was pumped through the system at 1 ml/min. Column and solvent temperatures were maintained at 40°C. In order to get sufficient material and to reduce possible losses during the prepreparation, samples from up to eight cultures were pooled.

Results

Tissue preservation in the perfusion culture depends on the culture period and stress factors applied. Thus, the duration of the culture and experimental conditions were adjusted to the needs of the subsequent investigation: for chemical analysis of fluorophores, tissues cultured for 7 days were used in order to obtain sufficient amounts of material produced by the treatment. Experiments investigating the tissue morphology and the fluorescence of the RPE cells were terminated after 3 days. This culture period was appropriate to produce sufficient fluorescence alterations at only minor loss of cell integrity. Figure 1 demonstrates the tissue preservation after the full 7-day culture period.

Figure 1a shows the histological micrograph of the untreated control after 7 days in culture. Choroidal vasculature, RPE, outer and inner nuclear layer as well as plexiform layers can be clearly distinguished, although some alteration in the inner retina occurred (for comparison see the micrograph of a native porcine fundus in Fig. 1g). Most remarkably, an atrophy of the ganglion cell layer, which is known to be extremely vulnerable, was found. The layer of photoreceptor outer segments is still present, but cellular structures were destroyed and only cell debris was found. A similar preservation of tissues was observed in the sample treated with hydrogen peroxide (Fig. 1c). After exposure to light as well as H_2O_2 , choroid, RPE, outer, and inner nuclear layer appeared morphologically preserved. However, in the layer of the photoreceptor outer segments, an accumulation of cell debris was found (Fig. 1e). Figure 1b,d, and f shows the autofluorescence of unstained tissue sections. These images were recorded with an automatic camera gain. Thus, the intensity of the fluorescence can not be compared from one image to another (for comparison of absolute fluorescence intensities see Fig. 2). In the control (Fig. 1b) there is only minor fluorescence primarily emerging from the RPE. Upon H_2O_2 treatment, an

additional rather intensive fluorescence in the outer nuclear layer appeared (Fig. 1d). An enhancement of the RPE autofluorescence was found in the sample additionally irradiated with blue light (0.053 mW/mm^2 , Fig. 1f). The strongest autofluorescence in this sample, however, was found in the layer of the photoreceptor outer segments.

