Retinal pigment epithelium cell damage by A2-E and its photo-derivatives

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Purpose: A2-E, a major component of the retinal pigment epithelium (RPE) lipofuscin, is a compound that can neither be degraded by nor eliminated from cells and is toxic as well as phototoxic to the cells. Illumination of A2-E with short wavelength light results in isomerization, photooxidation, as well as photolysis. Cytotoxic intermediates (free oxygen radicals) and reaction products (peroxides) are involved in this process.

Methods: A2-E solution (1.28 mM in ethanol or 10 μM phosphate-buffered saline) was kept in dark, exposed to blue light (450-490 nm, 0.2 mW/mm²) for 15 min, or to white light (8.9 mW/mm²) for 60 min, respectively and supplemented to the culture medium of primary porcine RPE cells for 24 h. Damaged cells were determined by staining with propidium iodide in 24 experiments. The photooxidation products of A2-E were analyzed by ultraviolet-visible spectroscopy and MALDI-TOF mass spectrometry.

Results: Supplementation of A2-E for 24 h resulted in a rate of damaged cells of 28%. Blue light exposure of A2-E before supplementation increased the rate to 91% whereas the exposure to high dosage white light reduced it to 14%. Irradiation of A2-E resulted in a dosage-dependent addition of one through four oxygen atoms.

Conclusions: The increase of the cell damage rate by A2-E irradiated with low dosage light supports the hypothesis of direct DNA damage by oxidized A2-E. Furthermore, we found a reduced cell damage rate from intensively irradiated A2-E resulting in a tetraoxidized molecule which was rather stable and thus less toxic.

A characteristic of aging of postmitotic cells such as cardiac muscle, neurons, and retinal pigment epithelium (RPE) is the accumulation of lipofuscin [1]. Lipofuscin is a mixture of compounds derived from lysids and posttranslationally modified proteins which are stored in the lysosomal compartment of cells. In most cell types, lipofuscin is a product of cellular autophagy. In RPE cells, however, a major component of the lipofuscin is derived from the phagocytosis of the rod outer segment (ROS) disc membranes. The accumulation of lipofuscin seems to be associated with the development of age-related macular degeneration (AMD) [2]. Whereas the pathogenetic role of proteins modified by highly reactive electrophilic aldehydes such as 4-hydroxynonenal and malondialdehyde, resulting from the breakdown of photooxidized lipids, needs further clarification [3,4], there is a growing body of evidence for the cytotoxic effects of retinal-derived compounds.

Among these, the most studied is the pyridinium bisretinoide A2-E, a Schiff-base product of retinaldehyde and phosphatidylethanolamine [5] which can be synthesized easily [6]. A2-E is known to damage lysosomal membranes and other cellular membranes due to its detergent-like properties [7,8]. Suter et al. [9] demonstrated the release of pro-apoptotic proteins such as cytochrome c and apoptosis-inducing factor from mitochondria after exposure of RPE cells to A2-E. Furthermore, A2-E is known to inhibit the lysosomal proton pumps, resulting in an increase in the physiologically acidic lysosomal pH and, subsequently, an inhibition of the lysosomal hydrolases [10]. Besides these cytotoxic effects, there is a growing body of evidence for the phototoxicity of A2-E. Supplementing the medium with A2-E Sparrow et al. found a dose- and time-dependent increase of nonviable ARPE-19 cells after blue-light irradiation [11]. The apoptotic nature of cell death was shown by the increase of DNA strand fragmentation? in terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) assays. Subsequently, the authors found increased caspase-3 activity [12] and, by alkaline single-cell gel electrophoresis (comet assay), marked DNA fragmentation in cells exposed to A2-E and blue light [13]. In an oxygen-depleted medium, however, almost all A2-E-laden cells survived blue-light irradiation [14]. This indicated the significant contribution of oxidative processes to the phototoxicity of A2-E. Investigations of irradiated A2-E by mass spectrometry (FAB-MS) revealed sequential addition of oxygen to A2E [14,15]. The authors expected this to be due to an oxidation of the double-bonds of the retinal-chains forming epoxides, also called oxiranes. The in vitro data was confirmed when, these oxiranes were also detected within eyes of ABCR-deficient mice, an animal model of Stargard’s disease, grown under bright light [16]. In contrast, Dillon et al. [17] assumed the formation of a furanoid oxide. The DNA of ARPE-19 cells incubated with A2-E-epoxides showed a much higher damage rate than that of cells incubated with A2-E of the same concentration [18]. Thus, blue-light irradiation of A2-E may induce photooxidation, and the resulting epoxides may trig-
ner the apoptosis cascade yielding DNA fragmentation. On the other hand, A2-E was shown to be a quencher of singlet oxygen with a rate constant comparable to that of ascorbic acid (vitamin C) or a-tocopherol (vitamin E) [19] in contrast to all-trans retinal, which produced singlet oxygen more efficiently than A2-E. In such a way, the conversion of two mol all-trans retinal into one mol A2-E would reduce the oxidative stress to the cells [19]. Therefore, the authors concluded that A2-E may protect RPE cells from damage by light-produced free radicals. This is in agreement with suggestions by Davies et al. [20] as well as Schütt et al. [21] that the production of reactive oxygen species such as hydrogen peroxide, superoxide anions, hydroxyl radicals, and singlet oxygen by A2-E under short wavelength irradiation is not the major cause of cell damage.