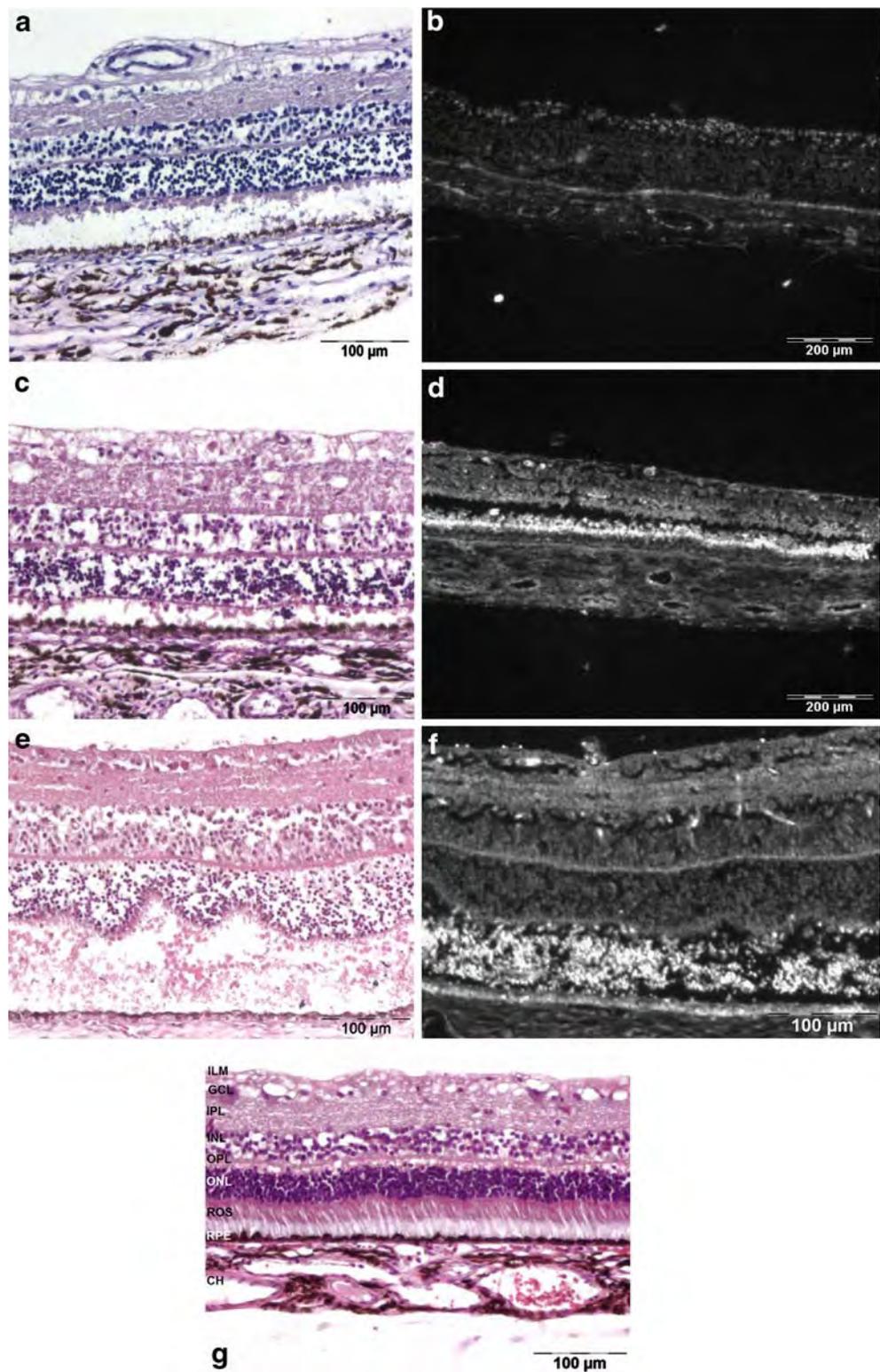
Figure 3 shows the fluorescence of the RPE, after removal of the neural retina, recorded with laser scanning microscopy in a green and in a red emission range simultaneously. The non-irradiated control (Fig. 3a), after 3 days in culture, looked like the noncultured RPE (Fig. 3c): the fluorescence was dominated by orange-red emitting granules. In contrast, the irradiated (0.41 mW/mm^2 , diurnal rhythm) culture showed a greenish fluorescence throughout the entirety of the cells, whereas the red fluorescence was reduced. Although there was no RPE detachment found in histology (Fig. 1), there was some loss of RPE cells after removal of the retina in the irradiated culture after 3 days. Thus, irradiation may have induced a decline of RPE adhesion to Bruch's membrane.

In order to investigate the fluorescence of single structures more quantitatively, separate emission spectra were recorded for retina as well as RPE. Figure 2 shows the fluorescence spectra (excited at 450–490 nm) measured from the RPE and the retina respectively. The increase of the fluorescence under irradiation and H_2O_2 -exposition is obvious. In order to recognize spectral shifts, normalized spectra are shown in Fig. 4. The spectra of RPE and retina, exposed to light and H_2O_2 , resemble very much those of lipofuscin granules, isolated from human donor eyes. Compared to those spectra, the emission of the RPE, not exposed to light, was bathochromic shifted with emission peaks at about 600 nm. In contrast, the peak of the retinal spectra was not changed by the treatment with H_2O_2 alone or in combination with blue-light irradiation. All specimens showed blue autofluorescence excited at 340–380 nm (data not shown). The measured peak wavelength (450 nm), however, may be the product of tissue emission and the blocking filter spectrum (cut-off wavelength 435 nm). Thus, the peak emission may be even shorter. As the longer wavelength fluorescence, excited at 450–490 nm, this blue fluorescence increased with exposure to H_2O_2 and light too.