Although there is no doubt in the phototoxic effect of lipofuscin, the mechanism, by which A2-E is involved in this process, is not completely clear yet. In this study we have characterized the photooxidation products of A2-E by ultraviolet-visible spectroscopy as well as mass spectrometry and elucidated their role in the photo-damage of RPE-cells.

**METHODS**

A2-E was synthesized as described by Parish et al. [6]. It was obtained from Lynkeus Bio Tech GmbH (Würzburg, Germany). Dulbecco’s modified Eagle medium (DMEM) with L-Glutamin and L/D-Glucose from Invitrogen (Karlsruhe, Germany) was supplemented with fetal bovine serum (FBS) from Biochrom AG (Berlin, Germany) and penicillin-streptomycin from Invitrogen. Dispase, fibronectin, and serum-free medium (SFM) were purchased from Invitrogen. Paraformaldehyde (PFA) was from Sigma (Steinheim, Germany). ZO-1 antibody (rat anti-ZO-1) and secondary antibody goat anti-rat IgG were obtained from Chemicon (Hampshire, United Kingdom) 4,6-diamidin-2-phenylindol-dihydrochloride (DAPI). The in situ cell death detection (TUNEL) kit (TMR red) and were from Roche (Mannheim, Germany). Propidium iodide (PI) was purchased from Molecular Probes (Eugene, OR).

**Preparation and cultivation of retinal pigment epithelium cells:** All experiments were done with primary cultures of porcine RPE. RPE cells were prepared from eyes supplied by a local slaughter house within 2 h postmortem. The eyes were dissected at the limbus and the anterior segment was discarded. After removal of the retina, the eye-cup was incubated with 2.5 U/ml dispase at 37 °C for 1.5 to 2 h. Then, RPE cells were abraded gently from Bruch’s membrane and resuspended in DMEM supplemented with 20% FBS and 1% penicillin-streptomycin. The cells of one eye were plated in one well of a fibronectin-coated 12-well plate (Greiner, Solingen, Germany). The cells were incubated at 37 °C with 5% CO₂ and achieved confluence after about three weeks. Upon confluence, the DMEM with FBS was replaced by SFM in four steps over eight days to induce cell differentiation. Cell differentiation was demonstrated by labeling of the tight junction protein ZO-1: To this aim, the cells were fixed with paraformaldehyde (3.3% for 15 min at room temperature). After permeabilization with Triton X-100 (0.1% for 5 min), they were washed three times with phosphate-buffered saline (PBS) and incubated with the rat anti-ZO-1 antibody (dilution: 1:1000) for one hour at 37 °C. After washing with PBS again, the cells were incubated with the FITC-labeled secondary antibody goat anti-rat IgG (dilution: 1:100) for one hour at 37 °C.

**Supplementation of A2-E, irradiation, and staining for viability of cultured retinal pigment epithelium cells:** A2-E was dissolved in ethanol to a concentration of 1.28 mM. This stock solution was diluted to 10 µM with SFM and fed to the cells for 24 or 48 h. In further experiments, the stock solution was irradiated with blue light (450-490 nm, 0.2 mW/mm²) for 15 min or with white light (tungsten halogen lamp at 6000 K, 8.9 mW/mm²) for 1 h before being diluted and fed to the cells. After incubation with A2-E, cells were washed with PBS three times and a portion of the cultures was irradiated with blue light (450-490 nm, 0.2 mW/mm²) for 15 min while the remaining cultures were kept in the dark. Thereafter, cells were incubated with PI (500 nM at 37 °C for 15 min). PI is excluded by viable cells, however, penetrates damaged cell membranes and thus binds to the DNA of damaged cells. Since it has a fluorescence excitation maximum at 535 nm and an emission maximum at 617 nm, the interference of its fluorescence with that of A2-E (excitation maximum: 418 nm [11])-is minimized. Subsequently, cells were fixed with PFA and stained with DAPI (1 µg/ml for 5-10 min at room temperature).