Fluorescence decays vs time are shown in Fig. 5. The decay of the irradiated tissues (fundus in toto), additionally exposed to H_2O_2 , was similar to that of lipofuscin granules, whereas that of the control and the specimen supplemented with H_2O_2 were shorter. A dual exponential fit of the data revealed decay times of 0.61 ns and 3.6 ns (irradiated and H_2O_2 exposed fundus), 0.26 and 2.73 ns (control), 0.31 and 2.89 ns (culture exposed to H_2O_2 in the dark), and 0.71 ns and 3.32 ns (lipofuscin granules).

Chloroform-methanol extracts of tissues, cultured under different conditions, were analyzed by HPLC. From the

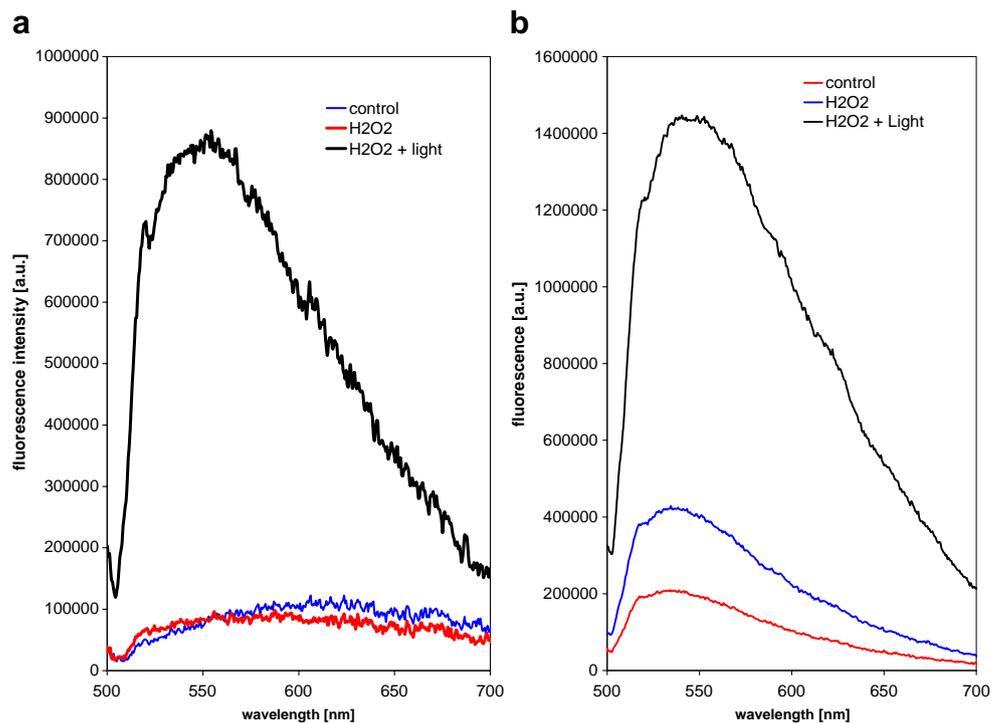
Fig. 1 Histologic micrographs (**a**, **c**, and **e**) and fluorescence micrographs (**b**, **d**, and **f**). **a**, **b** Control. **c**, **d** Sample exposed to 150 μM H_2O_2 . **e**, **f** Sample exposed to 0.053 mW/mm^2 blue light and 150 μM H_2O_2 . **g** Uncultured tissue: *ILM* inner limiting membrane, *GCL* ganglion cell layer, *IPL* inner plexiform layer, *INL* inner nuclear layer, *OPL* outer plexiform layer, *OPL* outer plexiform layer, *ROS* rod outer segments, *RPE* retinal pigment epithelium, *CH* choroid



RPE/choroid complex, the well-known fluorophores A2E, A2PE, and A2PE- H_2 were isolated and identified by their absorption spectra (Fig. 6a). Furthermore, fluorophores were found at retention times of about 6 and 11 min. These

species were much more abundant in the retina (Fig. 6b) than in the RPE, and showed a green fluorescence (peaks: 503 nm and 518 or 523 nm respectively, Fig. 6b, inset). Figure 6 shows the HPLC plots of the sample irradiated in a

Fig. 2 Fluorescence spectra of the RPE (a) and the retina (b) after seven days in culture. Excitation: 450–490 nm. H₂O₂–concentration: 150 μM, irradiance: 0.41 mW/mm²



diurnal rhythm. The results of all other samples were identical qualitatively but different in the content of the fluorophores.

The abundance of the retinal-derived fluorophores A2E, A2PE, and A2PE-H₂ was assessed by measuring the area under the HPLC peaks. For all three compounds, we found an increase under H₂O₂ compared to the control culture, but a dosage-dependent decrease under additional irradiation (Fig. 7, samples pooled from three culture experiments each).

Discussion

Porcine ocular fundi, explanted in toto, were successfully preserved in an organotypic perfusion culture for 7 days. Histology demonstrated the morphologic integrity of the tissues after culture, with some limitations known from previous investigations [22]. The administration of 150 μM H₂O₂ resulted in an atrophy of rod outer segments (ROS). The retina and ROS, rich in unsaturated fatty acids, are highly susceptible to autoxidation via free radical mechanisms. Whereas the inner retina seems to be well-protected against superoxide free radicals and hydrogen peroxide by virtue of superoxide dismutase, peroxidases and catalase activities, the ROS are only protected by superoxide dismutase [2]. Thus, ROS are more vulnerable to oxidative damage than other retinal cellular structures. This, together with the high concentration of PUFAs, would explain the early atrophy of ROS under H₂O₂ or high oxygen tension, a

condition generally found in the perfusion culture system [40]. The hypothesized free radical process is associated with a remarkable increase in autofluorescence (Fig. 1d). Also under blue-light irradiation, hyperfluorescence in the layers of the ROS and the RPE was observed (Fig. 1f). Hyperfluorescence of the outer retina indicates that, under our experimental conditions, the photo-oxidation rate was higher than the phagocytotic activity of the RPE-cells.

The spectrum of the control RPE (Fig. 4) resembles that reported by Delori et al. [8] from in vivo measurements at the ocular fundus of humans. Their spectra peak at 620 nm, while ours do so at about 600 nm. This difference may be explained by a shielding effect of the retinal blood in vivo, as demonstrated by the authors. While these spectra probably reflect the formation of the A2-E in the RPE [38] and its pre-cursor A2-PE in the retina, the spectra of irradiated samples exhibit shorter wavelength emissions. These may be attributed to lipid peroxidation products. Feeney-Burns et al. [13] found a fluorescence emission at 430 nm upon excitation at 360 nm from the non-vitamin A peak of the chromatographic separation of chloroform extracts of RPE cells. They attributed this fluorescence to a Schiff-base product of the lipid auto-oxidation product malondialdehyde with amines. A similar fluorescence emission (peak: 425 nm) was found by Spaide et al. [36] from peroxidised lipids extracted from Bruch's membrane. In both cases, the spectra were rather broad. Thus, the fluorescence we observed under UV excitation (340–380 nm) may be emitted by lipid peroxidation products,