To confirm the suggested apoptotic nature of cell damage, we performed TUNEL tests according to the guidelines of the manufacturer in separate experiments. Briefly, cells were fixed with 4% PFA, permeabilized with Triton X-100, and incubated with TUNEL-enzyme and labeling solution for one hour at 37 °C in the dark.

**Microscopy and image processing:** An inverted microscope Eclipse TS-100-F from Nikon (Tokyo, Japan) was used in conjunction with a CCD-camera F-view from Soft Imaging Systems (SIS, Münster, Germany). All image processing was done using the software analySIS by SIS. RPE cells were visualized in phase contrast whereas the A2-E was observed by its fluorescence using the FITC filter combination (excitation: 450-490 nm, emission >520 nm). The same filters were used for the detection of ZO-1 by the FITC-labeled antibody. The DAPI-stained nuclei were observed using the DAPI filter combination (excitation: 340-380 nm, emission >435 nm), whereas the PI was monitored with Texas Red filters (excitation: 540-580 nm, emission >595 nm). The cell damage rate was determined as the ratio of the PI-stained nuclei to all nuclei (DAPI-stained). All experiments were repeated three times. In additional experiments, A2-E-laden as well as control cells were TUNEL-stained and observed by fluorescence microscopy. FITC filter combination was used for TUNEL detection (emission maximum of the labeling dye: 580 nm).

**Mass-spectrometry and ultraviolet-visible spectroscopy:** MALDI-TOF measurements were performed on an Ultraflex II TOF/TOF mass spectrometer (Bruker Daltonics Inc., Bremen, Germany) in the reflectron mode. a-cyano-4-hydroxycinnamic acid (10 mg/ml in 50% acetonitril with 0,5% trifluoroacetic acid) was used as matrix. Samples were pre-
pared using the dried droplet method. Ultraviolet-visible spectra were recorded with a lambda-2 photometer (Perkin Elmer, Wellesley, MA).

RESULTS
RPE cells were grown to a confluent monolayer (Figure 1). The hexagonal morphology resembles that of RPE cells in vivo. These cultured primary cells were still slightly pigmented with melanin. Staining of the cell membranes with the ZO-1 antibody indicates the differentiated epithelial cells without contamination. Thus, the cells adequately reflect the situation in vivo. Since the cells were obtained from young pigs, there was almost no autofluorescence (Figure 2A). A2-E was internalized into the cells if supplemented to the medium in 10 µM concentration. After 24 h incubation with A2-E we found a diffuse A2-E fluorescence throughout the cells (Figure 2B). After 48 h the fluorescence was enhanced (Figure 2C). Removing the A2-E from the medium and incubating for three more days on SFM yielded a granular structure similar to that of lipofuscin granules in elderly human subjects (Figure 2D).

Incubation of RPE cells with 10 µM A2-E for 48 h showed a remarkable rate of cell death (data not shown). Therefore, all subsequent experiments on cell damage by A2-E or its photo-derivatives were performed after 24 h incubation on A2-E. The results are summarized in Figure 3. The control, cultured without supplementation of A2-E, showed a death rate of 5.3% which was increased slightly to 8.3% by blue light exposure of cells. Incubation with A2-E in the dark increased the damage rate to 28.1%. When A2-E-laden cells were irradiated with blue light, their death rate was significantly increased to 96.2% (p=0.02, T-test for independent samples). After incubation of RPE with A2-E, which was exposed before to blue light (0.2 mW/mm² for 15 min), these showed a death rate of 91.5% (significantly higher than after incubation with A2-E kept in the dark, p=0.018) which was only marginally increased to 93% after additional irradiation of the cells. Exposure of the A2-E to white light (8.9 mW/mm² for one h) prior to the supplementation, however, resulted in a death rate increased to 93%.

Figure 1. False-color image of a primary culture of porcine retinal pigment epithelium. The DAPI-labeled nuclei appear blue. The ZO-1 protein is shown green.

Figure 2. A2-E supplementation to retinal pigment epithelium cells. Red channel represents phase contrast image; green channel represents autofluorescence. A: Cells grown in the absence of A2-E (control). B: Cells after incubation with 10 µM A2-E for 24 h. C: Cells after incubation with 10 µM A2-E for 48 h. D: Cells 72 h after withdrawal of A2-E supplemented for 48 h.
Figure 3. Retinal pigment epithelium cell damage rates. Damage by A2-E and photo-derivatives to cells kept in dark and to irradiated cells indicated by propidium iodide staining.