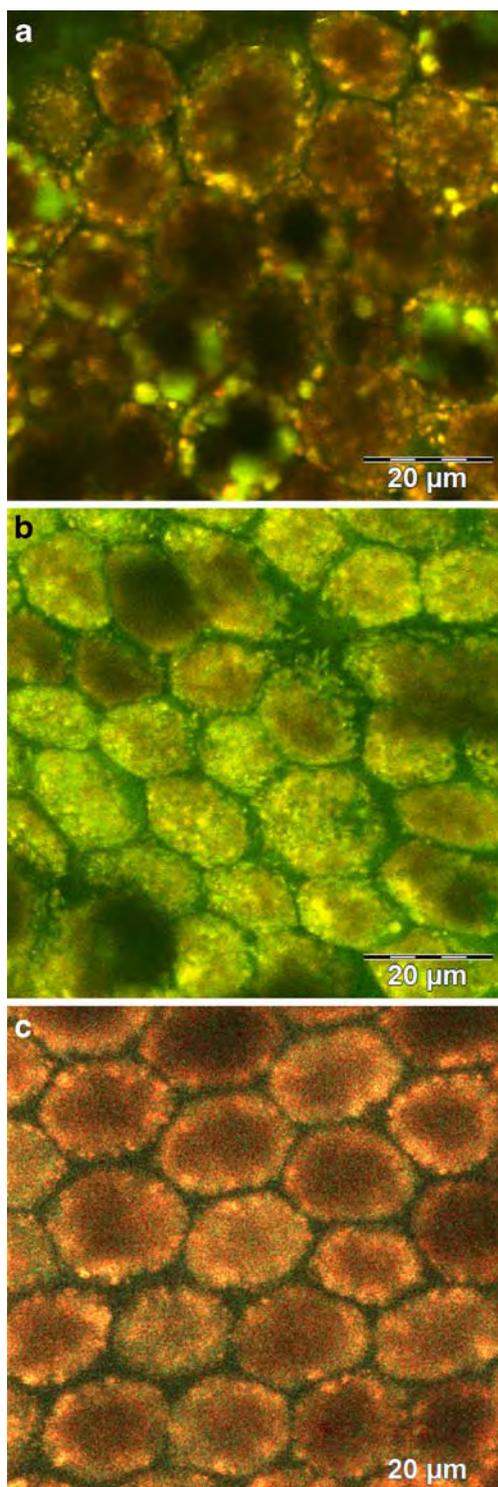


Fig. 3 Laser scanning fluorescence micrograph of the RPE after 3 days in culture in dark (a) and under 0.41 mW/mm² blue light diurnal irradiation (b). c Non-cultured RPE. Ex.: 456 nm, Em.: 505–550 nm (green channel) and >585 nm (red channel)

even if we were not able to observe the peak at 425–430 nm due to our cut-off filter at 435 nm.

It may be argued that the lipofuscin components generated here are different from that accumulated in

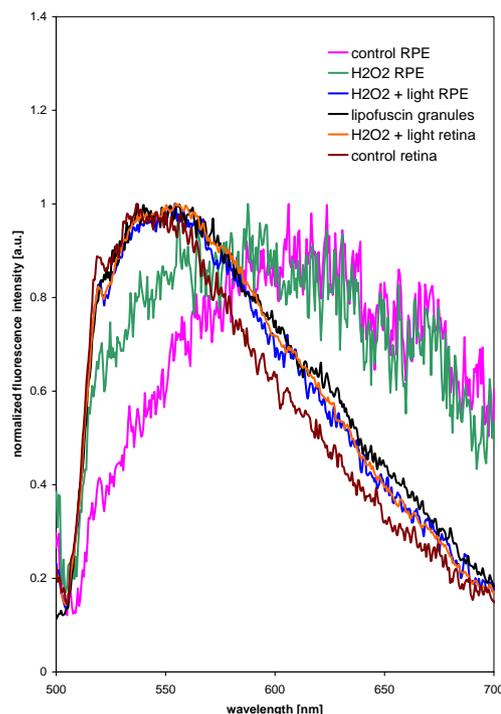


Fig. 4 Normalized fluorescence spectra of retina and RPE as well as lipofuscin granules. Excitation: 450–490 nm. H₂O₂-concentration: 150 µM, irradiance: 0.41 mW/mm²

humans over decades. However, a good agreement of the emission spectra, as well as the fluorescence decay of the irradiated tissue culture with the data from lipofuscin granules, was found (Figs. 4 and 5). Very similar spectra

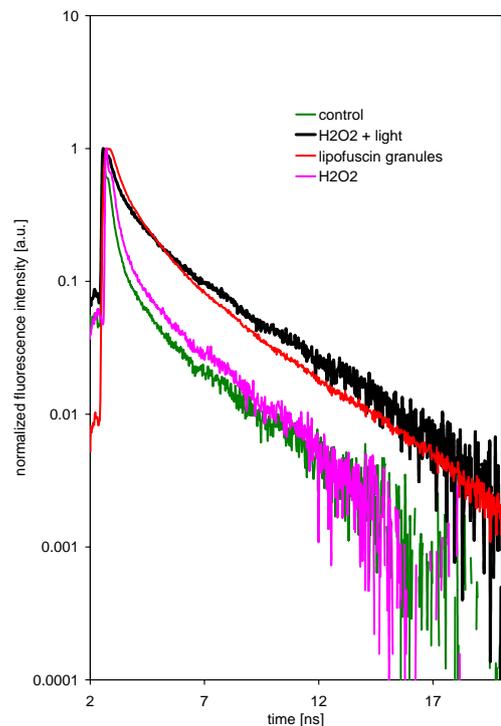


Fig. 5 normalized fluorescence decays vs time after short pulse laser excitation (446 nm, 100 ps)