Figure 4. Mass spectra of photo-derivatives of A2-E. MALDI-TOF spectra (Ultraflex II TOF/TOF) of A2-E photo-derivatives: A2-E irradiated with 0.2 mW/mm², dominated by the monooxo-A2-E (m/z=608). B: A2-E irradiated with 8.9 mW/mm², dominated by the tetraoxidized product (m/z=656). Peaks with differences of 16 m/z-units indicate gradual oxidation.
of only 15.2% which was about half the rate found with A2-E kept in dark. Irradiating these cells with blue light increased the rate to 42%. Thus, we found that the toxicity of A2-E depends on the dosage of light to which it was exposed before it was fed to RPE cells.

TUNEL staining was negative in control cells (cultured without A2-E-supplementation), whereas in A2-E-laden cells TUNEL positive cells were found indicating apoptosis as the mechanism of cell death. A quantitative analysis of the TUNEL-tests, however, was impossible because of the overlap of the TUNEL fluorescence with that of the A2-E.

Mass spectra (Figure 4) clearly revealed the photooxidation of A2-E (m/z=592). After low-dosage exposure (0.2 mW/mm², 15 min), the most abundant product was that of m/z=608, i.e., one oxygen atom was added to A2-E. However, two-, three-, and fourfold oxidized species were found, too. In contrast, the high dosage irradiation (8.9 mW/mm² for one hour) predominantly resulted in the A2-E tetroxide (m/z=656). Ultraviolet-visible spectra showed a hypsochromic shift by about 30 nm of the Ultraviolet-absorption peak after low dosage exposure of the A2-E (absorption maxima at 335 nm and 436 nm) whereas the absorption disappeared almost completely at the high light dosage (Figure 5).

**DISCUSSION**

Primary cultures of porcine RPE cells are an appropriate model to use to study the behavior of A2-E in vivo. Though not as easy to handle as the ARPE-19 cell line, they have some advantages. Their morphology resemble the in vivo situation better; the functional integrity of the cells could be demonstrated; and the cells were still pigmented with melanin. Though the pigmentation decreases with cell proliferation, it may be similar to that of human RPE in vivo since this is less pigmented than that of the pig [22].

A2-E was internalized into RPE cells if delivered to the culture medium. Based on the granular appearance of the fluorescence three days after A2-E supplementation, which is similar to that in patients, it is very likely A2-E accumulates in the lysosomal compartment as demonstrated by Holz et al. [10]. Although these authors argued that a coupling of A2-E to low-density lipoprotein (LDL) is necessary to pass the cell membrane via their LDL receptors, our data as well as that of others [23] show the ability of RPE cells to ingest A2-E from solution due to its amphiphilic structure [24].

The cytotoxicity of A2-E was clearly demonstrated by the increase of the cell death rate by a factor of five after incubation with 10 μM A2-E for 24 h. This observation confirms previous reports describing the detergent-like activity of A2-E [8,23], the inhibition of lysosomal enzymes [10], and the release of pro-apoptotic proteins from mitochondria [9]. Exposure of the A2-E-laden cells or the A2-E itself to blue light of 0.2 mW/mm² for 15 min resulted in death of almost all cells. The mean tail moment in the comet assay was increased significantly compared to that of cells fed with A2-E kept in dark, indicating DNA damage. TUNEL staining indicated DNA fragmentation and thus the occurrence of apoptosis. By immunostaining, an oxidation of guanine to 8-oxodeoxyguanosine (8-oxo-dG) was demonstrated [13,18], which can subsequently result in stalled transcription, fission of the base sugar linkage and DNA strand breaks observed in the comet assay. This mechanism of oxidative DNA damage, however, can be induced either by singlet oxygen, generated by photosensitized A2-E, or by A2-E epoxides.

We found similar damage rates in irradiated A2-E-laden cells as in the cells incubated with blue-light exposed A2-E. The light dosage, applied here, was similar to that used by Sparrow et al. [18] (430 nm, 0.36 mW/mm² for 10 min), who showed epoxidation (1-7 epoxides) of more than 50% of the A2-E under this conditions. Analysis of the irradiated A2-E solution revealed monooxidized A2-E (m/z=608, see Figure 4) as the most abundant compound. The hypsochromic shift of the absorption peak in the ultraviolet by about 30 nm (Fig-

![Figure 5. Absorption spectra of A2-E and photo-derivatives.](http://www.molvis.org/molvis/v12/a151/)

Ultraviolet-visible spectra of A2-E, recorded with a lambda-2 photometer, show a hypsochromic shift after 0.2 mW/mm² irradiation and the disappearance of the absorption bands after 8.9 mW/mm² irradiation.
ure 5) indicates oxidation at the shorter retinal chain of the molecule. The absorption spectrum of the blue light exposed A2-E, although clearly a mixture of different oxidation products, is in good agreement with that published recently by Jang et al. [25]. The initial epoxidation occurs at the 5.6 carbon double bond as this is the most electron rich one [25]. After ingestion into the RPE lysosomes, the epoxides may rearrange to furanoids under the acidic intra-lysosomal milieu [17,25]. The oxo-A2-E with m/z 624 might be a monoperoxide at the shorter retinal arm rather than a bisepoxid or bisfuranoid since there was no shift of the blue absorption peak of the spectrum. Because of the weakness of the O-O bond of the peroxide, its cleavage is likely, resulting in the formation of an unstable diradical and subsequent reactive intermediates such as aldehydes, epoxides, and epoxyketones [26]. The cytotoxicity of endoperoxide-containing compounds and their metabolites is well known [27,28]. Thus, intermediate peroxides may contribute to the toxicity of A2-E under light exposure.

In our experiments, high dosage irradiation of A2-E predominantly formed a fourfold oxidized product (m/z=656, see Figure 4). This might be an effect of the irradiated energy rather than of the extended spectral range compared to the blue light. The irradiation of the white light between 450 nm and 490 nm was 0.68 mW/mm² which was about 3.5 times as high as the irradiation of the blue light. Since the exposure time was four times as long as that of blue light, the radiant energy in the blue spectral range of the white light illumination was fourteen fold of that of the blue-light illumination. The spectral expansion may have additional effects; however, green light was proven to be much less efficient to damage A2-E-laden cells than blue light [11].

In similar experiments, Dillon et al. [17] found the m/z=656 compound to be a bisfuranoid with further oxygen additions at the cyclohexyl rings at either sides. Their ultraviolet-visible spectra showed two hypochromic shifted (compared to A2-E) absorption bands which are in agreement with the findings of Jang et al. [25]. Our spectra (Figure 5), however, do no show these absorptions. Thus, we rather assume an epoxidation breaking down the conjugated system of retinal chains which is responsible for the absorption. The differences in Dillon’s [17] and our findings may result from the different solvents used. Furthermore, a rearrangement after internalization by the cellular lysosomes can not be excluded. Interestingly, we found only scarce amounts of higher molecular weight ions. This may indicate that the m/z=656-species is a rather stable molecule and further oxidation is difficult under the experimental conditions employed here. This can be a consequence of either the loss of absorption of visible light of this oxidation product, or the reduced electron density of the remaining double bonds or steric hindrance, alone or in combination.

The most intriguing finding of this investigation was the reduced toxicity of the A2-E exposed to a high light dosage, probably resulting in a fourfold epoxidation. This possibly may be explained by the stability, and thus reduced reactivity, of this molecule. Another concept, to be followed in further investigations, is based on the assumption that unstable inter-mediates, rather than epoxides or furanoides may be responsible for oxidative cell damage and the induction of apoptosis.

Though the results shown in Figure 3 might reflect the effect of A2-E and oxo-A2-E with one through four additional oxygen atoms dominating the mass spectra shown in Figure 4, it should always be kept in mind that mixtures of oxidation products were applied to the RPE cells. Thus, effects of minor but highly toxic components such as lower weight decay products or higher order oxidation products cannot be ruled out completely. This problem should be addressed in subsequent investigations. While it is still a matter of debate whether A2-E produces singlet oxygen by photosensitisation or not [18,19,21,29], it is shown to quench singlet oxygen on the other hand [19]. Based on this finding, Roberts et al. [19] conclude that A2-E protects RPE cells from photo-damage by scavenging singlet oxygen. However, by capturing singlet oxygen molecules, A2-E epoxides, furanoids, and highly reactive intermediates (such as peroxides) are formed [15,17,25].

In conclusion, our data support the hypothesis of direct DNA damage by oxidized A2-E rather than a protective effect due to singlet oxygen scavenging. Certainly, oxidative processes are responsible for the detrimental effect of A2-E to RPE cells under irradiation. However, further research is needed to elucidate whether these are mediated by reactive oxygen species, generated by the photosensitization of A2-E, by A2-E epoxides, or by unstable intermediates generated in the oxidation process of A2-E.

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