Fig. 6 HPLC of the extracts from the RPE/choroid complex (a) and the retina (b), 7 days of culture under 0.053 mW/mm² blue light (diurnal rhythm). *Insets:* absorption spectra of A2E, A2PE, and A2PE-H₂ (a) and fluorescence spectra of retinal fluorophores (b)

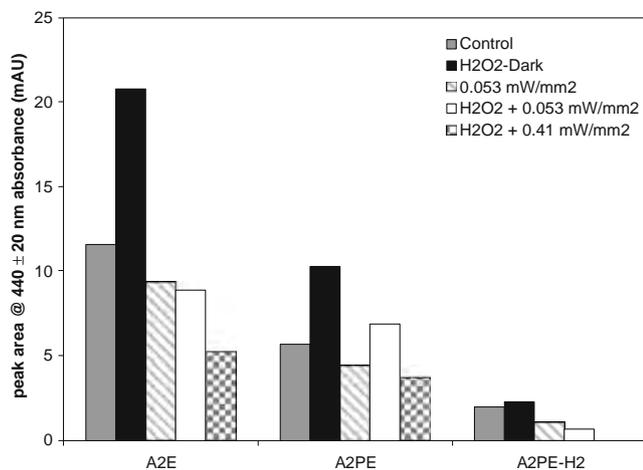
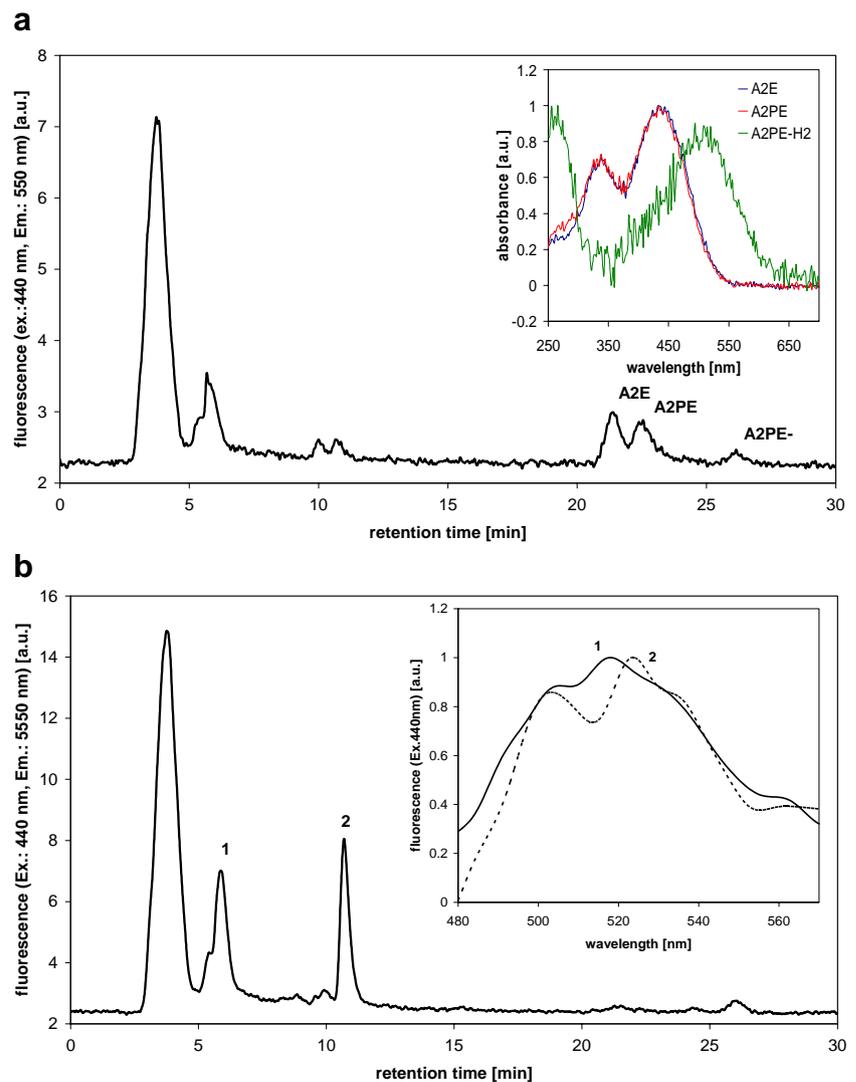


Fig. 7 HPLC absorption peak areas at 440 nm. Extracts from fundi in toto, cultured over 7 days under conditions specified

were measured by Haralampus-Grynaviski et al. [16] from lipofuscin granules. From their data, these authors concluded the existence of major chromophores as well as fluorophores other than A2E in the lipofuscin. Upon excitation at 488 nm, Marmorstein et al. [25] observed spectra from the lipofuscin in the RPE of donor eyes from AMD-patients as well as age-matched controls, which were very similar to the spectra of light-exposed cultures reported here.

Fluorescence microscopic findings (Fig. 3) as well as the spectra shown in Figs. 2 and 4 and HPLC results (Figs. 6 and 7), give hints to different processes: the vitamin A-related fluorophores A2E and its precursors A2PE and A2PE-H₂ were found in all cultures. Compared to the controls, their abundance was increased in the H₂O₂-treated cultures, but decreased in a dose-dependent manner under blue-light irradiation (Fig. 7). This result from the HPLC analysis agrees well with the fluorescence microscopic

finding of reduced fluorescence in the long-wavelength (>585 nm) channel (Fig. 3). This may be explained by a generation of these compounds under oxidative stress at one hand and, on the other hand, by their photo-bleaching. Bleaching of A2E was shown to be a photo-oxidation [15] resulting in epoxide and furanoid ring structures at the retinal side chains of the molecule [20]. This photo-oxidation of A2E results in a hypsochromic shift of the fluorescence excitation spectra of about 30 nm per additional oxygen atom [15]. Due to this shift, we did not see the oxidation products in our HPLC, and thus the peak areas of A2E and its precursors from the irradiated cultures are reduced (Fig. 7).

On the other hand, a dramatic increase of the fluorescence at short wavelengths under irradiation (Fig. 3, inset of Fig. 6b) was found, which showed much shorter HPLC retention times than A2E (Fig. 6), and thus may not be associated to retinal. The origin of this emission may be the peroxidation of lipids which finally results in 4-HNE HHE, CEP, and malondialdehyde adducts to proteins which can not be degraded by the lysosomal enzymatic system of the RPE cells, and thus accumulates in granules [34].

The data presented here demonstrate the ability of the ocular fundus organ culture model to generate and accumulate large amounts of fluorescent material under oxidative stress and blue-light irradiation. The spectral as well as temporal fluorescence characteristics, we found, were very similar to that of lipofuscin granules from human donor eyes. This supports the assumption that the fluorescent compounds generated in the organ culture model resemble the in vivo accumulated lipofuscin. However, the kinetics of the generation and the photo-bleaching of the fluorophores may be rather complicated, and the concentration of single lipofuscin constituents produced in our model may differ from that seen in elderly humans. Further investigations need to identify the molecular composition of the fluorescent species. The generation of lipofuscin-like fluorophores within 7 days only may result in immature lipofuscin constituents, possibly responsible for the blue-shift in the fluorescence emission [44].

Although further sophistications are necessary, the ocular fundus perfusion culture under irradiation by blue light may be an appropriate model of photo-oxidative processes occurring in aging and in the development of AMD. Because of its complexity, this model may be superior to RPE cell culture models. Provided that the apparent similarity of the fluorophores generated by this model with lipofuscin is established in chemical analysis currently under way, the perfusion culture model of the ocular fundus in toto might be a promising tool for the investigation of the pathogenesis of AMD, as well as for testing of future therapeutic approaches.

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Conflict of interests Hammer: none, Richter: none, Kobuch: none, Mata: employee of Sirion Therapeutics Inc., Schweitzer: none

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